A randomized controlled trial on the effect of incomplete milking during the first 5 days in milk on reproductive performance of dairy cows

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ABSTRACT

The main objective of the current study was to measure the effect of incomplete milking on luteal activity and on pregnancy hazard. We also aimed to study the effect of early-lactation hyperketonemia (i.e., β -hydroxybutyrate blood concentration ≥ 1.4 mmol/L during the first 3 wk in milk) on those reproductive outcomes. Multiparous Holstein cows ($n = 853$) from 13 commercial herds were enrolled in a randomized controlled trial. Cows were assigned to a control or a treatment group, incompletely milked (10–14 L of milk collected/d) from 1 to 5 DIM. Blood samples were collected once a week during weeks in milk 1 to 3 for β -hydroxybutyrate blood concentration, and a threshold of 1.4 mmol/L was used to define hyperketonemia. During weeks in milk 5 and 7, cows were sampled for progesterone blood concentration, and a threshold of 1 ng/mL was used to define luteal activity. Reproductive information and culling dates were obtained through herd records. Logistic regression models and survival analyses were used to assess the effect of treatment on luteal activity and on pregnancy hazard, respectively. Analogous models were used to investigate the effect of early-lactation hyperketonemia on reproductive outcomes. The odds of luteal activity for incompletely milked cows were 1.1 (95% confidence interval: 0.72–1.7) times those of conventionally milked cows. The effect of treatment on pregnancy hazard varied as a function of time, parity, and start of the breeding period. In second-parity cows that started the breeding period <55 d in milk, the pregnancy hazard (95% confidence interval) in incompletely milked cows was 576.3 (240.0–1,383.7), 36.9 (18.9–72.1), 6.8 (3.3–13.8), 2.5 (1.0–5.9), and 0.13 (0.07–0.26) times that of conventionally milked cows at 1 to 21, 22 to 43, 44 to 65, 66 to 87, and >87 d after the

voluntary waiting period, respectively. The treatment did not have an effect on pregnancy hazard in cows in third parity or greater or in those starting the breeding period ≥ 55 d in milk. Early-lactation hyperketonemia was not associated with any of the reproductive outcomes. In conclusion, the incomplete milking protocol had no effect on luteal activity and had a positive effect on pregnancy hazard in second-parity cows in herds with a short voluntary waiting period (<55 d). We did not observe an effect of early-lactation hyperketonemia on luteal activity or on pregnancy hazard.

Key words: dairy cattle, incomplete milking, pregnancy, luteal activity

INTRODUCTION

Negative energy balance (NEB) is considered to be an important reason for poor reproductive performance. Some studies have shown that cows with excessive NEB have longer interval from calving to first ovulation (Beam and Butler, 1998; Bossaert et al., 2008) and lower pregnancy risk (Walsh et al., 2007b; Wathes et al., 2007; Ospina et al., 2010). Therefore, limiting NEB in early lactation is important to ensure good subsequent reproductive performance.

The underlying mechanism of this relationship is not fully understood, but some aspects of it are documented. The lower blood glucose concentration resulting from NEB seems to inhibit pulse secretion of pituitary LH (Rossi et al., 2008; Knop and Cernescu, 2009), which subsequently prevents follicular ovulation (Boland et al., 2001). Insulin-like growth factor I, which acts synergistically with LH and stimulates steroidogenesis, is also inhibited (Clarenburg, 1992; Lucy et al., 2001; Fenwick et al., 2008); this negatively affects ovulation, which in turn leads to a reduction of cows' ability to conceive (Butler, 2003).

During NEB, body tissue reserves are mobilized as a result of intense galactopoiesis combined with an insufficient dietary intake. Several researchers hypothesized that increasing food energy density could limit the

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NEB (Vandehaar et al., 1999; Dewhurst et al., 2000), but only some investigated the effects of temporarily restricting milk production on NEB (McNamara et al., 2008; Loiselle et al., 2009). Reducing milk frequency during the first month in lactation had positive effects on interval to first ovulation in Patton et al. (2006). A novel strategy to temporarily reduce milk output without altering milking frequency is to milk cows incompletely during the first 5 DIM (Carbonneau et al., 2012; Morin et al., 2018).

