Title: Simultaneous free fatty acid elevations and accelerated desaturation in plasma and oocytes in early postpartum dairy cows under intensive feeding management

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Abstract

A severe negative energy balance and high circulating free fatty acids (FFA) in postpartum cows impair fertility. The lipotoxicity of FFA has been shown to decrease the quality of bovine oocytes *in vitro*. Therefore, excess FFA in cells is converted to triacylglycerol (TAG), a non-toxic form, to avoid lipotoxicity. We recently reported that the TAG content in oocytes was higher in postpartum lactating cows subjected to grazing management than in heifers (Theriogenology 176: 174-182, 2021). The present study investigated the compositions of the energy metabolism-related lipids, FFA and TAG, in the plasma and oocytes of cows at different lactation stages under indoor intensive feeding management in order to obtain insights into lipotoxicity in oocytes, particularly those in early postpartum cows. Blood and oocytes were collected from 20 lactating cows categorized into the following lactation groups: 20–30 days in milk (DIM) (n = 5), 40–50 DIM (n = 5), 60–80 DIM (n = 5), and 130–160 DIM (n = 5). Daily energy balance data were obtained for 3 weeks prior to oocyte collection using the ovum pick up (OPU) method. The contents and compositions of FFA and TAG in plasma and oocytes were analyzed using liquid chromatography-mass spectrometry. As expected, plasma FFA was high at 20–30 DIM, decreased by 50 DIM, and was maintained at a low level for the remainder of the experimental period. Similar changes were observed in oocyte FFA and TAG with DIM as plasma FFA. Oocyte FFA positively correlated with plasma FFA ($P < 0.05$), but negatively correlated with the mean energy balance 1 and 21 days before OPU ($P < 0.05$). Relationships were

noted between the composition and content of FFA in plasma and oocytes, with the FFA 16:1/16:0 and 18:1/18:0 ratios positively correlating with the total amount of FFA ($P < 0.05$). Elevated oocyte FFA in cows in the early postpartum period under intensive feeding management suggested that oocytes were at a high risk of FFA lipotoxicity. Furthermore, the present results implied that the severe negative energy balance in the previous few weeks was closely related to increases in oocyte FFA, which supports the importance of long-term cow feeding management for preserving the quality of oocytes in the early postpartum period. The present results provide insights into the effects of high circulating FFA on the fertility of postpartum cows.

Keywords

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

58 **1. Introduction**

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

68 Excessive lipids in cells are converted to triacylglycerol (TAG) and stored in lipid droplets
69 [14]. Lipid droplets serve as an energy source in cells, and function to maintain lipid and protein
70 homeostasis [15]. Furthermore, lipid droplets protect cells from the cytotoxicity of FFA, particularly
71 saturated FFA, by storing lipids in a non-toxic form [14]. This protective mechanism of lipid droplets
72 has been demonstrated in oocytes [16] and cumulus cells [17]. Cumulus cells are in direct contact with
73 follicular fluid and protect oocytes from elevated FFA by converting them to TAG and storing lipid
74 droplets in cumulus cells [17, 18]. Lipotoxicity, caused by excessive FFA, may impair the fertility of
75 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

outdoor grazing or indoor intensive feeding have been shown to alter the content and composition of lipids in animal products, *i. e.*, meat [34, 35] and milk [36], which may be attributed to variations in nutrition intake [37], milk production [37], body condition dynamics [36-38], and the lipid compositions of feed [39]. Therefore, the present study investigated the oocyte lipid profiles of cows that were under indoor intensive management and produced milk close to the average level of Japan (9,800 kg/305 days, 2020, Dairy Herd Performance Test data statistics, Livestock Improvement Association of Japan, Inc., Tokyo, Japan).

The objective of this study was to obtain insights into lipotoxicity in the oocytes of dairy cows, particularly in the early postpartum period. Therefore, the contents and compositions of the energy metabolism-related lipids (*i. e.*, FFA and TAG) in oocytes at different lactation stages were examined in cows under typical modern intensive management.

2. Materials and methods

2.1. Animals

The present study was implemented according to the animal experimental regulations of the Hokkaido University Animal Care and Use Committee (Approval No.: 18-0028 and 19-0127). Seven primiparous and 13 multiparous Holstein lactating cows (24–49 months of age, 1–3 parities) were 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 $$NE_L \text{ (Mcal/day) balance} = \text{energy intake (Mcal of } NE_L\text{/day)}$$

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,

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 °C; 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 °C; 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 $$Amount_{Fatty\ acyl\ in\ TAG} = \sum_{i=1}^{n} \left(Amount^{i}_{TAG\ species} \times Fatty\ acyl\ quantity^{i}_{TAG\ species} \right)$$

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

258 **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

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**

BCS, body condition score; COCs, cumulus–oocyte complexes; DIM, days in milk; D-PBS, Dulbecco's phosphate buffered saline; ESI, electrospray ionization; FFA, free fatty acid; IS, internal standard; LC/MS, liquid chromatography-mass spectrometry; NE_L, net energy for lactation; OPU, ovum pick up; PVA, polyvinyl alcohol; SCD, stearoyl-CoA desaturase; TAG, triacylglycerol.

