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1   Title: Simultaneous free fatty acid elevations and accelerated desaturation in plasma and oocytes in

2   early postpartum dairy cows under intensive feeding management

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26

27   **Abstract**

28   A severe negative energy balance and high circulating free fatty acids (FFA) in postpartum cows impair

29   fertility. The lipotoxicity of FFA has been shown to decrease the quality of bovine oocytes *in vitro*.

30   Therefore, excess FFA in cells is converted to triacylglycerol (TAG), a non-toxic form, to avoid

31   lipotoxicity. We recently reported that the TAG content in oocytes was higher in postpartum lactating

32   cows subjected to grazing management than in heifers (Theriogenology 176: 174-182, 2021). The

33   present study investigated the compositions of the energy metabolism-related lipids, FFA and TAG, in

34   the plasma and oocytes of cows at different lactation stages under indoor intensive feeding

35   management in order to obtain insights into lipotoxicity in oocytes, particularly those in early

36   postpartum cows. Blood and oocytes were collected from 20 lactating cows categorized into the

37   following lactation groups: 20–30 days in milk (DIM) (n = 5), 40–50 DIM (n = 5), 60–80 DIM (n =

38   5), and 130–160 DIM (n = 5). Daily energy balance data were obtained for 3 weeks prior to oocyte

39   collection using the ovum pick up (OPU) method. The contents and compositions of FFA and TAG in

40   plasma and oocytes were analyzed using liquid chromatography-mass spectrometry. As expected,

41   plasma FFA was high at 20–30 DIM, decreased by 50 DIM, and was maintained at a low level for the

42   remainder of the experimental period. Similar changes were observed in oocyte FFA and TAG with

43   DIM as plasma FFA. Oocyte FFA positively correlated with plasma FFA ($P < 0.05$), but negatively

44   correlated with the mean energy balance 1 and 21 days before OPU ($P < 0.05$). Relationships were

45 noted between the composition and content of FFA in plasma and oocytes, with the FFA 16:1/16:0 and

46 18:1/18:0 ratios positively correlating with the total amount of FFA (P <0.05). Elevated oocyte FFA in

47 cows in the early postpartum period under intensive feeding management suggested that oocytes were

48 at a high risk of FFA lipotoxicity. Furthermore, the present results implied that the severe negative

49 energy balance in the previous few weeks was closely related to increases in oocyte FFA, which

50 supports the importance of long-term cow feeding management for preserving the quality of oocytes

51 in the early postpartum period. The present results provide insights into the effects of high circulating

52 FFA on the fertility of postpartum cows.

53

54

55 **Keywords**

56 Dairy cow, Energy balance, Free fatty acid, Intensive feeding management, Oocyte, Triacylglycerol

57

1. Introduction

The fertility of modern high-yielding dairy cows was previously reported to be low [1, 2], and one of the leading causes was a malnourished status associated with high milk production after parturition [3, 4]. Dry cows show a zero or positive energy balance until the peripartum period, and then enter a negative energy balance concurrently with delivery and the start of lactation [5, 6]. The mobilization of fat in adipose tissue to produce energy increases the circulating levels of free fatty acids (FFA). Blood FFA levels start to increase in the peripartum period, peak within the first week of lactation, then decrease, and ultimately return to the basal level after 6–8 weeks of lactation [7-10]. High postpartum blood FFA levels have been reported to induce lipid disorders, such as fatty liver [11], immune malfunction [12], and lipotoxicity [13].

Excessive lipids in cells are converted to triacylglycerol (TAG) and stored in lipid droplets [14]. Lipid droplets serve as an energy source in cells, and function to maintain lipid and protein homeostasis [15]. Furthermore, lipid droplets protect cells from the cytotoxicity of FFA, particularly saturated FFA, by storing lipids in a non-toxic form [14]. This protective mechanism of lipid droplets has been demonstrated in oocytes [16] and cumulus cells [17]. Cumulus cells are in direct contact with follicular fluid and protect oocytes from elevated FFA by converting them to TAG and storing lipid droplets in cumulus cells [17, 18]. Lipotoxicity, caused by excessive FFA, may impair the fertility of lactating cows. When FFA levels increase beyond the ability of cells to synthesize TAG from FFA,

76   FFA are incorporated and accumulate in cells, which leads to reactive oxygen species production [19],

77   endoplasmic reticulum stress [20], ceramide accumulation [21], and, ultimately, apoptosis [22].

78   Oxidative stress was previously reported in the hepatocytes [23] and mammary glands [13] of lactating

79   cows with high circulating levels of FFA. Furthermore, the developmental competence of oocytes

80   matured with supplemental high FFA in culture medium was shown to be impaired due to lipotoxicity

81   [24].

82   Investigations on FFA and TAG profiles in blood and oocytes by high-resolution liquid

83   chromatography–mass spectrometry (LC/MS) using a small number of oocytes provide insights into

84   lipotoxicity in lactating cows. We recently reported that blood FFA and oocyte TAG were higher in

85   postpartum cows than in heifers [25]. TAG is the intracellular storage form of lipids that comprises

86   three fatty acyls linked to a glycerol backbone and is decomposed to FFA as required for energy

87   production and the synthesis of other lipids [26]. High TAG in the oocytes of postpartum cows

88   indicated that elevated circulating levels of FFA in the 3–6 weeks of the postpartum period increased

89   the amount of FFA reaching cumulus-oocyte complexes (COCs) via follicular fluid [27], and also that

90   TAG synthesis in COCs was accelerated to prevent FFA lipotoxicity. Elevated oocyte TAG indicated

91   that the ability of oocytes to manage lipotoxic FFA was reduced because their storage capacity for

92   TAG was surpassed [28].

93   Lipid characteristics are important for oocyte quality. Among the FFA present in bovine

94   oocytes, including 16:0, 16:1, 18:0, and 18:1 FFA [25], saturated FFA (FFA 16:0 and 18:0) are more

95   cytotoxic, while monounsaturated FFA (FFA 18:1) are less cytotoxic [16]. Monounsaturated FFA are

96   synthesized from saturated FFA, and this desaturation reaction is a key step in the synthesis of TAG

97   from FFA [29]. The conversion of monounsaturated FFA to TAG is one reason for monounsaturated

98   FFA being less cytotoxic [16]. Stearoyl-CoA desaturase (SCD) catalyzes desaturation reactions, and

99   one of the typical markers of its activity is the ratios of FFA 16:1/16:0 and FFA 18:1/18:0 as the product

100   and substrate ratios of desaturation reactions [30]. A previous study demonstrated that the FFA

101   18:1/18:0 ratio increased in blood and follicular fluid with elevations in blood and follicular fluid FFA

102   concentrations [18, 27]. These findings suggested that the increase in FFA transport from blood to

103   follicular fluid, which is attributable to elevation of the blood FFA level, affected the FFA

104   concentration and composition in follicular fluid. The FFA elongation reaction that long-chain fatty

105   acids family member 6 catalyzes also affects intracellular fatty acid profiles, and the activity of the

106   FFA elongation reaction interacts with the activities of desaturation reactions [31]. FFA 18:0/16:0 and

107   18:1/16:1 ratios, the product and substrate ratios of these elongation reactions, are used as elongase

108   markers [32, 33]. Examinations of these desaturase and elongase markers are useful for obtaining

109   information on the status of lipid metabolism in oocytes.

110   We previously reported the oocyte FFA and TAG profiles of grazed lactating cows with

111   relatively low milk production (~7,700 kg/305 days) [25]. The different livestock farming systems of

112 outdoor grazing or indoor intensive feeding have been shown to alter the content and composition of

113 lipids in animal products, *i. e.*, meat [34, 35] and milk [36], which may be attributed to variations in

114 nutrition intake [37], milk production [37], body condition dynamics [36-38], and the lipid

115 compositions of feed [39]. Therefore, the present study investigated the oocyte lipid profiles of cows

116 that were under indoor intensive management and produced milk close to the average level of Japan

117 (9,800 kg/305 days, 2020, Dairy Herd Performance Test data statistics, Livestock Improvement

118 Association of Japan, Inc., Tokyo, Japan).

119 The objective of this study was to obtain insights into lipotoxicity in the oocytes of dairy

120 cows, particularly in the early postpartum period. Therefore, the contents and compositions of the

121 energy metabolism-related lipids (*i. e.*, FFA and TAG) in oocytes at different lactation stages were

122 examined in cows under typical modern intensive management.

