Title: Postpartum cows showed high oocyte triacylglycerols concurrently with high plasma free fatty acids

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Abstract

Impaired oocyte quality is one of the main causes of low fertility in modern high-yielding dairy cows. One of the potential factors of the impaired oocyte quality is the effects of free fatty acids (FFA). In fact, high FFA supplementation to culture media exacerbated oocyte developmental competence *in vitro*. Meanwhile, artificially induced high blood FFA levels in heifers did not affect the lipid composition of oocytes *in vivo*; however, the oocyte lipid profile of postpartum cows has not yet been investigated. Therefore, the profile of lipids involved in energy metabolism, including FFA and triacylglycerols (TAG), and their relationship between plasma and oocytes were compared among cows at different lactation stages. Heifers were used as a control group that was not affected by lactation. Plasma and oocytes were collected from heifers (n = 4) and 14 Holstein cows categorized to the early lactation stage: 25–47 days in milk (DIM) (n = 6), peak lactation stage: 61–65 DIM (n = 4), and middle lactation stage: 160–202 DIM (n = 4). The FFA and TAG profiles of plasma and oocytes were examined by liquid chromatography mass spectrometry. Plasma FFA positively correlated with oocyte TAG ($P < 0.05$). Plasma FFA and oocyte TAG were significantly higher in cows in the early lactation stage than in heifers ($P < 0.05$), while the peak and middle lactation stage groups had intermediate levels. The proportion of oleic acid in plasma increased concurrently with elevations in total FFA, while the compositions of oocyte FFA and TAG fatty acyls were constant regardless of plasma FFA concentration or oocyte TAG content. The present results suggest that high postpartum

plasma FFA concentrations affect the quantity of oocyte TAG. Taken together with the adverse effects of high FFA concentrations on oocyte developmental competence *in vitro*, oocyte quality in postpartum cows may be impaired due to high circulating FFA concentrations. These results provide a more detailed understanding of the effects of postpartum high circulating FFA concentrations on the low fertility of cows.

Highlights

- Plasma FFA positively correlated with oocyte TAG in dairy cattle.
- The oocyte TAG content was higher in postpartum dairy cows than in heifers.
- The oocyte FFA quantity and composition were stable regardless of lactation stages.
- The fatty acyl composition of oocyte TAG was stable regardless of lactation stages.

Keywords

Dairy cow, Fatty acyls, Free fatty acid, Oocyte, Postpartum, Triacylglycerol

1. Introduction

Fertility in the modern dairy cow is becoming lower with increasing milk yields [1, 2]. One of the major factors contributing to compromised fertility in dairy cows is the malnourished status accompanying high milk production [3, 4]. Oocyte quality deteriorates due to postpartum nutritional depression [5], with impairments potentially being one of the main causes of low fertility in high-producing cows [6]. Previous studies reported no significant differences in oocyte morphology [7] or developmental competence to the blastocyst stage [8, 9] between different lactation stages, while another showed impaired oocyte morphology at approximately 100 days in milk (DIM) than at 30 DIM [10]. These findings suggest that differences in the oocyte quality of cows between different lactation stages may not be readily detectable by morphology and developmental competence to blastocysts. However, developmental competence beyond blastocysts may differ between morphologically normal blastocysts [11]. A previous study reported that these blastocysts exhibited differences at the molecular level [11]. Therefore, an investigation of oocytes at the molecular level, [12] such as proteins [13], lipids [14], and gene expression [12, 15], is warranted.

Lipids contribute to oocyte quality from multiple aspects [16-18]. Free fatty acids (FFA) are one of the potential factors adversely affecting oocyte quality [19], and triacylglycerols (TAG) are a harmless storage form of lipids synthesized from FFA [20]. Blood FFA concentrations rapidly increase from the base level (< 0.3 mM) to 0.8 – 1.2 mM at the time of parturition, concurrent with a depression

74 in the energy balance, and this is followed by a decrease and eventual return to the base level 6 weeks
75 after parturition [21]. Leroy et al. [22] indicated that the postpartum increase in blood FFA levels
76 reflected elevated follicular fluid FFA levels. Cows at 16 DIM showed a 3-fold higher blood FFA
77 concentration and 1.5-fold higher follicular fluid FFA concentration than those at 44 DIM [22]. In
78 contrast, limited information is currently available on FFA concentrations and TAG contents in the
79 oocytes of postpartum cows. A previous study examined the effects of a short-term exposure to high
80 FFA concentrations on oocyte TAG contents [23]. This study used heifers fasted for 4 days as a high
81 blood FFA model and demonstrated that TAG contents in oocytes did not increase despite high FFA
82 concentrations in blood and follicular fluid [23]. However, the effects of high plasma FFA on oocytes
83 for longer than several weeks remain unclear.
84 The final aim of the study was to examine the relationship between blood and oocyte energy
85 metabolism-related lipid profile in order to clarify whether and how high blood FFA concentrations
86 affect oocytes. Herein, the present study preliminarily confirmed blood and oocyte lipid profile of
87 cows in different lactation stages with different energy metabolic status and blood FFA levels.
88
89 **2. Materials and methods**
90 **2.1. Animals**
91 The present study was implemented according to the animal experimental regulations of the

92 Hokkaido University Animal Care and Use Committee (Approval No.: 18-0028). Holstein cows and
93 heifers were kept at the experimental farm of Hokkaido University (Sapporo, Japan). Eleven
94 multiparous and 3 primiparous non-pregnant lactating cows (26–85 months of age, 1–5 parities) and
95 4 non-pregnant heifers with normal ovarian cyclicity (22–31 months of age) were used, and the study
96 was conducted between June and August 2018. Cows were pastured all day and fed supplementary
97 corn silage or housed in the barn all day and fed corn silage, hay, and concentrated feed. Heifers were
98 kept in a free barn attached to a paddock and fed hay and wheat bran. Cows were milked twice daily
99 (9:00 and 15:30), and the mean 305-day milk yield of these cows was 7,710 kg.

100 To examine differences in the lipid compositions of plasma and oocytes between cows in
101 different lactation stages, cows were categorized into the following three lactation groups: the early
102 lactation group (38.7 ± 8.2, ranging between 25 and 47 DIM, n = 6); the period with a negative energy
103 balance and high plasma FFA [4, 24], peak lactation group (62.5 ± 1.5, ranging between 61 and 65
104 DIM, n = 4); the period with a recovering energy balance and normal plasma FFA, and middle lactation
105 group (175.8 ± 17.1, ranging between 160 and 202 DIM, n = 4); the period with a positive energy
106 balance and normal plasma FFA [9, 25] (Table 1). Each lactation group included 1 primiparous cow.
107 Mean daily milk yield for 7 days between 3 days before and after blood and oocyte collection is shown
108 in Table 1. Body condition scores (5-point scale) [26] were assessed by the same single assessor at the
109 time of blood and oocyte collection (Table 1).

