Interrelationships among plasma metabolites, production, and ovarian follicular function in dairy cows

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Interrelationships among plasma metabolites, production, and ovarian follicular function
in dairy cows

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ABSTRACT

Associations of blood metabolites and production variables with ovarian function and parity, specifically, interval to first ovulation (IFO) and the incidence of ovarian cysts (OC) and multiple ovulation (MOV) at the first ovulation postpartum, were determined in lactating dairy cows. This retrospective study involved data on 169 Holstein cows from 3 studies on the same herd. Blood samples were taken weekly from wk -1 to 4, relative to calving, and transrectal ultrasonography was performed twice weekly from d 7 to 60 postpartum. The overall IFO was 32.6 ± 1.9 d [mean ± SEM] and did not differ among lactations. Primiparous cows were at a greater risk of failing to ovulate before d 60 postpartum and cows in the 3rd or greater lactation were at a higher risk for OC and MOV. Blood metabolites and production variables indicating a negative energy balance were associated with cows failing to ovulate before d 35 postpartum, but were not related with OC. Increased DMI and milk yield, particularly in 2nd lactation cows, were associated with a higher incidence of OC. While BHBA and IGF-1 were associated with MOV, the relationship was weak. Reducing negative energy balance is necessary to reduce IFO but may increase the incidence of MOV.

Short Title: Metabolic Profile and Ovarian Function

Key Words: ovulation interval, ovarian cyst, multiple ovulation, metabolic profile, dairy cows

INTRODUCTION

The transition period for dairy cows is generally defined as the 3 wk before to 3 wk after calving, and is the most metabolically challenging time for the cow (Grummer et al. 2004). The onset of calving is associated with changes in hormonal status, and the onset of lactation drastically increases energy requirements, often leading to a period of negative energy balance.
This period of NEBAL is characterized by an increase in blood NEFA and BHBA concentrations and a decrease in glucose (GLU) and IGF-1 concentrations (Esposito et al. 2014). Increasing DMI is the primary factor in establishing positive energy balance, and is important from a reproductive standpoint because nutritional status in early lactation has a carry-over effect on fertility (Patton et al. 2007). The interval to first ovulation (IFO) postpartum is an indicator of reproductive efficiency as reducing the IFO is associated with a reduced interval to conception (Wathes et al. 2007) and increased conception rates (Butler 2000). The return to normal cyclicity occurs in early lactation during this metabolically stressful time, which makes nutritional status in the transition period very important. Previous studies have reported that energy balance is highly correlated with IFO (Beam and Butler 1998; Butler 2000), and a faster return to a positive energy balance is the primary characteristic associated with earlier ovulation (Patton et al. 2007).

A risk factor for a delay in IFO is the formation of ovarian cysts (OC), which interferes with normal cyclicity as the follicles fail to ovulate and continue to grow and persist on the ovary (Garverick 1997). Ovarian cysts also increase calving interval and number of AI per pregnancy (Vanholder et al. 2006). Previous studies report conflicting results on the role of blood metabolites related to nutritional status on OC in the transition period, with some indicating no effect of energy status on OC (Vanholder et al. 2005; Butler et al. 2006) and others indicating increased NEFA in cows with OC (Zulu et al. 2002). Milk production has also been identified as a risk factor for OC, in which cows with higher milk yield, as seen in early lactation, are more likely to have OC (López-Helguera et al. 2016).

The ovulation of more than one follicle per cycle, or multiple ovulations (MOV), is closely related to twinning in dairy cattle, which is undesirable due to the increased risk of health
problems, reproductive failures and reduced production (Andreu-Vázquez et al. 2012). Incidence of MOV is greater in the first ovulation postpartum (Lopez et al. 2005), which may be due to the energy balance at this time. Several possible factors associated with MOV in cattle have been described; however, reports have been controversial or inconclusive. Echternkamp et al. (2004) stated that increased plasma and follicular IGF-1 increased the incidence of MOV in multiparous beef cows. Kusaka et al. (2017) reported that milk yield (MY) was greater in MOV cows and Wiltbank et al. (2000) indicated that milk production was positively associated with increased incidence of twins, but López-Gatius et al. (2005) stated that increased MY reduced the risk for MOV. Therefore, the relationship between MOV, nutritional status and production during the transition period remains unclear.