123

124 **2. Materials and methods**

125 **2.1. Animals**

126 The present study was implemented according to the animal experimental regulations of the

127 Hokkaido University Animal Care and Use Committee (Approval No.: 18-0028 and 19-0127). Seven

128 primiparous and 13 multiparous Holstein lactating cows (24–49 months of age, 1–3 parities) were

129 used in this study, all of which were kept at the experimental farm of Dairy Research Center, Hokkaido

130   Research Organization (Nakashibetsu, Hokkaido, Japan). Enrolled cows had milk production ranging

131   between 19.8 and 43.5 kg/day (Table S1) and were without clinical issues requiring intensive treatment.

132   The present study was conducted between January and August 2020. Cows were kept in a freestall

133   barn and fed a total mixed ration containing a blend of grass silage, corn silage, rolled corn, soybean

134   meal, calcium carbonate, and dicalcium phosphate (Table S2). The mean 305-day 4% fat corrected

135   milk yield of lactation was 9,123 kg. To compare plasma and oocyte lipid profiles at different lactation

136   stages, 5 cows per lactation stage defined based on days in milk (DIM) with different energy balance

137   levels [40, 41] were used: 20–30 DIM (the severe negative energy balance group, 1 primi- and 4

138   multiparous), 40–50 DIM (the moderate negative energy balance group, 1 primi- and 4 multiparous),

139   60–80 DIM (the zero energy balance group, 3 primi- and 2 multiparous), and 130–160 DIM (the

140   positive energy balance group, 2 primi- and 3 multiparous).

141

142   **2.2. Assessment of the energy balance**

143   The energy balance was calculated using equations described in NRC (2001) [42] and

144   according to Ramos-Nieves et al. [43] with slight modifications. In brief, the energy balance per day

145   was calculated according to the following equation, where net energy for lactation is presented as NE_L

146   and megacalories as Mcal:

147   NE_L (Mcal/day) balance = energy intake (Mcal of NE_L/day)

148   − [maintenance requirement (Mcal of NE_L/day) + lactation requirement (Mcal of NE_L/day)

149   + growth requirement (Mcal of NE_L/day)]

150   The procedure used to calculate the energy balance was described in detail in Supplementary

151   Material. The daily energy balance for 3 weeks before ovum pick up (OPU) was calculated, and the

152   mean values of individual daily energy balances for each week (1–7, 8–14, and 15–21 days before

153   OPU) and for 3 weeks (1–21 days before OPU) were used in analyses.

154

155   **2.3. Sample collection**

156   Oocytes were collected and prepared for the lipid analysis according to our previous study

157   [25] with a slight modification. The ultrasound-guided OPU method was performed for oocyte

158   collection using an ultrasound imaging device (HS-1600V; Honda Electronics, Toyohashi, Japan)

159   equipped with a 9.0 MHz long-handled micro-convex probe (HCV-7710MV; Honda Electronics). All

160   follicles ≥2 mm in diameter were puncture targets, and COCs with follicular fluid were aspirated. The

161   mean numbers of follicles in a pair of ovaries before OPU were 19.7 ± 6.6 in total and 15.8 ± 6.4, 2.5

162   ± 2.2, and 1.5 ± 0.9 follicles with diameters of 2–4, 4–8, and ≥8 mm, respectively. Follicular fluid

163   containing COCs was diluted with Dulbecco's phosphate-buffered saline (D-PBS) (Nissui

164   Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA) (Sigma-

165   Aldrich, St. Louis, MO, USA) and 10 IU/mL heparin sodium (AY Pharmaceuticals Co., Ltd., Tokyo,

10

166   Japan) to avoid blood coagulation. An average of 8.2 ± 2.6 COCs were recovered per session. Cumulus

167   cells were removed from COCs by vortexing and gentle pipetting with a fine glass pipette. Oocyte

168   denudation was confirmed under a stereomicroscope, and 5 oocytes without the apparent atretic

169   appearance of ooplasms (classes 1–3 according to Blondin and Sirard [44]) were selected per cow and

170   a single sample of 5 oocytes/OPU was used in the lipidomic analysis. Five oocytes were transferred

171   to a 1.5-mL microcentrifuge tube (Eppendorf AG, Hamburg, Germany) with a small amount of D-PBS

172   + 0.1% PVA (<10 μL) and stored at −80 °C until analyzed.

173   Blood was collected at oocyte sampling by caudal venipuncture using

174   ethylenediaminetetraacetic acid–loaded vacuum tubes (VP-NA070K; Terumo Co., Tokyo, Japan) and

175   stored on ice until centrifugation. After plasma separation by centrifugation, 100 μL of plasma was

176   transferred to a 1.5-mL microcentrifuge tube and stored at −80 °C until analyzed.

177

178   **2.4. Lipid extraction of plasma and oocyte samples from cows**

179   Spectral grade solvents and reagents as well as authentic lipid standards were purchased from

180   Sigma-Aldrich for lipid extraction and LC/MS analyses. In LC/MS-based lipid profiling, appropriate

181   internal standards (IS) are essential for normalization and subsequent (semi-)quantitation. Therefore,

182   the authentic commercial compounds FFA 17:0 and TAG 11:0/11:0/11:0 used herein as IS were

183   prepared in methanol containing butylated hydroxytoluene (0.006%, w/v) and then stored at −80 °C

184   until analyzed. The lipid extraction procedure was performed as described in our previous studies [25,

185   45] within 1 h to avoid lipid oxidation or degradation. Five oocytes (in one microcentrifuge tube) were

186   extracted with 600 µL of ice-cold chloroform/methanol 2:1 (v/v) twice, and 100 µL plasma (in one

187   microcentrifuge tube) was extracted with 800 µL of ice-cold chloroform/methanol 1:1 (v/v). The

188   spiked amounts of IS are listed in Table S3. After extraction, the upper layer of the sample was dried

189   under vacuum. Total lipids were then dissolved in methanol and filtered to remove any residue prior

190   to injection.

191

192   **2.5. LC/MS-based lipid profiling of FFA and TAG**

193   To separate and measure each lipid molecular species, the LC/MS analysis was conducted

194   using a Prominence HPLC (Shimadzu Corp., Kyoto, Japan) and LTQ Orbitrap mass spectrometer

195   (Thermo-Fisher Scientific Inc., San Jose, CA, USA) under the conditions described in our previous

196   studies [25, 45]. Forty samples (2 types of samples [plasma and oocyte] × 5 cows × 4 lactation groups)

197   were analyzed for lipid profiling in a single LC/MS run. The main parameters used were as follows:

198   LC column, Atlantis T3 C18 (2.1 × 150 mm, 3 µm, Waters, Milford, MA, USA); column oven

199   temperature, 40 ℃; elution solvents, water (with 5 mM ammonium acetate) (A), isopropanol (B), and

200   methanol (C); solvent flow rate, 0.2 mL/min; MS ionization method, electrospray ionization (ESI)

201   under both positive and negative modes; spray voltage, 3 kV; capillary temperature, 330 ℃; MS^1 scan

202   range, *m/z* 150–1100 (positive), *m/z* 220–1650 (negative); MS[2] fragmentation method, collision-

203   induced dissociation; normalized collision energy, 35.0.