110

111 **2.2. Sample collection**

112 Oocyte collection was performed using the ovum pick up method [27, 28] by a single
113 operator. The ovary was depicted using an ultrasound imaging device (HS-2100; Honda Electronics,
114 Toyohashi, Japan) equipped with a 9.0 MHz long-handled micro-convex probe (HCV-4710MV;
115 Honda Electronics) designed for ovum pick up. Cumulus-oocyte complexes (COCs) with follicular
116 fluid were aspirated with the vacuum pressure of 100 mmHg using a single-lumen needle (17 gauge,
117 length of 490 mm; Misawa Medical, Kasama, Japan) from follicles of 2 mm or greater in diameter.
118 The single-lumen needle was connected to a 50-mL plastic conical tube (352070, Corning Inc.,
119 Corning, NY, USA) with a silicone tube, and the conical tube was joined to a vacuum pump with a
120 foot-pedal switch (K-MAR-5000, Cook Medical Technology, Bloomington, IN, USA). Follicular fluid
121 containing COCs was diluted with Dulbecco's phosphate-buffered saline (D-PBS) (Nissui
122 Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA) (Sigma-
123 Aldrich, St. Louis, MO, USA) and 10 IU/mL heparin sodium (AY Pharmaceuticals Co., Ltd., Tokyo,
124 Japan) to avoid blood coagulation. COCs were recovered under a stereomicroscope, and cumulus cells
125 were removed by gentle pipetting with a fine glass pipette. Between 4 and 6 denuded oocytes were
126 transferred to a 1.5-mL microcentrifuge tube (Eppendorf AG, Hamburg, Germany) with a small
127 amount of D-PBS + 0.1% PVA (<10 μL). Oocyte samples were stored at -80 ℃ until the lipidomic

analysis.

Blood was collected by caudal venipuncture using ethylenediaminetetraacetic acid–loaded vacuum tubes (Terumo Co., Tokyo, Japan) at oocyte sampling and stored on ice. Plasma was separated by centrifugation within 4 h of collection and 100 μL of plasma was transferred to a 1.5-mL microcentrifuge tube and stored at -80 ℃ until the lipidomic analysis.

2.3 Lipidomic analysis by liquid chromatography mass spectrometry (LC/MS)

The solvents for lipid extraction and the LC/MS analysis were of spectral grade and purchased from Sigma-Aldrich unless otherwise specified. Authentic lipid compounds as internal standards (IS) were obtained from Sigma-Aldrich. The mixture of IS for oocyte and plasma samples was newly prepared with methanol (containing 0.006% butylated hydroxytoluene, w/v). The details of these species and concentrations are listed in Table S1.1.

Total lipid extraction from oocytes were performed according to Folch's method [29] as previously described [30]. Briefly, 4-6 oocytes in one Eppendorf® tube were extracted with 600 μL of ice-cold chloroform/methanol 2:1 (v/v, with IS) twice. Plasma lipids were prepared using the method of Bligh and Dyer [31] with some modifications [32]. In brief, a 100-μL plasma sample was extracted with 800 μL of ice-cold chloroform/methanol 1:1 (v/v, with IS) twice. Extracted lipids were dried under a vacuum, dissolved in methanol, and filtered to remove any insoluble material prior to

146 the LC/MS injection. To avoid lipid degradation and auto-oxidation, the extraction procedure was
147 performed within 1 h.

148 LC/MS conditions were described in our previous study [30], with Prominence HPLC
149 (Shimadzu Corp., Kyoto, Japan) coupled to an LTQ Orbitrap mass spectrometer (Thermo-Fisher
150 Scientific Inc., San Jose, CA, USA) being utilized. An Atlantic T3 C18 column (2.1 × 150 mm, 3 μm,
151 Waters, Milford, MA, USA) was equipped, and the mobile phase consisted of water (with 5 mM
152 ammonium acetate), isopropanol, and methanol with gradient elution. MS data acquisition was
153 performed under electrospray ionization positive and negative modes. Details on hardware parameters
154 and LC/MS settings are shown in Supplementary Material 2. Spectrum processing was performed
155 using the workstation Xcalibur 2.2 (Thermo-Fisher Scientific Inc.) with comparisons to the
156 LIPIDMAPS database [33]. The species identified were annotated as "lipid class + total carbon
157 number (CN) in the fatty chain(s) + total double bond number (DB) in the fatty chain(s)" (*e.g.*, FFA
158 14:0 and TAG 46:1) [30, 34]. TAG fatty acyl compositions were elucidated using MS/MS
159 fragmentation as previously described [30]. The semi-quantitative amount of each lipid analyte was
160 calibrated by its corresponding internal standard as follows, and calculated data were exported for
161 further analyses.

162 $$Analyte\ amount = IS\ amount \times \frac{Analyte\ peak\ area}{IS\ peak\ area}$$

163 **2.4. Data analysis**

In the multivariate statistical analysis, a principal component analysis (PCA) and cluster analysis were processed using R software 4.0.2 (https://www.r-project.org) and R-package mixOmics [35]. Since some lipid species are of physiological significance despite their small contents (*e.g.*, polyunsaturated fatty acids), each variance was normalized to have a unit mean value and variance before the PCA and cluster analysis by mixOmics. The Euclidean distance was used for distance measurements, and the complete linkage method was employed for the clustering method in the cluster analysis. Other statistical analyses were performed using JMP Pro 14.3.0 (SAS Institute, Cary, NC, USA). Mean values were compared using the paired *t*-test between two groups or a one-way ANOVA (using Tukey's *post hoc* test) in multiple groups. The relationship between two parameters was analyzed using Pearson's correlation coefficient, and P-values were calculated by a regression analysis. All data are shown as means ± SD, and $P < 0.05$ was considered to be significant.