Disclosure

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Eri Furukawa: Methodology, Software, Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Zhen Chen: Methodology, Software, Investigation, Data Curation, Writing - Original Draft, Visualization. Tomoaki Kubo: Investigation, Resources. Yue Wu: Methodology, Validation, Investigation, Visualization. Koichiro Ueda: Formal analysis, Visualization. Madalitso Chelenga: Investigation. Hitoshi Chiba: Resources, Supervision. Yojiro Yanagawa: Resources, Writing - Review & Editing. Seiji Katagiri: Resources, Writing - Review & Editing. Masashi Nagano: Conceptualization, Supervision, Project administration, Funding acquisition. Shu-Ping Hui: Conceptualization, Supervision, Project administration.

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605

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

Two types of total mixed rations were fed to cows based on days in milk (DIM), and chemical components were analyzed using near-infrared reflectance spectroscopy at 2-week intervals. Daily energy balances of cows were calculated according to equations from NRC (2001). Data on the 3-day moving average of energy balances (the average values of the past 3 days) were used to remove short-term (within a few days) fluctuations in values. The values are shown in reference to the time of OPU (= Day 0). Symbols and error bars indicate mean values and SEM. The numbers in parentheses indicate the number of animals included in each lactation group.

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

Relationships between DIM and plasma FFA (A), oocyte FFA (B), and oocyte TAG (C). Blood sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation stages (20 cows in total), and FFA and TAG were analyzed using LC/MS with 100 μL of plasma and a single sample of 5 oocytes per cow. Different symbols indicate cows in different lactation groups, with numbers shown in parentheses indicating the number of animals in each lactation group.

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.

The upper panel (A–C) shows relationships between plasma FFA, oocyte FFA, and oocyte TAG and desaturase markers: plasma FFA concentrations and plasma FFA desaturase markers (A), oocyte FFA contents and oocyte FFA desaturase markers (B), and oocyte TAG contents and oocyte FFA desaturase markers (C).

The lower panel (D–F) shows relationships between desaturase markers of plasma FFA, oocyte FFA, and oocyte TAG: relationships of desaturase markers between plasma FFA and oocyte FFA (D), plasma FFA and oocyte TAG (E), and oocyte FFA and oocyte TAG (F).

Sampling of blood and oocytes from cows and LC/MS analysis of FFA and TAG were performed as described in Fig. 2. The ratios of fatty acid 16:1/16:0 and 18:1/18:0 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.

Fig. 6. Summary of the study: alterations in oocyte FFA and TAG profiles in early postpartum cows under intensive feeding management. A negative energy balance and elevated plasma FFA were observed in early postpartum cows (20–30 DIM). Plasma FFA desaturase markers (the 16:1/16:0 and 18:1/ 18:0 ratios) increased and elongase markers (18:0/16:0 and 18:1/16:1 ratios) decreased with 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

