Evaluation of various strategies to accelerate mammary gland involution at drying-off and study of the prolactin secretion regulation by glucocorticoids

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Abstract

The transitions between lactation and dry periods constitute a very critical phase in the reproduction cycle of dairy cows. After cessation of milking, the mammary gland is highly susceptible to new intra-mammary infections because of udder engorgement and milk leakage as well as a weakened immune system. In the absence of teat stimulation and udder emptying, the mammary gland regresses during a remodeling process known as involution. This physiological event involves a decrease in milk component synthesis and secretion by mammary epithelial cells, renewing of a portion of the mammary epithelium, activation of different proteases and an increase in the permeability of tight junctions linking epithelial cells together. However, exact elements triggering involution are not fully elucidated yet. Once mammary gland involution is completed, the udder is more resistant against bacterial infections. In addition, occurrence of involution is facilitated by the decrease in basal prolactin concentrations and in prolactin released during milking towards the end of lactation. Consequently, a strategy to improve cow's udder health would be to hasten the involution process by either increasing tight junction permeability or by decreasing the prolactinemic signal before drying-off. Another strategy to improve udder health around drying-off would be to increase lactation persistency by maintaining the prolactinemic signal over the course of lactation. In our first study, mammary gland involution was accelerated through the intra-mammary infusion of casein hydrolysates. These products of the natural degradation of caseins by plasmin during milk accumulation in the udder constitute a good candidate for triggering involution. In the second experiment, we shortened photoperiods or treated cows with melatonin aiming to reduce both prolactin concentrations and milk production before drying-off and to hasten involution. Only short day photoperiods slightly reduced milk production and tended to decrease milking-induced prolactin release but neither treatment affected the speed of the involution process. In the third experiment, the hypothesis that glucocorticoids participate in the regulation of the milking-induced prolactin release was tested in cows in mid-lactation. Glucocorticoid administration led to a depression in milk yield that was associated with a decrease in basal prolactin concentrations in both serum and milk and with a reduction in the milking-induced prolactin release. In summary, prolactin secretion could be maintained by glucocorticoid inhibitors during lactation and intra-mammary infusions of casein hydrolysates could be used to accelerate involution.

Résumé

Les transitions entre periodes de lactation et de tarissement constituent des phases critiques dans le cycle reproducteur de la vache laitière. Après l'arrêt de la traite, la glande mammaire est fortement susceptible de contracter de nouvelles infections intra-mammaires en raison d'un engorgement du pis causant des fuites de lait et d'un affaiblissement du système immunitaire. En absence de stimulation du trayon et de vidange du lait, la glande mammaire régresse au cours d'un processus de renouvellement appelé involution. Ce processus physiologique engendre une diminution de la synthèse et de la sécretion des constituants du lait par les cellules épithéliales mammaires, un renouvellement d'une portion de l'épithélium mammaire, l'activation de différentes protéases et l'augmentation de la perméabilité des jonctions serrées liant les cellules épithéliales entre elles. Cependant, les éléments précis déclencheurs de l'involution ne sont pas encore complètement déterminés. Une fois l'involution terminée, la glande mammaire devient plus résistante face aux infections bactériennes. D'autre part, le déroulement de l'involution est facilité par la baisse des concentrations basales de prolactine et de la quantité de prolactine sécrétée lors de la traite à l'approche de la fin de la lactation. Par conséquent, une bonne stratégie pour améliorer la santé des vaches serait d'accélérer le processus d'involution soit en administrant des traitements susceptibles d'augmenter la perméabilité des jonctions serrées, soit en réduisant le signal prolactinémique avant tarissement. Une autre stratégie pour éviter les problèmes infectieux lors du tarissement serait d'accroître la persistence de la lactation en maintenant le signal prolactinémique au cours de la lactation. Dans notre première étude, l'involution de la glande mammaire a été accélérée grâce à des infusions intra-mammaires d'hydrolysats de caséine. Ces produits de la dégradation naturelle des caséines par la plasmine lors de l'accumulation du lait dans le pis constituent de bons candidats comme éléments déclencheurs de l'involution. Dans la seconde experience, nous avons réduit la photopériode ou traité les vaches avec de la mélatonine afin de diminuer à la fois les concentrations de prolactine et la production de lait avant le tarissement dans le but d'accélérer l'involution. Seule l'exposition à une photopériode de jours courts a légèrement réduit la production de lait et a eu une tendance à décroître la libération de prolactine induite par la traite, mais aucun traitement n'a affecté la vitesse de l'involution. Lors de la troisième expérience, l'hypothèse que les glucocorticoides participent à réguler la libération de prolactine induite par la traite a été testée chez des vaches en milieu de lactation. L'administration de glucocorticoides a engendré une baisse de la production laitière, celle-ci ayant été associée à une diminution des

concentrations basales de prolactine dans le sérum et le lait ainsi qu'à une réduction de la sécretion de prolactine lors de la traite. En conclusion, la secretion de prolactine pourrait être maintenue par des inhibiteurs des glucocorticoides lors de la lactation et des infusions intra-mammaires d'hydrolysats de caséine pourraient être utilisées pour accélérer l'involution.

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Contributions of authors

Four co-authored manuscripts are included in this thesis.

Chapter 2: B. Ponchon and X. Zhao.

B. Ponchon wrote a review manuscript. X. Zhao reviewed and edited the manuscript.

Chapter 3: B. Ponchon, P. Lacasse, N. Silanikove, S. Ollier, and X. Zhao.

P. Lacasse and X. Zhao designed the study. N. Silanikove prepared one of the treatments and reviewed the manuscript. B. Ponchon realized the experiment, analyzed the samples and interpreted the results. S. Ollier helped to analyze the samples and reviewed the manuscript. P. Lacasse carriet out the statistical analyses. X. Zhao helped to realize the experiment. B. Ponchon wrote the manuscript. P. Lacasse and X. Zhao reviewed and edited the manuscript. Published in Journal of Dairy Science. 2014. Effects of intra-mammary infusions of casein hydrolysate, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA) and lactose at drying-off on mammary gland involution. 97: 779-788.

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P. Lacasse, X. Zhao and B. Ponchon designed the study. B. Ponchon realized the experiment, analyzed the samples and interpreted the results. S. Ollier helped to analyze the samples. P. Lacasse carried out the statistical analyses. X. Zhao helped to collect the samples. B. Ponchon wrote the manuscript. P. Lacasse and X. Zhao reviewed and edited the manuscript.

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P. Lacasse designed the experiment. B. Ponchon realized the experiment, analyzed the samples and interpreted the results. P. Lacasse helped to realize the experiment and performed the statistical analyses. S. Ollier helped to realize the experiment and to analyze the samples. B. Ponchon wrote the manuscript. X. Zhao and P. Lacasse reviewed and edited the manuscript.

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List of abbreviations

ACTH	adrenocorticotropic hormone
BCS	body condition score
BSA	bovine serum albumin
CNH	casein hydrolysate
CUD	close-up diet
DEXA	dexamethasone
DOMP	domperidone
ECM	energy corrected milk
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
FCM	fat corrected milk
FOD	far-off diet
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding proteins
JAK	janus kinase
LDPP	long day photoperiod
MAC-T	mammary alveolar cell-T
MEL	melatonin
METY	metyrapone

MMP	metalloproteinase
NEFA	non-esterified fatty acid
OM	once-daily milking
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PRL-R	prolactin receptor
pSTAT	phosphorylated signal transducer and activator of transcription
SCC	somatic cell count
SDPP	short day photoperiod
STAT	signal transducer and activator of transcription
TEER	transepithelial electrical resistance
TM	twice-daily milking
tPA	tissue-type plasminogen activator
TIMP	tissue inhibitor of metalloproteinase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
WAP	whey acidic protein

Chapter 1 General introduction

Canadian dairy industry significantly contributes to Canadian economy and ranks third in the Canadian agricultural sector following grains and oil seeds, and red meats. In 2014, dairy production in Canada generated total net farm receipts of more than $6 billion and sales of $17.3 billion, representing 16.4% of the Canadian food and beverage sector (Canadian Dairy Information Centre, 2015). As of January 1st 2014, the Canadian dairy cattle population totalled 1.4 million heads. Among them, 959,300 dairy cows produced a total of more than 78 million hectoliters of milk. Canada counts a total of 11,962 dairy farms with an average of 80 dairy cows per farm. Quebec possesses the largest number of dairy cows in Canada, with a total of 354,800 heads distributed on 5,894 dairy farms.

The dairy industry in Canada has changed dramatically over time. Genetic improvement and better nutrition have increased milk production per cow. The average milk production of Canadian Holstein cows in 2014 was 10,102 kg with 3.87% fat and 3.19% protein (Canadian Dairy Information Centre, 2015). Currently, some herds of Holstein cows can produce over 13000 kg of milk for a 305-day lactation period and it is quite common that milk yield reaches 50kg/day during early lactation. At the end of lactation, which lasts on average 357 days in Quebec, milking is stopped and the cow enters into a dry period. A dry period of 60 days is commonly practised to allow the mammary gland to rest, to renew its cell population by removing senescent cells and to prepare for the next lactation. In Quebec, the average length of the dry period was 63 days in 2014.

After the cessation of milking, the mammary gland continues to synthesize milk. This causes an engorgement of the mammary gland and induces milk to leak through the teat. Therefore, entry of microorganisms is facilitated and the risk of new intra-mammary infections, particularly mastitis, is elevated during the transition from a lactating state to a non-lactating state. Moreover, the immune system in the mammary gland is weakened at this time; for instance, the immune cells present in the mammary tissue are more devoted to remove casein micelles, lipid droplets and cellular debris due to the arrest of lactation than eliminating deleterious micro-organisms. Consequently, susceptibility to new intra-mammary infections is high during the early dry period.

Nowadays, it is not rare to dry-off high-producing cows which still secrete up to 28 kg of milk per day (Dingwell et al., 2001). Rajala-Schultz et al. (2005) have established that the

risk of intra-mammary infection at calving increases by 77% for every 5 kg of milk produced above 12.5 kg/d at the time when milking is stopped. Other works reported that uninfected quarters of cows producing more than 115 kg of milk during the last week of lactation (16.4 kg/d) were 7 times more susceptible to be infected at calving than uninfected quarters from cows producing less than 75 kg of milk (10.7 kg/d; Newman et al., 2009). Therefore, strategies which would decrease fluid accumulation and increase natural protective factors during early involution may improve mammary gland resistance against new intra-mammary infections during this critical period.

The mammary gland undergoes active involution after cessation of milking. Mammary gland involution is characterized by a decrease in milk protein synthesis and secretion, a replacement of a portion of mammary epithelial cells by apoptosis and proliferation, and a structural remodelling of the gland, which requires the activation of different proteases and the inactivation of their inhibitors. When involution is completed, the mammary gland is more resistant to new intra-mammary infections and the risk of mastitis is reduced. It is therefore of interest to find strategies that can accelerate the involution process after drying-off in order to improve udder health.

Dairy cows also have to face a nutritional challenge during transitions from lactation to dry period and then, from dry period to the following lactation. The 60 days-dry period implies to change diets for a cow at least three times during a short time period (a late lactation diet to a far-off dry (FOD) diet , a FOD diet to a close-up dry (CUD) diet and a CUD diet to an early lactation diet). The diet change is particularly problematic after calving, when a cow shifts from a non-lactating state to a high-producing state. Cows are generally in negative energy balance because of the high demand for energy during early lactation. They mobilize their energey reserves for lactation and less energy is devoted to the immune system which is therefore weakened during this period. Moreover, the hormonal status of periparturient cows does not favour the immune system. The natural increases in estrogens and cortisol close to parturition could be partly responsible for the immunosuppression observed at calving (Goff and Horst, 1997). Furthermore, most of the metabolic disorders, such as ketosis, milk fever, retained placenta or displacement of the abomasum, occur during the first 2 weeks of lactation (Goff and Horst, 1997).

Consequently, it would be beneficial for cows to be able to hasten the mammary gland involution process. Two strategies can be potentially used for this purpose. First, acceleration

of mammary gland involution could be achieved by utilization of agents stimulating the involution process itself. For example, this could be done by intra-mammary infusions through the teat canal of the cow of casein hydrolysates, EGTA or lactose iso-osmotic solutions. The second strategy is to decrease milk secretion before drying-off by reducing or suppressing the lactogenic signal driven by prolactin. It has been shown that inhibition of prolactin secretion through administration of quinagolide, a selective dopamine-2 receptor agonist with a long-lasting prolactin lowering activity, leads to a decrease in milk production in early and late lactating cows (Lacasse et al., 2011; Ollier et al., 2013). Alternatively, a natural way to act on prolactin release is to modulate photoperiods, as melatonin which mediates the effects of photoperiod influences prolactin secretion. Indeed, it has been shown that this hormone, produced by the pineal gland during the night, reduces blood prolactin concentrations and milk production in dairy cows (Auldist et al, 2007).

Finally, another approach to decrease the nutritional stress and the occurrence of health disorders during the transitions between lactation and dry period would be to increase lactation persistency. This would lengthen lactation and reduce the number of these transitions. However, mechanisms underlying the gradual decrease in milk production over the course of lactation are not fully elucidated yet. In both goats and cows, during the declining phase of the lactation curve after the peak of lactation, the decrease in milk production is first attributed to a reduction in mammary epithelial cell number and then to a reduction in mammary epithelial cell activity during late lactation (Knight and Peaker, 1984; Wilde et al., 1986; Capuco et al., 2001; Wareski et al., 2001). The factors triggering this decline are not fully elucidated.

This thesis project aimed to develop scientific approaches and techniques to improve the health and longevity of modern cows by accelerating the involution process of the mammary gland in dairy cows at drying-off.

1.1 Hypotheses

- The intra-mammary administration of treatments known for affecting tight junction permeability would accelerate the involution process.

- The inhibition of the lactogenic signal driven by prolactin by decreasing photoperiod or by administering melatonin would reduce milk production at the time of cessation of milking and accelerate mammary gland involution.
- Glucocorticoids would participate in the regulation of the milking-induced prolactin release.

1.2 Objectives

- To assess the relative speed of mammary gland involution by measuring involution markers after intra-mammary administration of selected treatments.
- To evaluate the effects of photoperiod modulation on the involution process.
- To study the effect of glucocorticoids on prolactin synthesis and/or secretion to elucidate factors responsible for the decline in the prolactinemic signal during the course of lactation.

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Chapter 2 Literature review

2.1 What is mammary gland involution?

Mammary gland involution is defined as a process through which the gland returns to a non-lactating state. There are three types of involution. First, gradual involution is the regression of the lactation function during progression of a normal lactation; second, senile involution occurs at the end of the reproductive life of the mammal; third, active involution is the process which takes place in the mammary gland after cessation of milking at drying off (Hurley, 1989).

A dry period can be typically divided into three stages. The first stage starts after the final milk removal and corresponds to the moment of active involution when the mammary gland undergoes a series of remodelling processes; the second stage is a stationary period when the mammary gland is in a resting state; the third stage is a period of preparation for the next lactation of approximately 14 days prior to parturition when the mammary gland undergoes redevelopment and begins colostrum formation (Hurley, 1989; Oliver and Sordillo, 1989). Active involution is fully initiated within 2 days after cessation of milking and seems to last approximately 21 days in the cow (Hurley, 1989). It is suggested that in goats, mammary gland involution is triggered by the increase in intra-mammary pressure whereas in rodents, withdrawal of the suckling stimulus seems to be more important (Fleet and Peaker, 1978). In cows, the event triggering the involution process is still debatable. A relevant hypothesis is that milk stasis occurring after cessation of milking could lead to the accumulation in the gland of factors inhibiting milk synthesis and secretion by decreasing mammary epithelial cell activity and triggering involution. It has been reported that a protein of 10-30 KDa present in the whey fraction of goat milk reduced lactose and casein synthesis in rabbit mammary explants (Wilde et al., 1987), decreased casein synthesis and fatty acid synthetase activity in mouse mammary epithelial cells (Wilde et al., 1991), increased intracellular degradation of caseins and reduced lactose secretion in goat mammary explants (Wilde et al., 1989). This protein was named feedback inhibitor of lactation (**FIL**). In vivo, intra-mammary infusions of FIL depressed milk secretion in rabbits (Wilde et al., 1987) and goats (Wilde et al., 1988; Peaker and Wilde, 1996). However, to our knowledge, FIL has not been identified in cows and the mechanisms triggering involution is still to be determined.

