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# Liver fatty acid metabolism associations with reproductive performance of dairy cattle

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ABSTRACT

The peri-calving period is characterized by a negative energy balance, which leads to lipid mobilization. Thus, during this period, the liver has important functions related to optimizing milk yield, preventing metabolic and infectious diseases, and improving fertility. To clarify the relationship between liver fatty acid metabolism and reproductive performance, the present study was conducted to assess the abundance of specific hepatic proteins related to lipid metabolism in both plasma and follicular fluid in dairy cattle with different days to conception (DC). Sixteen animals were grouped according to DC, as more and fewer DC (MDC and FDC, respectively). Blood and liver biopsies were sampled 14 days before the expected calving date and 4, 14 and 28 days after calving. The plasma beta-hydroxybutyric acid (BHBA) concentrations and the liver triacylglycerol (TAG) content were greater in the MDC group ($P < 0.05$), whereas the protein abundance of carnitine palmitoyl transferase 1 was greater in the FDC group ($P < 0.05$). Additionally, total bilirubin (TBil) concentration was less in the FDC than MDC group on day 28 ($P < 0.05$). These results indicate lipid mobilization and liver fatty acid oxidation capacity in dairy cows could contribute to the adaptations and reproductive performance.

## 1. Introduction

The reproductive efficiency of dairy cattle is one of the main factors that affects the profitability of a dairy farm (Giordano et al., 2011). In lactating cows, however, an increased genetic capacity for milk production has been associated with lesser fertility (Butler, 2000). Furthermore, reproductive disorders have been associated with an increased negative energy balance (NEB) during the peri-calving period. Also, NEB is related with lesser dry matter intake (DMI) and greater body condition score (BCS) at calving (Akbar et al., 2015). In this regard, non-esterified fatty acids (NEFA) and beta-hydroxybutyric acid (BHBA) concentrations have been used as indices of NEB (Ospina et al., 2010). The greater energy demand during the peri-calving period leads to an increase in the lipolysis of adipose tissue and consequently to an increase in the systemic concentration of NEFA. There have been several studies where there were associations of increased plasma NEFA concentrations with reduced oocyte developmental competence and granulosa cell

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viability in cattle (Kor and Moradi, 2013). In different species, results of several studies indicate there is an association between the concentration of metabolites in blood and follicular fluid (FF), which could ultimately compromise ovarian functionality (Leroy et al., 2004; Nandi et al., 2007; Abd Ellah et al., 2010; Albomohsen et al., 2011). The liver has important functions in the metabolic adaptation occurring during the peri-calving period (van Dorland et al., 2009). Within the liver, fatty acids can be partly oxidized to generate ATP, by both mitochondrial carnitine palmitoyl transferase 1 (CPT-1) and peroxisomal acyl-CoA oxidase 1 (ACOX-1) actions, or re-esterified to triacylglycerol (TAG) by diacylglycerol O-acyltransferase 1 (DGAT-1) actions (Bionaz and Loor, 2012). These enzymes are targets of the peroxisome proliferator-activated receptor alpha (PPAR-alpha) which has been extensively studied in non-ruminants but there are inconsistent findings in ruminants (Bionaz et al., 2013). There have been several studies where there has been metabolic adaptation of the liver, mainly through the mRNA abundance of different hepatic target genes (Loor, 2010; Roche et al., 2013a,b; Shahzad et al., 2014). Andersen et al. (2002) and Carlson et al. (2007) reported that with a greater hepatic oxidation capacity of long-chain fatty acids there was less TAG accumulation in the liver of dairy cattle. Nevertheless, there continues to be inconsistent thoughts regarding which mechanisms affect adaptations during early lactation and about the contribution of the liver to these processes and the possible effects on reproduction performance. In the present study, therefore, it was hypothesized that the reproductive performance of dairy cattle would be associated with changes in the abundance of liver proteins associated with fatty acid oxidation and the synthesis of TAG during the peri-calving period. Thus, in the present study, the aim was to analyze the possible relationship between days to conception (DC) and the different plasma and FF variables, liver TAG content, and the hepatic abundance of proteins related to fatty acid metabolism such as PPAR-alpha, CPT-1, ACOX-1 and DGAT-1 in grazing dairy cattle.

## 2. Materials and methods

### 2.1. Ethics

The present study was approved by the Ethics Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Protocol number 158/2013, Santa Fe, Argentina) and all procedures were conducted according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010).

### 2.2. Animals

Holstein cows ($n$ = 20; second to fourth lactation) were selected from a commercial dairy farm with a grass-based feeding system and an average milk production of approximately 26 liters per day. Fourteen days before the estimated calving date, the BCS of cows was recorded based on a 1 to 5 scale with 0.25 intervals, as described by Edmonson et al. (1989). Only cows with a BCS greater than 2.75 were considered for the study. Both before and during the experiments, animals were clinically evaluated for the diagnosis of retained placenta, clinical milk fever, mastitis, metritis, clinical ketosis, displaced abomasum, lameness and/or clinical gastrointestinal disorder. Two animals were excluded from the experiment because they developed metritis, therefore, there was not completion of the sampling schedule. Data of the other two animals were excluded from the final analysis because these cows were diagnosed with clinical endometritis. Thus, a total of 15 multiparous animals were assessed throughout the study. After parturition, cows were milked twice daily, and the milk yield was recorded monthly until 120 days in milk with milk meters (Waikato Milking Systems, Hamilton, New Zealand). During lactation, the days to conception (DC) were determined in each group. According to their DC, cows were allocated to two groups: relatively fewer days to conception (FDC, $n$ = 8; 82 ± 7 days, mean ± SEM) and a relatively greater number of days to conception (MDC, $n$ = 8; 156 ± 22 days, mean ± SEM).

