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Performance and health of dairy cows incompletely milked during the first five days in milk

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RÉSUMÉ

Pendant la période de transition chez les vaches laitières, la demande énergétique pour initier la production de lait est très élevée, tandis que l'apport d'énergie est faible, ce qui crée inévitablement une balance énergétique négative. Ce déséquilibre physiologique constitue l'un des principaux facteurs de risque dans le développement de maladies de transition, et ensemble, ils peuvent influencer les performances reproductrices et augmenter le taux de réforme. De nouvelles stratégies, aidant les vaches laitières à supporter une production intensive, doivent donc être mises en place. Bien que des progrès remarquables aient été réalisés pour garantir un apport énergétique élevé, peu de recherches ont évalué la possibilité de contrôler les dépenses énergétiques. La traite incomplète des vaches en début de lactation pourrait aider à limiter le déséquilibre énergétique chez les vaches laitières.

L'objectif de ce projet était de mesurer l'impact, sur la santé et la production, d'une traite incomplète durant les cinq premiers jours en lait (JEL ; sans changer la fréquence de traite), sur les vaches de fermes laitières commerciales. Plus spécifiquement, les éléments suivants ont été évalués : la production de lait, le taux de réforme, l'incidence de maladies infectieuses, les performances reproductrices et la sensibilité au niveau du pis. Un essai contrôlé randomisé a été réalisé chez les vaches multipares provenant d'un échantillon de convenance de 13 fermes laitières. Dans chaque troupeau, toutes les vaches multipares (n = 878), ayant mis bas entre décembre 2013 et mars 2015, ont été aléatoirement réparties entre un groupe traitement et un groupe témoin, à l'aide d'un générateur de nombres aléatoires. Les vaches du groupe traitement ont été soumises à une traite incomplète durant les cinq premiers JEL, avec une collecte maximale de 10, 10, 10, 12 et 14 litres de lait par jour aux JEL un, deux, trois, quatre et cinq, respectivement. Les vaches du groupe témoin ont été traites de manière conventionnelle.

Le taux de réforme des vaches et la production de lait ne différaient pas entre les deux groupes. Lorsqu'on s'intéresse aux différences de rendement en termes de lait corrigé en énergie, les vaches traites incomplètement produisaient de façon similaire aux vaches traites complètement. La traite incomplète n'affectait pas les cotes de nouvelles infections intramammaires du 11 au 18ème JEL, ni les cotes de maladies du système reproducteur à 35 JEL, ni l'incidence de mammite clinique durant les 90 premiers JEL. Les cotes d'élimination

d’une infection intramammaire du 11 au 18ème JEL chez les vaches traites incomplètement étaient 2,9 fois supérieures à celles des vaches traites complètement (intervalle de confiance à 95% : 1,4-6,0). La traite incomplète n'affectait pas non plus l'activité lutéale ; elle avait, cependant, un impact positif sur le risque de conception chez les vaches en deuxième lactation qui étaient dans des troupeaux avec une période d’attente volontaire inférieure à 55 jours (180/775 vaches). Chez ces vaches, le risque de conception (intervalle de confiance à 95%) pour les vaches traites incomplètement était 576,3 (240,0-1383,7), 36,9 (18,9-72,1), 6,8 (3,3-13,8), 2,5 (1,0-5,9), et 0,13 (0,07-0,26) fois celui des vaches traites normalement à 1-21, 22-43, 44-65, 66-87 et >87 jours respectivement après la période d'attente volontaire.

L'algomètre de pression a été validé pour mesurer des changements de sensibilité dus à une distension du pis. Cet instrument était modérément répétable pour quantifier le seuil nociceptif mécanique sur le pis et de nombreux facteurs externes influençaient également les valeurs obtenues. Par conséquent, son utilisation pour cet usage devrait être considéré avec prudence. Nous avons donc plutôt observé le comportement de repos pour évaluer une douleur éventuelle ressentie au niveau du pis des vaches traites de façon incomplète. Nous n'avons observé aucun effet sur le temps de repos. Cependant, l'impact de la traite incomplète sur la fréquence et la durée moyenne des phases de repos dépendait du nombre de lactations de la vache. Cette stratégie semble légèrement problématique pour les vaches de deuxième parité, mais potentiellement bénéfique pour les vaches plus âgées.

Mots-clés : vaches laitières, traite incomplète, taux de réforme, production de lait, mammite, maladies du système reproducteur, activité lutéale, conception, nociception, algomètre, comportement de repos, accéléromètre

ABSTRACT

During the transition period in dairy cows, energy demands for milk production are very high, while energy intake is low, leading to a physiologically unavoidable negative energy balance. Physiological imbalance or dysfunction appears to be one of the main factors leading to increased risk for transition diseases, and together, these problems have a great impact on subsequent reproductive performances and culling. This leads to the urgent need for alternative management strategies to help dairy cattle to cope with the intensive systems in which they are raised. Although there has been great improvements in managing the source of energy for the cow, little work has been done in controlling energy expenses to improve energy balance. An incomplete milking in early lactation could help limiting negative energy balance in dairy cattle.

The objective of this project was to measure, in a context of commercial dairy farms, the impact of an incomplete milking (without altering the milk frequency) during the first five days in milk (DIM), on performance and health. Specifically, the aims were to quantify its impact on: culling and milk production; on incidence of infectious diseases; on reproductive performance; and on udder sensitivity. A randomized controlled trial was conducted on multiparous cows from a convenient sample of 13 commercial dairy farms. In each herd, all multiparous cows (n = 878) calving between December 2013 and March 2015 were randomly allocated at the time of dry off to a treatment or a control group using a random number generator. Cows in the treatment group were milked incompletely during the first five DIM, with a maximum of 10, 10, 10, 12, and 14 L/d collected on DIM one, two, three, four and five, respectively. Cows in the control group were milked conventionally.

Culling hazard and milk yield did not differ among treatment groups. When investigating differences in energy corrected milk yield per week throughout the lactation, incompletely milked cows produced as much as conventionally milked cows during most weeks. Incomplete milking did not affect the odds of new intramammary infection from 11 to 18 DIM, the odds of reproductive tract disease at 35 DIM, or clinical mastitis incidence in the first 90 DIM. The odds of eliminating an existing intramammary infection from 11 to 18 DIM for incompletely milked cows were 2.9 (95% confidence interval: 1.4, 6.0) times those of conventionally milked cows. The incomplete milking protocol had no effect on postpartum luteal activity and it had a positive

impact on pregnancy hazard in second parity cows from herds with voluntary waiting period lower than 55 days (180/775 cows). The hazards of pregnancy (95% confidence interval) in incompletely milked cows were 576.3 (240.0, 1383.7), 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 1-21, 22-43, 44-65, 66-87 and >87 d after voluntary waiting period, respectively in second parity cows from herds with voluntary waiting period lower than 55 days.

The pressure algometer was validated for measuring changes in udder sensitivity due to udder distension. The instrument was shown to be only moderately repeatable for quantifying mechanical nociceptive threshold on the udder and was influenced by extraneous covariates, therefore its use should be considered cautiously or it should be further developed. Consequently, to assess potential increased udder pain due to incomplete milking, we used the resting behavior data of incompletely and conventionally milked animals. There was no effect of incomplete milking on lying time. However, the effect of incomplete milking on frequency of lying bouts and on mean lying bout duration varied by parity level, suggesting that an incomplete milking may be slightly problematic for second parity cows and, possibly, slightly beneficial for older cows.

Keywords : dairy cattle, incomplete milking, culling, milk production, mastitis, reproductive tract disease, luteal activity, pregnancy, nociception, algometer, resting behavior, accelerometer

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AMS	Automatic milking system
BCS	Body condition scoring
BHBA	β-hydroxybutyrate
CCC	Concordance correlation coefficient
CI	Confidence interval
CMT	California mastitis test
CRC	Controlled-release intraruminal capsule
DHI	Dairy Herd Improvement
DIM	Days in milk
ECM	Energy corrected milk
FAO	Food and Agriculture Organization
FSH	Follicle-stimulating hormone
HR	Hazard ratio
IFN-ɣ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukins
IMI	Intramammary infection
IQR	Interquartile range
LE	Leukocyte esterase
LH	Luteinizing hormone
MNT	Mechanical nociceptive threshold
NEFA	Non-esterified fatty acids
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells (agranulocytes such as lymphocytes and monocytes)
PCR	Polymerase chain reaction
PMNL	Polymorphonuclear leukocytes (granulocytes such as neutrophils, eosinophils, basophils)
PVD	Purulent vaginal discharge
P4	Progesterone
RCT	Randomized controlled trial

REFLECT	Randomized control trials in livestock and food safety statement
ROS	Reactive oxygen species
RR	Risk ratio
SCC	Somatic cell counts
SD	Standard deviation
Se	Sensitivity
Sp	Specificity
TCA	Tricarboxylic cycle
TMR	Total mixed ration
TNF-α	Tumor necrosis factor alpha
VLDL	Very low density lipoproteins
VWP	Voluntary waiting period
WIM	Weeks in milk

"Valeu a pena? Tudo vale a pena se a alma não é pequena."

(In: *Mensagem*. Fernando Pessoa, 1934)

Para os meus pais, Helena e Helder

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CHAPTER 1: INTRODUCTION

Most mammals undergo a state of negative energy balance around parturition, in which energy demands for the fetus or newborn exceed the energy obtained through the diet (Wade and Schneider, 1992). Dairy cows suffer particularly from it, as they were selected to produce as much milk as possible, far exceeding the milk needed for one calf (Jasper and Weary, 2002).

Selection for increased yield became the focus in dairy farming with the increased costs of land and labor, which tripled from 1960-95, compared to the price of milk, that only doubled (Veerkamp et al., 2009). Data from the United Nations Food and Agriculture Organization (**FAO**) food balance database (FAO, 2015) reveals that there has been a gradual increase in milk consumption per capita in the world from the 1960's (around 75 kg/capita/yr) until the 2000's (around 90 kg/capita/yr in 2013), and as the milk demand increases, so does the offer. The same database shows the enormous increase in global milk production from 350 billion liters in the 1960's to more than the double in 2013 (around 770 billion liters). That has been possible through an increment in the dairy cattle population, but also through genetic selection for increased milk production, through improved nutrition and feeding systems (Lucy, 2001; Vandehaar and St-Pierre, 2006; FAO, 2015). In the USA, at the cow level, milk production increased from 6,700 kg/yr in 1990, to 9,300 kg/yr (FAO, 2015). Nowadays, Holstein cows produce 9,000 to 10,000 kg/yr in most developed countries (CDIC, 2016), and according to Santos et al. (2011), the average North American milking cow will produce 14,000 kg/yr in 2050.

Along with this substantial increase in milk production, reproductive efficiency and longevity of dairy cows have decreased (Lucy, 2001; Oltenacu and Broom, 2010; Bicalho et al., 2014). Even though knowledge on dairy cattle feeding systems has increased considerably in the last decades, with higher levels of concentrate to meet dairy cow's nutrient requirements more adequately (Eastridge, 2006), the high-production settings have increased the risk for metabolic diseases (Zwald et al., 2004; Koeck et al., 2013; Pryce et al., 2016). These are sometimes called "production diseases", and they include ketosis, hypocalcemia (or milk fever), rumen acidosis, hepatic lipidosis and abomasal displacement. These production diseases often lead to immune suppression in early lactation, increasing the risk for infectious diseases such as

mastitis and metritis (Esposito et al., 2014). As Bauman and Currie (1980) mentioned, pregnancy and milk production are the priority for dairy animals, allowing these biological states to continue sometimes at the expense of other metabolic processes, even if a state of disease is created.

Physiological imbalance or dysfunction appears to be one of the main factors leading to increased risk for transition diseases (i.e. diseases occurring around parturition), and together, these problems have a great impact on subsequent reproductive performances (Walsh et al., 2007b; Wathes et al., 2007b; Ospina et al., 2010a) and culling (i.e. death or removal from herd). This leads to the urgent need of alternative management strategies to help dairy cattle to cope with the intensive systems in which they are raised (Esposito et al., 2014; Lacasse et al., 2017). Although there has been great improvements in managing the source of energy for the cow (e.g. increasing diet density, monensin, managing the transition period to maintain food intake; Eastridge, 2006), little work has been done in controlling energy expenses to improve energy balance.

Incomplete milking during the first five days in milk (**DIM**) is an innovative approach to limit the negative energy balance and its consequences. This strategy was mentioned for the first time by Carbonneau et al. (2012) in research settings. Carbonneau et al. (2012) showed that reducing the quantity of milk collected during the first five DIM, without reducing the milking frequency, allows to temporarily decrease energy demands in early lactation, thus helping cows to handle the high metabolic demands. Morin (2017) investigated the impact of a very similar milking protocol in a commercial dairy farms context, and showed that incomplete milking led to lower blood β-hydroxybutyrate (**BHBA**) in early lactation, along with lower odds of hyperketonemia, a commonly used indicator of negative energy balance (Whitaker et al., 1993). The cows enrolled in the randomized controlled trial (**RCT**) from Morin (2017) continued being followed until the rest of their lactations, allowing for the analyses of several other outcomes, besides BHBA, that will be described in the current thesis. Our hypothesis was that the reduction in hyperketonemia prevalence in early lactation would further improve dairy cows' performance and health throughout their lactation.

Research objectives

To help the reader to understand the problematic and the methodological choices, this thesis starts with a literature review in Chapter 2. The literature review is followed by the reports of a series of experimental epidemiological studies along with an observational study. The underlying objectives of the experimental studies were to quantify the impact of an incomplete milking during the first five DIM on:

1) Culling and milk production, presented in Chapter 3;
2) Incidence of infectious diseases, presented in Chapter 4; and
3) Reproductive performance, presented in Chapter 5.

Another aim of this project was to quantify the impact of an incomplete milking during the first five DIM on ketonemia and prevalence of hyperketonemia. That objective was developed and presented by Morin (2017) as part of a master program.

During the study, the fact that some producers were concerned about the potential discomfort caused by the treatment, led to the addition of two secondary objectives to evaluate pain or discomfort:

4) To quantify the reliability of the algometer for measuring mechanical nociceptive threshold (**MNT**) when applied to the udder of dairy cows, which led to the observational study described in Chapter 6;
5) To investigate the impact of the incomplete milking on behavior, which is commonly used to evaluate pain, presented in Chapter 7.

CHAPTER 2: LITERATURE REVIEW

In this review, the general life cycle of a cow is described, followed by the physiology of lactation and the negative energy balance. The latter section is further divided in the negative energy balance description, ways of measuring it, its consequences in terms of health and performance, and ways of decreasing its magnitude. This chapter finishes with the description of a novel strategy to improve cows' energy balance – to limit milk production in early lactation – along with its possible undesired effects.

The life cycle of a cow

The cow, like other mammals, reaches reproductive capacity while still growing. In an intensive dairy farming perspective, shorter times from birth to first calving, bring faster profits. Therefore, under an economic perspective, cows should have their first calf around 24 months of age (Esslemont and Kossaibati, 2000). After calving, the cow starts her lactation and a milk peak is achieved around four to eight weeks in milk (**WIM**). This peak may be held for several weeks, after which the milk production declines until the cow is dried off. The cow's ability to reduce or delay that decline in milk production is named *persistency* (Macciotta et al., 2005). Paradoxically, just at the time when the cow needs the most nutrients for milk production, the voluntary dry matter intake is at its lowest point (see *Negative energy balance – Description*). Maximum feed intake is only achieved around 8-22 WIM, so until that time the cow relies partialy on her body stores (Akers, 2002; Figure 1).

In primiparous animals (i.e. animals in their first lactation or parity), energy resources for growing will compete against resources for pregnancy and lactation, leading to a slower growing rate (about half of the previous rate). Energy allocation is prioritized for the fetus, followed by the body reserves and growth. After parturition, milk synthesis is prioritized and maternal growth is suspended until the energy balance is restored later in lactation (Whittemore, 2009). Compared to multiparous cows, in primiparous the lactation peak is lower and occurs a bit later (due to mammogenesis, see *Physiology of lactation*). Another difference is that the decline in milk yield after peak production is not as sharp as that of older cows (i.e., they show a better persistency; Stanton et al., 1992; Rekik et al., 2003; Coffey et al., 2004). These

differences in the lactation curve of primiparous animals, along with a shorter lag between peaks for milk yield and food intake, lead to better energy balance at the beginning of the lactation in this group of animals (see Figure 2). Cows in their second lactation are still growing, but almost reaching maturity. Full maturity is generally reached around the third lactation (Coffey et al., 2004; Van Hoeij et al., 2017).

After calving, producers allow for a period of around 50 DIM (voluntary waiting period, **VWP**) before first breeding, generally through artificial insemination (**AI**; Radostits et al., 2007). There might be several inseminations until the cow becomes pregnant again, but ideally, she becomes pregnant after one AI, and she will be calving again in one year (calving to calving interval of 341 to 403 d; Inchaisri et al., 2010). Approximately two months before calving, the cow has a pause, the *dry period*, in which she prepares for her next lactation, by maintaining and maybe slightly increasing her body reserves (Radostits et al., 2007).

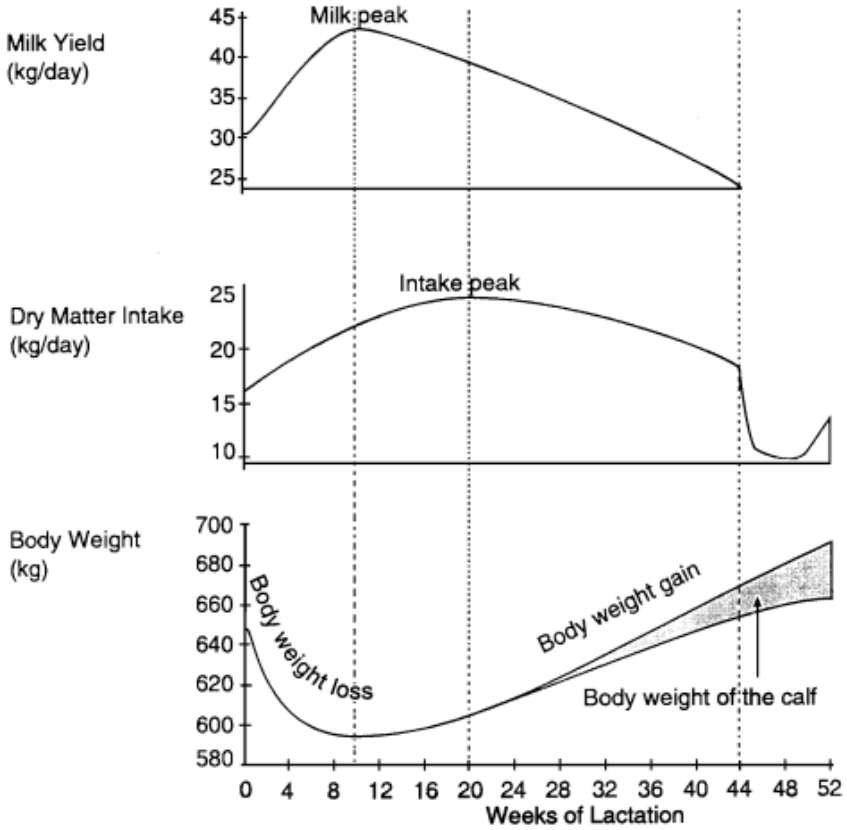

Figure 1. Phases of the lactation cycle with corresponding changes in milk yield, dry matter intake and body weight
(Source: NRC, 1989. Nutrient Requirements of Dairy Cattle. Washington, DC.)

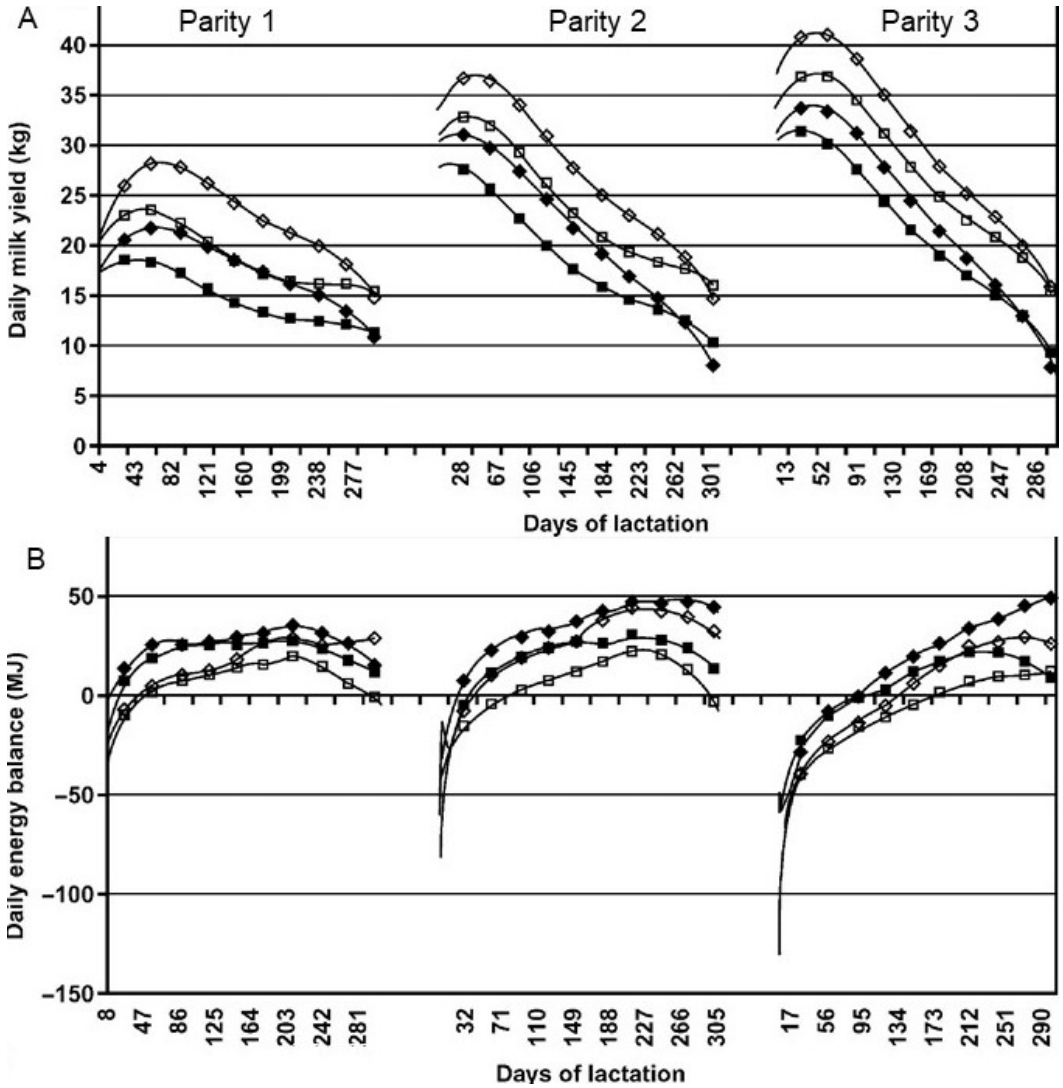

Figure 2. Average milk yield (kg; image in the top, A) and daily energy balance (MJ; image in the bottom, B) per by days of lactation for cows in first parity (left), second (middle), and third (right)

Symbols refer to different diets: low-concentrate control ■, low-concentrate selected for maximum production □, high-concentrate control ♦, and high-concentrate selected for maximum production ◊.

(Source: Coffey et al., 2004. Genotype and diet effects on energy balance in the first three lactations of dairy cows. J Dairy Sci. 87(12):4318-26.)

Physiology of lactation

Homeorhesis is, as defined by Bauman and Currie (1980), "the orchestrated or coordinated changes in metabolism of body tissues necessary to support a physiological state". The physiological state of lactation is an example of homeorhesis in which several physiological adaptations involving most of the body tissues (i.e. mammary tissue, digestive tract, liver, adipose tissue, etc) occur. These adaptations are related to the various hormonal changes that take place during mammogenesis (i.e. the development of the mammary gland), lactogenesis (i.e. the onset of milk secretion) and galactopoesis (maintenance of milk secretion). Most mammary growth occurs during pregnancy, but it lasts until the peak of lactation. Lactogenesis is divided in two phases, a first one (lactogenesis 1) that happens in midpregnancy, in which genes coding for proteins are activated in the mammary epithelial cells, allowing these to differentiate into active secretory cells; and a second one (lactogenesis 2), led by prolactin, that leads to secretion of colostrum and milk. After calving, prolactin and oxytocin maintain lactation, acting on milk secretion and ejection, respectively (Neville et al., 2002; Sjaastad et al., 2010).

Sexual hormones such as progesterone (**P4**) and estrogen are of primary importance for lactogenesis. Progesterone stimulates udder development during pregnancy, and it is a key hormone for lactogenesis. Its concentration falls around calving, allowing the mammary gland to respond to the presence of prolactin (Sjaastad et al., 2010).

On the other hand, blood estrogen concentration increases as calving approaches (Grummer et al., 1990; Ingvartsen, 2006). Estrogen stimulates insulin-like growth factor (**IGF)** I secretion from the udder, leading to growth of mammary epithelial cells (Svennersten-Sjaunja and Olsson, 2005). However, estrogen also has the capacity to increase the brain sensitivity to satiety hormones, more specifically to cholecystokinin (Geary, 2001) and to leptin (Ainslie et al., 2001), decreasing voluntary feed intake (Wade and Schneider, 1992). Leptin is a powerful regulator of appetite, being modulated not only by estrogen but also by insulin, glucocorticoids and thyroid hormones. It is secreted by the adipocytes and it remains low during the postpartum period (Vernon and Houseknecht, 1999; Wathes et al., 2007a).

Milk production is mainly controlled by prolactin and somatotropin (growth hormone), which are both lactogenic hormones. In early lactation, the presence of these two hormones leads to high rate of milk production despite the relatively low dry matter intake (Clarenburg, 1992). Somatotropin stimulates IGF-I production by the liver (but also in other organs such as the hypothalamus, ovaries, oviducts and uterus; Clarenburg, 1992). However, during early lactation, insulin concentrations remain low, which prevents an increase in receptors for somatotropin in the liver, therefore reducing IGF-I secretion and increasing somatotropin concentrations (Lucy, 2008). The rapid decline in IGF-I levels after parturition, is followed by a progressive increase as lactation persists and as energy balance improves (Baumrucker, 1999). This polypeptide has been positively correlated with dry matter intake (Francisco et al., 2002), and it is essential for normal follicular development (Zulu et al., 2002; Van Den Hurk and Zhao, 2005).

Somatotropin and insulin are primarily metabolic hormones and they consequently ensure adequate supplies of nutrients to the mammary gland. Somatotropin is a lypolitic agent, leading to utilization of protein and lipid stores for milk production, and it stimulates hepatic gluconeogenesis and decreases insulin receptors in the muscle, so that more glucose is available for milk production (Sjaastad et al., 2010). Low insulin levels reduce glucose utilization by non-mammary tissues (i.e. adipose and muscle) and facilitate greater uptake of glucose by the mammary gland. Cows have low capacity to absorb glucose directly from their diet, therefore gluconeogenesis is their main source to produce lactose (Herdt and Emery, 1992). If glucose supply fails to meet the needs associated to lactation, then several metabolic disorders occur, resulting in metabolic disease, ketosis and compromising the cow's well-being (Bauman, 1999). Insulin also affects reproduction by promoting follicular response to gonadotropins (i.e. follicle-stimulating hormone, **FSH** and luteinizing hormone, **LH**; Frajblat and Butler, 2000).

Glucocorticoids (e.g. cortisol and corticosterone) are adrenal hormones that are released in response to metabolic challenges (e.g. generalized stress, food deprivation) and have the capacity to increase the availability of endogenous glucogenic precursors depending on insulin status (Umpleby and Russell-Jones, 1996). Cortisol, particularly, is a primary hormone in lactogenesis, being essential for the effect of prolactin initiating lactation (Sjaastad et al., 2010). Its concentration starts to increase around three days before calving, and decreases around three

to five DIM (Patel et al., 1996). Similarly to cortisol, triiodothyronine (thyroid hormone) is required to maintain milk secretion, as in its absence the intensity and duration of milk secretion is reduced (Rose and Obara, 2000; Sjaastad et al., 2010).

Negative energy balance

Description

During the transition period (i.e. from three weeks before until three weeks after calving), the dry matter intake is at its lowest point while the energy requirements for fetal development (pre-calving period) and then for galactopoesis (post-calving period) are high (Grummer et al., 2004). The lower dry matter intake is a result of several factors such as fetal growth (based on the idea that the fetus restricts rumen volume) and body condition (i.e. lipid stores). For instance, high levels of concentrate in the diet appear to have a negative impact on voluntary intake when compared to diets with more fiber (Allen, 2000; Akers, 2002). Metabolism also plays an important role on food intake, through levels of estrogen, blood metabolites, corticosteroids, leptin, insulin, gut peptides, and cytokines (Grummer et al., 1990; Ingvartsen and Andersen, 2000; Ingvartsen, 2006). Therefore, the lag between peak yield and the increase in feed intake leads to a physiologically unavoidable negative energy balance, in which insulin-antagonist hormones (e.g. glucagon, glucocorticoids, epinephrine, and norepinephrine) are released, leading to proteolysis and lipolysis (Bauman and Currie, 1980; Clarenburg, 1992). Hence, cows rely partially on body reserves during early lactation, resulting in a 50–75 kg loss of body weight in early lactation (Roche, 2006).

Lipolysis leads to extensive mobilization of lipids from the adipose tissue, in the form of non-esterified fatty acids (**NEFA**). In the liver, NEFA are oxidized to form acetyl-CoA. In turn, acetyl-CoA can be further oxidized for energy in the Kreb's tricarboxylic cycle (**TCA**; complete oxidation; Ingvartsen, 2006). However, when there is an excess of NEFA to be oxidized by the liver, acetyl-CoA is instead partially oxidized into ketone bodies, which may be used as energy source by the heart, kidney, skeletal muscles, mammary gland and gastrointestinal tract (Whitaker et al., 1993; Figure 3). There are three ketone bodies, acetate, acetoacetate and BHBA, the latter being the predominant circulating ketone body. When liver's

capacity to oxidize NEFA is exceeded, NEFA are then re-esterified to form triglycerides that can be exported as very low density lipoproteins (**VLDL**) or stored in the liver. Ruminants ability to export VLDL is limited, therefore triglycerides liver storage is the main outcome (Grummer et al., 2004). Therefore, when lipid uptake by the liver is greater than its ability to oxidize NEFA's and to export VLDL's, hepatic lipidosis (or fatty liver) might develop, with consequent liver dysfunction (Bobe et al., 2004; Mulligan and Doherty, 2008).

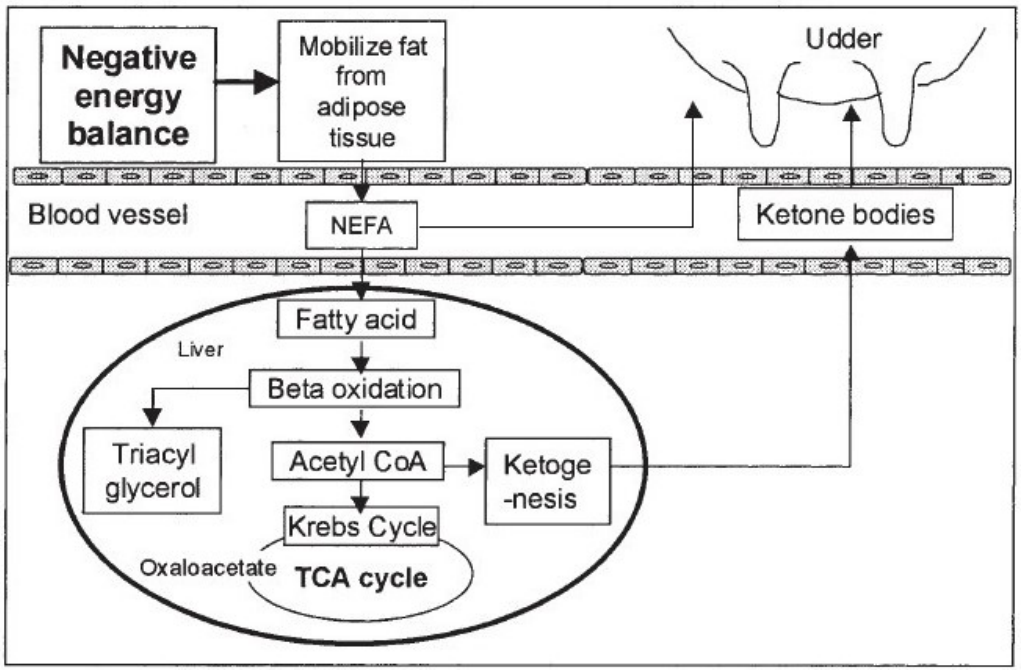

Figure 3. Metabolic pathways of ketogenesis in the blood circulation, the liver and the udder

NEFA: Non-esterified fatty acid; TCA: Kreb's tricarboxylic cycle.

(Source: Suriyasathaporn et al., 2000. Hyperketonemia and the impairment of udder defense: a review. Vet. Res. 31:397–412.)

Measuring the negative energy balance

Energy balance can be calculated by subtracting the energy utilization (e.g. for maintenance, growth, activity and milk yield) to the energy intake (the energy content in the diet; Emmans, 1994). Energy used for fetus and growth, along with the exact food intake by individual cow can be difficult to determine in practice, limiting the use of this method to measure energy balance on farm.

Another way to measure the level of negative energy balance, is to measure changes in cows' body condition during early lactation (Coffey et al., 2003). Body condition scoring (**BCS**) is a subjective evaluation of cows' body reserves made through observation of specific body regions such as the spinous and transverse processes of the lumber vertebrae, or the tail head regions (Edmonson et al., 1989; Ferguson et al., 1994). The 5-point scale method developed by Ferguson et al. (1994) is the most commonly used scale, in which 1 is thin and 5 is obese and increments are generally done by 0.25 or 0.5 units. According to Contreras et al. (2004), the ideal BCS at dry-off is 3.0. A BCS > 3.5 at calving, might lead to excessive fat mobilization in the early lactation (Clarenburg, 1992; Figure 4), which in turn can reduce cows' performance (Domecq et al., 1997). Since BCS is negatively associated with dry matter intake (through leptin production by the adipocytes), the higher the BCS at calving, the higher the negative energy balance and BCS loss (Roche et al., 2007). The problem with BCS is that within and between observers agreement might vary considerably (Ferguson et al., 1994; Morin et al., 2017).

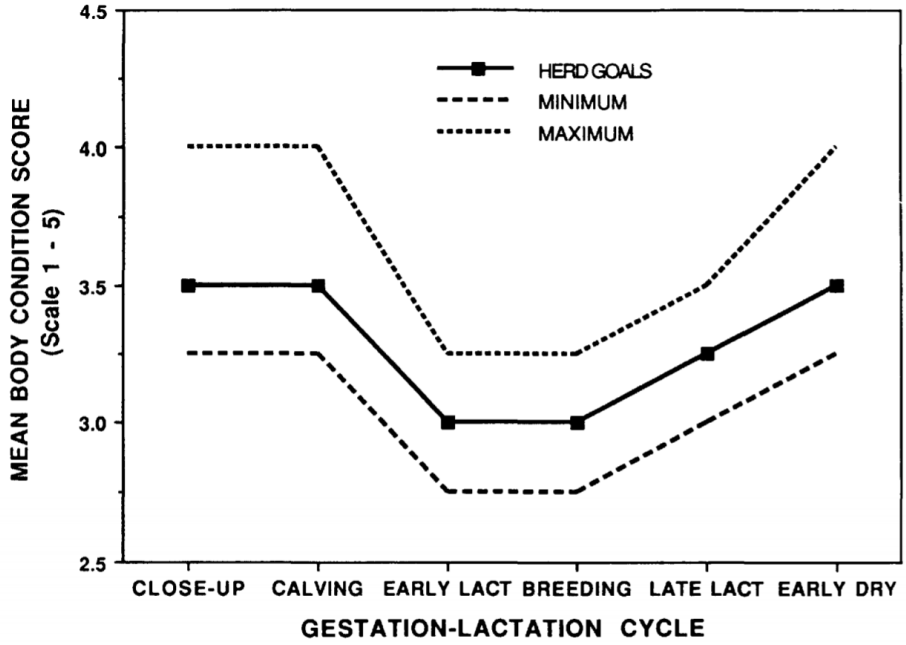

Figure 4. Goals, minimum, and maximum mean herd body condition scores for dairy cattle throughout the lactation cycle, based on a scoring system ranking cows from (emaciated) to 5 (obese)
LACT: Lactation; DRY: Dry period.
(Source: Data from Braun, et. al. 1986. Body condition scoring dairy cows as a herd management tool. Compend Contin Educ Pract Vet 8:62; and Ferguson and Otto 1988. Managing body condition in dairy cows. Page 75 in the Cornell Nutrition Conference for Feed Manufacturers Proceedings, Syracuse, NY.)

Blood NEFA concentration is a good indicator of lipid mobilization, and mirrors dry matter intake (Adewuyi et al., 2005; Ospina et al., 2010b; Seifi et al., 2011). A NEFA blood concentration ≥ 0.3 mmol/L detected 35 to 3 days before calving (Cameron et al., 1998) or ≥ 1.0 mmol/L detected between 1 and 7 DIM (LeBlanc et al., 2005) have been associated with increased risk for left displaced abomasum. Concentrations ≥ 0.6 mmol/L during 3 to 14 DIM have also been associated with increased risk for clinical ketosis, retained placenta and metritis (Ospina et al., 2010b). However, no cow-side test is available to measure NEFA yet, therefore to quantify it, samples have to be sent to a laboratory, making it less practical and more expensive to use.

Detection of ketone bodies in the blood, urine or milk can also be used as an indicator of the level of negative energy balance. As mentioned above, they reflect the completeness of oxidation of fat in the liver, thus they are only indirectly related to lipid mobilization. Compared to other ketone bodies, BHBA is the one with higher stability in serum, being therefore the most commonly measuring ketone body to diagnose ketosis (Oetzel, 2004; Iwersen et al., 2009). Although tests that detect ketone bodies in urine can reach good levels of sensitivity (**Se**) and specificity (**Sp**) to detect ketosis (Carrier et al., 2004), blood and milk samples are more easily obtained and therefore they are generally favored for ketosis diagnosis (Carrier et al., 2004; LeBlanc, 2010). Some examples of cow-side tests frequently used for ketone bodies measurement are Precision Xtra (for measurement of BHBA in blood, urine and milk; Abbott Laboratories, Abbott Park, IL), Ketostix Strip (for measurement of acetoacetate in urine; Bayer Corporation, Elkhart, IN) and KetoTest (for measurement of BHBA in milk; Sanwa Kagaku Kenkyusho Co. Ltd., Nagoya, Japan).