Previously, we demonstrated that incomplete milking for the first 5 DIM leads to lower odds of hyperketonemia up to 17 DIM (Morin et al., 2018) and a higher elimination rate of IMI (Krug et al., 2018a) without subsequent effects on milk yield and composition throughout the lactation (Krug et al., 2018b). We hypothesized that reproductive performance could be improved by limiting NEB through incomplete milking (Morin et al., 2018). Therefore, the aim of the current study was to use the large data set generated by Morin et al. (2018) and Krug et al. (2018a, b), to evaluate the effect of incomplete milking during the first 5 DIM on reproductive performance. Our specific objectives were to measure, on that same population of cows, the effects of incomplete milking during the first 5 DIM on luteal activity and on pregnancy hazard. Second, because the literature is sometimes inconsistent on this matter (Raboisson et al., 2014), we aimed to study the effect of early-lactation hyperketonemia (i.e., blood BHB concentration ≥ 1.4 mmol/L during the first 3 wk in milk) on those same reproductive outcomes.

MATERIALS AND METHODS

The study protocol was approved by the Animal Ethics Committee of the University of Montreal (rech-1701). The complete research protocol is described in detail in Morin et al. (2018).

Sample Size Calculations

The original study was designed to investigate, using a randomized controlled trial (**RCT**), the effect of incomplete milking on odds of hyperketonemia (Morin et al., 2018) and on other outcomes such as ketone blood concentration, culling hazard, milk production, infectious diseases, and fertility (Krug et al., 2018a, b). Sample size calculations were computed for all these different outcomes, but odds of hyperketonemia was the outcome requiring the largest sample size ($n/\text{group} = 400$), and therefore it determined the number of animals used in the RCT described in this study.

Nevertheless, using the POWER procedure in SAS 9.4 (SAS Institute Inc., Cary, NC), we calculated the

minimal difference in proportion of cows exhibiting luteal activity that could be detected using a 95% confidence level and 80% power. Using a sample size of 400 animals per group and expecting a prevalence of luteal activity of 74% around weeks in milk 5 and 7 in conventionally milked animals (Dubuc et al., 2012), we estimated the minimal detectable difference between groups (i.e., incompletely vs. conventionally milked) at 8.5 percentage points. Expecting a prevalence of 18% (144/800) of early-lactation hyperketonemia (Dubuc and Denis-Robichaud, 2017) and 74% of luteal activity in normoketonemic animals (Dubuc et al., 2012), the minimal detectable difference between cows with or without hyperketonemia was 12.5 percentage points. Moreover, to calculate sample size and power calculations for time to pregnancy (survival analysis), the package *epiR* 0.9-93 (Stevenson et al., 2012) from RStudio 1.1.383 (R Core Team, 2013) was used. The minimal detectable pregnancy hazard ratio that could be detected between conventionally and incompletely milked cows was estimated at 1.2.

Herds, Animals, and Experimental Design

This study was an RCT conducted in 13 commercial dairy farms selected by convenience. There were several requirements for selection of farms: being near Saint-Hyacinthe (Québec, Canada), agreeing to apply the research protocol, participating in a DHIA program, having computerized health records, having a milking system that allows for measurement of milk collected in real time, and agreeing to share herd health and DHIA information with the research team. Herds had an average (SD) of 103 (51) lactating cows and a 305-d milk yield of 9,973 (660) kg/cow. Cows were mainly housed in freestall barns (9/13 herds). Milking frequency was 3 times a day in 1 farm, 1 herd used an automatic milking system, and the other herds (11/13 herds) milked cows twice a day. The majority of herds fed TMR (11/13 herds) and used monensin supplementation (11/13 herds). Cows were bred by AI after a voluntary waiting period (**VWP**) of 40 d on 1 farm ($n = 37$ cows included in this RCT), 50 d on 6 farms ($n = 440$ cows), 55 d on 2 farms ($n = 111$ cows), 60 d on 3 farms ($n = 237$ cows), and 70 d on 1 farm ($n = 28$ cows). Eight farms systematically enrolled their animals in an ovulation synchronization protocol: Double-Ovsynch protocol was used on 4 farms, Presynch-Ovsynch protocol was used on 3 farms, and Ovsynch was used along with an intravaginal progesterone (P4)-releasing insert (CIDR 1380, Zoetis Canada Inc., Kirkland, QC, Canada) on another farm.

Holstein cows of second parity or greater were enrolled 1 mo before expected calving in a control or a

treatment group. Cows were randomly allocated to each group using a random number generator. Cows in the control group were milked conventionally according to their herds' milking practices. In 6 herds, farmers reported applying incomplete milking during the first 2 DIM as part of their regular milking procedures. The treatment was applied by dairy producers and farm personnel and consisted of limiting the quantity of milk collected to a maximum of 10, 12, and 14 L/d on DIM 1 to 3, 4, and 5, respectively. The thresholds chosen were derived from Carbonneau et al. (2012) but slightly modified so they would be more easily applied by farmers. The herd that used an automatic milking system maintained cows in a separate pen during the first 5 DIM, where they were milked manually twice a day. On the remaining farms, milking frequency was not altered; therefore, the maximum amount allowed per day was simply divided by milking frequency. Producers were not blinded to group allocation.