204   Raw data were processed using Xcalibur 2.3 (Thermo-Fisher Scientific Inc.). Lipids were

205   identified based on retention behavior and high-resolution MS[1] signals (in the Fourier transform mode,

206   with tolerance ≤5.0 ppm). The annotation of lipid species was performed according to the following

207   format: "lipid class + total carbon number in the fatty chain(s) + total double bond number in the fatty

208   chain(s)" (*e. g.*, FFA 14:0 and TAG 46:1). The semi-quantitation of each lipid species was calibrated

209   with IS using the following equation:

210
$$Amount_{Analyte} = Amount_{IS} \times \frac{Peak\ area_{Analyte}}{Peak\ area_{IS}}$$

211   To accurately identify the molecular species (*i. e.*, fatty acyl composition) of each TAG, the

212   acquired MS[2] fragments (in ion-trap mode) were compared with the LIPIDMAPS database

213   (https://www.lipidmaps.org/) and our in-house library [25, 45]. Therefore, each TAG was identified

214   using MS (both MS[1] and MS[2]), and the amount of every fatty acyl was individually measured [45].

215   The total amount of each fatty acyl in TAG was then calculated as the sum of its amount in each TAG

216   species, as shown in the equation:

217
$$Amount_{Fatty\ acyl\ in\ TAG} = \sum_{i=1}^{n} \left(Amount_{TAG\ species}^{i} \times Fatty\ acyl\ quantity_{TAG\ species}^{i} \right)$$

218

219   **2.6. Data analysis**

220   All statistical analyses were conducted using the statistical software JMP Pro 15.2.0 (SAS

221   Institute, Cary, NC, USA). The mean values of the FFA desaturase markers (FFA 16:1/16:0 and

222   18:1/18:0 ratios) in plasma and oocytes were compared using the paired *t*-test. Other statistical

223   analyses of the relationships between two parameters were performed using Pearson's correlation

224   coefficient, and P-values were calculated by a regression analysis. A P-value <0.05 was considered to

225   be significant, and data are shown as means ± SD, except for data on the energy balance, which were

226   expressed as means ± SEM.

227

228   **3. Results**

229   **3.1. Energy balance transition in cows at different lactation stages**

230   The energy balance status is a basic factor that affects lipid metabolism. Therefore, we

231   initially examined the energy balance transition before OPU in the different lactation groups.

232   Nutritional parameters (*e. g.*, milk production and components) were similar between the lactation

233   groups (Table S1). The 20–30 DIM group showed a negative energy balance over 3 weeks before OPU

234   (Fig. 1); however, it consistently increased during this period. The 40–50 DIM group showed a

235   negative energy balance 3 weeks before OPU, followed by a consistent increase, and a nearly zero

236   energy balance at the time of OPU. The energy balance remained near zero in the 60–80 DIM group

237   and was maintained at a constant positive value in the 130–160 DIM group in the 3 weeks before OPU

238   (Fig. 1). These results confirmed the different energy balance transition conditions between the

239   lactation groups.

240

241   **3.2. High plasma FFA, oocyte FFA, and oocyte TAG at 20–30 DIM**

242   To clarify the relationship between lactation stages and energy metabolism-related lipids in

243   plasma and oocytes, we examined alterations in the distribution of plasma FFA, oocyte FFA, and

244   oocyte TAG with DIM. We confirmed the expected transition of plasma FFA with DIM, namely, an

245   increase in the early postpartum period at 20–30 DIM (194.7 ± 53.1 μmol/L), a decrease by 50 DIM

246   (125.5 ± 54.0 μmol/L at 40–50 DIM), and maintenance at a low level after 50 DIM (126.3 ± 28.7

247   μmol/L at 60–80 DIM and 85.2 ± 29.4 μmol/L at 130–160 DIM) (n = 5, respectively) (Fig. 2A).

248   Alterations in oocyte FFA and TAG with DIM were similar to those in plasma FFA. Oocyte FFA was

249   319.1 ± 26.5 pmol/oocyte at 20–30 DIM, decreased by 50 DIM (278.0 ± 20.6 pmol/oocyte at 40–50

250   DIM), and was maintained at a low level after 50 DIM (241.2 ± 25.0 pmol/oocyte at 60–80 DIM and

251   234.3 ± 25.0 pmol/oocyte at 130–160 DIM) (n = 5, respectively) (Fig. 2B). Oocyte TAG was 57.4 ±

252   13.5 pmol/oocyte at 20–30 DIM, decreased by 50 DIM (39.6 ± 3.5 pmol/oocyte at 40–50 DIM), and

253   was maintained at a low level after 50 DIM (37.2 ± 6.1 pmol/oocyte at 60–80 DIM and 42.9 ± 22.0

254   pmol/oocyte at 130–160 DIM) (n = 5, respectively) (Fig. 2C). Despite similar transitions in plasma

255   FFA, oocyte FFA, and oocyte TAG, 2 out of 5 cows at 130–160 DIM showed high oocyte TAG of 78.5

256 and 59.1 pmol/oocyte, respectively (Fig. 2C).

257

3.3. Concurrent increase in oocyte FFA with a long-term negative energy balance and elevated

259 **plasma FFA**

260 We examined the relationships between plasma FFA, oocyte FFA, and oocyte TAG, which

261 showed similar transition patterns with DIM. A correlation analysis using Pearson's correlation

262 coefficient confirmed positive correlations between plasma FFA and oocyte FFA (Fig. 3A) and

263 between oocyte FFA and oocyte TAG (Fig. 3C) (r = 0.63 and r = 0.46, respectively, P <0.05), indicating

264 relationships between the metabolic balance of plasma FFA, oocyte FFA, and oocyte TAG. However,

265 no correlation was observed between plasma FFA and oocyte TAG (r = 0.27, P = 0.24) (Fig. 3B).

266 We then assessed the daily energy balance in different periods before OPU to clarify the

267 relationship between the long-term nutritional status and oocyte lipid profiles. A correlation analysis

268 using Pearson's correlation coefficient was performed, and the results obtained showed that oocyte

269 FFA negatively correlated with the mean energy balance 1 and 21 days before OPU (r = -0.70, P <0.05)

270 (Fig. 4A3), 1 and 14 days before OPU (r = -0.64, P <0.05) (Fig. 4A2), and 1 and 7 days before OPU

271 (r = -0.58, P <0.05) (Fig. 4A1). Therefore, the mean energy balance for a longer period before OPU

272 showed a stronger negative correlation with oocyte FFA (r = -0.70, P <0.05) (Fig. 4A3). In contrast,

273 oocyte TAG did not correlate with the mean energy balance 1 and 21 days before OPU (r = -0.34, P =

274   0.14) (Fig. 4B3), 1 and 14 days before OPU (r = -0.32, P = 0.17) (Fig. 4B2), and 1 and 7 days before

275   OPU (r = -0.24, P = 0.30) (Fig. 4B1).

276

277   **3.4. Positive correlations between plasma and oocyte FFA metabolism markers**

278   To investigate the relationships between the contents and compositions of FFA and TAG in

279   plasma and oocytes, we investigated markers of desaturase (the 16:1/16:0 and 18:1/18:0 ratios) (Fig.

280   S1) and elongase (the 18:0/16:0 and 18:1/16:1 ratios) activities, which are associated with the

281   metabolism of FFA and synthesis of TAG [30, 46]. As expected, the results obtained showed increases

282   in plasma FFA desaturase markers with elevations in plasma FFA (P <0.05, respectively) (Fig. 5A).