Studies that examined a large range of blood metabolites and production variables on IFO and the incidence of OC and MOV before or at first ovulation are lacking. Additionally, the interaction with the previously mentioned variables and lactation number is lacking in previous literature. As cows in different parities, particularly primiparous vs. multiparous, have both production and metabolic differences (Wathes et al. 2007), the association between blood metabolites, production variables and ovarian function likely differs as well. The objective of this retrospective study was to establish the relationship between blood metabolites and production variables, with parity and the resumption of normal ovarian function after calving. We hypothesized that a higher level of production in multiparous cows, also resulting in increased concentrations of blood metabolites related to a NEBAL in early lactation, would be associated with increased interval to first ovulation, as well as increased incidence of OC and MOV at the first ovulation postpartum.
MATERIALS AND METHODS

Animals and Diets

Data from 169 Holstein cows from 3 studies (Colazo et al. 2009; Dyck et al. 2011; Subramaniam et al. 2016) conducted between 2006 and 2010 (Table 1) were compiled to examine the relationship between plasma metabolic profiles as well as production parameters and ovarian follicular function in dairy cows. The studies were conducted on the same herd at the Dairy Research and Technology Center at the University of Alberta (Edmonton, Canada), with all experimental procedures approved by the University of Alberta Animal Care and Use Committee and animals cared for in accordance with the requirements of the Canadian Council on Animal Care (2009). Cows were housed in a tie-stall barn, allowed 2 h of exercise each day and milked twice daily from 0400 to 0600 h and 1530 to 1730 h.

Cows were fed once daily at 07:30 h and had unrestricted access to water. The amount of feed offered and refused was recorded daily for each cow. Detailed ingredient composition of the diets has been published (Colazo et al. 2009; Dyck et al. 2011; Subramaniam et al. 2016). Briefly, in study 1 (Colazo et al. 2009) cows were fed a standard far-off dry diet until 28 d before parturition, and then were fed 1 of 6 diets in a 2 x 3 factorial arrangement until parturition. The close-up diets were offered either ad libitum or at 70% of ad libitum intake, and contained 1 of 3 rolled oilseeds (canola, linola or flax) at 8% of dietary DM. After calving, cows were fed a common lactation diet that did not contain oilseeds. In study 2 (Dyck et al. 2011) cows were assigned to 1 of 3 diets immediately following calving: alfalfa silage, barley silage, or barley silage with 4% preof the barley silage replaced by corn starch on a DM basis. All diets were fed ad libitum. In study 3 (Subramaniam et al. 2016) cows were fed one of 2 barley silage-based
diets ad libitum containing either 17% wheat dried distillers grain with solubles or 17% rolled barley.

**Production and Metabolites**

Dry matter intake (DMI), DMI relative to BW (DMIBW), and MY were determined daily postpartum. Cow BW and BCS were determined on d 1 (BW1 and BCS1, respectively) and d 28 (BW28 and BCS28, respectively) after calving. The same technician assigned BCS to each cow on a scale of 1 (emaciated) to 5 (over-conditioned) (Edmonson et al. 1989). Milk yield was recorded twice daily for 28 d.

Blood was sampled wk 1 before calving (wk -1) and wk 1, 2, 3, and 4 after calving from a coccygeal blood vessel into evacuated tubes containing sodium heparin (Vacutainer; Becton Dickinson and Co., Franklin lakes, NJ). Samples were immediately placed on ice and centrifuged at 3,000 x g for 20 min at 4°C; plasma was separated and stored at -20°C until further analysis.

Plasma NEFA was determined colorimetrically using a commercial kit [NEFA-HR (2), Wako Chemicals, Richmond, VA]. Plasma GLU was analyzed using glucose oxidase/peroxidase enzyme (P7119, Sigma-Aldrich, St. Louis, MO) and dihydrochloride (F5803, Sigma-Aldrich). An RIA kit was used to determine concentrations of plasma insulin (INS, Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) and plasma IGF-1 (Immulite, Diagnostic Products Corporation). Plasma BHBA was determined in all cows in Study 1 and 2 (n=109) by the procedure of Williamson and Mellanby (1974) adapted to a 96-well microtiter plate format.

**Ultrasonography**

Transrectal ultrasonography (Aloka-500V scanner equipped with a 7.5-MHz linear transducer, Aloka Co., Tokyo, Japan) was performed twice weekly from d 7 to d 60 after calving.
to determine IFO and incidence of first MOV and OC. Ovarian maps were drawn for each cow, which included the diameter and location of follicles and the corpus luteum (CL) recorded as described by Pierson and Ginther (1984). Ovulation was confirmed by the absence of a large follicle (diameter ≥10 mm) that had been detected at the previous examination, and subsequent CL formation (Pierson and Ginther 1984). The ovulation of 2 or more follicles between scannings was considered MOV. Anovulatory follicles, ≥25 mm in diameter, which persisted for at least 10 d in the absence of a CL, were defined as OC (Garverick 1997).

**Statistical Analyses**

Cows were assigned a “+, positive” or “-, negative” value for ovarian function, *i.e.* cows that ovulated before 35 d postpartum were classified as OV+ and those that did not as OV-, cows that developed a OC were classified as OC+ and those that did not as OC-, and cows that ovulated more than one follicle at first ovulation were classified as MOV+ and those that did not as MOV-. The IFO was recorded as number of d between calving and first ovulation, up to 60 d postpartum. All data were analyzed using SAS (version 9.3, SAS Institute, Cary, NC).

Pearson’s correlation coefficients (PROC CORR) were used to test the strength of the relationship between IFO and production variables including DMI, DMIBW, MY, BW1, BW28, the difference in BW between d 1 and 28 (BWD), BCS1, BCS28, and the difference in BCS between d 1 and 28 (BCSD). A survival analysis (PROC PHREG) was conducted to compare the IFO between lactations and the hazard ratio for failure to ovulate before 60 d postpartum.