During the pre-calving period, cows were fed a diet formulated using the NRC 2001 diet software, with 17% corn silage, 17% cracked corn grain, 22% sunflower meal, 26% wheat straw and 18% grass hay on a dry matter (DM) basis. The diet was composed of: 13% CP, 49% NDF, 32% ADF, 33% NFC, and 1.40 Mcal/kg of NEL, with an estimated DMI of 12 kg/day. After calving, animals were fed a diet based on 10% and 24% DM of alfalfa and ryegrass grazing, whereas the other components of the mixed diet were 24% corn silage, 22% cracked corn grain, 10% soybean silage, 5% soybean meal and 5% wheat grain on a DM basis. This diet was composed of: 16% CP, 36% NDF, 21% ADF, 41% NFC, and 1.63 Mcal/kg of NEL, with an estimated DMI of 15 kg/day. The consumption of alfalfa and ryegrass was *ad libitum* and was estimated as the difference between the pre-grazing and post-grazing mass per $m^2$ and multiplied by the area allocated/cow. Both diets were supplemented with a mineral and vitamin block.

### 2.3. Blood, FF and liver sampling

Samples were collected and managed using the procedures described by Bertoni and Trevisi (2013), to try to minimize extraneous sources of variation in blood analyte concentrations. Blood and liver samples were obtained at 14 ( ± 3) days before the expected calving date and on days 4 ( ± 3), 14 ( ± 3) and 28 ( ± 3) after calving. Blood samples were collected from the jugular vein after milking, between the 1600 and 1900 h. Part of the sample was used to measure BHBA in whole blood by using reactive strips as subsequently described in this manuscript. The rest of the blood was collected in tubes with EDTA and cooled at 5 °C. The blood was then centrifuged for 10 min to obtain the plasma and then stored at - 20 °C until analyzed. The liver was sampled *via* puncture biopsy in the right 11th intercostal space at the greater trochanter, using local anesthesia by infiltration of 15 mL of lidocaine hydrochloride 2% (OVER, Santa Fe, Argentina) into the muscular and subcutaneous tissues. To this end, a percutaneous biopsy needle, described by Buckley et al. (1986) and modified *ad hoc*, was inserted through a skin incision of 1.5 cm, and approximately 500 mg of liver tissue was collected in a tube that was snap-frozen in liquid nitrogen and subsequently stored at – 80 °C. The stab incision was closed with

non-absorbable suture.

After 28 days of lactation, cows were estrous synchronized using the protocol described by Rivera et al. (2005) with modifications: 100 μg of gonadotropin-releasing hormone (GnRH) (Gestar, OVER, Santa Fe, Argentina) on day 0, insertion of an intravaginal device containing 1 g of progesterone (Sincrover 1000, OVER, Santa Fe, Argentina) from days 0 to 6, and 25 mg of prostaglandin F2-alpha (PGF2-alpha) (Prostal, OVER, Santa Fe, Argentina) on day 6. On day 8, pre-ovulatory follicles were aspirated by using a digital ultrasonic system 8300vet Chison equipped with a micro-convex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, Sao Paulo, Brazil) (Díaz et al., 2018). Part of the FF was immediately used to measure BHBA by using reactive strips as described later. The rest of the FF was immediately refrigerated, transported to the laboratory and stored at - 20 °C until analyzed.

### 2.4. Plasma and FF metabolites and hormone measurements

Plasma and FF concentrations of NEFA, glucose and TAG were determined enzymatically with commercial kits (NEFA: RANDOX Laboratories LTD, UK; glucose and TAG: WIENER Lab, Rosario, Argentina) using a spectrometer (ultra-fast UV/Vis spectrometer SPECTROstar Nano, BMG LABTECH GmbH, Ortenberg, Germany). The BHBA was quantified in whole blood and FF using reactive strips (FreeStyle Optium Xceed, Abbott Diabetes Care Ltd., Oxon, UK), as previously described (Gareis et al., 2018). Plasma and FF concentrations of insulin were quantified using a radioimmunoassay (RIA) that included utilization of an anti-bovine insulin antibody (Product No. I6136, Sigma, St. Louis, MO, USA) and a human insulin standard provided by Laboratorios Beta (Buenos Aires, Argentina), as previously described (Becú-Villalobos et al., 2007; Hein et al., 2015). The minimum detectable concentration of insulin was 0.05 ng/mL. The intra- and inter-assay coefficients of variation for insulin were always less than 8% and 11%, respectively. Volumes of plasma, FF or IGF-1 standards were subjected to an acid-ethanol extraction (Rodríguez et al., 2017) previous to the RIA. The concentration of IGF-1 was determined using an antibody (UB2-495, Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases, Rockville, MD, USA), and a recombinant human IGF-1 (rhIGF1, Chiron Corp., Emeryville, CA, USA) was used as a radioligand and unlabelled ligand (Giuliodori et al., 2011). The minimum detectable concentration of IGF-1 was 0.4 ng/mL. The intra- and inter-assay coefficients of variation for IGF-1 were always less than 6% and 9%, respectively. Plasma total protein and albumin, aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), total bilirubin (TBil) and alkaline phosphatase (ALP) were determined enzymatically, using commercial kits (WIENER Lab, Rosario, Argentina).

### 2.5. Liver TAG content analysis

Total lipids were extracted from liver biopsy homogenates using a mixture of chloroform and methanol (2:1 v/v) (Folch et al., 1957) and the liver TAG content was determined enzymatically using a commercial kit (WIENER Lab, Rosario, Argentina).