3. Results

3.1. LC/MS analysis

In the present study, we detected and annotated 6 FFA and 45 TAG species from plasma and/or oocyte samples (listed in Table S1. 2) according to their retention behavior on reversed-phase HPLC, as well as their protonated, ammoniated, or deprotonated ion signals on high-resolution MS. The lipid profile obtained by high-resolution LC/MS and MS/MS provided the identities of lipid

182 species and their fatty acyl chains. Separated chromatographic peaks along with IS enabled the semi-
183 quantitation of lipids for further multivariable analyses.
184
185 **3.2. Lipid profiles of plasma and oocytes in different lactation groups**
186 **3.2.1. Positive correlation between plasma FFA and oocyte TAG**
187 To investigate the relationship between total plasma FFA and total oocyte TAG, we
188 performed a correlation analysis, and the results obtained revealed a positive correlation between
189 plasma FFA and oocyte TAG ($r = 0.55$, $P < 0.05$) (Fig. 1). Plasma FFA and oocyte TAG were higher
190 in all cows in the early lactation group than in heifers (Fig. 1). Plasma FFA and plasma TAG as well
191 as plasma TAG and oocyte TAG showed negative correlations ($r=-0.62$ and $r=-0.72$, $P<0.05$,
192 respectively) when an outlier which showed high plasma TAG (Early lactation (1) in Fig. 2AC) was
193 excluded.
194
195 **3.2.2. Characteristic clustering of FFA and TAG species in relation to lactation groups**
196 To examine differences in lipid profiles between the lactation groups, we performed PCA
197 with total FFA and TAG species in plasma and oocytes. Lactating cows and heifers were located in
198 separate areas in the plasma and oocyte PCA, respectively, which indicated different lipid profiles
199 between cows and heifers (Fig. 2AB). A hierarchical clustering analysis was performed to investigate

differences in lipid profiles between the lactation groups (Fig. 2CD). Lipid species were categorized into 5 groups based on clustered lipid profiles in relation to different lactation groups: (1) saturated FFA (DB = 0); (2) unsaturated FFA (DB = 1 or 2); (3) 44–48 carbon–TAG (CN ranging between 44 and 48); (4) 50–54 carbon–TAG; and (5) 56–58 carbon–TAG (Fig. 2CD). In the cluster analysis of plasma, plasma FFA were higher in the early and peak lactation groups than in the middle lactation group and heifers (Fig. 2C). The cluster analysis of oocytes revealed that heifers showed the lowest contents of all lipids, except for saturated FFA (Fig. 2D). Among lactating cows, 50–54 carbon–TAG and 56–58 carbon–TAG were high in clusters including the early lactation group (*i.e.*, Early lactation (1)(4)(6) and (2)(3)(5), respectively, as indicated on the right side of the heat map, shown in Fig. 2D), and 44–48 carbon–TAG was high in the cluster including the peak lactation group (*i.e.*, Peak lactation (1)(3)(4)) (Fig. 2D). These results indicated that differences in lactation stages and experience of delivery were reflected in the composition of FFA and TAG species in plasma and oocytes.

3.2.3. Plasma FFA and oocyte TAG were higher in the early lactation group than in heifers

FFA and TAG levels were compared between lactation groups (Fig. 3). In plasma, saturated and unsaturated FFA as well as total FFA were higher in the early lactation group than in the middle lactation group and heifers ($P < 0.05$) (Fig. 3A). In oocytes, 50–54 carbon–TAG and total TAG were higher in the early lactation group than in heifers ($P < 0.05$) (Fig. 3D). However, oocyte FFA were

similar between lactation groups (Fig. 3B). In terms of the composition of these lipids, the proportion of plasma unsaturated FFA was higher in the early and the peak lactation groups (49.6% ± 6.3% and 49.9% ± 5.8%, respectively) than in the middle lactation group and heifers (32.6% ± 4.4% and 34.1% ± 5.4%, respectively) ($P < 0.05$) (Fig. 4A). In oocytes, 50–54 carbon–TAG accounted for approximately 70% of total TAG, which was similar between lactation groups (Fig. 4D).

3.2.4. Oocyte FFA and TAG fatty chain compositions were stable during lactation

We examined differences in the fatty chain compositions of FFA and TAG between lactation groups. All FFA in plasma were significantly higher in the early lactation group than in the middle lactation group and heifers ($P < 0.05$) (Table S3. 1). Oocyte fatty acyls 16:0, 16:1, 18:0, 18:1, 18:2, and 20:3 in TAG were significantly higher in the early lactation group than in heifers ($P < 0.05$) (Table S3. 2). Fatty chain compositions were compared between lactation groups. Among plasma FFA, the early and peak lactation groups showed a significantly lower proportion of stearic acid (FFA 18:0) and higher proportion of oleic acid (FFA 18:1) than the middle lactation group and heifers ($P < 0.05$) (Fig. 5). However, only slight differences were observed in oocyte FFA and TAG fatty acyls between lactation groups (Fig. 5). The dominant fatty chains differed between plasma FFA (18:0, 18:1, and 16:0), oocyte FFA (18:0 and 16:0), and oocyte TAG (16:0, 18:1, and 16:1) (Fig. 5).

4. Discussion

This is the first study to describe the lipid profiles of plasma and oocytes from cows at different lactation stages. Total oocyte TAG positively correlated with total plasma FFA. When FFA and TAG levels were compared between lactation groups, the early lactation group showed higher plasma FFA and oocyte TAG than heifers. FFA are substrates for energy production and important components for biosynthesizing other lipids, including TAG and phospholipids, in cells [36, 37]. A previous study that used fasted heifers as a high blood FFA model indicated that when blood FFA levels were high, FFA levels in follicular fluid and TAG contents in cumulus cells increased, whereas TAG in oocytes did not [23]. These findings suggested that the effects of elevated blood FFA were buffered at cumulus cells; therefore, oocytes were protected against the effects of high FFA [14, 23]. However, the present study suggested that high blood FFA affected and altered the quantity of oocyte TAG. Differences in the present results and previous findings [23] may be attributed to the different durations of the high blood FFA exposure period before oocyte sampling. Heifers were subjected to 4 days of fasting in the previous study [23], while cows in the early lactation stage in the present study were under high blood FFA conditions for more than 3–6 weeks between the time of peripartum and oocyte collection. Therefore, it may take between 4 days and 3 weeks for high plasma FFA levels to affect oocyte TAG contents. After the period from 4 days to 3 weeks under circulating high FFA condition, TAG contents in cumulus cells may exceed the capacity of storage in these cells,

accordingly incorporated FFA in cumulus cells do not convert to TAG but overflow, and as the result, FFA may be directly or indirectly transferred from cumulus cells to oocytes via transporters such as transzonal projections [38] and/or CD36 [39]. Meanwhile, plasma and oocyte TAG showed a negative correlation, probably not because plasma TAG directly affected oocyte TAG, but because plasma TAG correlated negatively with plasma FFA which were plausible to directly influence the increase of oocyte TAG content in the postpartum period. A detailed analysis of the relationship between blood FFA profiles after parturition and oocyte lipid compositions is needed.