283   Similarly, oocyte FFA positively correlated with the oocyte FFA 16:1/16:0 ratio (r = 0.79) and

284   18:1/18:0 ratio (r = 0.56) (P <0.05, respectively) (Fig. 5B). Oocyte FFA desaturase markers also

285   positively correlated with oocyte TAG (P <0.05, respectively) (Fig. 5C). When desaturase markers

286   were compared between plasma and oocytes, the plasma FFA 16:1/16:0 ratio (0.153 ± 0.040, n=20)

287   and 18:1/18:0 ratio (1.415 ± 0.436, n=20) in all cows were significantly higher than the oocyte FFA

288   16:1/16:0 ratio (0.077 ± 0.059, n=20) and 18:1/18:0 ratio (0.094 ± 0.038, n=20), respectively (P <0.05),

289   indicating an inherently different saturated and monounsaturated FFA balance between plasma and

290   oocytes regardless of lactation stages. Positive correlations were observed between the plasma and

291   oocyte FFA 16:1/16:0 ratio (r = 0.70, P <0.05) and the plasma and oocyte FFA 18:1/18:0 ratio (r =

292 0.51, P <0.05) (Fig. 5D), which indicated relationships between lipid compositions in plasma and

293 oocytes. On the other hand, regarding elongase markers, negative correlations were noted between

294 plasma FFA and plasma FFA elongase markers (Fig. S2A), and between oocyte FFA and oocyte FFA

295 elongase markers (Fig. S2B). Collectively, these results confirmed the relationships of lipid contents

296 and specific metabolism markers between plasma and oocytes.

297

298 **4. Discussion**

299 **4.1. Increases in oocyte FFA in early postpartum cows and potential effects on oocyte quality**

300   The present results revealed a positive correlation between plasma and oocyte FFA and

301 between oocyte FFA and desaturase markers. The analysis of specific lipid metabolism markers in the

302 present study revealed a distinctly altered oocyte lipid composition with an increase in FFA. Plasma

303 FFA showed the expected transition with DIM: an increase at 20–30 DIM, a decrease by 50 DIM, and

304 maintenance at a low level after 50 DIM. Oocyte FFA and TAG showed similar transitions to plasma

305 FFA. The postpartum increase in oocyte TAG was consistent with our previous findings [25], whereas

306 that in oocyte FFA was not; oocyte FFA was similar among heifers and cows in the early lactation (~40

307 DIM), peak lactation (~60 DIM), and middle lactation (~180 DIM) stages. This discrepancy was

308 attributed to cows in the present study being subjected to more intensive management with higher milk

309 production. We also focused on the earlier lactation stage accompanied by higher plasma FFA in the

18

310   present study (20–30 DIM) than in our previous study (25–47 DIM). Furthermore, we selected oocytes

311   based on their morphology for the LC/MS analysis, whereas oocytes without apparent morphological

312   collapse were specifically used in our previous study. The present results suggest that high plasma FFA

313   in postpartum cows influenced the contents and compositions of oocyte FFA and TAG.

314   Oocyte FFA increased at 20–30 DIM with high plasma FFA, which indicated that oocytes

315   in the early postpartum period were at a high risk of FFA lipotoxicity. FFA lipotoxicity may induce

316   oocyte degeneration, which inhibits folliculogenesis and anovulation [47]. A severe negative energy

317   balance in postpartum cows delays the first postpartum ovulation by suppressing the hypothalamic

318   pituitary gonadal axis [48]. In addition to this mechanism, FFA lipotoxicity in the oocytes of

319   postpartum cows may partly account for the negative energy balance and delay in the first postpartum

320   ovulation [49-53]. Furthermore, the increase in oocyte FFA may explain the previously reported

321   epigenetic changes in metabolism-associated genes in the oocytes of postpartum cows [54].

322   Supplemental high FFA in *in vitro* oocyte maturation was previously shown to alter the DNA

323   methylation fingerprints of the resultant embryos [55]. This is the first study to examine the

324   relationship between long-term energy balance and oocyte lipid profiles in lactating cows. Oocyte FFA,

325   but not TAG, negatively correlated with the energy balance 1 and 21 days before OPU, and this

326   correlation was stronger than those on 1 and 7 days and on 1 and 14 days before OPU. In the 20–30

327   DIM group, 3 weeks before OPU corresponded to the period between parturition and oocyte collection.

328   The present results suggest that postpartum cow management to avoid a severe negative energy

329   balance will reduce risk of elevated oocyte FFA at 20–30 DIM.

330   The desaturase markers, fatty acid 16:1/16:0 and 18:1/18:0 ratios, reflect the synthesis of

331   TAG from FFA in cells [29, 30]. FFA desaturase markers are generally lower in oocytes than in tissues

332   with a high TAG synthesis capacity, such as the liver (FFA 18:1/18:0 ratio of ~4 [56]). The low values

333   obtained for FFA desaturase markers in oocytes in the present study were consistent with our previous

334   findings [25]. Additionally, the protein expression of SCD1 (an abundantly expressed SCD isoform in

335   bovine cumulus cells) was lower in bovine oocytes than in cumulus cells [17]. Therefore, the present

336   results confirmed the inherently low ability of bovine oocytes to synthesize TAG from FFA due to low

337   SCD activity. This low FFA-processing ability of oocytes additionally supported oocytes in the early

338   postpartum period being vulnerable to FFA lipotoxicity. When the amount of TAG in non-adipocytes

339   surpasses the storage capacity of cells, excess FFA are provided to cells [57]. Therefore, the increase

340   observed in oocyte FFA in early postpartum cows in the present study may be attributed to (1)

341   excessive FFA beyond the capacity of SCD in oocytes to convert FFA to TAG, resulting in the

342   accumulation of FFA, and/or (2) TAG surpassing the storage capacity of oocytes, which has a negative

343   impact on its synthesis from FFA, resulting in excess FFA in oocytes.

344   Cumulus cells play an important role in oocyte viability by providing nutrients and

345   regulatory signals [58, 59]. They also exhibit high SCD activity and actively synthesize TAG in

346 response to FFA supplementation [17]. TAG in cumulus cells were higher in heifers subjected to

347 fasting than in control animals [18]. Accordingly, the present results showing elevated oocyte FFA in

348 early postpartum cows prompted us to speculate that FFA and TAG profiles in cumulus cells may also

349 be affected by high FFA in this period. Future studies are needed to investigate the role of cumulus

350 cells in the regulation of oocyte lipids and the protection of oocytes from elevated FFA in early

351 postpartum cows.

352

353 **4.2. Altered plasma and oocyte lipid compositions with increases in plasma and oocyte lipids**

354 The lipid analysis performed in the present study revealed a close relationship between the

355 content and composition of lipids. A positive correlation was observed between oocyte FFA desaturase

356 markers and oocyte TAG. Monounsaturated FFA are essential for the synthesis of TAG [30]; therefore,

357 an increase in the proportion of monounsaturated FFA may directly contribute to active TAG synthesis.

358 In contrast, oocyte FFA elongase markers decreased with increases in oocyte FFA in the early

359 postpartum period. The decrease in FFA elongase markers indicates an increase in the proportion of

360 the shorter chain FFA species, *i. e.*, less-matured FFA containing less energy [60]. Since we only

361 examined lactating cows in the present study, further studies are needed to compare the relationship

362 between oocyte lipid compositions and oocyte quality in lactating cows with those in heifers [61, 62]

363 and dry cows [62], which potentially have high-quality oocytes. Plasma FFA desaturase markers

364   positively correlated with plasma FFA and oocyte FFA desaturase markers. This result suggests that

365   the composition of plasma FFA reflected that of oocyte FFA due to the transport of abundant FFA from

366   blood to oocytes via follicular fluid [18, 27]. Another potential explanation for the simultaneous

367   elevations observed in oocyte desaturase markers (FFA 16:1/16:0 and 18:1/18:0 ratios) and oocyte

368   FFA levels was an increase in oocyte SCD activity in response to abundantly incorporated FFA,

369   resulting in higher FFA 16:1/16:0 and 18:1/18:0 ratios and oocyte TAG contents. Although we used

370   product and substrate ratios as markers of desaturase and elongase activities in the present study,

371   further studies on the expression of these enzymes and their genes are needed in order to obtain a more

372   detailed understanding of lipid metabolism in cow oocytes.