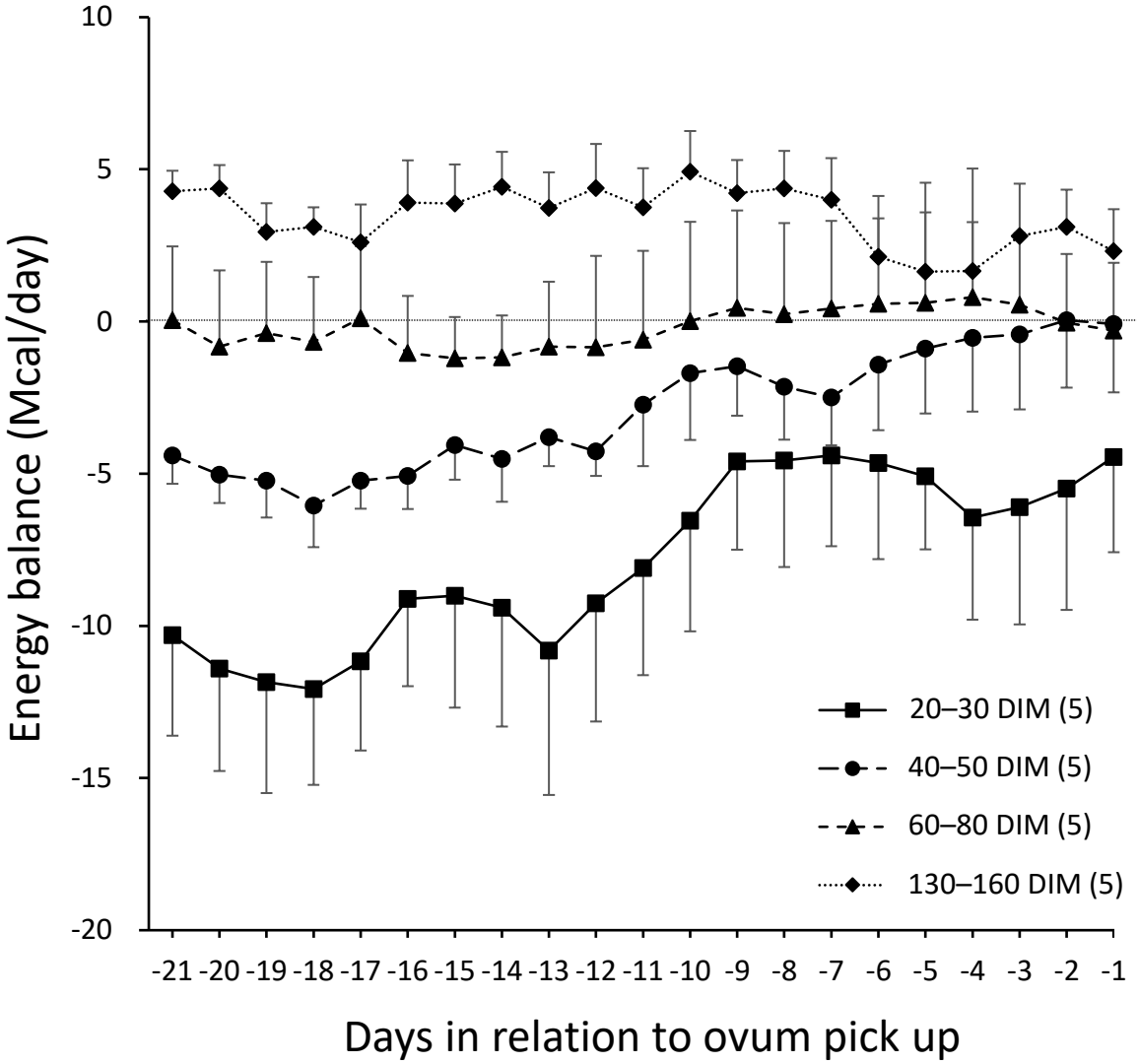
10
5
0
-5
-10
-15
-20
Energy balance (Mcal/day)
-21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1
Days in relation to ovum pick up
20–30 DIM (5)
40–50 DIM (5)
60–80 DIM (5)
130–160 DIM (5)

Fig. 1

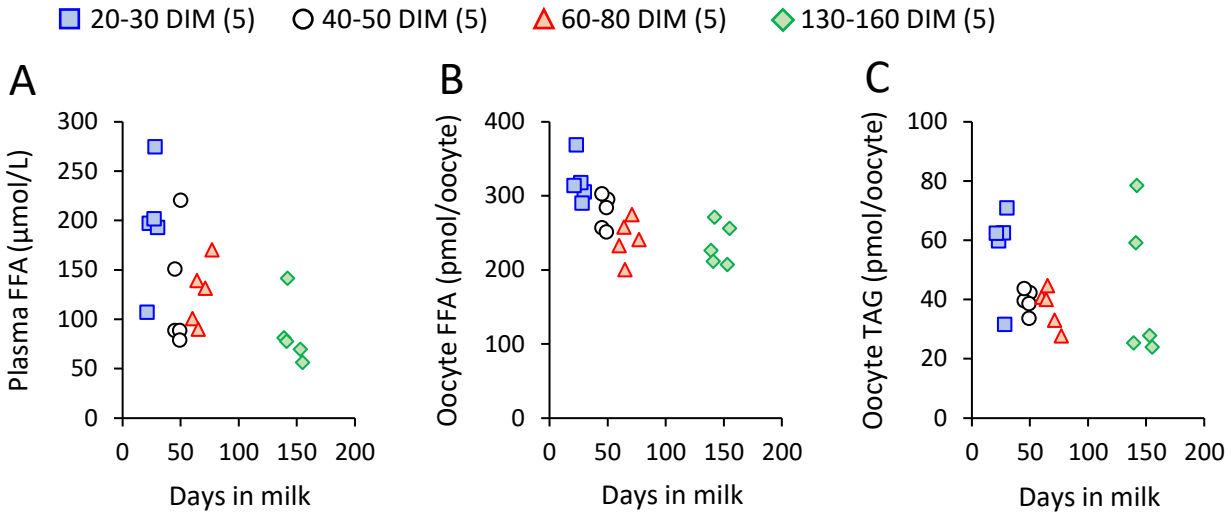
20-30 DIM (5)
40-50 DIM (5)
60-80 DIM (5)
130-160 DIM (5)
A
Plasma FFA (µmol/L)
300
250
200
150
100
50
0
0
50
100
150
200
Days in milk
B
Oocyte FFA (pmol/oocyte)
400
300
200
100
0
0
50
100
150
200
Days in milk
C
Oocyte TAG (pmol/oocyte)
100
80
60
40
20
0
0
50
100
150
200
Days in milk