Data were analyzed using the GENMOD procedure to examine the effect of experiment and lactation on ovarian function (i.e. OV, OC and MOV); model specifications included a binomial distribution, logit link function and an exchangeable correlation structure. Mixed model analyses
(MIXED PROC) were conducted to examine associations between ovarian function and production variables, and between ovarian function and blood metabolites (NEFA, BHBA, INS, GLU and IGF-1) including lactation, wk, and lactation by wk interaction as fixed effects with dietary treatment nested within experiment as a random effect to account for differences among studies. Treatment differences were considered significant if $P \leq 0.05$ and as a tendency if $0.05 < P \leq 0.10$.

RESULTS

Ovulation Interval

Cows in their 1st lactation had an IFO of $35.6 \pm 2.0\text{ d}$, and a 12.7% failure to ovulate before 60 d. For 2nd lactation cows, IFO was $31.6 \pm 1.9\text{ d}$ and failure to ovulate was 9.6%, whereas for 3rd+ lactation cows, IFO was $30.6 \pm 1.9\text{ d}$ and failure to ovulate was 1.9%. In the overall model, there was a tendency for difference between lactations and failure to ovulate ($P = 0.10$), which was due to 1st lactation cows being 1.5 times more likely to fail to ovulate before 60 d (Hazard Ratio = 0.66; $P = 0.03$) compared to 3rd lactation cows (Figure 1). For 1st lactation cows there was a negative correlation between IFO and BW1 ($r = -0.39; P = 0.002$), BCS1 ($r = -0.26; P = 0.04$), BW28 ($r = -0.43; P = 0.0004$), and BCS28 ($r = -0.36; P = 0.004$) i.e., the higher the BW and BCS, the shorter the IFO. Second lactation cows had similar results with negative correlations between IFO and BW1 ($r = -0.27; P = 0.05$), BCS1 ($r = -0.30; P = 0.03$), BW28 ($r = -0.33; P = 0.02$), and BCS28 ($r = -0.30; P = 0.03$). For 3rd+ lactation cows there was a negative correlation between IFO and DMI ($r = -0.38; P = 0.004$) and DMIBW ($r = -0.36; P = 0.007$) and a positive correlation between IFO and BWD ($r = -0.30; P = 0.03$). For all lactations, there was no correlation between IFO and MY ($P = 0.54$).
Ovulation

There was no relationship between lactation number and the incidence of ovulation before d 35 postpartum \((P = 0.29)\). Cows that were OV+ tended to have higher concentrations of GLU (56.5 vs. 54.9 mg/dL, \(P = 0.09\); Figure 2a), and there was an interaction between OV, wk and lactation \((P = 0.007)\), in which OV+ cows in their 3\textsuperscript{rd+} lactation had higher GLU concentrations in wk 1 (52.5 vs. 47.5 mg/dL) and wk 4 (56.2 vs. 50.1 mg/dL) compared to 3\textsuperscript{rd+} lactation OV- cows (Figure 4a). Cows that were OV+ had lower concentrations of NEFA (563 vs. 645 mEq/L, \(P = 0.01\); Figure 2c), and there was an interaction between OV and lactation \((P = 0.04)\) where only OV+ cows in their 3\textsuperscript{rd+} lactation had significantly lower concentrations of NEFA (570 vs. 765 mEq/L) compared to OV- cows (Figure 3a). Additionally, there was an interaction between OV, wk and lactation for NEFA \((P = 0.02)\), in which 1\textsuperscript{st} lactation OV+ cows had lower NEFA concentrations in wk -1 (429 vs. 581 mEq/L) and 3\textsuperscript{rd+} lactation OV+ cows had lower NEFA concentrations from wk 1 to wk 4 compared to 1\textsuperscript{st} and 3\textsuperscript{rd+} OV- cows, respectively (Figure 4b). Cows that were OV+ had lower concentrations of BHBA (9.67 vs. 13.1 mg/dL, \(P = 0.008\); Figure 2b), and there was an interaction between OV, wk and lactation \((P = 0.02)\), in which 1\textsuperscript{st} lactation OV+ cows had lower BHBA concentrations in wk -1 (6.34 vs. 9.18 mg/dL) and 3\textsuperscript{rd+} lactation OV+ cows had lower BHBA concentrations in wk 1, 2 and 4 compared to 1\textsuperscript{st} and 3\textsuperscript{rd+} OV- cows, respectively (Figure 4c). Cows that were OV+ had higher IGF-1 concentrations (53.4 vs. 47.7 ng/dL, \(P = 0.04\); Figure 2d), and there was a tendency for interaction between OV and wk \((P = 0.06)\) where OV+ cows had greater IGF-1 concentrations in wk -1 and wk 1, and tended to have greater IGF-1 concentrations in wk 2 compared to OV- cows (Figure 3b). There was no association of INS with ovulation by 35 d \((P = 0.59)\). Cows that were OV+ had a higher BCS1 and BSC28, as well as a higher BW28 and lower BWD compared to OV- cows (Table 2). There
was no association of BW1 and BCSD with ovulation by 35 d (Table 2). Cows that were OV+ had greater DMI and DMIBW, and there was a weak tendency for OV+ cows to have higher MY compared to OV- cows (Table 2). There was also an interaction between OV, wk and lactation for MY, in which OV+ cows in their 1\textsuperscript{st} lactation had greater milk yield ($P = 0.009$) compared to 1\textsuperscript{st} lactation OV- cows in wks 1 (24.0 vs. 21.4 kg/d) and 2 (27.7 vs. 25.0 kg/d; Figure 4).