### 2.6. Western blot analysis

Approximately 50 mg of frozen liver sample was homogenized in a lysis buffer containing 1% v/v octylphenyl-polyethylene glycol (IGEPAL CA630), 50 mmol/L sodium fluoride, 1 mmol/L EDTA, 0.1% w/v sodium dodecyl sulphate (SDS), 0.5% w/v sodium desoxycholate (all from Sigma-Aldrich Corp., St. Louis, MO, USA), 0.1 mol/L PBS, and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Tissue lysates were subsequently centrifuged at 12,000 *g* for 20 min and the supernatant was separated and collected at -80 °C. Protein concentrations were quantified using the Lowry method with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) (Hein et al., 2010). Each sample was denatured in a Laemmli sample buffer by boiling for 5 min. Proteins were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) 10% (w/v) and then transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), as previously described (Rodríguez et al., 2017). The membranes were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST, Sigma–Aldrich Corp.) at room temperature and then incubated overnight at 4 °C with the specific primary antibodies (Table 1). Membranes were subsequently washed with TBST and incubated for 1.5 h at room temperature with the corresponding secondary peroxidase-conjugated antibody (Table 1). Furthermore, membranes were washed with TBST and the specific reactions were detected using a chemiluminescence detection kit (ECL Prime Western Blotting System, GE Healthcare) on hyperfilm-ECL film (GE Healthcare). Beta-actin was used as internal control by using the same protocol as previously described in this manuscript using the antibodies detailed in Table 1. Results from a preliminary study indicated there was linearity of the Western blot assay from 20 to 80 μg of protein and at increasing exposure times. The intensity of the bands was quantified using the IMAGE PRO-PLUS 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) and a standard sample from a pool of six animals was blotted on each membrane to adjust band values from different membranes.

### 2.7. Statistical analysis

The data were analyzed using the statistical software package SPSS 22.0 for WINDOWS (SPSS Inc., Chicago, IL, USA). The distribution of data was tested for normality by using the Kolmogorov-Smirnov test. Data for most variables were not normally distributed. Thereby, for BHBA in whole blood, plasma and liver determinations, a repeated-measures analysis was performed using the Generalized Linear Model (GLM) approach with the gamma distribution and a log link function. For outcome variables with normal distribution, a GLM with linear log link function was used. The model consisted of DC, time (T), and DC × T as fixed effects,

**Table 1**
Antibodies and conditions used for western blot assays.

| Antibodies | Type | Suppliers | Dilution | Protein/lane |
|---|---|---|---|---|
| Primary Antibodies | | | | |
| PPAR-alpha | Rabbit Polyclonal H-98: sc-9000 | Santa Cruz Biotechnology. Inc. CA, USA | 1/250 | 60 µg |
| CPT-1-L | Rabbit Polyclonal H-95:sc-20669 | Santa Cruz Biotechnology. Inc. CA, USA | 1/400 | 60 µg |
| ACOX-1 | Rabbit Polyclonal H-140: sc-98499 | Santa Cruz Biotechnology. Inc. CA, USA | 1/500 | 40 µg |
| DGAT-1 | Rabbit Polyclonal ab54037 | Abcam. Cambridge, UK | 1/1000 | 40 µg |
| Beta–actin | Mouse monoclonal JLA20 | DSHB. Iowa City, IA, USA | 1/1500 | |
| Secondary Antibodies | | | | |
| Mouse anti-rabbit IgG | Polyclonal sc-2357 | Santa Cruz Biotechnology. Inc. CA, USA | 1/10,000 | |
| Goat anti-mouse IgG | Monoclonal sc-2005 | Santa Cruz Biotechnology. Inc. CA, USA | 1/10,000 | |

and cow as the random effect. For FF determinations, a GLM was used to assess associations described previously and with DC as fixed effect. A value of $P < 0.05$ was considered significant. The results are expressed as mean ± SEM.

## 3. Results

### 3.1. Milk production, BCS, plasma variables and liver TAG content

There was no difference in milk production after 120 days in lactation between MDC (3506 ± 177 L) and FDC groups (3410 ± 177 L; $P > 0.05$). Regarding BCS, there was a progressive reduction in values for this variable ($P < 0.05$). There, however, was no difference between groups and there was no interaction among values for DC and T during the study ($P > 0.05$; Fig. 1a). In addition, NEFA concentration increased during the study, peaking on day 4 ($P < 0.05$), but there was no difference between groups or interaction among values for DC and T during the study ($P > 0.05$; Fig. 1b). The pattern of BHBA concentration was similar to that of NEFA, with a post-calving increase with the greater concentration being maintained throughout the period in which evaluations occurred ($P < 0.05$). The BHBA concentration was less, however, in the FDC than MDC group ($P < 0.05$), but there was no interaction between values for DC and T during the study ($P > 0.05$; Fig. 1c). Similar to the BHBA pattern, the liver TAG content increased after calving and was less in the FDC group than MDC group ($P < 0.05$). In addition, there was an interaction among values for DC and T during the study, and the liver TAG content was less in the FDC than MDC group on days 14 and 28 post-calving ($P < 0.05$; Fig. 1f).