Fig. 2

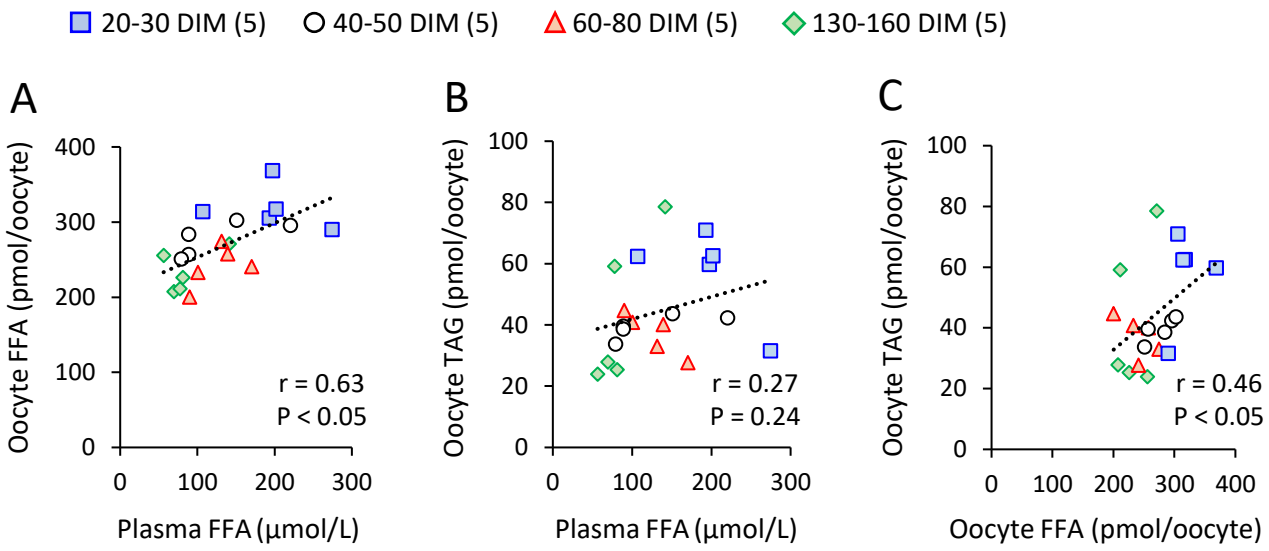
20-30 DIM (5)
40-50 DIM (5)
60-80 DIM (5)
130-160 DIM (5)
A
Oocyte FFA (pmol/oocyte)
Plasma FFA (µmol/L)
r = 0.63
P < 0.05
B
Oocyte TAG (pmol/oocyte)
Plasma FFA (µmol/L)
r = 0.27
P = 0.24
C
Oocyte TAG (pmol/oocyte)
Oocyte FFA (pmol/oocyte)
r = 0.46
P < 0.05