Hyperketonemia is frequently defined as blood BHBA concentrations ≥ 1.4 mmol/L during WIM one (Duffield et al., 2009) or ≥ 1.2 during the first two WIM (Suthar et al., 2013), because both thresholds have been associated with increased risk of left abomasal displacement and lower milk production. Hyperketonemia generally occurs during the first two WIM, with a peak incidence at DIM five (McArt et al., 2012) and its prevalence is generally between 16 and 25% (Duffield et al., 2009; Chapinal et al., 2012b). Some authors prefer to use the terminology *subclinical* or *clinical ketosis*, when hyperketonemia is presented without or with signs of disease (i.e. lower appetite, obvious rapid weight loss, decrease in milk production), respectively

(Oetzel, 2007; Radostits et al., 2007). The term hyperketonemia will be used throughout the current document because clinical cases were not excluded.

Consequences of severe negative energy balance

Numerous studies have demonstrated an association between negative energy balance and its indicators (hyperketonemia) and several transition diseases. These diseases are inter-related, and most of them, along with negative energy balance, increase the risk of poor reproductive performance and culling (Mulligan and Doherty, 2008). It is therefore very difficult to determine the level of causality between all these variables. Those relationships will be described in detail in the following sections.

Increased risk for metabolic diseases

Besides ketosis, another condition that occurs due to high demands for fetal maturation and milk production is peripartum hypocalcemia. It results from the increased requirement for calcium combined with a poorer response of osteocytes to the hormones responsible for triggering bone resorption and calcium homeostasis (Radostits et al., 2007) and it is more common in multiparous animals (Reinhardt et al., 2011). Hypocalcemia can be subdivided in clinical (also known as milk fever) or subclinical, depending on presence of clinical signs. Incidence rate of clinical hypocalcemia varies between 3.5 and 7% (DeGaris and Lean, 2008) while that of subclinical hypocalcemia (blood calcium concentration < 2.0 mmol/L) may vary between 25 and 54% (Reinhardt et al., 2011). Hypocalcemia can put an important toll on the cow ability to survive. It is not only positively associated with development of ketosis, but also with several other conditions such as dystocia, uterine prolapse, retained placenta, displacement of the abomasum, downer cow syndrome, immunosuppression, infectious diseases (mastitis and endometritis) and infertility (Goff and Horst, 1997; Houe et al., 2001).

Displacement of the abomasum is a multifactorial syndrome occurring mostly in high yielding cows, during the first month after parturition. Gastrointestinal stasis, due to hypocalcemia, is a prerequisite for its development. Other risk factors are hyperinsulinemia and ketosis (Fecteau and Guard, 2015). Several studies have shown an association between ketosis and development of left abomasal displacement (Duffield et al., 2009; Ospina et al., 2010b;

McArt et al., 2012). In Duffield et al. (2009) study, BHBA blood concentration ≥ 1.4 mmol/L detected during the first WIM increased the odds of abomasal displacement by 2.8 times (95% confidence interval, **CI**, for the odds ratio, **OR**: 1.3, 6.0). Ospina et al. (2010b) found an increased risk by 6.9 (95% CI for the risk ratio, **RR**: 3.7, 12.9) in cattle presenting BHBA blood concentration ≥ 1.7 mmol/L around 3 to 14 DIM. Finally, McArt et al. (2012) found that BHBA blood concentration between 1.2 and 2.9 mmol/L detected around 3 to 16 DIM increased the risk of abomasal displacement by 1.1 times (95% CI for the RR: 1.0, 1.2). Left displaced abomasum is the most common form of the disease, with an incidence ranging from 5 to 7% (LeBlanc et al., 2005).

Increased risk for infectious diseases

The transition period is accompanied by some degree of immunosuppression in most dairy cows (Goff, 2008). The etiology of this immunosuppression is not completely understood, but it seems to be caused by an association of factors that include negative energy balance and increased cortisol (Preisler et al., 2000). Cortisol, estrogen and P4 have negative effects on the immune system, while somatotropin, prolactin, insulin and IGF-I have positive effects (Ingvartsen et al., 2003). The low levels of IGF-I during early lactation, particularly, decrease the immune defenses, due to the IGF-I role in modulating the pro-inflammatory response. Moreover, during the periparturient period, as a result of stresses associated to calving (injuries during calving, mammary gland edema and uterus involution), there is often release of pro-inflammatory cytokines (tumor necrosis factor alpha, **TNF-α**; interleukins, **IL** 1 and 6; Trevisi et al., 2011) and of reactive oxygen species (**ROS**; Abuelo et al., 2014). Release of pro-inflammatory cytokines leads to an increase of some acute-phase proteins and a decrease in other liver proteins such as albumins, lipoproteins and retinol binding protein (Trevisi and Bertoni, 2008). Reactive oxygen species play an important role in the host immune response, being involved in expression of immunoregulatory substances and inflammatory response optimization. But when produced in excess, ROS can lead to tissue damage (through oxidation of DNA, cellular proteins and lipids) and impairment of cell functions (Valko et al., 2007). Cow's homeostasis plays, therefore, a major role in modulating the immune system.

Another reason for the immunessupression observed during the transition period is hypocalcemia. Essentially, a key feature in peripheral blood mononuclear cells (**PBMC**) activation is an increase in intracellular ionized calcium concentration ($[Ca^{2+}]_i$). This increase in $[Ca^{2+}]_i$ after an activation signal is an indicator of PBMC responsiveness and function (Baus et al., 1996; Grafton and Thwaite, 2001; Kimura et al., 2006). Therefore, hypocalcemic cows have generally decreased immune defenses (Ducusin et al., 2003; Bréchard and Tschirhart, 2008; Martinez et al., 2012).

Cows with elevated blood concentration of NEFA and BHBA (indicators of negative energy balance), have impaired polymorphonuclear leukocytes (**PMNL**) function (Suriyasathaporn et al., 1999; Suriyasathaporn et al., 2000; Hammon et al., 2006; Scalia et al., 2006; Ster et al., 2012). These functions include cell adhesion, molecules expression, chemotaxis and oxidative burst. Also, NEFA seem to decrease *in vitro* cytokine production by lymphocytes (examples of cytokines: interferon gamma, **IFN-γ**; TNF-α; IL-4; Lacetera et al., 2004; Ster et al., 2012) and to decrease PBMC proliferation (Ster et al., 2012). The cytokine IFN-γ produced by lymphocytes is responsible for the activation of macrophages and it is important for an effective cell-mediated immune response, therefore its shortage leads to immunosuppression (Loiselle et al., 2009). The action of BHBA on IFN-γ production and in PBMC proliferation is less pronounced than NEFA's, and it might be caused by the close association between NEFA and BHBA (Ster et al., 2012). Nevertheless, it is not only the innate immune system that is affected during the transition period, the acquired immune responses are also negatively affected (Mallard et al., 1997). As consequence, the periparturient dairy cow becomes more susceptible to infectious diseases (e.g. metritis and mastitis).

Increased risk for reproductive tract disease

Development of infectious diseases is dependent on the balance between host immunity and the bacteria. Therefore, although contamination of the uterus during calving or in early postcalving is very common, development of uterine disease, however, depends heavily on type and number of microorganisms and on the immunological status of the cow (Sheldon et al., 2006). Conditions that lead to low immune defenses such as those related to negative energy

balance are important factors to development of reproductive tract diseases. Other risk factors are retained placenta, twins, dystocia, parity, season and BCS (LeBlanc et al., 2002).

Uterine diseases in dairy cattle can be classified as metritis or endometritis. Metritis is an acute inflammation of the uterus, occurring < 21 DIM and being often accompanied by fetid brown-red watery vaginal discharge and systemic signs (i.e. fever, lower milk yield, lower feed intake). Endometritis occurs later (≥ 21 DIM) and does not include systemic signs (Sheldon et al., 2006). Reproductive tract diseases also include purulent vaginal discharge (**PVD**), which is sometimes referred to as *clinical* endometritis (LeBlanc et al., 2002; Kaufmann, 2010).

Cytological endometritis is diagnosed by endometrial cytobrush (Kasimanickam et al., 2004), endometrial flush (Sheldon et al., 2006; Galvão et al., 2009) or endometrial biopsy (Bonnett et al., 1993; Chapwanya et al., 2010) and it is defined as an increased proportion of PMNL in the endometrium. When testing (with endometrial cytobrush) animals without clinical signs from 20-33 DIM, PMNL count > 18% was associated with reduced subsequent reproductive performance (Kasimanickam et al., 2004). When testing all animals from 28-42 DIM, regardless of presence of clinical signs, PMNL count ≥ 6% had the highest Se (42%) and Sp (80%) to predict non-pregnancy status at first AI (Denis-Robichaud and Dubuc, 2015). These tests generally involve microscopic PMNL count, unless when used along with leukocyte esterase (**LE**), which is performed with a colorimetric semi-quantitative strip that becomes violet in presence of PMNL (Cheong et al., 2012). This dye occurs through the oxidation of diazonium salt in presence of leukocyte esterase released from the neutrophilic cells, thus darker colors are correlated to higher levels of leukocytes and recorded as: 0, negative, 0.5, trace of leukocytes, 1, small amount of leukocytes, 2, moderate amount of leukocytes, 3, large amount of leukocytes (Couto et al., 2013). The LE test is highly correlated to endometrial cytology according to Santos (2006; kappa = 0.60), but moderately correlated according to Denis-Robichaud and Dubuc (2015; kappa = 0.43). According to Denis-Robichaud and Dubuc (2015), when testing all animals from 28-42 DIM, regardless of presence of clinical signs, LE ≥ 1 had the highest Se (52%) and Sp (60%) to predict non-pregnancy status at first AI. Cheong et al. (2012), tested cows from 40-60 DIM and concluded that only LE ≥ 3 had an association with decreased hazard of pregnancy (increased calving to conception interval), presenting a good Se (34%) and Sp (90%). In Dubuc and Denis-Robichaud (2017), the median herd-level prevalence

(126 herds) of cytological endometritis between 30 and 43 DIM diagnosed by PMNL count (≥ 6%) or by LE (≥ 1) was 29.4% (range: 5.3, 80.0%) and 43.8% (range: 0, 77.8%), respectively. In summary, several definitions of cytological endometritis are present in the literature and various tools including cytobrush and leukocyte esterase are available to evaluate its presence.

Purulent vaginal discharge can be diagnosed by visual examination (Miller et al., 1980), manual examination (Sheldon et al., 2002), vaginoscopy (Barlund et al., 2008) or by Metricheck (McDougall et al., 2007). The Metricheck test (Simcro, Hamilton, New Zealand) has six levels from 0 to 5 depending on vaginal secretion's appearance (McDougall et al., 2007): 0, no discharge; 1, clear mucus; 2, mucus with flecks of pus; 3, mucopurulent discharge; 4, purulent discharge; or 5, foul smelling discharge. When using Metricheck, Dubuc et al. (2010a) found that the threshold ≥ 3 was the best to predict non-pregnancy status during the first 120 DIM, showing a Se and Sp of respectively, 18% and 90% when used at 32-38 DIM, and of 15% and 92%, when used at 53-59 DIM. On the other hand, Denis-Robichaud and Dubuc (2015) found that a Metricheck score ≥ 4 around 28-42 DIM had the highest Se (20%) and Sp (91%) to predict non-pregnancy status at first AI. There is only a slight agreement between endometrial cytology and presence of PVD (kappa = 0.20; Denis-Robichaud, 2013). Furthermore, both disorders have a cumulative impact on reproductive performance (Dubuc et al., 2010a), which shows that they have additive detrimental impacts, and that therefore they should not be considered as different levels of the same disease (endometritis). In Dubuc and Denis-Robichaud (2017), the median herd-level prevalence of PVD (≥ 4) between 30 and 43 DIM was 5.0% (range: 0, 45.0%).

Several studies have showed an association between reproductive tract diseases and negative energy balance. In Hammon et al. (2006), cows with metritis and cows with endometritis had significantly higher NEFA and higher BHBA blood concentrations from one to four WIM, compared to unaffected cows. Similarly, in Dubuc et al. (2010b), a blood BHBA concentration ≥ 1.1 mmol/L from one to seven DIM increased the odds of cytological endometritis and of PVD by 1.4 times (95% CI for the OR: 1.1, 2.0 and 1.0, 1.8, respectively), but it did not affect metritis risk.

Increased risk for mastitis

Mastitis is an inflammation of the mammary gland that is generally caused by bacterial pathogens. Clinical mastitis is characterized by local clinical signs such as abnormal secretion (clots or serum in milk), size, consistency or temperature of the mammary gland (one or more quarters). Systemic clinical signs might be present, depending on severity of mastitis (Ruegg et al., 2009). In Canada, similarly to other countries, the bacteria most commonly associated to clinical mastitis are *Staphylococcus aureus* streptococci, and coliforms (Reyher et al., 2011; Ruegg et al., 2015). To monitor clinical mastitis, besides the observation of local clinical signs, farmers frequently make a visual observation of foremilk stripping against a black-colored strip plate (George et al., 2008). The susceptibility of the mammary gland to an intramammary infection (**IMI**) is the result of several factors (i.e. parity, stage of lactation, season; Rodriguez-Zas et al., 1997). For instance, there is generally higher rate of new infection just after drying off and around calving (Figure 5). Similarly, a peak in clinical mastitis incidence rate generally occurs just after calving (WIM one), followed by a declining toward the end of the lactation (Riekerink et al., 2008; Figure 6). The reasons for the higher susceptibility to IMI and clinical mastitis at the beginning of the lactation include dilution of the protective factors (e.g. lactoferrin) in milk, along with keratin plug break down and negative energy balance (Bradley and Green, 2004). In Riekerink et al. (2008) study, conducted in 106 herds from 10 Canadian provinces, the median herd-level incidence rate of clinical mastitis was 16.7 (range: 0.7, 97.4) cases per 100 cow-years.

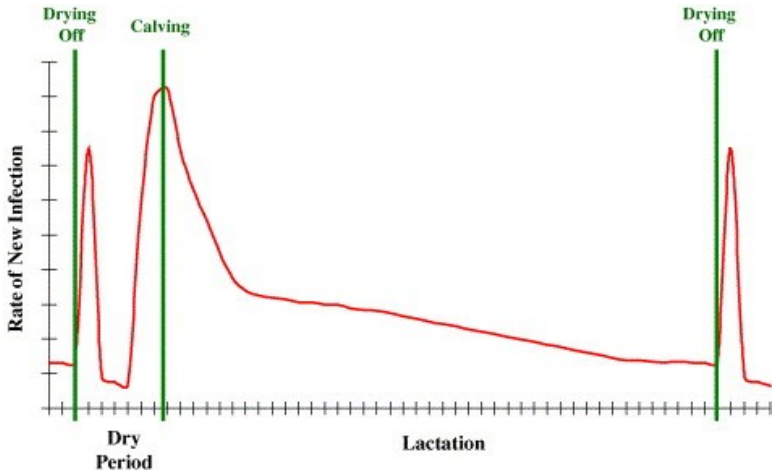

Figure 5. Incidence of new intramammary infection during the dry period and lactation

The peak in new infection rate, after drying off, is considerably higher in cows not receiving any form of dry cow therapy.

(Source: Bradley and Green, 2004. The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. Veterinary Clinics: Food Animal Practice. 20(3):547-68.)

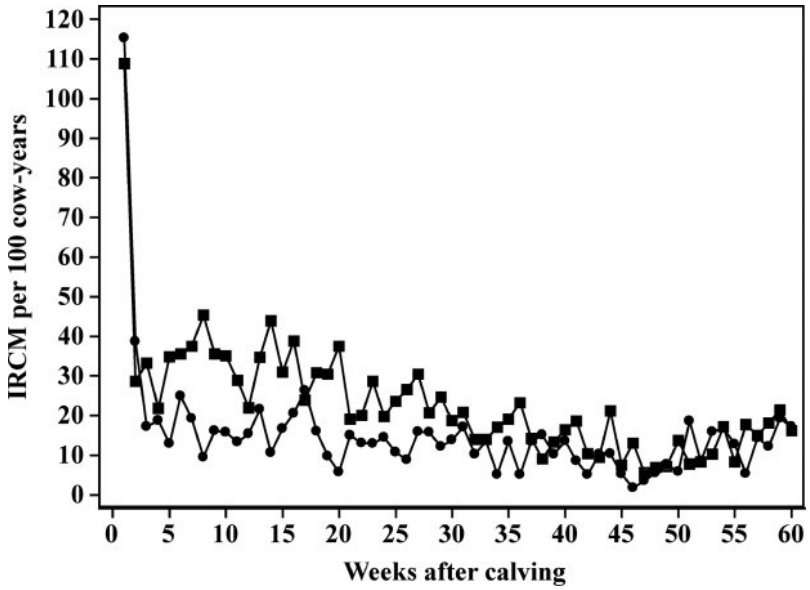

Figure 6. Distribution of incidence rate of clinical mastitis per week after calving

The participating producers recorded clinical mastitis cases, defined as udders/quarters with visible signs of inflammation. IRCM: Incidence rate of clinical mastitis; ● primiparous, ■ multiparous.

(Source: Riekerink et al., 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J Dairy Sci. 91(4):1366-77.)

In subclinical mastitis, milk appearance is normal even though an IMI is usually present. This form of mastitis is accompanied by increased milk somatic cell counts (**SCC**; i.e. inflammatory cells), which are mainly composed by phagocytes (macrophages and PMNL) and are essential for pathogen removal from the udder (Suriyasathaporn et al., 2000; Ruegg et al., 2009). Generally, IMI diagnosis would be made by milk bacteriological culture or polymerase chain reaction (**PCR**), but SCC is often used as a proxy for IMI. Using individual quarter milk samples, Schepers et al. (1997) concluded that a SCC of ≥ 200,000 cells/mL could be used to diagnose an IMI (defined using bacteriological milk analyses) with a Se and a Sp of 75% and 90%, respectively. The SCC should be measured using milk collected from individual quarters, since composite milk samples might impede ability to identify infected quarters' due to dilution of inflammatory cells with milk from healthy quarters (Schepers et al., 1997). There are several other factors, besides IMI, affecting SCC: stage of lactation (late lactation), parity (older cows), season (summer) or time of the day (high just after milking up to four hours afterwards; Sharma et al., 2011). For instance, SCC from morning or from evening milking can vary considerably within the same quarter (Eisenberg et al., 2016). Another way to detect subclinical mastitis is to perform a California Mastitis Test (**CMT**), which makes a qualitative estimate of the amount of DNA present in milk (i.e. CMT disrupts the cell membrane of milk cells, allowing the DNA to react with the reagent, resulting in the formation of a gel), and correlates to the quantity of SCC in milk. It scores in five levels: negative, trace, +1, +2 and +3 (George et al., 2008). However, the CMT is not very sensitive in detecting infected quarters (Se = 0.27 and Sp = 0.80 using threshold ≥ +1) from cows without clinical mastitis (Kandeel et al., 2018). The bacteria most commonly associated to subclinical mastitis are coagulase-negative staphylococci (Reyher et al., 2011; Ruegg et al., 2015). In Haine (2016) study, the median herd-level prevalence of cows with SCC ≥ 200,000 cells/mL was 20.1% (range: 10.4, 33.3%).

Since the negative energy balance affects many of the functions exerted by phagocytes (the main factors for a successful udder defense), it leads to inefficient pathogen removal and, consequently, to an increased risk for mastitis. Table I summarizes the results obtained in some studies evaluating the relationship between hyperketonemia and mastitis. Collectively, these studies suggest that hyperketonemia is associated with increased odds of mastitis.

Table I. The impact of hyperketonemia on clinical mastitis and on high somatic cell counts

Outcome	n	Prevalence of subclinical ketosis (%)	Diagnostic		Prevalence of disease	OR (95%CI)	Reference
			Threshold (mmol/L)	WIM of detection			
Clinical mastitis	951	3	BHBA ≥1.4	1	11.5	1.8 (1.2, 2.6)	Leslie (2000)
Clinical mastitis	951	3	BHBA ≥1.4	1 and 2	11.5	1.5 (1.0, 2.2)	Leslie (2000)
Clinical mastitis	5,041	22	BHBA ≥1.2	1 and 2	6.1	1.3 (0.38, 4.4)	Suthar et al. (2013)
"Elevated" SCC	552	11	BHBA ≥1.4	1 to 9	15.7	1.7 (1.2, 2.5)	Leslie (2000)
SCC > 250	1,720	NA	Acetoacetic acid in urine ≥1.5	NA	21.9	1.4 (1.2, 1.7)	Van Straten et al. (2009)

SCC, somatic cell count, n, sample size, BHBA, β-hydroxybutyrate, WIM, weeks in milk, OR, odds ratio

Reduced reproductive performance

Following parturition, the increase in plasma FSH concentration induces the first wave of follicular development around 5-7 DIM. Following that follicular wave, there are three possibilities for the dominant follicle (Beam and Butler, 1997): (1) ovulation (16-20 DIM); (2) non-ovulation followed by new follicular wave; or (3) non-ovulation followed by a cystic follicle. Ovulation occurs in 30-80% of cows, while options (2) and (3) delay the first ovulation to 40-50 DIM and they occur 15-60% and 1-5% of the time, respectively (Beam and Butler, 1997; Sartori et al., 2004). The frequency of LH pulses is crucial for the fate of the dominant follicle. For ovulation to occur, the dominant follicle must produce high levels of estradiol to stimulate the release of one LH pulse per hour (Roche, 2006). However, during negative energy balance, the hypothalamus-pituitary-ovary axis is hampered in several ways.

The lower IGF-I levels, along with the increased somatotropin affect not only the sensitivity of the ovary to the gonadotropins, but also the LH pulse frequency (Jorritsma et al., 2003; Lucy, 2003; Patton et al., 2007; Fenwick et al., 2008a). Moreover, the dominant follicle produces less estradiol and P4 after ovulation (Britt, 1992; Sangsritavong et al., 2002; Diskin et al., 2003). The lower estradiol produced leads to extra time needed for the follicle to increase its diameter until the adequate LH pulse frequency is finally triggered (Lopez et al., 2004; Sartori

et al., 2004). Cows that recover well from the peripartum negative energy balance and that have normal ovulatory follicles, have generally higher blood IGF-I concentration (Lucy et al., 1992; Roche, 2006) and higher estradiol production by the dominant follicle, contrarily to cows with non-ovulatory or cystic follicles (Beam and Butler, 1997; Beam and Butler, 1998). Moreover, these cows have generally two follicular waves every 18-23 d period (Sartori et al., 2004).

The IGF-I also promotes embryo formation and quality (Sirisathien and Brackett, 2003), therefore lower IGF-I levels associated to negative energy balance will impair early stages of embryo development (Wathes et al., 2003). Similarly to IGF-I, high plasma P4 concentrations are imperative for the embryo quality (Green et al., 2005; Mann et al., 2006). However, in cows experiencing prolonged negative energy balance, P4 concentrations take longer time to rise (Vasconcelos et al., 2003; Sartori et al., 2004).

Besides the impact on hypothalamus-pituitary-ovary axis, the negative energy balance can also interfere with fertility through a direct effect of blood metabolites on the oocyte. When follicles are in pre-ovulatory stage, they only possess a discontinuous unilaminar wall separating the oocyte from the blood, which makes the blood-oocyte barrier highly permeable. Thus, blood metabolites such as NEFA and BHBA may easily penetrate in the follicular fluid and exert a direct toxic effect in the oocyte (Jorritsma et al., 2004; Leroy et al., 2004; Fernandez-Fernandez et al., 2006).

There are several parameters that can be used to measure reproductive performance (Radostits et al., 2007): average time from calving to first-service; average time from calving to pregnancy; calving to calving interval; estrus detection rate; first-service pregnancy rate; overall pregrancy rate; services per pregnancy. Some of these are biased for not accounting for unbred cows (e.g. first-service pregnancy risk), for being highly influenced by intensity of estrus detection (e.g. average time from calving to first-service) or by culling policy (e.g. time from calving to conception; Radostits et al., 2007). Table II summarizes the effects of blood (or milk) BHBA and NEFA concentration on some of these parameters. Most studies are at cow level, with the exception of Ospina et al. (2010a), which was at herd level. In a recent meta-analysis conducted by Abdelli et al. (2017), BHBA and NEFA decreased significantly the pregnancy hazard (95% CI for the hazard ratio, **HR**: 0.61, 0.97), but had no impact on luteal activity (95% CI for the OR: 0.83, 1.1).

Table II. The impact of high non-esterified fatty acids and of hyperketonemia on reproductive performance

Outcome	n	%	Diagnostic		Measure of association	Value (95%CI)	Reference
			Threshold (mmol/L)	WIM of detection			
No luteal activity (CL absence at ultrasound)	957	20	NEFA ≥0.7	1 and 2	OR	2.3 (1.3, 4.0)	Ribeiro et al. (2013)
No luteal activity (CL absence at ultrasound)	957	35	BHBA ≥1.0	1 and 2	OR	0.75 (0.41, 1.4)	Ribeiro et al. (2013)
No luteal activity (blood P4 < 1 ng/mL)	2,178	NA	NEFA ≥0.9	1	OR	1.4 (1.1, 1.8)	Dubuc et al. (2012)
No luteal activity (milk P4 < 1 ng/mL)	1,341	NA	BHBA milk	1	OR	1.7 (1.2, 2.4)	Walsh et al. (2007a)
First-service pregnancy rate	203	17	BHBA 1.2-2.9	2	OR	0.23 (0.07, 0.63)	Rutherford et al. (2016)
First-service pregnancy rate	796	36	BHBA ≥1.0	1	OR	0.73 (0.54, 0.99)	Walsh et al. (2007b)
Time from calving to first-service	796	13	BHBA ≥1.0 and ≥1.4	1 and 2	HR	0.93 (0.76, 1.1)	Walsh et al. (2007b)
Time from calving to pregnancy	796	13	BHBA ≥1.0 and ≥1.4	1 and 2	HR	0.03 (0.004, 0.21)	Walsh et al. (2007b)
Time from calving to pregnancy	1,095	NA	BHBA ≥1.0	1 and 2	HR	0.87 (0.71, 1.1)	Ospina et al. (2010a)
Time from calving to pregnancy	1,095	NA	NEFA ≥0.7	1 and 2	HR	0.84 (0.75, 1.0)	Ospina et al. (2010a)

CL, corpus luteum, P4, progesterone, n, sample size, %, prevalence of cows with high metabolites, NEFA, non-esterified fatty acids, BHBA, β-hydroxybutyrate, WIM, weeks in milk, OR, odds ratio, HR, hazard ratio

Reduced milk production, reduced longevity, and higher costs

The previous sections clearly show that severe negative energy balance increases the risk of disease (both metabolic and infectious), and these have been associated with decreased milk production, so it is logical to assume that hyperketonemia would have an indirect effect on milk production through its effects on disease. Indeed, Duffield et al. (2009) found that a BHBA blood concentration ≥ 1.4 mmol/L on the first WIM was associated with lower milk yield at the first Dairy Herd Improvement (**DHI**) test and concentrations ≥ 1.8 mmol/L were associated with 300 kg of milk yield losses over the whole lactation. Negative impacts of peripartum hyperketonemia on daily milk production in early lactation (≤ 30 DIM; McArt et al., 2012) or

in milk performance during the whole lactation (Harrison et al., 1990; Ospina et al., 2010a; Chapinal et al., 2012a) were supported by other authors. A recent meta-analysis conducted by Raboisson et al. (2014) used 13 models from four studies and concluded that high BHBA levels in early lactation were associated with 340 kg of milk losses over the lactation. However, this relationship might become ambiguous depending on the time at which the BHBA sample is taken (Duffield et al., 2009), or when comparing primiparous to multiparous animals (Ospina et al., 2010a).

Since milk production is the main source of income in a dairy farm, it is also logical to assume that if a cow produces less milk, she will be at higher risk of being removed from the farm (i.e. culled). In Quebec, infertility is the main cause of culling for primiparous and second lactation animals (Durocher, 2007), while mastitis is the main cause for culling of older animals. Hence, severe negative energy balance can influence culling through several pathways. In fact, according to McArt et al. (2012), cows that present hyperketonemia (BHBA concentration between 1.2 and 2.9 mmol/L) at 2-16 DIM are 3.0 times more likely (95% CI for the RR: 2.2, 4.2) to be culled or die during the first 30 DIM, than unaffected cows. In Roberts et al. (2012) study, the results were quite similar. The odds of culling (95% CI of the OR) within 60 DIM were increased by 1.8 (1.4, 2.2) if hyperketonemia (1.2 mmol/L threshold) was detected on the first WIM (Roberts et al., 2012). It is relatively easy to demonstrate that there is a relationship between hyperketonemia and early culling, because these events occur close in time. However, if we want to study its association with late culling, there are generally many events interfering between the hyperketonemia status and the actual culling decision, which makes it hard to infer causality.

Finally, the costs associated to severe negative energy balance can be very high. Besides the direct effects of ketosis (costs for monitoring, treating and milk losses), we can also add the indirect effects (increased risk of disease, decreased fertility, and higher culling). McArt et al. (2015) developed an economic model to estimate 1) costs directly associated to hyperketonemia, and 2) costs due to hyperketonemia-attributed diseases, in which the authors accounted for displaced abomasum, metritis (and retained placenta), as well as reproduction losses. When considering only the direct causes of hyperketonemia, the cost per case was estimated at US$111 in multiparous cows. When also accounting for the losses associated to hyperketonemia-

attributed diseases, the total cost per case of hyperketonemia was estimated at US$256 in multiparous cows. The factors with the highest contribution for the total cost were reproductive losses followed by culling and milk losses. Expenses were even higher in primiparous cows (McArt et al., 2015).

Limiting the negative energy balance

To limit the negative energy balance, one can either increase the cow's energy intake, or decrease the energy demand. Alternative ways to increase the energy balance include genetic improvement (Oikonomou et al., 2008) and shorter or inexistent dry periods (Van Hoeij et al., 2017). In the following sections, the most frequent methods will be described.

Increasing source of energy

Increasing energy density of the diet during the prepartum is the classic method to limit development of severe negative energy balance in dairy cows (Eastridge, 2006). Diet energy content should be increased progressively during the prepartum period, with lower content in the beginning of the dry period (1.25 Mcal/kg) and increased content (1.54-1.62 Mcal/kg) during the last three weeks before calving (NRC, 2001). If the change from a forage-based diet to a high energy diet occurs abruptly, there is high risk of development of ruminal acidosis (Rabelo et al., 2003). Moreover, if the energy content is too high during the last month of pregnancy, the cow might become too fat, which intensifies the decrease in dry matter intake (Ingvartsen and Andersen, 2000; Grummer et al., 2004).

To increase the energy density of the diet, glycogenic nutrients such as grain, concentrates, starch, non-fiber carbohydrates, propylene glycol or glucose infusion can be used. In the rumen, these sugars are fermented by the rumen microbes, allowing for the production of volatile fatty acids (mostly propionate), that are absorbed and used for hepatic gluconeogenesis and as a source of energy. Consequently, there is lower need to mobilize fat reserves, which lowers plasma NEFA and liver triglycerides (Vandehaar et al., 1999) and increases prepartum dry matter intake (Hayirli et al., 2002). The positive effects on the energy balance also reflect on better reproductive performances (Gong, 2002; van Knegsel et al., 2005). Propylene glycol can be administered orally to cows (Studer et al., 1993; Stokes and Goff, 2001), but when used

as a feed additive, the benefits are no longer observed (Nielsen and Ingvartsen, 2004; Kristensen and Raun, 2007).

Ionophores such as monensin are also largely used feed additives. Monensin can be added directly to the cows' diet, or it can be administered using a controlled-release intraruminal capsule (**CRC**). It is a product of fermentation of *Streptomyces cinnamonensis* that affects the ionic gradients of gram-positive bacteria's cellular membrane. Since gram-negative bacteria are positively influenced, there is an increase of propionate-producing bacteria and a reduction of acetate and butyrate-producing bacteria (Grummer, 2008). Duffield et al. (1998) showed that using monensin as a feed additive can reduce the incidence of hyperketonemia.

The energy content of the diet can also be increased with fat or with lipogenic nutrients (van Knegsel et al., 2005). The conjugated linoleic acids (i.e. a specific type of fatty acids) are a group of several isomers of linoleic acid. Its use seems to decrease milk yield (Overton and Waldron, 2004) and to increase dry matter intake, therefore improving the energy balance. Animals supplemented with those isomers have therefore lower NEFA and BHBA blood concentration (Trevisi and Bertoni, 2008; Esposito et al., 2013). The use of extra lipogenic nutrients such as alfalfa silage, seem to increase plasma NEFA and BHBA blood concentrations compared to the use of glucogenic nutrients or to the isomers of linoleic acid (van Knegsel et al., 2005). The main reason behind this difference is that: while glucogenic nutrients decrease the ratio of plasma lipogenic/glucogenic (through increased insulin and glucose blood levels), thus stimulating body fat deposition and energy partitioning into body tissue; lipogenic nutrients increase the ratio of plasma lipogenic/glucogenic compounds (through increased production of butyrate and acetate in the rumen), thus increasing energy partition into milk and consequently limiting the energy partition into body reserves (van Knegsel et al., 2005, 2007).

Reducing energy requirements in early lactation

By temporarily reducing milk production through milk frequency

To limit the negative energy balance, one can either increase cow's energy intake, or decrease the energy demand. Since the main goal of dairy farmers is to get as much milk as possible from their cows, using the best of the cows' maximum potential, then it would be almost

illogical to think about strategies to reduce milk output during early lactation. However, the numerous negative consequences of severe negative energy balance (mentioned throughout this literature review) emphasize the urgent need for new management strategies designed to prevent or at least mitigate these problems. Therefore, a short-term reduction of milk output, during the most critical period of the production cycle could be considered, for example reducing milking frequency.

The relationship between milking frequency and milk yield has been studied for several years. Increased milking frequency has a positive impact on the amount of milk produced (Hale et al., 2003; Bernier-Dodier et al., 2010), certainly due to prolactin stimulation (Lacasse et al., 2011). Higher milk production, in turn, lowers blood glucose concentration and probably intensifies lipolysis, leading to greater NEFA and BHBA concentrations in blood (Andersen et al., 2004; Patton et al., 2006; McNamara et al., 2008). To the best of my knowledge, Rémond et al. (1999) was the first group of researchers investigating the potential of temporarily altering milking frequency during early lactation to improve energy balance. They allocated dairy cows in several groups including: a control group, milked 2x/d during all the study duration; a group milked 1x/d for the first three WIM; and another milked 1x/d for the first six WIM. These last two groups were milked 2x/d after each treatment period. The energy balance was better in cows milked 1x/d, leading to higher blood glucose concentration and lower NEFA and BHBA (measured on WIM three). Moreover, there were no differences in dry matter intake between groups. After this first study from Rémond et al. (1999), several other took place. Table III resumes some of the findings.

Studies from Loiselle et al. (2009) and Ster et al. (2012) have also evaluated the effect of reduced milk frequency on the immune system. In Loiselle et al. (2009) study, the level of IFN-γ produced by lymphocytes (on DIM five and 14) was higher within cows milked 1x/d than the level in cows milked 2x/d, reflecting a better lymphocytic response. However, chemotaxis, phagocytosis and oxidative burst of PMNL were unaffected. In Ster et al. (2012) study, IFN-γ production was not affected, but PBMC (i.e. lymphocytes and monocytes) proliferation was greater in cows milked 1x/d (than in cows milked 2x/d). Larger studies seem to be necessary to establish if reduced milk frequencies in early lactation can actually reduce disease incidence.

Table III. Comparison between serum metabolites and milk yield and composition obtained in different studies interested in altering milking frequency in early lactation as a strategy to limit negative energy balance

Research group	Rémond et al. (1999)		Patton et al. (2006)		McNamara et al. (2008)		Loiselle et al. (2009)	
Treatment group (n)	T (11)	C (11)	T (22)	C (22)	T (21)	C (21)	T (11)	C (11)
Milking frequency (/d)	1	2	1	3	1	3	1	2
Protocol duration (weeks)[1]	3		4	4	4	4	1	
WIM of measurement for serum metabolites	3	3	2	2	2	2	2	2
NEFA (mmol/L of blood)	0.35	0.62 (3)	~0.55*	~0.80	~0.65*	~1.00	~0.50*	~0.80
BHBA (mmol/L of blood)	1.07	1.43 (3)	~0.45*	~0.70	~0.60	~1.00	~0.80*	~1.40
WIM of measurement for milk and components	7-12	7-12	5-6	5-6	5-6	5-6	2-13	2-13
Milk production (kg/d)	33.2*	35.0	26.8	26.7	28.3	30.9	~37.0	~40.0
Milk fat (g/kg)	43.2*	40.7	42.0	40.0	41.7	41.4		
Milk protein	30.6*	28.1	30.8*	28.6	29.6	28.5		

[1] After that time, cows in the treatment group would be submitted to the same milking frequency as the control group, except in Patton et al. (2006) and in McNamara et al. (2008), where groups were submitted to a 2x/d milking frequency at the end of the protocol duration

* Means differ significantly between treatment groups ($P < 0.05$)

~ Numbers derived from figures

WIM, week in milk, T, treatment, C, control

The impact of reduced milk frequency on reproductive performances was investigated in both Patton et al. (2006) and McNamara et al. (2008) studies. In Patton et al. (2006) study, although there was earlier resumption of ovarian activity (18.3 days in cows milked 1x/d; *vs.* 28.6 days in cows milked 3x/d), interval from calving to conception did not vary. Similarly, in McNamara et al. (2008) study, mean calving to conception interval did not vary across different milking frequencies. However, survival analysis would have been a more appropriate analysis to study time to conception data (Lean et al., 2016), but it was not used in any of these studies.

The main problem that arose from the lower milk frequency in most studies was the negative carryover effect on milk production for the rest of the lactation. For instance, in Patton et al. (2006) study, cumulative milk prod was significantly lower in cows milked 1x/d *vs.* 3x/d on 10 WIM (1,682 *vs.* 1,880 kg) but not on 20 WIM (3,333 *vs.* 3,703 kg). Moreover, the 305-d lactation total was lower in cows milked 1x/d *vs.* 3x/d (6,198 *vs.* 6,813 kg). In McNamara et al. (2008), although daily milk production was not significantly lower in cows milked 1x/d *vs.* 3x/d on WIM five to six, it was on WIM 10. Similarly, in Loiselle et al. (2009), daily milk production tended to be lower (P-value = 0.06) over WIM 2-13 in cows milked 1x/d *vs.* 2x/d. Still, this

practice seems promising, and the less abrupt rise in milk production provoked by the lower milking frequency in early lactation might even be more similar to what would happen in nature. In presence of the calf, the quantity of milk sucked by the offspring would be low in the first weeks of life, increasing later on, along with the calf growth (Jasper and Weary, 2002).

By temporarily reducing milk production through incomplete milking

One way to overcome the negative carryover effect on milk production caused by lower milking frequency, but still being able to improve the cows' energy status, would be to provoke the same slower increase in milk production by limiting the amount of milk collected in early lactation, but without altering milking frequency (i.e. milking stimulus).