Although the proportion of oleic acid to total FFA in plasma was higher in the early lactation stage than in heifers, the FFA profile and fatty acyl composition of TAG in oocytes were similar between these lactation groups. Previous studies indicated that the most abundant FFA species in follicular fluid was oleic acid when the dominant blood FFA species was stearic acid [22, 23]. One possible explanation for this phenomenon is the high activity of stearoyl-CoA desaturase (SCD) at granulosa and cumulus cells [40]. SCD is an enzyme that converts saturated fatty acids (*e.g.*, 16:0 and 18:0) to monounsaturated fatty acids (*e.g.*, 16:1 and 18:1, respectively). These findings suggest that the effects of the blood FFA composition was buffered at the level of cumulus cells after blood FFA were incorporated into follicles, and support the present results showing that oocyte FFA profiles and TAG fatty acyl compositions were stable. On the other hand, oocytes showed a higher palmitic acid proportion than plasma. The reason for this phenomenon may be the fatty acid preference of binding

proteins [41] or the balance between the elongation and decomposition [42] of oocyte FFA. The amounts of fatty acyls of palmitic (16:0), stearic (18:0), and oleic (18:1) acids in oocyte TAG increased in cows in the early lactation stage compared to heifers, while the ratios of these fatty acyls in oocyte TAG were similar between lactation groups. Although it is known that oleic acids can compensate the adverse effects of palmitic and stearic acids on oocyte developmental competence *in vitro* [43], it is not clear whether the ratio or the absolute amounts of these fatty acids are more crucial to oocyte quality in oocytes in living cows. The present result suggested either possible change of oocyte quality; one possible change was that the increase of palmitic and stearic acids adversely affected oocyte quality, and the other possible change was that the increase of oleic acid by as the same ratio as palmitic and stearic acids sufficiently contributed to protecting oocytes from the adverse effects of saturated fatty acids.

Cows in the early lactation stage showed high plasma FFA and oocyte TAG, which suggested an increase in the conversion of FFA to TAG in COCs. Since FFA lipotoxicity induces harmful effects, such as endoplasmic reticulum stress [44], mitochondrial dysfunction [45], and apoptosis [46], in various non-adipocyte cells [14, 45, 47, 48], FFA are converted to TAG, which are a harmless form of lipids [40, 49]. TAG may be actively synthesized from FFA to avoid lipotoxicity in cows in the early lactation group; therefore, oocyte FFA in the early lactation group remained at similar levels to other lactation stages and heifers. Meanwhile, the low ratios of FFA 16:1 / 16:0 (0.051 ± 0.037, n=18) and

18:1 / 18:0 (0.072 ± 0.043, n=18) in oocytes regardless of lactation groups suggested that the capacity of oocytes to synthesize TAG from FFA was inherently low compared to other tissues. FFA 16:1 / 16:0 and 18:1 / 18:0 are the product / substance ratios of the reactions SCD catalyzes, and these reactions are key steps for TAG synthesis [50]. Therefore, FFA 16:1 / 16:0 and 18:1 / 18:0 are the markers of the capacity of cells to synthesize TAG from FFA [50, 51]. As indicated in the high FFA 18:1 / 18:0 ratio (~4) in murine liver tissue [52], SCD activity varies between different tissues and high in such as adipocytes and hepatocytes [53]. Furthermore, a previous study indicated the lower ratios of 16:1 / 16:0 and 18:1 / 18:0 of TAG and diacylglycerols in oocytes than cumulus cells [23, 54]. The low ratios of FFA 16:1 / 16:0 and 18:1 / 18:0 in oocytes suggested the low capacity of oocytes to avoid lipotoxicity by converting FFA to TAG. In previous studies using bovine COCs, the developmental competence of oocytes decreased when they were cultured with a supplemental high FFA mixture (palmitic, stearic, and oleic acid) [55], suggesting the adverse effects of lipotoxicity on oocyte quality. Additionally, in metabolism disorder model mice fed a high lipid diet, an increase in oocyte TAG, the up-regulation of lipotoxicity biomarkers (*e.g.*, endoplasmic reticulum stress marker genes) in COCs, and a reduction in the fertilization rate have been reported [56]. A previous report found that embryos collected from lactating cows showed the darker cytoplasm, which indicated higher TAG contents in these embryos, and the lower developmental competence than those of heifers [57]. This finding suggested the relevance of lactation, TAG accumulation in oocytes, and the low quality of oocytes,

and accordingly supported the potential low quality of oocytes with high TAG content in the postpartum lactating cows in the present study. The accumulated TAG in oocytes in postpartum cows are plausible to be utilized as the stored energy for oocyte and embryo development [58, 59]; however, once the incorporation of FFA into cumulus cells/oocytes exceeds their metabolizing ability, oocyte quality will be deteriorated by their lipotoxicity. The investigation of the expression of lipotoxicity was beyond the scope of this paper, accordingly it is our future problem to examine lipotoxicity in oocytes in living lactating cows by assessing endoplasmic reticulum stress marker [60] or ceramide [14].

The present study utilized 11 multiparous and 3 primiparous cows together. Although it was reported primi- and multiparous cows in the early lactation stage showed different blood FFA levels (0.3–0.4 and 0.4–0.5 mmol/L, respectively) [61-64], the pattern of blood FFA levels between the different lactation stages was similar between primi- and multiparity according to previous reports. Namely, blood FFA level was higher in the early lactation stage (0.3–0.5 mmol/L) [61-64] compared to the peak and the middle lactation stage (0.1–0.3 mmol/L) [62, 63, 65], and blood FFA level in the peak and the middle lactation stage was similar to or higher than that of heifers (~0.1 mmol/L) [66, 67]. Therefore, it was considered that the inclusion of primiparous cows in addition to multiparous cows did not compromise the objective of the present study. Ovum pick up was performed at random stages of ovarian cycles in the present study. However, quality of oocytes may differ depending on the

phase of a follicular wave, specifically being better in the early than the late phase [68, 69]. Therefore, quality of collected oocytes was plausible to vary to the similar degree in all lactation groups. In order to make oocyte quality as even as possible, oocytes need to be collected at the same stage of a follicular wave by using hormonal synchronization, follicular ablation, or monitoring ovarian cycles in future studies.

In conclusion, the present results indicated a positive correlation between plasma FFA and oocyte TAG, and postpartum cows with high plasma FFA had higher oocyte TAG than heifers. The accumulation of TAG in oocytes during the postpartum period may have adverse effects of lipotoxicity on oocyte quality and the negative effects may surpass the beneficial effects of the increased energy storage in oocytes. The present study provides insights to increase the fertility of high-yielding cows by improving oocyte quality through feeding and housing management for appropriate circulating FFA.