Plasma glucose and TAG concentrations were not different between groups over time and there was no interaction among values for DC and T during the study ($P > 0.05$; Fig. 1d and e). Regarding liver function biomarkers, for values of TBil there was an interaction among values for DC and T during the study due to the lesser concentration in the FDC group than in the MDC group on day 28 post-calving ($P < 0.05$). The AST and GGT increased after calving ($P < 0.05$), however, there were no differences in values between groups or interaction among the values for these two variables during the study ($P > 0.05$). In addition, IGF-1 concentrations changed over time with there being a lesser concentration on day 4 ($P < 0.05$), and without differences between groups or interaction among values for the two groups during the study ($P > 0.05$). There were no differences in concentrations of insulin, total protein and albumin and the activity of ALP between groups nor was there an interaction of values for these variables during the study ($P > 0.05$; Table 2).

### 3.2. Protein abundance

The protein abundance of CPT-1 was greater in the FDC than MDC group ($P < 0.05$). Furthermore, there was an interaction among values for the two groups during the study due to the greater protein abundance in cows of the FDC group on day 4 ($P < 0.05$) but there was no difference on days 14 and 28 ($P > 0.05$; Fig. 2b). The ACOX-1 protein abundance increased over time ($P < 0.05$), but there were no differences in values between groups or interaction of values among the two groups during the study ($P > 0.05$; Fig. 2c). Similar to ACOX-1, the pattern of PPAR-alpha protein the abundance tended to increase over time ($P = 0.09$), but there were no differences in the DC or interaction among values for the two groups during the study ($P > 0.05$; Fig. 2a). Furthermore, DGAT-1 there were no differences in protein abundance between groups over time or an interaction among values for the two groups during the study ($P > 0.05$; Fig. 2d).

### 3.3. Follicular fluid variables

There were no differences in concentrations of NEFA, BHBA, glucose, TAG, insulin and IGF-1 in FF between groups ($P > 0.05$; Fig. 3).

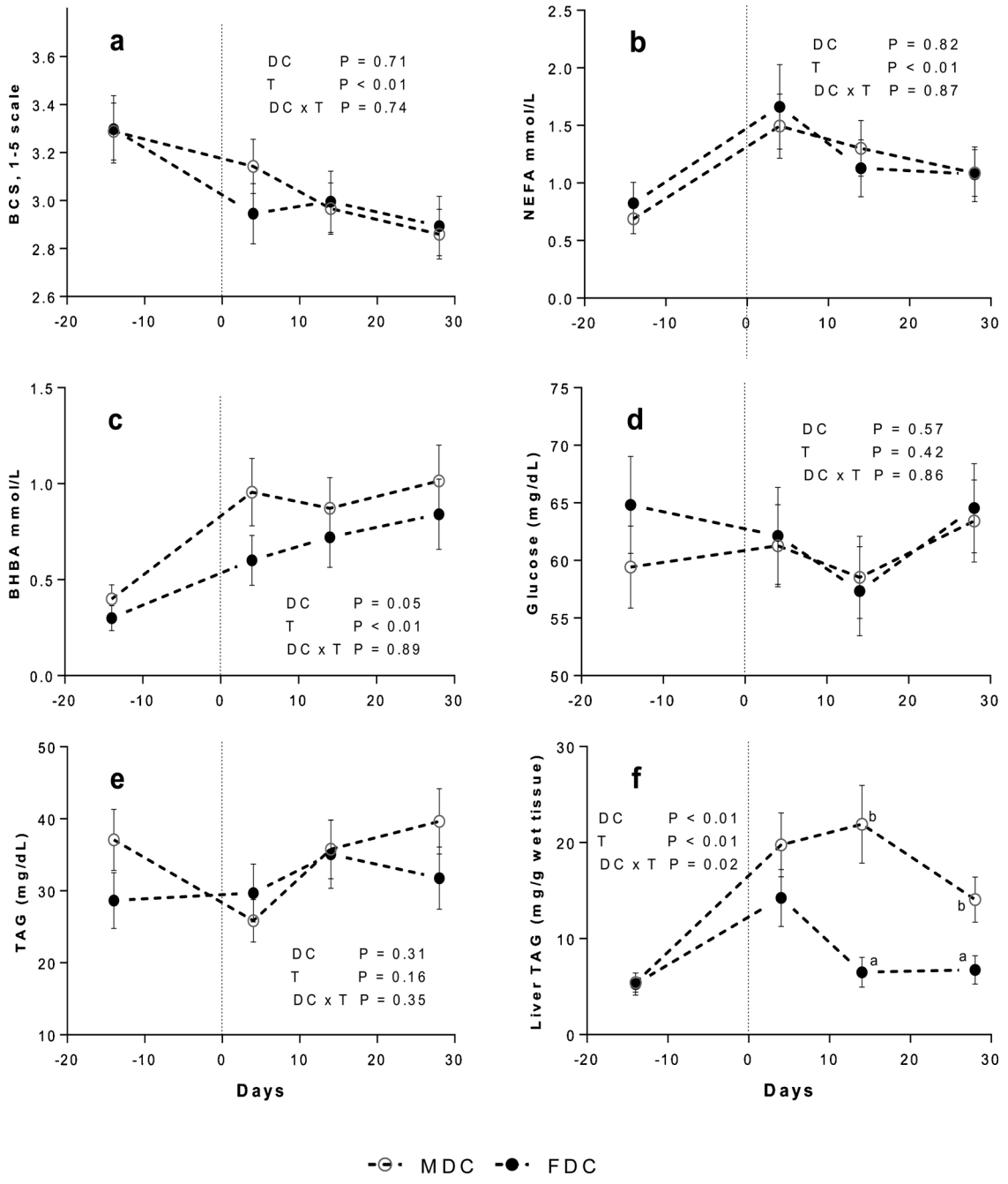


**Fig. 1.** Body condition score (BCS, panel a), plasma concentrations of non-esterified fatty acids (NEFA, panel b), beta-hydroxybutyric acid (BHBA, panel c), glucose (panel d), and triacylglycerol (TAG, panel e) and liver TAG content (panel f) between day 14 pre-calving and day 28 post-calving in animals grouped as cows with more days to conception (MDC; $n = 8$) or fewer days to conception (FDC; $n = 8$); Values represent the mean ± SEM; Statistical effects of DC, T and DC x T are indicated; $^{a\text{-}b}$DC × T ($P < 0.05$) among DC at a given week.