Generally *incomplete milking* is referred to an automatic milking without hand- or machine-stripping. The milk remaining in the gland after teat-cup detachment is sometimes named *strip* yield or milk *strippings* (Clarke et al., 2004; Clarke et al., 2008). In the current document, the term incomplete milking refers to a milking protocol designed to withdraw a specific amount of milk that voluntarily leads to varying quantities of milk remaining in the mammary gland, depending on the individual animal production level. The incomplete milking (also mentioned as *partial* or *restricted milking*) performed during the first day in milk has been referred before as a management strategy to prevent hypocalcemia (Hoard et al., 1993; Salgado-Hernández et al., 2014; Zaidi, 2016), although there are not many scientific findings supporting this method.

It is generally accepted that milking should be complete, to avoid teat-end congestion and edema, which may result in increased risk of bacterial invasion (Reinemann, 2012). However, there is little evidence that incompletely milking dairy cows causes mastitis. In fact, most studies evaluating the impact of shorter milking times (supposedly accompanied by higher quantities of milk remaining in the udder) on mastitis, did not find a relationship (Rasmussen, 1993; Clarke et al., 2008; Jago et al., 2010). Other factors that contribute to decreased amount of bacteria at the teat end seem to be more determinant for mastitis development (Reinemann, 2012).

To the best of my knowledge, Carbonneau et al. (2012) were the first to investigate the use of an incomplete milking during the first five DIM as a strategy to improve energy balance.

In their study, the incomplete milking was defined as the withdrawal of 6, 8, 10, 12 and 14 L/d on DIM one to five, respectively, which ended up being about 43%, 35%, 34%, 38% and 36% of the milk collected in conventionally milked cows. Through this milking protocol, Carbonneau et al. (2012) were able to improve glucose blood concentration, resulting in lower BHBA and NEFA concentrations. These blood concentrations were likely caused by an improved energy balance, which persisted until 28 DIM. Although several PBMC functions were evaluated, incomplete milking only tended to increase PBMC proliferation on DIM five, but the effect was opposite on DIM 61. Moreover, the authors found that milk production in the treated group was similar to the control-group by 14 DIM, and no differences were found between groups during the remaining weeks of the study (until WIM nine). Fat concentration was lower, and protein concentration was higher in the milk of incompletely milked cows compared with the control group, but only at WIM two (Carbonneau et al., 2012). The lack of carry-over effect on subsequent milk production was probably a result of maintained stimulation of oxytocin and other galactopoietic hormones (e.g. prolactin and glucocorticoids) by maintenance of milking frequency (Lacasse and Ollier, 2014). Another reason for the lack of effect on milk production could be the short duration of the treatment, because both milk removal and milking (or suckling) stimulus are needed for maintenance of milk secretion (Schmidt, 1971). With this study, Carbonneau et al. (2012) showed the enormous potential of the incomplete milking as a strategy to improve energy balance. However, the results cannot be generalized to commercial farms, because the trial was conducted on a limited number of animals (16 treated and 15 control cows) and in an experimental research station conditions. Moreover, the impacts on diseases incidence and reproduction were not evaluated. Consequently, it would be of great interest to repeat the study performed by Carbonneau et al. (2012), but in commercial-farms and with a greater sample size.

Assessing udder pain associated to lower milk withdrawal in early lactation

Practices such as abrupt cessation of milking at drying-off (Zobel et al., 2015), prolonged milking intervals (Österman and Redbo, 2001), and incomplete milking at the beginning of the lactation (Carbonneau et al., 2012; Morin et al., 2018), may lead to udder distension, milk leakage and inflammatory responses (Davis et al., 1998; Rovai et al., 2007). Under such

conditions, the animals may experience increased udder sensitivity, perhaps to a level where their welfare is negatively affected (FAWC, 2009).

Handheld pressure algometers have been used to assess changes in sensitivity in dairy cattle in cases of mastitis (Fitzpatrick et al., 2013), lameness (Dyer et al., 2007), or after dehorning (Heinrich et al., 2010). The rater exerts pressure with the device in the body region of interest until the animal responds with an avoidance reaction (i.e. kicking, shifting weight). The MNT represents the amount of force (in kg) necessary to trigger animal avoidance response, measuring animals' sensitivity. Although algometers have been shown to be reliable in humans (Potter et al. 2006) and in some domestic animals (e.g. dogs: Kaka et al., 2015; horses: Haussler and Erb, 2006; piglets: Janczak et al., 2012), formal evaluation and validation of handheld algometers for quantifying MNT on the udder of dairy cows appears to be lacking.

Behavior observation can also be used to evaluate animal pain (Weary et al., 2006), which is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by The International Association for the Study of Pain (IASP; Merskey, 1991). Internal or external challenges that lead to pain often produce differences in cows' behavioral activities, namely changes in activity and resting, gait, posture and vocalization (Wechsler, 1995; Molony and Kent, 1997). Österman and Redbo (2001) showed that cows milked 2x/d vs. cows milked 3x/d had higher number of lying bouts of shorter duration and fewer long lying bouts 4 h before milking, possibly due to discomfort associated with udder distention. In another study, Tucker et al. (2007) showed that cows milked 1x/d had higher udder firmness, but similar grazing activity and a tendency for longer lying times compared to cows milked 2x/d. The authors concluded that there was not enough evidence to state that cows milked 1x/d were in pain. Two other studies (O'Driscoll et al., 2010, 2011) reported that cows milked 1x/d had similar lying times and improved hoof health and locomotion score compared to cows milked 2x/d, supporting the previous findings that cows milked in lower frequency did not suffer from pain. However, in these studies, cows were not assessed in early lactation, when milk yield is increasing. Rémond et al. (1999), that applied lower milking frequencies only in early lactation, mentioned no impact of the milking protocol on cow's behavior, but they did not mention further details on the type of data recorded. No studies were found on the impacts of incomplete milking on pain or behavior.

CHAPTER 3: A RANDOMIZED CONTROLLED TRIAL ON THE EFFECT OF INCOMPLETE MILKING DURING THE FIRST FIVE DAYS IN MILK ON CULLING HAZARD AND ON MILK PRODUCTION AND COMPOSITION OF DAIRY COWS

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Abstract

An incomplete milking in early lactation could help limit negative energy balance in dairy cattle, but its potential effects on culling hazard and on milk production and composition throughout the entire lactation are unknown. The objective of this study was to evaluate the effect of an incomplete milking during the first five DIM on culling hazard, milk weight, milk fat and protein concentrations, and energy corrected milk (**ECM**) yield during the whole lactation. A RCT was conducted in 13 dairy farms near St-Hyacinthe, Quebec, Canada. Approximately one month before expected calving, Holstein multiparous cows calving between December 2013 and March 2015 (n = 846 cow-lactations) were randomly assigned to a control or a treatment group. Cows in the control group were milked conventionally, whereas cows in the treatment group were submitted to an incomplete milking protocol (maximum of 10, 12, and 14 L/d of milk was collected on DIM one to three, four and five, respectively). All farms were registered on DHI, which was used to obtain records on culling, monthly milk yield, and milk fat and protein concentrations. In addition, daily milk yield records were available for six farms. A Cox proportional hazards model with a herd frailty term was fitted to the data to compare culling hazard among treatment groups. Regarding milk production and composition, four linear mixed models with herd as a fixed effect, cow as a random effect, and using an autoregressive covariance structure were used to study the effect of the incomplete milking on (1) milk weight, (2) milk fat concentration, (3) milk protein concentration, and (4) ECM yield. Culling hazard did not differ among treatment groups (HR = 1.0; 95% CI for the HR = 0.82, 1.3). We observed no differences in milk weight, milk fat, or protein concentration among treatment groups between WIM two and 44 (the studied period). We noted difference in ECM between treatment groups for WIM 38, with incompletely milked cows producing less milk than conventionally milked cows (−2.7 kg/d; 95% CI = −0.02, −5.2 kg/d), but no differences were found for any of the other WIM. These results suggest that this strategy for controlling the negative energy balance has negligible effect on cow productivity.

Key words: dairy cattle, incomplete milking, culling, milk production

Introduction

In dairy cows, the transition period is marked by substantial nutritional, metabolic, hormonal, and immunological changes (van Knegsel et al., 2007; Ster et al., 2012). During this period, cows are in a state of negative energy balance that occurs because the demand for nutrients for milk production increases rapidly and exceeds the supply of nutrients provided by food intake (Grummer et al., 2004). This negative energy balance results in lower blood glucose levels and the mobilization of body reserves to provide additional energy, leading to elevated blood concentration of metabolites, such as NEFA and BHBA (Busato et al., 2002). High concentrations of these metabolites have been associated with a state of immunosuppression (van Knegsel et al., 2007; Ster et al., 2012), increased risk of infectious diseases (Suriyasathaporn et al., 2000), metabolic diseases, reduced milk production (Duffield et al., 2009), and higher culling risk (Roberts et al., 2012). Decreasing the imbalance between nutrient requirements and energy intake (i.e., the negative energy balance) should reduce the incidence of metabolic and infectious diseases in dairy cows. The classic approach to decrease that imbalance is to increase energy density of the diet offered during this period (Grummer et al., 2004). Another option is to temporarily decrease energy demands, by slowing down the increase in milk production for a few days just after calving. Once a day milking during the first WIM seems to have this effect, by temporarily reducing the energetic requirements and, therefore, the negative energy balance (Loiselle et al., 2009; O'Driscoll et al., 2012). However, it also has a negative carryover effect on milk production for the rest of the lactation, possibly due to the lower stimulation of prolactin hormone (Lacasse et al., 2011).

Carbonneau et al. (2012) have shown that reducing milk output by milking cows incompletely (collecting about one third of expected milk production), 2x/d until day five after calving while maintaining the stimulus of frequent milk removal, improves metabolic status and immune functions without having a carryover effect on subsequent milk production. Although the results of their study are very promising, it was conducted on a limited number of animals (16 treated and 15 control cows) in experimental research station conditions. These results cannot, consequently, be generalized to commercial farms. Therefore, a RCT was conducted on 13 commercial dairy farms to evaluate the effect of an incomplete milking protocol (Morin et

al., 2018). The RCT showed a marked decrease in BHBA blood concentration and in odds of hyperketonemia among cows milked incompletely. The aim of the present research was to investigate the effect of the incomplete milking protocol on culling hazard and on milk production and composition using the large data set generated by the Morin et al. (2018) study.

Materials and methods

Sample size calculations

The original study was designed to investigate the effect of an incomplete milking protocol on ketonemia, odds of hyperketonemia, reproductive performances, odds of infectious diseases (e.g., mastitis, metritis), culling hazard and, finally, milk production and composition (Krug et al., 2017; Morin et al., 2018). Among all the outcomes studied, the outcome requiring the largest sample size was odds of hyperketonemia, which required the recruitment of 400 cows per treatment group (Morin et al., 2018). The sample size for the RCT described in our study was, therefore, determined for answering this latter research objective. Nevertheless, power calculations using the POWER procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) were conducted to estimate the differences in milk weight, fat and protein concentrations, and ECM that could be detected using the available data. Using an α of 0.05, and assuming a standard deviation (**SD**) of 5.0 kg/d for milk weight and ECM and of 0.5 percentage points for milk fat and protein concentrations, we estimated that differences in milk weight and ECM $\geq$ 1.2 kg/d and of fat and protein concentration $\geq$ 0.12 percentage points could be detected with > 90% power with the available data. Clustering of observations by cow and herd was not considered for these calculations; therefore, the minimal detectable differences presented are likely to be slightly optimistic.

Herds and cows

Our study was a RCT conducted on multiparous cows from a convenient sample of 13 commercial dairy farms. The complete research protocol is described by Morin et al. (2018). Briefly, to be selected, farms had to be in the vicinity of Saint-Hyacinthe (Quebec, Canada); to accept to follow the standardized research protocol; to participate in a DHI program; to have

computerized health records; to use a milking system allowing measurement of the harvested milk in real time during milking; and to share their herd health, DHI, and daily milk weight (if available) data with the research group. The study protocol was accepted by the Animal Ethics Committee of the Université de Montréal (rech-1701).

In each herd, all multiparous cows calving between December 2013 and March 2015 were randomly allocated at the time of dry off to a treatment or a control group using a random number generator. Cows in the treatment group were milked incompletely during the first five DIM, with a maximum of 10, 10, 10, 12, and 14 L/d collected on DIM one, two, three, four and five, respectively. This protocol was derived from the one investigated by Carbonneau et al. (2012); 6, 8, 10, 12, and 14 L/d on DIM one, two, three, four and five, respectively) in an attempt to make the protocol more practical for milk producers. More specifically, farms with 2 milkings/d were informed to collect 5, 5, 5, 6, and 7 L/milking on DIM one, two, three, four and five, respectively. Farms with 3 milkings/d were informed to divide the maximum amount allowed per day in 3. In the herd using an automatic milking system (**AMS**), cows from the treated and control groups were milked 2x/d in the maternity pen during the first five DIM and then sent to the AMS for the remaining of the lactation. In all other herds, cows in the control group were milked conventionally according to the farm practices. The majority of farms (12/13) had automatic teat cup removers, with detachment at a mean milk flow rate of 600 g/min (range = 300 to 1,200 g/min). In six farms, producers reported not milking cows completely during the first two DIM as part of their conventional milking routine. Because group allocation influenced how cows were milked, dairy producers could not be blinded to treatment group.

Milk production and composition data

In six of the 13 participating farms, daily milk production was recorded automatically by the herd parlor or automatic milking system software and these data were retrieved directly from the producers' computerized records. In the remaining herds, the participating producers were asked to record milk production manually, at least once a week, for the first four WIM. In these herds, subsequent monthly milk production data were retrieved from DHI records. Additionally, DHI records were used to obtain monthly milk components (milk fat and protein concentration, in %), culling date, and lactation duration for cows not culled during the lactation.

For each cow and for each day with a milk yield record, ECM was calculated using the following formula (NRC, 2001):

ECM (kg/d) = 12.55 × fat (kg/d) + 7.39 × protein (kg/d) + 0.2595 × milk yield (kg/d)

[Equation 1]

To calculate fat yield (kg/d), fat concentration (%) was multiplied by milk yield (kg/d) of the same test date. Protein yield (kg/d) was calculated in the same way. For farms with daily milk records, means for fat and protein yields from the cow's previous and following DHI tests were used to compute ECM whenever a milk yield record was available on a given day, but without fat and protein yield data (i.e., between DHI tests). Due to higher oscillations in fat and protein yields at the beginning of the lactation, this approximation was only used after 30 DIM. Before 30 DIM, only milk yield, fat, and protein data from the actual DHI test day (for which both milk production and composition data were available) were used for estimating ECM in farms with daily milk records. Therefore, before 30 DIM, approximately one ECM observation was available per cow (the DHI record) for all herds. After 30 DIM, herds with daily milk yield had daily ECM observations until the date of the last DHI test, whereas herds with monthly milk yield had generally one observation per month.

Data management and statistical analyses

Culling hazard

First, we assessed whether treatment group affected culling hazard. This first step was important, as the association between treatment and milk production could be biased if a higher proportion of cows were excluded (i.e., culled) earlier in their lactation in one group rather than in the other. Time to culling was defined as the number of days from calving until the cow was culled (left the farm or died). Cows were followed until the end of their lactation or until a maximum of 450 DIM (right-censoring) or culling (whichever comes first). A Cox model with a herd frailty term, to account for the data structure, was fitted to the data using the PHREG procedure in SAS. The general model was

$$h_i(t) = \lambda_0(t) \exp(\beta_1 Tx_{i1}) + \varepsilon_i \qquad \text{[Equation 2]}$$

where $h_i(t)$ is the culling hazard for the i^{th} cow at time t; $\lambda_0(t)$ is the baseline hazard function; β_1 is the coefficient for the treatment group (Tx_{i1}; conventional vs. incomplete milking); and ε_i represents the unobserved heterogeneity, accounting for difference between herds. Using this model, the culling HR between conventionally and incompletely milked cows can be computed simply by exponentiating β_1 using the natural base logarithmic transformation.

Parity group (categorized as second parity and third parity or greater) was tested as a potential effect modifier of the treatment-culling hazard relationship by adding the main term and 2-way interaction term with treatment group in the model. To evaluate if the treatment effect varied as function of time (i.e., the proportional hazards assumption), the significance of a 2-way interaction term between a time variable and the treatment group variable were added to the model. More specifically, we investigated whether the effect of treatment on culling hazard would increase or decrease exponentially (using DIM natural logarithm as time variable), or if it would change at a given time point (testing several dichotomous time variables with cutoffs at 21, 28, and 35 DIM). In all cases, 2-way interaction terms (effect modifiers or time variables) were retained if *P*-value < 0.05. The assumption of independent censoring was checked as described by (Dohoo et al., 2009). Kaplan-Meier survival curves for each treatment group were produced using STATA/MP 12.0 (StataCorp, College Station, TX).

Milk production and composition

Four linear mixed models were used to quantify the effect of treatment group on (1) milk weight (kg/d), (2) milk fat (%), (3) milk protein (%), and (4) ECM (kg/d). The SAS MIXED procedure was used to estimate all four models. Herd was included in the models as a fixed effect to account for correlation between cows within each farm. A random cow intercept with an autoregressive covariance structure was used to account for dependency between milk measurements from the same cow, and for the higher correlation between measurements collected closer in time (i.e., a repeated measures model). Treatment group and an interaction term between treatment group and WIM were forced into all models to investigate the effect of the treatment for each WIM. The Tukey adjustment was used to adjust for multiple comparisons. The general model was:

$$Y_{ij} = \beta_0 + \beta_1 Tx_j + \beta_2 WIM_{ij} + \beta_3 Tx_j \times WIM_{ij} + \beta_4 Herd + u_{0j} + e_{0ij} \qquad \text{[Equation 3]}$$

where Y_{ij} is the predicted milk weight (model 1), milk fat (model 2), milk protein (model 3) concentrations, or ECM (model 4) for the i^{th} day from the j^{th} cow; β_0 is the intercept; β_1 is the regression coefficient for the treatment group (Tx; conventional vs. incomplete milking); β_2 is the WIM effect; β_3 is the treatment × WIM interaction; β_4 is the herd fixed effect included to account for clustering of cows by herd; and u_{0j} and e_{0ij} are the cow random intercept and measurement error term, respectively (assumed to follow approximately normal distributions). Using this model, least squares means can be estimated per treatment group for each WIM using the β_1, β_2, and β_3 terms.

Parity group was tested as a potential effect modifier of the treatment-milk yield or treatment-composition relationships by adding the main term and 2- and 3-way interaction terms with treatment group and WIM in the models. Parity was retained as an effect modifier if the 3-way interaction term yielded *P*-value < 0.05 on the *F* test. Residuals were visually examined for each model to evaluate normality using quantile-quantile plot and histogram of residuals. Assumption of homoscedasticity was assessed visually using plot of the residuals against predicted values.

Results

Description of study population

Participating herds had a mean (SD) number of 103 (51) milking cows and a mean (SD) 305-d milk yield of 9,973 (660) kg/cow. Herds were mainly housed in freestalls (9/13 herds). In the majority of herds, cows were milked 2x/d (11/13 herds), in one herd cows were milked 3x/d, and 1 herd used an AMS. Most farms fed totally mixed ration (**TMR**; 11/13 herds) and used monensin supplementation (11/13 herds). Conventionally milked cows 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 on DIM one, two, three, four and five, respectively.

In total, 846 cow-lactations were enrolled in the project (838 animals, in which eight animals were enrolled for two different lactations), but 25 cow-lactations were not included in the study because farmers inadvertently changed their treatment group (14/25) or because cows entered the study before formal randomization (11/25). From the 14 cows that were

inadvertently changed from one treatment group to another, seven were from the conventional milking group and seven were from the incomplete milking group.

Culling

Two herds stopped DHI recording during the RCT. Consequently, time to culling or lactation duration could not be computed for 33 cow-lactations (15 incompletely and 18 conventionally milked). In addition, 40 cow-lactations (21 incompletely and 19 conventionally milked) enrolled in the project could not be matched to their DHI record. Finally, for 16 cow-lactations (nine incompletely and seven conventionally milked) ending up with censuring (i.e., no culling), exact lactation duration was missing. In the end, 732 (360 incompletely and 372 conventionally milked) could be used for the survival analysis. On average 56 cow-lactations were available per farm (range = 12 to 148).

A total of 37% (136/372) of cows among the conventional milking group and 38% (136/360) in the incomplete milking group were culled before 450 DIM. After observing the plot of the log cumulative hazard against the log time, three cut points were identified as possible moments for change in culling hazard ratio between treatment groups: 21, 28, and 35 DIM. None of the 2-way interaction terms between time (dichotomous variable, with 0 and 1 representing culling DIM lower and higher than the cut point, respectively) and treatment were statistically significant. Furthermore, the 2-way interaction term between time natural logarithm and treatment was not statistically significant. Therefore, we concluded that the effect of treatment on culling hazard did not vary as function of time (i.e., the proportional hazards assumption was respected). Culling hazard in incompletely milked cows was 1.0 (95% CI = 0.82, 1.3) times that of conventionally milked cows (*P*-value = 0.74). The effect of treatment on culling hazard did not vary across parity (*P*-value = 0.31). Kaplan-Meier survival curves are presented in Figure 7.

Milk weight

From the 821 cow-lactations enrolled in the project, observations from seven cow-lactations (1%; one conventionally and six incompletely milked) could not be matched to any production record and were, therefore, missing. The data structure of the data set used for the

milk weight analyses is presented in Table IV. For analysis, 814 cow-lactations were available; on average, 119 observations were available per cow-lactation (range = 2 to 305).

The linear mixed model with milk weight as outcome showed some level of heteroscedasticity; therefore, several transformations of the milk weight variable were evaluated to improve homoscedasticity. The squared root transformation (√milk weight) led to a certain improvement of the normality and homoscedasticity assumptions, but some degree of heteroscedasticity could still be observed. The results presented below should, therefore, be interpreted with caution.

Figure 8 illustrates distribution of milk weight least squares means (in √kg/d), after applying the square root transformation, for conventionally and incompletely milked cows between WIM 2 and 44. The effect of the treatment on square-rooted milk weight varied as function of WIM (*P*-value = 0.02; Figure 8). However, after adjusting for multiple comparisons, we found no significant differences between treatment groups for any of the WIM. During WIM 2 to 9, 10 to 17, 18 to 25, 26 to 33, and 34 to 44, the conventionally milked cows produced, on average, 40.9, 39.6, 34.8, 29.8, and 23.4 kg/d, respectively. During the same time periods, incompletely milked cows produced, on average, 39.9, 39.1, 34.2, 29.1, and 22.4 kg/d, respectively. The effect of treatment on square-rooted milk weight was not modified by parity level (*P*-value = 0.91 for the 3-way interaction).

Fat and protein concentrations

For the same reasons pointed in the culling section, from the 821 cow-lactations enrolled in the project, observations from 84 cow-lactations (10%; 45 from the conventional milking group and 39 from the incomplete milking group) were missing. The structure of the data set used for fat and protein concentration analyses is presented in Table V. From the 737 cow-lactations that were included in the analysis, on average, seven observations were available per cow-lactation (range: 1 to 17).

Fat

The effect of the incomplete milking on milk fat concentration did not vary across WIM and it was not modified by parity level (*P*-value = 0.12 for the triple interaction). Figure 9

illustrates distribution of milk fat least squares means (in %) for conventionally and incompletely milked cows between WIM 2 and 44. We found no statistically significant differences in fat concentration between conventionally and incompletely milked cows for any of the WIM studied.

Protein

The effect of the incomplete milking on milk protein concentration did not vary across WIM and it was not modified by parity level (*P*-value = 0.99 for the triple interaction). Figure 10 illustrates distribution of milk protein least squares means (%) for conventionally and incompletely milked cows between WIM 2 and 44. As with fat concentration, we found no statistically significant differences in protein concentration between conventionally and incompletely milked cows for any of the WIM studied.

Energy corrected milk

For the previously mentioned reasons, from the 821 cow-lactations enrolled in the project, observations on milk composition of 134 cow-lactations (17%; 70 conventionally and 64 incompletely milked) were missing. The data structure of the data set used for the ECM analyses is presented in Table VI. From the 687 cow-lactations that entered the analysis, on average, 79 daily ECM measures were available per cow-lactation (range = 1 to 264).

Figure 11 illustrates ECM least squares means (in kg/d), for conventionally and incompletely milked cows between WIM 2 and 44. The treatment-ECM relationship varied as function of WIM (*P*-value < 0.01; Figure 11). After adjusting for multiple comparisons, the only difference in ECM between groups was found during WIM 38, in which incompletely milked cows had a predicted ECM 2.7 kg/d inferior to that of conventionally milked cows (95% CI = −0.2, −5.2 kg/d). In the other periods, the ECM was comparable between treatment groups. The effect of treatment on ECM varied by parity level (*P*-value < 0.01 for the 3-way interaction term). However, after adjusting for multiple comparisons, we found no difference between treatment groups for none of the parity levels for each WIM.

Discussion

Our study is the first to illustrate the effect of an incomplete milking protocol during the first five DIM on culling and milk production and composition throughout the whole lactation. Assuming that the treatment protocol was applied strictly following recommendations, the level of milk withdrawal in the incompletely milked group was about 48, 39, 42, and 47% of the conventional group on DIM two, three, four, and five, respectively.

In general, our results suggest that the incomplete milking has no effect on milk production and composition, except for WIM 38, for which incompletely milked cows seem to have a lower ECM than conventionally milked cows. No obvious explanation exists for this finding, as we would expect a higher effect of treatment shortly after its application rather than at the end of the lactation. However, in our study, ECM could only be calculated between the first and the last DHI test, which resulted in a smaller number of ECM observations at the beginning and toward the end of the lactation (i.e., after the last DHI test). As ECM records were scarce in the end of the lactation, some individual observations may have had a higher influence on the results, and so the difference found on WIM 38 could be caused by a few deviated observations. Differences in ECM observed toward the end of the lactation could be investigated in future research, for instance, using AMS herds collecting daily milk yield, fat, and protein milk concentrations.

To our knowledge, only one study evaluated the effect of an incomplete milking for a short period of time in the early lactation on dairy cattle milk production and composition (Carbonneau et al., 2012). In that study, the quantity of milk collected in cows in the incompletely milked group was about 43, 35, 34, 38, and 36% of the conventional group on DIM one, two, three, four and five, respectively. Those authors only observed milk production levels between WIM one and nine and concluded that milk weight of cows incompletely milked rapidly reached similar levels to that of control cows soon after the end of treatment. Regarding milk protein and fat concentrations, Carbonneau et al. (2012) only found differences during WIM 2, in which fat concentration was lower and protein concentration was higher in the milk of incompletely milked cows compared with the control group, but no differences were found in the remaining of the study period.

Other studies were conducted using a half-udder design to understand the effect of an incomplete milking on milk production. Wilde et al. (1989) showed that an incomplete milking in dairy goats (defined as a gland with around 100 mL left behind at the end of milking) affects local enzyme activity with a reduction of total protein synthesis and partial secretory cellular involution if it lasts 24 weeks, but not if it lasts only 2 weeks. Although ran in a different species and with a different study design, the results observed by Wilde et al. (1989) also support the findings of the current study, where a relatively short period of incomplete milking was used. A more recent study from Penry et al. (2017), conducted in dairy cows, showed that an incompletely milked half-udder (defined as approximately 30% of total milk yield left behind at the end of milking) had lower milk production rate (0.73 kg/h) than a completely milked half-udder (0.97 kg/h), without an effect on milk composition. In that study, however, the treatment was applied for a longer period (from 5 to 47 DIM) compared with our study (1-5 DIM). Moreover, due to the half-udder design used by Penry et al. (2017), we could hypothesize the levels of galactopoietic hormones to be the same for both treated and control quarters; thus, the response observed in treated quarters in their study was possibly solely due to local quarter-level response (rather than local and central changes). In our study, the treatment could have induced both local and central responses, but minimal differences in milk yield were observed between groups. Thus, a discrepancy seems to exist between Penry et al. (2017) and the current study, and this divergence might have been caused by the very short treatment duration used in our study.

Considerable research has been produced on automatic teat cup removers and their effect on residual milk and subsequent milk production. In general, milking is considered optimal if it is conducted as gently, quickly, and completely (no residual milking) as possible (Reinemann, 2012), and without a negative effect on health and productivity (Lollivier et al., 2002). However, Hultén (2016) showed that take-off levels (i.e., milk flow rate threshold that leads to the detachment of the teat cups) of 100 or of 500 g/min led to similar amounts of residual milking (approximately 1 kg/quarter), and that this level of residual milk did not affect daily milk yield or composition. Jago et al. (2010) and Clarke et al. (2004) also compared similar cutoff levels and did not find any negative consequences on milk production, even with higher residual milking levels varying from 2 to 7 kg/quarter in Clarke et al. (2004). Although these studies

were conducted under different scenarios (i.e., different treatment durations or moments in lactation), none showed significant effects on milk yield. The literature on automatic teat cup removers supports, therefore, the absence of a negative effect of our very short duration incomplete milking protocol on milk production.

Moreover, Bach and Busto (2005) found that teat cup attachment failures in an AMS occurring at the beginning of the lactation had a lower effect on subsequent milk yield than if they occurred near the end of the lactation. Their conclusion was that milking failure affects milk emptying but not mammary cell number. These results might also explain, in part, the absence of a sustained effect of our incomplete milking protocol on overall milk production in the current study.

Finally, Roberts et al. (2012) found that odds of culling within the first 60 DIM were increased by 1.8 in cows presenting hyperketonemia (BHBA blood concentration ≥1.2 mmol/L) during the first WIM. As the incomplete milking was found to reduce odds of hyperketonemia (Carbonneau et al., 2012; Morin et al., 2018), we would have expected to observe a preventive effect of the incomplete milking on culling; however, this was not the case in our study. Many internal (e.g., parity, health status) and external (e.g., need for producing more milk, milk/price) factors will affect the producer's decision whether to cull a cow (Bell et al., 2010). The herds followed in this RCT were shown to have a relatively low prevalence of hyperketonemia in the early lactation (10.7% between four and seven DIM and 19.4% between eight and 17 DIM; Morin et al., 2018) compared with research conducted on 4,242 DHI herds in the same Canadian region (25.8 and 34.6% during WIM two for cows in second parity and in third parity or greater, respectively; Santschi et al., 2016). Culling reasons in the herds followed in the current study were, therefore, possibly less influenced by hyperketonemia and, instead, driven by other internal or external factors.

Conclusion

In the current study, an incomplete milking during the first five DIM did not lead to significant differences in culling hazard, milk weight, and milk fat and protein concentrations when compared with conventional milking. However, a lower ECM yield was observed for WIM 38 in incompletely milked cows. The incomplete milking during the first five DIM is an

interesting strategy for controlling the negative energy balance with relatively small negative effects on cow productivity.

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Table IV. Structure of the dataset used for investigating the effect of incomplete milking during the early lactation on milk weight. Data obtained from a randomized controlled trial conducted on 846 cow-lactations from 13 commercial dairies

Level	Number of units	Replication at level above[a]	
		Mean	Range
Farm	13	-	-
Cow-lactations	814	62.6	12-159
Daily milk yield observations	97,487	119.7	2-305

[a] Number of observations per cluster. For instance, number of daily milk yield observations per cow, or number of cow-lactations per farm.

Table V. Structure of the dataset used for investigating the effect of incomplete milking during the early lactation on milk protein and fat concentrations. Data obtained from a randomized controlled trial conducted on 846 cow-lactations from 13 commercial dairies

Level	Number of units	Replication at level above[a]	
		Mean	Range
Farm	13	-	-
Cow-lactations	737	56.7	12-155
Daily milk composition observations	5,825	7.9	1-17

[a] Number of observations per cluster. For instance, number of daily milk yield observations per cow, or number of cow-lactations per farm.

Table VI. Structure of the dataset used for investigating the effect of incomplete milking during the early lactation on energy corrected milk. Data obtained from a randomized controlled trial conducted on 846 cow-lactations from 13 commercial dairies

Level	Number of units	Replication at level above[a]	
		Mean	Range
Farm	13	-	-
Cow-lactations	687	52.8	12-155
Daily milk yield observations	54,326	78,9	1-264

[a] Number of observations per cluster. For instance, number of daily milk yield observations per cow, or number of cow-lactations per farm.

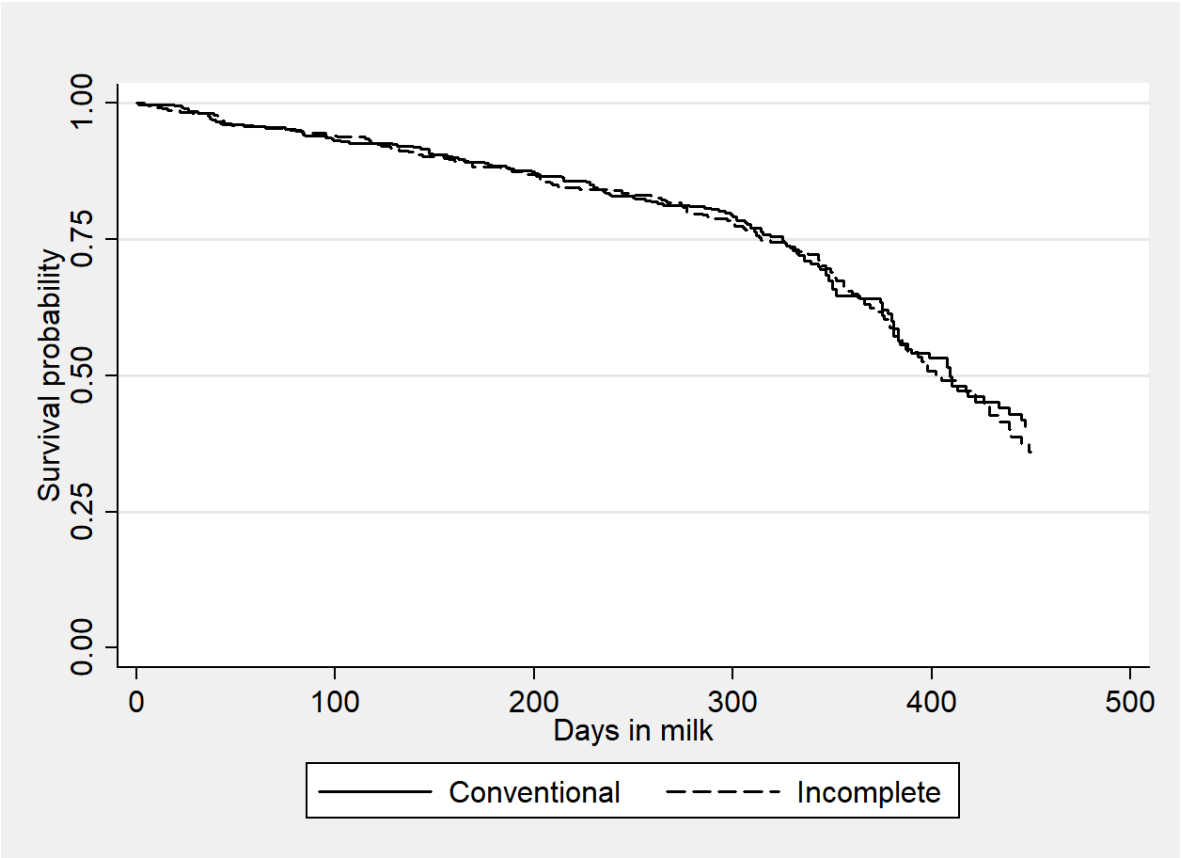

Figure 7. Kaplan-Meier survival curves illustrating survival probability for 732 multiparous cow-lactations from 13 commercial herds enrolled in a randomized controlled trial (dash line represents cows incompletely milked; full line represents cows conventionnally milked)

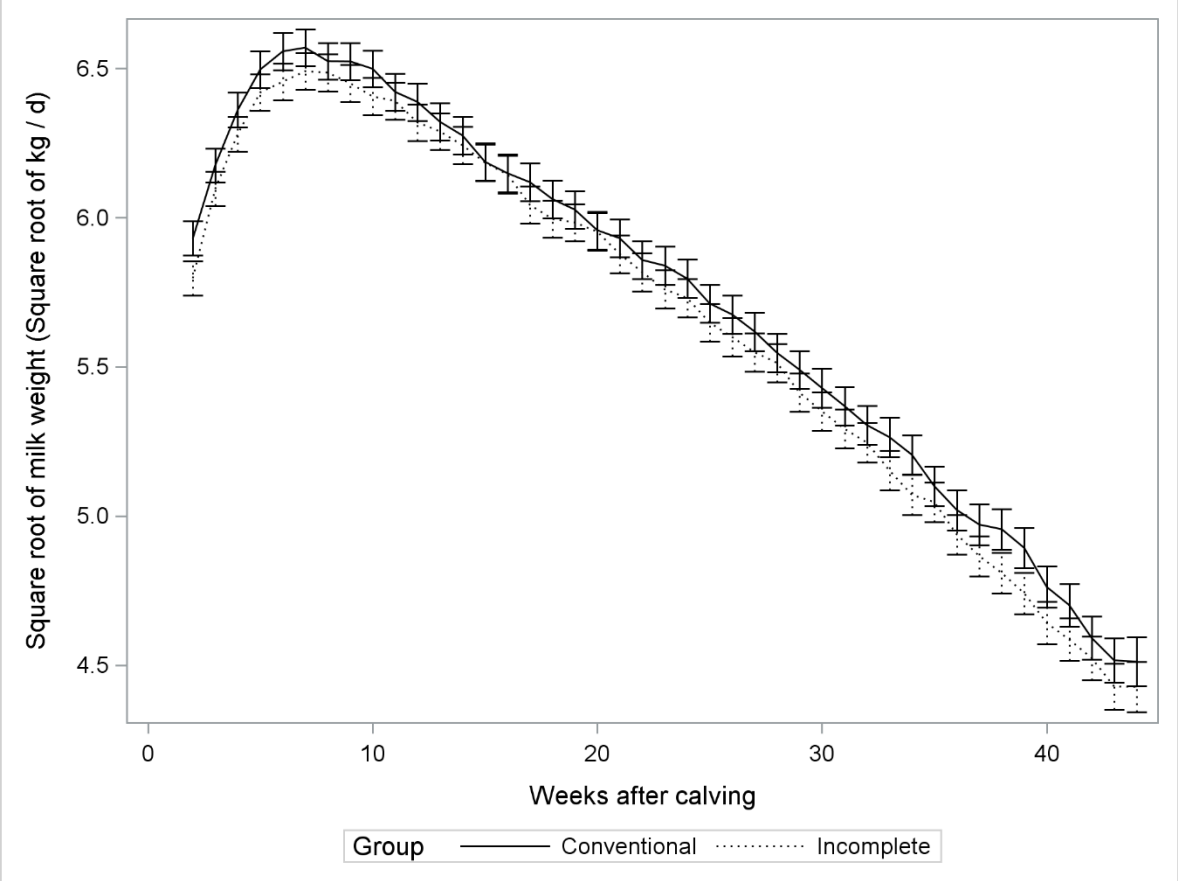

Figure 8. Square root of milk weight least square means (in kg/d) in conventionally milked cows (dash line) and cows incompletely milked during the first five days in milk (full line)