Samples and Data Collection

Farms were visited weekly by an animal health technician and 2 veterinarians (authors C. K. and P.-A. M.) from November 2013 until February 2015, and data were collected until the end of the lactation of the last cow enrolled in the RCT (October 2015). Both technician and veterinarians were blinded to treatment assignment of animals.

Early-Lactation Hyperketonemia. Blood samples (coccygeal vessels; 10 mL) were collected using an uncoated BD Vacutainer (Becton Dickinson, Franklin Lakes, NJ) once a week during the first 3 wk in milk starting at least 36 h after calving. Blood samples were immediately analyzed on farm with a Precision Xtra handheld device (Abbott Diabetes Care, Alameda, CA) for measurement of BHB concentration. Early-lactation hyperketonemia was considered if blood BHB concentration was ≥ 1.4 mmol/L (Duffield et al., 2009; Iwersen et al., 2009). Only animals with at least 3 blood samples were used for the analysis. With that information, we created a categorical variable for early-lactation hyperketonemia status with 4 levels: 1 = never experiencing hyperketonemia; 2 = hyperketonemia in 1 out of 3 wk; 3 = hyperketonemia in 2 out of 3 wk; 4 = hyperketonemia in all weeks.

Luteal Activity. Blood samples (coccygeal vessels; 10 mL) were collected at weeks in milk 5 and 7 using a BD Vacutainer (Becton Dickinson) at a 14-d interval. After centrifugation ($1,400 \times g$ for 10 min at 20°C), serum was collected, frozen (-20°C), and then sent to the diagnostic laboratory of the Faculté de médecine vétérinaire of the Université de Montréal (Saint-Hya-

cinthe, QC, Canada) to measure P4 serum concentration. Serum P4 was measured using a sequential competitive immunoassay (Immulite; Siemens, Mississauga, ON, Canada). The inter- and intra-assay coefficients of variation for P4 were 8.1 and 9.1%, respectively.

If a P4 serum concentration ≥ 1 ng/mL was observed at least once (weeks in milk 5 or 7), the cow was considered to have luteal activity (Stevenson et al., 2006). A cow was considered to have no luteal activity if both blood samples were available and both had P4 serum concentration < 1 ng/mL and if no prostaglandin administration was recorded within 7 d before the second P4 measurement. Cows' computerized health records (DSHR Inc., Saint-Hyacinthe, QC, Canada) were verified to ensure that no prostaglandin was administered during that period. Consequently, luteal activity status was considered missing whenever (1) both measurements yielded a P4 concentration < 1 ng/mL and prostaglandin was administered within 7 d before the second P4 measurement or (2) one of the P4 measurements was missing and the other measure would yield a P4 serum concentration < 1 ng/mL. We therefore obtained a binary outcome for luteal activity.

Time to Pregnancy. The reproductive information collected consisted of herd use of ovulation synchronization program and timed AI, calving dates, VWP, AI dates, DIM at pregnancy, and culling dates. These were obtained from the herds' computerized record systems (calving and AI dates), DHIA (culling dates), and a questionnaire completed by farmers (synchronization program and VWP). Pregnancy diagnosis was performed by the herd veterinarian by rectal palpation between 35 and 49 d after last AI, and it was recorded by the veterinarian or the herd personnel. We considered the pregnancy date as the date of the last AI happening before a positive pregnancy diagnosis. Furthermore, we considered the first pregnancy diagnosis only, meaning that even if there was pregnancy loss with a new AI period, we recorded only the first pregnancy. To avoid penalization of farms with longer VWP, time to pregnancy was defined as the number of days from the end of the herd's VWP until the successful AI.

Potential Effect Modifiers. Morin et al. (2018) observed different effects of incomplete milking on odds of hyperketonemia in cows in second parity versus those in third parity or greater; therefore, parity (2 vs. ≥ 3) was tested as an effect modifier of the relationship between the incomplete milking protocol and reproductive outcomes. Parity records were obtained through computerized record systems (DSHR Inc.). We also hypothesized that treatment effect could be greater in herds that started breeding cows sooner after calving (and, thus, closer in time to treatment). Consequently,

we tested whether VWP acted as an effect modifier of the relationship between incomplete milking and reproductive outcomes. Because there were 13 herds, there could be a maximum of 13 different VWP values; therefore, VWP was categorized in 2 levels based on a value that best divided the observations in half (55 DIM).