**Ovarian Cysts**

There was a difference in incidence of OC ($P = 0.02$) between lactations, in which 1\textsuperscript{st} lactation cows had a lower incidence (3\%) of OC than did 2\textsuperscript{nd} (15\%) and 3\textsuperscript{rd+} (17\%) lactation cows. There was no main association between OC and blood metabolites (Figure 2c); however, there was an interaction for NEFA ($P = 0.05$) and tendency for interaction for GLU ($P = 0.06$) with OC, wk and lactation. For GLU, the only difference occurred in 1\textsuperscript{st} lactation cows where OC+ cows tended to have greater GLU concentrations in wk 2 compared to OC- (69.6 vs. 57.4 mg/dL). For NEFA, 1\textsuperscript{st} lactation OC+ cows tended to have lower NEFA concentrations in wk 1 (190 vs. 518 mEq/L) and had higher concentrations in wk 1 (1348 vs. 794 mEq/L) and wk 2 (1156 vs. 629 mEq/L) compared to OC- cows. There was no association of BW and BCS with incidence of OC, with the exception of a tendency for OC+ cows to have greater BW28 compared to OC- cows (Table 2). Cows that were OC+ had greater MY and tended to have greater DMI compared to OC- cows, with no main association between OC and DMIBW (Table 2). There was an interaction between OC and lactation for both DMI ($P = 0.04$) and DMIBW ($P = 0.03$), in which OC+ cows in their 2\textsuperscript{nd} lactation had greater DMI (19.8 vs. 16.5 kg/d) and DMIBW (3.33 vs. 2.81 \%) compared to 2\textsuperscript{nd} lactation OC- cows. For MY, there tended to be an interaction between OC, wk and lactation ($P = 0.08$). Cows that were OC+ in their 2\textsuperscript{nd} lactation
had greater MY in wk 2 (40.9 vs. 36.7 kg/d), wk 3 (44.3 vs. 38.6 kg/d) and wk 4 (45.6 vs. 40.0 kg/d).

**Multiple Ovulations**

There was a difference in incidence of MOV ($P = 0.006$) between lactations, in which cows in the 3rd+ lactation had a higher incidence of MOV (46%) than 2nd (21%) and 1st (21%) cows. The only main association between MOV and blood metabolites was a tendency for MOV+ cows to have greater IGF-1 concentrations compared to MOV- cows (55.5 vs. 49.9 ng/mL, $P = 0.07$; Figure 2d). The interaction between MOV, wk and lactation was significant for GLU ($P = 0.04$) and BHBA concentrations ($P = 0.01$). For GLU, there was no difference between MOV+ and MOV- cows for any lactation at any time point. For BHBA, 2nd lactation MOV+ cows had greater concentrations of BHBA in wk -1 compared to 2nd lactation MOV- cows (9.98 vs. 6.92 mg/dL) and 3rd+ lactation MOV+ cows had a lesser concentration of BHBA in wk 4 compared to 3rd+ lactation MOV- cows (11.1 vs. 19.7 mg/dL; Figure 5a). There was no main association between incidence of MOV and any of the production variables. There was an interaction between MOV, wk and lactation for MY ($P = 0.02$); however, there was no significant difference between MOV+ and MOV- cows within lactation at any time point (Figure 5b).