373

374   **4.3. Oocyte TAG contents in the middle lactation stage**

375   Two cows in 130–160 DIM showed higher oocyte TAG than the remaining 3 cows in the

376   same lactation stage, while oocyte FFA was similar. These 2 cows, which were primiparous, showed

377   larger reductions in BCS during early lactation after parturition (*i. e.*, approximately 120 days before

378   OPU) than the remaining 3 cows, which were multiparous (data not shown). This intensive adipose

379   tissue mobilization during early lactation, the period corresponding to the retrospective duration of the

380   folliculogenesis of oocytes collected at 130–160 DIM [63-65], may be associated with the elevation

381   observed in oocyte TAG at 130–160 DIM in these two cows. The number of cows in the middle

382 lactation stage in the present study was small; therefore, we need to investigate the long-term effects

383 of reductions in BCS in postpartum cows on oocyte lipid profiles and their relationship with oocyte

384 quality in a larger number of cows.

385

386 **5. Conclusion**

387   The present study revealed that cows subjected to typical modern intensive management had

388 high oocyte FFA and TAG in the early postpartum period (Fig. 6). Consistent with the increase in

389 oocyte FFA, the 16:1/16:0 and 18:1/18:0 ratios as desaturase markers were elevated. These lipid

390 changes were associated with increases in plasma FFA. Based on increases in oocyte FFA in

391 combination with the inherently low ability of oocytes to synthesize TAG from FFA, oocytes appeared

392 to be at a high risk of FFA lipotoxicity in the early postpartum period. Milk production by the cows

393 examined in the present study was slightly lower than the average in Japan; therefore, oocyte lipid

394 compositions may be affected more in cows from farms with higher milk production and a high

395 incidence of postpartum metabolic diseases than in those used in this study. High circulating FFA in

396 the early postpartum period may result in low fertility in modern high-yielding cows via the

397 deterioration of oocyte quality due to lipotoxicity.

398

399 **Abbreviations**

400   BCS, body condition score; COCs, cumulus–oocyte complexes; DIM, days in milk; D-PBS,

401   Dulbecco's phosphate buffered saline; ESI, electrospray ionization; FFA, free fatty acid; IS, internal

402   standard; LC/MS, liquid chromatography-mass spectrometry; NE_L, net energy for lactation; OPU,

403   ovum pick up; PVA, polyvinyl alcohol; SCD, stearoyl-CoA desaturase; TAG, triacylglycerol.

404

405 **Disclosure**

406   The authors declare no conflicts of interest.

407

408 **CRediT authorship contribution statement**

409   Eri Furukawa: Methodology, Software, Formal Analysis, Investigation, Data Curation,

410 Writing - Original Draft, Visualization. Zhen Chen: Methodology, Software, Investigation, Data

411 Curation, Writing - Original Draft, Visualization. Tomoaki Kubo: Investigation, Resources. Yue Wu:

412 Methodology, Validation, Investigation, Visualization. Koichiro Ueda: Formal analysis, Visualization.

413 Madalitso Chelenga: Investigation. Hitoshi Chiba: Resources, Supervision. Yojiro Yanagawa:

414 Resources, Writing - Review & Editing. Seiji Katagiri: Resources, Writing - Review & Editing.

415 Masashi Nagano: Conceptualization, Supervision, Project administration, Funding acquisition. Shu-

416 Ping Hui: Conceptualization, Supervision, Project administration.

417

422

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605

1   Fig. 1. Energy balance transition between 1 and 21 days before OPU.

2   Two types of total mixed rations were fed to cows based on days in milk (DIM), and chemical

3   components were analyzed using near-infrared reflectance spectroscopy at 2-week intervals. Daily

4   energy balances of cows were calculated according to equations from NRC (2001). Data on the 3-day

5   moving average of energy balances (the average values of the past 3 days) were used to remove short-

6   term (within a few days) fluctuations in values. The values are shown in reference to the time of OPU

7   (= Day 0). Symbols and error bars indicate mean values and SEM. The numbers in parentheses indicate

8   the number of animals included in each lactation group.

9

10   Fig. 2. Plasma FFA, oocyte FFA, and oocyte TAG profiles in relation to days in milk.

11   Relationships between DIM and plasma FFA (A), oocyte FFA (B), and oocyte TAG (C). Blood

12   sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation

13   stages (20 cows in total), and FFA and TAG were analyzed using LC/MS with 100 µL of plasma and

14   a single sample of 5 oocytes per cow. Different symbols indicate cows in different lactation groups,

15   with numbers shown in parentheses indicating the number of animals in each lactation group.

16

17   Fig. 3. Relationships between plasma FFA concentrations and oocyte FFA and TAG contents.

18 Relationships between plasma FFA and oocyte FFA (A), plasma FFA and oocyte TAG (B), oocyte FFA

19 and oocyte TAG (C). Sampling of blood and oocytes from cows and LC/MS analysis of FFA and TAG

20 were performed as described in Fig. 2. Different symbols indicate cows in different lactation groups,

21 with numbers shown in parentheses indicating the number of animals in each lactation group.

22 Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines

23 represent the regression lines.

24

25 Fig. 4. Relationships between the energy balance and oocyte FFA and TAG contents.

26 Relationships between energy balance during the indicate time before OPU and oocyte FFA (A) and

27 oocyte TAG (B). Oocyte collection and LC/MS analysis of FFA and TAG were performed as described

28 in Fig. 2. Energy balance was calculated daily as described in Fig. 1, and the mean energy balance

29 during the indicated periods, 1 and 7 days before OPU (A1B1), 1 and 14 days before OPU (A2B2),

30 and 1 and 21 days before OPU (A3B3) were calculated. Different symbols indicate cows in different

31 lactation groups, with numbers shown in parentheses indicating the number of animals in each

32 lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the

33 dotted lines represent the regression lines.

34

35 Fig. 5. Desaturase markers of fatty acid 16:1/16:0 ratio and 18:1/18:0 ratio in plasma and oocytes.

36   The upper panel (A–C) shows relationships between plasma FFA, oocyte FFA, and oocyte TAG and

37   desaturase markers: plasma FFA concentrations and plasma FFA desaturase markers (A), oocyte FFA

38   contents and oocyte FFA desaturase markers (B), and oocyte TAG contents and oocyte FFA desaturase

39   markers (C).

40   The lower panel (D–F) shows relationships between desaturase markers of plasma FFA, oocyte FFA,

41   and oocyte TAG: relationships of desaturase markers between plasma FFA and oocyte FFA (D), plasma

42   FFA and oocyte TAG (E), and oocyte FFA and oocyte TAG (F).

43   Sampling of blood and oocytes from cows and LC/MS analysis of FFA and TAG were performed as

44   described in Fig. 2. The ratios of fatty acid 16:1/16:0 and 18:1/18:0 were calculated. Different symbols

45   indicate cows in different lactation groups, with numbers shown in parentheses indicating the number

46   of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each

47   subfigure, and the dotted lines represent the regression lines.

48

49   Fig. 6. Summary of the study: alterations in oocyte FFA and TAG profiles in early postpartum cows

50   under intensive feeding management. A negative energy balance and elevated plasma FFA were

51   observed in early postpartum cows (20–30 DIM). Plasma FFA desaturase markers (the 16:1/16:0 and

52   18:1/ 18:0 ratios) increased and elongase markers (18:0/16:0 and 18:1/16:1 ratios) decreased with

53   elevations in plasma FFA concentrations. Alterations in the content and composition of oocyte FFA

54   were similar to those in plasma FFA. The effects of abundant FFA in oocytes were reflected as an