Active involution is a complex event transforming the structure and the composition of the mammary gland after a state of intense milk production during the lactating period. This remodelling process is characterized by numerous cellular and molecular changes leading to the renewing of the mammary epithelial cell population. These changes involve a decrease in milk component synthesis, an increase in secretory alveolar cell apoptosis, an increase in tight junction permeability, activation of several proteases in combination with inactivation of their inhibitors and the loss of cell-cell and cell-extracellular matrix contacts. These different physiological events in regards with involution will be discussed next.

2.1.1 Mammary gland involution induces morphological changes in the mammary epithelial cell and in the mammary tissue

In rodents, mammary gland involution is characterized by a reduction in milk component synthesis (Quarrie et al., 1996), by a marked decrease in the size and the number of ducts and by a drastic reduction in epithelial cell numbers due to apoptosis two days after weaning (Walker et al., 1989; Guenette et al., 1994, Quarrie et al., 1996). Indeed, the secretory tissue regresses dramatically during involution and returns to a state close to that of the virgin mammary gland. Even if a small number of epithelial cells are directly shed into the alveolar lumen, the reduction in mammary epithelial cell number during involution in rodents is due to extensive apoptosis (Walker et al., 1989). The apoptotic epithelial cells are mainly phagocytosed by macrophages in which they are enzymatically degraded in heterolysosomes (Walker et al., 1989). The number of macrophages is effectively enhanced in the mammary tissue during this period to support the elimination of the apoptotic cells (Walker et al., 1989). A small proportion of apoptotic epithelial cells are also phagocytosed by other epithelial cells (Walker et al., 1989). This is why there is an increase in the activity of lysosomal enzymes, such as cathepsin D, acid phosphatase, aryl sulphatase and β–glucuronidase occurring in both epithelial cells and macrophages during early involution in the rat (Helminen et al., 1968; Helminen and Ericsson, 1970). During the first 3 days of involution, apoptosis of mammary epithelial cells increases dramatically in both mice and rats (Walker et al., 1989; Guenette et al., 1994; Quarrie et al., 1996). This intense phase of apoptosis of the mammary epithelium is considered as the first step of active involution in rodents (Lund et al., 1996). Three to four days after weaning, the second step of active involution is triggered. This phase is irreversible and involves an intense tissue remodeling through the action of different proteases and

macrophages. Another feature of mammary gland involution in rodents is the increase in the appearance of large cytoplasmic vacuoles and vesicles containing protein globules and lipids (Walker et al., 1989; Guenette et al., 1994). This is due to decrease in milk component secretion leading to their accumulation in the secretory epithelial cell.

In ruminants and particularly in cows, the lobulo-alveolar structure is maintained during involution (Akers et al., 1990; Capuco and Akers, 1999) and the mammary gland does not regress to the same extent as it can be seen in rodents. There is no apparent loss of mammary cells during this remodeling process (Capuco and Akers, 1999). This difference relative to rodents may be partly explained by the fact that dairy cows are usually pregnant during the time of drying-off, thus during the time of involution. Therefore, the hormonal and local signals acting on mammary gland development during pregnancy may counteract those acting during involution in the cow. Indeed, it has been shown in rodents that the size and the integrity of alveoli were maintained after 3 d of involution in pregnant mice and that concomitant pregnancy decreased the apoptosis rate and increased the proliferation rate of mammary epithelial cells (Capuco et al., 2002). However, it has been shown that even in non-pregnant cows, the number of apoptotic bodies was only slightly increased during involution compared to the massive cell death occurring in rodent involution, suggesting that there is a real species difference between rodents and ruminants.

In ruminants, during lactation and the first hours of milk accumulation, the mammary epithelium is constituted by a monolayer of cuboidal shaped mammary epithelial cells, well polarized, surrounded by myoepithelial cells and circumscribed by a basement membrane (Holst et al., 1987; Singh et al., 2005). The area occupied by the alveolar lumen in the secretory tissue is predominant, the stromal area is at its minimum and the epithelial area is at its maximum (Holst et al., 1987; Singh et al., 2005; Colitti and Farinacci, 2009). The alveoli are uniform, contain a large proportion of epithelial cells, and are distended and fulfilled with milk (Wilde et al., 1997; Singh et al., 2005). The epithelial cells contain numerous small secretory vesicles and fat droplets, an extensive rough endoplasmic reticulum, a well-developed Golgi apparatus and many mitochondria (Holst et al., 1987; Singh et al., 2005). They also contain many small vacuoles in their apical half and the nucleus is centrally or basally located (Holst et al., 1987; Colitti and Farinacci, 2009). The microtubules are significantly more located in the apical side of the epithelial cells and are oriented perpendicularly to the cell apex (Nickerson et al., 1982), indicative of their roles in the process of milk component secretion.

Once milking is stopped, numerous modifications in the composition of the mammary tissue and in the ultrastructure of the epithelial cells occur. These modifications reflect the changes in the secretory activity of the mammary gland. Globally, the number of organelles involved in milk component synthesis and secretion and the number of secretory vesicles decline abruptly, indicative of the decrease in mammary epithelial cell activities. In bovine, two days after cessation of milking, the rough endoplasmic reticulum is intact but the number of mitochondria is reduced and the Golgi apparatus is less detectable than during lactation (Holst et al., 1987). Large vacuoles appear within mammary epithelial cells and their number increases as involution advances, leading to a condensation of the other organelles in the intracellular space (Holst et al., 1987). These vacuoles contain casein micelles or lipids and some of them arise from the coalescence of both lipid droplets and secretory vesicles (Holst et al., 1987). Casein micelles and lipid droplets accumulate in the cell because the fusion of the secretory vesicles to the apical membrane is altered. This is due to impairment of the cytoskeleton function, and particularly that of microtubules, highly involved in the secretion process. Nickerson et al (1982) showed in cows that mammary epithelial cells contained less microtubules during involution compared with lactation and that the epithelial cells lost the perpendicular organization of the microtubules with the apical cell membrane. As involution continues to progress, the rough endoplasmic reticulum is reduced and sparser (Holst et al., 1987) and the alveoli become smaller and collapsed (Singh et al., 2005).

After one week of involution, the relative proportion of epithelial tissue and stromal tissue is markedly changed. Whereas the epithelial area does not vary a lot during involution (Akers et al., 1990; Capuco et al., 1997), the luminal area decreases and the stromal area increases considerably (Capuco et al., 1997; Capuco and Akers, 1999). Finally, by 21 days in the cow, the majority of epithelial cells are in a non-secretory state (Akers et al., 1990). The luminal area reaches a minimum of 9.5% on d 35 prepartum whereas the stromal area reaches a maximum on d 35 (Capuco et al., 1997).

The involution process of bovine mammary glands is heterogeneous. The alveolar tissue is heterogeneous with regressing alveoli and still some alveoli with active lactating appearance during the first 3 days of cessation of milking (Singh et al., 2005). Even one week after cessation of milking, some alveoli remain intact whereas others degenerate, containing a high proportion of sloughed cells and a high proportion of apoptotic cells (Wilde et al., 1997). The mammary parenchyma does not regress uniformly throughout the udder: involution begins in lower regions of the udder and extends progressively (Akers et al., 1990). Moreover,

even though the cytoplasmic organelles involved in milk component synthesis are reduced, they are still present and the epithelial cells seem to be capable of metabolic activity (Holst et al., 1987). This explains why mammary gland involution is partially reversible after 11 days of milk stasis in the cow, after the involution process was fully established (Noble and Hurley, 1999). This flexibility of the bovine mammary gland is likely due to the fact that intact alveoli are still present in the non-lactating tissue throughout involution. Furthermore, contrary to rodents, there is no extensive sloughing of epithelial cells and the lobulo-alveolar structure of the gland is maintained in cows throughout the dry period (Akers et al., 1990; Capuco and Akers, 1999).

Mammary gland involution is a period of intense cell turnover in ruminants. It has been shown in cows that mammary cell proliferation as determined by Ki-67 staining is greater during the dry period than during lactation and that the percent of Ki-67 positive nuclei is higher during the late dry period compared with the early dry period (Sorensen et al., 2006; Nørgaard et al., 2008). The proliferation rates of both epithelial and stromal cells increase from late lactation to late dry period (Wall et al., 2005; de Vries et al., 2011). On the other hand, the proportion of mammary epithelial cells positively stained by the TUNEL assay, identifying the cells in apoptosis, is greater during the early dry period (0.37%) than during the late dry period (0.17%; Sorensen et al., 2006). About 50% of the epithelial cells are eventually lost during involution in bovine (Akers et al. 1990). It has been hypothesized that this apoptosis process during early dry period aims to discard nonfunctional or senescent cells (Sorensen et al., 2006).

This is why a dry period is mandatory to renew the epithelial cell population of the mammary gland. In bovine mammary tissue, the incorporation rate of [^{3}H] thymidine, used to evaluate cell proliferation, was 80% higher in cows that were dried 60 days before the expected calving date compared with continuously milked cows (Capuco et al., 1997). Among cells labeled with [^{3}H] thymidine, 96% and 86% were epithelial cells in dry and lactating cows, respectively (Capuco et al., 1997). At 35 days prepartum, the percentage of mammary epithelial cells incorporating [^{3}H] thymidine was greater with than without a dry period. By 7 days prepartum, epithelial cells represented 83 % of the total mammary cells in dry cows compared with 74 % in continuously milked animals (Capuco et al., 1997). In conclusion, during the dry period, the involution process allows the mammary gland to renew the epithelial cell population by removing old and senescent cells through an intense cell turnover in cattle.

2.1.2 Mammary gland involution dramatically modifies the composition of mammary secretions by altering tight junction integrity

The composition of milk starts to change during late lactation as drying-off approaches and is greatly modified after cessation of milking in dairy ruminants. These modifications in fluid composition are the consequence of a reduction in synthesis and secretion of milk components, of the action of the protease plasmin in milk, and of the increase in tight junction permeability.

Firstly, epithelial cell activities are greatly reduced during mammary gland involution. At the time of drying-off, mammary secretions are rich in specific milk fat, caseins, α-lactalbumin and β-lactoglobulin and contain a very low proportion of lactoferrin (Noble and Hurley, 1999). After cessation of milking, the composition is inverted with lactose, milk fat, caseins, α-lactalbumin and β-lactoglobulin declining rapidly and lactoferrin being up-regulated (Hurley, 1989; Noble and Hurley, 1999). As said previously, secretion of milk components is altered during involution because of disorganization of the microtubule network. Moreover, the abundance and the functionality of the cytoplasmic organelles involved in milk component synthesis are reduced. The percentage of fat in mammary secretions decreases progressively as involution advances (Sordillo et al., 1987) due to reduction in specific milk fat secretion and synthesis. The production and release of milk-specific proteins are greatly decreased for the same reason. The concentration of caseins, α-lactalbumin and β-lactoglobulin in milk are markedly decreased during the first half of the dry period (Hurley and Rejman, 1986, Aslam et al., 1994; Noble and Hurley, 1999). For instance, 1 week after cessation of milking in cows, expression of α-lactalbumin and α_{s1}-casein dropped by 99% and 85%, respectively, when compared to lactation (Wilde et al., 1997). The concentration of citrate, which is also an indicator for mammary epithelial cell activities, is reduced during the first 2 weeks of involution (Nonnecke and Smith, 1984). By day 11, the composition of milk is inverted: caseins, α-lactalbumin and β-lactoglobulin are at their lowest level whereas lactoferrin becomes the major protein present in mammary secretions (Noble and Hurley, 1999). These modifications in milk composition during involution reflect the decrease in epithelial cell activities highlighted by the ultrastructural changes mentioned above.

Secondly, another reason for such a change in milk composition originates from the fact that the different subtypes of caseins present in milk, in particular β-casein, are degraded into

smaller fragments under the action of the serine protease plasmin. This enzymatic catabolism contributes to the decrease in casein concentrations in mammary secretion throughout involution.

Thirdly, the modification in milk composition is also due to the change in the permeability of the tight junction barrier. This topic will be further discussed next.

2.1.2.1 Mammary gland involution involves the impairment of tight junction integrity

The mammary epithelium, besides its secretory function, allows the delimitation between two different fluids: the milk secreted by the mammary epithelial cells and the interstitial fluid. Milk is rich in lactose, milk proteins such as caseins, and contains low concentrations of sodium and chloride (Nguyen and Neville, 1998). On the other hand, the interstitial fluid contains plasma proteins such as bovine serum albumin (**BSA**), and high concentrations of sodium and chloride (Nguyen and Neville, 1998). In order to maintain the separation between these two fluids, the mammary epithelium needs to be impermeable and acts as a barrier. For that purpose, mammary epithelial cells are joined steadily by tight junctions. These junctions prevent the transit of milk components into the interstitial fluid and, conversely, prevent the transit of interstitial components into milk (Stelwagen et al, 1997).

Tight junctions make a part of the junctional complex which also includes adherent junctions and desmosomes (Nguyen and Neville, 1998). They are extracellular structures located close to the apical pole of the epithelial cells and are associated with a certain number of cytoplasmic proteins such as ZO-1, and with a transmembrane protein, occludin (Nguyen and Neville, 1998). They also appear to be associated with elements of the cytoskeleton, especially with actin filaments (Nguyen and Neville, 1998). Actually, the ZO-1 protein links the integral tight junction occludin to the actin-based cytoskeleton (Fanning et al, 1998).

Tight junction permeability varies during a reproductive cycle and has an impact on milk composition. During pregnancy, mammary tight junctions are leaky in goats and cows (Fleet and Peaker, 1978). Before parturition, mammary secretions contain more proteins, sodium and chloride and less lactose than true milk (Nguyen and Neville, 1998) and the transepithelial potential difference between milk and blood decreases during late pregnancy compared to lactation (Peaker, 1977). These observations indicate there is a diffusion of the interstitial components into milk and vice versa because mammary tight junctions are

permeable. The composition of mammary secretions changes around calving and this phenomenon is attributed to the strengthening of the tight junctions (Linzell and Peaker, 1972). In contrast, during lactation, mammary tight junctions are strongly sealed and form a highly impermeable barrier between milk and the interstitial fluid. The mammary epithelium thus allows maintenance of ionic gradients between these two compartments. After cessation of milking, mammary tight junction integrity starts to be compromised after 20-21 h of milk accumulation in the ewe and the goat (Stelwagen et al., 1994; Castillo et al., 2008).

As mentioned above, disruption of the integrity of the tight junction barrier allows the transfer of milk components into interstitial fluid and blood and reciprocally the transfer of blood components into milk. Consequently, after weaning or cessation of milking, there is an exchange of different elements (ions, proteins, carbohydrates) according to their gradients of concentrations across the disrupted tight junction barrier. This phenomenon leads to an increase in concentrations of different proteins of blood origin such as lactoferrin, bovine serum albumin and immunoglobulin (**Ig**) in mammary secretions during the first week of involution (Nonnecke and Smith, 1984; Sordillo et al., 1987). During lactation, BSA is almost absent in milk. After drying-off, the BSA concentration increases until 14 days (Tremblay et al., 2009; Ollier et al., 2013) and remains high throughout the dry period (Nonnecke and Smith, 1984; Sordillo et al, 1987). In a similar way, the concentration of lactoferrin in milk is low during lactation and rises abruptly during the first 2 weeks of involution before declining thereafter (Nonnecke and Smith, 1984; Hurley and Rejman, 1993; Ollier et al., 2013). The content of IgG in mammary secretions also increases progressively during the first days of the dry period (Nonnecke and Smith, 1984; Sordillo et al., 1987). The ionic composition of milk is also altered as the gland enters the involution process. In particular, the concentration of potassium in milk decreases after cessation of milking in cows whereas that of sodium in milk is enhanced after drying-off (Ollier et al., 2013). Moreover, there is a decrease in milk concentrations of lactose and α-lactalbumin, two specific milk components, associated with an increase in lactose and α-lactalbumin concentrations in blood during milk stasis (Sordillo et al., 1987; Stelwagen et al., 1994). All of these modifications are facilitated by the elevated tight junction permeability during involution and constitute good indicators for measuring the extent of the involuting process. Another change in mammary secretion composition concerns the reduction in milk citrate concentration after cessation of milking. Whereas citrate content is relatively high during lactation, which is indicative of the level of activity of mammary epithelial cells, it decreases at least during the first 2 weeks of involution (Nonnecke and

Smith, 1984; Sordillo et al., 1987; Ollier et al., 2013). The citrate:lactoferrin molar ratio can be calculated to give an indication on the extent of the involution process. During lactation, this ratio is relatively elevated, whereas it is progressively reduced during the first week of involution and remains low throughout the dry period (Nonnecke and Smith, 1984; Sordillo et al., 1987; Ollier et al., 2013). The evolution of all of these parameters induced by the impairment of tight junction integrity makes them good markers to measure mammary gland involution rate.