Data Management and Statistical Analyses

Effect of Incomplete Milking on Reproductive Performance. To study the effect of incomplete milking on luteal activity, a generalized mixed regression model using a logit link, with herd as a random effect and odds of luteal activity as outcome, was fitted to the data using the GLIMMIX procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). The model was as follows:

$$Y_{ij} \sim \text{bin} [P(Y_{ij})] \text{ logit } [P(Y_{ij})] = \beta_{0ij} + \beta_1 \text{Tx}_i + v_{0ij}, \quad [1]$$

where $P(Y_{ij})$ is the probability of luteal activity for the i th cow from the j th herd—it is a function of treatment group (Tx) through the logit function, and it follows a binary distribution; β_0 is the intercept; β_1 is the regression coefficient for Tx and corresponds to the natural logarithm of the odds ratio between incompletely milked and conventionally milked; and v_{0ij} is the herd random error term. Parity (2 vs. ≥ 3) was tested as effect modifier of the relationship treatment and odds of luteal activity by adding the main term and an interaction term (treatment $\times$ parity) in the model. Parity was retained as effect modifier if the interaction term yielded a P -value of <0.05 on the F -test.

To investigate the effect of incomplete milking on pregnancy hazard, a Cox proportional hazards model with a herd frailty term was fitted to the data using the PHREG procedure in SAS. Cows were followed until their first pregnancy status, until the cow was culled (left the farm or died), or until 200 d from the end of the herd's VWP, whichever came first (right censoring). Animals inseminated or culled before the start of the breeding period were left censored. The general model was as follows:

$$h_i(t) = \lambda_0(t) \exp(\beta_1 \text{Tx}_i) + \varepsilon_i, \quad [2]$$

where $h_i(t)$ is the pregnancy hazard for the i th cow at time t ; $\lambda_0(t)$ is the baseline hazard function; β_1 is the coefficient for treatment group (Tx); and ε_i represents the unobserved heterogeneity, accounting for difference between herds. Using this model, the pregnancy haz-

ard ratio between treatment groups was computed by exponentiation of β_1 using the natural base logarithmic transformation.

To evaluate whether the effect of the incomplete milking protocol on pregnancy hazard varied as a function of time (i.e., the proportional hazards assumption), we investigated whether an interaction term between time and treatment yielded a P -value <0.05 . Several time variables were offered to the model, and if many were significant, the one leading to the lowest Akaike information criterion was chosen. The time variables investigated were (1) time in days (i.e., in its original form); (2) the natural logarithm of time; (3) a time categorical variable with 2 levels (1–21 and >21 d following the end of the VWP), differentiating the first breeding cycle from the subsequent ones; and (4) a time categorical variable with 5 levels (1–21, 22–43, 44–65, 66–87, and >87 d after the end of VWP), thus differentiating the first 4 breeding cycles from the subsequent ones.

We hypothesized that the relationship between the incomplete milking protocol and pregnancy hazard could change by parity level. Therefore, interactions between treatment and parity (2-way) and, if the proportional hazard assumption was not met, between the time variable and parity (2-way) and between treatment, the time variable, and parity (3-way) were added to the model. Interaction terms were considered significant and retained in the model whenever the F -test yielded a P -value <0.05 . If the 3-way interaction was deemed significant, then all 2-way and main terms were kept in the model to allow interpretation.

We also hypothesized that the relationship between the incomplete milking protocol and pregnancy hazard could change by VWP level. Therefore, we checked 2-way and 3-way interactions between VWP and the other predictors, as described for parity. If the proportional hazards assumption was not met, and if both 3-way interactions were deemed significant, we tested the 4-way interaction between exposure, the time variable, parity, and VWP. We retained that interaction, along with all 3-way, 2-way, and main terms, if the F -test for the 4-way interaction yielded a P -value <0.05 . Finally, the assumption of independent censoring was checked as described by Dohoo et al. (2009). The cow was considered as the experimental unit and as the unit of statistical analysis at all times.

Effect of Early-Lactation Hyperketonemia on Reproductive Performance. To study the effect of early-lactation hyperketonemia on luteal activity, a generalized mixed regression model using a logit link was ran using the GLIMMIX procedure in SAS. Herd was included as a random effect. The model was as follows:

$$Y_{ij} \sim \text{bin} [P(Y_{ij})] \text{ logit} [P(Y_{ij})] = \beta_{0ij} + \beta_1 \text{hyperketonemia}_i + v_{0ij}, \quad [3]$$

where $P(Y_{ij})$ is the probability of luteal activity for the i th cow from the j th herd—it is a function of early-lactation hyperketonemia status through the logit function, and it follows a binary distribution; β_0 is the intercept; β_1 is the regression coefficient for hyperketonemia status and corresponds to the natural logarithm of the odds ratio between hyperketonemia levels; and v_{0ij} is the herd random error term.