Fig. 3

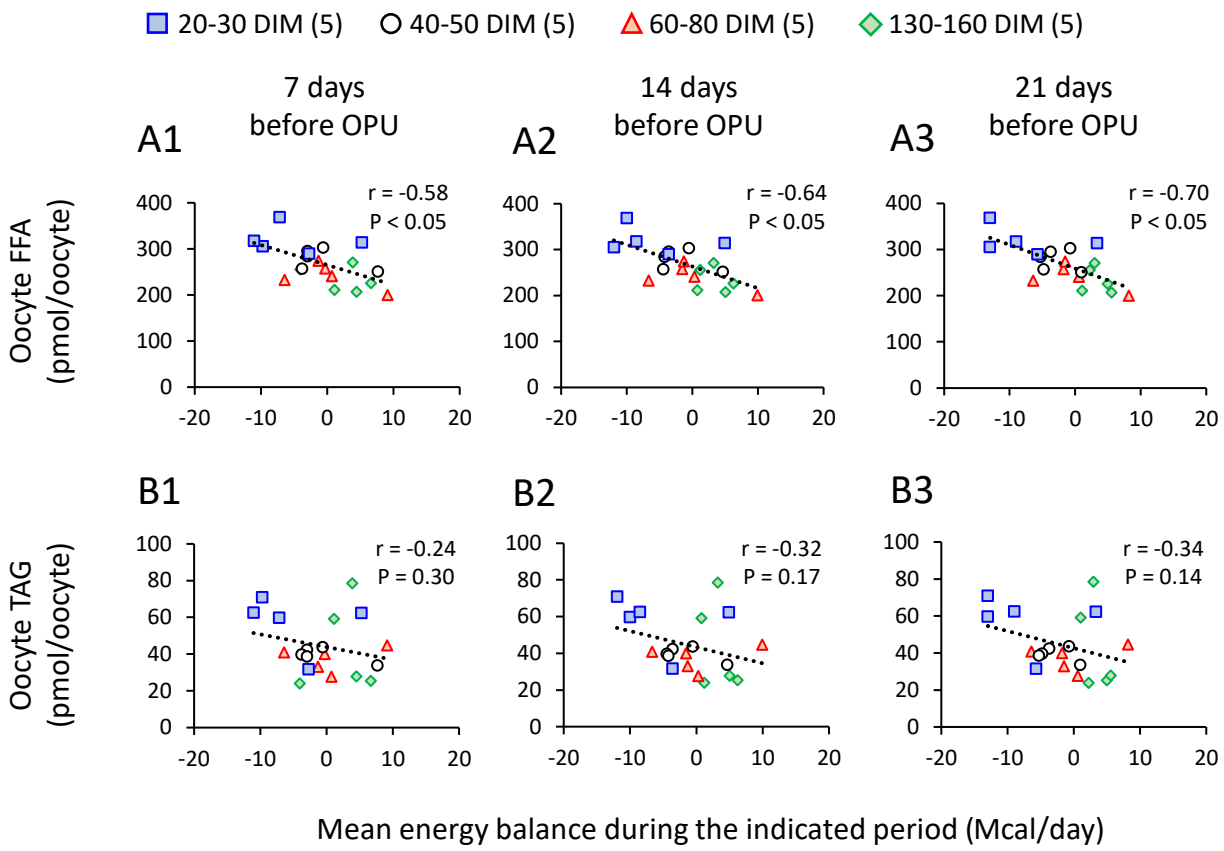
20-30 DIM (5)
40-50 DIM (5)
60-80 DIM (5)
130-160 DIM (5)
7 days before OPU
14 days before OPU
21 days before OPU
A1
A2
A3
B1
B2
B3
Oocyte FFA (pmol/oocyte)
Oocyte TAG (pmol/oocyte)
r = -0.58
P < 0.05
r = -0.64
P < 0.05
r = -0.70
P < 0.05
r = -0.24
P = 0.30
r = -0.32
P = 0.17
r = -0.34
P = 0.14
Mean energy balance during the indicated period (Mcal/day)