55   increase in TAG, the non-toxic form of lipids synthesized from FFA.
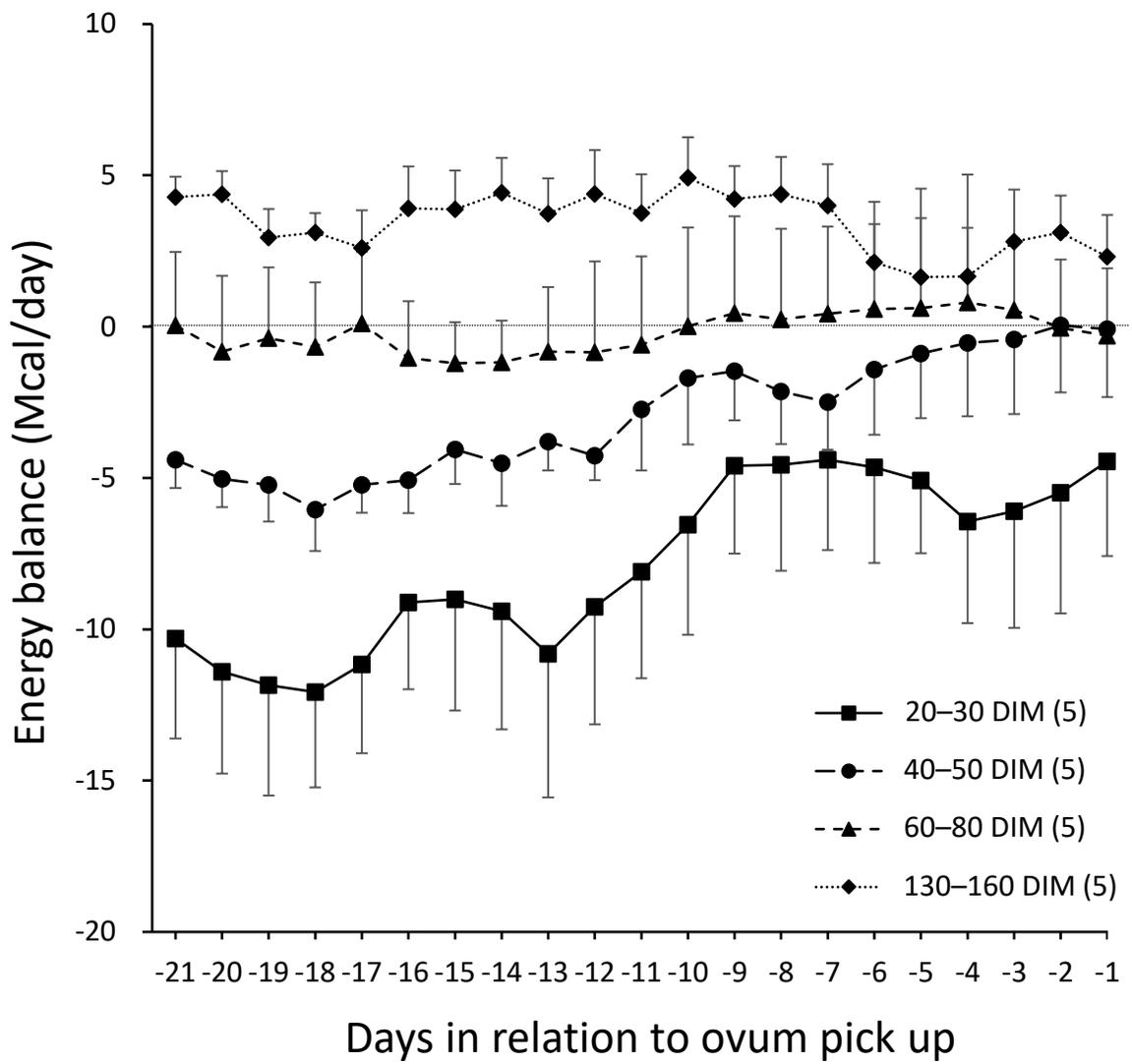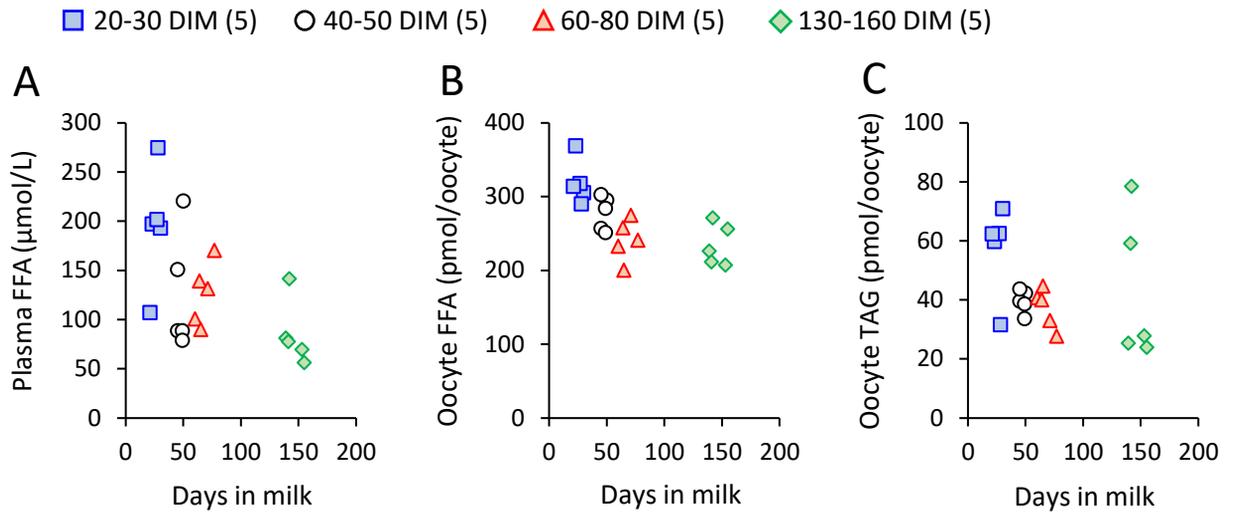
56

Fig. 1

■ 20-30 DIM (5)   ○ 40-50 DIM (5)   △ 60-80 DIM (5)   ◇ 130-160 DIM (5)
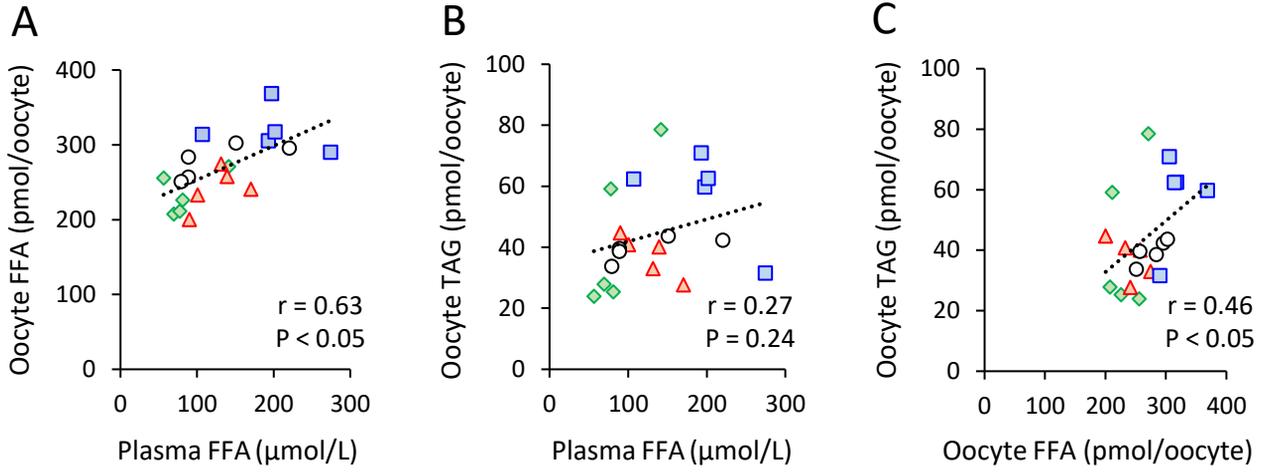
A