The effect of early-lactation hyperketonemia on pregnancy hazard was described using Kaplan–Meier analyses. Median days to pregnancy were obtained from Kaplan–Meier models using the LIFETEST procedure in SAS. The cow was considered as the experimental unit and as the unit of statistical analysis at all times.

RESULTS

Overall, 878 cow-lactations (870 animals, in which 8 were enrolled in 2 contiguous lactations) calved during the project. The sample size in the current study was higher than the previously reported sample size ($n = 846$; Krug et al., 2018b; Morin et al., 2018) because sampling continued after the end of those studies. The flow of cows in the current RCT is illustrated in Figure 1.

The proportion of cows with luteal activity (weeks in milk 5 or 7) was 84% (565/669), and it varied from 76 to 93% between herds. The proportion of cows conceiving within 200 d from the start of the breeding period was 71% (551/776). The mean (SD) interval from the start of the breeding period to pregnancy or to censoring was 61.9 (52.2) and 157.2 (60.9) d, respectively. Of those that did not conceive, 64% (143/225) had at least 1 AI before the end of the follow-up period.

Effect of Incomplete Milking on Reproductive Performance

Cows conventionally milked produced a mean (SD) of 6.0 (3.3), 20.9 (9.9), 25.9 (8.9), 28.8 (7.9), and 30.1 (9.1) L of milk/d on DIM 1, 2, 3, 4, and 5, respectively. Average (SD) parity of cows enrolled was 3.1 (1.4) and 3.1 (1.3) for conventionally and incompletely milked cows, respectively. Of the total 853 animals, 357 animals were in second parity (185 conventionally milked and 172 incompletely milked), 495 animals were in third parity or greater (249 conventionally milked and 246 incompletely milked), and 1 animal for which exact parity number was unknown. From that total, 477 were bred after a VWP of <55 d (245 conventionally milked

and 232 incompletely milked) and 376 were bred after a VWP of ≥ 55 d (189 conventionally milked and 187 incompletely milked).

Luteal Activity. The proportion of cows presenting luteal activity was 84% (286/341) and 85% (279/328) in conventionally and incompletely milked cows, respectively. Odds of luteal activity for incompletely milked cows were 1.1 (95% CI: 0.72–1.7; $P = 0.67$) times those of conventionally milked cows. Parity ($P = 0.79$) did not act as an effect modifier on the relationship between treatment and luteal activity.

Time to Pregnancy. There were 72% (283/392) and 70% (268/384) pregnancies among conventionally and incompletely milked cows, respectively. Table 1 shows median (interquartile range) interval from the start of the breeding period to pregnancy across variables of interest. The interactions between the time variables and treatment variable were all significant ($P < 0.01$; the proportional hazards assumption was violated), and the time categorical variable with 5 levels (1–21, 22–43, 44–65, 66–87, and >87 d after the end of the VWP) was selected. The effect of treatment on pregnancy hazard varied not only across time but also across parity (P -value for the 3-way interaction term <0.01) and across VWP levels (P -value for the 3-way and 4-way interaction terms <0.01). Table 2 shows the final model, and Table 3 shows the estimated hazard ratio by parity, VWP, and time. Briefly, in second-parity cows with start of breeding period <55 d, incomplete milking led to higher pregnancy hazards. Moreover, the effect was stronger early after the end of the VWP and persisted until 87 d following the end of the VWP. In cows in third parity or greater and in cows with start of breeding period ≥ 55 d, incomplete milking did not affect pregnancy hazard.

Effect of Early-Lactation Hyperketonemia on Reproductive Performance

In the sample of 853 animals, prevalence of early-lactation hyperketonemia was 10% (69/690) in wk 1, 21% (148/703) in wk 2, and 26% (180/701) in wk 3. Reproductive outcomes across different hyperketonemia levels are described in Table 4.

Hyperketonemia status in early lactation was not associated with odds of luteal activity ($P = 0.48$). The odds of luteal activity for cows experiencing hyperketonemia in 1 out of 3 wk were 1.7 (95% CI: 0.87–3.1; $P = 0.12$) times those of cows never experiencing hyperketonemia. The odds of luteal activity for cows experiencing hyperketonemia in 2 out of 3 wk were 1.2 (95% CI: 0.56–2.7; $P = 0.60$) times those of cows never experiencing hyperketonemia. The odds of luteal activity for cows experiencing hyperketonemia in all weeks were 1.3

(95% CI: 0.35–4.5; $P = 0.72$) times those of cows never experiencing hyperketonemia.

Survival curves for time (from the end of the herd's VWP to 60 d following that period) to pregnancy by hyperketonemia status are presented in Figure 2. Survival curves were not significantly different across early-lactation hyperketonemia status (Wilcoxon test P -value = 0.89) even though cows never experiencing hyperketonemia (during the first 3 wk in milk) appeared to have numerically fewer days to pregnancy

compared with the other hyperketonemia levels, especially between 0 and 60 d following the end of the VWP (Figure 2).