Abbreviations

CN, carbon number; COCs; cumulus–oocyte complexes; DB, double bond number; DIM, days in milk; D-PBS, Dulbecco's phosphate buffered saline; FFA, free fatty acids; IS, internal standard; LC/MS, liquid chromatography–mass spectrometry; MS/MS, tandem MS; PCA, principal component analysis; PVA, polyvinyl alcohol; SCD, stearoyl-CoA desaturase; TAG, triacylglycerols.

Disclosure

The authors declare no conflict of interests.

Author contributions

Eri Furukawa: Methodology, Software, Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Zhen Chen: Methodology, Software, Investigation, Data Curation, Writing - Original Draft, Visualization. Hiroki Ueshiba: Methodology, Investigation, Visualization. Yue Wu: Methodology, Validation, Investigation, Visualization. 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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Table 1. Days in milk, daily milk production, and body condition scores of animals in different lactation groups.

Item	Early lactation, n=6	Peak lactation, n=4	Middle lactation, n=4	Heifer, n=3[†]
Days in milk	38.7 ± 8.2 (25 – 47)	62.5 ± 1.5 (61 – 65)	175.8 ± 17.1 (160 – 202)	–
Milk production (kg/day)	30.6 ± 6.3 (17.0 – 36.5)	33.1 ± 7.1 (24.5 – 41.9)	19.4 ± 1.1 (18.2 – 20.6)	–
Body condition score	2.6 ± 0.2 (2.5 – 3)	2.5 ± 0.4 (2 – 3)	2.8 ± 0.1 (2.75 – 3)	3.3 ± 0.0 (3.25 – 3.25)

Values are presented as the mean ± SD. Values in parentheses indicate minimum and maximum values.

Milk production (kg/day) was mean daily milk production for 7 days between 3 days before and after blood and oocyte sampling.

[†]The body condition score of one heifer was missing.

Figure legends

Fig. 1. Relationship between plasma FFA and oocyte TAG ($r = 0.55$, $P < 0.05$).

Fig. 2. Score plot in a principal component analysis and heat map of FFA and TAG lipid species in heifers and cows at different lactation stages.

(A) Score plot of plasma, (B) score plot of oocytes, (C) heat map of plasma, and (D) heat map of oocytes.

A principal component analysis was performed after the quantitative value was normalized.

Quantitative data were normalized before the heat map clustering procedure. The red color indicates a high value, while the blue color indicates a low value. Cows are indicated with the lactation stage [XX lac.] + the individual number [(X)]. The lactation groups to which cows belong are indicated on the left side of the column with different colors, and the lipid groups to which the lipid species belong are indicated on the top of the rows with different colors.

Saturated FFA: no DB in the fatty acyl chain, unsaturated FFA: 1-2 DB(s) in the fatty acyl chain, 44–48 carbon–TAG: a total of 44–48 carbons in the fatty acyl chains, 50–54 carbon–TAG: a total of 50–54 carbons in the fatty acyl chains, 56–58 carbon–TAG: a total of 56–58 carbons in the fatty acyl chains.

Fig. 3. Quantity of FFA/TAG lipid groups categorized based on CNs and DBs in fatty acyl chains in different lactation groups; (A) FFA in plasma, (B) FFA in oocytes, (C) TAG in plasma, and (D) TAG in oocytes.

^{AB} Different letters indicate a significant difference in total FFA/TAG between lactation groups ($P < 0.05$).

^{abc} Different letters indicate a significant difference in lipid groups between lactation groups ($P < 0.05$).

Error bars indicate the SD of total FFA/TAG. The numbers in parentheses indicate the number of animals included in each lactation group.

Fig. 4. Composition of FFA/TAG lipid groups categorized based on CNs and DBs in fatty acyl chains to total FFA/TAG in different lactation groups.

(A) FFA in plasma, (B) FFA in oocytes, (C) TAG in plasma, and (D) TAG in oocytes.

^{ab} Different superscripts indicate a significantly different composition between lactation groups ($P < 0.05$).

* Asterisks indicate significantly different compositions between plasma and oocytes ($P < 0.05$).

Mean values were compared using the paired *t*-test between plasma and oocytes or a one-way ANOVA (using Tukey's *post hoc* test) between lactation groups. The numbers in parentheses indicate the

number of animals included in each lactation group.

Fig. 5. Composition of fatty chains to total FFA/TAG in plasma and oocytes in different lactation groups.

[ab] Different superscripts indicate significantly different compositions between lactation groups ($P < 0.05$).

* The asterisks indicate significantly different compositions between plasma and oocytes ($P < 0.05$). Results are shown as means ± SD (error bars). Mean values were compared using the paired *t*-test between plasma and oocytes or a one-way ANOVA (using Tukey's *post hoc* test) between lactation groups. The numbers in parentheses indicate the number of animals included in each lactation group.

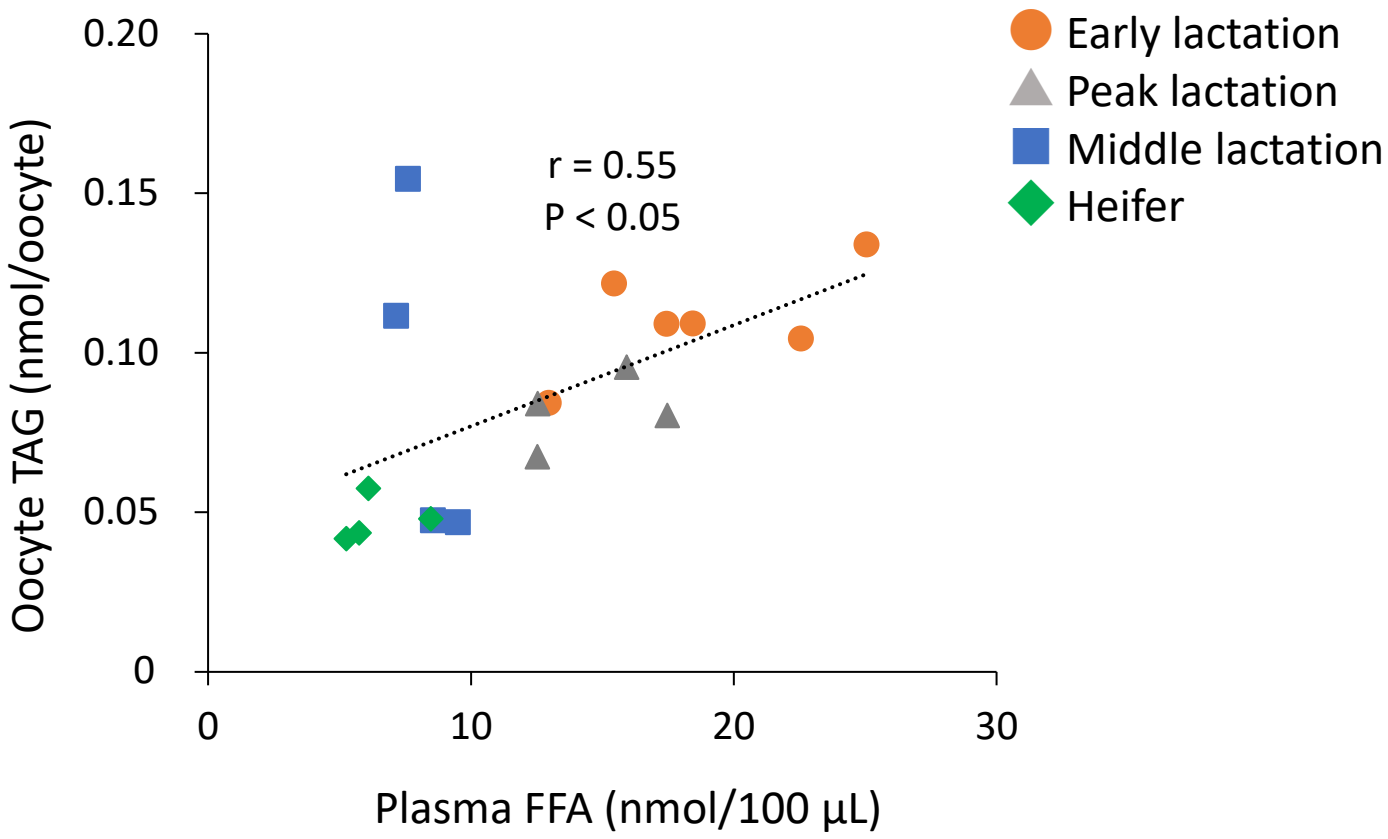
0.20
0.15
0.10
0.05
0
Oocyte TAG (nmol/oocyte)
r = 0.55
P < 0.05
0
10
20
30
Plasma FFA (nmol/100 µL)
Early lactation
Peak lactation
Middle lactation
Heifer