Plasma FFA (µmol/L) vs Days in milk

B

Oocyte FFA (pmol/oocyte) vs Days in milk

C

Oocyte TAG (pmol/oocyte) vs Days in milk

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Negative energy balance

Blood

FFA ⬆

16:1 / 16:0 ⬆
18:1 / 18:0 ⬆
18:0 / 16:0 ⬇
18:1 / 16:1 ⬇

Oocyte

FFA ⬆

16:1 / 16:0 ⬆
18:1 / 18:0 ⬆
18:0 / 16:0 ⬇
18:1 / 16:1 ⬇

TAG ⬆
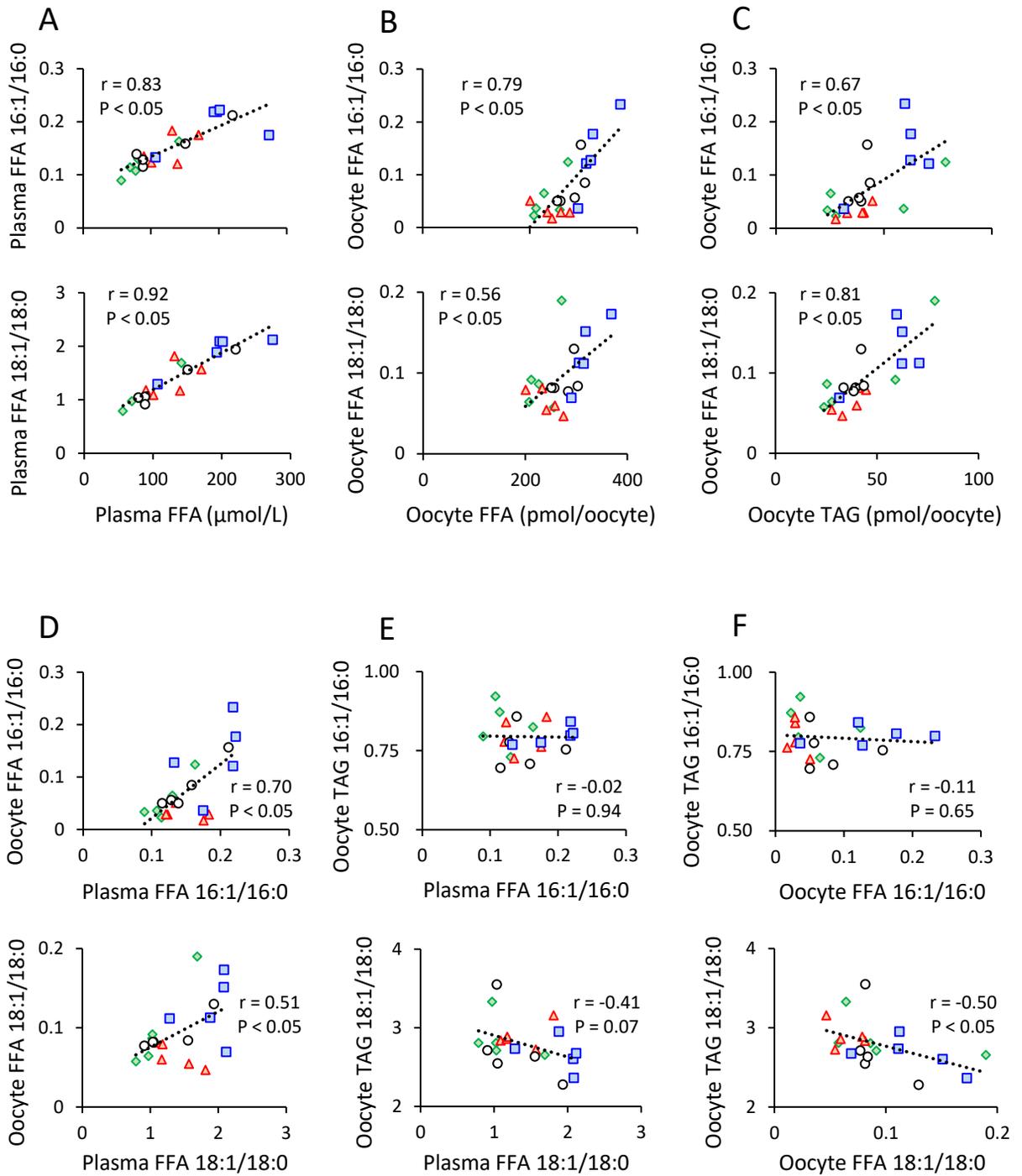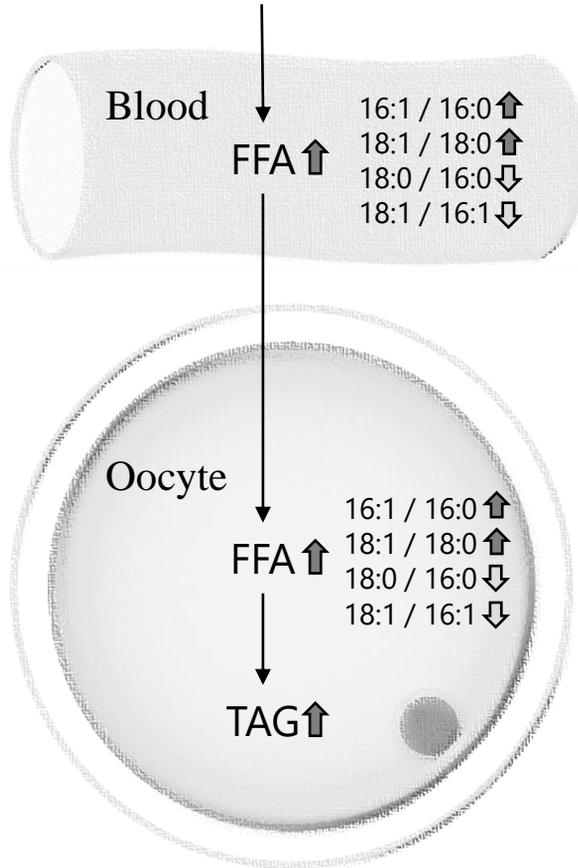
Fig. 6

1 SUPPLEMENTAL EXPERIMENTAL PROCEDURE

2 **Feeding, sampling, and energy balance calculation**

3   Feeding management and sampling procedures of the farm used in the present

4 study are descried elsewhere (Nishiura et al. Anim Sci J 93(1): e13757, 2022). Namely,

5 two types of total mixed rations were fed to cows depending on days in milk (DIM); one

6 is formulated for high producing cows (1–149 DIM) and the other is for low producing

7 cows (≥150 DIM) (Table S2). Amounts of feed intake were determined daily by an

8 automated feeder (Roughage Intake Control System, Insentec BV, The Netherlands). Dry

9 matter contents of grass silage and corn silage were determined by drying at a 60 ℃

10 forced air oven for 48 hours weekly, and the inclusion rates of ration ingredients were

11 adjusted to account for differences in dry matter contents. Dried samples of the silages

12 were ground in a mill to pass through a 1 mm screen, composited at 2-week intervals, and

13 analyzed for chemical components at the Dairy Research Center, Hokkaido Research

14 Organization. Crude protein (CP), neutral detergent fiber (NDF), ether extract (EE),

15 nonfiber carbohydrate (NFC), and lignin were measured by using near-infrared

16 reflectance spectroscopy (NIRS 6500, Foss, Denmark). Milk production was recorded at

17 every milking, and milk fat, protein, and lactose concentrations by infrared spectroscopy

18 (MilkoScan FT2, Foss Electric, Denmark), and body weight (BW) were recorded weekly.

19   Calculation of energy balance per day was performed according to the following

20   equation, where net energy for lactation is presented as NE_L, megacalorie as Mcal, and

21   day as d:

22   NE_L (Mcal / d) balance = energy intake (Mcal of NE_L / d)

23   − [maintenance requirement (Mcal of NE_L / d)

24   + lactation requirement (Mcal of NE_L / d) + growth requirement (Mcal of NE_L / d)],

25   where

26   energy intake (Mcal / d) = (0.0245 $\times$ total digestible nutrients (TDN) (%) − 0.12)

27                $\times$ dry matter intake (kg / d),

28   maintenance requirement (Mcal) = metabolic BW ($kg^{0.75}$) $\times$ 0.08 (Mcal / $kg^{0.75} \cdot$ d),

29   lactation requirement (Mcal) = [0.0929 $\times$ fat (%) + 0.0547 $\times$ crude protein (%)

30   + 0.0395 $\times$ lactose (%)] $\times$ milk production (kg).