2.1.2.2 Mammary gland involution solicits some elements of the immune system

One of the characteristic of active involution is recruitment of immune cells. Globally, the total number of leukocytes increases rapidly in bovine milk during the first 3 days of involution and remains elevated until calving (Nonnecke and Smith, 1984; Hurley, 1989) and SCC increases in mammary secretions from the last milk removal to at least 10 to 14 days of involution (Sordillo et al., 1987; Ollier et al., 2013). During the early stages of involution, from 2 to 4 days after drying-off, polymorphonuclear neutrophils (**PMN**) are the predominant leukocytes in mammary secretions followed by macrophages and lymphocytes (Sordillo et al., 1987; Tatarczuch et al., 2000). As involution progresses, macrophages replace PMN as the predominant immune cell type (Monks et al., 2002; Atabai et al., 2007). Neutrophils and macrophages are thought to play a role in clearance of casein micelles, lipid droplets and cellular debris (Monks et al., 2002; Atabai et al., 2007). This explains the high susceptibility of the mammary gland to infections during the early dry period because of the engulfment and incapacitation of the phagocytic cells.

2.1.3 Mammary gland involution activates different proteases systems

2.1.3.1 The plasmin/plasminogen/PA system

Mammary gland involution also involves activation of different proteases. Among those proteases, plasmin is a major enzyme responsible for cleavage of different casein subtypes leading to formation of casein breakdown products. The majority of the peptidic fragments obtained by plasmin activity during bovine mammary gland involution come from β-casein degradation (Aslam and Hurley, 1998). Plasmin is a serine protease which is found

in milk during lactation in its inactive form, plasminogen. Plasmin and plasminogen concentrations in milk increase as lactation advances and increase further during bovine mammary gland involution (Politis et al., 1989a; Politis et al., 1990; Aslam et al., 1994). During the dry period, the plasminogen:plasmin ratio is markedly decreased, which is indicative of a conversion of plasminogen into active plasmin (Politis et al., 1990; Athie et al., 1997). Plasmin and plasminogen have been found to be associated with casein micelles at 82% and 80%, respectively (Politis et al., 1992). The conversion of plasminogen into plasmin is regulated by other enzymatic agents, namely plasminogen activators (**PA**) and plasminogen activator-inhibitors (**PAI**). The balance between PA and PAI controls the activation of the plasminogen/plasmin/PA system. In cows, plasmin, plasminogen and PA activities are greatly enhanced during mammary gland involution (Aslam and Hurley, 1997; Athie et al., 1997). Two forms of PA are found in the mammary gland: the tissue-type plasminogen activator (**tPA**) and the urokinase-type plasminogen activator (**uPA**) (Heegard et al., 1994a). The tPA is associated with casein micelles and binds more specifically to αs2- and κ-caseins whereas the uPA is associated with milk somatic cells in bovine and binds to uPA receptor (**uPAR**) (Heegard et al., 1994a; Ismail et al., 2006; Weng et al., 2006). The immune system also contributes to activation of plasminogen as uPA and uPAR are associated with PMN in cows (Chou et al., 2009). It has also been shown that bovine mammary epithelial cells produce both PA and PAI (Heegard et al., 1994b).

During involution, levels of tPA and uPA increase sharply (Ossowski et al., 1979; Strange et al., 1982, Flint et al., 2006), stimulating therefore the activation of plasmin. Once activated, plasmin degrades mainly α_s- and β-caseins but also κ-casein and lactoferrin (Aslam and Hurley, 1997; Politis et al., 1989b). Alpha-lactalbumin and β-lactoglobulin seem to be resistant to proteolysis (Aslam and Hurley, 1997). This action of plasmin on milk proteins contributes to the change in milk composition occurring during mammary gland involution. Furthermore, plasmin, through generation of casein breakdown products, could be responsible for triggering of the involution process. Indeed, it has been reported that intra-mammary infusions of casein hydrolysates in cows and goats were able to cause a physiological response in the gland (tight junction opening, change in milk composition, decrease in milk secretion) similar to what is observed during involution (Shamay et al., 2002; Shamay et al., 2003).

2.1.3.2 The metalloproteinase system

Matrix metalloproteinases (**MMP**) constitute a family of proteases playing a major role in mammary gland involution, besides the plasminogen/plasmin/PA system. These enzymes are more specifically involved in tissue, extra-cellular matrix and/or basement membrane degradation. Many of those proteases, such as MMP-1, MMP-2, MMP-14 and MMP-19 are upregulated during involution in cattle (Rabot et al., 2007). Among the MMP family, 3 enzymes, MMP-3, MMP-2 and MMP-9, have been intensively studied in relation to mammary gland involution. Stromelysin-1 (MMP-3) is a metalloproteinase exclusively expressed in the mammary gland and particularly in myoepithelial cells. In the cow, its expression is increased during late lactation (de Vries et al., 2011). Boudreau et al. (1995) reported that degradation of the basement membrane by MMP-3 caused a substantial increase in apoptosis both in vitro and in vivo. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are two other proteases that both have the property to degrade the basement membrane collagens and that possess gelatinolytic activity (Matrisian, 1990). MMP-2, which is mostly produced by myoepithelial cells, is also enhanced during bovine mammary gland involution (Tremblay et al., 2009). Some epithelial cells express MMP-2 and MMP-3 in the form of cytoplasmic granules (Dickson and Warburton, 1992). MMP-9 levels in mammary tissue are low during lactation and increase markedly during involution in both rodents and cows (Lund et al., 2000; Tremblay et al., 2009). The expression of MMP can be regulated at the systemic level. Indeed, it has been shown that the mRNA expression of MMP-3 was markedly inhibited in vitro by lactogenic hormones, i.e. insulin, hydrocortisone and prolactin (Li et al., 1994). In addition, activation of those MMP is regulated by the tissue inhibitor of metalloproteinases (**TIMP**). In cattle, TIMP-1 and -2 are up-regulated concomitantly with some MMP, uPA, tPA, uPAR and PAI during involution (Rabot et al., 2007). Actually, the ratio between MMP and TIMP drives both the fate of the basement membrane and the extra-cellular matrix and the remodeling process of the mammary gland after drying-off. When the ratio of MMP to TIMP is low, degradation of the extra-cellular matrix is prevented and involution is slow (Talhouk et al., 1992). When the ratio is high, the extra-cellular matrix is degraded and mammary gland remodeling takes place. In conclusion, degradation of the extra-cellular matrix and basement membrane is due to a shift in the balance between MMP and TIMP which favors a marked increase in MMP and thus, proteolysis.

2.1.3.3 The loss of cell-extracellular matrix communication triggers mammary epithelial cell apoptosis

During mammary gland involution, proteolytic degradation of the extracellular matrix by MMP and the plasmin/plasminogen systems leads to a reduction in the communication between mammary cells and the extracellular compartment. This loss of attachment induces and accelerates apoptosis (Merto et al., 1997). In vivo, mammary epithelial cells lie on a specialized extracellular matrix, the basal membrane. In particular, survival of mammary epithelial cells requires that they adhere to extracellular matrix proteins of a basement membrane rich in laminin (Boudreau et al., 1995; Pullan et al., 1996; Farrelly et al., 1999). The beneficial effect of the basement membrane on mammary epithelial cell survival is direct and mediated through proteins called integrins. Integrins are glycoproteins essentials for communication between mammary epithelial cells and the extracellular matrix. In bovine mammary glands, expression of β1-, α6- and α5-integrins mRNA decreased by 24h of milk accumulation relative to 6 h post-milking (Singh et al., 2005). The disruption of the interaction between mammary epithelial cells and the extracellular matrix by addition of an antibody directed against β1 or α6 integrins led to an increase in apoptosis (Boudreau et al., 1995; Pullan et al, 1996; Farrelly et al., 1999). Consequently, down-regulation of integrins in the bovine mammary tissue disrupts the communication between mammary epithelial cells and the extracellular matrix and participates to trigger apoptosis during involution.

2.1.4 IGFB-5 and STAT3 are two key effectors of mammary gland involution

2.1.4.1 The IGF/IGFBP system

Survival of mammary epithelial cells is driven and regulated coordinately by the basal membrane and by signals from insulin, IGF-1 and IGF-2 (Farrelly et al., 1999). In vitro, IGF-1 suppresses mammary epithelial cell apoptosis (Farrelly et al., 1999) and prevents mammary gland involution in rodents (Neuenschwander et al., 1996). In bovine mammary gland, IGF-1 is up-regulated during involution. Actually, IGF-1 up-regulation corresponds to periods of high cell turnover. Indeed, mRNA IGF-1 expression is the highest during mammogenesis, decreases during lactogenesis and galactopoiesis, and increases during involution (Path-Gabler et al., 2001; Sorensen et al., 2006). Conversely, mRNA IGF-2 expression, while relatively high during mammogenesis and lactation, decreases after drying-off (Path-Gabler et

al., 2001). The mammary tissue is also more sensitive to IGF-1 as expression of the IGF-1 receptor is increased after drying-off in cows (Path-Gabler et al., 2001). During the dry period itself, IGF-1 mRNA expression does not significantly vary (Wall et al., 2005). Nonetheless, it seems that IGF-1, given its role in stimulating cell proliferation, is involved during mammary epithelial cell renewal during involution.

In contrast, IGFBP-5 expression is up-regulated during the first two weeks of involution in cows (Sorensen et al., 2006). IGFBP-5 promotes mammary epithelial cell apoptosis (Tonner et al., 2002; Flint et al., 2006) likely by inhibiting the survival signal driven by IGF-1 (Marshman et al., 2003). IGFBP-5 also activates the proteolytic degradation of the mammary epithelium (Tonner et al., 2002) and can interact with the plasmin/plasminogen system. It has been shown that IGFBP-5, probably by binding PAI-1 (Nam et al., 1997), counteracted the inhibitory effect of PAI-1 on plasminogen activation by stimulating tPA (Sorrell et al., 2006). Therefore, the IGF/IGFBP system is implied in the regulation of mammary gland involution and IGFBP-5 seems to be a key mediator in the occurrence of this process.

2.1.4.2 The JAK/STAT signaling pathway

Finally, another feature of mammary gland involution is modulation of expressing the signal transducer and activator of transcription (**STAT**) factors. After their activation by phosphorylation and dimerization, STATs can induce the transcription of genes involved in cell proliferation throughout activation of the JAK/STAT pathway. Two members of the STAT family evolve significantly during mammary gland involution: STAT5, with its subtypes STAT5a and STAT5b, and STAT3. More specifically, after weaning or cessation of milking, STAT5 is down-regulated whereas STAT3 is up-regulated. STAT5 is involved in cell division and differentiation, favors cell-cell adhesion (Miyoshi et al., 2001) and is inversely related with apoptosis. As it has been shown in the mouse, overexpression of STAT5 accelerated mammary development during pregnancy, enhanced mammary cell differentiation, stimulated mammary epithelial cell proliferation and delayed mammary gland involution (Iavnilovitch et al., 2002). During lactation, the concentrations of STAT5 and its phosphorylated activated form, phospho-STAT5 (**pSTAT5**) are high but after cessation of milking, they both decline as soon as 2 days of involution (Flint et al., 2006). The abundance and phosphorylation STAT5a are both markedly reduced during involution, leading to a

decrease in the pSTAT5a/STAT5a ratio in mice (Chapman et al., 1999; Bertucci et al., 2010). Mammary epithelial cells are therefore deprived of a proliferative signal during that time. Conversely, STAT3 is barely detectable during lactation but by d 2 of involution, its concentration and its phosphorylation are markedly up-regulated, leading to an increase in the pSTAT3/STAT3 ratio (Chapman et al., 1999, Flint et al., 2006; Bertucci et al., 2010). Contrary to STAT5, STAT3 is highly associated with involution and apoptosis. For instance, in mice with a conditional knockout of STAT3, involution was delayed and mammary epithelial cells apoptosis was inhibited (Chapman et al., 1999). The prevention of milk removal activated Stat 3, inhibited Stat5a and Stat5b phosphorylation and heterodimerization and locally induced apoptosis of alveolar cells (Li et al., 1997). Finally, STAT3 would also stimulate IGFBP-5 expression (Chapman et al., 1999).

2.2 Is there a need for re-evaluating dry period length?

The interest to hasten mammary gland involution can also be found in limiting the dietary changes occurring during the dry period. Indeed, the transition from the lactation to the dry period and then, from the dry period to the following lactation, is a stressful challenge for dairy cows. During a 60-day dry period, 3 diet changes usually occur (late lactation diet to FOD diet, FOD diet to CUD diet and CUD diet to early lactation diet). The rumen microbial population has to adapt to these nutritional changes and this adaptation could require several days to weeks (Goff and Horst, 1997). Shortening the dry period length from 60 days to 30 or 35 days could be associated with the suppression of the FOD diet. The animals would therefore be fed only with the CUD diet during the dry period, directly after the late lactation diet. This would reduce the frequency of diet changes, reducing stress for the cows and this may improve the maintenance of beneficial rumen microbial flora (Pezeshki et al., 2007). Goff and Horst (1997) have suggested that the early lactation diet could be fed as soon as 5 weeks before calving instead of just 2 to 3 weeks before calving in order to increase the adaptation of the rumen. This management practice could be done with a shorter dry period with just one dry-off diet. Another advantage for a shorter dry period is the potential improvement of reproduction in the subsequent lactation. Pezeshki et al. (2007) showed that frequent and sudden diet changes (late lactation, FOD, CUD and early lactation diets) within a short time during a dry period (42 days) decreased reproductive performance of the animals. Thus, reducing dietary changes by shortening a dry period to 35 days could be beneficial

against reproduction problems in dairy cows. For that purpose, 2 strategies can be developed: hastening the involution process during the first weeks of the dry period or decreasing the number of transitions between lactation and dry period by increasing lactation persistency.

The main purpose of a dry period is to allow the mammary gland to rest and to produce high quantity of milk during the following lactation. Dry period length should be sufficient to allow the gland to complete involution and to renew mammary epithelial cell population. Currently, a 60-day dry period is a common practice in dairy management and is considered to be adequate in order to maximize milk production during the subsequent lactation.

The 60-day dry period was adopted during 1930s as the optimal dry period length for maximal milk yield and genetic progress. Since its adoption, the 60-day dry period has been maintained as the dry period length that best maintains the balance between lost milk income during the dry period and production levels achieved in the subsequent lactation. Numerous earlier studies showed that a reduction in dry period length from 60 days resulted in a decrease in milk yield during the following lactation. Swanson (1965), in an experiment using identical twin cows, showed that cows dried during 60 days produced more milk during the second lactation than during their first lactation and produced more milk than cows which were continuously milked. Moreover, the average lactation yields of the cows given a 60-day dry period increased each year whereas the average of those milked continuously decreased each year (Swanson, 1965). Coppock et al. (1974) have stated that a dry period of less than 40 days would result in a substantial loss in milk production.

Nowadays, dairy cows produce substantially more milk than 80 years ago thanks to the advance in genetics and nutrition management. In the 1930s, a typical dairy cow produced about 2000 kg of milk yearly, whereas a well-nourished modern cow can produce more than five times this amount. So, there is a need to re-evaluate optimal dry period length in the context of current high producing cows.