## 4. Discussion

In several studies, there has been analysis of the gene expression and concentration of relevant molecules affecting different pathways of the hepatic metabolism of dairy cattle in relation to the effects of different diets or treatments with the aim to improve milk production or health status (Loor et al., 2006; Selim et al., 2015; Vailati Riboni et al., 2015; Mann et al., 2018). In the present study, there was evaluation of how the fertility of dairy cattle could be associated with liver fatty acid metabolism during the peri-calving period, based on selected indicators in plasma, FF and liver biopsies.

The genetic ability to increase for milk production has been associated with lesser fertility (Lucy, 2007; Mee, 2007). The close

**Table 2**
Concentration of insulin, IGF-1, protein, albumin, TBil, and activity of AST, GGT and ALP in plasma of dairy cattle.

| | | Prepartum days | Postpartum days | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | −14 | 4 | 14 | 28 | DC | T | DC x T |
| Insulin | FDC | 0.98 ± 0.32 | 0.69 ± 0.20 | 0.46 ± 0.14 | 0.53 ± 0.15 | 0.46 | 0.57 | 0.24 |
| (ng/mL) | MDC | 0.53 ± 0.13 | 0.51 ± 0.13 | 0.76 ± 0.19 | 0.45 ± 0.11 | | | |
| IGF-1 | FDC | 126.08 ± 22.50 | 91.29 ± 22.50 | 176.99 ± 22.50 | 140.82 ± 22.50 | 0.79 | < 0.01 | 0.29 |
| (ng/mL) | MDC | 144.26 ± 19.01 | 69.79 ± 19.01 | 135.84 ± 19.01 | 169.59 ± 19.01 | | | |
| Protein | FDC | 7.16 ± 0.37 | 6.96 ± 0.36 | 7.21 ± 0.37 | 7.49 ± 0.38 | 0.19 | 0.07 | 0.73 |
| (g/dL) | MDC | 7.55 ± 0.33 | 6.83 ± 0.29 | 7.63 ± 0.33 | 8.15 ± 0.35 | | | |
| Albumin | FDC | 3.18 ± 0.15 | 3.23 ± 0.15 | 3.06 ± 0.15 | 3.01 ± 0.15 | 0.41 | 0.95 | 0.40 |
| (g/dL) | MDC | 3.07 ± 0.12 | 2.92 ± 0.12 | 3.04 ± 0.12 | 3.15 ± 0.12 | | | |
| TBil | FDC | 1.26 ± 0.16 | 1.60 ± 0.16 | 1.71 ± 0.16 | 1.10 ± 0.16 [a] | 0.66 | 0.10 | 0.05 |
| (mg/dL) | MDC | 1.34 ± 0.13 | 1.58 ± 0.13 | 1.37 ± 0.13 | 1.57 ± 0.13 [b] | | | |
| AST | FDC | 119.05 ± 13.25 | 106.24 ± 13.25 | 138.77 ± 13.25 | 136.69 ± 13.25 | 0.76 | 0.05 | 0.83 |
| (IU/L) | MDC | 111.16 ± 11.20 | 120.61 ± 11.20 | 137.64 ± 12.10 | 142.11 ± 11.20 | | | |
| GGT | FDC | 20.37 ± 2.56 | 27.24 ± 2.56 | 28.17 ± 2.56 | 23.54 ± 2.56 | 0.49 | 0.01 | 0.86 |
| (IU/L) | MDC | 20.00 ± 2.16 | 27.55 ± 2.16 | 24.60 ± 2.16 | 22.54 ± 2.16 | | | |
| ALP | FDC | 24,98 ± 3,92 | 28,44 ± 3,92 | 24,68 ± 3,92 | 26,15 ± 3,92 | 0.19 | 0.94 | 0.75 |
| (IU/L) | MDC | 28,47 ± 3,31 | 27,17 ± 3,31 | 30,20 ± 3,31 | 31,91 ± 3,31 | | | |

Animals were grouped as cows with more days to conception (MDC; n = 8) or fewer days to conception (FDC; n = 8) at 14 days pre-partum, and at 4, 14 and 28 days post-partum; Values are expressed as mean ± SEM; Statistical effects of DC, T and DC x T are indicated; [a-b]DC × T ($P \leq 0.05$) among DC at a given week; TBil: total bilirubin; AST: aspartate transaminase; GGT: gamma-glutamyl transpeptidase; ALP: alkaline phosphatase.