Least square mean estimates were obtained using a linear mixed model using data from a randomized controlled trial conducted on 814 dairy cow-lactations from 13 commercial herds; error bars represent unadjusted 95% confidence interval (i.e. not adjusted for multiple comparisons)

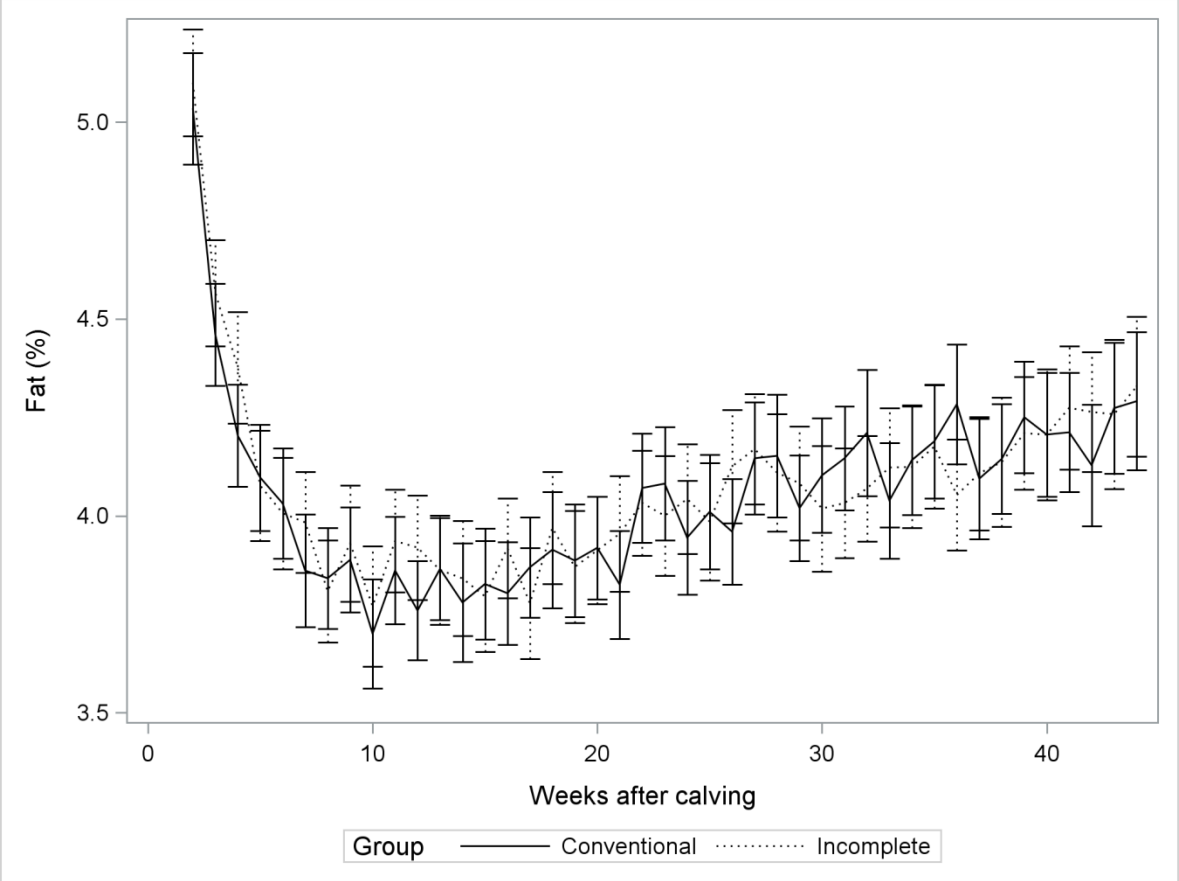

Figure 9. Milk fat concentration least square means (in %) in conventionally milked cows (dash line) and cows incompletely milked during the first five days in milk (full line)
Least square mean estimates were obtained using a linear mixed model using data from a randomized controlled trial conducted on 737 dairy cow-lactations from 13 commercial herds; error bars represent unadjusted 95% confidence interval (i.e. not adjusted for multiple comparisons)

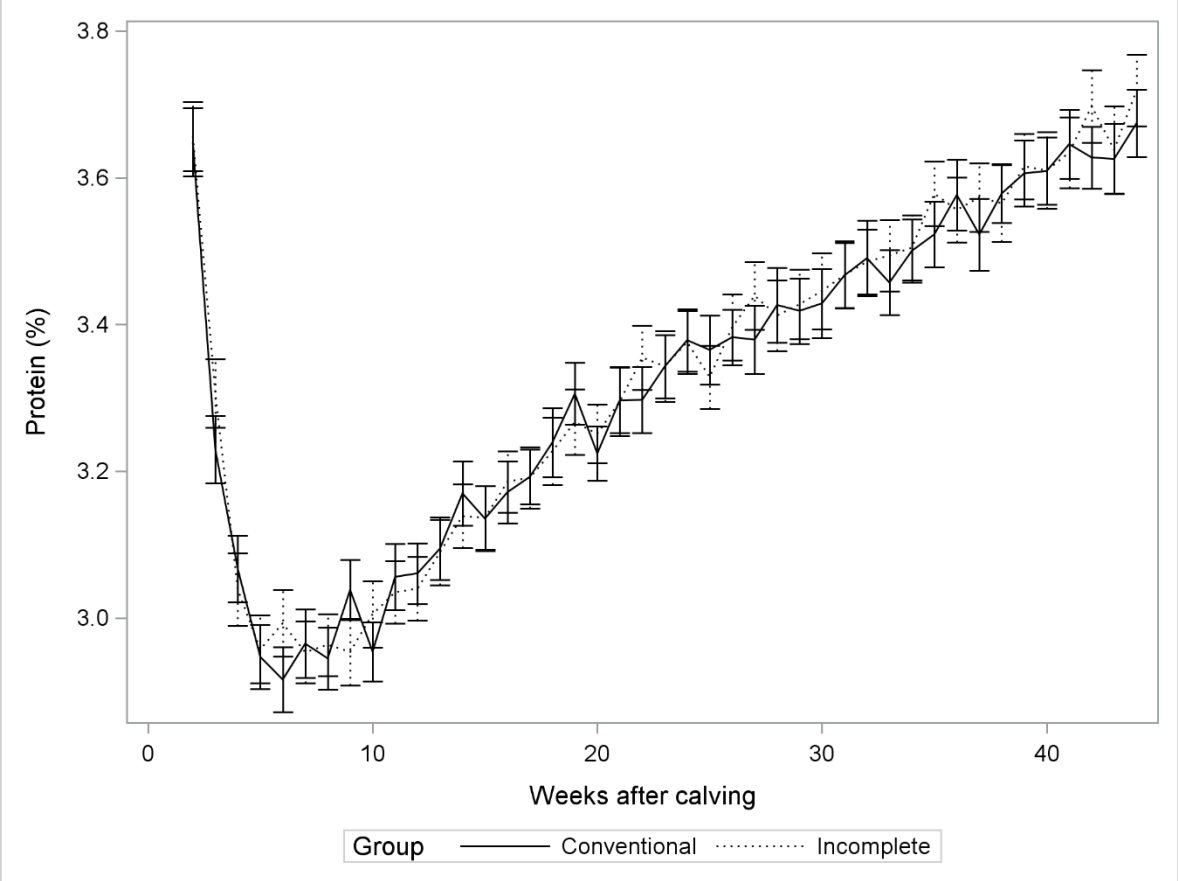

Figure 10. Milk protein concentration least square means (in %) in conventionally milked cows (dash line) and cows incompletely milked during the first five days in milk (full line)

Least square mean estimates were obtained using a linear mixed model using data from a randomized controlled trial conducted on 737 dairy cow-lactations from 13 commercial herds; error bars represent unadjusted 95% confidence interval (i.e. not adjusted for multiple comparisons)

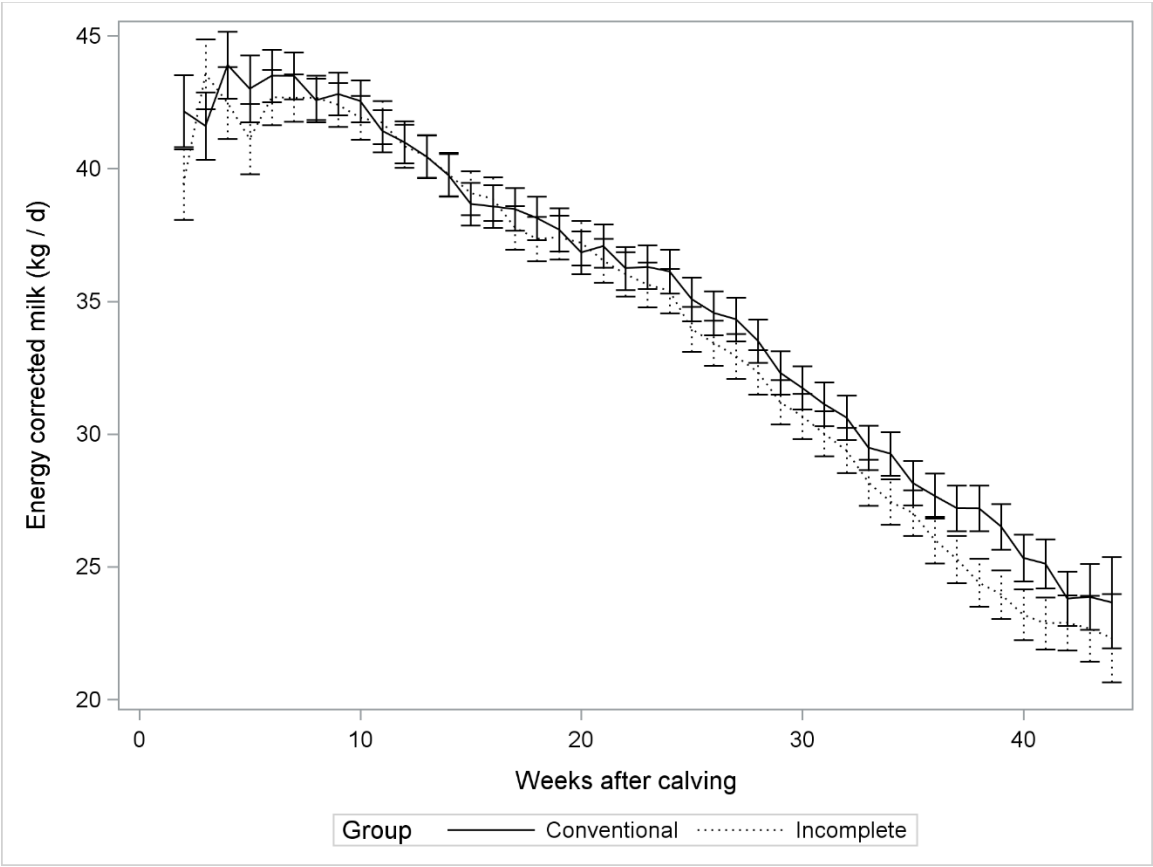

Figure 11. Energy corrected milk least square means (in kg/d) in conventionally milked cows (dash line) and cows incompletely milked during the first five days in milk (full line)
Least square mean estimates were obtained using a linear mixed model using data from a randomized controlled trial conducted on 687 dairy cow-lactations from 13 commercial herds; error bars represent unadjusted 95% confidence interval (i.e. not adjusted for multiple comparisons)

CHAPTER 4: EFFECT OF INCOMPLETE MILKING DURING THE FIRST FIVE DAYS IN MILK ON UDDER AND REPRODUCTIVE TRACT HEALTH: RESULTS FROM A RANDOMIZED CONTROLLED TRIAL

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Abstract

The aim of this study was to investigate the impact of an incomplete milking on risk of mastitis and reproductive tract disease. Multiparous dairy cows (n = 878) from 13 commercial herds were enrolled in a RCT. Cows were randomly assigned to either a control (milked conventionally) or a treatment group, which consisted of an incomplete milking (10-14 L of milk collected /d) from DIM one to five. Quarter milk samples were collected at approximately 11 DIM and 18 DIM to measure SCC. Quarters were considered negative for IMI if SCC < 100,000 cells/mL, and positive if SCC ≥ 200,000 cells/mL. To calculate IMI incidence, negative quarters of the initial samples collected were tested again one week later. This was done in order to deter incidence of positive quarters. To calculate elimination rate, positive quarters were tested again one week later to detect mastitis elimination. Farmers recorded clinical mastitis events. Cows were also examined at approximately 35 DIM with a Metricheck device for detection of PVD, and with an endometrial cytobrush for presence of leukocytes (ENDO and LE). A threshold ≥ 3 was used to define a positive PVD or LE test, while a PMNL count ≥ 6% was used to define a positive ENDO. Five generalized mixed models with cow and/or herd as random intercepts were used to determine the effects of the incomplete milking on odds of new IMI, on odds of IMI elimination, and on odds of a positive PVD, LE or ENDO status. To investigate time until first clinical mastitis event, a Cox model with a herd frailty term was used. The odds of new IMI and of IMI elimination for incompletely milked cows were 0.90 (95% CI: 0.49, 1.7) and 2.9 (95% CI: 1.4, 6.0) times those of conventionally milked cows, respectively. The hazard of clinical mastitis in incompletely milked cows was 0.96 (95% CI: 0.59, 1.6) times that of conventionally milked cows. The odds of PVD, LE and ENDO for incompletely milked cows were 1.4 (95% CI: 0.89, 2.1), 1.3 (95% CI: 0.88, 1.8) and 1.2 (95% CI: 0.81, 1.7) times those of conventionally milked cows. These results suggest that the incomplete milking during the first five DIM increases the odds of drop in SCC from 11 to 18 DIM, but does not affect odds of increase in SCC in the same period. The incomplete milking had no effect on clinical mastitis incidence in the first 90 DIM and on reproductive tract health at 35 DIM.

Key words: dairy cow, incomplete milking, mastitis, reproductive tract disease

Introduction

Dairy cows produce a large quantity of milk very quickly after parturition, and their dietary intake in early lactation is not sufficient to meet their energy requirements, which leads to mobilization of body reserves and a negative energy balance (Bertoni et al., 2009). This is especially true for multiparous cows, which have a very steep increase in milk yield early in lactation (Wathes et al., 2007a). The resulting negative energy balance can lead to several negative metabolic and health issues in periparturient dairy cows, including increased susceptibility to infectious diseases such as mastitis (Suriyasathaporn et al., 2000, Holtenius et al., 2004) and metritis (Reist et al., 2003, Hammon et al., 2006, Huzzey et al., 2007).

Elevated blood concentrations of NEFA and ketone bodies (hyperketonemia) are commonly used indices for the presence of negative energy balance, as they are related to increased mobilization of fat. Several studies have shown an association between elevation of these metabolites and immunosuppression (Lacetera et al., 2005, Mulligan and Doherty, 2008, Ster et al., 2012). Non-esterified fatty acids appear to exert a direct negative effect on the immune system, but the relationship between the presence of ketone bodies and decreased immune function appears to be due to the link between ketone bodies and NEFA (Hegardt, 1999, Drackley et al., 2001, Ster et al., 2012). Decreasing the cow's energetic demand during the first days post-partum might improve resistance to infection by decreasing the release of NEFA into the blood circulation (Carbonneau et al., 2012, Morin et al., 2018).

Since negative energy balance is associated with immunosuppression, we hypothesized that preventing negative energy balance by milking dairy cows incompletely in early lactation would result in a reduction of the subsequent risk of mastitis and reproductive tract diseases. Therefore, the aim of this study was to use the large dataset generated by Morin et al. (2018) and Krug et al. (2018), which was a RCT to evaluate the impact of incomplete milking during the first five DIM on mastitis and reproductive tract disease. Using the previous studies, we demonstrated that incomplete milking for the first five DIM leads to a decreased odds of experiencing hyperketonemia through 17 DIM (Morin et al., 2018), without subsequent impacts on milk yield and composition throughout the lactation (Krug et al., 2018). Our specific objectives for the current study were to measure, on that same population of cows, the impacts of an

incomplete milking on: (1) incidence of IMI; (2) IMI elimination rate; (3) time until first clinical mastitis event; (4) incidence risk of clinical mastitis in the first 90 DIM; (5) prevalence of PVD; (6) prevalence of ENDO; and (7) prevalence of LE endometritis.

Materials and methods

Sample size calculations

The original study was designed to investigate the impact of the incomplete milking on ketonemia and odds of hyperketonemia (Morin et al., 2018), on odds of infectious diseases, on fertility, on culling hazard and on milk production (Krug et al., 2018). Sample size calculation were computed for all these different outcomes. Odds of hyperketonemia was the outcome requiring the largest sample size (n/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 determined the minimal differences that could be detected for each of the outcomes examined in this experiment. For these calculations we used the predetermined sample size of 400 animals per group, a 95% confidence level, 80% power, and various udder health and reproductive tract health parameters provided by the previously published literature (see Table VII). Moreover, to calculate sample size/power calculations for time to clinical mastitis (survival analysis), the package epiR 0.9-93 (Stevenson et al., 2012) from RStudio 1.1.383 (R Core Team, 2013) was used. Results from the sample size/power calculations are presented in Table VII. The minimal detectable clinical mastitis hazard ratio that could be detected between groups was estimated at 1.2.

Herds, animals and experimental design

This study was a RCT conducted on 13 commercial dairy farms selected by convenience. There were several requirements for selection of farms: proximity to Saint-Hyacinthe (QC, Canada); agreeing to follow the research protocol; participating in a DHI program; having computerized health records; having a milking system that allows measurement of milk collected in real time; and agreeing to share their herd health and DHI info with the research team. The study protocol was approved by the

Animal Ethics Committee of the Université de Montréal (Rech-1701). The complete research protocol is described with more detail in Morin et al. (2018).

Briefly, one month before the expected calving, all multiparous cows from participating farms were randomly allocated to a control or a treatment group, using a random number generator. Cows in the control group were milked conventionally according to regular farm practices. On six farms, producers reported that they milked cows incompletely during the first two DIM as part of their conventional milking procedures. The treatment consists of limiting milking to a maximum of 10, 12 and 14 L/d in DIM one to three, four and five, respectively, without altering milking frequency (i.e. the maximum amount allowed per day was divided by milking frequency). In one herd with an AMS, cows were milked manually 2x/d during the first five DIM and then were sent to the AMS. The milk volumes thresholds for incompletely milked cows were adapted from the study of Carbonneau et al. (2012). Dairy producers were not blinded to group allocation, as they were the ones applying the treatment protocol.

Samples and animal measurements

Udder health

Impact of incomplete milking on udder health was investigated using three outcomes: IMI incidence, IMI elimination, and clinical mastitis. To evaluate IMI incidence and elimination, quarter-milk samples were collected, after fore-stripping the quarters, around 2-8h after morning milking on mean (SD) 11.5 (2.3) and 18.5 (2.2) DIM. Samples were analyzed by Valacta (Ste-Anne-de-Bellevue, QC, Canada) using the Fossomatic cell counter (Fossomatic 4000 series; Foss Electric A/S, Denmark) to determine SCC of quarter-milk samples. To calculate IMI incidence, only quarters without IMI (defined as having a SCC < 100,000 cells/mL) at first sampling were considered at risk of acquiring an IMI. These were monitored again one week later to detect quarters that developed an IMI (defined as SCC ≥ 200,000 cells/mL). To calculate elimination rate, only quarters with an IMI (defined as SCC ≥ 200,000 cells/mL) at first sampling were considered at risk of eliminating an IMI, and thus were monitored again on second measurement to observed whether that IMI was eliminated (defined as SCC < 100,000 cells/mL). These thresholds were utilized based on Schepers

et al. (1997) and Dohoo and Leslie (1991). We therefore obtained binary outcomes for development and elimination of IMI.

Cases of clinical mastitis were recorded by participating producers in their electronic health records. A clinical mastitis case definition was presented to participating producers before the start of the study. Clinical mastitis was defined as presence of abnormal milk or typical inflammation signs (swelling, redness, pain) of the mammary gland (Reyher et al., 2011). Weekly farm visits were conducted over the course of the study by the research team for sample collection and to ensure proper recording of clinical mastitis events by producers.

Reproductive tract health

On mean (SD) 32.9 (2.9) DIM, cows enrolled in the RCT were tested for PVD, LE and ENDO. The Metricheck test (Simcro, Hamilton, New Zealand) was used for clinical examination of cows and resulted in a score from 0 to 5 depending on vaginal secretion's appearance (McDougall et al., 2007): 0, no discharge; 1, clear mucus; 2, mucus with flecks of pus; 3, mucopurulent discharge; 4, purulent discharge; or 5, foul smelling discharge. Positive PVD was considered when scores ≥ 3 were observed (Dubuc et al., 2010a).

Cows were also examined using an endometrial cytology with cytobrush (VWR CanLab, Mississauga, Canada), as described by Kasimanickam et al. (2004). A stainless steel instrument for endometrial cytology was sterilized before each use; the cytobrush was threaded onto the solid steel rod and placed in the steel tube; prior to examination, the instrument was placed in a sanitary plastic sleeve that was then lubricated; the vulva was cleaned using paper towel; the instrument was guided from the vagina to the external cervical, using rectal palpation; the sanitary sleeve was punctured before entering the cervix; the cytobrush was exposed for sampling against the uterine wall; the cytobrush was again placed in the steel tube before being removed from the cervix. The cytobrush was then rolled on a microscope slide to create a smear for PMNL count and it was immediately plunged into a 3 mL vial with 1 mL of 0.9% saline (NaCl 0.9%) to perform the LE test (Multistix 10 SG; Bayer Corporation, Elkart, IN, USA). Endometrial cytology slides were stained with modified Wright-Giemsa stain (Protocol-Hema3®; Biochemical Sciences Inc., Swedesboro, NJ, USA). Slides were then examined independently by two observers, using a microscope at 400x

magnification to count the number of PMN within 100 cells (PMN%) in two different locations of the slide. If the PMN count was between 1 and 49% in at least one of the slide locations, two more places within the same slide were examined. Two observers that were blinded to treatment group and to on-farm examination findings conducted microscopic evaluations independently. The average PMNL% for each slide and for each observer was calculated. If PMNL > 30% for both observers, the mean between both observers was calculated to be used as final measurement. If the average PMNL obtained was between 0 and 30% for at least one of the observers and differed by > 10 percentage-points between observers, a third reading was performed by a third observer and a final PMNL average was calculated between the two most similar results. Finally, if the final PMNL average was < 6%, the cow was considered negative for ENDO, and if the final PMNL was ≥ 6%, the cow was considered positive for ENDO (Denis-Robichaud and Dubuc, 2015).

The LE test was performed with a colorimetric semi-quantitative strip (Multistix 10 SG, Bayer Corporation, Elkart, IN, USA) that changes color in presence of leukocytes. Since darker colors are correlated to higher levels of leukocytes, interpretation of this test is as follows: 0, negative, 0.5, trace of leukocytes, 1, small amount of leukocytes, 2, moderate amount of leukocytes, 3, large amount of leukocytes (Couto et al., 2013). A score of 3 was considered positive for the LE test (Cheong et al., 2012).

Data management and statistical analyses

The experimental unit was cow for all experiments. The observational unit and the unit of statistical analysis was cow in all outcomes except for IMI outcomes, when observational unit and the unit of statistical analysis was quarter within cow.

Udder health

The effects of the incomplete milking on odds of acquiring an IMI, and on odds of eliminating an existing IMI, were determined using generalized mixed models. The GLIMMIX procedure was used in SAS 9.4 (SAS Institute Inc., Cary, NC) to run these models. Cow and herd were considered as random intercepts to account for the higher correlation among quarter-milk samples from the same cow and among cows from the

same herd. Treatment group was forced as the main predictor in the models. The general model was the following:

$Y_{ijk} \sim \text{bin}\ [P(Y_{ijk})]$

$\text{Logit}\ [P(Y_{ijk})] = \beta_0 + \beta_1 Tx_{jk} + v_{0k} + u_{0jk} + e_{0ijk}$ [Equation 4]

where $P(Y_{ijk})$ is the probability of new IMI or of IMI elimination for the i^{th} quarter from the j^{th} cow from the k^{th} herd. It is a function of treatment group (Tx) through the logit function, and it approximately follows a binomial distribution; β_0 is the intercept; β_1 is the regression coefficient for treatment group (conventionally vs. incompletely milked); v_{0k}, u_{0jk} and e_{0ijk} are the herd, cow and quarter error terms, respectively (random parts of the model).

Parity (categorized as second parity and third parity or greater) was tested as effect modifier of the relationship between treatment group and odds of IMI or of IMI elimination by adding the main terms and a 2-way interaction term (treatment group × parity) in the model. Parity was retained as an effect modifier if the 2-way interaction term yielded a *P*-value < 0.05 on the *F* test.

Residuals were visually examined for each model to evaluate normality using quantile-quantile plot of residuals. Assumption of homoscedasticity was assessed visually using plot of the residuals against predicted values.

To investigate time until first clinical mastitis case, a Cox proportional hazard model with a herd frailty term to account for the data structure was used (PHREG procedure in SAS). Time until clinical mastitis was defined as the number of days from calving until the cow developed clinical mastitis or until the cow was culled (left the herd or died). Cows were followed until 90 DIM (right-censoring) or culling (whichever comes first), as we did not expect an effect of treatment in later stages of lactation. The general model was as follows:

$h_i(t) = \lambda_0(t) \exp(\beta_1 Tx_{i1}) + \varepsilon_i$ [Equation 5]

where $h_i(t)$ is the clinical mastitis hazard for the i^{th} cow at time t; $\lambda_0(t)$ is the baseline hazard function; β_1 is the coefficient for the treatment group (conventional vs. incomplete milking); and ε_i represents the unobserved heterogeneity, accounting for difference between herds. Using this model, the clinical mastitis HR between

conventionally and incompletely milked cows can be computed by exponentiation of β_1, using the natural base logarithmic transformation.

We also hypothesized that the relationship between treatment and clinical mastitis hazard could change by parity level, therefore the interaction between treatment group and parity level was again tested. The 2-way interaction term was kept in the model if it yielded a *P*-value < 0.05. To evaluate if the treatment effect varied as function of time (i.e. the proportional hazards assumption), the log-cumulative hazard plot for incompletely and conventionally milked cows was visually inspected. The assumption of independent censoring was checked as described by Dohoo et al. (2009). Kaplan-Meier survival curves for each treatment group were produced using STATA/MP 12.0.

Reproductive tract health

Three generalized mixed models using herd as random intercept were used to study the effect of the incomplete milking on odds of a positive PVD, LE and ENDO test. The general model was as follows:

$Y_{ij} \sim \text{bin}\ [P(Y_{ij})]$

$\text{Logit}\ [P(Y_{ij})] = \beta_{0ij} + \beta_1 Tx_{ij} + u_{0j} + e_{0ij}$ [Equation 6]

where $P(Y_{ij})$ is the probability of PVD, LE or ENDO for the i^{th} cow from the j^{th} herd. It is a function of treatment group (Tx) through the logit function, and approximately follows a binomial distribution; β_0 is the intercept; β_1 is the regression coefficient for the treatment group (conventionally vs. incompletely milked); u_{0j} and e_{0ij} are the herd and cow error terms, respectively (random parts of the model).

In a similar way to the one described for IMI, parity (categorized as second and third or greater) was tested as effect modifier of the relationship between treatment group and odds of PVD, LE or ENDO by adding the main terms and a 2-way interaction term (treatment group × parity) in the model. Interaction terms were retained if the 2-way interaction term yielded a *P*-value < 0.05 on the *F* test. Normality and homoscedasticity were assessed as described for IMI.

Adjustment for misclassification bias

In the current study, quarter SCC was used to define the quarter IMI status. However, SCC accuracy for diagnosing IMI status is far from perfect. Similarly, using PVD, LE, or ENDO to define reproductive tract health status is associated to a certain level of outcome misclassification. Using these imperfect measurements could lead to biased measures of association between incomplete milking and diseases (Dufour et al., 2012, Dohoo, 2014). To correct our estimates of association for this systematic error, the Se and Sp (i.e. bias parameters) of the diagnostic test used for each outcome was obtained either through external or internal data sources. Then, the method proposed by Lash et al. (2009a,b) was used to compute the measure of association adjusted for misclassification bias.

Udder health bias adjustment

To correct the OR obtained with the generalized mixed models, the Se and Sp of the different SCC thresholds to exclude or detect IMI were obtained through literature (i.e. external data source). The threshold of 100,000 cells/mL of milk to define absence of IMI at the quarter level was reported to have a Se of 0.81 and a Sp of 0.83, while the threshold of 200,000 cells/mL of milk to detect IMI was reported to have a Se of 0.75 and a Sp of 0.90 (Schepers et al., 1997).

Reproductive tract health bias adjustment

The studies conducted on the validity of diagnostic tests for reproductive tract health generally estimate validity in diagnosing future reproductive performance, as opposed to estimating validity in diagnosing current reproductive tract health status (LeBlanc et al., 2002, Dubuc et al., 2010b). To compute estimates of Se and Sp of these tests for diagnosing current reproductive tract health status, we used a Bayesian latent class model for three tests (PVD, LE and ENDO) and one population, as described by Branscum et al. (2005). The model was ran using the MCMC procedure from SAS, with uniform beta prior distributions for all parameters (Se and Sp of all tests) and for prevalence of reproductive tract disease. We then used median Se and Sp estimates of each test to adjust the OR of each of the three models.

Results

Description of study population

The herds enrolled in this project had a mean (SD) number of 103 (51) lactating cows and a mean (SD) 305-d milk yield of 9,973 (660) kg/cow. Cows were predominantly housed in free-stall barns (9/13 herds) and were milked 2x/d (11/13 herds). Cows from one herd were milked 3x/d and, in another herd, an AMS was used. The majority of herds fed TMR (11/13 herds) and used monensin supplementation (11/13 herds). Cows from control group 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 one, two, three, four and five, respectively. The flow of quarters and cows in the current RCT is illustrated in Figure 12.

Udder health

From a total of 3,291 sampled quarters from 847 animals, 9% (301/3,291) were sampled only once and were, therefore, not used in the IMI incidence and elimination analyses. Consequently, two successive quarter-milk samples from 2,990 (1,511 conventionally milked and 1,479 incompletely milked) quarters pertaining to 779 cows were available for the udder health analyses. The mean number of sampled quarters per cow was 3.8 (Range: 1 to 4; Table VIII). Among these double sampled quarters, 3% (102/2,990) had a SCC between 100,000 and 200,000 cells/ml on the first sample and, therefore, did not met the healthy (< 100,000 cells/ml) nor the infected (> 200,000 cells/ml) case-definitions. Consequently, these quarters were not considered for the IMI incidence and elimination analyses. At the first sampling, 2,735 quarters (1,387 conventionally milked and 1,348 incompletely milked) were free from IMI, and, therefore, at risk of acquiring a new IMI (Table IX). New IMI rates of 1.7 and 1.5% were observed in conventionally and incompletely milked groups, respectively. The odds of new IMI for incompletely milked cows were 0.90 (95% CI: 0.49, 1.7; $P = 0.70$) times those of conventionally milked cows. Therefore, the impact of incomplete milking on new IMI was non-significant. Regarding IMI elimination, 153 quarters (81 conventionally milked and 72 incompletely milked) were considered infected on first sampling (Table IX). Intramammary infection elimination rates of 24.7 and 44.4% were

observed in conventionally and incompletely milked groups, respectively. Odds of IMI elimination for incompletely milked cows were 2.9 (95% CI: 1.4, 6.0; $P < 0.01$) times those of conventionally milked cows.

A total of 769 cows (387 conventionally milked and 382 incompletely milked) could be used for clinical mastitis analysis. The mean number of cows included per farm was 59 (range: 16 to 153). The hazard of clinical mastitis in incompletely milked cows was 0.96 (95% CI: 0.59, 1.6; $P = 0.88$) times that of conventionally milked cows. Kaplan-Meier survival curves of conventionally milked cows and incompletely milked cows are illustrated in Figure 13. Since curves of the log-cumulative hazard plot for incompletely and conventionally milked cows were approximately parallel, we concluded that the effect of treatment on clinical mastitis hazard did not vary as function of time (i.e. the proportional hazards assumption was respected). The effect of treatment on clinical mastitis hazard did not vary across parity (P-value = 0.30). Table X shows clinical mastitis incidence risk per herd, along with other herd characteristics such as mean herd SCC.

Reproductive tract health

The odds of PVD, LE and ENDO for incompletely milked cows were, respectively, 1.4 (95% CI: 0.89, 2.1; $P = 0.15$), 1.3 (95% CI: 0.88, 1.8; $P = 0.20$) and 1.2 (95% CI: 0.81, 1.7; $P = 0.38$) times those of conventionally milked cows. The effect of treatment on PVD, on LE, and on ENDO, did not vary across parity ($P = 0.27$, $P = 0.07$ and $P = 0.65$, respectively).

Adjustment for misclassification bias

The Se and Sp found in literature for IMI definition using the specified SCC thresholds, were used to adjust the OR obtained with Equation 4 for misclassification bias. The adjusted OR for new IMI and for IMI elimination are presented in Table IX. Briefly, a bias-adjusted OR could not be computed for IMI incidence due to the presence of negative count in some cells of the adjusted two by two table. For IMI elimination, the true OR was greater (OR: 5.5) than the unadjusted OR (OR: 2.9), indicating that the misclassification bias caused a bias toward the null value.

The cross-classified test results used in the Bayesian latent class model to compute estimates of Se and Sp for each reproductive tract health test (PVD, LE and ENDO), are presented in Table XI.

The posterior means and 95% probability intervals for the Se and Sp of PVD, LE and ENDO are presented in Table XII. True reproductive tract disease prevalence was estimated at 15% (95% CI: 12, 18). All three diagnostic tests appeared to have very good Sp (0.97, 0.95, and 0.94 for PVD, LE, and ENDO, respectively), but PVD's Se (0.57) was lower than that of LE (0.89) and ENDO (0.89). The (misclassification) adjusted OR for all three tests computed using the Se and Sp estimates are presented in Table IX. In this case, the adjusted and unadjusted OR were similar, indicating a rather small impact of misclassification bias for the reproductive tract disorder outcomes.

Discussion

In a previous article (Morin et al., 2018), we demonstrated that the incompletely milked cows enrolled in the current RCT had lower odds for hyperketonemia compared to conventionally milked animals. Assuming that the treatment protocol was applied as indicated, on first DIM there was no additional residual milk for most cows from the treatment group compared to conventionally milked cows. On DIM two, three, four and five, the level of uncollected milk in the incompletely milked cows corresponded to 52%, 61%, 58%, and 53% of the milk collected in the conventional group, respectively. Although energy balance was only indirectly measured in the cows enrolled in the current RCT (through BHBA measurement; Morin et al., 2018), it is reasonable to assume that energy balance was improved in incompletely milked cows.

Our results on IMI elimination support the idea that the incomplete milking has a positive impact on the immune system, probably through its action on limiting NEB and therefore decreasing NEFA blood concentration. Accordingly, Carbonneau et al. (2012) demonstrated that 16 cows that were submitted to an incomplete milking protocol with a residual milk of 57%, 65%, 66%, 62% and 64% on one, two, three, four and five DIM, had a lower mean SCC (43,000 cells/mL) than 15 control cows (190,000 cells/mL) during weeks 2 to 9 after calving. On the other hand, the study from Penry et al. (2017) conducted in 12 dairy cows, showed that incompletely milking an half-udder from 5-47 DIM, led to around 30% residual milk and to an increased mean SCC (48,300

cells/mL) compared to control half-udders (26,300 cells/mL). However, we believe that these differences are very small, considering the range of SCC that can be observed in the mammary gland. Nevertheless, differences between the study of Penry et al. (2017) and the one from Carbonneau et al. (2012) are probably a result from the different treatment lengths, different moments in lactation, and of central versus local (half-udder) response.

Our results did not show an effect of the incomplete milking on preventing new IMI, which might have been due to the very low IMI incidence among the cows followed (1.6%). With such a low incidence, it is very difficult to have enough statistical power to conclude a significant effect of treatment.

Although the literature on incomplete milking as a management strategy to prevent mastitis is very scarce, many studies investigated the impact of automatic teat cup remover settings on residual milk and udder health. For example, some studies (Rasmussen, 1993, Clarke et al., 2008, Hultén, 2016) reported no differences in SCC of cows with different take-off levels (i.e., milk flow rate threshold that leads to the detachment of the milking unit), but one study reported increased SCC in cows milked with higher take-off levels (Jago et al., 2010). Impacts on clinical mastitis were also non-significant in most studies (Rasmussen, 1993, Jago et al., 2010). The hypothesis present in most of these studies was that higher take-off levels could lead to increased residual milking and therefore an increased risk for IMI and clinical mastitis. The studies investigating residual milk (Clarke et al., 2008; Jago et al., 2010; Hultén, 2016) did not find any differences in the volume of milk remaining in the udder in cows milked with high versus low take-off levels. Although these studies can be of interest for the incomplete milking topic, we believe that the quantities of milk remaining in the udder in the current RCT, although not directly quantified, were probably considerably higher.

Clinical mastitis was the only outcome that was obtained through farmers' records. Although we encouraged farmers to record cases of clinical mastitis by visiting them on a weekly basis (during the study period), clinical mastitis incidence might have been underestimated in the current study. Moreover, each farmer was provided with a clinical mastitis case definition, but there was no validation of the actual application of that definition by each farmer. Differences in management strategies, in bacterial

populations, but also in case definition might have led to the high discrepancy observed in clinical mastitis incidence risk between farms.

As mentioned earlier in this paper, we hypothesized that the positive impacts of the incomplete milking on energy balance and NEFA, deduced from the decrease in BHBA (Carbonneau et al., 2012, Morin et al., 2018), could reduce the odds of reproductive tract disease. However, the current study does not support that hypothesis. It is possible that metabolic disturbances occurring prior to calving are more determinant than those occurring after calving, as suggested by Hammon et al. (2006). An additional explanation could be due to the time at which reproductive tract health diagnosis was evaluated. Sheldon et al. (2006) and Huzzey et al. (2007) detected a relationship between hyperketonemia and development of reproductive tract disease diagnosed at 21-28 DIM or < 15 DIM, respectively. However, Kaufmann (2010) used the time from 28-35 DIM for reproductive tract health diagnosis, which is similar to the time used in the current study, did not find an association between hyperketonemia and reproductive tract disease. With the current study design, we could not demonstrate an effect of the milking protocol on the development of reproductive tract disease earlier in lactation (0-28 DIM). This hypothesis could be investigated in future research. The lack of positive impact of the incomplete milking on reproductive tract diseases on week 5 after calving could also be explained by a greater time lag between the actual treatment (1-5 DIM), its effects (lowering odds of hyperketonemia up to 17 DIM; Morin et al., 2018), and, finally, disease diagnosis around 30 DIM. That long period between treatment and disease event could have decreased our ability to detect positive effects of the treatment due to presence of other important disease determinants occurring in the course of those weeks. The same reasoning applies for clinical mastitis up to 90 DIM.