Results from most recent studies seem to support the notion that current dairy cows show a different response to changes of dry period length compared to many earlier studies. A study conducted with 122 primiparous and multiparous Holstein cows showed that a 35 day-dry period tended to increase body condition score (**BCS**) and significantly decreased non-esterified fatty acids (**NEFA**) concentrations postpartum compared to a 56 day-dry period (Pezeshki et al., 2007). A lower NEFA concentration indicates that the organism mobilizes

less of its body reserves. Watters et al. (2008) have also observed that postpartum BCS was higher and that postpartum NEFA concentrations were lower in cows dried-off during 34 days compared to those dried-off for 55 days. Consequently, cows that experienced a short dry period of 34 or 35 days had a lower rate of fat mobilization and a less negative energy balance during early lactation than cows experiencing a dry period of 55 or 56 days. Gulay et al. (2003) also confirmed that a 60 day-dry period led to a lower BCS and a higher negative energy balance postpartum compared to a 30 day-dry period. In addition, no detrimental effects of a 30-day dry period on milk yield, 3,5% fat corrected milk (**FCM**) or 305-day milk yield of the following lactation have been shown compared to a 60 days-dry period (Bachman, 2002; Gulay et al., 2003). Milk production of multiparous Holstein cows, when compared to a 56-day dry period, decreased with a dry period of 42 days but no difference was observed when compared with a dry period of 35 days (Pezeshki et al., 2007).

The modulation of the dry period length does not seem to impact other parameters such as milk composition or cow health status to a great extent. Dry period length affects slightly milk composition. Milk fat percentage is not affected by shortening the dry period (Gulay et al., 2003; Annen et al., 2004; Pezeshki et al., 2007; Watters et al., 2008; Santschi et al., 2011a). The effects of dry period management strategy on milk protein percentage are more variable. Milk protein percentage can be unaffected by decreasing dry period from 56 to 35 days (Gulay et al., 2003; Pezeshki et al., 2007) or can be increased when a dry period is decreased from 55/60 days to 34/35 days (Watters et al, 2008; Santschi et al., 2011a). In other works, a reduction in dry period length from 65 days to 35 or 30 days led to an increase in milk protein concentration (Annen et al., 2004; Bernier-Dodier et al., 2011). Furthermore, shortening dry period length does not affect animals' health status. Coppock et al. (1974) did not detect any difference of 20, 30, 40, 50 or 60 days-dry periods on the incidence of ketosis, milk fever or retained placenta. The incidences of severe ketosis, metritis, mastitis, milk fever, retained placenta and displaced abomasum were not affected by a 34/35 days-dry period compared with a 55/60 days-dry period (Watters et al., 2008; Santschi et al., 2011b).

However, numerous other studies did report the negative impact of shortened dry periods with modern dairy cows. Sorensen and Enevoldsen (1991) showed that a dry period of 7 weeks increased 4% FCM compared to a 4-week dry period but decreased 4% FCM relative to a 10-week dry period. In experimental herds (Watters et al., 2008; Bernier-Dodier et al., 2011) or in commercial herds (Santschi et al., 2011a), a decrease in milk production occurs with a 34 or 35-day dry period compared with a 55 or 60-day dry period. A decrease in dry

period length from 60 days to 30 days or a complete omission of the dry period caused a reduction in daily milk yield in primiparous cows during the first days to 17 weeks postpartum (Annen et al., 2004; Fitzgerald et al., 2007). The decrease in milk secretion during the lactation following a shorter dry period could be due to a reduced mammary epithelial cell activity or reduced numbers of epithelial cells. Both whole animal and half-udder model studies showed that mammary epithelial cells proliferation was greatly depressed before parturition in the absence of a dry period (Capuco et al., 1997; Annen et al., 2007). Mammary epithelial cell apoptosis was also decreased by 60% after parturition in continuously milked gland compared with dry glands (Annen et al., 2007). Therefore, although one study showed that mammary cell dynamics on days 21 and 154 of lactation was not affected by shortening dry period from 65 to 35 days (Bernier-Dodier et al. 2011), mammary epithelial cells turnover is thought to be reduced in the complete absence of a dry period and the replacement of senescent cells is altered compared with a 60-day dry period. Moreover, this means that there are more senescent or non-functional secretory cells at the beginning of the next lactation which may be the causative factor of reduced milk functionality in continuously milked cows. It is thus necessary to allow the mammary gland to complete its involution and to optimize the renewal of the epithelial cell population.

Two strategies could facilitate the use of a short dry period while allowing a complete involution of the mammary gland. One strategy would be to accelerate the involution process. This strategy has double advantages : by taking into account the fact that the mammary gland is more resistant when its involution is completed, it could increase the resistance of the cow to new intra-mammary infections and decrease metabolic disorders thanks to a fewer change of diets related to a dry period shorter than 40 days or less. Another strategy is to inhibit the lactogenic signal to decrease milk secretion before the beginning of the dry period and therefore to decrease the risk of intra-mammary infections due to milk leakage. These two strategies will be discussed next.

2.3 Strategies to decrease health problems due to the transition between lactation and dry period

As previously said, dry cows become more resistant when mammary gland involution is completed. Therefore, it would be particularly interesting to allow the mammary gland to complete its involution as soon as possible. The faster the mammary gland involutes, the faster the gland would be resistant to new intra-mammary infections.

One of the strategies to reach this goal is to administer treatments that are known to increase tight junction permeability in the mammary gland through the teat canal. As tight junctions are more permeable during involution, these treatments could affect the involution process. Different treatments have been infused in mammary glands of dairy goats or dairy cows and affected tight junction integrity.

2.3.1 Use of intra-mammary infusions to accelerate the involution process

Besides endocrine control, mammary gland involution is also locally controlled at the level of the mammary gland. First, studies using a half-udder model demonstrated that unilateral teat sealing led to an increase in DNA-end labelling and induced a disruption of the alveolar integrity in lactating mice and goats (Quarrie et al, 1994; Quarrie et al, 1995).

Under the stimulation by pup suckling, apoptosis of alveolar cells was induced, Stat5a and Stat5b phosphorylation and heterodimerization were inhibited and STAT3 was activated in glands in which milk removal was prevented (Li et al., 1997). This suggests that, even in the presence of the same hormonal environment due to the suckling stimulus, local signals specific to the mammary gland were able to induce the first step of involution in rodents. The systemic lactogenic hormones maintained however the lobulo-alveolar structure of the gland and prevented the entrance in the second proteolytic degradation phase of involution (Li et al., 1997). In cows, in experiments using a unilateral milking model, apoptosis transiently increased and the percentage of proliferating epithelial cells was greater in mammary tissue after milk stasis compared with mammary tissue originated from the contralateral milked quarters (Capuco et al., 1990; Tremblay et al, 2009). Mammary cell turnover is thus partly dependent on local mammary factors. Involution is therefore regulated not only at a systemic level but also at the mammary gland level.

2.3.1.1 Casein hydrolysates, a potential candidate to hasten involution

As previously mentioned, activation of the plasminogen/plasmin/PA system during mammary gland involution leads to degradation of the β-, αs1- and αs2- caseins. It has been proposed that formation of casein-breakdown products could constitute a signal for the mammary epithelial cells to stop milk synthesis. A research team from Israel has tried to mimic the accumulation of casein-breackdown products during milk stasis by the administration of casein hydrolysates into the mammary gland. Silanikove et al. (2000) found that a proteose-peptone phosphopeptide produced by plasmin digestion of activity on casein blocked the potassium channel activity of vesicles isolated from skim milk. This peptide is composed of residues 1-28 of β-casein. It has been reported in goats and cows that the depression in milk production induced by water deprivation or by dexamethasone was associated with an increase in PA and plasmin concentrations and with a rise in the potassium channel blocking activity (Silanikove et al., 2000), suggesting a role for casein breakdown products as a feedback inhibitor of milk secretion.

The casein breakdown products may also engender the opening of mammary tight junctions and induce involution in goats and cows and seem to mimic the involution process. Shamay et al. (2002) have shown that the intra-mammary infusion of fragments of caseins hydrolyzed by trypsin into goat udder led to a decrease in milk production. In the treated gland, infusion of casein hydrolysates (**CNH**) enhanced plasmin and PA concentrations and caused an increase in sodium and bovine serum albumin concentrations in milk as well as a decrease in potassium and lactose concentrations in milk which are characteristic of an increase in tight junction permeability.

In late lactating Holstein cows, intra-mammary infusions of CNH were able to reduce mammary gland secretions and to mimic the modifications in milk during the cessation of milking at drying-off (Shamay et al., 2003). The CNH treatment affected mammary tight junction integrity and led notably to an increase in the lactoferrin concentration, another characteristic of mammary gland involution (Shamay et al., 2003). Lactoferrin is a protein found in blood which is known to catch iron and deprive iron-dependent bacteria from the substrate.

As a mammary gland which has undergone involution is more resistant to new mammary infections, it is therefore of interest to use the CNH as a treatment at drying-off in order to hasten involution. Moreover, the fast increase in lactoferrin due to the CNH intra-

mammary infusion could also bring a supplemental anti-bacterial effect beneficial for the mammary gland. It has indeed been shown that intra-mammary infusions of CNH in combination with antibiotic treatment had the potential to cure coagulase negative staphylococci intra-mammary infections in dairy cows (Leitner et al., 2011). The effects of intra-mammary infusions of CNH have been highlighted only by one team of researchers and need to be confirmed. Furthermore, the results concern tight junction integrity and more parameters such as lactoferrin concentration or protease activity should be measured in order to analyse the effects of CNH intra-mammary infusions on the involution process of the mammary gland.

2.3.1.2 EGTA

In order to maintain integrity of the mammary epithelial cells' tight junctions, the extracellular concentration of calcium needs to be kept at a certain level (Pitelka et al., 1983). The compound ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (**EGTA**) is a chelator of calcium. EGTA has the ability to induce the opening of cell tight junctions in vitro. Rothen-Rutishauser et al. (2002) showed that MDCK cells which were incubated 20min in a culture medium containing 2mM of EGTA resulted in a decrease in 30% of the transepithelial electrical resistance (**TEER**) suggesting opening of the tight junctions. The removal of EGTA led to a complete recovery of TEER after 3 hours (Rothen-Rutishauser et al., 2002). They also observed that the cells are more detached from each other with EGTA treatment and that the opening of the tight junctions due to the treatment induced large changes in colocalization of transmembrane proteins such as ZO-1, occludin and claudin-1.

EGTA induces disruption of tight junctions in mammary epithelial cells in lactating goats. Administration of a solution of 68 mM of EGTA (final concentration into the mammary gland) led to an increase in sodium and chloride concentrations in milk as well as an increase in potassium and lactose concentrations in blood suggesting that tight junctions' permeability was compromised (Stelwagen et al., 1995). The EGTA treatment caused also a change in milk composition: milk protein percentage increased, may be due to the passage of blood proteins such as serum albumin (Stelwagen et al., 1995). Moreover, EGTA treatment was efficient to decrease milk production (Stelwagen et al., 1995). To our knowledge, intra-mammary infusions of EGTA have not been used in dairy cows and the effects of such a treatment on involution's parameters are not known.

2.3.1.3 Lactose

Lactose is a milk-specific component which represents the main osmotic molecule in milk. In an experiment with dairy goats, Ben Chedly et al. (2010) have observed that intra-mammary infusions of lactose solution via the teat canal led to an increase in the milk Na: K ratio and a tendency towards an increase in milk BSA concentration. These authors also observed that isolated mammary epithelial cells from milk treated with 120mM of lactose showed a decrease in the transepithelial electrical resistance, similar to what have been found by Rothen-Rutishauser et al. (2002) with EGTA treatment in vitro. Together, these results suggest that mammary epithelial cells tight junctions' integrity could have been impaired by the lactose treatment. Therefore, it could be of interest to use intra-mammary infusion of lactose in dairy cows in order to evaluate the effect on the integrity of mammary epithelial cells' tight junctions and on involution in dairy cows.

2.3.2 Modulation of photoperiods to accelerate the involution process

2.3.2.1 Short day photoperiods may help hastening involution by decreasing the prolactinemic signal

Release of prolactin by the pituitary gland is influenced by the environment in which the animal is living. The external temperature is known to affect blood prolactin concentrations in cattle (Wettemann and Tucker, 1974; Peters and Tucker, 1978). Another well-known environmental condition that affects prolactin secretion is the photoperiod. The photoperiod corresponds to the daily alternation between episodes of light, the photophase, and episodes of darkness, the scotophase.

It is well established that photoperiods affect body growth and sexual maturity in cattle. Indeed, it has been reported in heifers that a long day photoperiod (**LDPP**; more than 16 h of light/day) enhanced average daily gain (Peters et al., 1980; Petitclerc et al., 1983; Rius et al., 2005), body growth rate (Peters et al., 1978) and lean body growth (Rius and Dahl, 2006) compared with a short day photoperiod (**SDPP**; less than 12 h of light/day). The onset of puberty was also accelerated when heifers were exposed to LDPP (Petitclerc et al., 1983; Rius et al., 2005). It has been reported that the increase in body growth observed in heifers

exposed to LDPP occurred without affecting body composition and mammary growth (Rius et al., 2005).

A change in the photoperiod, occurring either during lactation or during the dry period, exerts a marked impact on milk synthesis. Generally, dairy animals exposed during lactation to LDPP produce more milk than those exposed to SDPP. The photoperiod can therefore be artificially manipulated in order to stimulate milk production. Numerous studies demonstrated that an increase in light exposure during lactation in cows enhanced milk production compared with a shorter photoperiod (Peters et al., 1978; Peters et al., 1981; Bilodeau et al., 1989; Dahl et al., 1997; Miller et al., 1999) with an average gain of 2.5 kg of milk per cow per day (Dahl et al., 2000; Dahl, 2008). A long day photoperiod also enhanced milk yield in lactating goats (+15% kg of milk/goat/day; Garcia-Hernandez et al., 2007) and in lactating ewes (+15.4% after 8 weeks of treatment; Morrissey et al., 2008). Conversely, a decrease in light exposure during lactation causes a reduction in milk production.

The photoperiod can also be modified during the dry period. In this case, effects of a photoperiodic manipulation are opposite to those occurring during lactation. Indeed, cows exposed to LDPP during the dry period produced less milk during the following early lactation than cows exposed to SDPP (Miller et al., 2000; Auchtung et al, 2005; Velasco et al., 2008; Lacasse et al., 2014). Therefore, exposing cows to SDPP during the dry period is an interesting manner to increase milk production during the following lactation and this technique can be used in combination with a reduced dry period length. It has recently been shown in Holstein cows that a SDPP applied to a short dry period of 42 days improved milk production during the subsequent lactation compared with LDPP (Velasco et al., 2008).

Except an absence of effect in one study (Dahl et al., 1997), dry matter intake is usually increased in response to LDPP during lactation (Peters et al., 1981; Bilodeau et al., 1989; Miller et al., 1999) or to SDPP during the dry period (Miller et al., 2000; Velasco et al., 2008; Lacasse et al., 2014). In contrast, milk composition is barely affected by photoperiod changes in most studies in cows and ewes during lactation (Peters et al., 1978; Peters et al., 1981; Bilodeau et al., 1989; Dahl et al., 1997; Miller et al., 1999, Morrissey et al., 2008) and during the dry period (Miller et al., 2000; Auchtung et al., 2005; Velasco et al., 2008). Only in some cases, milk fat concentration can be reduced after supplemental lighting in the lactating cow (Stanisiewski et al., 1985; Phillips and Schofield, 1989) and SDPP can increase protein and ECM yields in dry cows (Lacasse et al., 2014). On the other hand, an increase in light

exposure enhanced milk fat concentration in lactating goats (Garcia-Hernandez et al., 2007) and dry goats (Mabjeesh et al., 2007).

Besides its effect on milk production, photoperiod manipulation during lactation or during the dry period influences the endocrine status of the cow. Photoperiods affect prolactin and IGF-1 secretions. Generally, blood prolactin concentrations are higher when cattles are exposed to LDPP compared to those that are exposed to SDPP (Peters and Tucker, 1978; Peters et al., 1981; Stanisiewski et al., 1987; Stanisiewski et al., 1988b; Newbold et al., 1991; Auchtung et al., 2003; Miller et al., 2000; Kendall et al., 2003). For instance, steers exposed to LDPP during 9 weeks displayed a higher prolactin concentration in plasma from wk 2 to wk 9 than steers exposed to SDPP (Auchtung et al., 2003). An exposure to 16 h of light increased prolactin concentration in heifers compared with 8 h of light (Rius et al., 2005). Dry cows exposed to LDPP displayed greater plasma prolactin concentrations (Miller et al., 2000; Auchtung et al, 2005; Velasco et al., 2008; Lacasse et al., 2014) and a greater periparturient prolactin surge (Newbold et al., 1991; Auchtung et al., 2004; Lacasse et al., 2014) than dry cows exposed to SDPP. Blood prolactin concentration was also higher in primiparous heifers exposed to LDPP compared with those exposed to SDPP (Peters and Tucker, 1978; Lacasse et al., 2014). A long day photoperiod also stimulated prolactin secretion in small ruminants. In goats exposed to LDPP during their third trimester of gestation, plasma prolactin concentration was greater than that of dry goats exposed to SDPP, an effect that persisted until the first week of lactation (Mabjeesh et al., 2007). In lactating ewes, LDPP induced an increase in plasma prolactin concentration after 6 weeks of treatment (Morrissey et al., 2008).