DISCUSSION

The main objective of our study was to evaluate the effects of an incomplete milking during the first 5 DIM on prevalence of luteal activity (weeks in milk 5 or 7) and on pregnancy hazard. Our data suggest

Figure 1. Diagram illustrating flow of cows and missing observations for cows from 13 commercial herds enrolled in a randomized controlled trial comparing conventionally (C) and incompletely (I) milked cows. VWP = voluntary waiting period.

that the incomplete milking protocol does not influence prevalence of luteal activity, but it does increase the pregnancy hazard in second-parity cows from 1 to 87 d after the end of the herd's VWP in animals bred <55 d. The fact that we found an effect of the incomplete milking protocol at the beginning of the breeding period but not at the end is not surprising because the milking protocol was of short duration and applied very early in the lactation.

The current RCT only includes multiparous animals because in primiparous cows, the lactation peak is lower and occurs a bit later compared with multiparous cows. Moreover, the decline in milk yield after peak production is not as sharp as that of older cows (i.e., primiparous animals show a better persistency; Stanton et al., 1992; Rekik et al., 2003). These differences in the lactation curve of primiparous animals, along with a shorter lag between peaks for milk yield and for food intake, lead to better energy balance at the beginning of the lactation in this group of animals (Coffey et al., 2004).

The increased effect in second-parity cows compared with cows in third parity or greater is in agreement with results from Morin et al. (2018), in which the effect of the incomplete milking protocol on odds of early-lactation hyperketonemia in second-parity cows was twice that of cows in third parity or greater from 8 to 17 DIM. In that study, the effect of the treatment on odds of hyperketonemia was similar for all parities during the period immediately preceding and immediately following the end of the milking protocol (i.e., 4–7 DIM). However, the residual effect of treatment on odds of hyperketonemia in the following days (8–17 DIM), also observed by Carbonneau et al. (2012), was present only in second-parity cows (Morin et al.,

2018). That short-term prevention of hyperketonemia in cows in third parity or greater (without a sustained residual effect) could have restricted a positive effect of incomplete milking on subsequent health events. Another possibility is that because cows in third parity or greater produce generally more milk than second-parity cows, the treatment protocol might have to be longer than stipulated, for example, to produce an effect in cows in third parity or greater. Finally, it is possible that because second-parity animals are still growing, their bodies might be more susceptible or more malleable to an intervention than those of older animals.

The fact that incomplete milking led to a stronger effect in cows starting the breeding period before 55 DIM compared with cows starting ≥ 55 DIM can be explained by the fact that the first group of animals were bred starting at a time closer to the milking intervention (1–5 DIM). Another reason might be that later than 55 DIM, most animals that suffered from severe NEB at the beginning of the lactation would already have recovered and so their reproductive performances would be similar no matter their state at the onset of lactation.

This is the first study investigating the effect of incomplete milking on reproductive performance, but other studies investigated the effect of reduced milk output in early lactation by temporarily reducing milk frequency (Patton et al., 2006; McNamara et al., 2008). In Patton et al. (2006), although there was earlier resumption of ovarian activity (18.3 d in cows milked 1×/d vs. 28.6 d in cows milked 3×/d), interval from calving to pregnancy did not vary. Similarly, in McNamara et al. (2008), mean calving to pregnancy interval did not vary across different milking frequencies applied during the first month after calving. Survival analysis

Table 1. Median time between the end of the voluntary waiting period (VWP) and pregnancy (in days) by breeding cycle and by treatment, parity level, and herd VWP¹

Treatment	Parity	VWP, d	n ²	Median time (interquartile range) per breeding cycle ³				
				1–21 d	22–43 d	44–65 d	66–87 d	>87 d
Conventionally milked	2	<55	96	13 (10–16)	28 (26–38)	55 (51–58)	71 (67–74)	117 (105–144)
		≥ 55	72	9 (6–15)	36 (32–37)	50 (48–56)	77 (73–79)	125 (115–143)
	≥ 3	<55	137	12 (8–13)	30 (24–34)	50 (46–54)	75 (71–85)	136 (110–159)
		≥ 55	87	14 (8–17)	32 (27–36)	51 (49–53)	76 (71–77)	100 (91–126)
Incompletely milked	2	<55	84	9 (5–14)	34 (29–37)	53 (49–57)	70 (69–76)	113 (91–131)
		≥ 55	77	8 (5–13)	27 (24–36)	50 (48–52)	75 (74–83)	111 (94–140)
	≥ 3	<55	139	11 (3–15)	30 (25–35)	53 (45–55)	74 (69–81)	105 (94–123)
		≥ 55	83	9 (6–16)	30 (23–39)	53 (45–55)	74 (71–80)	117 (92–158)

¹Values were calculated using data from 853 multiparous Holstein cows from 13 herds participating in a randomized controlled trial evaluating the effects of an incomplete milking during the first 5 DIM compared with conventional milking.