relationship between milk yield and fertility is not always obvious (López-Gatius et al., 2006; Patton et al., 2007). In the present study, there was no association between DC and milk yield. Furthermore, results of other studies have indicated there is a greater BCS with greater milk yield (Roche et al., 2013a,b). In this regard, as with milk yield, there were no differences in BCS as a result of cow group. The BCS has been associated with a lesser DMI and greater NEB in the peri-partum period (Roche et al., 2015), which could resulted in compromised fertility. There was evaluation of plasma concentrations of NEFA and BHBA as biomarkers of NEB in the present study. The results indicate concentrations of NEFA in both groups were slightly greater than the recommendations (Ospina et al., 2010; Chapinal et al., 2012), suggesting that there was a fat mobilization in these cows. In the present study, there was no association between the plasma concentrations of NEFA and the DC. There were also no differences in milk yield, BCS and plasma NEFA concentrations between groups, so the expectation was that there would not be differences in DMI and NEB between groups. Results of other studies in grazing cows indicate there is an association between greater NEFA concentrations and delayed ovulation, possibly resulting from a negative effect at the hypothalamic-hypophysis-gonadal axis (Giuliodori et al., 2011). Furthermore, results of previous *in vitro* studies indicate NEFA can have harmful effects on ovarian functionality, such as inhibition of granulosa cell survival in cattle, alteration in follicular cell development and proliferation, and alteration in oocyte development (Leroy et al., 2012; Van Hoeck et al., 2013). In the present study, BHBA concentration of cows of the MDC group was slightly less than the reference value for subclinical ketosis (Ospina et al., 2010), which indicates that these animals had not undergone severe NEB. Consistent with results in previous studies in which there was an association between lesser reproductive performances and subclinical ketosis (McArt et al., 2013; Abdelli et al., 2017), there was an association between BHBA and DC in the present study. Furthermore, Leroy et al. (2006) reported that there was an *in vitro* toxic effect of BHBA with a moderate glucose concentration (similar to the concentrations recorded in the present study) on the oocyte maturation. In this regard, it is thought that greater BHBA concentrations suppress the glycolytic pathway in the cumulus cells leading to a lack of metabolic energy for oocyte functions, compromising early embryonic development (Sarentonglaga et al., 2013).

Results of previous studies also indicate there is an association between the liver TAG content and a NEB (Bobe et al., 2004; Loor et al., 2006). Bobe et al. (2004) reported there was a decrease in DMI with a greater liver TAG content, but only when there was hepatic lipidosis (greater than 10% wet weight). In contrast, in the present study, the liver TAG content of cows was less than 10% wet weight. Results of the present study, therefore, indicate there are no differences in BCS and NEFA concentration between groups and there was a liver TAG content of less than10% wet weight; it is possible, therefore, that the DC are not associated with a NEB. In addition, in the present study there was a greater liver TAG content in the animals of the MDC group. Particularly, on days 14 and 28, the cows of the MDC group had a mildly fatty liver compared to those of the FDC group, which had a normal liver morphology (Bobe et al., 2004). These findings are consistent with those of Jorritsma et al. (2000), where there was a negative relation between greater liver TAG content and DC. As described previously in this manuscript, the plasma NEFA concentration was not different between groups, but the lesser BHBA concentration and the liver TAG content in cows of the FDC group could indicate there was a greater adaptation to the post-calving lipid mobilization.

In the present study, there was also evaluation of plasma concentrations of glucose, insulin and IGF-1 in all groups, but there were no associations between the values for these variables and DC. While results of some studies indicate there is an association between the greater concentrations of these compounds and greater reproductive performance (Velazquez et al., 2008; Garverick et al., 2013), results of other studies have not been conclusive (van Knegsel et al., 2005; Wathes, 2012). As described previously, in the present study in the different groups there were no differences between BCS and NEFA, so there were similar plasma concentrations of