Although LDPP increases milk production and prolactin secretion, SDPP enhances the sensibility of different organs and cells to prolactin. In mammary tissue and lymphocytes, expression of both short and long forms of prolactin receptor (**PRLR**) was increased in cows exposed to SDPP during the dry period compared with pre-exposure levels and was greater than those of cows exposed to LDPP (Auchtung et al., 2005; Velasco et al., 2008). Similarly, SDPP increased expression of both forms of PRLR in mammary tissue, liver and lymphocytes of steers (Auchtung et al., 2003). Conversely, LDPP decreased their expressions compared with pre-exposure values in mammary tissue and lymphocytes (Auchtung et al., 2005). Furthermore, in mammary tissue, prolactin seems to downregulate PRLR as evidenced by the fact that the inhibition of prolactin secretion by bromocriptine increased the number of prolactin binding sites in rats (Di Carlo et al., 1995) and rabbits (Djiane et al., 1977) or that exogenous prolactin administration decreased the mammary expression of the long form of

PRLR mRNA in rats (Di Carlo et al., 1995) and cows (Wall et al., 2006). Therefore, it is not surprising to observe that SDPP, which decreases prolactin release, stimulates PRLR expression. The effects of SDPP on cellular immune function seem also to be driven by prolactin. Indeed, when prolactin secretion was inhibited by bromocriptine in bovine exposed to LDPP, the levels of PRLR mRNA expression, lymphocyte proliferation and neutrophil chemotaxis were similar to those of animals exposed to SDPP (Auchtung et al., 2003). These results indicate that changes in photoperiod affect prolactin sensitivity and cellular immune function through the modulation of prolactin secretion.

Another consequence of a modification of the light regimen is the change in blood IGF-1 concentrations in ruminants. Indeed, it has been shown that blood IGF-1 concentrations are significantly increased in reindeers (Suttie et al., 1991), steers (Kendall et al., 2003), heifers (Spicer et al., 2007) and lactating cows (Dahl et al., 1997) during exposition to LDPP. Dahl et al. (1997) reported that LDPP did not affect plasma IGFBP-2 and IGFBP-3 concentrations suggesting that during lactation, the positive effect of LDPP on IGF-1 concentration is mediated at the level of IGF-1 secretion rather than on its clearance. However, when applied during the dry period, the modification of the photoperiod did not affect circulating concentrations of IGF-1 in cows (Miller et al., 2000; Wall et al., 2005) whereas LDPP induced an increase in plasma IGF-1 concentration in dry goats (Mabjeesh et al., 2007) and SDPP increased mammary tissue IGF-2 mRNA expression in cows (Wall et al., 2005). In addition, the expression of IGFBP-5 mRNA in mammary tissue was not affected when the light regimen was changed in dry cows (Wall et al., 2005). This suggests that neither the synthesis/secretion nor the clearance of IGF-1 is influenced by the photoperiod during the dry period in bovine.

Photoperiod did not affect growth hormone and glucocorticoid concentrations in heifers (Peters and Tucker, 1978; Peters et al., 1980; Petitclerc et al., 1983; Kendall et al., 2003), in lactating cows (Peters et al., 1981; Newbold et al., 1991; Miller et al., 1999; Dahl et al., 1997) and in dry cows (Miller et al., 2000; Auchtung et al., 2004). Therefore, the increase in prolactin concentration due to LDPP does not involve a regulation via glucocorticoids. Additionally, as LDPP does not affect circulating growth hormone, the increases in IGF-1 and in milk production due to LDPP are not mediated through somatotropin.

2.3.2.2 Use of melatonin could mimic a short day photoperiod

Besides prolactin, melatonin is another hormone tightly associated with photoperiods. This hormone is released by the pineal gland (Stanisiewski et al., 1988a) and its secretion displays a circadian profile and a pulsatile secretory pattern (Critser et al., 1987). In the cow, melatonin concentrations in blood are high during the night and low during daylight (Berthelot et al., 1990). As duration of melatonin release is correlated to duration of darkness, it has been suggested that duration of the melatonin surge drives the photoperiodic information in cattle (Buchanan et al., 1992). Thus, this hormone is supposed to be more secreted during SDPP. Melatonin can therefore be administered to ruminants in order to mimic the effects of SDPP exposure.

The way that a photoperiod affects blood prolactin concentrations is not elucidated yet. Early works tended to show that photoperiod increased circulating prolactin concentrations independently of melatonin regulation. Indeed, it has been shown that pinealectomy did not affect serum prolactin concentrations in calves (Stanisiewski et al., 1988a; Stanisiewski et al., 1988b). Moreover, LDPP caused an increase in plasma prolactin concentrations in both pinealectomized and sham pinealectomized calves (Stanisiewski et al., 1988a), suggesting that this increase was not mediated, at least not only, by melatonin.

However, more recent studies based on slow release of melatonin through subcutaneous implants or oral administration of the hormone in ruminants showed that melatonin influenced circulating prolactin concentrations and IGF-1 in some cases. In red deer, the utilization of subcutaneous implants delivering melatonin has been shown to suppress IGF-1 secretion (Suttie et al, 1992). In dairy cows, subcutaneous implants of melatonin reduced plasma prolactin concentration by 4 weeks of treatment and decreased milk production by 6 weeks, an effect that was sustained until the end of the experiment (Auldist et al., 2007). Melatonin administration did not affect plasma IGF-1 concentrations but affected milk composition by increasing milk fat, protein and casein concentrations and decreasing that of lactose by 6 or 8 weeks (Auldist et al., 2007). These changes on milk composition resembled those occurring during late lactation as cows approach involution. Moreover, oral administration of melatonin induced a reduction in serum prolactin concentrations in heifers (Sanchez-Barcelo et al., 1991), lactating cows (Dahl et al., 2000) and dry cows (Lacasse et al., 2014). However, in the case of this mode of melatonin treatment, milk production was not affected (Dahl et al., 2000; Lacasse et al., 2014).

To our knowledge, no experiments have been done to assess the effects of the modulation of the photoperiod on involution parameters. Indeed, as prolactin is involved in maintaining lactation and in delaying involution, it could be hypothesized that its inhibition would facilitate and enhance mammary gland involution. In conclusion, because of their consequences on circulating prolactin concentrations, SDPP and melatonin treatment constitute two strategies that need to be evaluated in relation to involution.

2.3.3 Understanding the mechanisms favoring lactation persistency

Prolactin is one of the major hormones involved in promoting and maintaining lactation and in controlling involution. Prolactin is a multifunctional hormone mainly synthesized by the lactotrophs of the anterior pituitary which is highly involved in the preparation of the mammary gland for lactation and in the lactation process itself in mammals.

Prolactin is involved in mammogenesis towards the end of pregnancy, in epithelial cell differentiation during lactogenesis and in galactopoiesis (Delouis et al., 1980; Houdebine et al., 1985; Lacasse et al., 2014). This hormone constitutes one of the endocrine signals, with oxytocin and glucocorticoids, to be released upon the stimulation of suckling or milking. At the time of suckling or milking, plasma prolactin concentrations rise suddenly and decrease thereafter (Jacquemet and Prigge, 1990). This milking-induced prolactin surge is greatest during early lactation and decreases as lactation advances (Bernier-Dodier et al., 2011). After drying-off, serum prolactin concentrations decrease whereas prolactin concentrations in milk increase, as reported in cows during the 2 first weeks of the dry period (Ollier et al., 2014). Prolactin is also released in a pulsatile manner throughout the day (Koprowski et al., 1972) and follows a circadian rhythm (Mollett and Malven, 1982). At the level of the nervous central system, its secretion is regulated by the dopaminergic neurons of the hypothalamus. The dopamine released by the dopaminergic neurons binds to the D2 receptor located on the surface of the lactotrophs of the anterior pituitary, leading to an inhibition of prolactin release.

After being secreted by the pituitary gland and reaching its target tissue, prolactin binds to prolactin receptor. Two forms of the prolactin receptor are expressed in the mammary gland: the short and the long PRLR isoforms (Jahn et al., 1991). The long form predominates in the mammary gland (Jahn et al., 1997), is more abundant during pregnancy and lactation than the short form (Cassy et al., 1998) and seems to be responsible for the transduction of the

prolactin signal stimulating mammary epithelial cell differentiation and activity (Neville et al., 2002). It has been shown in a transfected bovine mammary gland epithelial cell line with long or short rat PRLR that only the long isoform was able to transduce the signal induced by prolactin such as promotion of β-casein expression (Berlanga et al., 1997). In another study, prolactin strongly stimulated milk protein transcription when the long PRLR form was expressed whereas no stimulation was reported in the presence of the short PRLR isoform (Lesueur et al., 1991). It seems that the short form of PRLR acts as a negative dominant isoform, repressing the lactogenic effect of prolactin by forming heterodimer with the PRLR long form (Berlanga et al., 1997; Cassy et al., 1998). Olazabal et al. (2000) confirmed this repressive effect by demonstrating that the short PRLR isoform blocked the prolactin signaling pathway to β-casein promoter. However, the short PRLR form is able to induce mammogenesis and lactogenesis in the absence of the full expression of the long PRLR form (Binart et al., 2003).

When prolactin binds to its receptor, the Janus Kinase 2 (**JAK2**) linked to PRLR is activated by phosphorylation and phosphorylates then PRLR in return. This leads to STAT5 phosphorylation, allowing STAT5 dimerization and translocation in the nucleus to further regulate the transcription of genes involved in cell division and milk component synthesis. Prolactin mainly induces the phosphorylation of STAT5a compared with STAT5b (Gallego et al., 2001). Once the JAK2/STAT5 signaling pathway has been activated, the complex prolactin-PRLR is internalized into the cell, and prolactin follows a transcellular pathway into endosomes to be either degraded or liberated at the apical side of the cell, i.e. in milk (Forsyth et al, 1995; Lkhider et al., 1996).

2.3.3.1 The role of prolactin in mammogenesis and lactogenesis

Prolactin is essential for mammogenesis and lactogenesis. Generally, when prolactin release is inhibited during the periparturient period, a time when the mammary epithelium typically prepares for the following lactation during the lactogenesis process, it negatively affects the activity and the differentiation of the alveolar secretory cells and the volume of milk produced thereafter. Early works showed that a prepartum treatment with bromocriptine, an inhibitor of prolactin release, lowered prolactin levels, abolished the prolactin surge occurring at parturition, delayed the onset of milk secretion and reduced milk production postpartum (Schams et al., 1972). Numerous other studies reported a decrease in milk

production following a prepartum bromocriptine treatment in cows (Johke and Hodate, 1978), Murrah buffaloes (Prasad and Singh, 2010) and sows (Taverne et al., 1982) highlighting the role of prolactin in preparing the mammary epithelium for lactation. When prolactin release was inhibited by bromocriptine from 12 days before to 10 days after parturition in Holstein cows, total mammary RNA content was decreased by 36%, the RNA/DNA ratio was reduced and the activities of acetylcoenzyme A carboxylase, fatty acid synthetase and α-lactalbumin (Akers et al., 1981a), enzymes essential for milk component synthesis, were inhibited. The relative area occupied by Golgi membranes, vacuoles and RER were also all reduced (Akers et al., 1981b). These two studies demonstrated that the differentiation and the activity of mammary epithelial cells were compromised in the absence of prolactin during the periparturient period in bovine. Conversely, exogenous prolactin administration for the first 3 weeks of lactation increased milk yield and α-lactalbumin mRNA expression in cows compared with untreated animals (Wall et al., 2006; Shao et al., 2013). Prolactin is therefore crucial for complete structural differentiation of the mammary epithelium and thus for the full initiation of lactation.

Many other reports showed that prolactin plays a critical role in differentiation of the mammary epithelium and in activation of key biochemical steps involved in milk production. Firstly, prolactin highly stimulates casein synthesis. In vitro, prolactin stimulated α-casein secretion in bovine mammary epithelial cells (Sakamoto et al., 2005). In goat mammary explants, prolactin stimulated total casein synthesis (Skarda et al., 1982). In bovine mammary explants, the trio of hormones (prolactin, insulin and glucocorticoids), also called the lactogenic complex, stimulated β-casein and α-lactalbumin gene expression (Shao et al., 2013) but prolactin alone also increased β-casein mRNA expression (Choi et al., 1988; Yang et al., 2005). Insulin and glucocorticoids amplifies the lactogenic action of prolactin on mammary epithelial cells in vitro (Choi et al., 1988). Secondly, prolactin is highly implicated in milk fatty acid synthesis. It has been shown that the lactogenic complex is essential for milk-specific fatty acid synthesis from bovine mammary explants (Collier et al., 1977b). Prolactin, together with insulin and glucocorticoids, increased the expression of fatty acid synthase, acetyl-coA carboxylase and stearoyl-coA desaturase (Shao et al., 2013). Finally, prolactin greatly enhances the activity of numerous enzymes involved in milk component synthesis. In MAC-T cells, prolactin increased the activity of 2 types of acyltransferases naturally more active in lactating tissue than in non-lactating tissue (Morand et al., 1998). Prolactin, in association with insulin and glucocorticoids, is also involved in the activation of

galactosyltransferase and α-lactalbumin (Vonderhaar et al., 1973), the 2 key enzymes of lactose synthesis. In vivo, exogenous prolactin administration led to an increase in α-lactalbumin concentration in milk (Plaut et al, 1987) and to an increase in α-lactalbumin mRNA expression in mammary tissue (Wall et al., 2006; Shao et al., 2013).

2.3.3.2 The role of prolactin in galactopoiesis

Until recently, the role of prolactin in galactopoiesis in ruminants is not clear. Attempts to stimulate milk secretion during an established lactation by treatments with exogenous prolactin were not conclusive. It has been reported that intramuscular prolactin administration increased markedly plasma prolactin concentrations and decreased the milking-induced prolactin release but did not affect milk yield and milk composition both before and after the peak of lactation in Holstein cows, suggesting an absence of a galactopoietic role for prolactin in ruminants (Plaut et al., 1987). However, this may mean that endogenous prolactin release is sufficient to elicit a maximal response by the mammary gland. Alternatively, prolactin receptors could be saturated and could not be further stimulated by prolactin. Thus, above a certain threshold of concentration, prolactin would not have a stimulatory effect on milk production.

Attempts to inhibit prolactin secretion generated conflicting results according to the experimental design, to the number of individuals used, and to the type and the dose of inhibitor tested. Most of the studies used bromocriptine, a dopaminergic analogue, to suppress the release of prolactin in the blood. In bovine, several old studies reported that bromocriptine inhibited prolactin release without affecting milk yield (Karg et al., 1972; Hart, 1973; Smith et al., 1974; Peel et al. 1978). On the other hand, more recent studies with bromocriptine tended to show an inhibition of milk secretion in ruminants. When bromocriptine was administered for 8 d in established lactation, plasma prolactin concentration was reduced and milk yield was decreased for one week after treatment (Forsyth and Lee, 1993). In another report, bromocriptine treatment (5 mg per day for 5 consecutive days) decreased milk yield by 16.8% to 25.8% and affected milk composition by reducing protein and lactose content in goats (Singh et Ludri, 1999). In addition, when bromocriptine was injected prepartum, the postpartum milk production was reduced (Johke and Hodate, 1978; Akers et al., 1981a; Akers et al., 1981b). The absence of effect by bromocriptine on milk production in old studies may be due to the small number of animals studied. The different doses of the inhibitor also

certainly contributed to this discrepancy. Studies in which milk production was reduced after bromocriptine treatment may be due to higher doses of the inhibitor, by completely abrogating the prolactin release. However, it was not possible to detect a galactopoietic role for prolactin bovine, only based on works with bromocriptine.