²Number of pregnant cows by cows at risk.

³Number of days following the end of the VWP.

Table 2. Results from the final Cox proportional hazard model on the effect of incomplete milking during the first 5 DIM on time from the end of the herd's voluntary waiting period (VWP) to pregnancy¹

Predictor	Level ²	β (SE)	P-value ³	β for the time component parameters (SE) per breeding period cycle ⁴			
				1–21 d	22–43 d	44–65 d	66–87 d
Treatment			<0.01				
Parity	C	Referent					
	I	6.357 (0.447)		Referent	–2.747 (0.420)	–4.445 (0.497)	–5.459 (0.581)
VWP, d	2	Referent					
	≥ 3	6.304 (0.410)		Referent	–2.475 (0.367)	–4.376 (0.414)	–5.891 (0.554)
	<55	Referent					
	≥ 55	6.300 (0.447)		Referent	–3.031 (0.534)	–4.265 (0.464)	–5.462 (0.601)
Treatment $\times$ parity	C $\times$ 2	Referent					
	I $\times$ ≥ 3	–6.242 (0.544)		Referent	2.707 (0.634)	4.449 (0.670)	5.547 (0.839)
Treatment $\times$ VWP	C $\times$ <55	Referent					
	I $\times$ ≥ 55	–5.793 (0.590)		Referent	2.741 (0.766)	4.139 (0.786)	4.778 (0.900)
Parity $\times$ VWP	2 $\times$ <55	Referent					
	3 $\times$ ≥ 55	–6.405 (0.551)		Referent	3.028 (0.711)	4.498 (0.696)	6.115 (0.919)
Treatment $\times$ parity $\times$ VWP	C $\times$ 2 $\times$ <55	Referent					
	I $\times$ ≥ 3 $\times$ ≥ 55	5.900 (0.740)		Referent	–2.780 (1.114)	–4.770 (1.094)	–5.555 (1.263)
Herd variance		0.042 (0.029)					

¹Values were calculated using a Cox model with a herd frailty term using data from 776 multiparous Holstein cows from 13 herds participating in a randomized controlled trial evaluating the effects of incomplete milking during the first 5 DIM compared with conventional milking.

²C = conventionally milked; I = incompletely milked.

³Joint P-value obtained using an F-test.

⁴Number of days following the end of the VWP.

Table 3. Estimated pregnancy hazard ratios (95% CI) between incompletely milked cows and conventionally milked cows stratified by voluntary waiting period (VWP), parity, and breeding cycle period¹

Breeding cycle period ²	VWP <55 d		VWP ≥55 d	
	Parity 2	Parity ≥3	Parity 2	Parity ≥3
1–21 d	576.3 (240.0–1,383.7)	1.1 (0.61–2.1)	1.8 (0.81–3.8)	1.2 (0.67–2.3)
22–43 d	36.9 (18.9–72.1)	1.1 (0.5–2.2)	1.7 (0.65–4.7)	1.1 (0.38–3.4)
44–65 d	6.8 (3.3–13.8)	1.1 (0.60–2.1)	1.3 (0.51–3.3)	0.66 (0.24–1.8)
66–87 d	2.5 (1.0–5.9)	1.2 (0.44–3.4)	0.89 (0.29–2.7)	0.63 (0.21–1.9)
>87 d	0.13 (0.07–0.26)	1.1 (0.63–1.77)	1.46 (0.68–3.1)	1.1 (0.53–2.3)

¹Values were calculated using a Cox model with a herd frailty term using data from 776 multiparous Holstein cows from 13 herds participating in a randomized controlled trial evaluating the effects of incomplete milking during the first 5 DIM compared with conventional milking.

²Number of days following the end of the VWP.

would have been a more appropriate analysis to study time to pregnancy data (Lean et al., 2016), but it was not used in any of these studies.