Fig. 4

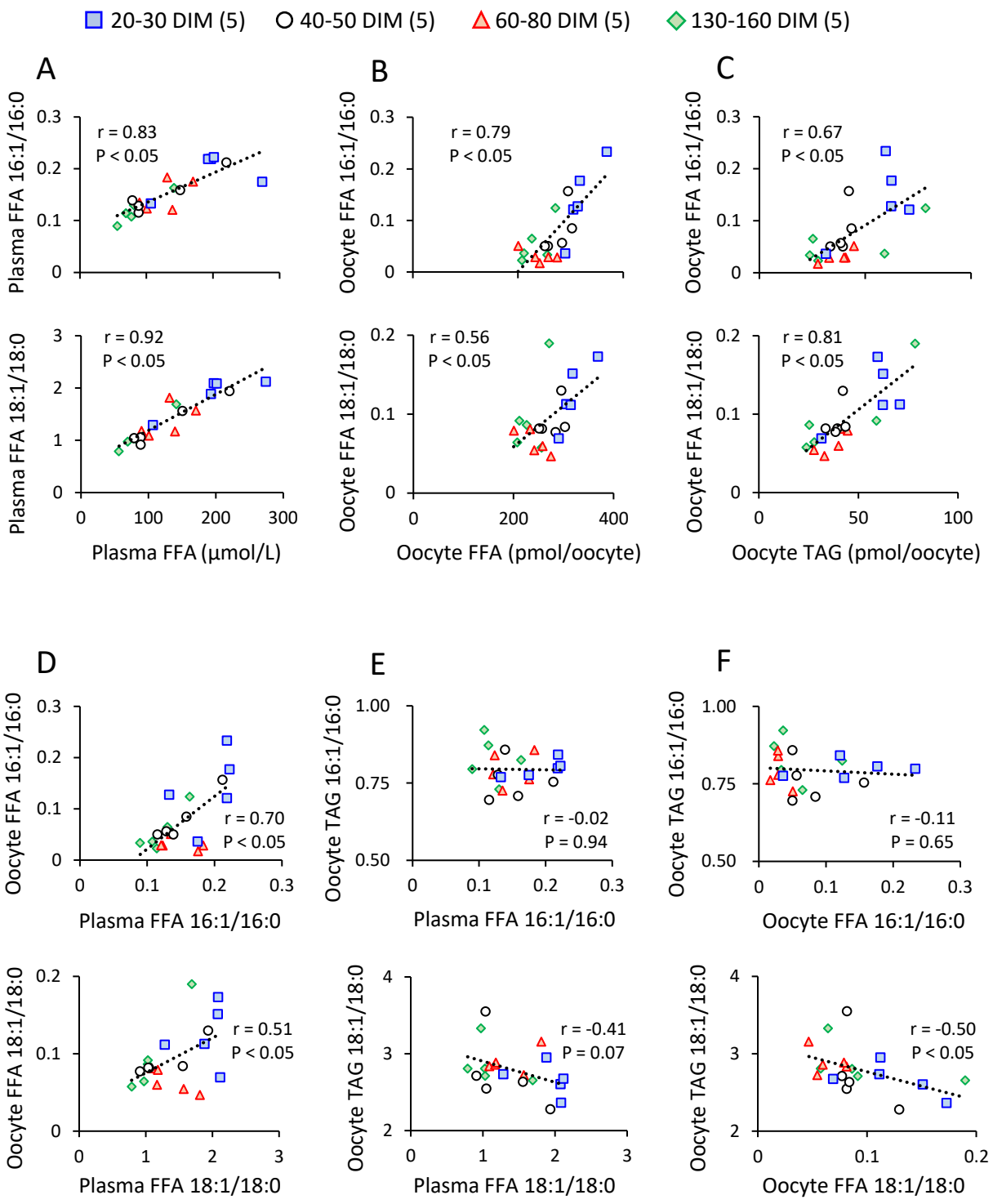
20-30 DIM (5)
40-50 DIM (5)
60-80 DIM (5)
130-160 DIM (5)
A
B
C
D
E
F
Plasma FFA 16:1/16:0
Plasma FFA 18:1/18:0
Oocyte FFA 16:1/16:0
Oocyte FFA 18:1/18:0
Oocyte TAG 16:1/16:0
Oocyte TAG 18:1/18:0
Plasma FFA (µmol/L)
Oocyte FFA (pmol/oocyte)
Oocyte TAG (pmol/oocyte)
Plasma FFA 16:1/16:0
Plasma FFA 18:1/18:0
Oocyte FFA 16:1/16:0
Oocyte FFA 18:1/18:0
r = 0.83
P < 0.05
r = 0.79
P < 0.05
r = 0.67
P < 0.05
r = 0.92
P < 0.05
r = 0.56
P < 0.05
r = 0.81
P < 0.05
r = 0.70
P < 0.05
r = -0.02
P = 0.94
r = -0.11
P = 0.65
r = 0.51
P < 0.05
r = -0.41
P = 0.07
r = -0.50
P < 0.05