Other observation tended to prove that prolactin is involved in the maintenance of lactation in ruminants. For instance, it has been shown that multiparous cows, producing more milk than primiparous cows, display greater serum prolactin concentrations (Koprowski and Tucker, 1973a). On the other hand, use of reserpine, a dopamine antagonist, increased serum prolactin concentration, peak milk yield and 100-day milk production in cows (Bauman et al., 1977; Collier et al., 1977a). Furthermore, a treatment with perphenazine, a D2-dopamine receptor antagonist, prevented the normal decline in milk yield in late lactation goats (Vandeputte-Van Messom and Peters, 1982). The most convincing evidence that prolactin may play a role in galactopoiesis in ruminants comes from recent studies in cows using a new inhibitor of prolactin secretion. Quinagolide is a dopaminergic agonist binding to the D2-dopamine receptor of lactotrophs in the hypothalamus, which is more specific, has a longer half-life, has fewer side effects and is 200 times more potent than bromocriptine for inhibiting lactation in rodents (Brownell, 1998; Lacasse et al., 2012). In cows in mid lactation, daily injections of quinagolide for 9 wk reduced the milking-induced prolactin release (Lacasse et al., 2011). This effect was associated with a faster decline in milk yield compared with control animals (Lacasse et al., 2011). The suppression of prolactin secretion by quinagolide decreased the transcription of α-lactalbumin and κ-casein genes in lactating cows (Boutinaud et al., 2012). Quinagolide treatment also led to a decrease in the milking-induced prolactin release in cows in late lactation, associated with a 28% reduction in milk yield during the 4 last days of lactation compared with control animals (Ollier et al., 2013). In quinagolide-treated cows, a correlation between the total amount of prolactin released during milking and milk production was reported (Lacasse et al., 2011; Ollier et al., 2013). These recent experiments demonstrated that a specific and strong inhibition of prolactin secretion was successful to elicit a depression in milk production in cows, suggesting that prolactin may play a role in galactopoiesis in cattle.

2.3.3.3 The role of prolactin during mammary gland involution

Besides its involvement in mammogenesis, lactogenesis and even galactopoiesis, prolactin plays also a critical role during mammary gland involution. This hormone acts as a survival factor for the epithelial cells by preventing apoptosis and maintaining cell activity. It was suggested that prolactin prevented mammary epithelial cell loss by potentially acting on mammary stromal cells, besides its known direct effects on mammary epithelial cells (Flint et al., 2006). This can be analogous to the indirect action of GH on cell survival through the stimulation of IGF-1 secretion by stromal cells. The inhibition of mammary epithelial cell loss by prolactin is further supported by the fact that prolactin deficiency induced involution of the rat mammary gland by increasing apoptosis and decreasing the DNA content of the gland, an effect that was prevented by prolactin administration (Travers et al., 1996). Moreover, it has been shown that prolactin injections reduced significantly the dramatic increase in IGFBP-5 observed during mammary gland involution (Tonner et al., 1997). Accorsi et al. (2002) reported that cow mammary explants cultured in a medium containing no prolactin displayed a significant increase in DNA laddering, characteristic of cellular apoptosis, and in IGFBP-5 mRNA expression compared with cow mammary explants cultured in a medium containing prolactin, GH, insulin, IGF-1, hydrocortisone, oestradiol 17β and progesterone. Prolactin is also able to counteract the increase in proteolytic activity observed during mammary gland involution. In rodents, the increase in plasmin and PA activities triggered by unilateral teat sealing or litter removal was inhibited by prolactin (Tonner et al., 2000). It has been suggested that prolactin enhances PAI-1 release by inhibiting IGFBP-5 synthesis thus leading to tPA inactivation while GH increases IGF-1 synthesis which would in turn increase PAI-1 mRNA expression (Fattal et al., 1992; Tonner et al., 2000). Moreover, the decreases in mammary gland weight and DNA content and the increase in PA concentration naturally observed during involution were all reduced by injections of prolactin in the mouse (Ossowski et al., 1979).

Furthermore, when basal prolactin concentration and the milking-induced prolactin release were inhibited by quinagolide in late lactation cows, milk production was decreased before drying-off (Ollier et al., 2013; Ollier et al., 2014). Moreover, mammary gland involution rate was markedly accelerated as it was shown by the fact that quinagolide treatment hastened the normal evolution of several involution markers such as SCC, BSA, citrate:lactoferrin molar ratio, Na^+:K^+ ratio or MMP-2 activity (Ollier et al., 2014). Therefore,

an inhibition of prolactin secretion in late lactation cows accelerated the involution process, suggesting a protecting role of prolactin against mammary gland remodelling.

In conclusion, prolactin seems to be involved in both galactopoiesis and involution in cows. Inhibiting the prolactinemic signal to either decrease milk production before drying-off or to remove an important survival factor for the mammary epithelium after drying-off would facilitate and/or hasten the involution process, consequently reducing udder health problems associated with the transitions between lactation and dry period. Furthermore, it is of interest to understand the mechanisms underlying the regulation of prolactin synthesis/secretion in order to be able to increase lactation persistency. The knowledge about prolactin regulation and activity has progressed significantly since the past decades. However, a lot of physiological aspects of the lactation regulation remain to be elucidated. In particular, it is not known how and why both basal blood prolactin concentrations and the milking-induced prolactin released are progressively decreased as lactation advances. One candidate for this gradual decrease in the prolactinemic signal over the course of lactation could be glucocorticoids.

2.3.3.4 The role of glucocorticoids during the lactation cycle

2.3.3.4.1 Glucocorticoids promotes lactogenesis and inhibits involution

Besides prolactin and oxytocin, milking induces release of glucocorticoids, notably cortisol (Koprowski and Tucker, 1973b; Lacasse and Ollier, 2014). Glucocorticoids are steroid hormones synthesized in the zona fasciculata of the cortex of the adrenal glands. Their release is stimulated by the secretion of adrenocorticotropin hormone (**ACTH**) by the anterior pituitary gland. Like prolactin, glucocorticoids are involved in preparing the mammary epithelium for active milk secretion during lactogenesis. As so, they make up a part of what is called the lactogenic complex with insulin and prolactin. Glucocorticoids are necessary with insulin to induce proper response of the mammary tissue to prolactin such as milk-specific fatty acid synthesis (Collier et al., 1977b; Carrington et al., 1983), α-casein synthesis (Sakamoto et al., 2005), activations of galactosyltransferase and α-lactalbumin, the enzymes responsible for lactose synthesis (Vonderhaar et al., 1973; Shao et al., 2013) in mammary gland explants from mice, rabbits, goats and cows. Specifically in bovine mammary explants, glucocorticoids participate with insulin and prolactin to the induction of β-casein and α-

lactalbumin gene expressions and to the regulation of genes involved in lipogenesis (Shao et al., 2013). It has also been shown that dexamethasone enhanced the prolactin-induced β-casein transcription in bovine mammary epithelial cells (Olazabal et al., 2000). Consequently, cortisol, in combination with insulin, constitutes a crucial endocrine element that enhances the tissue sensitivity to the prolactin stimulus (Skarda et al., 1982).

Interestingly, besides the fact that prolactin and glucocorticoids are released upon mammary gland stimulation through milking, secretion of glucocorticoids is also under control of the dopaminergic system. It has been shown in early lactating cows that injections of fluphenazine, a dopamine receptor antagonist, elicited a nearly 3-fold increase in serum cortisol concentration (Ahmadzadeh et al., 2006).

Glucocorticoids play also a role in maintaining tight junction integrity of the mammary gland epithelium. It has been shown in cows that an ACTH treatment caused a 6-fold increase in plasma cortisol concentration and this effect was associated with a decrease in plasma lactose and an increase in milk K^+ content, indicating a decrease in tight junction permeability (Stelwagen et al., 1997). In another experiment in cows, twice daily milking (**TM**), once daily milking (**OM**), and once daily milking with ACTH injections (OM+ACTH) were compared (Stelwagen et al., 1998). Adrenocorticotrophin treatment increased rapidly plasma cortisol concentration (Stelwagen et al., 1998). Whereas plasma concentration of lactose rose markedly in OM cows, it remained relatively stable for TM and OM+ACTH treatments (Stelwagen et al., 1998). Therefore, once daily milking increased tight junction permeability and ACTH, via the stimulation of cortisol release, reduced it (Stelwagen et al., 1998). Consequently, glucocorticoids seem to maintain the integrity of mammary epithelial cell tight junctions in vivo.

2.3.3.4.2 Glucocorticoids affect milk production

Glucocorticoids seem to be involved in the regulation of milk production in ruminants. It has already been shown that exogenous glucocorticoid administration is able to depress milk production and can affect milk composition in the cow. For instance, intramuscular injections of the glucocorticoid analogue dexamethasone induced a decrease in milk yield (Hartmann and Kronfeld, 1973) and reduced lactose, whey protein and total protein secretions in cows (Shamay et al., 2000). Moreover, glucocorticoids led to a reduction in sodium,

chloride, potassium and calcium secreted in milk during a 24 h period (Shamay et al., 2000). The negative effect of dexamethasone on milk yield seems to occur through a decreased glucose uptake from the mammary gland leading to a reduction in lactose synthesis, lactose being the major osmotic component of milk with monovalent ions (Hartmann and Kronfeld, 1973; Shamay et al., 2000). This hypothesis is further supported by a study showing that cows milked once daily and treated with ACTH displayed a higher plasma glucose concentration than untreated cows (Stelwagen et al., 1998). These authors suggested that glucose mammary uptake was reduced in ACTH-treated cows, given the fact that they displayed a higher circulating glucose concentration than the untreated group. Therefore, glucocorticoids seem to alter the glucose partitioning between the mammary gland and other organs notably by decreasing its supply to the mammary gland (Hartmann and Kronfeld, 1973).

2.3.3.4.3 Glucocorticoids influence prolactin synthesis and/or secretion

The mechanism by which glucocorticoids alter milk production may involve an interaction with prolactin synthesis or secretion. It has been shown in vitro that low doses of corticosterone applied to pituitary cells increased prolactin mRNA expression whereas high doses of corticosterone down-regulated prolactin mRNA expression (Yokoyama et al., 2008). A dual role of glucocorticoids has been reported in several experiments. For instance, it has been shown in rodents that adrenalectomy suppressed the source of glucocorticoids and inhibited the suckling-induced prolactin release (Horváth et al., 2001a), suggesting that glucocorticoids are necessary for prolactin secretion. On the other hand, dexamethasone given to control animals decreased the suckling-induced prolactin response (Horváth et al., 2001a), showing that glucocorticoids can also affect negatively prolactin secretion. Another experiment showed that both adrenalectomy and dexamethasone treatment increased significantly plasma prolactin levels in the rat and that the combination of adrenalectomy and dexamethasone treatment did not alter plasma prolactin concentrations (Horváth et al., 2001b). Domperidone, a D2 dopamine receptor antagonist, increased plasma prolactin concentration in the rat, and this increase was further enhanced by adrenalectomy or dexamethasone treatment (Horváth et al., 2001b). However, dexamethasone treatment decreased prolactin and oxytocin concentrations 15 min after suckling and reduced milk secretion in the lactating rat (Vilela and Giusti-Paiva, 2011). In conclusion, the effects of glucocorticoids on prolactin secretion are complex but it appears that they are involved at

least indirectly on the regulation of the prolactinemic signal: glucocorticoids seem to negatively regulate prolactin secretion but are necessary to maintain basal prolactin release. It appears that circulating glucocorticoid concentration and the time of the lactation period are two factors determining the glucocorticoid effects on prolactin secretion but this still need to be elucidated, and particularly in cattle.

Glucocorticoids may also act on the IGF system in bovine as it has been reported that dexamethasone treatment decreased plasma concentrations of IGF-1 and IGF-2 in Holstein cows without affecting IGFBP levels (Maciel et al., 2001).

In conclusion, it is of interest to study the influence that glucocorticoids may have on the regulation of prolactin release in the dairy cow. It would represent a new step in the understanding of the decline in prolactin secretion over the course of lactation.

2.4 Summary

The transition between lactation and a dry period is a very critical phase for the cow during which the mammary gland is highly susceptible to new intra-mammary infections. In order to improve udder health, different strategies can be used. The end of the lactation cycle, after cessation of milking, is characterized by the involution of the mammary gland which lasts about 21 days in the cow. Once involution is achieved, the gland is more resistant against bacterial infections. The first strategy would be to hasten the involution process of the gland in order to reach the resistant state of the gland more rapidly. This could be done through administration at drying-off of treatments that are known to increase tight junction permeability. Another way to increase the involution rate would be to depress prolactin secretion by modulating the photoperiod. As prolactin inhibits involution and short day photoperiod reduces prolactin concentrations in blood, a decrease in light exposure around drying-off could affect the speed of the involution process. The third strategy to improve the health status of the cow would be to reduce the occurrence of the transitions between lactation and dry period by increasing lactation persistency. As prolactin seems to be galactopoietic in cows, it is of interest to elucidate the reasons of the progressive decrease in prolactin released during milking as lactation progresses. This milking-induced prolactin release might be under the regulation by glucocorticoids.

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Chapter 3 Effects of intra-mammary infusions of casein hydrolysate, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA) and lactose at drying-off on mammary gland involution

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"Effects of intra-mammary infusions of casein hydrolysate, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA) and lactose at drying-off on mammary gland involution." Journal of Dairy Science. 97 (2014): 779-788.

3.1 Abstract

The transition from the lactation to the dry period in dairy cows is a period of high risk for acquiring new intra-mammary infections. This risk is reduced when involution of mammary glands is completed. Consequently, strategies that accelerate the involution process after drying-off could reduce the incidence of mastitis. The objective of this study was to assess the effect of 3 different treatments on mammary gland involution. Each quarter of 8 Holstein cows in late lactation was randomly assigned at drying-off to an intra-mammary infusion of casein hydrolysate (CNH; 70 mg), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA; 5.7 g), lactose (5.1 g) or saline 0.9% (control) solutions. Milk samples were collected on the last 2 d before and 1, 3, 5, 7, 10 and 14 d after the last milking for determining concentrations of mammary gland involution markers. Lactoferrin, somatic cell counts (SCC), BSA and Na^+ concentrations, as well as matrix metalloproteinase-2 and -9 activities gradually increased in mammary secretions during the first 2 wk following the last milking whereas milk citrate and K^+ concentrations decreased. As involution advanced, the Na^+:K^+ ratio increased whereas the citrate:lactoferrin ratio decreased. Compared with mammary secretions from control quarters, mammary secretions of quarters infused with CNH had higher SCC on d 1, 3, 5, and 7, and greater BSA concentrations on d 1, 3, and 5. Similarly, the CNH treatment induced a faster increase in lactoferrin concentrations, which were greater than in milk from control quarters on d 3, 5, and 7 after drying-off. Milk citrate concentrations were unaffected by CNH but the citrate:lactoferrin ratio was lower in CNH-treated quarters on d 3 and 5 than in control quarters. Moreover, CNH treatment hastened the increase in Na^+ concentration and in the Na^+:K^+ ratio on d 1. Infusion of CNH also led to an increase in proteolytic activities, with greater metalloproteinase 9 activities on d 1 and 3. The EGTA infusion increased SCC above that of control quarters on d 1 and 3 but it had no effect on the other parameters. Lactose infusion had no effect on any of the involution markers. In this study, intra-mammary infusions of CNH were the most efficient treatment to accelerate mammary gland involution, suggesting a potential role of CNH as a local milk secretion inhibitor during milk stasis.

3.2 Introduction

The transition from a lactating to a non-lactating state represents a challenge for modern dairy cows. Although milk is not removed anymore, the mammary gland continues to synthesize milk for a few days, leading to an engorgement of the gland and to milk leakage, facilitating the entry of microorganisms into the udder through the teat canal. Accordingly, the risk of new intra-mammary infections in dairy cows is enhanced during this period, especially for high-yielding cows (Rajala-Schultz et al., 2005). It has been estimated that 16.7% of quarters which were bacteriologically negative before cessation of milking become infected during the dry period, regardless of antibiotic treatments (Dingwell et al., 2002).