Secondarily, we aimed to describe the effect of early-lactation hyperketonemia on luteal activity and on pregnancy hazard. Early-lactation hyperketonemia was not associated with odds of luteal activity or with pregnancy hazard. Previous studies obtained similar results regarding luteal activity (Ribeiro et al., 2013; Abdelli et al., 2017) and pregnancy hazard (Ospina et al., 2010; McArt et al., 2013). However, opposing results have also been found (Walsh et al. 2007a,b; Dubuc et al., 2012). The lack of association between early-lactation BHB and reproductive outcomes could have been caused by the low prevalence of early-lactation hyperketonemia that probably resulted in decreased power to detect a difference between groups. Also, hyperketonemia was measured only once a week from weeks in milk 1 to 3, but twice-weekly diagnosis would probably have been better for accurate diagnosis, as suggested by McArt et al. (2012). Our sampling schedule may thus have led to some misclassification of hyperketonemia status due to lower sensitivity in detecting positive cows. There was, however, a high specificity (i.e., a large proportion of the healthy cows were diagnosed as such); due to that,

the effect on measure of association was very small, as shown by Haine et al. (2018). Finally, we only accounted for early-lactation hyperketonemia, but if both hyperketonemia and luteal activity had been measured around the same time (e.g., wk 5 and 7), the results would probably have differed. Even though there was no relationship in BHB and time to pregnancy in the population studied, we still found an association between incomplete milking and pregnancy hazard. The reason for it is probably related to the stronger association between reproductive outcomes and other indicators of NEB (e.g., blood concentration of non-esterified fatty acids; Ospina et al., 2010).

The RCT is the best study design to reveal a causal effect (Dohoo et al., 2009). Nevertheless, herds were recruited from a convenience sample. The overall prevalence of hyperketonemia (10% in the first week after calving) and of luteal inactivity (16% at weeks in milk 5 and 7) was low compared with previous studies. For instance, the study of Dubuc and Denis-Robichaud (2017) showed a median prevalence of hyperketonemia of 18.8% between 1 and 14 DIM, and the prevalence of cows without luteal activity was much higher in Walsh et al. (2007a; 57% around week in milk 7), Dubuc et al. (2012; 26% around week in milk 7), and Dubuc and

Table 4. Number of cows, proportion of cows presenting luteal activity, proportion of pregnant cows, and median days from calving to pregnancy by early-lactation hyperketonemia level¹

Variable	Early-lactation hyperketonemia level ²			
	1	2	3	4
Cows, no. (%)	412/638 (65)	113/638 (18)	89/638 (14)	24/638 (4)
Luteal activity, no. (%) of cows	292/333 (88)	69/85 (81)	52/61 (85)	17/20 (85)
Pregnant cows, no. (%)	274/387 (71)	75/106 (71)	61/81 (75)	16/22 (72)
Median (IQR ³) days between end of VWP ⁴ and pregnancy	43 (17–82)	44 (21–90)	48 (24–88)	44 (22–58)

¹Values were calculated using data from 638 multiparous Holstein cows from 13 herds participating in a randomized controlled trial evaluating the effects of an incomplete milking during the first 5 DIM compared with conventional milking.

²1 = never experiencing hyperketonemia in the first 3 wk in milk; 2 = hyperketonemia in 1 out of first 3 wk in milk; 3 = hyperketonemia in 2 out of 3 wk in milk; 4 = hyperketonemia in weeks in milk 1 to 3.

³Interquartile range.

⁴Voluntary waiting period.

Figure 2. Survival curves of time to pregnancy for 596 lactating Holstein cows classified in different early-lactation hyperketonemia levels based on serum BHB concentration determined in weeks in milk 1 to 3. 1 = never experiencing hyperketonemia in first 3 wk in milk; 2 = hyperketonemia in 1 out of first 3 wk in milk; 3 = hyperketonemia in 2 out of first 3 wk in milk; 4 = hyperketonemia in weeks in milk 1 to 3.

Denis-Robichaud (2017; 35% around weeks in milk 5 and 7). The lower prevalence of hyperketonemia and luteal inactivity in the current study might have been caused by better management strategies for controlling these health events in the herds selected in our study compared with herds considered for other studies. Finally, discrepancies might also have been caused by disease definitions (i.e., type of test used, time of sampling) used in the different studies. Prevalence of pregnant cows (71% by 200 d after the start of the breeding period) was similar to the results obtained elsewhere (78% by 150 DIM in McArt et al., 2012).

Other limitations of the current study were the fact that producers were not blinded to treatment allocation and that compliance could not be quantified. However, we believe that knowledge of treatment allocation could

hardly influence objective reproductive outcomes such as P4 measurement or reproduction data (i.e., pregnancy dates). We also believe that the frequent on-farm visits (weekly during the total duration of the study) minimized possible noncompliance during data collection.

CONCLUSIONS

The current study shows, for the first time, that the incomplete milking protocol has no effect on luteal activity and a positive effect on pregnancy hazard in second-lactation cows in herds that started their breeding period <55 d. We did not observe an effect of early-lactation hyperketonemia on luteal activity or on pregnancy hazard.

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