Fig. 5

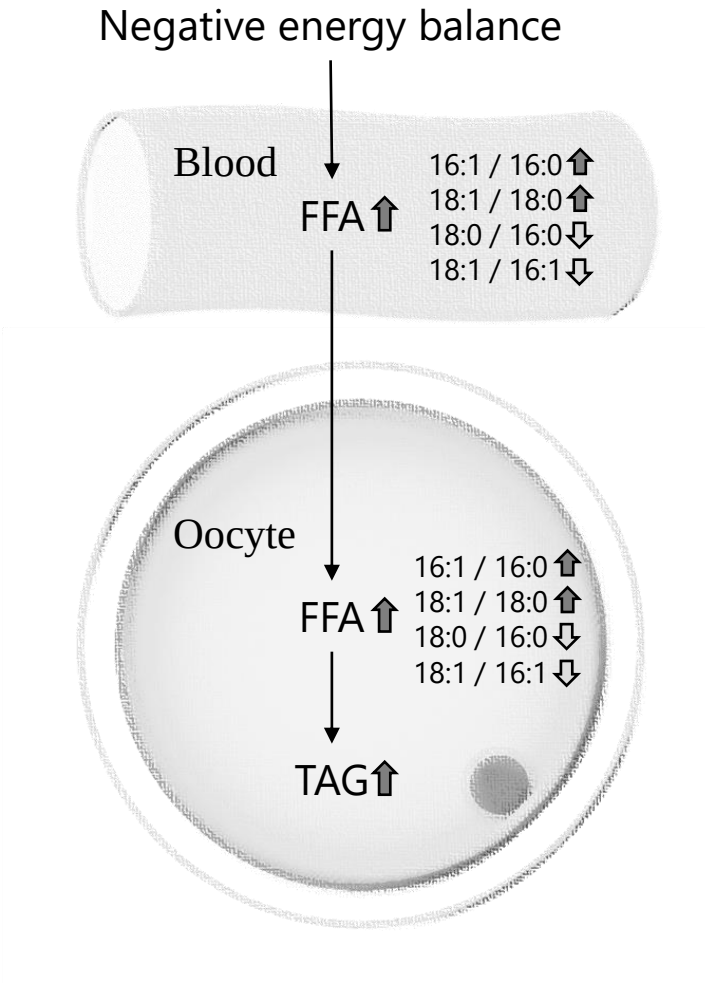
Negative energy balance
Blood
FFA
16:1 / 16:0
18:1 / 18:0
18:0 / 16:0
18:1 / 16:1
Oocyte
FFA
16:1 / 16:0
18:1 / 18:0
18:0 / 16:0
18:1 / 16:1
TAG

Fig. 6

SUPPLEMENTAL EXPERIMENTAL PROCEDURE

Feeding, sampling, and energy balance calculation

Feeding management and sampling procedures of the farm used in the present study are descried elsewhere (Nishiura et al. Anim Sci J 93(1): e13757, 2022). Namely, two types of total mixed rations were fed to cows depending on days in milk (DIM); one is formulated for high producing cows (1–149 DIM) and the other is for low producing cows (≥150 DIM) (Table S2). Amounts of feed intake were determined daily by an automated feeder (Roughage Intake Control System, Insentec BV, The Netherlands). Dry matter contents of grass silage and corn silage were determined by drying at a 60 ℃ forced air oven for 48 hours weekly, and the inclusion rates of ration ingredients were adjusted to account for differences in dry matter contents. Dried samples of the silages were ground in a mill to pass through a 1 mm screen, composited at 2-week intervals, and analyzed for chemical components at the Dairy Research Center, Hokkaido Research Organization. Crude protein (CP), neutral detergent fiber (NDF), ether extract (EE), nonfiber carbohydrate (NFC), and lignin were measured by using near-infrared reflectance spectroscopy (NIRS 6500, Foss, Denmark). Milk production was recorded at every milking, and milk fat, protein, and lactose concentrations by infrared spectroscopy (MilkoScan FT2, Foss Electric, Denmark), and body weight (BW) were recorded weekly.

Calculation of energy balance per day was performed according to the following equation, where net energy for lactation is presented as NE_L, megacalorie as Mcal, and day as d:

$$NE_L \text{ (Mcal / d) balance} = \text{energy intake (Mcal of } NE_L \text{ / d)}$$
$$- [\text{maintenance requirement (Mcal of } NE_L \text{ / d)}$$
$$+ \text{lactation requirement (Mcal of } NE_L \text{ / d)} + \text{growth requirement (Mcal of } NE_L \text{ / d)}],$$

where

$$\text{energy intake (Mcal / d)} = (0.0245 \times \text{total digestible nutrients (TDN) (\%)} - 0.12)$$
$$\times \text{ dry matter intake (kg / d)},$$
$$\text{maintenance requirement (Mcal)} = \text{metabolic BW (kg}^{0.75}) \times 0.08 \text{ (Mcal / kg}^{0.75} \cdot \text{d)},$$
$$\text{lactation requirement (Mcal)} = [0.0929 \times \text{fat (\%)} + 0.0547 \times \text{crude protein (\%)}$$
$$+ 0.0395 \times \text{lactose (\%)}] \times \text{milk production (kg)}.$$

Growth requirement was considered in the calculation regarding first and second parity;

$$\text{growth requirement (Mcal)} = 0.0635 \times \text{equivalent empty BW (EQEBW)}^{0.75}$$
$$\times \text{ equivalent empty body tissue gain (EQEBG)}^{1.097},$$

where

$$\text{EQEBW} = 0.891 \times \text{equivalent shrunk BW (EQSBW)},$$

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

$$\text{mature SBW} = 0.96 \times \text{mature weight (MW)},$$

$$EQEBG = 0.956 \times \text{weight gain (WG)}.$$

MW was defined as 700 kg, and WG was defined as 0.2 kg for first parity and 0.1 kg for second parity. Daily energy balance between 1 and 21 days before OPU were calculated and used for the analysis.