Fig. 1

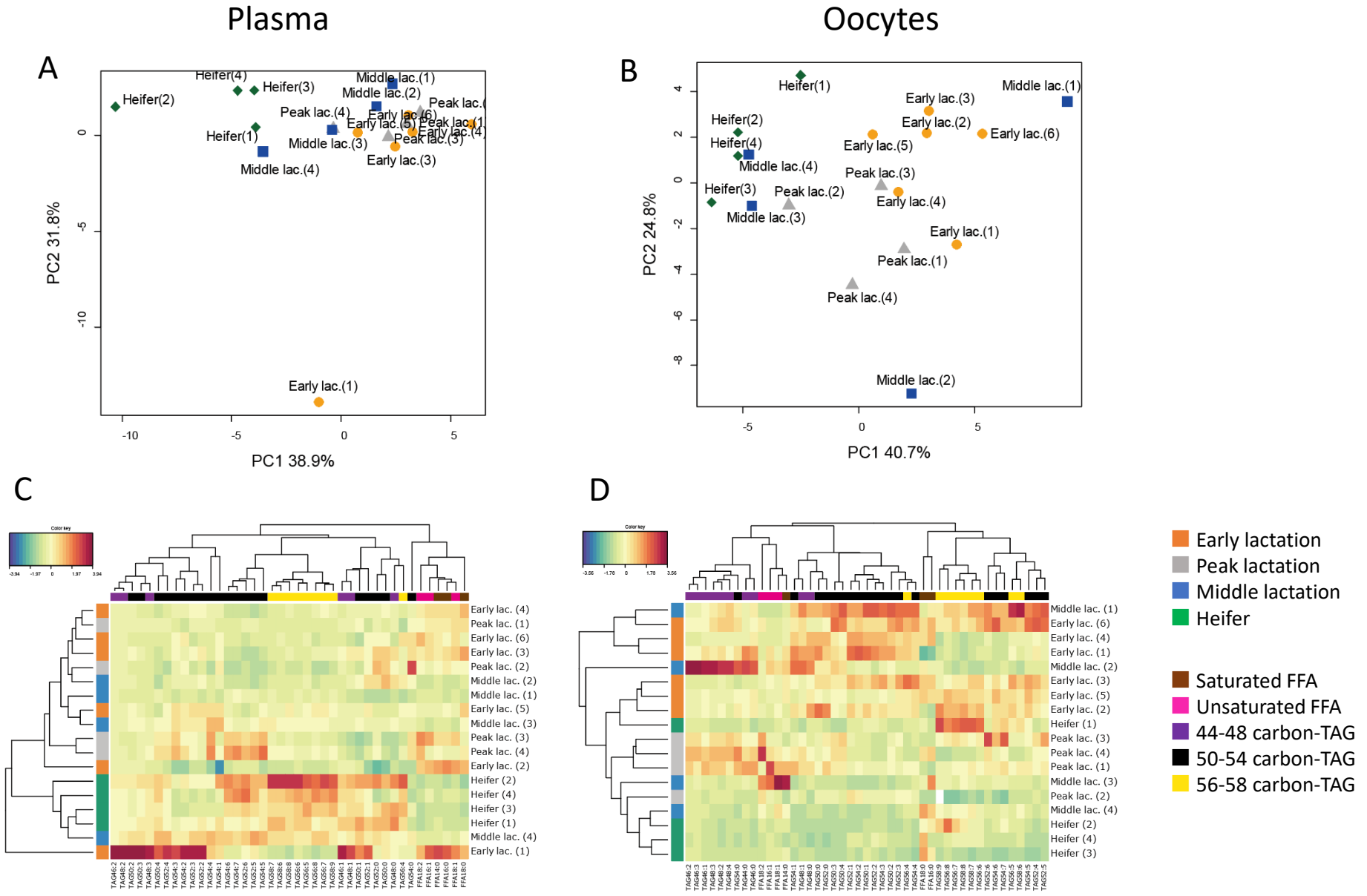
Plasma
Oocytes
A
B
C
D
PC1 38.9%
PC2 31.8%
PC1 40.7%
PC2 24.8%
Heifer(1)
Heifer(2)
Heifer(3)
Heifer(4)
Middle lac.(1)
Middle lac.(2)
Middle lac.(3)
Middle lac.(4)
Early lac.(1)
Early lac.(2)
Early lac.(3)
Early lac.(4)
Early lac.(5)
Early lac.(6)
Peak lac.(1)
Peak lac.(2)
Peak lac.(3)
Peak lac.(4)
Early lactation
Peak lactation
Middle lactation
Heifer
Saturated FFA
Unsaturated FFA
44-48 carbon-TAG
50-54 carbon-TAG
56-58 carbon-TAG