**DISCUSSION**

The objective of this retrospective study was to determine the relationships among plasma metabolites and production variables with parity and ovarian function, specifically the IFO after calving, and incidence of OC and MOV during the early postpartum period. In the data set, 50.9 % of cows ovulated before d 35 postpartum and 91.7% ovulated before d 60
postpartum. This is in agreement with the results of Jeong et al. (2015), who reported 58% of cows resumed cyclicity by 6 wk postpartum, and Ambrose and Colazo (2007), who reported 90% of cows resumed cycling by 9 wk postpartum. Reducing the IFO is advantageous as it is associated with a shortened interval to conception (Wathes et al. 2007) and increased conception rates (Butler 2000). In the current study, there was no difference among lactations in IFO with a mean interval of 32.6 d; however, 1st lactation cows were at a greater risk of failing to ovulate before d 60 postpartum. Previous studies have reported that primiparous cows have a longer IFO (Balogh et al. 2009; Zhang et al. 2010; López-Helguera et al. 2016), as well as a longer interval to involution of the gravid uterine horn (Zhang et al. 2010). In 1st and 2nd lactation cows, IFO decreased with an increased BW and BCS on d 1 and 28 postpartum, whereas reduced BWD was associated with a decreased IFO in 3rd+ lactation cows. These results indicate that in 1st and 2nd lactation, cows may need to calve with a higher BW and BCS to reduce IFO, whereas reducing the extent of fat mobilization and weight loss is more important to reduce IFO in older cows. In this regard, a review by Roche et al. (2009) reported that the ideal pre calving BCS is 3 to 3.25 and coming into calving with a higher amount of body fat, i.e. >3.5 BCS, increases fat mobilization and reduction of BCS post calving. Both the 1st and 3rd+ lactation cows had an average BCS at calving of 3.55, which may be more detrimental to the older cows with increased MY, as evidenced by the increase in postpartum BHBA concentrations. Although there was no association between IFO and MY for all cows in the current study, 3rd+ lactation cows had a decreased IFO with increased DMI and DMIBW. Dry matter intake has been reported to have a positive effect on IFO and is a primary factor in establishing a positive energy balance (Patton et al. 2007), which is more important in cows with higher parities and MY, due to a higher risk of NEBAL in the transition period. Promoting BW/BCS maintenance and DMI is important for
reducing the IFO and improving fertility and ensuring that cows are not over conditioned at calving can help prevent excessive fat mobilization and loss of body condition in early lactation.

In the current study, there was no difference among lactations and incidence of ovulation before d 35 postpartum. Cows that did not ovulate before d 35 had higher plasma NEFA and BHBA concentrations, indicative of NEBAL and excessive fat mobilization. Butler (2000) reported that NEBAL in early lactation is highly correlated with days to first ovulation and the presence of NEFA reduces the proliferation of granulosa cells and delay the maturation of the oocyte in vitro (Jorritsma et al. 2004), which might also have negative consequences on fertility if ovulation occurs. However, in the current study, for both NEFA and BHBA there was an interaction between OV, wk and lactation, which was significant for 1st and 3rd lactation cows. While previous studies have reported that cows with prolonged IFO had increased NEFA and BHBA concentrations (Jeong et al. 2015) and increased NEBAL (Patton et al. 2007), looking at different parities separately may alter the management strategies for each group. In the current study, cows in the 1st lactation that were OV- had higher NEFA and BHBA concentrations only in week -1 compared to 1st lactation OV+ cows. This supports the conclusion made earlier for IFO that it is important for primiparous cows to calve at a higher BW, and perhaps be fed a higher energy diet prepartum to avoid NEBAL, to ensure a timely postpartum ovulation. However, Wathes et al. (2007) reported the opposite, in which primiparous cows had a negative association between BHBA and IFO prepartum and no association of prepartum BCS on IFO. The authors inferred that increased tissue mobilization prepartum reduced IFO in primiparous cows, in contrast to our results, which is unexpected. Cows in the 3rd+ lactation that were OV- had higher NEFA and BHBA concentrations postpartum, which was supported by the result of Wathes et al. (2007), indicating that reducing NEBAL in early lactation for older cows is
important to reduce IFO. In the current study, OV+ cows had higher plasma IGF-1 concentrations from wk -1 to 2 compared to OV– cows, which is supported by results from previous studies (Taylor et al. 2004; Patton et al. 2007; Castro et al. 2012). Cows in a severe NEBAL have reduced circulating IGF-1 concentrations and reduced expression of growth hormone (GH) receptors in the liver (Fenwick et al. 2008), which reduces the stimulatory effect that IGF-1 has on growth and differentiation of the follicle and can delay return to cyclicity (Spicer et al. 1993). The only association between GLU and OV in the current study occurred in 3rd+ lactation cows, in which OV+ cows had higher GLU in wk 1 and 4. While previous studies reported no relationship between GLU and resumption of cyclicity (Patton et al. 2007; Castro et al. 2012; Jeong et al. 2015), a higher GLU in wk 1 may indicate reduced fat mobilization and in wk 4 may indicate that OV+ cows recovered from NEBAL faster, in the older, higher producing cows that are more susceptible to NEBAL. In the current study, cows that did not ovulate before d 35 postpartum had reduced DMI and DMIBW and increased BWD, which all would indicate an increased NEBAL. Shrestha et al. (2005) reported that a decrease in BCS of 1 unit or more at wk 7 postpartum increased the occurrence of delayed first ovulation. There was only a weak association of OV and MY, where previous studies have found no association between OV and MY (Shrestha et al. 2005; Castro et al. 2012) and an association with peak MY and delayed return to cyclicity (Taylor et al., 2004). In the current study, OV+ cows in their 1st lactation had greater milk yield than OV- 1st lactation cows and as DMI and MY were positively correlated, this may indicate that 1st lactation cows that were eating more and thus had a greater MY had a better chance of a shorter IFO. From these results we can infer that reducing NEBAL and promoting DMI is important for a timely return to cyclicity, particularly in older cows, and promoting DMI both pre and postpartum is important for primiparous cows.
Cystic ovaries are a common cause of impaired fertility as they reduce the pregnancy per AI and increase calving interval (Vanholder et al. 2006). Cysts are generally thought to be associated with dysfunction in the hypothalamic-pituitary-ovarian (HPG) axis, in which the pre-ovulatory LH surge is absent, insufficient in magnitude, or mistimed and the dominant follicle fails to ovulate (Peter 2004; Vanholder et al. 2006). In the current study, the incidence of OC was 11.2 %, which is within the range of 5.6 to 18.5 % reported in previous studies (Zulu et al. 2002; Butler et al. 2006; Jahani-Moghadam et al. 2015). Cystic ovaries are seen more frequently in multiparous cows (Peter 2004; López-Helguera et al. 2016) and the results from the current study also indicate that cows in the 3rd + lactation had a higher incidence of OC. In a review by Vanholder et al. (2006), NEBAL was identified as a potential stressor for OC, in which prolonged exposure to high concentrations of NEFA may be toxic, and in addition to low circulating IGF-1 and INS, may interfere with follicular development (Spicer et al. 1995). The current study observed no significant difference in GLU, BHBA, INS or IGF-1 concentrations between +OC and –OC cows. Butler et al. (2006) also reported no strong relationship between any of the above metabolites and OC formation. However, in the current study OC+ 1st lactation cows had higher NEFA concentrations, indicating higher NEBAL, in wks 1 and 2 compared to OC- cows. Although OC+ 1st lactation cows had a numerical increase in milk production of 5.5 kd/d in wks 1 and 2 compared to OC- 1st lactation cows, the incidence of OC was 3% in 1st lactation and results should be interpreted with caution. Overall, +OC cows had higher MY and DMI compared to OC- cows. Previous studies have reported that higher milk production increases the risk for OC (Hooijer et al. 2003; Gábor et al. 2016; López-Helguera et al. 2016). Based on the tendency for interaction in the current study, the relationship between OC, MY and DMI was only significant for 2nd lactation cows. This could be due to 2nd lactation cows having
the greatest DMI and MY, which was significantly different from 1\textsuperscript{st} lactation but only numerically greater than 3\textsuperscript{rd+} lactation cows. Increased DMI, contributing to increased MY, also leads to an increased metabolic rate and clearance of steroid hormones (Sangsritavong et al. 2002), which could contribute to OC formation. Sartori et al. (2004) observed that lactating cows have lower peak and circulating estradiol and progesterone concentrations, which could delay the LH surge and account for ovulation failure and OC formation. The results of the current study indicate that OC formation has a strong relationship with high milk production and a weaker relationship with DMI, particularly with the highest producing group, but not a clear relationship with NEBAL.