Accurate measurements are necessary to assess relationship between exposures and outcomes. Errors in measurement are generally divided in random error, or variance, and systematic error, also known as bias. Systematic error, however, is often downplayed, even in very large datasets where it can surpass greatly random error (Lash et al., 2009a). We believe, however, that it is important to quantify systematic error too and therefore we adjusted the OR obtained with our models for the misclassification bias. In general, the real effect of incomplete milking was higher than the one reported without corrections (i.e. for IMI elimination). However, it was not possible to compute

an adjusted OR for all outcomes of interest. Another method that could have been used to control for misclassification bias, is the use of Bayesian latent class models, that would allow to obtain not only adjusted OR, but also an adjusted 95% CI for the OR (McInturff et al., 2004). In the current study, we tried using the latter approach, but we had to change to a simpler methodology due to convergence issues with the Bayesian latent class model approach.

Conclusion

Our results suggest that the incomplete milking during the first five DIM increases the odds of IMI elimination, but does not affect development of new IMI, clinical mastitis incidence, or reproductive tract health.

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Table VII. Sample size/power calculations for evaluating the impact of an incomplete milking during the first five days in milk on udder and reproductive tract health using a 95% confidence level and 80% power

Outcome of interest	n/group	Expected prevalence or incidence in control animals	Minimal detectable difference between groups (in percentage-points)
Udder health			
New IMI	1,440 quarters[a]	3.5% per quarter-week (Dufour and Dohoo, 2013)	± 1.7
IMI elimination	160 quarters[b]	15%, 30%, 50%[c]	± 14, ± 16, ± 16
Reproductive tract health			
Purulent vaginal discharge	400 cows	15% (Dubuc et al., 2010a)	± 6.5
Leukocyte esterase test	400 cows	10% (Denis-Robichaud, 2013)	± 5.5
Endometrial cytology	400 cows	20% (Dubuc et al., 2010b)	± 7.5

[a] According to Canadian bovine mastitis and milk quality research network (St-Hyacinthe, Québec, personal communication), 10% out of 3,000 quarter-milk samples had ≥ 200,000 cells/mL at week 2 after calving, therefore we would expect to have 1,440 quarters at risk of new intramammary infection out of a total of 1,600 (4 quarters×400 cows)

[b] For the same reasons mentioned in a, we would expect to have 160 quarters at risk of intramammary infection elimination out of a total of 1,600 (4 quarters×400 cows)

[c] We did not find references on intramammary infection elimination rates, therefore we reported minimal detectable differences for three different scenarios.

Intramammary infection (IMI)

Table VIII. Structure of the dataset for evaluating the impact of an incomplete milking during the first five days in milk on intramammary infection incidence and elimination[a]

Level	Number of units	Replication at level above[b]	
		Mean	Range
Farm	13	-	-
Cows	779	59.9	15-143
Quarters	2,990	3.8	1-4

[a] Data obtained from a randomized controlled trial conducted on 779 cows from 13 commercial dairies.
[b] Number of observations per cluster. For instance, number of quarters followed per cow, or number of cow-lactations per farm.

Table IX. Results obtained from a randomized controlled trial (853 cows, 13 herds) on the association between an incomplete milking during the first five days in milk and udder and reproductive tract health

Outcome of interest	Conventionally milked group			Incompletely milked Group			Measure of association	Value (95% CI)[a]	True value[b]
	Cases	N	%	Cases	N	%			
Udder health									
New IMI	24	1,387 quarters	1.7	20	1,348 quarters	1.5	OR	0.90 (0.49, 1.7)	---[c]
IMI elimination	20	81 quarters	24.7	32	72 quarters	44.4	OR	2.9 (1.4, 6.0)	5.5
Clinical mastitis	33	387 cows	8.5	31	382 cows	8.1	HR	0.96 (0.59, 1.6)	---[d]
Reproductive tract health									
Purulent vaginal discharge	42	417 cows	10.1	53	398 cows	13.3	OR	1.4 (0.89, 2.1)	1.6
Leukocyte esterase	67	415 cows	16.1	77	395 cows	19.5	OR	1.3 (0.88, 1.8)	1.4
Endometrial cytology	63	375 cows	16.8	67	347 cows	19.3	OR	1.2 (0.81, 1.7)	1.3

[a] Estimates obtained using logistic models accounting for clustering by cow and/or herd

[b] Estimates adjusted for outcome misclassification and obtained using the method described by Lash et al. (2009a) and exemplified in Lash et al. (2009b), without considering clustering by herd

[c] Adjusted odds ratio could not be computed due to presence of negative cells in the adjusted two-by-two table

[d] No adjustment was made for the error associated to clinical mastitis diagnosis

Intramammary infection (IMI); Total number of quarters or cows followed (N); Odds ratio (OR); Hazard ratio (HR); confidence interval (CI)

Table X. Description of the 13 participating farms regarding udder health

Herd	Mean number of milking cows[a]	Mean herd SCC (x 1,000 cells/ml)[a]	Number of cows for clinical mastitis analyses	Number of cows with ≥ 1 clinical mastitis case between 0-90 DIM	0-90 DIM clinical mastitis incidence risk (%)	Clinical mastitis risk in incompletely milked group in % (# of cases)	Clinical mastitis risk in conventionally milked group (%)
A	110	288	93	19	20.4	22.5 (9/40)	18.9 (10/53)
B	62	228	16	2	12.5	1.0 (1/10)	16.7 (1/6)
C	153	224	91	5	5.5	6.3 (3/48)	4.7 (2/43)
D	50	223	32	8	25.0	31.3 (5/16)	18.8 (3/16)
E	64	207	34	1	2.9	6.3 (1/16)	0 (0/18)
F	76	207	52	4	7.7	26.1 (2/23)	6.9 (2/29)
G	44	191	26	3	11.5	7.7 (1/13)	15.4 (2/13)
H	194	184	28	3	10.7	12.5 (2/16)	8.3 (1/12)
I	68	146	62	12	19.4	14.8 (4/27)	22.9 (8/35)
J	87	143	54	4	7.4	3.6 (1/28)	11.5 (3/26)
K	101	123	63	0	0	0 (0/26)	0 (0/37)
L	189	93	153	0	0	0 (0/81)	0 (0/72)
M	139	89	65	3	4.6	5.3 (2/38)	3.7 (1/27)
Total	-	-	769	64	8.3	8.1 (31/382)	8.5 (33/387)

[a] Calculated from DHIA records from year 2014

Somatic cell count (SCC); Days in milk (DIM); Number (#)

Table XI. Cross-classified test results for reproductive tract health from purulent vaginal discharge, leukocyte esterase test, and endometrial cytology

	ENDO positive		ENDO negative	
	PVD positive	PVD negative	PVD positive	PVD negative
LE positive	49	37	6	34
LE negative	6	36	17	533

Leukocyte esterase test (LE); Endometrial cytology (ENDO); Purulent vaginal discharge (PVD)

Table XII. Mean and 95% credibility interval for the sensitivity and specificity of three tests for detection of reproductive tract disease in dairy cows, obtained using a Bayesian latent class model comparing three tests in one population

	Mean	95% credibility interval
Purulent vaginal discharge		
Sensitivity	0.57	0.47, 0.68
Specificity	0.97	0.95, 0.98
Leukocyte esterase test		
Sensitivity	0.89	0.79, 0.97
Specificity	0.95	0.92, 0.97
Endometrial cytology		
Sensitivity	0.89	0.79, 0.97
Specificity	0.94	0.92, 0.96
Reproductive tract disease prevalence	0.15	0.12, 0.18

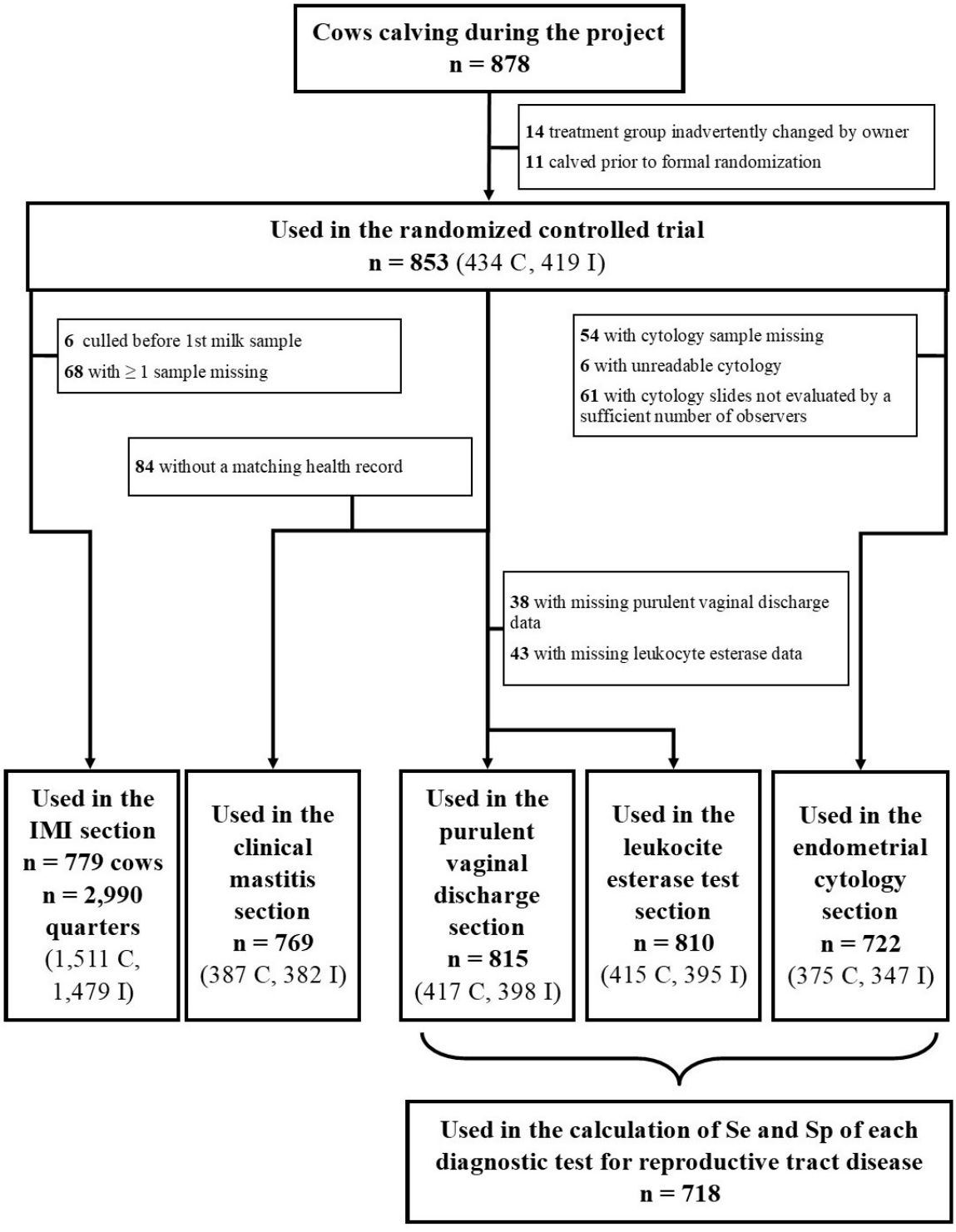

Figure 12. Flow of cows in a randomized control trials conducted on 13 commercial dairies and evaluating impact of incomplete milking during the first five days in milk (I) compared to conventionnal milking (C) on udder and reproductive tract health

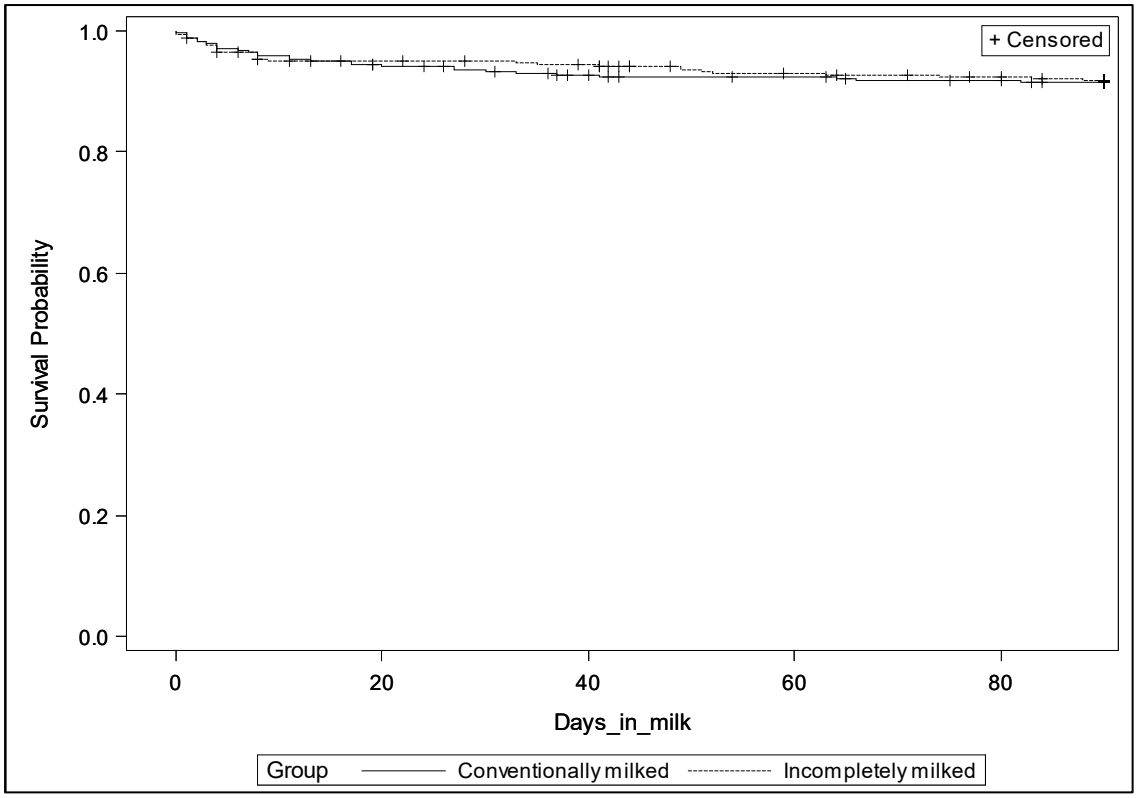

Figure 13. Kaplan-Meier survival curves illustrating clinical mastitis hazard for 769 multiparous cows from 13 commercial herds enrolled in a randomized controlled trial comparing conventionally and incompletely milked cows

CHAPTER 5: A RANDOMIZED CONTROLLED TRIAL ON THE EFFECT OF INCOMPLETE MILKING DURING THE FIRST FIVE 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 impact of incomplete milking on luteal activity and on pregnancy hazard. Secondarily, we aimed to study the impact of early lactation hyperketonemia (i.e. BHBA blood concentration ≥ 1.4 mmol/L during the first three WIM) on those reproductive outcomes. Multiparous Holstein cows (n = 853) from 13 commercial herds were enrolled in a RCT. Cows were assigned to a control or a treatment group, incompletely milked (10-14 L of milk collected /d) from DIM one to five. Blood samples were collected once a week during WIM one to three for BHBA blood concentration and a threshold of 1.4 mmol/L was used to define hyperketonemia. During WIM five and seven, cows were sampled for P4 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 impact of early lactation hyperketonemia on reproductive outcomes. The odds of luteal activity for incompletely milked cows were 1.1 (95% CI: 0.72, 1.7) times those of conventionally milked cows. The effect of treatment on pregnancy hazard varied as function of time, parity and start of breeding period. In second parity cows from herds with a VWP < 55 DIM, the pregnancy hazard (95% CI) in incompletely milked cows were 576.3 (240.0, 1383.7), 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 1-21, 22-43, 44-65, 66-87 and >87 d after VWP, respectively. The treatment did not have an effect on pregnancy hazard in cows in third parity or greater or in those from herds with a VWP ≥ 55 DIM. Early lactation hyperketonemia was not associated with any of the reproductive outcomes. In conclusion, the incomplete milking protocol had no effect on luteal activity it had a positive impact on pregnancy hazard in second parity cows in herds having a short VWP (< 55 DIM). We did not observe an impact of early lactation hyperketonemia on luteal activity or on pregnancy hazard.

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

Introduction

Negative energy balance is considered as an important reason for poor reproductive performance. Some studies have shown that cows with excessive negative energy balance 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., 2010a), therefore limiting negative energy balance 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 negative energy balance 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, that acts synergistically with LH and stimulates steroidogenesis, is also inhibited (Clarenburg, 1992, Lucy et al., 2001, Fenwick et al., 2008b), affecting negatively ovulation which in turn leads to a reduction of cows' ability to conceive (Butler, 2003).

During negative energy balance, body tissue reserves are mobilized as a result of intense galactopoiesis combined with an insufficient dietary intake. Hence, several researchers hypothesized that increasing food energy density could limit the negative energy balance (Vandehaar et al., 1999, Dewhurst et al., 2000), but only some investigated the impacts of temporarily restricting milk production on negative energy balance (McNamara et al., 2008, Loiselle et al., 2009). Reducing milk frequency during the first month in lactation had positive impacts on interval to first ovulation in Patton et al. (2006) study. A novel strategy to temporarily reduce milk output without altering milking frequency is to milk cows incompletely during the first five DIM (Carbonneau et al., 2012, Morin et al., 2018).

Previously, we demonstrated that incomplete milking for the first five DIM leads to lower odds of hyperketonemia up to 17 DIM (Morin et al., 2018), a higher elimination rate of IMI (Krug et al., In Press), without subsequent impacts on milk yield and composition throughout the lactation (Krug et al., 2018). We hypothesized that, by limiting negative energy balance through incomplete milking (Morin et al., 2018), reproductive performances could be improved. Therefore, the aim of the current study was to use the large dataset generated by Morin et al. (2018) and Krug et al. (2018

and *In Press*), to evaluate the impact of incomplete milking during the first five DIM on reproductive performance. Our specific objectives were to measure, on that same population of cows, the impacts of an incomplete milking during the first five DIM on luteal activity and on pregnancy hazard. Secondarily, because literature is sometimes inconsistent on this matter (Raboisson et al., 2014), we aimed to study the impact of early lactation hyperketonemia (i.e. blood BHBA concentration ≥ 1.4 mmol/L during the first three WIM) 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 with more detail in Morin et al. (2018).

Sample size calculations

The original study was designed to investigate, using a RCT, the impact 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., 2018 and In Press). Sample size calculations were computed for all these different outcomes, but odds of hyperketonemia was the outcome requiring the largest sample size (n/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 WIM five and seven 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 of 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/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 HR that could be detected between conventionally and incompletely milked cows was estimated at 1.2.

Herds, animals and experimental design

This study was a RCT conducted in 13 commercial dairy farms selected by convenience. There were several requirements for selection of farms: being near to Saint-Hyacinthe (Quebec, Canada); agreeing to apply the research protocol; participating in a DHI program; having computerized health records; having a milking system that allows for measurement of milk collected in real time; and agreeing to share their herd health and DHI info 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 free-stall barns (9/13 herds). Milking frequency was 3x/d in one farm, one herd used an AMS, and the other herds (11/13 herds) milked cows 2x/d. The majority of herds fed TMR (11/13 herds) and used monensin supplementation (11/13 herds). Cows were bred by AI after a VWP of 40 d in one farm (n = 37 cows included in this RCT), 50 d in six farms (n = 440 cows), 55 d in two farms (n = 111 cows), 60 d in three farms (n = 237 cows) and 70 d in one farm (n = 28 cows). Eight farms systematically enrolled their animals in an ovulation synchronization protocol: Double-Ovsynch protocol was used in four farms; Presynch-Ovsynch protocol was used in three farms; and Ovsynch was used along with an intravaginal P4-releasing insert (CIDR®1380, Zoetis Canada Inc., Kirkland, QC) in another farm.

Holstein cows of second parity or greater were enrolled one month before expected calving in a control or a treatment group. Cows were randomly allocated in each group using a random number generator. Cows in the control group were milked conventionally, according to their herds' milking practices. In six herds, farmers reported to apply an incomplete milking during the first two DIM as part of their regular milking procedures. The treatment was applied by dairy producers and farm personnel, and consisted in limiting the quantity of milk collected to a maximum of 10, 12 and 14 L/d on DIM one to three, four and five, respectively. The thresholds chosen were derived from Carbonneau et al. (2012) article, but slightly modified so they would be more easily applied by farmers. The herd using an AMS, maintained cows in a separated pen during the first five DIM, where they were milked manually, 2x/d. On the remaining farms, milking frequency was not

altered and, therefore, 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 two 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 and Company, Franklin Lakes, USA) once a week during the first three WIM starting at least 36 h after calving. Blood samples were immediately analyzed on farm with a Precision Xtra handheld device (Abbott Diabetes Care, Alameda, USA) for measurement of BHBA concentration. Early lactation hyperketonemia was considered if blood BHBA concentration ≥ 1.4 mmol/L (Duffield et al., 2009, Iwersen et al., 2009). Only animals with at least three blood samples were used for the analysis and, with that information, we created a categorical variable for early lactation hyperketonemia status with four levels: 1 = never experiencing hyperketonemia; 2 = hyperketonemia in one out of three weeks; 3 = hyperketonemia in two out of three weeks; 4 = hyperketonemia in all weeks.

Luteal activity

Blood samples (coccygeal vessels; 10 mL) were collected at WIM five and seven using a BD vacutainer and at exactly 14 d of interval. After centrifugation (1,400 x 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* (St-Hyacinthe, 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%.

If a P4 serum concentration ≥ 1 ng/mL was observed at least once (WIM five and/or seven), 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 seven days prior to the second P4 measurement. Cow's computerized health records (DSAHR Inc., Saint-Hyacinthe, QC, Canada) were verified to ensure that there was no prostaglandin administration during that period. Consequently, luteal activity status was considered missing whenever: 1) both measurements yielded a P4 concentration < 1 ng/mL and there was prostaglandin administration within seven days prior to the second P4 measurement; or 2) one of the P4 measurement 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 through 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 only considered the first pregnancy diagnosis, meaning that even if there was pregnancy loss with new AI period, we only recorded the first pregnancy. To avoid penalization of farms with longer VWP, time to pregnancy was defined as the number of days from end of the herd's VWP until the successful AI.

Potential effect modifiers

Since Morin et al. (2018) observed different effects of incomplete milking on odds of hyperketonemia in second parity cows vs. cows in third parity or greater, parity 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 (DSAHR Inc., Saint-Hyacinthe, QC, Canada). 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 if VWP acted as an effect modifier of the relationship between incomplete milking and reproductive outcomes. Since there were 13 herds, there could be a maximum of 13 different VWP values and so VWP was categorized in two levels based on a value that best divided the observations in half (55 DIM).

Data management and statistical analyses

The impact of incomplete milking on reproductive performance

To study the impact 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:

$Y_{ij} \sim \text{bin}\ [P(Y_{ij})]$

$\text{Logit}\ [P(Y_{ij})] = \beta_{0ij} + \beta_1 Tx_i + v_{0j}$ [Equation 7]

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 OR between incompletely milked and conventionally milked; and v_{0j} is the herd random error term. Parity was tested as effect modifier of the relationship treatment and odds of luteal activity by adding the main term and an interaction term (treatment × parity) in the model. Parity was retained as effect modifier if the interaction term yielded a *P*-value < 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 end of the herd's VWP (whichever come first; right censoring). Animals inseminated or culled before the start of the breeding period were left censored. The general model was as follows:

$h_i(t) = \lambda_0(t) \exp(\beta_1 Tx_i) + \varepsilon_i$ [Equation 8]

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

To evaluate if the effect of the incomplete milking protocol on pregnancy hazard varied as 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 two levels, 1-21 and >21 d following the end of the VWP, differentiating the first breeding cycle from the subsequent ones; 4) a time categorical variable with five levels: 1-21, 22-43, 44-65, 66-87, and >87 d after end of VWP, thus differentiating the first four 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.

The impact of early lactation hyperketonemia on reproductive performance

To study the impact of early lactation hyperketonemia status 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:

$Y_{ij} \sim \text{bin}\ [P(Y_{ij})]$

$\text{Logit}\ [P(Y_{ij})] = \beta_{0ij} + \beta_1 \text{Hyperketonemia}_i + v_{0j}$ [Equation 9]

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 OR between hyperketonemia levels; and v_{0j} 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 eight were enrolled in two contiguous lactations) calved during the project. The sample size in the current study was higher than previously reported sample size (n = 846; Krug et al., 2018, 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 14.

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

The impact 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 one, two, three, four and five, respectively. Average (SD) parity of cows enrolled was 3.1 (1.4) and 3.1 (1.3) for conventionally and incompletely milked cows, respectively. From the total 853 animals, there were 357 animals in second parity (185 conventionally milked and 172 incompletely milked), 495 animals in third parity or greater (249 conventionally milked and 246 incompletely milked) and one animal for which exact parity number was unknown. From that total, there were also 477 cows from herds with a VWP < 55 DIM (245 conventionally milked and 232 incompletely milked) and 376 cows from herds with a VWP ≥ 55 DIM (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-value = 0.79) did not act as 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 XIII shows median (IQR) interval from start of breeding period to pregnancy across variables of interest. The interaction 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 five levels (1-21, 22-43, 44-65, 66-87, and >87 d after end of VWP) was selected. The effect of treatment on pregnancy hazard did not only vary across time but it also varied 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 XIV shows the final model, while Table XV shows the estimated HR by parity, by VWP and by time. Briefly, in second parity cows from herds with a VWP < 55 DIM, 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 from herds with a VWP ≥ 55 DIM, incomplete milking did not affect pregnancy hazard.

The impact of early lactation hyperketonemia on reproductive performance

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

Hyperketonemia status in early lactation was not associated to odds of luteal activity (P = 0.48). The odds of luteal activity for cows experiencing hyperketonemia in one out of three weeks 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 two out of three weeks 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 end of herd's VWP to 60 d following that period) to pregnancy, by hyperketonemia status are presented in Figure 15. 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 three WIM) appeared to have numerically shorter number of days to pregnancy compared to the other hyperketonemia levels, especially between 0-60 d following end of VWP (Figure 15).

Discussion

The main objective of our study was to evaluate the effects of an incomplete milking during the first five DIM on prevalence of luteal activity (WIM five and seven) and on pregnancy hazard. Our data suggest that the incomplete milking protocol does not influence prevalence of luteal activity, but it does increase the pregnancy hazard in second parity cows from 1-87 d after end of the herd's VWP in animals from herds with a VWP < 55 days. The fact that we only found an impact of the incomplete milking protocol at the beginning of the breeding period but not at the

end is not surprising, as the milking protocol was of short duration and applied very early in the lactation.

The current RCT only includes multiparous animals. The reasons for that decision were based on the fact that in primiparous cows, the lactation peak is lower and occurs a bit later compared to multiparous. Moreover, the decline in milk yield after peak production is not as sharp as that of older cows (i.e., primiparous 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 to cows in third parity or greater is in agreement to 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 8-17 DIM. In that study (Morin et al., 2018), 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 only present 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 impact of incomplete milking on subsequent health events. Another possibility is that since cows in third parity or greater produce generally more milk than second parity cows, it might be that the treatment protocol has to be longer than stipulated, for example, to produce an effect in cows in third parity or greater. Finally, it is also possible that since second parity animals are still growing, their bodies might be more susceptible, or more "malleable" to an intervention than that of older animals.

The fact that incomplete milking led to a stronger effect in cows from herds with a VWP < 55 DIM compared to cows from herds with VWP ≥ 55 DIM can be explained by the fact that the first group of animals start being bred at a time closer to the milking intervention (1-5 DIM). Another reason might be that later than 55 DIM, most animals that experienced severe negative energy balance 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 impact 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) study, although there was earlier resumption of ovarian activity (18.3 d in cows milked 1x/d; vs. 28.6 d in cows milked 3x/d), interval from calving to pregnancy did not vary. Similarly, in McNamara et al. (2008) study, mean calving to pregnancy interval did not vary across different milking frequencies applied during the first month after calving. However, survival analysis 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 impact of early lactation hyperketonemia on luteal activity and on pregnancy hazard. Early lactation hyperketonemia was not associated with odds of luteal activity nor 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. 2010a; McArt et al. 2013). However, opposing results have also been found (Walsh et al. 2007a and 2007b; Dubuc et al. 2012). The lack of association between early lactation BHBA 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 only measured once a week from WIM one to three, but twice-weekly diagnosis would probably have been better for accurate diagnosis, as suggested by McArt et al. (2012) results. Our sampling schedule may thus have led to some misclassification of hyperketonemia status due to lower Se in detecting positive cows. There was, however, a high Sp (i.e. a large proportion of the healthy cows were diagnosed as such), and due to that, the impact on measure of association is very small, as showed 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. WIM five and seven), the results would probably differ. Even though there was no relationship in BHBA 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 negative energy balance (e.g. blood concentration of non-esterified fatty acids; Ospina et al. 2010a).

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% on 1st week after calving) and of luteal inactivity (16% at WIM five and/or seven) were low compared to previous studies. For instance, the study of Dubuc and Denis-Robichaud (2017) showed a median prevalence of hyperketonemia of 18.8% between 1-14 DIM, and prevalence of cows without luteal activity were much higher in Walsh et al. (2007a; 57% around WIM seven), in Dubuc et al. (2012; 26% around WIM seven), or in Dubuc and Denis-Robichaud (2017; 35% around WIM five and/or seven) studies. 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 to 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 start of 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 length of the study) minimized possible non-compliance during data collection.

Conclusion

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

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Table XIII. Median time between end of voluntary waiting period and pregnancy (in days) by breeding cycle and by treatment, parity levels and herd's voluntary waiting period. Values 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 five days in milk compared to conventional milking

				Median time (IQR) per breeding cycle[a]				
Tx	Parity	VWP	n	1-21	22-43	44-65	66-87	> 87
C	2nd	< 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)
	≥ 3rd	< 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)
I	2nd	< 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)
	≥ 3rd	< 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)

[a] Number of days following end of voluntary waiting period

Tx, Treatment; C, conventionally milked; I, incompletely milked; VWP, Voluntary waiting period (d); IQR, interquartile range

Table XIV. Results from the final Cox proportional hazard model on the effect of an incomplete milking during the first five days in milk on time from the end of the herd's voluntary waiting period to pregnancy[a]

				β for the time component parameters (SE) per breeding period cycle[c]				
Predictor	Level	β (SE)	*P*[b]	1-21	22-43	44-65	66-87	> 87
Treatment			<0.01					
	C	Ref.						
	I	6.357 (0.447)		Ref.	-2.747 (0.420)	-4.445 (0.497)	-5.459 (0.581)	-8.396 (0.551)
Parity			<0.01					
	2nd	Ref.						
	≥ 3rd	6.304 (0.410)		Ref.	-2.475 (0.367)	-4.376 (0.414)	-5.891 (0.554)	-7.890 (0.440)
VWP								
	< 55	Ref.						
	≥ 55	6.300 (0.447)		Ref.	-3.031 (0.534)	-4.265 (0.464)	-5.462 (0.601)	-8.227 (0.517)
Treatment × Parity			<0.01					
	C × 2nd	Ref.						
	I × ≥ 3rd	-6.242 (0.544)		Ref.	2.707 (0.634)	4.449 (0.670)	5.547 (0.839)	8.334 (0.685)
Treatment × VWP			<0.01					
	C × < 55	Ref.						
	I × ≥ 55	-5.793 (0.590)		Ref.	2.741 (0.766)	4.139 (0.786)	4.778 (0.900)	8.208 (0.778)
Parity × VWP			<0.01					
	2nd × < 55	Ref.						
	3rd × ≥ 55	-6.405 (0.551)		Ref.	3.028 (0.711)	4.498 (0.696)	6.115 (0.919)	8.267 (0.702)
Treatment × Parity × VWP			<0.01					
	C × 2nd × < 55	Ref.						
	I × ≥ 3rd × ≥ 55	5.900 (0.740)		Ref.	-2.780 (1.114)	-4.770 (1.094)	-5.555 (1.263)	-8.270 (1.006)
Herd variance		0.042 (0.029)						

[a] Values 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 an incomplete milking during the first five days in milk compared to conventional milking

[b] Joint *P*-value obtained using a *F* test

[c] Number of days following end of voluntary waiting period

Estimates, β; Reference level, Ref.; Conventionally milked, C; Incompletely milked, I; VWP, Voluntary waiting period (d)

Table XV. Estimated pregnancy hazard ratios (95% confidence interval) between incompletely milked cows and conventionally milked cows stratified by voluntary waiting period, parity and by breeding cycle period[a]

Breeding cycle	VWP < 55		VWP ≥ 55	
period[b]	Parity 2	Parity ≥ 3	Parity 2	Parity ≥ 3
1-21	576.3 (240.0, 1383.7)	1.1 (0.61, 2.1)	1.8 (0.81, 3.8)	1.2 (0.67, 2.3)
22-43	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	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	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	0.13 (0.07, 0.26)	1.1 (0.63, 1.77)	1.46 (0.68, 3.1)	1.1 (0.53, 2.3)

[a] Values 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 an incomplete milking during the first five DIM compared to conventional milking

[b] Number of days following end of voluntary waiting period

VWP, Voluntary waiting period (d)

Table XVI. Number of cows, proportion of cows presenting luteal activity, proportion of pregnant cows and median days from calving to pregnancy by early lactation hyperketonemia levels. Values 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 five days in milk compared to conventional milking

	Early lactation hyperketonemia levels[a]			
Variable	1	2	3	4
Number of cows (%)	412/638 (65%)	113/638 (18%)	89/638 (14%)	24/638 (4%)
Luteal activity (%)	292/333 (88%)	69/85 (81%)	52/61 (85%)	17/20 (85%)
Pregnant cows (%)	274/387 (71%)	75/106 (71%)	61/81 (75%)	16/22 (72%)
Median (IQR) days between end of VWP and pregnancy	43 (17-82)	44 (21-90)	48 (24-88)	44 (22-58)

[a] 1 = never experiencing hyperketonemia in the first three WIM; 2 = hyperketonemia in one out of first three WIM; 3 = hyperketonemia in two out of first three WIM; 4 = hyperketonemia in WIM one to three
IQR, interquartile range; VWP, voluntary waiting period

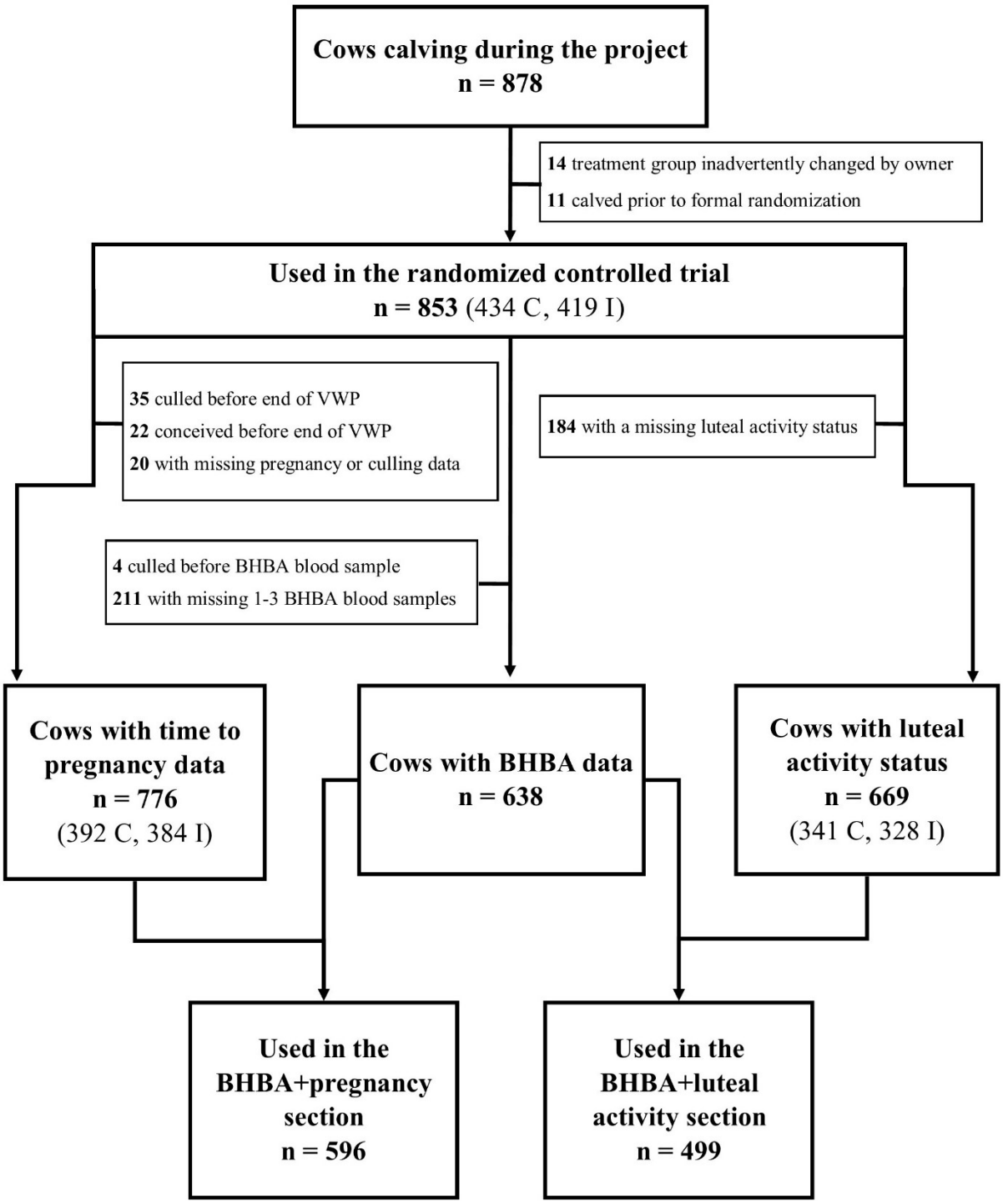

Figure 14. Diagram illustrating flow of cows and missing observations for cows from 13 commercial herds enrolled in a randomized controlled trial comparing conventionally (C) and incompletely (I) milked cows

C, conventionally milked; I, incompletely milked; VWP, voluntary waiting period

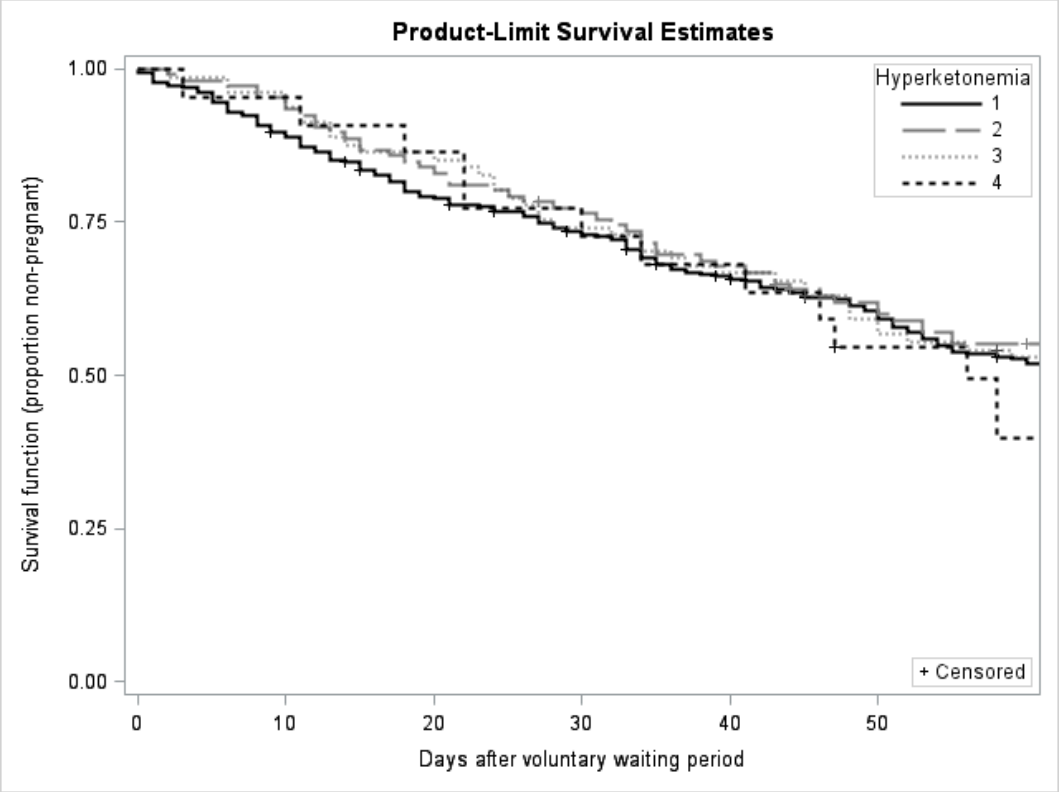

Figure 15. Survival curves of time to pregnancy for 596 lactating Holstein cows classified in different early lactation hyperketonemia levels based on serum β-hydroxybutyrate concentration determined in first to third weeks in milk

1 = never experiencing hyperketonemia in first three WIM; 2 = hyperketonemia in one out of first three WIM; 3 = hyperketonemia in two out of first three WIM; 4 = hyperketonemia in WIM one to three

CHAPTER 6: ALGOMETER PRECISION FOR QUANTIFYING MECHANICAL NOCICEPTIVE THRESHOLD WHEN APPLIED TO THE UDDER OF LACTATING DAIRY COWS

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Abstract

Objectives of this study were to: (1) quantify the reliability of an algometer for measuring mechanical nociceptive thresholds (**MNT**) when applied to the udder of dairy cows; and (2) evaluate whether covariates, such as cow characteristics or time of the day, would influence the algometer measurements. This prospective study was performed in a university herd of 37 lactating cows during five consecutive days, involving two raters. Two types of measurement were obtained: one qualitative binary measure (i.e. reaction vs. no reaction) and one quantitative measure presented in kilograms (i.e. MNT) for the cows that reacted. Kappa statistics were used to investigate test-retest and inter-rater reliability for the qualitative measure, while concordance correlation coefficient (**CCC**) and limits of agreement plot were used for the quantitative measure. Whether algometer measurements were influenced by several covariates (i.e. time of the day, level of milk production, DIM, and parity) was then evaluated using logistic or linear regression models, depending on the outcome. The algometer was moderately reliable; there was moderate test-retest reliability (Kappa = 0.53; CCC = 0.58) and inter-rater reliability (Kappa = 0.42; CCC = 0.54). The MNT varied substantially as a function of time of the day and parity. This is the first study reporting reliability of a pressure algometer for quantifying MNT and investigating covariates possibly affecting this measurement when applied to the udder of dairy cows. It is concluded that the use of the algometer for quantifying MNT on the udder is only moderately repeatable and is influenced by extraneous covariates. Its usage in research setting to quantify changes in sensitivity at the udder level should, therefore, be considered very cautiously or it should be further developed.