31   Growth requirement was considered in the calculation regarding first and

32   second parity;

33   growth requirement (Mcal) = 0.0635 $\times$ equivalent empty BW $(EQEBW)^{0.75}$

34          $\times$ equivalent empty body tissue gain $(EQEBG)^{1.097}$,

35   where

36   EQEBW = 0.891 $\times$ equivalent shrunk BW (EQSBW),

37                    $EQSBW = SBW \times (478 / \text{mature SBW})$,

38   mature $SBW = 0.96 \times$ mature weight (MW),

39                    $EQEBG = 0.956 \times$ weight gain (WG).

40   MW was defined as 700 kg, and WG was defined as 0.2 kg for first parity and

41   0.1 kg for second parity. Daily energy balance between 1 and 21 days before OPU were

42   calculated and used for the analysis.

43

44   SUPPLEMENTAL TABLES

45

46   Table S1. Average body condition score (BCS) and nutritional parameters of cows in

47   different lactation stages

Item	20–30 DIM (n = 5)	40–50 DIM (n = 5)	60–80 DIM (n = 5)	130–160 DIM (n = 5)
BCS[1]	2.9 ± 0.5	2.9 ± 0.4	2.9 ± 0.4	3.0 ± 0.3
BW[23] (kg)	622 ± 53	603 ± 37	584 ± 45	649 ± 83
Dry matter intake[24] (kg/day)	17.1 ± 2.0	19.1 ± 2.2	19.2 ± 2.7	20.9 ± 2.1
Milk production[245] (kg/day)	35.5 ± 8.2 (19.8–43.5)	34.2 ± 5.7 (24.7–40.6)	32.4 ± 7.6 (23.7–42.1)	29.2 ± 3.6 (25.7–35.8)
Milk fat[23] (%)	4.57 ± 0.18	4.38 ± 0.45	4.19 ± 0.21	4.29 ± 0.27
Milk protein[23] (%)	3.17 ± 0.47	2.96 ± 0.10	2.82 ± 0.23	3.43 ± 0.26
Milk lactose[23] (%)	4.12 ± 0.27	4.30 ± 0.19	4.50 ± 0.26	4.37 ± 0.13

48   Values are presented as the mean ± SD. Values were compared using nonparametric

49   Kruskal-Wallis test with Steel-Dwass *post hoc* test between lactation groups.

50   [1]BCS was assessed in the week ovum pick up (OPU) was performed.

51   [2]Mean values during 21 days before OPU

52   [3]BW and milk components were measured weekly.

53   [4]Dry matter intake and milk production were calculated daily.

54   [5]Values in parenthesis indicate the minimum and the maximum values.

55

56   Table S2. Ingredient and chemical composition of the total mixed ration fed to cows

Total mixed ration	1–149 DIM[1]	≥150 DIM[2]
Ingredient (%DM)		
Grass silage	38.7	47.1
Corn silage	22.5	20.2
Rolled corn	24.0	20.8
Soybean meal	13.1	10.5
Calcium carbonate	1.6	1.4
Dicalcium phosphate	0.0	0.1
Chemical		
DM (%)	35.0	32.8
TDN[3] (%DM)	75.3	71.8
CP (%DM)	15.7	15.1
NDF (%DM)	38.1	41.6
NFC (%DM)	35.4	31.7

57   [1]Mean values were calculated from 83 samples.

58   [2]Mean values were calculated from 10 samples.

59   [3]TDN were calculated following NRC 2001 with digestibility estimated by total feces

60   collection:

61   TDN = digestible CP + 2.25 × digestible EE + digestible NFC + digestible NDF

62   Each component was calculated using the digestibility parameters as indicated in

63   Appendix A of Nishiura et al. Anim Sci J 93(1): e13757, 2022.

64

65   Table S3. Amounts of spiked lipid internal standards for LC/MS analysis.

Lipid standard	Spiked amount (nmol) per tube of oocyte	Spiked amount (nmol) per tube of plasma
FFA 17:0	0.281	0.627
TAG 11:0/11:0/11:0	0.141	0.313

66   FFA, free fatty acid; TAG, triacylglycerol.
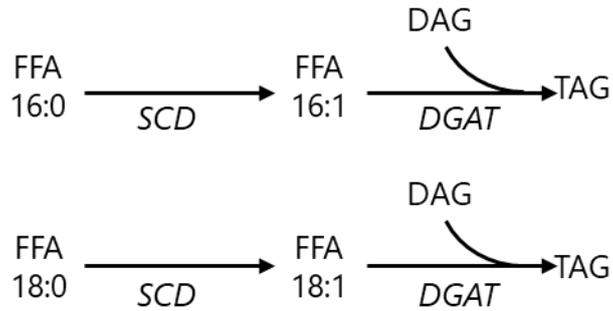
67

68   SUPPLEMENTAL FIGURES

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78   Fig. S1. Diagram of FFA desaturation reactions towards TAG synthesis in cells. FFA 16:1

79   and 18:1 are produced from FFA 16:0 and 18:0, respectively, by desaturation reactions

80   which *SCD* catalyzes, and are subsequently utilized for TAG synthesis (Paton and Ntambi

81   Am J Physiol Endocrinol Metab 297(1):E28-37, 2009). FFA, free fatty acid; DAG,

82   diacylglycerol; TAG, triacylglycerol; *SCD*, stearoyl-CoA desaturase; *DGAT*,

83   diacylglycerol acyltransferase
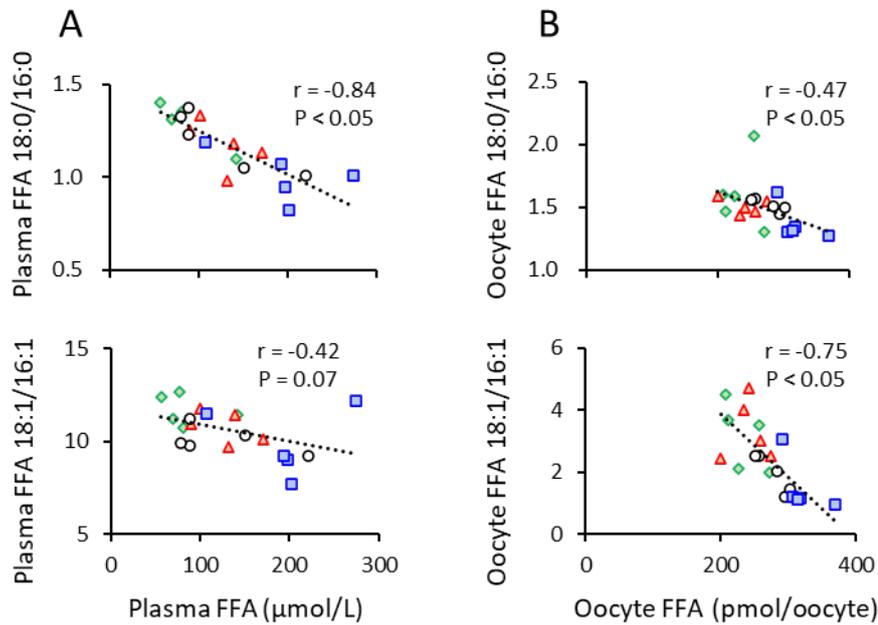
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Fig. S2. Elongase markers of fatty acid 18:0/16:0 ratio and 18:1/16:1 ratio in plasma and oocytes. Plasma FFA concentrations and elongase markers (A), and oocyte FFA contents and elongase markers (B). Blood sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation stages (20 cows in total). FFA and TAG were analyzed using LC/MS with 100 μL of plasma and a single sample of 5 oocytes per cow, and the ratios of fatty acid 18:0/16:0 and 18:1/16:1 were calculated. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group. Pearson's correlation coefficient and P-values are shown in each subfigure, and the dotted lines represent the regression lines.