Mammary gland involution is a remodeling process that starts as soon as 2 d after cessation of milking in dairy cows (Holst et al., 1987) and through which the gland returns to a non-lactating state. During this process, among other physiological events, there is a reduction in the synthesis and secretion of milk components, recruitment of immune cells, and anti-bacterial proteins such as immunoglobulins and lactoferrin (Fleet and Peaker, 1978; Sordillo et al., 1987; Monks et al., 2002) as well as an increase in enzyme activities, such as plasmin, plasminogen activator (**PA**) and matrix metalloproteinases (**MMP**; Talhouk et al., 1992; Politis, 1996). Another characteristic of mammary gland involution is the increase in the permeability of the tight junctions that link the mammary epithelial cells together (Nguyen and Neville, 1998). Tight junctions are located at apical sides of epithelial cells and make the mammary epithelium impermeable to paracellular transport between interstitial fluid and milk. Tight junctions are strongly closed during lactation and become permeable during involution, thereby causing a change in mammary gland fluid composition (Nguyen and Neville, 1998), which can be used to measure the involution rate (Shamay et al., 2003).

When involution is advanced, the mammary gland is more resistant to new intra-mammary infections (Oliver and Smith, 1982). Nonnecke and Smith (1984) have reported that inhibition of *Escherichia coli* growth by whey increased significantly during the dry period and the inhibition was maximal in wheys collected d 15 of the dry period. In addition, treatments accelerating the rate of involution, such as intra-mammary infusions of colchicine, endotoxin or a combination of both, reduced the rate of new intra-mammary infections during the first week of involution (Oliver and Smith, 1982). Consequently, it would be interesting to find certain strategies which could hasten mammary gland involution process.

Intra-mammary infusions of casein hydrolysates (**CNH**) in dairy goats and dairy cows decrease milk synthesis (Silanikove et al., 2000; Shamay et al., 2002) and increase tight junction permeability (Shamay et al., 2002; Shamay et al., 2003). In goats, intra-mammary infusion of the calcium chelator ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (**EGTA**) also affected the integrity of tight junctions between mammary epithelial cells and milk secretion (Ben Chedly et al., 2010; Stelwagen et al., 1995). In Madin-Darby canine kidney (MDCK) cells, addition of EGTA to the culture milieu led to a decrease in transepithelial electrical resistance and disrupted the continuity of the tight junction network (Rothen-Rutishauser et al., 2002). In vivo, infusions of EGTA into goat mammary glands caused a decrease in milk potassium concentration, an increase in milk sodium concentration, and an increase in blood lactose concentration, as well as a decrease in milk secretion (Stelwagen et al, 1995). Further, Ben Chedly et al. (2010) have reported that intra-mammary infusions of lactose led to a disruption of the mammary epithelium. Therefore, the objective of this study was to assess the effect of intra-mammary infusions of CNH, EGTA and lactose on mammary gland involution in dairy cows. To measure the extent of involution, parameters that are known to be affected during involution were measured, such as SCC, lactoferrin, BSA, citrate, Na^+, and K^+ concentrations, and MMP activities.

3.3 Materials and methods

3.3.1 Animals and experimental design

The experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993). Eight first- to fourth-parity Holstein dairy cows in late lactation (373 ± 25 DIM at drying-off), housed in the Howard Webster Center of McGill University (Sainte-Anne-de-Bellevue, QC, Canada), were used. The animals were housed in a tie stall barn and were fed a late-lactating diet before drying-off and hay after drying-off. One week before drying-off, SCC was measured and bacterial cultures were performed for each quarter. The cows whose quarters were not infected by bacteria and contained less than 200 somatic cells per µL were selected. Cows were producing 21.2±2.3 kg of milk at drying-off.

After the last milking (d -1), the 4 quarters of each cow were randomly assigned to 1 of the following intra-mammary infusions: a 50-mL solution of 0.9% saline as a control, a 50-mL solution of distilled water containing 5.7 g of EGTA (pH=8), a 50-mL solution of distilled

water containing 5.1 g of lactose, or a 50-mL solution of saline 0.9% containing 70 mg of CNH (Volcani Center Institute of Animal Science, Israel). Casein hydrolysates were prepared as described by Shamay et al. (2003). All solutions were prepared under sterile conditions and teats were scrubbed with gauzes soaked in 70% ethanol before infusion through the teat canal using a thin, rounded plastic needle.

Two days before cessation of milking (d -2 and -1 relative to drying-off), 50 mL of milk was manually collected from each quarter at the a.m. milking. During the dry period, mammary secretions (50 mL) were manually collected on the morning on d 1, 3, 5, 7, 10, and 14 after drying-off. Milk samples were samples collected before drying-off, whereas mammary secretion samples were samples collected from the gland after drying-off. After the last sampling, on d 14, cows were infused with antibiotics (Novodry Plus, Pfizer Canada Inc., Kirkland, QC) and a teat sealant (Orbeseal, Pfizer Canada Inc., Kirkland, QC). After milk and mammary secretion collection, samples were mixed by inversion and 2 mL were used to measure SCC. The remaining fluid was skimmed by centrifugation (1900 x *g*, 4ºC, and 15 min) and stored at -20ºC until analyses of lactoferrin, citrate, BSA, Na^+, and K^+ concentrations, as well as determination of gelatinase activities.

3.3.2 SCC in milk and mammary secretions

Somatic cell count was analyzed with an automatic cell counter (DeLaval International AB, Tumba, Sweden). Mammary secretion samples were diluted with commercial skimmed microfiltered milk until SCC reached a value between 100 and 200 cells/µL.

3.3.3 Lactoferrin and citrate concentrations in milk and mammary secretions

The concentration of lactoferrin in skim milk and mammary secretions was measured by ELISA using a commercial bovine lactoferrin ELISA quantitation set (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions. Milk and mammary secretion samples were diluted to obtain a concentration of lactoferrin comprising between 31.25 and 500 ng/mL. The absorbance for each sample was measured at 450 nm using a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The intra- and inter-assay coefficients of variation were 3.0 and 3.4%, respectively.

The concentration of citrate in skim milk and mammary secretions was determined by an enzymatic assay using a commercial citrate assay kit (Megazyme International Ireland, Bray, Ireland). Before analysis, the skimmed samples were deproteinized by adding an equal volume of cold 1 *M* perchloric acid. After centrifugation (1500 x *g*, 4ºC, 10 min), they were neutralized with 1 *M* KOH. The citrate concentration was then measured according to the manufacturer's recommendations. The intra- and inter-assay coefficients of variation were 1.6 and 3.2%, respectively.

The molar citrate:lactoferrin ratio was calculated by dividing the molar concentration of citrate [citrate concentration/citrate molecular weight (192.1 g/mol)] with the molar concentration of lactoferrin [lactoferrin concentration/lactoferrin molecular weight (78,056 g/mol)].

3.3.4 BSA concentration in milk and mammary secretions

The concentration of BSA in milk and mammary secretions was analyzed by a colorimetric assay as previously described by Bouchard et al. (1999), with some modifications. Briefly, 200 µL of skim milk or mammary secretion was mixed with 450 µL of distilled water and 450 µL of a solution containing 1 volume of 1.2 m*M* of bromocresol green dissolved in 5 m*M* NaOH, 3 volumes of 0.2 *M* of succinic acid (pH 4.0), and 0.8% Brij 35. After mixing by inversion and centrifugation at 1900 x *g* for 10 min at room temperature, 150 µL of the supernatant was added to a 96-well microplate and the optical density was read at 640 nm using a SpectraMax 250 microplate reader.

3.3.5 Na^+ and K^+ concentrations in milk and mammary secretions

The concentrations of Na^+ and K^+ in skim milk and mammary secretions were analyzed by flame atomic emission spectrometry. Commercial Na^+ and K^+ reference standard solutions (Fisher Scientific, Ottawa, ON, Canada) were used to establish standard curves, ranging from 0.2 to 1 mg/mL for Na^+ and from 0.5 to 2 mg/mL for K^+. Samples were diluted in a solution containing 0.02 *N* HCl and 0.5 g/L CsCl and concentrations of Na^+ and K^+ were measured by an atomic absorption spectrophotometer (Analyst 300 Spectrometer, Perkin

Elmer Instruments, Woodbridge, ON, Canada). The intra- and inter-assay coefficients of variation were 0.35 and 0.44% respectively for Na^+ and 0.19 and 0.85% respectively for K^+.

The molar Na^+:K^+ ratio was calculated by dividing the molar concentration of Na^+ [Na^+ concentration/Na^+ molecular weight (23.0 g/mol)] with the molar concentration of K^+ [K^+ concentration/K^+ molecular weight (39.1 g/mol)].

3.3.6 Gelatinase activities of milk and mammary secretions

Gelatinase activity in milk and mammary secretions was analyzed by performing gelatin zymography. Briefly, gelatinases were separated on a 10% polyacrylamide gel containing 2 mg/mL of gelatin. Two microliters of skim milk from d -2 and -1, and 1 µL of skimmed mammary secretions from d 1, 3, 5, and 7 or 0.5 µL of skimmed mammary secretions from d 10 and 14 were used for migration. Migration was performed at 4°C during 20 min at 90 V and during 150 min at 150 V. The gels were then washed with gentle shaking for 30 min in 100 mL of 2.5% Triton X-100 and washed for 30 min in 50 mL of developing buffer (50 m*M* Tris base, 200 m*M* NaCl, 5 m*M* $CaCl_2$, and 0.02% Brij 35) before incubation for 22 h at 37°C in developing buffer. The gels were stained with a 50-mL solution containing 0.5% of Coomassie blue G-250, 40% methanol, and 10% acetic acid, and then washed with gentle shaking 3 times for 30 min with a 50-mL solution containing 50% methanol, 10% acetic acid and 40% distilled water. Pictures of the gels were taken using a FluorChem SP imaging system (Alpha Innotech, San Leandro, CA) and integrated density of each band (pixel intensity x area) was analyzed with AlphaEase FC Software (Alpha Innotech). Due to the factor of dilution, integrated density of bands from d 1, 3, 5, and 7, and bands from d10 and 14 were multiplied by 2 and 4, respectively.

3.3.7 Statistical analysis

Data were analyzed by ANOVA using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Time was used as a repeated effect and quarter (treatment) was used as the subject. When variances were not homogeneous, data were log_{10}-transformed before analyses.

Differences were considered statistically significant when $P \leq 0.05$. Orthogonal contrasts were performed to compare the effect of each treatment to control.

3.4 Results

Before drying-off, SCC, lactoferrin, citrate, BSA, Na^+ and K^+ concentrations, and MMP activities were similar for all treatments. Somatic cell counts increased ($P < 0.001$) after drying-off and an interaction was observed between time and treatments ($P < 0.001$; Figure 3.1). Intra-mammary CNH infusion induced a faster increase in SCC, which was higher ($P < 0.05$) than that of control quarters from d 1 to d 7. Infusion of EGTA increased SCC above that of control quarters at d 1 and 3 ($P < 0.05$). Lactose infusion did not affect SCC at any time.

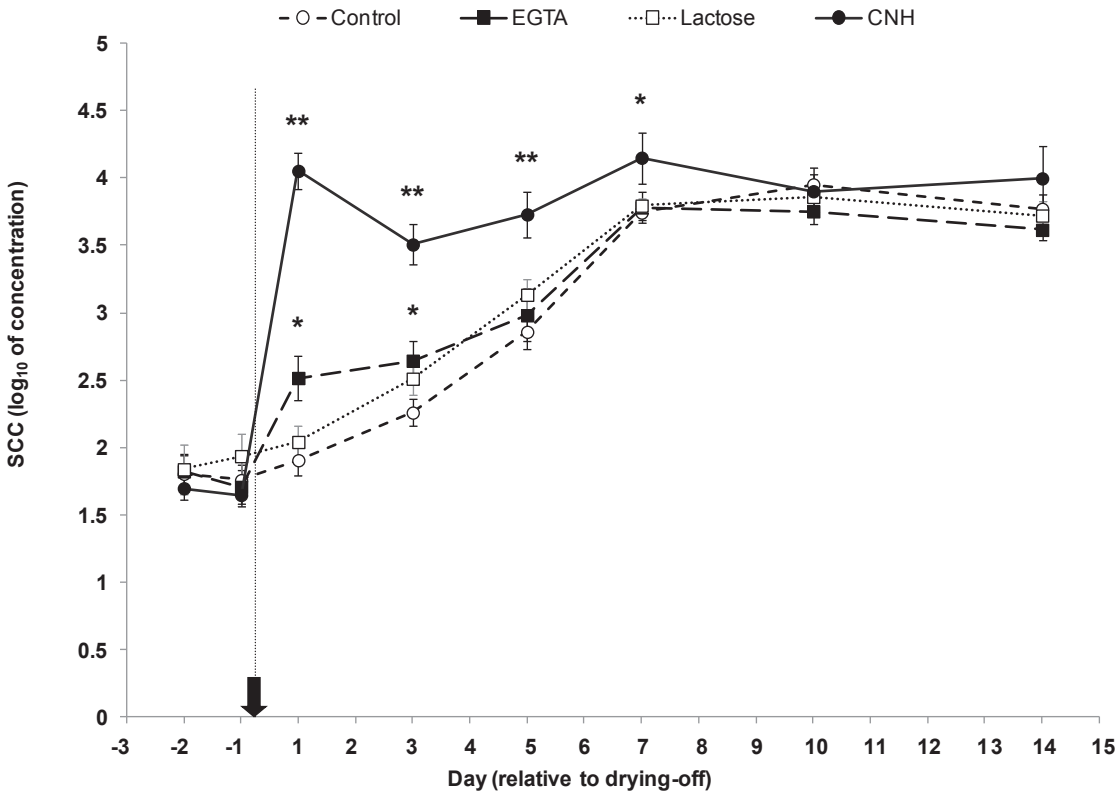

Figure 3.1. Somatic cell count (SCC) in milk and mammary secretions (cells/µL) from quarters of dairy cows (n=8) infused with a casein hydrolysate (CNH), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA), lactose or saline (Control) at drying-off. The time of the intra-mammary infusions is represented by the black arrow. Data are presented as least squares means ± SEM of log_{10}-transformed values and significant differences relative to the control are indicated as follows: *$P < 0.05$, **$P < 0.01$

Similarly to SCC, concentrations of BSA in mammary secretions increased (P < 0.001) after drying-off and an interaction was observed between time and treatments (P < 0.001; Figure 3.2). Intra-mammary infusions of CNH hastened the increase in BSA concentrations in mammary secretion, which were higher (P < 0.01) than those of control quarters from d 1 to d 5 and tended to be higher on d 7 (P = 0.086). The EGTA and lactose infusions did not affect BSA concentrations.

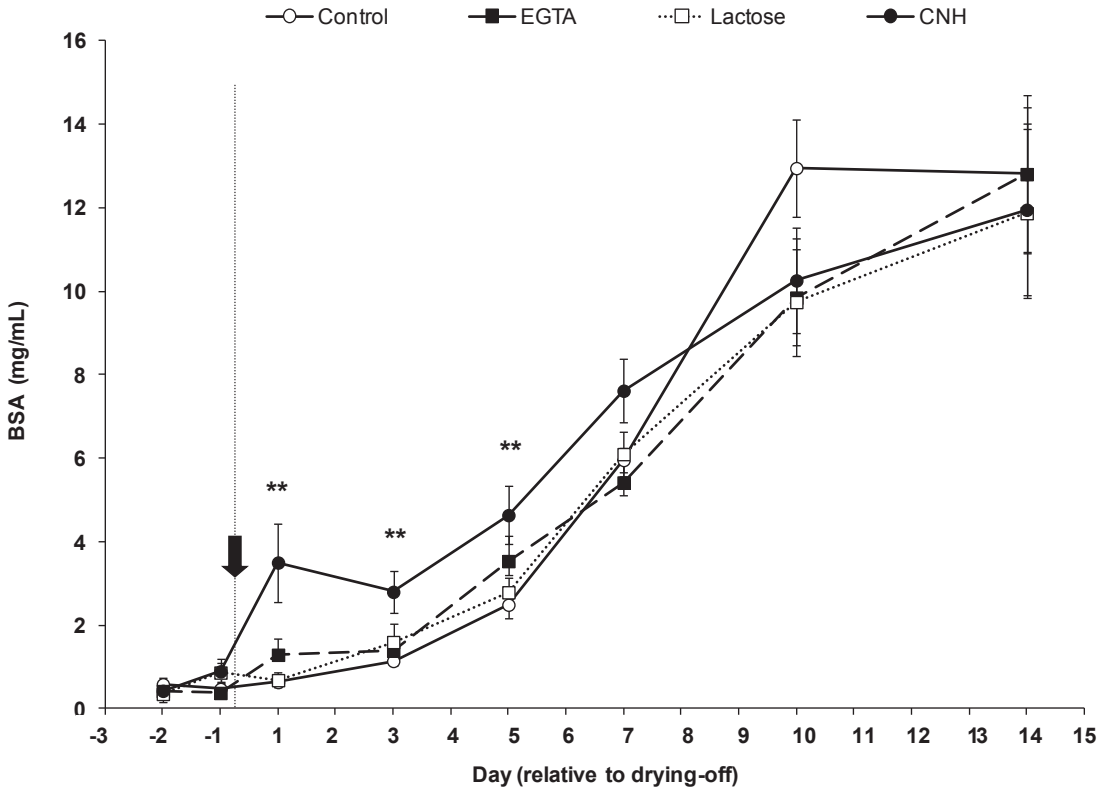

Figure 3.2. Bovine serum albumin concentration in milk and mammary secretions from quarters of dairy cows (n=8) infused with a casein hydrolysate (CNH), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA), lactose or saline (Control) at drying-off. The time of the intra-mammary infusions is represented by the black arrow. Data are presented as least squares means ± SEM and significant differences relative to the control are indicated as follows: $^{**}P < 0.01$.