Multiple ovulation occurs when more than one follicle is selected for continued growth (codominance) from the pool of growing follicles, resulting in more than one ovulation. The direct effect of MOV on reproductive outcomes is varied, with studies reporting no effect (Kusaka et al. 2017), increased conception rates (Colazo et al. 2014; Mussard et al. 2007), and increased pregnancy loss (Colazo et al. 2014; Stevenson et al. 2006). However, MOV increases the risk for twin pregnancy (Colazo et al. 2014) which has been reported to increase calving issues, uterine disease, and pregnancy loss, as well as reduce conception rates and mean productive lifespan (Andreu-Vázquez et al. 2012). In the current study, MOV at first ovulation after calving occurred in 29 % of cows, which is between the 11.6 % reported by Kuska et al. (2017) and 35.7 % reported by Sartori et al. (2004). Incidence of MOV was higher in 3\textsuperscript{rd+} lactation cows compared to 1\textsuperscript{st} and 2\textsuperscript{nd} lactation (46 vs. 21 vs. 21 %), which has also been reported in other studies (López-Gatius et al. 2005; López-Helguera et al. 2016; Kusaka et al. 2017). In the current study, no main relationship between any production variables and MOV was observed, and the interaction between MOV, wk and lactation for MY indicated no
significant difference between MOV+ and MOV- cows. Previous studies have reported conflicting results, with increased MY decreasing MOV risk (López-Gatius et al. 2005), increasing MOV risk (Kusaka et al. 2017), or having no effect (López-Helguera et al. 2016). In a study that measured milk production specifically around the time of follicle selection, incidence of MOV was higher in cows with higher milk production (Lopez et al. 2005), but incidence of MOV in the first ovulation postpartum was not related to MY. The first ovulation following anestrous occurs in a low progesterone environment, which increases concentrations of E2 and LH (Cerri et al. 2011a), as well as increased incidence of MOV (Cerri et al. 2011b). This is also likely why incidence of OC increases the likelihood of MOV (López-Helguera et al. 2016). In the current study, there was a tendency for MOV+ cows to have greater IGF-1 concentrations compared to MOV-, but no interaction with lactation. In an experimental herd in which cows were selected for twinning, the primary difference between twinner and non-twinner cows was higher blood and follicular fluid concentrations of IGF-1 in twinner cows (Echternkamp et al. 1990; Echternkamp et al. 2004). As mentioned earlier, IGF-1 stimulates proliferation, differentiation and steroidogenesis in follicles (Spicer et al. 1993), indicating possible mechanisms that could be facilitating the incidence of MOV in the first ovulation. The other difference between MOV+ and MOV- cows observed was for MOV+ cows to have greater BHBA concentrations in wk -1 for the 2nd lactation and lesser concentrations of BHBA in wk 4 for the 3rd lactation. In the 3rd lactation cows, increased NEBAL may reduce the production of IGF-1 by the liver (Fenwick et al. 2008), which would contribute to a lower incidence of MOV with increased levels of BHBA. From our data set, we are unable to explain why increased tissue mobilization prepartum would increase incidence of MOV in 2nd lactation cows; further study in
this area is required. According to the results there may be a connection between MOV and NEBAL; however, the greatest association is with older, higher producing cows.