Key words: dairy cattle, udder, nociception, algometer, precision, reliability

Introduction

Practices such as abrupt cessation of milking at drying-off (Zobel et al., 2015), prolonged milking intervals (Österman and Redbo, 2001), and incomplete milking at the beginning of the lactation (Carbonneau et al., 2012; Morin et al., 2018), may lead to udder distension, milk leakage and inflammatory responses (Davis et al., 1998b; Rovai et al., 2007). Under such conditions, the animals may experience increased mechanical sensitivity, perhaps to a level where their welfare is negatively affected (FAWC, 2009).

Handheld pressure algometers have been used to assess sensitivity in dairy cattle in cases of mastitis (Fitzpatrick et al., 2013), lameness (Dyer et al., 2007), or after dehorning (Heinrich et al., 2010). The rater exerts pressure with the device in the body region of interest until the animal responds with an avoidance reaction (i.e. kicking, shifting weight). The MNT represents the amount of force (in kg) necessary to trigger animal avoidance response, measuring animals' sensitivity. Although algometers have been shown to be reliable in humans (Potter et al. 2006) and in some domestic animals (e.g. dogs: Kaka et al., 2015; horses: Haussler and Erb, 2006; piglets: Janczak et al., 2012), formal evaluation and validation of handheld algometers for quantifying MNT on the udder of dairy cows appears to be lacking.

The aims of the current study were to: (1) quantify the reliability of algometer measurement for quantifying MNT when applied to the udder of dairy cows; and (2) evaluate whether extraneous covariates, such as time of the day or cow characteristics (parity, stage of lactation, or production level), influence algometer results. Specifically, we hypothesized that if algometer measurement was influenced by these covariates, then care should be taken in standardizing, if possible, for those variables when using pressure algometers.

Materials and methods

This experiment was performed with the permission of the Animal Ethics Committee of the *Université de Montréal* under reference number Rech-1701. Observations from animals presenting clinical mastitis during the study were excluded from the analyses, because clinical mastitis may influence algometer measurements (Fitzpatrick et al., 2013). Clinical mastitis was diagnosed by the farm manager and defined as presence of abnormal milk or typical inflammation signs (swelling, redness, pain) of the mammary gland (Reyher et al., 2011). Only animals clinically healthy were selected for the study.

Algometer measurements

An observational prospective study was conducted at the *Institut de Technologie Agroalimentaire* teaching farm (Saint-Hyacinthe, QC, Canada) from July 6 to 10, 2015. The teaching farm had 37 milking cows that were housed in a tie-stall facility, and produced a mean 305-d milk yield of 11,301 kg. Stalls' resting surface were 1.52 m wide by 1.80 m long and

consisted of a rubber mat covered with a small amount of straw. Most cows had two neighbours, except four cows located at the very end of the two alleys. Milking occurred twice a day, at 07:00 h and 16:00 h. An algometer (Force Ten FDX 50; Wagner Instruments, Greenwich, CT) was used to measure the MNT. A concave probe head with 24.2 cm2 was added to the pressure point of the algometer to assure a good adaptation to the udder anatomy, and to avoid discomfort due to algometer surface (Figure 16). Before exerting pressure with the algometer, the rater would touch at the cow's upper hind leg, so that she would perceive the rater's presence. If lying, the cow would be encouraged to stand up for the measurement. Using the algometer device, the pressure would be exerted perpendicular to the skin of the inferior third of the left hind quarter, while the animal was standing, as shown on Figure 16. When the cow reacted by kicking or shifting weight, the quantitative measure on the algometer (in kg) was noted. When the cow did not react at the raters' maximum pressure, the lack of reaction would be noted, along with the pressure applied (for descriptive reasons), but this latter measure was not considered as the MNT. Therefore, two types of result were recorded, one qualitative binary measure (reaction vs. no reaction) and one quantitative measure (i.e. MNT, in kg) for the cows that reacted.

To investigate the algometer reliability, two veterinary students used the device for measuring (twice each) the MNT of all individual cows immediately prior to the afternoon milking, for five consecutive days (Monday to Friday). Before farm sampling begun, the raters reviewed how to use the algometer based on the methodology of Fitzpatrick et al. (2013). Rater one always recorded before rater two, and all measurements were taken within a short time frame (i.e. 2-5 min). Raters were neither blinded to their own results, nor to the other rater's results.

To evaluate whether the algometer measurement was affected by covariates, additional algometer measurements were collected by one of the raters 5x/d: immediately after morning milking (time 0), + 4:00 h, + 5:30 h, + 7:30 h following morning milking, and immediately prior to afternoon milking (+ 9:00 h). Days in milk, parity, and milk production obtained in the afternoon milking of each of the five days of study were also recorded. Parity was categorized in 1, 2 or ≥ 3. Days in milk were categorized in early (0-100 DIM), mid (101-200 DIM) and late lactation (> 200 DIM).

Definition of terms

Reliability refers to the consistency of different observations/measurements of the same object by the same rater (test-retest reliability, or repeatability) or by different raters (inter-rater reliability, or concordance). When investigating tests for which the rater may influence the results, both inter-rater reliability and test-retest reliability should be assessed (Nielsen et al., 2004), as the lower the reliability is, the higher the measurement error. Measurement error includes both systematic error, or bias, and random error. Precision is a measure of variability due to random error, and therefore, it is related to reliability (Thrusfield, 2005).

Agreement is distinguished from reliability, as the former corresponds to how close the results of the repeated measures are. Both reliability and agreement are important when assessing measurement properties of an instrument such as the algometer, since validity would be impaired if a measurement would not be adequately consistent (Weir, 2005).

The algometer validity relates to how close the MNT obtained with the algometer is to the actual level of udder pain/discomfort of a cow. The importance of any diagnostic technique, including the algometer, is judged in terms of its reliability and validity. Since there is no gold standard to measure udder pain, direct assessment of validity is impossible. However, if a measurement is affected by other extraneous factors (i.e. factors not associated with what we want to measure), then the validity of the measurement can be questioned (Thrusfield, 2005).

Statistical analyses

Reliability

Kappa statistics, CCC, and limits of agreement plots were used to determine test-retest reliability and inter-rater reliability (Dohoo et al., 2009). Kappa statistics were used to assess agreement of qualitative outcomes within and between raters. Kappa values ≤ 0 were considered poor agreement, 0.01-0.20 slight, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial, and 0.81-1.00 almost perfect agreement (Dohoo et al., 2009).

Reliability of the algometer's quantitative result was calculated using CCC and limits of agreement plots. The CCC measure a linear association between two measurements and classifies in several degrees of agreement (Dohoo et al., 2009): poor, < 0.20; fair, 0.21-0.40; moderate, 0.41-

0.60; good, 0.61-0.80; and very good, 0.81-1.00. The limits of agreement plot presents graphically the difference against the mean between two measurements. The latter is especially useful in detecting patterns of disagreement between raters/measurements, consequently helping to understand the origin of discrepancies (Dohoo et al., 2009).

To further detail test-retest reliability and inter-rater reliability, the effect of raters (A or B) and of order of the four measurements (1st, 2nd, 3rd, or 4th) collected repeatedly on one time point on both the qualitative binary measure (reaction vs. no reaction) and on the MNT were investigated independently (i.e. in univariate models). Mixed logistic and linear regression models were used to investigate effect of these two covariates on the qualitative binary measure (reaction vs. no reaction) and on the MNT, respectively. Clustering of algometer measurements by day, and by cow (4 measurements / d / cow) was accounted for using random day and cow intercepts. The logistic mixed models were as follows:

$$Y_{ijk} \sim \text{bin}\,[P(Y_{ijk})]$$

$$\text{Logit}\,[P(Y_{ijk})] = \beta_{0ijk} + \beta_1 X_{ijk} + v_{0k} + u_{0jk} + e_{0ijk} \qquad \text{[Equation 10]}$$

where Y_{ijk} was the reaction (or lack of reaction) at the i^{th} measurement of the j^{th} day of the k^{th} cow, which was a function of a predictor X (raters A or B; or sampling order, 1^{st}, 2^{nd}, 3^{rd}, or 4^{th}) through the logit function, and followed a binomial distribution with prevalence P of presentation of avoidance reaction. β_0 was the intercept and β_1 the regression coefficient for the effect of the predictor X. The cow, day, and measurement error terms, were represented as v_{0k}, u_{0jk}, and e_{0ijk}, respectively.

The linear mixed models were as follows:

$$MNT_{ijk} \sim N(\mu, \sigma)$$

$$MNT_{ijk} = \beta_{0ijk} + \beta_1 X_{ijk} + v_{0k} + u_{0jk} + e_{0ijk} \qquad \text{[Equation 11]}$$

where MNT_{ijk} was the MNT (in kg; mean μ and variance σ) for the i^{th} measurement of the j^{th} day from the k^{th} cow. β_0 was the intercept and β_1 was the regression coefficient for the predictor X (raters A or B; or sampling order, 1^{st}, 2^{nd}, 3^{rd}, or 4^{th}). v_{0k}, u_{0jk}, and e_{0ijk} were the cow, day, and measurement error terms, respectively, all assumed to follow an approximately normal distribution.

For the latter model, the fit of different covariance structures (compound symmetry, autoregressive, autoregressive moving average, Toeplitz, and heterogeneous variance compound symmetry) was compared using the Akaike Information Criterion. The compound symmetry correlation structure (equivalent to a conventional random intercept) was shown to provide a fit similar to that of more complex structures and was, therefore, retained for these analyses.

Relationship between algometer measure and covariates

Similarly to the previous section, logistic and linear regressions were used to investigate effect of covariates on the qualitative binary measure (reaction *vs.* no reaction) and on the MNT, respectively. Clustering of algometer measurements by day, and by cow (5 measurements / cow / 5 d) was accounted for using random day and cow intercepts. Causal diagrams were made between algometer measurement and each covariate (Dohoo et al., 2009; diagrams not shown). Based on those causal diagrams, it was deemed reasonable to use unconditional models for all covariates (i.e. no important confounders were identified for any of the covariates under investigation).

The logistic mixed models were as presented in Equation 10, where Y_{ijk} was the reaction (or lack of reaction) at the i^{th} measurement of the j^{th} day of the k^{th} cow, which was a function of the covariate (X) through the logit function, and followed a binomial distribution with prevalence P of presentation of avoidance reaction. β_0 was the intercept and β_1 the regression coefficient for X. The cow, day, and measurement error terms, were v_{0k}, u_{0jk}, and e_{0ijk}, respectively.

The linear mixed models were as presented in Equation 11, where MNT_{ijk} was the MNT (in kg; mean μ and variance σ) for the i^{th} measurement of the j^{th} day from the k^{th} cow. β_0 was the intercept and β_1 was the regression coefficient for the covariate (X). v_{0k}, u_{0jk}, and e_{0ijk} were the cow, day, and measurement error terms, respectively, all assumed to follow an approximately normal distribution.

Again, for the latter model, the fit of different covariance structures (compound symmetry, autoregressive, autoregressive moving average, Toeplitz, and heterogeneous variance compound symmetry) was compared using the Akaike Information Criterion. The compound symmetry correlation structure (equivalent to a conventional random intercept) was shown to provide a fit similar to that of more complex structures and was, therefore, chosen.

Estimates of variances were obtained using a model without predictors. Then, as described by Dohoo et al. (2009), we used those estimates to calculate the proportion of the variation in the MNT that was explained by the characteristics of the observation, day, or cow.

Descriptive statistics were performed using SAS version 9.4 (SAS Institute Inc., Cary, NY). Statistical analyses regarding algometer reliability were performed with the same software. All mixed effect models were fitted using MlWin 2.3 (Rasbash, London, UK).

Sample size calculation

Regarding reliability, we estimated that a sample size of 175 observations (35 cows observed 1x/d for five days) would be sufficient to detect a difference in Kappa values between 0.60 and 0.40, with an alpha of 0.05, a power of 80% and assuming that 90% of cows would respond to the algometer (Sim and Wright, 2005).

Regarding the association between covariates and the algometer measurement, we estimated that with a confidence level and a power of 95% and 80%, respectively, a sample of 28 cows, and assuming a SD of 0.68 kg in MNT (Fitzpatrick, 2011), we would be able to detect a difference of at least 0.77 kg between covariate levels.

Results

The total number of cows enrolled in the project was 36, and five cows had some missing observations. From the total source population of 37 cows, one was excluded from the sampled population due to aggressiveness. Twenty six percent were first parity cows (9/36), 23% were second parity cows (8/36) and the majority, 51% (18/36), were third parity or greater. Furthermore, 28% (10/36) were in early lactation, 31% (11/36) in mid and 42% (15/36) were in late lactation. Mean (± SD) milk production at afternoon milking was 14.0 (± 3.6) L.

Reliability

Three hundred and forty three pairs of observations were available for the test-retest reliability (36 cows x five days x two raters, minus 17 pairs of missing observations). An avoidance reaction was observed in half of the first measurement observations (49%, 169/343) and in 45% (156/343) of second measurements. Figures 17 and 18 show, respectively, the pressure applied in

case of reaction (i.e. MNT) and in case of no-reaction (i.e. maximum pressure). Moderate test-retest agreement was observed (Kappa = 0.53; 95% CI: 0.45, 0.63) for the reaction or no reaction to the pressure algometer.

Three hundred and thirty six pairs of observations were available for inter-rater reliability, (36 cows x five days x two observations, minus 24 pairs of missing observations). An avoidance reaction was observed in 48% (160/336) of rater one and 48% (160/336) of rater two observations. Again, moderate inter-rater agreement was observed (Kappa = 0.42; 95% CI: 0.45, 0.63) for the reaction or no reaction to the pressure algometer.

Regarding the MNT (only in cows reacting to the algometer), limits of agreement plots were inspected both for the test-retest and inter-rater analyses and no discernible pattern of disagreement was observed (data not shown). The mean difference between the MNT evaluated in two measurements from the same rater (test-retest reliability) was -0.56 kg (95% CI: -5.40, 4.29). The mean difference between the MNT evaluated by both raters (comparison between the first measurement of both raters; and comparison between the second measurement of both raters) was 0.81 kg (95% CI: -4.44, 6.06). Figures 19 and 20 show the CCC plots for inter-rater and test-retest reliability, respectively. Only a small shift of the slopes and, consequently, of the intercepts were observed. Moderate test-retest agreement (CCC = 0.58; 95% CI: 0.46, 0.68) and inter-rater agreement (CCC = 0.54; 95% CI: 0.27, 0.73) were observed.

To further detail test-retest reliability and inter-rater reliability, the effect of raters (A or B) and of order of measurements (1st, 2nd, 3rd, or 4th) on both the qualitative binary measure (reaction vs. no reaction) and on the MNT were investigated. There was a total of 686 observations available for analyses (36 cows x 5 days x 2 raters x 2 observations, minus 34 pairs of missing observations). Raters ($P = 0.79$) and sampling order ($P = 0.66$) did not affect odds of reacting to pressure with an algometer. On the other hand, both rater ($P < 0.01$) and sampling order ($P = 0.03$) affected the MNT (among the 325 cow-observations that reacted). One rater recorded MNT that were on average 0.63 kg (95% CI: 0.17, 1.10) higher than MNT recorded by the other rater. After adjustment for multiple comparisons using the Tuckey-Kramer adjustment, the effect of order of measurements on MNT was only significant between 2nd and 3rd measurements ($P = 0.03$; mean difference 0.92, 95% CI 0.06 to 1.78) but not for any of the other comparisons (i.e. 1st *vs.* 2nd *vs.* 3rd *vs.* 4th). Mean (non-adjusted 95% CI) MNT was 5.25 (4.48, 6.02), 5.35 (4.56, 6.13), 4.43 (3.65,

5.21) and 4.92 (4.12, 5.71) kg for the 1st, 2nd, 3rd, and 4th measurements, respectively. Note that, as per study design, rater A always collected his two measurements (1st and 2nd) prior to rater B's measurements (3rd and 4th).

Relationship between the algometer measurements and covariates

Parity was associated to odds of reacting to the algometer (*P*-value = 0.02). Primiparous had equal odds of reacting compared to second parity cows (95% CI for the OR: 0.74, 6.0; P = 0.20) and higher odds of reacting than cows in third parity or greater (OR: 3.5; 95% CI: 1.5, 8.2; *P* < 0.01). Unconditional associations between covariates and probability of presenting an avoidance reaction are shown in Table XVII.

The variation in MNT was 59% due to characteristics of the measurement (e.g. time of the day and/or level of activity of the cow at that moment), 10% due to characteristics of the day (e.g. Monday vs. Tuesday), and 31% due to cow characteristics (e.g. production level, age).

Mechanical nociceptive threshold varied with time after milking (*P*-value < 0.01). The mean (95% CI) MNT, in kg, was 5.45 (4.6, 6.3) immediately after milking, 4.83 (4.0, 5.7) at + 4:00 h, 5.62 (4.8, 6.5) at + 5:30 h, 4.40 (3.6, 5.2) at + 7:30 h, and 5.53 (4.7, 6.4) at + 9:00 h post-milking (Table XVII). Therefore, MNT did not increase or decrease constantly during the day. Milk production and DIM were not associated to MNT, but parity was (*P*-value < 0.01). Compared to primiparous cows, cows in second parity and in third parity or greater had an increment of 1.7 (0.1, 3.4) and 2.5 (1.1, 3.9) on the MNT, respectively (Table XVII).

Discussion

In large animal studies, algometers have been used to assess sensitivity, but their use in assessing udder sensitivity of dairy cows is relatively recent (Fitzpatrick et al., 2013) and has never been validated. This study describes, for the first time, the reliability of using a pressure algometer to assess MNT when applied to the udder. The instrument is simple to apply, moderately reliable, and the MNT appears to be affected by many factors.

The number of reactions and no reaction to the algometer was approximately the same within and between raters. However, in previous studies (Fitzpatrick et al., 2013) reporting using an algometer on mastitic cows, the lack of reaction to the pressure exerted with the algometer was

not mentioned. It was therefore unclear if the use of the algometer led to a reaction in all cows (regardless of evaluation in an infected or uninfected quarter), if the maximum pressure applied was recorded as MNT in cows not showing a reaction, or if cows that did not react were not used in the analyses. In the current study, the high proportion of observations with no reaction may have been caused by the fact that milking cows are accustomed to being handled, touched and milked by humans, and if all cows showed avoidance each time they were milked, the milking process would be difficult for both the cow and the person milking. Other reason could be a very low udder sensitivity, or the development of some degree of tolerance due to repeated sampling. However, other studies in humans have showed that repeated stimulus can also provoke progressive intensification of the perceived pain (Arendt-Nielsen et al. 1994). In future studies, modifying the area of skin contact could be investigated. The use of different types of probe could also be investigated. In the current study, we fixed a 24.2 cm^2 concave probe head on the pressure point of the algometer to assure a good adaptation to the udder anatomy, and to avoid discomfort due to algometer surface. We could hypothesize that this type of probe does not create a level of discomfort that is sufficient to elicit a response from the majority of cows. A narrower, pencil-shaped, but smooth probe, for instance, could have elicited a higher proportion of response among cows.

Within the same rater, the second measurement lead to slightly lower, but not significant, odds of reaction, which suggests that some cows might have only reacted due to the initial stimulation, therefore gaining some tolerance at the 2^{nd} measurement. Thus, there was some bias due to test repetition (Coldwells et al., 1994). If, in the future, the device is used by other researchers, perhaps only 2^{nd} measurements should be used. Moreover, Raundal et al. (2015) suggested that a period of habituation to handheld devices used to evaluate MNT improves reliability.

The moderate test-retest and inter-rater reliability, both in Kappa and CCC, showed that the measurement is still somehow reliable. Our results showed that odds of reaction were not influenced by rater nor order of measurements. On the other hand, the quantitative outcome of the pressure algometer stimulation was associated to both factors. There may be some subjectivity for the rater to decide when pressure has to be stopped for reading (i.e. to judge when the cow initiated a reaction). Moreover, differences between raters may also have been caused by difference in promptness of response (i.e. withdrawal of the handheld algometer) once the cow's reaction is

noticed. Nevertheless, for research purposes, the use of a single observer would possibly be preferred. In our study, the MNT was also significantly influenced by whether it was the 1st, 2nd, 3rd, or 4th consecutive measurement, but not in a linear way. Since all 1st and 2nd measurements were collected by rater A and all 3rd and 4th were collected by rater B, the rater effect could not be dissociated from the order effect. Nevertheless, cows may have become sensitized by the repeated stimuli and, thus, started to respond more promptly to the pressure applied on 3rd and 4th measurements.

In the current study, raters were not blinded to their own results, nor to the other rater's results, and operator induced variation is a known challenge of handheld tools (Raundal et al., 2015). When designing the current study, we were not aware of the possibility of some cows (51% in our case) not reacting, thus blinding was not considered in the current study. The results obtained, however, suggest that the algometer measurement is not as objective as initially hypothesized. As suggested before, further studies involving different shape of probes may help reducing the proportion of cows not responding to the stimuli. Regarding blinding, we can hypothesize that the absence of blinding in the current study may have led to either no bias or to bias leading to more similar measurements between raters. Thus, the repeatability measure obtained should be considered as a "best case scenario". As a result, we suggest the inclusion of blinding procedures in future studies.

Within all the covariates tested in the unconditional analysis, the only one that influenced the qualitative outcome was parity of the cow, with cows from lower parities having higher odds of reacting than older cows. Analogous results were obtained for the quantitative analysis, since primiparous cows had lower MNT compared to multiparous. The higher chances of reaction along with the lower MNT observed in primiparous cows might be a result of their lower experience in being milked, handled or touched in the udder area compared to multiparous cows. If this hypothesis is correct, then algometer results would be an indicator of the cow's experience, instead of an indication of increased sensitivity due to udder distension. Another possibility is that the lower MNT in primiparous were caused by less quantity of secretory tissue (Klaas et al., 2004) and smaller udder than multiparous cows. In such a case, algometer results would actually be a measure of cow increased sensitivity due to udder distension. These results are in agreement with those of Fitzpatrick (2011), who found that multiparous cows tolerated on average 0.77 kg more pressure

than primiparous cows. Future studies could help confirming these differences and clarifying the reasons behind those.

Although time after milking did not affect the qualitative algometer outcome, it did affect the MNT. However, MNT was not proportional to udder repletion (i.e. number of hours post-milking). It is hypothesized that algometer result was modulated by cows' activity when the measurement was taken (i.e. if they were lying down *vs.* up and eating at the moment of sampling), similarly to what was found previously in piglets (Janczak et al., 2012). In the current study, the interval between the morning and afternoon milking (time of sampling process) was of approximately nine hours. However, according to Ayadi et al. (2003), even though cisternal and alveolar milk volume increased proportionally to milking interval, cisternal and alveolar repletion plateau were only reached 20 h and 16 h after milking, respectively. Thus, it is possible that the study duration (i.e. nine hours) was not sufficient to lead to the accumulation of a volume milk that would cause an increased udder sensitivity. Future studies evaluating the effect of time since milking, but using longer milking interval, would possibly lead to different conclusions. Algometer measurements following abrupt cessation of milking at drying-off (Zobel et al., 2015) or following incomplete milking at the beginning of the lactation (Carbonneau et al., 2012; Morin et al., 2018), could also possibly lead to different conclusions.

According to Caja et al. (2004), cisternal milk volume decreased (by 49%) between early and mid-lactation, while alveolar milk volume decreased mostly between mid and late lactation (by 68%). So if MNT is related to milk volume in the udder, we would expect to have cows in early lactation reacting faster than cows in late lactation. In the current study, in the same way that high-producing cows did not show lower MNT to the pressure algometer, differences between stages of lactation were not observed.

In conclusion, the algometer had moderate test-retest and inter-rater reliability on both qualitative (reaction *vs.* no reaction) and quantitative outcomes (i.e. MNT). Cow MNT was influenced by various extraneous covariates, including time of the day at which the measurement was taken, and cow characteristics such as parity. These results suggest that these factors should be taken into account when using an algometer to measure MNT on the udder of dairy cows. Algometer results seem to be highly variable and may actually measure concepts that are quite different than udder sensitivity. Algometer usage in research setting to quantify sensitivity when applied to the udder of dairy cows should, therefore, be considered cautiously or this methodology

should be further developed. At the very least, if using these devices, an attempt to match animal studied (e.g. exclusion, pairing, conditional models) on time of the day and cow characteristics should be made. In future research, understanding what influences the changes in MNT during the day (e.g. the cow, the type of activity such as lying down or eating, the time since last milking, diurnal cycles, etc.) could help standardizing the algometer MNT measurement.

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Author contributions statement

CK: conception or design of the work, data collection, data analysis and interpretation, drafting the article, final approval of the version to be published. TD: critical revision of the article, final approval of the version to be published. J-PR, JD, and SD: conception or design of the work, data analysis and interpretation, critical revision of the article, final approval of the version to be published.

Conflict of interest

None.

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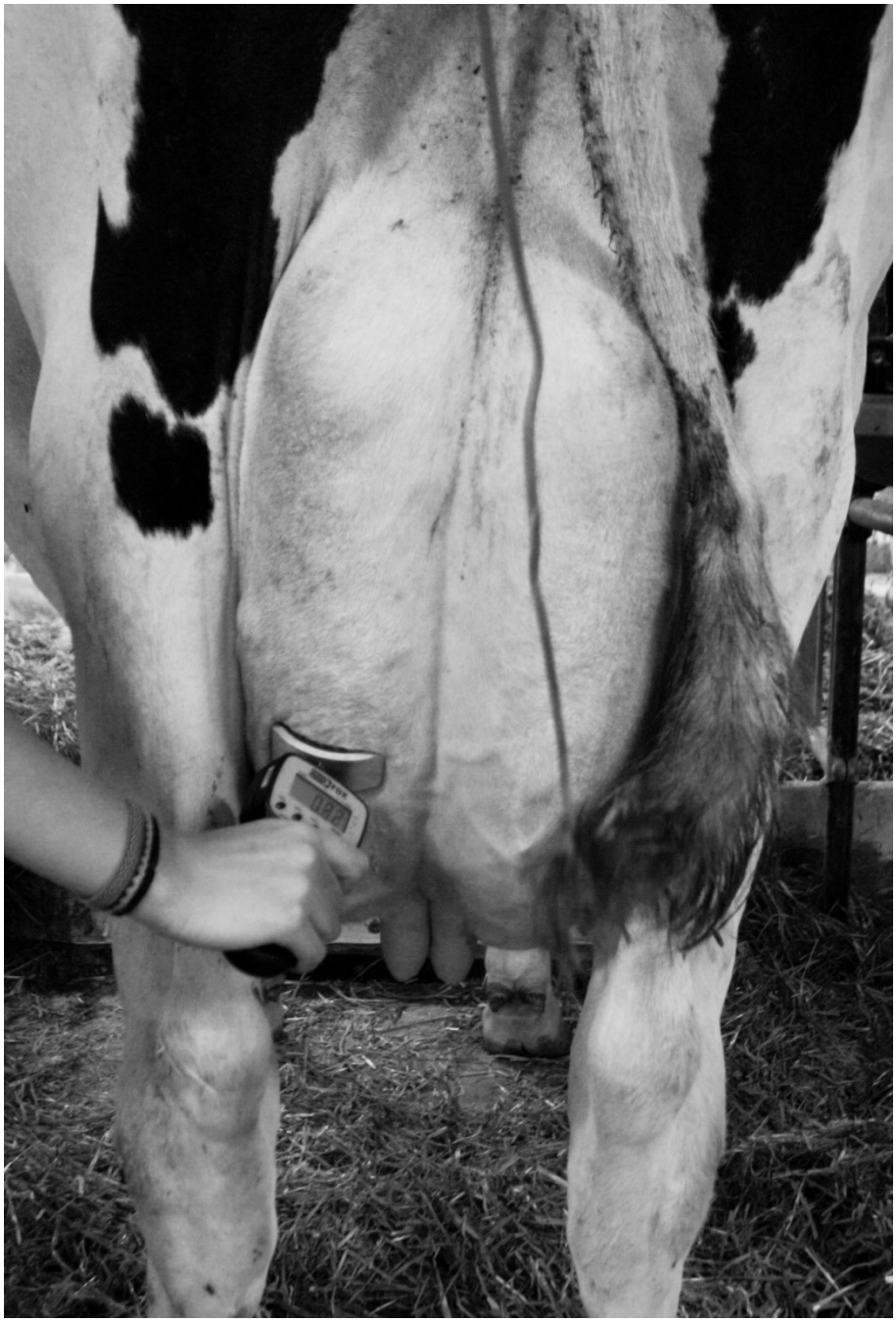

Figure 16. Illustration of the placement of a pressure algometer for quantifying mechanical nociceptive threshold in dairy cows.

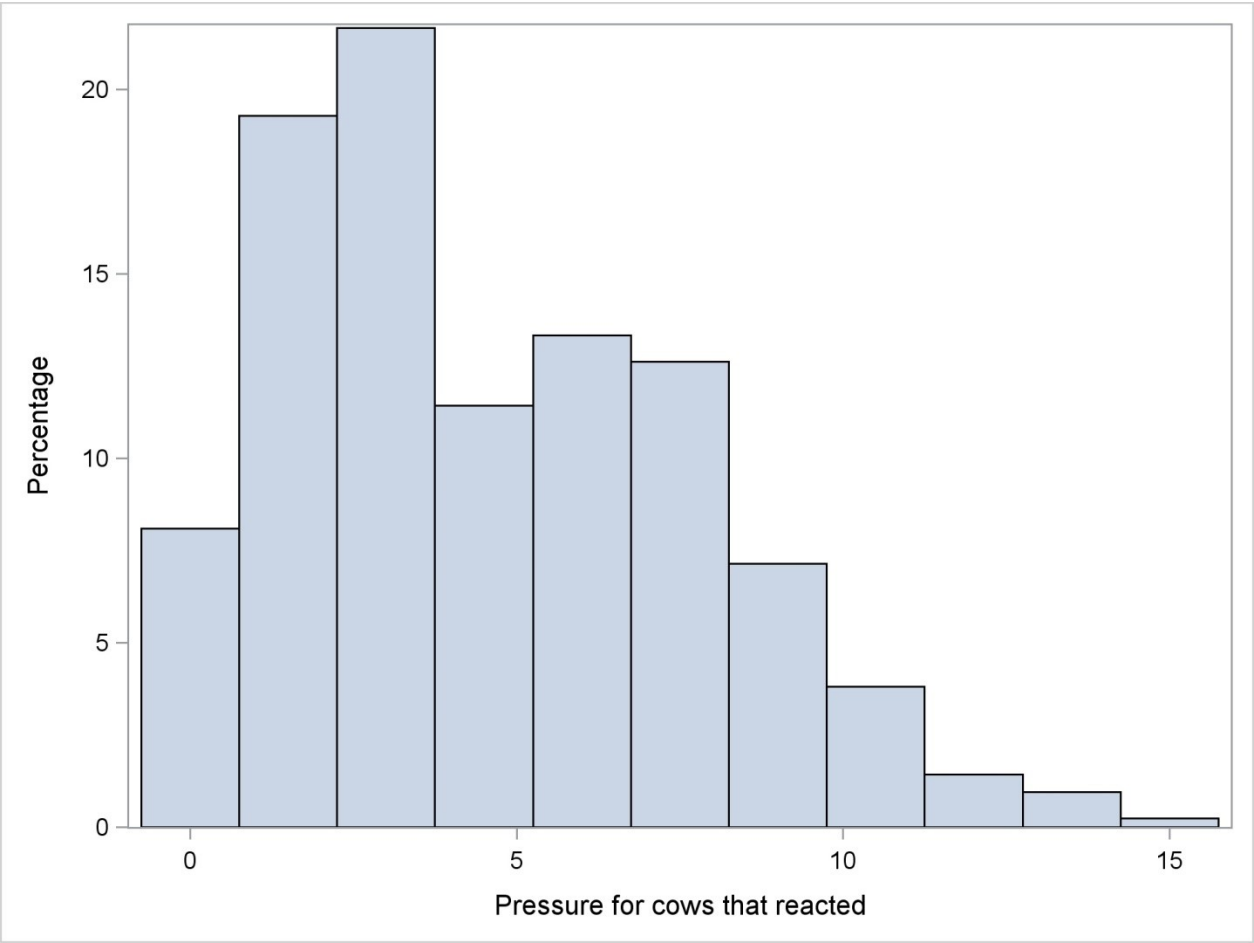

Figure 17. Distribution of the mechanical nociceptive threshold (in kg) measured using a handheld pressure algometer. Data obtained using one measure per day for five consecutive days on 36 milking cows.

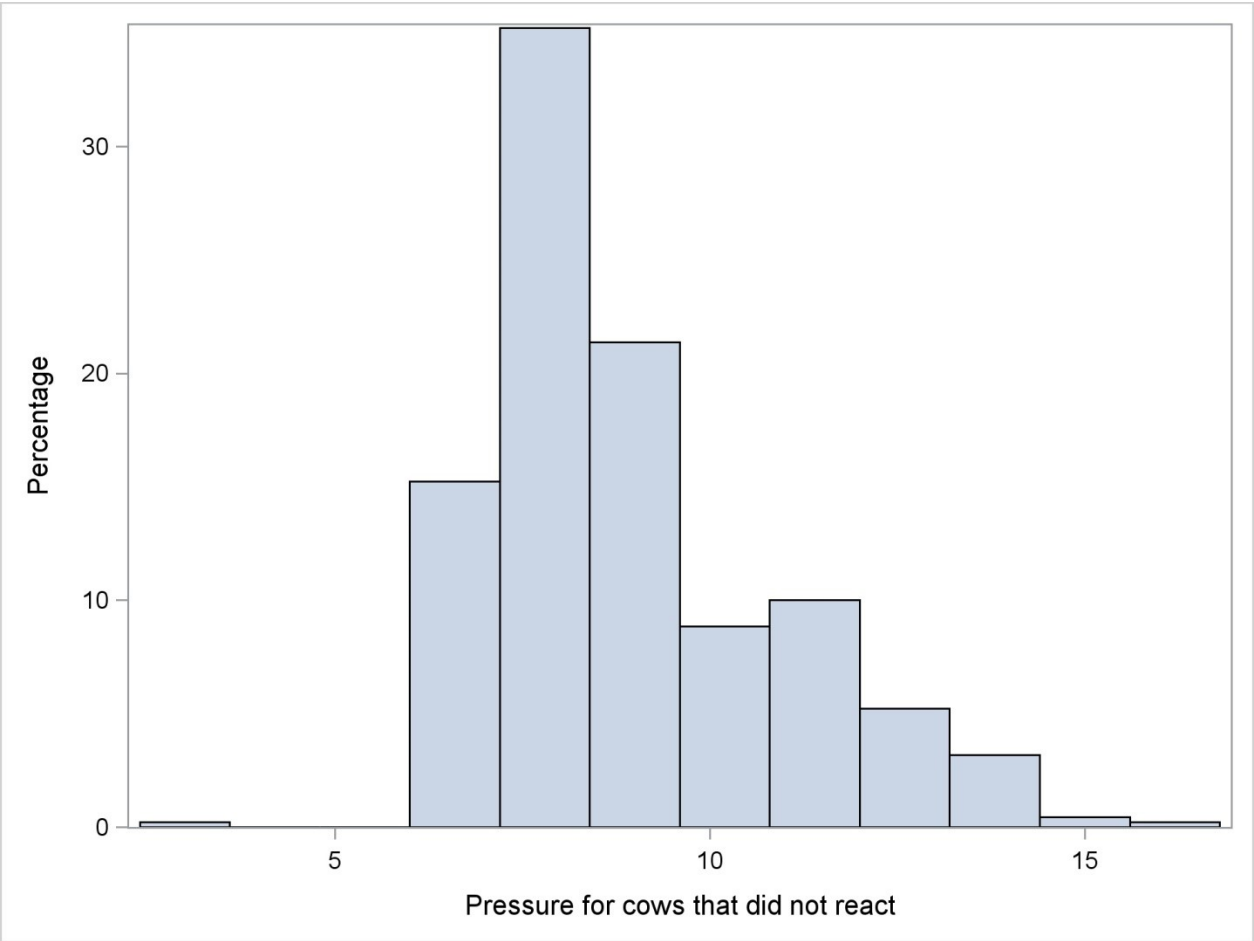

Figure 18. Distribution of the amount of pressure applied using a handheld pressure algometer in cases where cows did not react (in kg; maximum pressure). Data obtained using one measure per day for five consecutive days on 36 milking cows.