Fig. 2

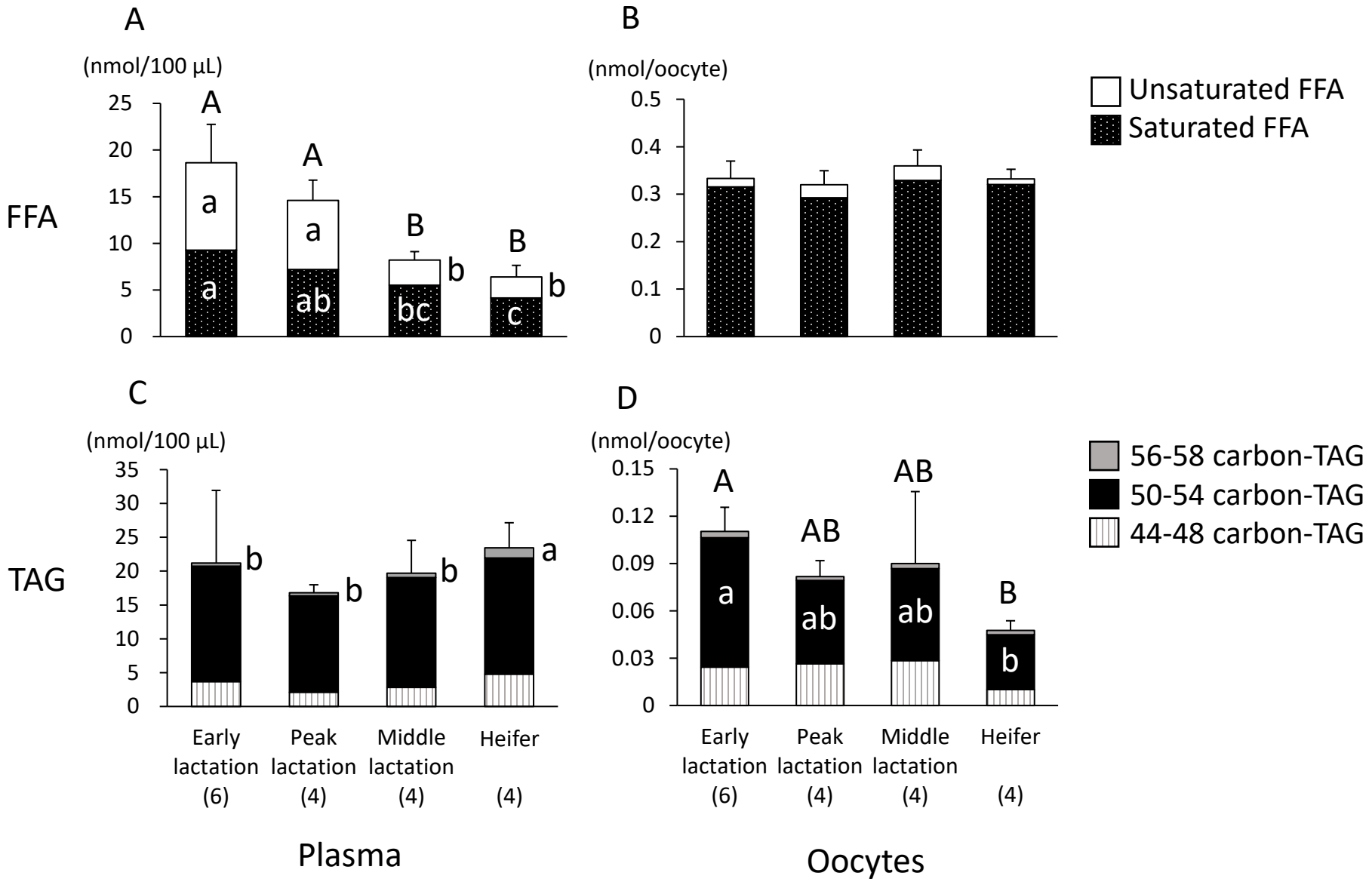
A
(nmol/100 μL)
25
20
15
10
5
0
A
A
B
B
a
a
a
ab
bc
c
b
b
B
(nmol/oocyte)
0.5
0.4
0.3
0.2
0.1
0
Unsaturated FFA
Saturated FFA
FFA
C
(nmol/100 μL)
35
30
25
20
15
10
5
0
b
b
b
a
D
(nmol/oocyte)
0.15
0.12
0.09
0.06
0.03
0
A
AB
AB
B
a
ab
ab
b
56-58 carbon-TAG
50-54 carbon-TAG
44-48 carbon-TAG
TAG
Early lactation (6)
Peak lactation (4)
Middle lactation (4)
Heifer (4)
Plasma
Oocytes