In conclusion, there was no difference among lactations in IFO, primiparous cows were at a greater risk of failing to ovulate before d 60 postpartum; conversely, cows in the 3\textsuperscript{rd} or greater lactation were at a higher risk for OC and MOV. A higher NEBAL, based on metabolites and production variables, was the primary factor for cows failing to ovulate before d 35 postpartum. Ovulation at this time was also correlated with BW and BCS in 1\textsuperscript{st} and 2\textsuperscript{nd} lactation cows, whereas DMI and BWD were correlated with ovulation in 3\textsuperscript{rd}+ lactation cows. Negative energy balance was not strongly related with OC, but increased DMI and MY, particularly in 2\textsuperscript{nd} lactation cows, was associated with a higher incidence of OC. Incidence of MOV was increased in older cows and there tended to be a positive relationship between IGF-1 and MOV. Additionally, BHBA was associated with MOV in the 2\textsuperscript{nd} lactation prepartum and in the 3\textsuperscript{rd} lactation in wk 4 postpartum. While this may imply that MOV is related to NEBAL in the transition period, the association was specific to only 2 time points and should be interpreted with caution. These results indicate that reducing NEBAL in early lactation, and prepartum for primiparous cows, is essential for a timely return to cyclicity after calving, but incidence of OC and, to some extent MOV, in high-producing dairy cows may be independent of energy balance in the transition period.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Description of 3 studies included in the combined data set for the current retrospective study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Lact (n)</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Lact (n)</th>
<th>3&lt;sup&gt;rd+&lt;/sup&gt; Lact (n)</th>
<th>MY&lt;sup&gt;a&lt;/sup&gt; (kg/d)</th>
<th>DMI&lt;sup&gt;b&lt;/sup&gt; (kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2006</td>
<td>25</td>
<td>17</td>
<td>27</td>
<td>33.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>2008</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>32.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>2010</td>
<td>22</td>
<td>23</td>
<td>15</td>
<td>35.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: Means within a column not sharing a lowercased italic letter differ significantly at the $P < 0.05$ level.

<sup>a</sup>MY = average milk yield.

<sup>b</sup>DMI in the postpartum period.
Table 2. The relationship between production variables and incidence of ovulation (OV) before 35 d postpartum and ovarian cyst formation (OC) before first ovulation postpartum.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OV-</th>
<th>OV+</th>
<th>SEM</th>
<th>P-Value</th>
<th>OC-</th>
<th>OC+</th>
<th>SEM</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW1, kg</td>
<td>635</td>
<td>648</td>
<td>5.91</td>
<td>0.12</td>
<td>640</td>
<td>664</td>
<td>15.3</td>
<td>0.12</td>
</tr>
<tr>
<td>BCS1, kg</td>
<td>3.41</td>
<td>3.53</td>
<td>0.12</td>
<td>0.03</td>
<td>3.46</td>
<td>3.48</td>
<td>0.15</td>
<td>0.86</td>
</tr>
<tr>
<td>BW28</td>
<td>565</td>
<td>587</td>
<td>6.94</td>
<td>0.004</td>
<td>574</td>
<td>600</td>
<td>14.9</td>
<td>0.08</td>
</tr>
<tr>
<td>BCS28</td>
<td>2.80</td>
<td>2.94</td>
<td>0.05</td>
<td>0.01</td>
<td>2.86</td>
<td>3.00</td>
<td>0.10</td>
<td>0.21</td>
</tr>
<tr>
<td>BWD, kg</td>
<td>70.4</td>
<td>-59.1</td>
<td>7.48</td>
<td>0.03</td>
<td>-64.7</td>
<td>-60.9</td>
<td>6.92</td>
<td>0.70</td>
</tr>
<tr>
<td>BCSD</td>
<td>-0.61</td>
<td>-0.59</td>
<td>0.14</td>
<td>0.67</td>
<td>-0.59</td>
<td>-0.49</td>
<td>0.17</td>
<td>0.31</td>
</tr>
<tr>
<td>DMI, kg/d</td>
<td>14.0</td>
<td>15.8</td>
<td>0.44</td>
<td>&lt;0.001</td>
<td>14.8</td>
<td>16.3</td>
<td>0.88</td>
<td>0.09</td>
</tr>
<tr>
<td>DMIBW, %</td>
<td>2.39</td>
<td>2.59</td>
<td>0.07</td>
<td>0.004</td>
<td>2.48</td>
<td>2.65</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>MY, kg/d</td>
<td>33.0</td>
<td>34.3</td>
<td>0.77</td>
<td>0.10</td>
<td>33.5</td>
<td>36.3</td>
<td>1.46</td>
<td>0.05</td>
</tr>
</tbody>
</table>