SUPPLEMENTAL TABLES

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

Values are presented as the mean ± SD. Values were compared using nonparametric Kruskal-Wallis test with Steel-Dwass *post hoc* test between lactation groups.

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

[2]Mean values during 21 days before OPU

[3]BW and milk components were measured weekly.

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

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

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

[1]Mean values were calculated from 83 samples.

[2]Mean values were calculated from 10 samples.

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

$$\text{TDN} = \text{digestible CP} + 2.25 \times \text{digestible EE} + \text{digestible NFC} + \text{digestible NDF}$$

Each component was calculated using the digestibility parameters as indicated in Appendix A of Nishiura et al. Anim Sci J 93(1): e13757, 2022.

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.

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68 SUPPLEMENTAL FIGURES

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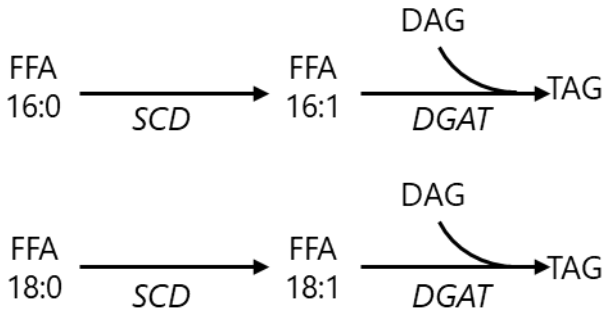

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78 Fig. S1. Diagram of FFA desaturation reactions towards TAG synthesis in cells. FFA 16:1
79 and 18:1 are produced from FFA 16:0 and 18:0, respectively, by desaturation reactions
80 which *SCD* catalyzes, and are subsequently utilized for TAG synthesis (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

84

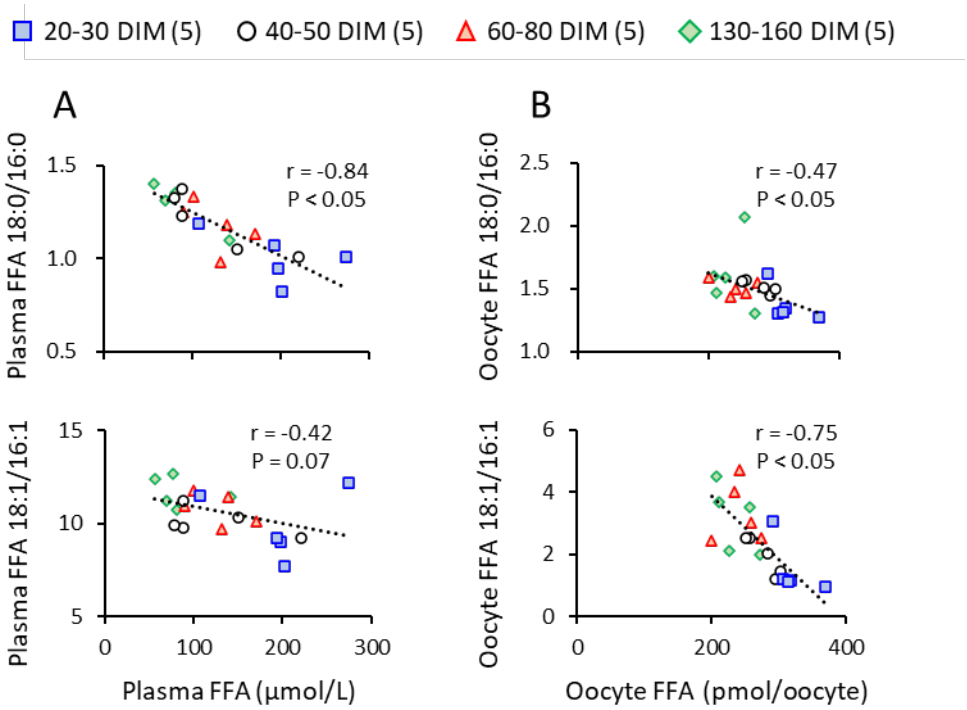

Fig. S2. Elongase markers of fatty acid 18:0/16:0 ratio and 18:1/16:1 ratio in plasma and oocytes. Plasma FFA concentrations and elongase markers (A), and oocyte FFA contents and elongase markers (B). Blood sampling and oocyte collection by OPU were performed in a group of 5 cows at 4 different lactation 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.