Fig. 3

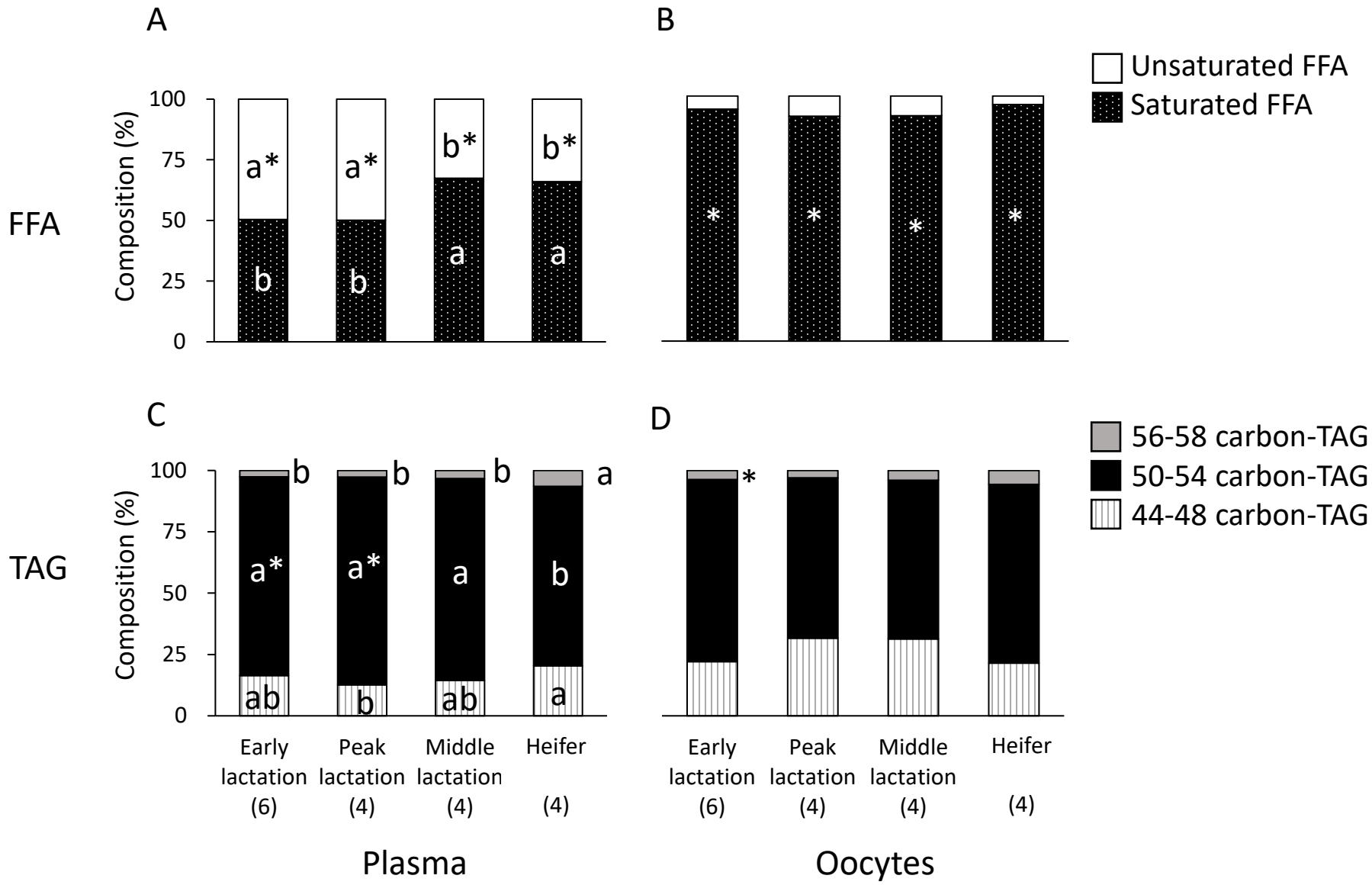
A
B
FFA
Composition (%)
100
75
50
25
0
a*
b
a*
b
b*
a
b*
a
*
*
*
*
Unsaturated FFA
Saturated FFA
C
D
TAG
Composition (%)
b
b
b
a
a*
a*
a
b
ab
b
ab
a
*
56-58 carbon-TAG
50-54 carbon-TAG
44-48 carbon-TAG
Early lactation (6)
Peak lactation (4)
Middle lactation (4)
Heifer (4)
Plasma
Oocytes

Fig. 4

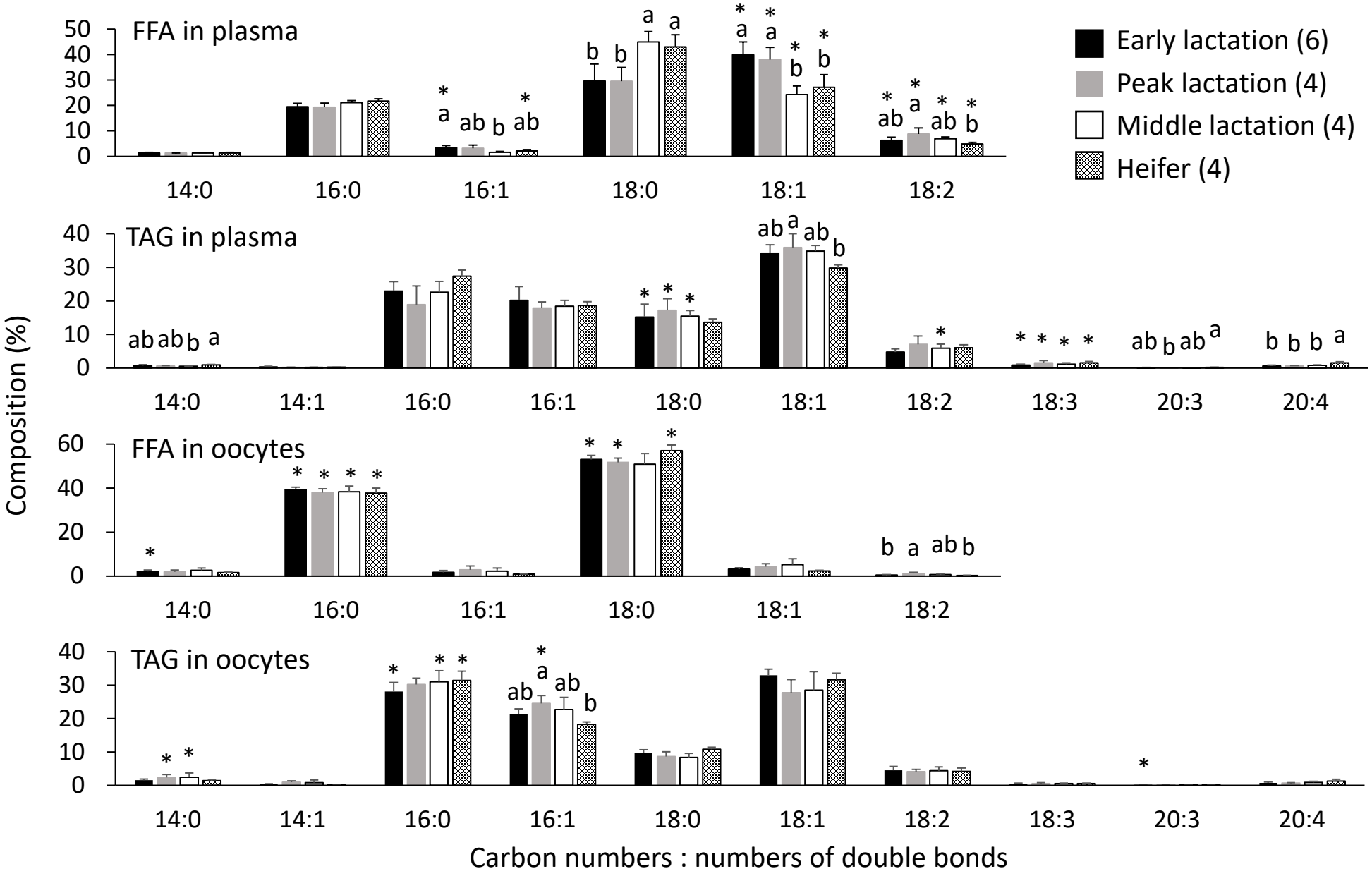
FFA in plasma
TAG in plasma
FFA in oocytes
TAG in oocytes
Early lactation (6)
Peak lactation (4)
Middle lactation (4)
Heifer (4)
Composition (%)
14:0
14:1
16:0
16:1
18:0
18:1
18:2
18:3
20:3
20:4
Carbon numbers : numbers of double bonds

Fig. 5