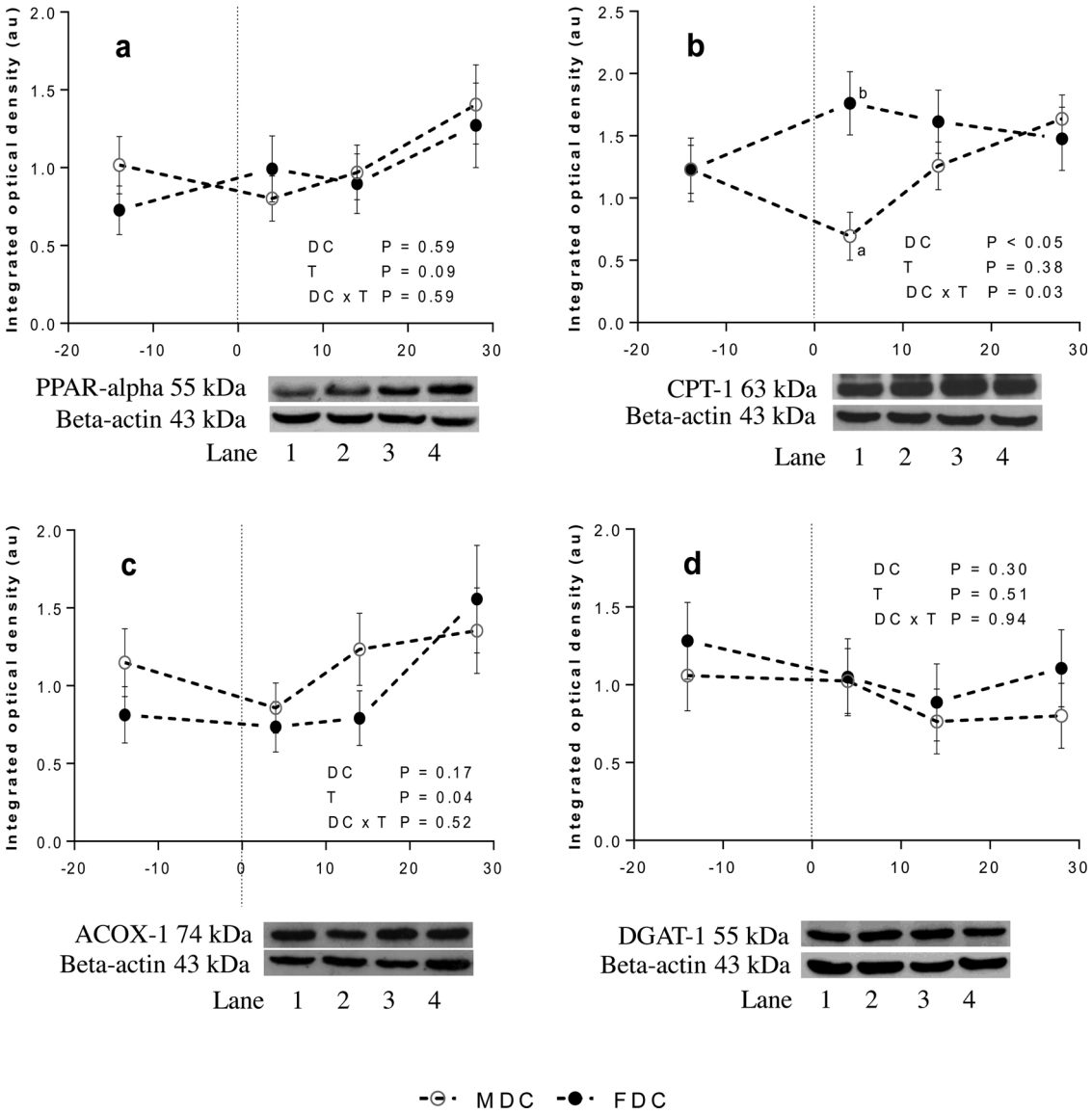


**Fig. 2.** Protein abundance of hepatic PPAR-alpha (panel a), CPT-1 (panel b), ACOX-1 (panel c) and DGAT-1 (panel d) between day 14 pre-calving and day 28 post-calving in animals grouped as cows with more days to conception (MDC; $n = 8$) or fewer days to conception (FDC; $n = 8$); Top of each panel: Integrated optical density (au: arbitrary units) determined by Western blot analysis; Values represent the mean ± SEM; Statistical effects of DC, T and DC x T are indicated; $^{a\text{-}b}$DC × T ($P < 0.05$) among DC at a given week; Bottom of each panel: Representative immunoblots of each determination; Molecular weight is shown on the right. Lane 1, day 14 precalving; lanes 2–4, days 4, 14 and 28 post-calving.

glucose, insulin and IGF-1 in these cows indicating there were no differences in the NEB (Kawashima et al., 2012).

In other studies, there has been determination of the importance of other blood compounds and associations with liver functions (Bertoni et al., 2009; Bertoni and Trevisi, 2013). Consistent with these previous results of Bertoni et al. (2008) where there was lesser concentrations of negative acute-phase-proteins and clearance of bilirubin that was associated with a relatively lesser reproductive performance, in the present study there was a greater plasma concentration of TBil in cows of the MDC group. For the activities of AST and GGT, there was no association with the DC of cows. Although no liver damage was evident in the cows in the present study, the plasma concentration of TBil could be related to the greater liver TAG content in the MDC group, compromising liver functions.

In the present study, there was also an analysis of some relevant metabolites in the FF of preovulatory follicles due to the close contact of this fluid with developing oocytes and because it is an indicator of the secretory activities, metabolism and nutrient availability of follicular cells. In a previous *in vivo* study in cows with cystic ovarian disease, there was less glucose and TAG concentrations with greater NEFA concentration in FF of cows with this disease (Gareis et al., 2018). Cystic ovarian disease in dairy cattle occurs most frequently during the early post-partum period, at the time normal ovarian activity resumes (Ortega et al., 2016). In the present study, however, the FF concentration of NEFA, BHBA, glucose, TAG, insulin and IGF-1 were not associated with the DC

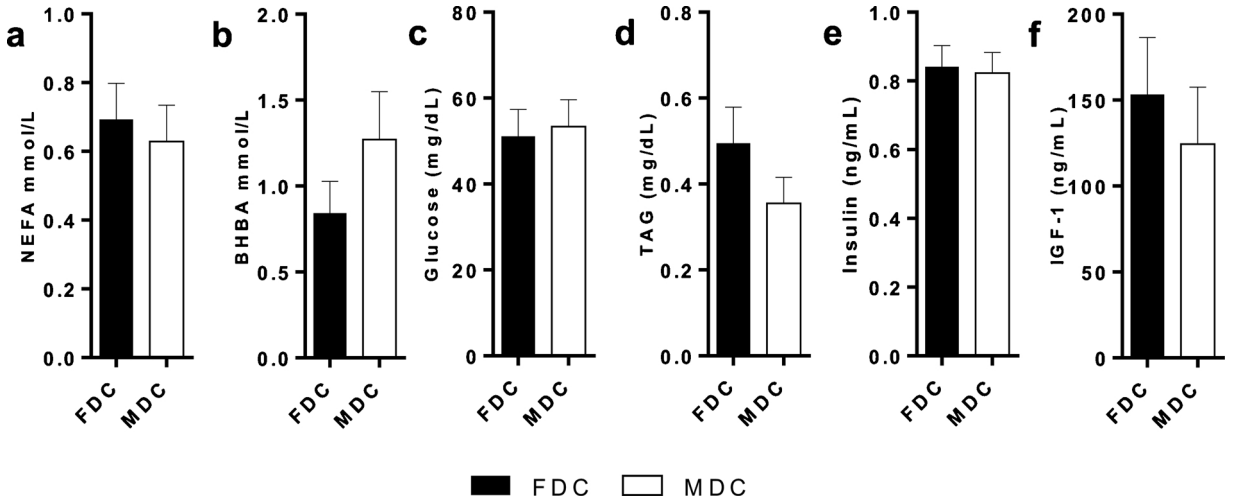


**Fig. 3.** Follicular fluid (FF) concentrations of non-esterified fatty acids (NEFA, panel a), beta-hydroxybutyric acid (BHBA, panel b), glucose (panel c), triacylglycerol (TAG, panel d), insulin (panel e) and IGF-1 (panel f) on day 37( ± 3) post-calving; Animals were grouped as cows with relatively greater days to conception (MDC; $n = 8$) or lesser days to conception (FDC; $n = 8$); Values represent the mean ± SEM.