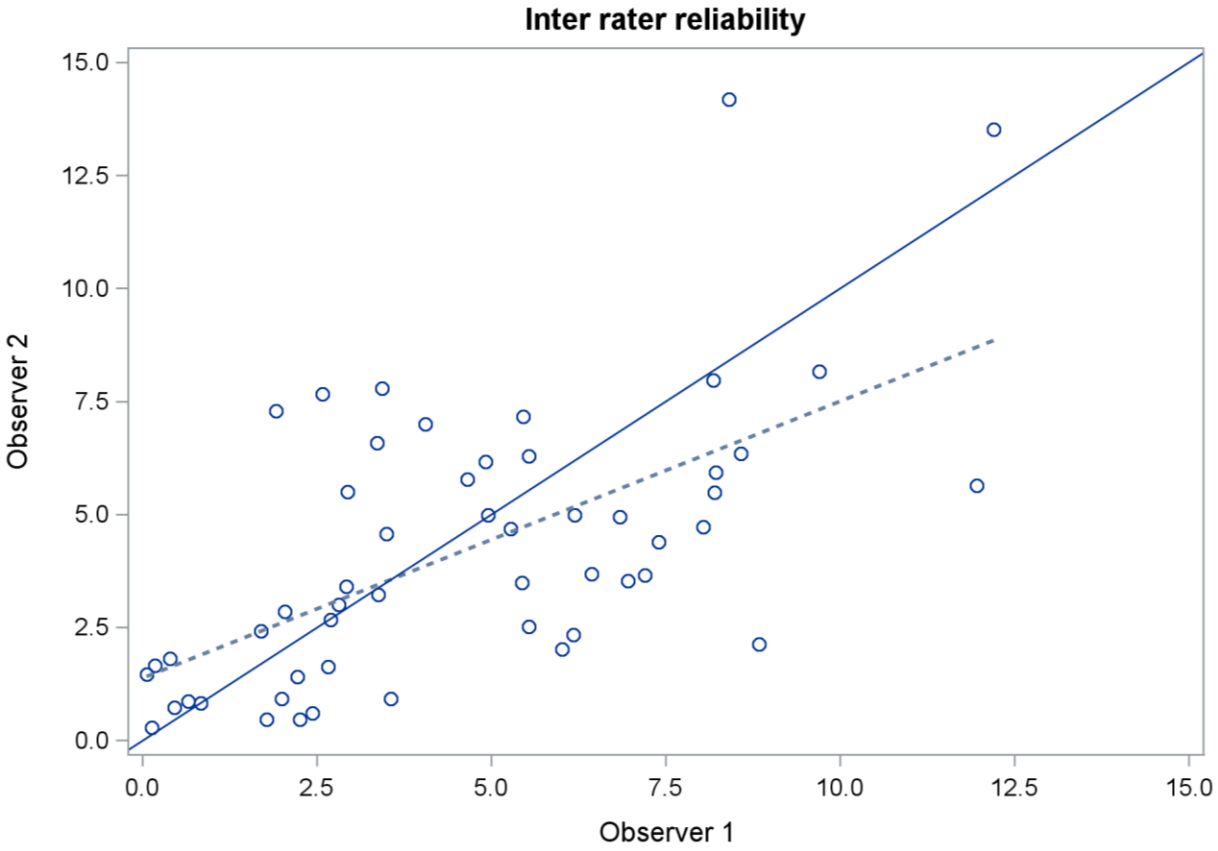

Figure 19. Concordance correlation plot comparing inter-rater reliability for mechanical nociceptive threshold quantified using the algometer. Data obtained using one measure per day for five consecutive days on 36 milking cows. The full line represents the line of perfect concordance and dashed line represents reduced major axis.

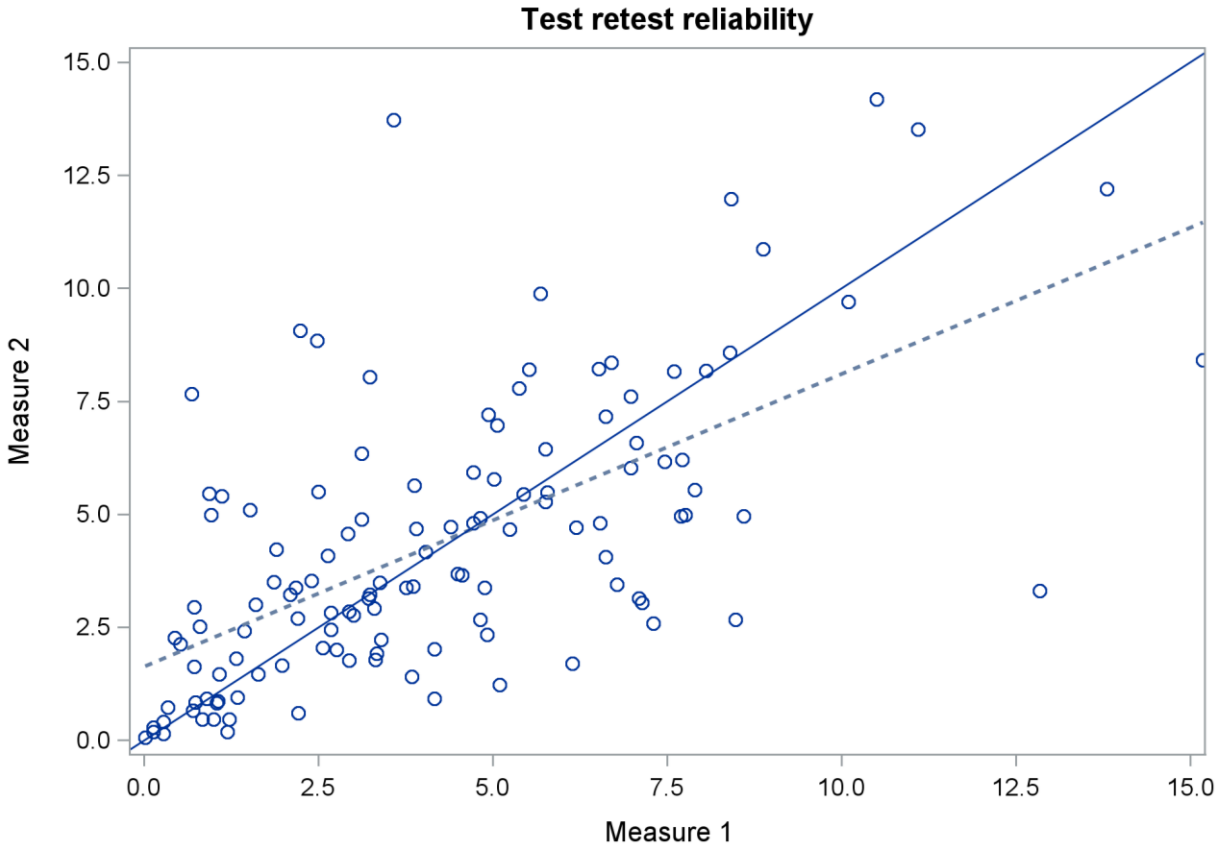

Figure 20. Concordance correlation plot comparing test-retest reliability for mechanical nociceptive threshold quantified using the algometer. Data obtained using two consecutive measures per day for two raters and for five consecutive days on 36 milking cows. The full line represents the line of perfect concordance and dashed line represents reduced major axis.

Table XVII. Unconditional associations between predictors and odds of reacting to the algometer (reaction *vs.* no reaction) and between predictors and mechanical nociceptive threshold (in kg; for cows reacting to the algometer). Data generated from an observational study conducted on 36 dairy cows from a teaching farm. Estimates were obtained using logistic (n = 860 observations) and linear (n = 420 observations) mixed regression models accounting for clustering by day and by cow.

		Reaction *vs.* no reaction (logistic mixed regression)				Mechanical nociceptive threshold (in kg; linear mixed regression)			
Parameter	Level	β	SE	95% CI	Joint *P*-value	β	SE	95% CI	Joint *P*-value
Model 1									
Intercept		-0.03	0.23			5.45	0.42		
Time after morning milking	0 h	Ref.	Ref.	Ref.	0.92	Ref.	Ref.	Ref.	<0.01
	4:00 h	-0.02	0.22	-0.4, 0.4		-0.62	0.38	-1.4, 0.1	
	5:30 h	-0.11	0.22	-0.5, 0.3		0.17	0.39	-0.6, 0.9	
	7:30 h	0.10	0.22	-0.3, 0.5		-1.05	0.37	-1.8, -0.3	
	9:00 h	0.02	0.22	-0.4, 0.4		0.08	0.38	-0.7, 0.8	
Model 2									
Intercept		0.22	0.47			6.17	0.95		
Milk production [a]		-0.02	0.03	-0.1, 0.0	0.56	-0.07	0.06	-0.2, 0.1	0.25
Model 3									
Intercept		-0.12	0.33			4.92	0.62		
Days in Milk	≤ 100	Ref.	Ref.	Ref.	0.79	Ref.	Ref.	Ref.	0.88
	101-199	-0.03	0.42	-0.8, 0.8		0.39	0.77	-1.1, 1.9	
	≥ 200	0.22	0.43	-0.6, 1.1		0.23	0.80	-1.3, 1.8	
Model 4									
Intercept		0.75	0.36			3.52	0.56		
Parity	1	Ref.	Ref.	Ref.	0.02	Ref.	Ref.	Ref.	<0.01
	2	-0.75	0.53	-1.8, 0.3		1.74	0.84	0.1, 3.4	
	≥ 3	-1.24	0.44	-2.1, -0.4		2.47	0.71	1.1, 3.9	

[a] Milk production in kg per milking

Regression model coefficient estimate (β)

Standard error of the mean (SE)
Confidence interval (CI)
Reference level (Ref.)

CHAPTER 7: INCOMPLETE MILKING IN EARLY LACTATION DOES NOT AFFECT DAIRY COWS RESTING BEHAVIORS: RESULTS FROM A RANDOMIZED CONTROLLED TRIAL

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Abstract

The objective of this study was to investigate the effect of incomplete milking during the first five DIM on the resting behavior of commercial dairy cows. The hypothesis was that the elevated intramammary pressure resulting from milk retained in the udder in incompletely milked cows could lead to a change in lying behavior. This study was a RCT in which cows from two farms were randomly allocated into a treatment (n = 18) or a control group (n = 14). Cows in the treatment group were milked incompletely (10-14 L/day) during the first five DIM, while cows in the control group were milked as usually done on farm. Resting behaviors were recorded with a data logger. Linear mixed models were used to quantify the effects of treatment group on three dependent variables measured between two and 14 DIM: daily duration of lying time (h/d), lying bout frequency (bouts/day), and mean duration of lying bouts (min/bout). There was no significant effect of treatment on lying time. However, the effect of treatment on frequency of lying bouts and on mean lying bout duration varied by parity level. Incompletely milked cows in second parity had a higher number of lying bouts (11.9 vs. 9.2 bouts/day) and shorter mean lying bout duration (57.8 min/bout vs. 66.7 min) than control cows. In third parity or more, the opposite happened. Therefore, our results suggest that an incomplete milking may be slightly problematic for second parity cows and, possibly, slightly beneficial for older cows. Whether the differences observed resulted from a biologic process (discomfort due to the incomplete milking) or from random error will have to be determined by future research.

Keywords: dairy cattle, animal welfare, resting behavior, data logger, incomplete milking

Introduction

Milking cows incompletely in early lactation is a novel way to reduce the negative energy balance and its detrimental effects in dairy cows (Carbonneau et al., 2012). However, reducing the volume of milk harvested might potentially be associated with a sustained udder distention, especially in high producing cows, which could lead to a modification of the cow's lying behaviors. Unfortunately, there are currently no published studies on this topic.

Internal or external challenges that lead to poor animal welfare often produce differences in cows' behavioral activities, including resting behavior (Wechsler, 1995). For instance, a study

by Österman and Redbo (2001) showed that cows milked 2x/d vs. cows milked 3x/d had higher number of lying bouts of shorter duration and fewer long lying bouts four hours before milking. Such difference in behavior was hypothesized by these authors to be caused by pain due to udder distension. However, welfare was not impaired by a lower milking frequency in other studies. For example, two studies (O'Driscoll et al., 2010, 2011) reported that cows milked 1x/d had similar lying times and improved hoof health and locomotion score compared to cows milked 2x/d. However, in these studies, cows were not assessed in early lactation, when milk yield is increasing. In another study (Tucker et al., 2007), cows milked 1x/d had higher udder firmness, but similar grazing activity and a tendency for longer lying times compared to cows milked 2x/d.

Work conducted at dry off may be useful in determining the potential impacts of incompletely milking cows in early lactation. For example, Zobel et al. (2013) presented a review on the effects of abrupt cessation of lactation on animal welfare. According to these authors, the elevated intramammary pressure resulting from milk retained in the udder after milking cessation could lead to tissue damage and pain. The sum of articles reviewed in that paper, however, did not lead to a conclusive answer regarding changes in lying behavior following abrupt cessation of milking at drying off, and the authors suggested that level of milk production at dry off should be considered when conducting such analysis.

For the current study, our hypothesis was that the elevated intramammary pressure resulting from milk retained in the udder in incompletely milked cows could lead to a measurable change in resting behavior compared to cows milked completely. Therefore, the objective of the current study was to investigate the effect of an incomplete milking during the first five DIM on daily duration of lying time, lying bout frequency, and mean duration of lying bouts of commercial dairy cows up to 14 DIM.

Materials and methods

Sample size estimation/Power

The current study was initiated following discussions with producers participating in a larger RCT. Producers were concerned about the potential discomfort of the treatment procedure

for their cows. With the larger RCT already ongoing, a limited number of cows were available for studying impact of treatment on resting behaviors. We expected to be able to recruit approximately 32 cows (16 in each group) before the end of the study, which would contribute to around 448 daily observations (32 cows multiplied by 14 d). Rather than a sample size estimation, we estimated the minimal difference that could be detected with the available sample size using SAS power procedure. A power of 90% and a level of confidence of 95% were used. For lying time, assuming a SD of 1.3 h/d in the control group, it was deemed possible to detect with a power of 90% a difference ≥ 0.4 h/d between treatment groups. For lying bouts frequency, with an expected SD of 3.8 bouts/d in the control group, it was deemed possible to detect a different of at least 1.2 bouts/day between treatment groups. For mean lying bout duration, with an expected SD of 12 min/bout, it was judged possible to detect a difference of at least 3.7 min/bout between treatment groups.

Animals and treatments

This study was part of a larger RCT that was conducted on multiparous cows from a convenient sample of 13 commercial dairy farms in the province of Quebec, Canada. Morin et al. (2018) describes this larger RCT. The eligibility criteria for these farms included: being enrolled in a DHI, having a milking system that allows measurement in real time of the volume of milk harvested from the cow, having computerized records of disease, having at least around 70 multiparous cows calving per year, and being willing to apply the methodology necessary for the study and to share their herd records with the research group. The study protocol was accepted by the Animal Ethics Committee of the Université de Montréal (rech-1701). For this RCT, all multiparous cows, in the study herds, calving during the 14-month period comprised between January 2013 and March 2015 were recruited. For the current study, cows from two of the participating herds that calved in the last five months of the RCT (i.e., from October 2014 to February 2015) were recruited. In these two herds, cows were housed in free stall barns (mattress-based stalls covered with wood shavings as bedding). Herds were milked 2x/d (04:00 and 16:00 h; herd A) and 3x/d (04:30, 12:30, and 20:30 h; herd B). Herd A had a mean number of 68 milking Holstein cows and a mean 305-day milk yield of 10,091 kg per cow whereas herd B had a mean number of 189 milking Holstein cows and a mean 305-day milk yield of 9,155 kg

per cow. During the study, cows were randomly allocated to a treatment or a control group using a random number generator. Cows in the treatment group were milked incompletely during the first five DIM: 10 L on DIM one, 10 L on DIM two, 10 L on DIM three, 12 L on DIM four, and 14 L on DIM five. The decision on the quantity of milk withdrawn per day was based on the study from Carbonneau et al. (2012). Cows in the control group were milked completely, as usually done on these farms. Because treatment influenced how cows were milked, dairy producers could not be blinded to the group allocation (treatment or control).

Animal-based measures

Parity and calving date were obtained through farm records. Resting behavior was recorded with Hobo Pendant Acceleration data loggers (Onset Computer Corporation, Bourne, MA, USA) validated by Ledgerwood et al. (2010). The data logger was installed one week before expected calving and replaced every week until the end of the second WIM. The device was set to record g-force and slope of the x, y, and z-axes in a scale of −3.2–3.2 at intervals of 60 s (UBC, 2013). The data loggers were attached with bandage to the left hind leg above the metatarsophalangeal joint of cows for easy access in the milking parlor during the following weeks. The three axes were drawn on the exterior of the data logger and, when attached to the leg, the data logger was placed with the illustrated x-axis parallel to the ground pointing to the head of the animal, the y-axis perpendicular to the floor pointing to cow's back, and the z-axis parallel to the floor pointing to the lateral of the cow (UBC, 2013).

Data management and statistical analyses

To extract the data from the data logger, the Onset Hoboware Pro Software (Onset Computer Corporation, Bourne, MA, USA) was used. Data were then imported as comma separated values files in SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA) to be edited using the standard operating procedures described by the University of British Columbia (UBC, 2013). Three outcomes (daily duration of lying time, h/d; lying bout frequency, bouts/day; and mean duration of lying bouts, min/bout) were computed for each cow-day of observation. These outcomes were considered as the dependent variables in this study. Since there is usually a drop in lying time around calving (see Figure 21, for example), only

observations from 2-14 DIM were used in the models. The predictor of interest in the current study was treatment group (i.e., incomplete vs. complete milking). Researchers assessing the outcome were not blinded to treatment allocation.

Prior to modeling, quantitative variables were tested for normality by visual inspection of histograms. Variables were then screened individually for their association with the three dependent variables using linear mixed regression models using the MIXED procedure of SAS 9.4. Models were then developed to investigate conditional associations. The 13 daily observations were clustered by cow; therefore, a cow random intercept was used in the models; herd was included in the models as a fixed effect to control for clustering of cows by herd. Furthermore, a treatment × DIM interaction term was forced into the models to capture the daily variance and to investigate the effect of the treatment for each day of observation. In these models, DIM was treated as categorical variable (i.e., DIM two to DIM 14). The Tukey adjustment was used to account for multiple comparisons.

There was an equal proportion of cows in second parity and in third parity or greater (16 cows in each category). The distribution of parity, however, was different between treatment groups (*P*-value = 0.03); with 12 second parity cows (67%) in the treated group and four second parity cows (29%) in the control group. Consequently, parity was kept as a fixed effect in all models to account for confounding by parity of the relationship between treatment and resting behavior. By keeping parity in each model, the reported effect of treatment on each outcome can then be interpreted as the effect of treatment on resting behavior if parity level had been held constant (i.e., if parity level was the same in treated and control cows). The linear mixed models were as follows:

$$RestBv_{ij} = \beta_0 + \beta_1 Tx_j + \beta_2 Herd_j + \beta_3 Parity_j + \beta_4 DIM_{ij} + \beta_5 Tx_j \times DIM_{ij} + v_{0j} + e_{0ij} \text{ [Equation 12]}$$

where $RestBv_{ij}$ is the predicted resting behavior (i.e., either daily duration of lying time, lying bout frequency, or mean duration of lying bouts), for the i^{th} day from the j^{th} cow; β_0 is the intercept; β_1 is the regression coefficient for treatment group; β_2 is the herd fixed effect included to account for clustering of cows by herd; β_3 is the effect of parity and is included strictly to account for confounding of the treatment effect by parity; β_4 is the DIM effect; β_5 is the

treatment × DIM interaction; and v_{0j} and e_{0ij} are the cow random intercept and measurement error term, respectively (all assumed to follow an approximately normal distribution).

Parity (categorized as second parity and third parity or greater) was tested as a potential effect modifier by adding the main term and an interaction term with treatment group in the models. Parity was retained as an effect modifier if the interaction term yielded a *P*-value < 0.20 on the *F* test. The interaction between parity and DIM was also tested and retained if the interaction term yielded a *P*-value < 0.20 on the *F* test. Residuals were visually examined for each model to evaluate normality using quantile-quantile plot and histogram of residuals. Assumption of homoscedasticity was assessed visually using plot of the residuals against predicted values.

Results

Data loggers were attached to a total of 38 cows, but six cows (four from control group and two from treatment group) were excluded due to abnormal data records indicating misplacement of the logger (n = 3), or due to sickness/death (n = 3). In the end, 32 cows (22 from herd A and 10 from herd B) had usable resting behavior data: 14 were from control group and 18 from treatment group. Daily data were missing for some cows due to logger failure, therefore, out of a potential number of 448 cow-day observations, there were 331 usable cow-day observations and a mean number of 10.3 d of observation per cow.

The average daily lying time was 11.0 ± 2.2 h/d, with an average frequency of 13.1 ± 6.4 bouts/d, and a mean lying bout duration of 56.9 ± 18.1 min/bout when considering only the 2-14 DIM period. Figures 21–23 illustrate distributions of non-adjusted least square means for lying time (h/d), frequency of lying bouts (bouts/d), and mean lying bout duration (min/bout), for control and treatment groups between six days before calving and up to 14 DIM.

The treatment-lying time relationship varied as function of DIM (Table XVIII; Figure 21). However, after adjusting for multiple comparisons, there were no significant differences between treatment groups for none of the DIM. Lying times were, therefore, comparable between treatment groups throughout the 2–14 DIM period. The effect of treatment on lying time was not modified by parity level (*P*-value: 0.77).

When investigating lying bouts frequency, the effect of treatment did not vary as a function of DIM (Table XIX; Figure 22), but it varied as a function of parity (*P*-value = 0.10; Table XIX). For second parity cows, we observed, in incompletely milked cows, 11.9 bouts/d (95% CI: 9.3, 14.4) compared to 9.2 bouts/d (95% CI: 4.4, 13.9) for control cows. For cows in third parity or greater, incompletely milked cows had 12.2 bouts/d (95% CI: 8.5, 15.9) compared to 15.4 bouts/d (95% CI: 12.7, 18.1) for cows in the control group. So treatment was associated with a higher number of lying bouts in second parity cows, while it was associated with a lower number of bouts in older cows.

Similar results were obtained for mean lying bout duration; the relationship between treatment and lying bout duration did not vary as function of DIM (Table XX; Figure 23), but varied as a function of parity (*P*-value = 0.10; Table XX). For second parity cows, we observed 57.8 min/bout (95% CI: 49.9, 65.6) in incompletely milked cows, compared to 66.7 min/bout (95% CI: 52.1, 81.4) for control cows. For cows in third parity or greater, incompletely milked cows had 60.9 min/bout (95% CI: 49.4, 72.4) compared to 51.8 min/bout (95% CI: 43.4, 60.2) for cows in the control group. So treatment was associated with shorter bouts in second parity cows, while it was associated with longer bouts in older cows.

Discussion

To our knowledge, this is the first study investigating the impact of an incomplete milking during the first five DIM on resting behavior. The mean total lying time for cows was within the range of previously reported studies (9.3–13.9 h/d, DeVries et al., 2011; 9.7–12.9 h/d, Westin et al., 2015). Number of lying bouts and mean lying bout duration were also in agreement with other studies (29-115 min/bout, Tucker et al., 2009; 6–20 bouts/d and 48-96 min/bout, Gomez and Cook, 2010). The population studied, therefore, appears to be comparable to that of other studies. In the current study, there were no differences in resting behaviors among groups for none of the DIM. So, in general, we could conclude that an incomplete milking during the early lactation does not lead to alteration of cows' resting behaviors.

Similarly to what was observed by Calderon and Cook (2011), lying time was decreased around calving, and then started increasing to reach a plateau around DIM six. Lying time would usually be maintained for the remainder of the lactation after DIM eight (Calderon and Cook,

2011). In the current study, cows from the incomplete milking group seemed to reach this level of lying time earlier than conventionally milked cows, which could be interpreted as a positive effect of the incomplete milking. These differences, however, were not statistically significant and could, therefore, result simply from random error.

Although lying time was not altered by the milking protocol used, lying patterns differed by parity level. In second parity cows, we observed higher number of bouts and bouts of shorter duration in incompletely milked cows compared to control cows, while in third parity cows, incomplete milking resulted in a lower number of bouts and in longer mean lying bouts compared to the control cows. Whether these observed statistical interactions are truly the result of an existing biological interaction will have to be confirmed in future research using a larger sample size (and/or fewer degrees of freedom in the model). Nevertheless, we could hypothesize that a cow with a high number of bouts of short duration may be experiencing some level of discomfort. In fact, in a study from Siivonen et al. (2011), cows with mastitis had lower lying times and a higher number of bouts of shorter duration per day. In that study, such a lying pattern was possibly caused by some level of discomfort due to inflammation of the udder. Therefore, our results suggest that an incomplete milking may not act in the same way for second compared to third parity cows and that it may be, somehow, slightly problematic for second parity cows and, possibly, slightly beneficial for third parity cows. These potential interpretations, however, must be considered cautiously.

The incomplete milking could also have altered cows' metabolic status, which would, in turn, alter their feeding behaviors, and, consequently, their resting behaviors. The observed change in behaviors cannot, therefore, be directly interpreted as a sign of pain or discomfort. For instance, Carbonneau et al. (2012) showed that the cow's negative energy balance could be improved by reducing milk output during the first days of the lactation. We may hypothesize that an improved energy balance may have resulted in a reduced nutrient demands of the incompletely milked cows, and thus, in an alteration of their feeding behaviors. Thus, incompletely milked cows would have a greater amount of time that can be dedicated to activities other than feeding. Indeed, several researchers showed that cows with higher milk production have different resting patterns, mainly shorter lying times per day, than cows with lower milk production (Fregonesi and Leaver, 2001; Løvendahl and Munksgaard, 2005; Deming

et al., 2013). This is probably a result of the higher energy requirements in cows that produce more milk, leading to an increased time standing while feeding at the feed bunk to meet those needs (Bewley et al., 2010; DeVries et al., 2011). Tucker et al. (2007) compared lying time from cows milked 1x/d (n = 20) and 2x/d (n = 40) from 52 to 55 DIM and found that cows milked 1x/d had a tendency to spend more time lying down (9.8 h/d) than cows milked 2x/d (8.3 h/d) in a 24 h basis. No differences in resting behavior were found in the four hours before morning milking in that study.

In future research, recording resting and feeding behaviors altogether will possibly help understanding the effect of the milking protocol on the complete activity patterns of dairy cows. Furthermore, resting behaviors during the four hours prior to milking could be specifically investigated, since milk accumulation is maximal during that period (Davis et al., 1998a). In the current study, it could not be investigated because the exact time a cow was milked (or time at which she left the pen) was not recorded. Finally, it would also be valuable, in future research, to capture information regarding time spent standing in the stall and social behaviors (Cook et al., 2008).

There was no significant effect of treatment on lying time. However, the effect of treatment on frequency of lying bouts and on mean lying bout duration varied by parity level. Whether the differences observed resulted from a biologic process (pain or discomfort due to the incomplete milking) or from random error will have to be determined by future research.

Ethics statement

The study protocol was accepted by the Animal Ethics Commit tee of the Université de Montréal (rech-1701).

Author contributions

CK: conception or design of the work, data collection, data analysis and interpretation, drafting the article, final approval of the version to be published. TD: data analysis and interpretation, critical revision of the article, final approval of the version to be published. J-PR,

JD, and SD: conception or design of the work, data analysis and interpretation, critical revision of the article, final approval of the version to be published.

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Table XVIII. Conditional association between predictors and daily duration of lying down (h/d) from 32 dairy cows (two commercial herds) enrolled in a randomized controlled trial; estimates were obtained using linear mixed regression models

Variable	Level	β	SE	95% CI	*P*-value[a]
Intercept		10.0	0.9	8.1, 11.9	
Treatment					0.75
	Control	Reference			
	Incomplete	0.6	0.9	–1.2, 2.4	
DIM					<0.01
	2	Reference			
	3	0.2	0.7	–1.1, 1.5	
	4	–1.0	0.6	–2.3, 0.2	
	5	–0.3	0.6	–1.5, 1.0	
	6	0.3	0.6	–1.0, 1.0	
	7	1.8	0.6	0.5, 3.0	
	8	1.4	0.6	0.1, 2.6	
	9	1.2	0.6	–0.0, 2.5	
	10	2.1	0.6	0.8, 3.3	
	11	1.1	0.6	–0.1, 2.4	
	12	1.1	0.6	–0.2, 2.3	
	13	1.0	0.6	–0.2, 2.2	
	14	1.4	0.6	0.2, 2.7	
Treatment × DIM					<0.01
	Incomplete×2	Reference			
	Incomplete×3	0.2	0.9	–1.5, 1.9	
	Incomplete×4	1.4	0.8	–0.2, 3.1	
	Incomplete×5	0.8	0.8	–0.9, 2.4	
	Incomplete×6	0.4	0.8	–1.2, 2.1	
	Incomplete×7	–1.3	0.8	–2.9, 0.3	
	Incomplete×8	–0.8	0.8	–2.5, 0.8	
	Incomplete×9	–0.6	0.9	–2.2, 1.1	
	Incomplete×10	–1.7	0.8	–3.3, 0.0	
	Incomplete×11	–0.2	0.8	–1.9, 1.4	
	Incomplete×12	–0.7	0.8	–2.3, 0.9	
	Incomplete×13	–0.5	0.8	–2.1, 1.2	
	Incomplete×14	–1.5	0.8	–3.1, 0.1	
Parity					0.49
	2	Reference			
	3	0.5	0.7	–0.9, 1.9	
Farm					0.74
	2	Reference			
	1	–0.2	0.7	–1.6, 1.1	
Cow level variance	---	3.0	---	---	---

[a] Joint *P*-value obtained using an *F* test
Coefficient (β)
Standard error of the mean (SE)

Confidence interval (CI)
Days in milk (DIM)

Table XIX. Conditional association between predictors and lying bout frequency (bouts / d) from 32 dairy cows (two commercial herds) enrolled in a randomized controlled trial; estimates were obtained using linear mixed regression models

Variable	Level	β	SE	95% CI	*P*-value[a]
Intercept		5.0	3.0	–1.2, 11.2	
Treatment					0.89
	Control	Reference			
	Incomplete	4.6	3.2	–1.7, 11.0	
DIM					<0.01
	2	Reference			
	3	0.6	2.2	–3.7, 4.9	
	4	2.1	2.1	–2.1, 6.2	
	5	0.8	2.1	–3.4, 5.0	
	6	1.5	2.1	–2.6, 5.6	
	7	3.6	2.0	–0.4, 7.6	
	8	2.1	2.1	–2.0, 6.3	
	9	5.0	2.1	0.8, 9.1	
	10	4.0	2.0	–0.0, 8.0	
	11	4.2	2.0	0.2, 8.2	
	12	5.3	2.1	1.2, 9.4	
	13	6.2	2.0	2.2, 10.2	
	14	8.9	2.0	4.9, 12.9	
Treatment × DIM					0.13
	Incomplete×2	Reference			
	Incomplete×3	–0.1	2.8	–5.7, 5.5	
	Incomplete×4	–0.4	2.8	–5.9, 5.1	
	Incomplete×5	0.6	2.8	–4.9, 6.0	
	Incomplete×6	–0.8	2.8	–6.2, 4.6	
	Incomplete×7	–2.1	2.7	–7.3, 3.2	
	Incomplete×8	0.2	2.8	–5.3, 5.6	
	Incomplete×9	–3.7	2.8	–9.3, 1.8	
	Incomplete×10	–1.2	2.7	–6.6, 4.2	
	Incomplete×11	–2.3	2.7	–7.7, 3.0	
	Incomplete×12	–4.2	2.7	–9.5, 1.2	
	Incomplete×13	–3.9	2.7	–9.4, 1.5	
	Incomplete×14	–7.2	2.7	–12.6, –1.8	
Parity					0.06
	2	Reference			
	≥ 3	6.3	2.8	0.9, 11.7	
Treatment × Parity					0.10
	Incomplete×2	Reference			
	Incomplete×≥3	–5.9	3.6	–12.9, 1.1	
Farm					0.39
	2	Referent			
	1	1.5	1.7	–1.9, 4.9	
Cow level variance	---	16.7	---	---	---

[a] Joint *P*-value obtained using an *F* test

Coefficient (β)
Standard error of the mean (SE)
Confidence interval (CI)

Table XX. Conditional association between predictors and mean lying bout duration (min/bout) from 32 dairy cows (two commercial herds) enrolled in a randomized controlled trial; estimates were obtained using linear mixed regression models

Variable	Level	B	SE	95% CI	*P*-value[a]
Intercept		79.3	9.1	60.7, 97.9	
Treatment					0.99
	Control	Reference			
	Incomplete	–16.8	9.6	–35.7, 2.1	
DIM					0.03
	2	Reference			
	3	0.2	0.7	–1.1, 1.5	
	4	–1.0	0.6	–2.3, 0.2	
	5	–0.3	0.6	–1.5, 1.0	
	6	0.3	0.6	–1.0, 1.0	
	7	1.8	0.6	0.5, 3.0	
	8	1.4	0.6	0.1, 2.6	
	9	1.2	0.6	–0.0, 2.5	
	10	2.1	0.6	0.8, 3.3	
	11	1.1	0.6	–0.1, 2.4	
	12	1.1	0.6	–0.2, 2.3	
	13	1.0	0.6	–0.2, 2.2	
	14	1.4	0.6	0.2, 2.7	
Treatment × DIM					0.55
	Incomplete×3	2.9	7.7	–12.3, 18.1	
	Incomplete×4	10.5	7.6	–4.4, 25.5	
	Incomplete×5	8.5	7.5	–6.3, 23.2	
	Incomplete×6	9.4	7.5	–5.4, 24.2	
	Incomplete×7	6.1	7.3	–8.3, 20.5	
	Incomplete×8	2.1	7.5	–12.7, 16.9	
	Incomplete×9	18.3	7.6	3.4, 33.3	
	Incomplete×10	5.6	7.4	–9.0, 20.2	
	Incomplete×11	12.3	7.4	–2.2, 26.8	
	Incomplete×12	11.0	7.4	–3.5, 25.6	
	Incomplete×13	4.7	7.4	–9.9, 19.4	
	Incomplete×14	10.5	7.4	–4.0, 25.0	
Parity					0.28
	2	Reference			
	≥ 3	–14.9	8.5	–31.6, 1.7	
Treatment × Parity					0.10
	Incomplete × ≥3	18.1	11.0	–3.6, 39.7	
Farm					0.50
	2	Reference			
	1	–3.6	5.9	–13.8, 9.3	
Cow level variance	---	166.55	---	---	---

[a] Joint *P*-value obtained using an *F* test
Coefficient (β)
Standard error of the mean (SE)
Confidence interval (CI)

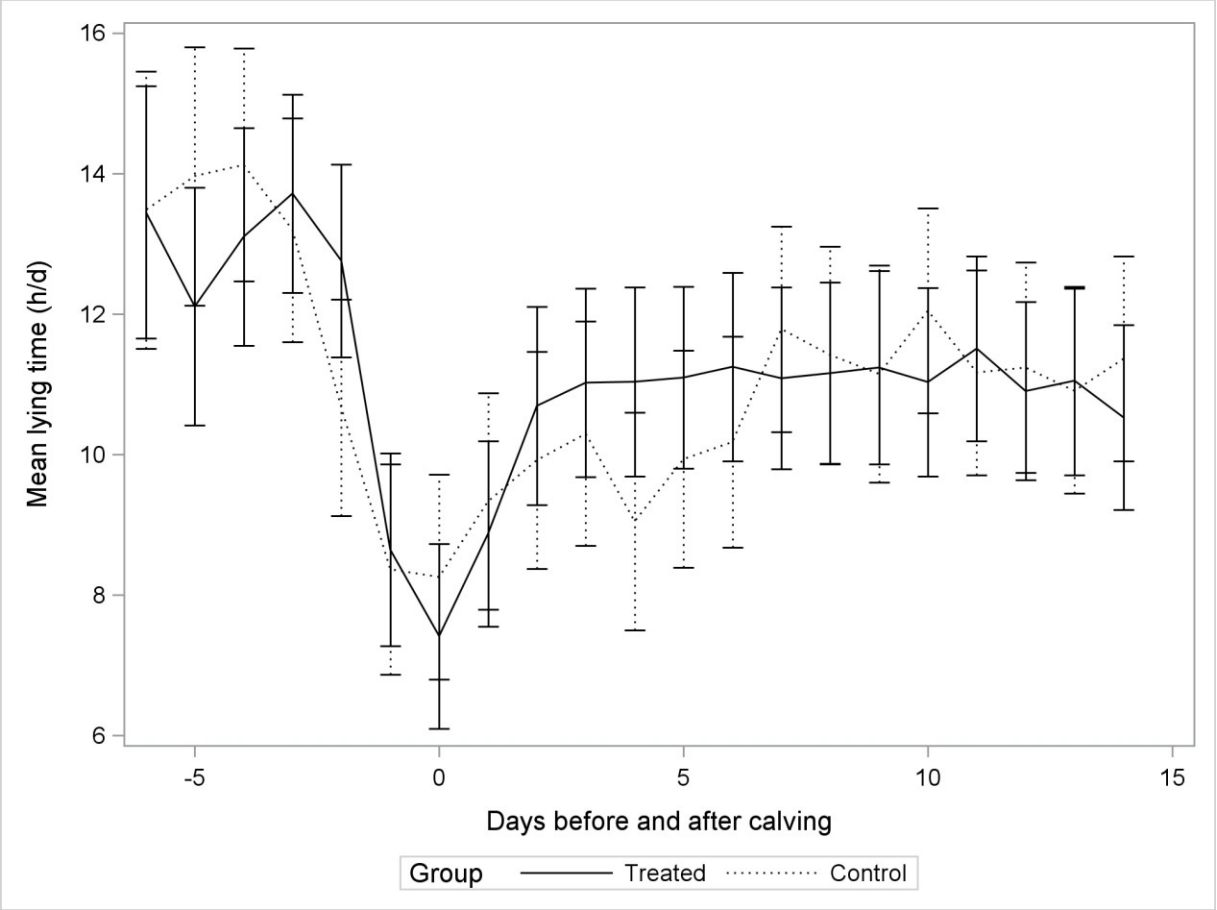

Figure 21. Non adjusted least square means for lying time (h/d) in incompletely milked (Treated) cows and control cows (Control) in a randomized controlled trial conducted on 32 dairy cows from two commercial herds