BW1 = body weight at 1 DIM; BW28 = body weight at 28 DIM; BCS 1 = body condition score at 1 DIM; BCS 28 = body condition score at 28 DIM; BWD = difference in body weight between 1 and 28 DIM; BCSD = difference in body condition score between 1 and 28 DIM; DMI = dry matter intake; DMIBW = DMI relative to BW; MY = milk yield.

OV- = failed to ovulate before 35 DIM; OV+ = ovulated before 35 DIM.
OC- = no occurrence of ovarian cyst; OC+ = occurrence of ovarian cyst.
SEM = standard error of mean.
**Figure 1.** Survival analysis of interval to first ovulation (IFO) between 1\textsuperscript{st} (▲), 2\textsuperscript{nd} (■) and 3\textsuperscript{rd+} (●) lactations.

Average IFO was not different between lactations ($P > 0.10$), but failure to ovulate before 60 d postpartum tended to differ ($P = 0.10$) between lactations. The 1\textsuperscript{st} lactation cows were 1.5 times more likely to fail to ovulate compared to 3\textsuperscript{rd+} lactation cows (Hazard ratio $= 0.66$; $P = 0.03$).

**Figure 2.** Associations between blood metabolites and ovarian function.

In each figure, OV = incidence of ovulation by 35 DIM, OC = incidence of ovarian cysts and MOV = incidence of multiple ovulations. The light colored bars represent the failure of occurrence (-) and the dark bars the occurrence of each variable (+). For each comparison a * denotes a significant difference ($P < 0.05$) and a † denotes a tendency for difference ($0.05 < P < 0.10$).

**Figure 3.** Interaction of incidence of ovulation by 35 DIM (OV) with lactation (L1, L2, L3+) for blood NEFA concentrations and with wk relative to calving (-1 to 4) for blood IGF-1 concentrations.

a) The light colored bars represent the failure of OV (-) and the dark bars the occurrence of OV (+). There was an interaction between OV and lactation ($P = 0.03$), in which only OV+ cows in their 3\textsuperscript{rd+} lactation had lower NEFA concentrations than OV- 3\textsuperscript{rd+} lactation cows. b) There was a tendency for interaction between OV and wk ($P = 0.06$) where OV+ cows had greater IGF-1 concentrations in wk -1 and wk 1, and tended to have greater IGF-1 concentrations in wk 2 compared to OV- cows. For each comparison a * denotes a significant difference ($P < 0.05$) and a † denotes a tendency for difference ($0.05 < P < 0.10$).
**Figure 4.** Associations of blood metabolites and milk yield (MY) with the incidence or failure of ovulation by 35 DIM (OV+ vs. OV-) and interactions with lactation (L1, L2, L3+) and week relative to calving (-1 to 4).

a) There was an interaction \((P = 0.007)\) between OV, wk and lactation in which glucose concentrations in 3rd+ lactation OV+ cows were higher in wks 1 and 4 compared to 3rd+ lactation OV- cows. b) The interaction between OV, wk and lactation was significant \((P = 0.02)\) for NEFA, in which 1st lactation OV+ cows had lower NEFA in wk -1 and 3rd+ lactation OV+ cows had lower NEFA wk 1 to wk 4. c) There was an interaction \((P = 0.02)\) between OV, wk and lactation, in which 1st lactation OV+ cows had lower BHBA in wk -1 and 3rd+ lactation OV+ cows had lower BHBA in wk 1, 2 and 4. d) There was a significant interaction for MY \((P = 0.009)\), in which 1st lactation OV+ cows had a greater MY in wks 1 and 2 than 1st lactation OV- cows.

**Figure 5.** Association of BHBA and milk yield (MY) with the incidence or lack of multiple ovulations (MOV+ vs. MOV-) and interactions with lactation (L1, L2, L3+) and wk relative to calving (-1 to 4).

a) There was a significant interaction for BHBA \((P = 0.01)\), in which MOV+ 2nd lactation cows had greater BHBA in wk -1 than MOV- 2nd lactation cows. Also, MOV+ 3rd+ lactation cows had less BHBA in wk 4 than MOV- 3rd+ lactation cows. b) Despite a significant interaction \((P = 0.02)\) for MY and MOV, there was no difference between MOV+ and MOV- for any lactation at any time.
Figure 2.
Figure 3.
Figure 4.
Figure 5.