of cows. It is well known that some metabolites and metabolic hormones affect fertility, not only directly in the ovary and follicle environment, but also indirectly by modulating the somatotropic/gonadotropic axis (Leroy et al., 2008; Ortega et al., 2016). Nevertheless, in the present study, there was an increase in plasma BHBA before the FF sampling. Thus, and consistent with findings in a previous study (McArt et al., 2013), changes occurring in relevant systemic metabolites around parturition could have direct and indirect effects on the ovarian functions and there may subsequently be consequences in the reproductive performance.

An increase in the concentration of NEFA post-calving affects not only ovarian functions but also liver metabolism, with possible consequences in milk production and reproduction (Bobe et al., 2004). One metabolic pathway for NEFA in the liver could be mitochondrial oxidation, where CPT-1 has an important function. In the present study, although there were no differences in the plasma NEFA concentrations in cows with different DC, CPT-1 protein abundance was greater in the cows of the FDC group. Li et al. (2012) reported that the liver of cows with clinical ketosis had less CPT-1 gene expression even though there was a greater serum NEFA concentration. In the present study, there was a greater abundance of hepatic CPT-1 protein in cows of the FDC group on day 4, previous to the time when there was detection of the lesser TAG liver content detected in these cows (on days 14 and 28). Thus, the greater CPT-1 protein abundance, which is a mitochondrial oxidation index, could be involved in the positive effects on the health and DC of cows. Louet et al. (2001) reported that the oxidation of fatty acids is controlled by changes in the activity of CPT-1, concentration of malonyl-CoA, and sensitivity of CPT-1 to malonyl-CoA inhibition. Furthermore, malonyl-CoA has been proposed as a regulator of the activity of CPT-1 in both ruminants and non-ruminants (Han van der Kolk et al., 2017). Nevertheless, results in a previous study with peri-parturient dairy cows indicated with a restricted diet during the pre-partum period there was an increase in CPT-1 activity but not malonyl-CoA sensitivity (Dann and Drackley, 2005). In addition, in an *in vitro* study with hepatocytes of cattle (Louet et al., 2001) there was a greater mRNA transcript and protein abundance of CPT-1 associated with an increase in NEFA concentrations when concentrations were less than 1.6 mmol/L but a decrease when there was an increase in NEFA concentration if the concentrations were greater than 1.6 mmol/L (Li et al., 2013). In the present study, the NEFA concentration was about 1.6 mmol/L on day 4 postpartum and decreased later in the study; nevertheless, in the present study, there was not an evaluation of the effects on the CPT-1 in animals with different DC, and thus additional research is needed to elucidate possible associations of CPT-1 to biological functions.

In cattle, both mitochondrial and peroxisomal oxidation are important (Grum et al., 1994). In the present study, ACOX-1 protein abundance increased during the study but there were not associations of this abundance with DC. Furthermore, peroxisomal ACOX-1 is a target enzyme of the nuclear receptor PPAR-alpha. Considering this fact and similar to findings in the present study for ACOX-1, there was a tendency for PPAR-alpha protein abundance to increase during the study, but there were not associations with respect to DC. In non-ruminants animals, PPARs are involved in major metabolic and inflammatory regulations (Varga et al., 2011), but, in ruminants, the data are not conclusive. Regarding this issue, results of previous studies indicate there are no differences in *PPAR-alpha* gene expression in animals with different BCS (Akbar et al., 2015), or in peri-partum cows with different dietary lipid and energy content (Akbar et al., 2013; Khan et al., 2014). Also, results of some studies indicate that even though there was an increase in NEFA concentration, the greater mRNA abundance around the time of calving was not a consistent finding (Loor et al., 2005; Graber et al., 2010).

Furthermore, in the present study there was evaluation of DGAT-1 protein abundance, but there were no differences as a result of treatment. The content of TAG in the ruminant liver is not related to the synthesis of TAG but rather content is modulated by the capacity for secretion of very low density lipoproteins (Kessler et al., 2014).

In several studies, there has been assessment of a relationship between liver functionality and fertility. Jorritsma et al. (2000)

reported that there was a greater liver TAG content associated with lesser fertility rather than with milk production. Bertoni et al. (2008) proposed that the impairment of metabolic liver function could be related to a reduced fertility. The present study is the first in evaluating the abundance of relevant proteins involved in hepatic fatty acid metabolism associated with a parameter of reproductive performance. Results of the present study indicate there is a greater abundance of proteins related to liver fatty acid oxidation in the FDC group possibly contributing to the lesser TAG content and lesser plasma BHBA concentration. The lesser TAG content could contribute to modulation of liver functions leading to a greater clearance of bilirubin. It is also concluded from results of the present study that an enhanced reproductive performance of lactating cows could be associated with a greater fatty acid oxidation in the liver, based on the findings that there was a lesser TAG content and the greater protein abundance of CPT1. Further studies of other pathways involved in lipid metabolism are necessary for gaining a more precise understanding of liver adaptation during the transition period from not lactating to lactating in dairy cattle.

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