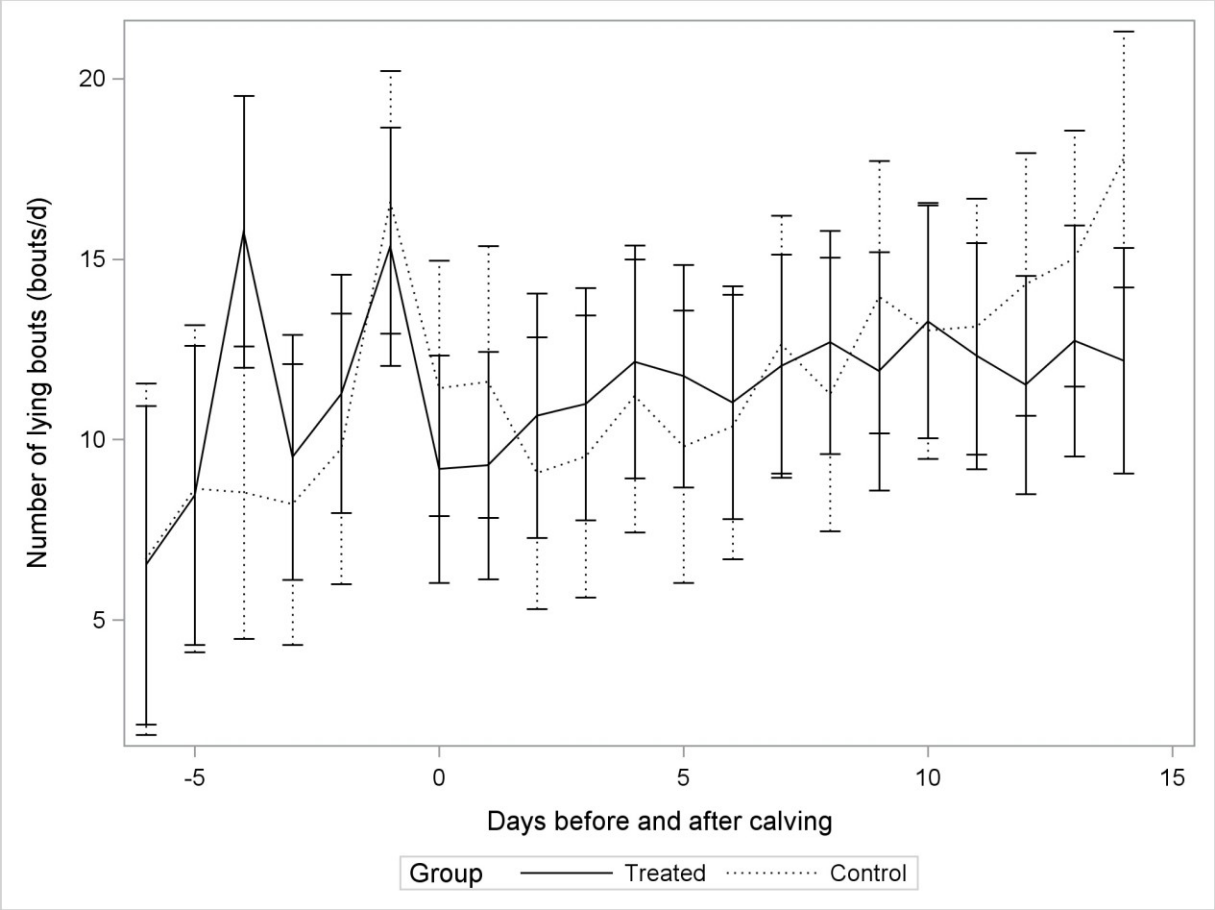

Figure 22. Non adjusted least square means for lying bout frequency (bouts/d) in incompletely milked (Treated) cows and control cows (Control) in a randomized controlled trial conducted on 32 dairy cows from two commercial herds

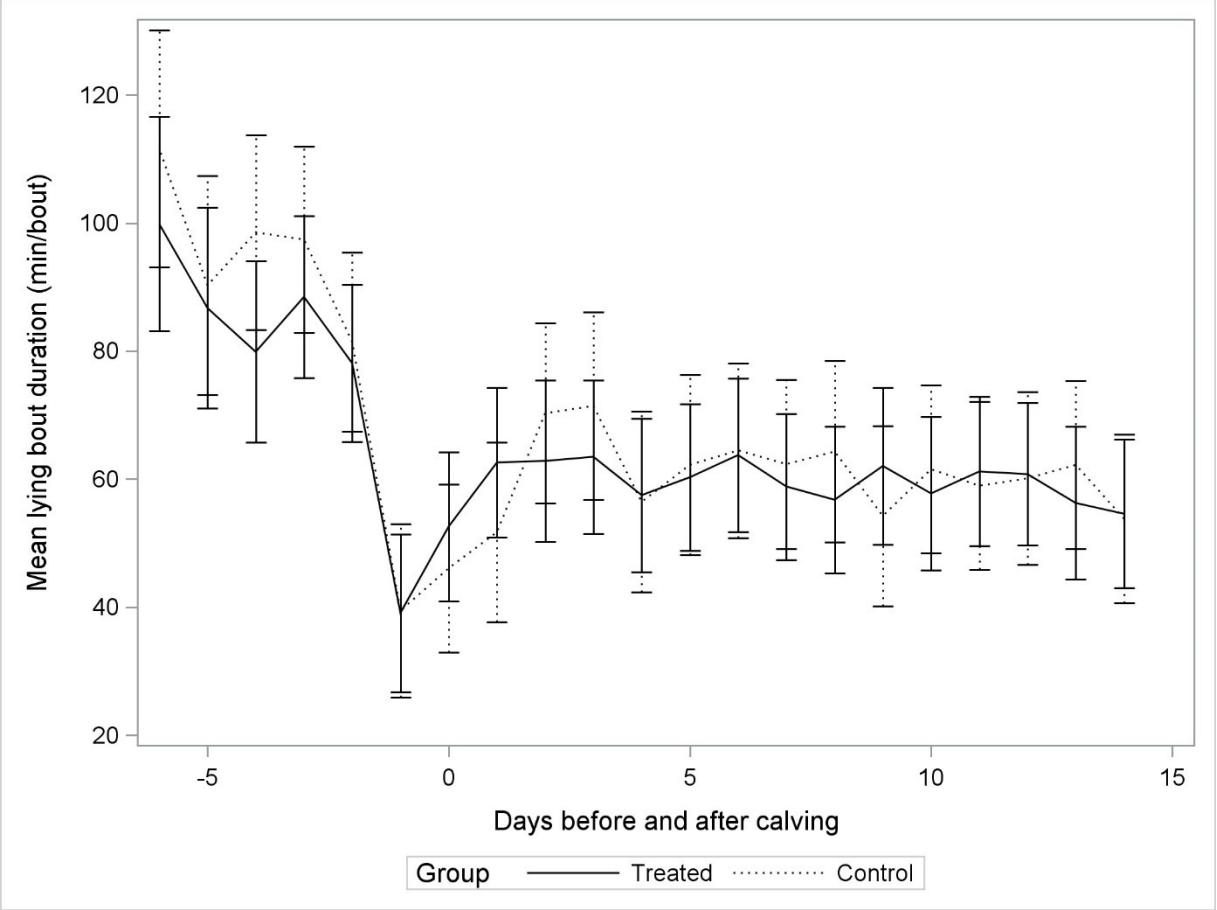

Figure 23. Non adjusted least square means for mean lying bout duration (min/bout) in incompletely milked (Treated) cows and control cows (Control) in a randomized controlled trial conducted on 32 dairy cows from two commercial herds

CHAPTER 8: GENERAL DISCUSSION

This thesis aimed to evaluate de usefulness of an incomplete milking during the first five DIM in improving the health and performance of dairy cows. Previously, Morin et al. (2018) showed that the incomplete milking led to lower blood BHBA concentrations and lower odds of hyperketonemia during 4-7 DIM. In second parity cows, a residual effect of the incomplete milking on odds of hyperketonemia was also observed during 8-17 DIM. Since hyperketonemia during 3-7 DIM leads to higher risk of disease, lower milk production and culling later in lactation (McArt et al., 2012), we hypothesized that the reduction in early hyperketonemia prevalence during that period would further improve dairy cows' performance and health.

Main findings

Our first objective was to investigate the effect of the incomplete milking protocol on culling hazard and on milk production and composition. First, we assessed whether treatment group affected culling hazard. This first step was important, as the association between treatment and milk production could be biased if a higher proportion of cows were excluded (i.e. culled) earlier in their lactation in one group. For example, if most of the animals were culled in the incompletely milked group (due to poor milk production), but only a small proportion in the conventionally milked group, we could obtain better milk yields among the incompletely milked cows because the poor performers would have been removed from the herd. Our results suggested no impact of the milking protocol on culling. In fact, the culling hazard was constant throughout the 450 DIM follow-up period and it did not differ between treatment groups. Since odds of culling within the first 60 DIM are increased in cows presenting hyperketonemia during the first WIM (BHB blood concentration ≥1.2 mmol/L; Roberts et al., 2012), we could have expected to observe a preventive effect of the incomplete milking on culling through its preventive effects on odds of hyperketonemia (Carbonneau et al., 2012; Morin et al., 2018). However, herds followed in this RCT had a relatively low prevalence of early lactation hyperketonemia (10.7% and 19.4% at 4-7 and 8-17 DIM, respectively; Morin et al., 2018) compared with research conducted on 4,242 DHIA herds in the same Canadian region (25.8 and 34.6% during second WIM for second and third parity cows, respectively; Santschi et al., 2016).

Therefore, culling reasons in the herds followed in the current study were possibly less influenced by hyperketonemia and, instead, driven by other internal (e.g. parity, health status) or external factors (e.g. need for producing more milk, milk/price; Bell et al., 2010).

Carbonneau et al. (2012) applied a similar protocol to the one used in the present research and did not observe any carry over effects on milk production. They did however observe lower fat and higher protein concentrations among incompletely milked cows, but only on second WIM (no effect from WIM 3-9). Based on those results, we hypothesized that no effect on milk yield would occur in commercial dairies settings, and we did not expect substantial differences in milk composition. Indeed, none of the variables studied (milk weight, fat and protein concentrations, and ECM) were affected by the treatment protocol, with the exception of ECM on WIM 38. This late effect was probably caused by the small number of observations at the end of the lactation, with greater influence of outliers. Since both milk removal and milking (or suckling) stimulus are needed for maintenance of milk secretion (Schmidt, 1971), the lack of carry-over effect on milk yield was probably a result of maintenance of milking frequency (Lacasse and Ollier, 2014) along with the short treatment duration. Other studies were conducted using a half-udder design to understand the effect of an incomplete milking on milk production. Wilde et al. (1989) showed that an incomplete milking in dairy goats (defined as a gland with around 100 mL left behind at the end of milking) affects local enzyme activity with a reduction of total protein synthesis and partial secretory cellular involution if it lasts 24 wk, but not if it lasts only 2 wk. Although ran in a different species and with a different study design, the results observed by Wilde et al. (1989) also support the findings of the current study, where a relatively short period of incomplete milking was used.

Since most management strategies that effectively improve energy balance, generally also lead to better immune defenses (Loiselle et al., 2009; Ster et al., 2012), we hypothesized that cows incompletely milked would have a better immune system, which would in turn decrease infectious diseases risk. We focused on udder and reproductive tract health. Our results suggested no impact of the treatment protocol on development of mastitis and reproductive tract diseases, but it substantially increased the chances of eliminating an existent IMI. The lack of positive impact on IMI prevention could have resulted from a lack of statistical power associated to the very low prevalence of disease (1.6%). The lack of positive impact of the incomplete

milking on reproductive tract diseases on WIM five could also be explained by a greater time lag between the actual treatment (1-5 DIM), its effects (lowering odds of hyperketonemia up to 17 DIM; Morin et al., 2018), and, finally, disease diagnosis around 30 DIM. That long period between treatment and disease diagnosis could have decreased our ability to detect positive effects of the treatment due to presence of other important disease determinants occurring in the course of those weeks. The same reasoning applies for clinical mastitis up to 90 DIM.

Finally, among the positive effects of the incomplete milking, we also expected better reproductive performances resulting from the improved energy balance. We measured the impact of an incomplete milking during the first five DIM on luteal activity at WIM five and/or seven and on pregnancy hazards. There was no difference between groups in terms of proportions of luteal activity (84 and 85% in conventionally and incompletely milked cows, respectively), and no treatment effect on odds of luteal activity. The relationship between conception hazard and treatment, on the other hand, was complex. The relationship was affected by time, parity and by the DIM at start of breeding period. Briefly, in second parity cows from herds with VWP < 55 DIM, 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 from herds with VWP ≥ 55 d, incomplete milking did not affect pregnancy hazard.

In Morin et al. (2018), we observed a similar effect of the treatment on odds of hyperketonemia 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 in the following days (8-17 DIM), also observed by Carbonneau et al. (2012), was only observed in second parity cows by Morin et al. (2018). Older cows are generally at higher risk of ketosis compared to younger cows (Duffield et al., 1997; Rasmussent et al., 1999; McArt et al., 2013), even though that risk difference is not always observed among herds (McArt et al., 2013). The higher risk for ketosis observed in older animals (third parity or greater) is probably related to their higher milk yield with consequent lower energy balance in early lactation (Coffey et al., 2004; Van Hoeij et al., 2017). Younger animals have, therefore, lower ketonemia, and greater insulin and IGF-I blood levels (Van Hoeij et al., 2017), which have positive influence

on reproductive performances (Lucy et al., 1992; Roche, 2006). Thus, there are several hypothesis for the lack of effect in older animals compared to second parity:

— The short-term prevention of hyperketonemia in older cows, without a residual effect, could have restricted any positive impact of incomplete milking on subsequent health events. Since cows of third parity or greater produce generally more milk than second parity cows, the treatment protocol might be more appropriate for the latter. It might be that the treatment protocol has to be longer than stipulated, for example, to produce an effect in older cows.

— The risk factors for hyperketonemia could differ between second parity animals and older animals, leading to less susceptibility among the latter to the incomplete milking strategy.

— Since second parity animals are still growing, their bodies might be more susceptible, or more "malleable" to an intervention than in an older animal.

— The only animals that reach their third lactation (not culled) might be metabolically superior, being at their maximum potential, without much space for improvement (i.e. culling bias).

The fact that incomplete milking led to a stronger effect in cows from herds with VWP < 55 DIM compared to cows from herds with VWP ≥ 55 DIM can be explained by the fact that the first group of animals start being bred at a time closer to the milking intervention (1-5 DIM).

We expected an effect of treatment on luteal activity due to its impact in preventing hyperketonemia (Morin et al., 2018), which is a risk factor for absence of luteal activity (Walsh et al., 2007a). However, literature is not always in agreement regarding hyperketonemia and luteal activity relationship (Abdelli et al., 2017). In our study, we verified the relationship between BHBA and luteal activity and we did not observe an association. It might be that the low prevalence of hyperketonemia (10% on WIM one) and of cows without luteal activity (16% at WIM five and seven) decreased the power of our study, making it hard to find an association between treatment and luteal activity. We could also argue that the small numerical difference in luteal activity between treatment groups (in the source population) is not clinically relevant.

Even though we did not find an association between the incomplete milking and luteal activity, we did find one between the treatment protocol and pregnancy hazard. In a recent meta-analysis conducted by Abdelli et al. (2017), BHBA and NEFA decreased significantly the pregnancy hazard (95% CI for the HR: 0.61, 0.97), but had no impact on luteal activity (95% CI for the OR: 0.83, 1.1). So it might be that the relationship is weaker between the energy balance and luteal activity than between the energy balance and pregnancy hazard, consequently affecting the way incomplete milking modulated these reproductive indicators. We know, through our results, that the differences found in pregnancy hazard were not caused by higher odds of endometritis in conventionally milked cows nor by lower luteal activity. It might be that pregnancy hazard is more influenced by the rapidity in resumption of ovarian activity (which was not evaluated in the current RCT) than with the odds of that activity at a specific point in time.

We hypothesized that reducing the volume of milk harvested through incomplete milking could be associated with a sustained udder distention, which could lead to increased udder sensitivity. However, we noticed that methodologies for evaluating changes in udder sensitivity were lacking. Handheld pressure algometers have been shown to be reliable for assessing sensitivity in humans (Potter et al. 2006) and in some domestic animals (e.g. dogs: Kaka et al., 2015; horses: Haussler and Erb, 2006; piglets: Janczak et al., 2012), but formal evaluation and validation of handheld algometers for quantifying MNT on the udder of dairy cows appears to be lacking. Therefore, we aimed to evaluate the usefulness of the algometer as an instrument to quantify MNT when applied to the udder. Our results showed that the algometer is simple to apply, moderately reliable, but the MNT appears to be influenced by many factors, including time of the day at which the measurement is taken, and cow characteristics such as parity. The algometer results seem to be variable and might measure concepts that are different from udder sensitivity. Therefore, we concluded that the use of the algometer to evaluate pain when applied to the udder of dairy cows should be considered cautiously or it should be further developed and therefore we did not use it to evaluate the impacts of an incomplete milking.

Animal behavior, on the other hand, is often used as a tool to assess animal welfare in various animal species including cows (Weary et al., 2006). Therefore, we decided to use it to investigate the impact of incomplete milking on udder pain. Our hypothesis was that if

incompletely milked cows experienced pain, they would have a change in lying behaviour compared to conventionally milked cows. When using DIM as a discrete variable, we did not find any differences in lying time among groups at any DIM. However, when running the same models but with DIM as a three-level variable (2-5 DIM, corresponding to treatment; 6-10 DIM, corresponding to the following four days after treatment; and 11-14 DIM), cows incompletely milked had longer lying time per day (95% CI: 10.5, 11.6 h/d) than cows conventionally milked (95% CI: 8.9, 10.3 h/d) during the treatment period (2-5 DIM). These latter analyses were not published in the article presented in Chapter 6. Nevertheless, I believe that these results describe well what was observed in Figure 21. Lying time was decreased around calving, and then started increasing to reach a plateau of around 11 h/d (Calderon and Cook, 2011), but cows from the incomplete milking group seemed to reach this plateau earlier than conventionally milked cows, which could be interpreted as a positive effect of the incomplete milking. Cows with udder pain would be expected to have shorter lying times. In opposition to our hypothesis, incompletely milked cows had the opposite behavior of what would be expected if they were in pain, particularly regarding lying time per day. However, in the published model, analyzed at day-level, these differences were not statistically significant. Lying bouts and mean lying bout duration differed significantly between treatment groups, but only by parity level. Second parity cows had higher number of bouts and shorter mean lying bout duration in incomplete vs. conventional milking, respectively. Cows of greater parities had the opposite results. Indicating that incomplete milking could be slightly disadvantageous for second parity cows. These potential interpretations, however, must be considered cautiously. Whether the differences observed resulted from a biologic process (discomfort or pain due to the incomplete milking) or from random error will have to be determined by future research.

Although no economical study was conducted within this RCT, the incomplete milking appears to be economically viable. Most farmers only add the milk to the bulk tank after 3-5 DIM since the legislation states that farmers cannot sell milk from animals < 3 DIM, or greater, if colostrum is still present. Another reason for milk rejection during the first days after calving is the presence of antibiotic residues originating from the dry-period therapy (i.e. intramammary administration of antibiotics at the end of lactation). Again, legislation does not allow selling milk containing any residues of drugs (CFIS, 1997, revised September 2015). Consequently, the

only milk losses effectively caused by the incomplete milking occur at DIM four and five, in most farms. These milk losses are of 11 to 23 kg per animal on DIM four and of 10 to 23 kg on DIM five (Figure 24). Since the cost for production of one kg of milk is CA$0.77 (CDC, 2017), that loss would be of CA$16 to 35 per animal. Then there might be the cost for buying a transparent milk recipient allowing for real-time measurement of the milk withdrawal (if not already available on farm). On the other hand, the economic gains associated to the incomplete milking appear to be numerous: decreased the odds of hyperketonemia; higher odds of IMI elimination; and a higher pregnancy hazard resulting in less days open. The cost of a case of hyperketonemia was estimated at US$256 in a multiparous cow (McArt et al., 2015), and the cost of subclinical mastitis was estimated at CA$370 per cow-year (Aghamohammadi et al., 2018). Moreover, Meadows et al. (2005) estimated the cost of a day open at US$0.44 to US$1.71 at 130-190 DIM. These numbers give an idea of the possible economic benefits associated to incomplete milking.

Finally, although the incomplete milking protocol seems to have several advantages, the incomplete milking protocol should only be seen as an alternative practice to deal with negative energy balance, but it cannot substitute an appropriate feeding system or a comfortable and clean environment for dairy cattle.

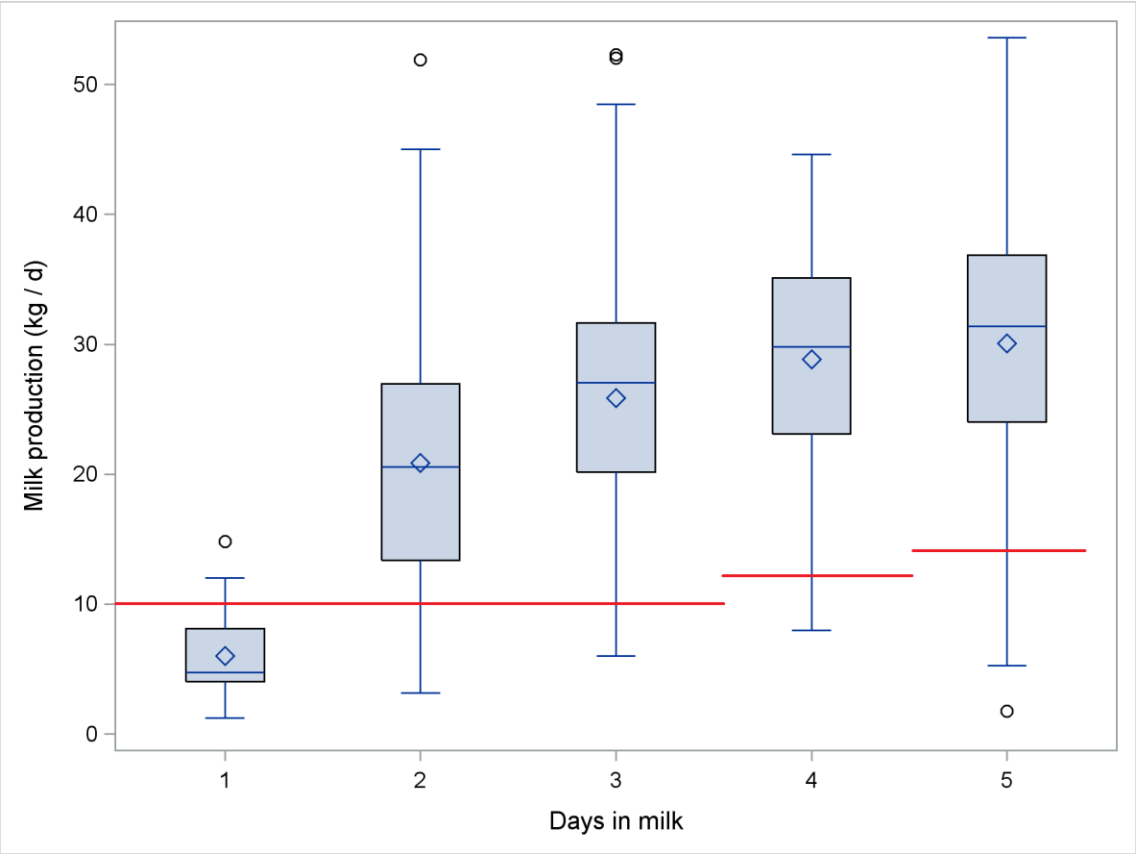

Figure 24. Distribution of milk production on days in milk one (n = 30), two (n = 107), three (n = 178), four (n = 236) and five (n = 286) in conventionally milked cows (i.e. complete milking) compared to incomplete milking (red line)

Study limitations

The RCT is the best study design to provide evidence of a causal effect. However, like other types of study, internal and external validity might be disrupted through presence of bias (Dohoo et al., 2009). Internal validity refers to being able to make unbiased inferences about the associations of interest in the source population. External validity (also called *generalizability* by some authors such as Rothman et al., 2008b), refers to being able to make unbiased inferences about the target population (Dohoo et al., 2009). As defined by (Dohoo, 2014), "the discrepancy between the true value and the estimated value is attributable to systematic error", also known as *bias*.

The articles that constitute this thesis were written on the basis of the reporting guidelines from Randomized Control Trials in Livestock and Food Safety statement (**REFLECT**; O'Connor et al., 2010). Another checklist adapted from REFLECT to report research experiments in dairy cattle reproduction was used for the corresponding manuscript on reproductive performances (Lean et al., 2016). We believe that the use of REFLECT was essential for planning the RCT and helped readers to assess the validity of our studies.

In the current RCT we used random numbers (of 0 and 1) generated by a computer, to generate the sequences for allocation of the multiparous Holstein cows enrolled in the study. This method allows to control for confounding bias, by distributing the confounders evenly across study groups, therefore improving the study accuracy (i.e. parameter estimated with little error, both random or systematic; Rothman et al., 2008b). However, there are several types of bias that can influence the composition and characteristics of study groups (Dohoo et al., 2009).

Selection bias happens when the study group is not representative of the source population (Dohoo, 2014), which was, in our case, multiparous Holstein cows from commercial dairies near the *Faculté de médecine vétérinaire* of the *Université de Montréal* (Montérégie, Quebec, Canada). This type of bias could have arisen through the convenient sampling of the farms. The average Quebec herd has 64 cows, while the 13 farms from the current study had a mean of 103 cows. Moreover, the farms selected appeared to have lower prevalence of diseases when compared to other studies conducted in this region (Morin et al., 2018; Chapter 5). Since the incomplete milking is a very innovative practice, that required committed and open minded producers, it is possible that those who accepted to participate in this RCT were farmers with management above average.

It is generally suggested that masking/blinding should be used to ensure validity. It refers to a process that attempts to keep the treatment group unknown. Blinding can be applied to several groups – the farmer, the technicians and veterinarians who measure the outcomes or the researchers analyzing the data. Blinding was not possible at the farmer or farm worker level, because they were the ones applying the intervention. That could have influenced the study validity if potential study subjects were selectively excluded from the study (selection bias) or if the producer behaved differently toward the treatment group or the control group, for example by increasing disease monitoring and recording in one of the groups but not in the other.

Technicians and veterinarians measuring the outcome were blinded to treatment group, but not those who analyzed the data. Since the outcomes were mostly very objective (rather than subjective), the validity was hardly biased by the lack of masking among technicians and researchers (Egglin and Horwitz, 1996; Downs and Black, 1998).

Other limitations of the current study were the fact that compliance could not be objectively quantified. In other words, we could not know if every farmer had performed the intervention exactly as requested (i.e. 10 L, 12 L and 14 L at one to three DIM, four DIM and five DIM, respectively). Through frequent communication with farmers, during on-farm visits, we do know that there were sometimes mistakes such as completely milking a cow from the incompletely milked group, by mistake, or withdrawing some extra milk than what was proposed. Data obtained through a questionnaire to the participating farmers, reveals that only four cows from the incomplete milking group received a treatment of less than five days (Morin et al., 2018), so it seems to have been an uncommon event. These mistakes, however, are likely to occur in routine practice if the incomplete milking during the first five DIM is used by other farmers in the future. In our analysis, we included cases of nonadherence, by using the *intent-to-treat* analysis, therefore eliminating respective confounding, the only drawback is that the effect of the intervention could have been slightly underestimated (Weiss, 2008).

As in most RCT, we had missing data at several steps of our analyses. Nevertheless, we believe that data were missing completely at random, with missing values randomly distributed throughout the dataset. Missing data might have depended on some predictors such as herd, or age of the cow, but since these were generally present in our models, that allows us to control for any confounding present. The statistical software used, conducted a complete-case analysis, allowing to discard any observations with missing values for the outcome variable or for the predictors. This type of analysis allows for unbiased estimates under the use of data missing completely at random (Rothman et al., 2008a; Dohoo et al., 2009).

Since there are no perfect tests for some of the outcomes studied (e.g. IMI, uterine health, luteal activity), information bias due to misclassification (categorical outcome) could have been present. In the infectious diseases manuscript we dealt with that problem by adjusting the OR for the Se and Sp of each test using Lash et al. (2009) approach. In general, the real effect of incomplete milking was higher than the one reported without corrections (i.e. for IMI

elimination). However, it was not possible to compute an adjusted odds ratio for all outcomes of interest. Another method that could have been used to control for misclassification bias, is the use of Bayesian latent class models, that would allow to obtain not only adjusted odds ratio, but also an adjusted 95% CI for the odds ratio (McInturff et al., 2004). Due to problems in convergence, these models were not used.

In the current RCT, most comparisons were superiority studies, for which the null hypothesis (H_0) was that there was no difference between the effects of an incomplete (μ_{TX}) or a conventional milking (μ_{CON}) in the population (H_0: $\mu_{TX} - \mu_{CON} = 0$), and for which the alternative hypothesis (H_1) was that there was one (H_1: $\mu_{TX} - \mu_{CON} \neq 0$). Conversely, the studies investigating the impact of incomplete milking on milk production and resting behaviors were non-inferiority studies. For those two studies, the hypothesis was that the incomplete milking was at least as good/effective as the conventional milking (i.e. non-inferior). Specifically, the null hypothesis was that the difference between groups was higher than a specified non-inferiority margin or margin of indifference (H_0: $\mu_{TX} - \mu_{CON} \leq -\delta$), while the alternative hypothesis was that the difference was smaller than that margin (H_1: $\mu_{TX} - \mu_{CON} > -\delta$, $\delta > 0$). The margin should be defined *a priori*, and the smaller it is, the larger the sample size needed (Freise et al., 2013). In the current RCT, since the sample size chosen was that for the outcome requiring the largest sample size (i.e. odds of hyperketonemia), we performed power calculations to estimate the differences in milk weight, milk composition, and in resting behaviors that could be detected using the available data (the margin of indifference was defined by the study sample). We could only subjectively determine if those differences are clinically relevant. For example in the milk production study, the sample size/power calculations allowed us to conclude that the minimal detectable difference between groups with the available sample size was 1.2 kg/d. Since we were not able to reject H_0, we could only conclude with 80% confidence that the difference between groups was probably lower than 1.2 kg/d.

Finally, this was a cow-level study, therefore we can only draw conclusions at the cow level, and we do believe that the incomplete milking performed according to our protocol, in other Holstein multiparous cows from well-managed commercial dairy farms would lead to similar conclusions to the ones obtained here. We should not assume that individual-level results (as those reported here) can be generalized to the herd-level, as we could be provoking an

atomistic fallacy (Dohoo et al., 2009). To know the impact of the incomplete milking at the herd level, a herd-level study would have to be conducted.

We believe that we minimized bias as much as possible, but we do not expect with this single RCT to have obtained results that are applicable to all multiparous Holstein cows and to all commercial dairies. Further well-design studies (experimental or observational) should be used in the future to confirm or reject some of the results observed here. This trial can only help individual farmers and veterinarians to guide their management strategies and their practice.

Suggestions for future research

Since this was the first RCT to investigate the impact of an incomplete milking on health and performance, under commercial conditions, there are, of course, many possibilities for future research. The first one is on the definition of the procedure. As mentioned above, the duration and the level of withdrawal were determined based on the previous study from Carbonneau et al. (2012) and based on the results obtained here, it appears, in fact, to be an effective management strategy to limit the negative energy balance and its consequences. Future research could perhaps explore the effects of determining the level of withdrawal based on the previous lactation. In some of the outcomes studied, second parity animals seem to benefit more from the incomplete milking than older animals. It might be that since older animals produce more milk (Coffey et al., 2004; Lee and Kim, 2006; Van Hoeij et al., 2017), the treatment protocol has to be longer than stipulated, for example, to produce an effect in cows from third parity or greater, compared to the original protocol. This hypothesis could be explored in the future.

If, in the future, a great number of farms end up adhering to the incomplete milking strategy during the first five DIM, herd-level studies could be conducted. For instance, a cross-sectional study (i.e. comparison between herds applying or not the milking protocol) or a RCT at herd level (i.e. allocation of treatment at the herd level) could be conducted to evaluate the association between the use of an incomplete milking protocol (exposure) and development of disease, or outcomes, at the herd level. Herd-level studies would have several advantages such as allowing to quantify the effects of the incomplete milking under different management practices (e.g. type of system, diet) and to study the possible modification of effect that these

herd-level variables might have on the relationship of interest (incomplete milking and various outcomes). These herd level studies would also allow to understand which herds would benefit the most from the incomplete milking protocol, and they would allow to clarify if the individual-level results obtained in this thesis also reproduce at the herd-level, or not.

Farms with AMS and those with modern automated milk parlors are probably the ones where an incomplete milking could more easily be putted into practice. For which the producer could select the animals that he/she thinks that could benefit from the incomplete milking and set the milking machine to perform the incomplete milking during the first five DIM. To set the milking protocol, the farmer could program the AMS to withdraw a specific amount of milk per milking or per day. Furthermore, the use of AMS allows for recording of valuable information that could increase accuracy, validity and power of future research. For instance, an RCT conducted in farms with AMS would allow for masking/blinding of the farmer, decreasing the chances of bias (higher accuracy and validity). Moreover, the AMS has the ability to withdraw a massive amount of information about the animal, such as daily milk yield, fat, and protein milk concentrations, along with variations at the quarter level or per milking, which could increase the power of the study, in detecting a difference in milk yield and composition between groups, if there is one.

There are also numerous outcomes that were not studied in the current RCT and that could possibly be explored in the future. For instance, the incomplete milking during the first five DIM might decrease the chances of subclinical hypocalcemia, by decreasing the quantity of calcium needed for milk production. The incomplete milking increased the odds of IMI elimination, and therefore it could possibly also increase the odds of cure from reproductive tract disease, which is yet to be explored. Regarding reproductive performance, no effects of the incomplete milking were observed on luteal activity on WIM five and seven. However, resumption of ovarian activity was not investigated and it could maybe beneficiate from treatment. The same could be expected on embryonic death, which appears to be higher in presence of severe negative energy balance (Wathes et al., 2003).

Based on the results obtained on Chapter 6, regarding the algometer validation, we concluded that the handheld pressure algometer methodology would have to be further developed for measuring changes in udder sensitivity in dairy cows. Some possibilities would

be modifying the area of skin contact or investigating the use of different types of probe heads. The other possibility would be to investigate the use of other types of nociceptive tests. For instance, Rasmussen et al. (2011) have used thermal nociceptive tests in cows following experimentally induced *Escherichia coli* mastitis. These tests have also been largely used in humans (Djouhri et al., 2006; Agostinho et al., 2009) and in other animals (rodents: Barrot, 2012; cat: Dixon et al., 2002; sheep: Musk et al., 2014), making them a potential candidate for evaluation of changes in sensitivity in the udder of cows.

The impacts of the incomplete milking on behavior could also be further explored. Specifically, resting behaviors during the four hours prior to milking could be specifically investigated, since milk accumulation is maximal during that period (Davis et al., 1998a). Information regarding time spent standing in the stall and social behaviors could also be of interest (Cook et al., 2008). Moreover, as mentioned above, incompletely milked cows appeared to be lying for longer time than conventionally milked cows, corresponding to an opposite behavior to what would be expected if they were in pain. This result might have been related to feeding behaviour, which is also often used as an indicator of discomfort and welfare (Weary et al., 2006; González et al., 2008). Results on feeding behavior could help understanding the effect of the milking protocol on the complete activity patterns of dairy cows.

Finally, the effects of the incomplete milking on antibiotic residues in early lactation was not explored. Dry cow therapy is a common practice to prevent mastitis. The presence of antibiotic in the udder decreases during the dry period, but residues can still be found in the first days after calving, which can be a public health issue and negatively influences cheese and yogurt production (Mitchell et al., 1998). Therefore, the legislation asks for the rejection of the milk (i.e. non addition to the bulk tank) while antibiotics are present. The number of days with antibiotic residues depends on the pharmacokinetics of the drug. According to Whittem et al. (2012), pharmacokinetics of drugs administered via intramammary infusion can be influenced by milking interval and by volume capacity. The longer the milking interval and/or the lower the volume capacity, the longer the time needed to eliminate the drug from the udder. Pharmacokinetics is quite complex and depends on the drug concentration on both cisternal and alveolar compartments. Incompletely milking dairy cows during the first five DIM might influence these concentrations. During the RCT presented in this thesis, tests for detection of

antibiotic residues in the bulk tank were performed on each milk pick up as part of routine on farm procedures, and no positive results were observed. It might be that the incomplete milking procedure does not lead to antibiotic residues, or maybe there was milk dilution of the positive tests. Therefore, before letting farmers fully adhere to the incomplete milking protocol, the impacts of this strategy on antibiotic residues in milk should be investigated. Similarly to the RCT presented in this document, cows could be randomized within herd to an incomplete or to a conventional milking, and then, following calving, cow-level milk samples could be collected 1x/d for 10 d and analyzed for antimicrobial residues using a broad spectrum microbial inhibition test such as Delvotest®. Number of milkings or days presenting antibiotic residue-positive could then be compared between groups.

CHAPTER 9: GENERAL CONCLUSIONS

This thesis has allowed to describe the impacts of incomplete milking during the first five days in milk on performance and health of dairy cows. The main conclusions are the following:

— The milking protocol did not lead to significant differences in culling hazard, and it had negligible negative effects on cow productivity (since a lower ECM yield was only observed at WIM 38 in incompletely milked cows);

— The milking protocol did not affect development of new IMI, clinical mastitis incidence, or reproductive tract, but it increased the odds of elimination of existing IMI;

— The milking protocol had no effect on luteal activity and it had a positive impact on pregnancy hazard in cows in second parity from herds with VWP < 55 DIM;

— The use of the pressure algometer to quantify MNT in the udder of dairy cows should be considered cautiously, as although the instrument had moderate test-retest and inter-rater reliability, it was influenced by various extraneous covariates (e.g. time of the day, parity of the cow);

— The milking protocol had little effect on lying time. Our results regarding frequency of lying bouts and mean lying bout duration suggest that the milking protocol may be slightly problematic for second parity cows and, possibly, slightly beneficial for older cows.

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