Lactoferrin concentrations increased (P < 0.001) gradually in mammary gland secretions, and a trend existed for an interaction between time and treatments (P = 0.08; Figure 3.3). Once again, CNH intra-mammary infusions led to a faster increase in lactoferrin concentrations in mammary secretions, which were higher than those of control quarters from d 3 to d 7 (P < 0.05). In contrast, concentrations of citrate in mammary secretions varied over time but were not affected by any of the treatments (data not shown). The citrate:lactoferrin

ratio decreased ($P < 0.001$) during involution, and a trend was observed for an interaction between time and treatments ($P = 0.08$; data not shown). This ratio was lower in CNH-treated quarters on d 3 and 5 ($P < 0.05$) than in control quarters. The EGTA and lactose intra-mammary infusions did not affect concentrations of lactoferrin and citrate or the molar citrate:lactoferrin ratio.

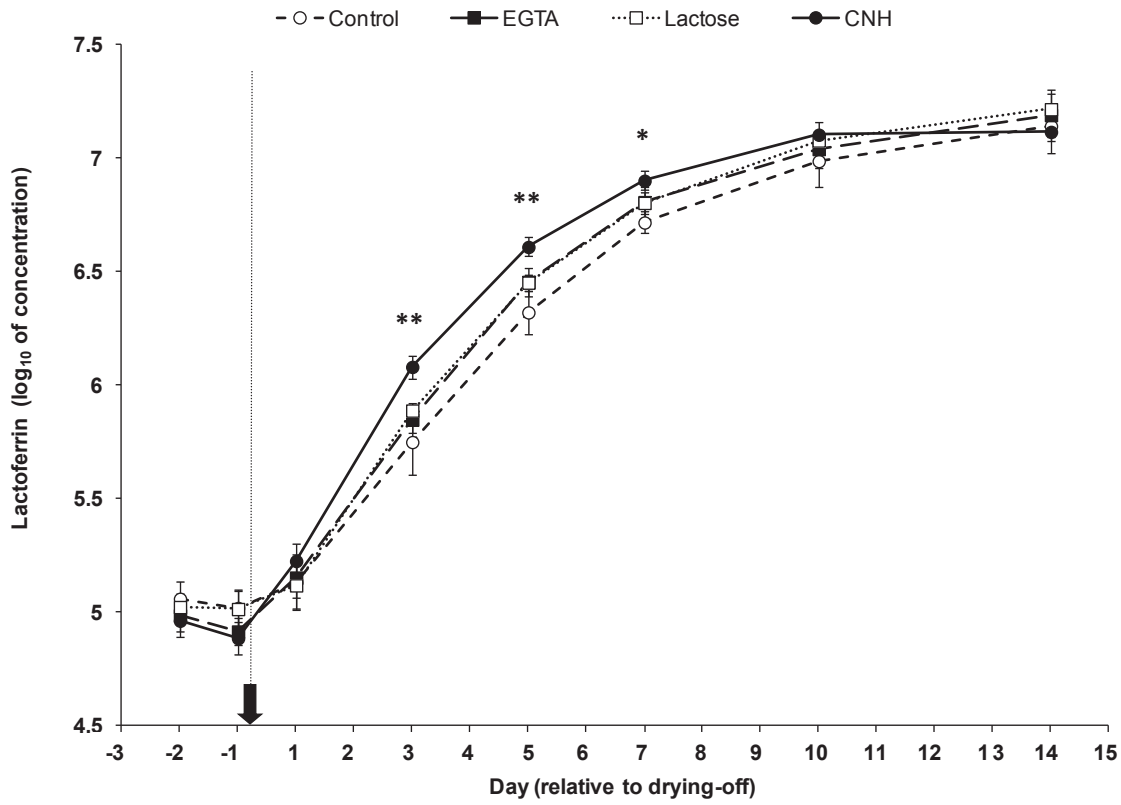

Figure 3.3. Lactoferrin concentration in milk and mammary secretions (ng/mL) from quarters of dairy cows (n=8) infused with a casein hydrolysate (CNH), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA), lactose or saline (Control) at drying-off. The time of the intra-mammary infusions is represented by the black arrow. Data are presented as least squares means ± SEM of $\log_{10}$-transformed values and significant differences relative to the control are indicated as follows: $*P < 0.05$, $**P < 0.01$.

Potassium and sodium concentrations in mammary gland fluids exhibited 2 different patterns. After cessation of milking, K^+ concentrations in mammary secretions decreased ($P < 0.001$; data not shown in the original paper but shown in the supplemental figures in this thesis), whereas Na^+ concentration increased ($P < 0.001$; data not shown in the original paper but shown in the supplemental figures in this thesis). Treatments did not affect K^+ concentration, but an interaction existed between time and treatments ($P < 0.001$) for Na^+ concentration. Infusion of CNH hastened the increase in Na^+ concentration, which was higher in CNH-treated quarters than in control quarters on d 1 ($P < 0.05$) and tended to be higher on d 3 ($P = 0.06$). The Na^+:K^+ ratio increased ($P < 0.001$) during involution, and an interaction was observed between time and treatments ($P < 0.05$; data not shown in the original paper but shown in the supplemental figures in this thesis). The ratio was higher in CNH-treated quarters on d 1 ($P < 0.05$) than in control quarters. The Na^+ and K^+ concentrations as well as Na^+:K^+ ratio were not affected by EGTA or lactose infusions.

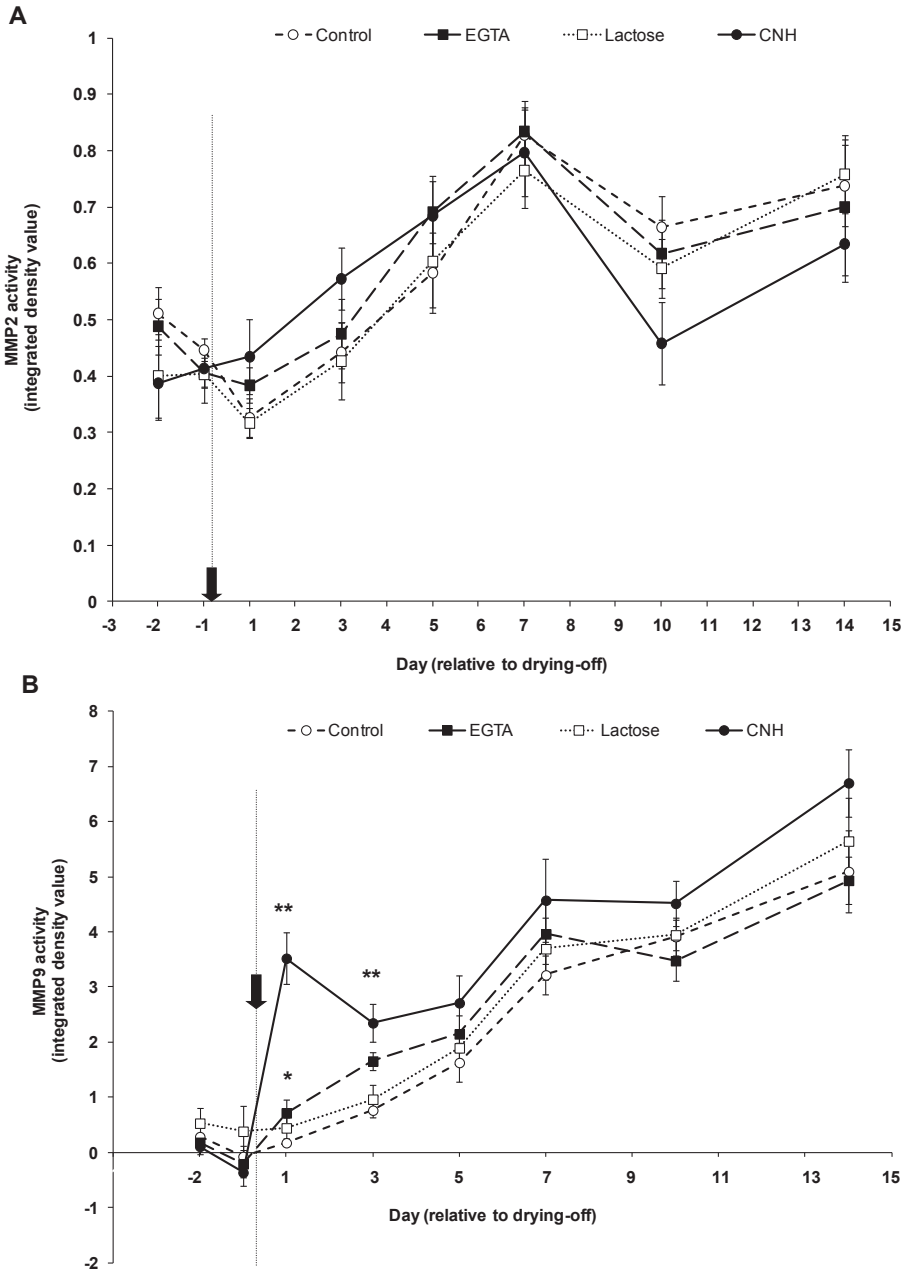

Figure 3.4. Matrix metalloproteinase-2 (MMP-2; A) and matrix metalloproteinase-9 (MMP-9; B) activities in milk and mammary secretions from quarters of dairy cows (n=8) infused with a casein hydrolysate (CNH), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA), lactose or saline (Control) at drying-off.
The time of the intra-mammary infusions is represented by the black arrow. Data are presented as least squares means ± SEM of integrated density values and significant differences relative to the control are indicated as follows: $*P < 0.05$, $**P < 0.01$.

Milk and mammary secretion proteolytic activities were assessed by measuring gelatinase activity by zymography. Activities of MMP-2 (Figure 3.4A) and MMP-9 (Figure 3.4B) increased ($P < 0.001$) progressively after drying-off. Treatments did not affect MMP-2 activity, but an interaction was observed between time and treatments ($P < 0.001$) for MMP-9 activity. The increase in MMP-9 activity after cessation of milking was hastened by CNH treatment. Indeed, MMP-9 activity was higher ($P < 0.001$) in mammary secretions from CNH-treated quarters compared to those from control quarters on d 1 and 3, and tended to be higher on d 5 ($P = 0.06$). Intra-mammary infusion of EGTA increased MMP-9 activity on d 3 ($P < 0.05$). Lactose treatment did not affect MMP-9 activity.

3.5 Discussion

The objective of the present study was to test whether intra-mammary infusions of CNH, EGTA and lactose, which may increase tight junction permeability, could affect the speed of mammary gland involution process. Different cellular or molecular markers related to mammary gland involution were measured to assess its rate of occurrence.

One of the major characteristic of mammary gland involution is epithelium tight junction impairment. Tight junctions, which seal mammary epithelial cells at their apical sides, allow the delimitation between two different compartments. In the lumen of the alveoli above the apical side of the epithelium, milk is rich in K^+ and milk specific proteins such as caseins and α-lactalbumin, and low in Na^+ and blood components such as BSA (Nguyen and Neville, 1998). Conversely, the interstitial fluid below the basolateral side of the epithelium is rich in Na^+ and BSA, and low in K^+ and milk specific proteins (Nguyen and Neville, 1998). During an established lactation, mammary tight junctions are closed and prevent paracellular transport between mammary epithelial cells (Fleet and Peaker, 1978). In contrast, tight junctions become leaky during involution, and milk components can pass through the epithelium to the interstitial fluid and vice-versa (Fleet and Peaker, 1978). Mammary tight junctions are impaired as soon as 18 h and 21 h of milk stasis in cows and goats, respectively (Stelwagen et al., 1994; Stelwagen et al., 1997). This opening explains the increase in milk Na^+ and BSA concentrations and the decrease in milk K^+ concentration. In our study, CNH treatment hastened the increase in BSA concentration until 5 d after cessation of milking. Although CNH infusion did not affect K^+ concentration, this treatment hastened the increase in Na^+ concentration and in Na^+/K^+ ratio on d 1. Therefore, we suggest that mammary tight

junction integrity was compromised more rapidly in the CNH-treated quarters than in the control quarters after drying-off. These results are concordant with previous studies in which CNH intra-mammary infusions were performed in lactating goats and late lactation cows. Indeed, repeated doses of CNH infused in lactating goats (4 doses of 300 mg) induced almost a 5-fold increase in milk BSA and Na^+ concentrations and a rapid decrease in milk K^+ concentration (Shamay et al., 2002). In cows, repeated doses (6 doses of 67.5 mg) of CNH intra-mammary infusions before drying-off mimicked the involution process by increasing milk BSA and Na^+ concentrations and by decreasing milk K^+ concentration (Shamay et al., 2003). In our study, we demonstrated that a single dose of 70 mg CNH infused at drying-off was able to increase tight junction permeability in the cow. In contrast, infusions of EGTA and lactose did not affect BSA, Na^+ and K^+ concentrations in mammary secretions. Therefore, CNH infusion was the most effective treatment to affect the integrity of mammary tight junctions.

Changes in lactoferrin and citrate concentrations represent two additional indicators for mammary gland involution. Lactoferrin concentrations naturally increase in mammary secretions during the first week of involution, reach a peak around 14 d and remain high throughout the dry period (Nonnecke and Smith, 1984; Sordillo et al., 1987). Conversely, citrate concentrations and the citrate:lactoferrin ratio decrease during the first 7 d of involution and remain low during the dry period (Sordillo et al., 1987). The fact that citrate concentrations decrease after cessation of milking is indicative of a reduction of the secretory activity of alveolar cells. In our study, none of the treatments affected citrate concentrations. This may suggest that mammary epithelial cell secretory activity was not affected by the treatments. However, CNH intra-mammary infusion hastened the increase in lactoferrin and the decrease in citrate:lactoferrin ratio on d 3, 5 and 7, and on d 3 and 5, respectively. These results suggest that mammary involution has been accelerated by CNH.

In all the treated quarters, SCC increased after cessation of milking and reached a plateau around d 7 of the dry period. An increase in total leukocyte concentrations in mammary secretions is characteristic of active involution (Hurley, 1989). Sordillo et al. (1987) reported that SCC naturally increases in mammary secretions from the last milk removal to at least d 7 of involution. The rise in SCC observed during mammary gland involution is mainly due to recruitment of immune cells, particularly PMNL and macrophages (Monks et al, 2002). These immune cells are involved in ingestion and clearance of cellular debris and residual milk components such as casein micelles and lipid droplets (Tatarczuch et

al., 2000; Monks et al., 2002; Atabai et al., 2007). The faster increase in SCC with CNH intra-mammary infusions provides additional evidence that the involution process was accelerated by this treatment.

Another feature of active involution is the increase in the activity of different proteases involved in the degradation of the extracellular matrix and basement membrane components. Matrix metalloproteinase 2 and MMP-9 are 2 proteases that are able to degrade the basement membrane (Matrisian, 1990). Matrix metalloproteinase 2 is mostly produced by the basal myoepithelial cells and epithelial cells and its activity increases during rodent mammary gland involution (Dickson and Warburton, 1992; Talhouk et al., 1992) and during gradual involution in cows (Miller et al., 2006). Matrix metalloproteinase 9 levels in mammary gland are low during lactation and increase markedly during involution in the mouse (Lund et al., 2000). In the present study, MMP-2 activity increased during the first week of involution but this increase was not affected by any of the treatments. Matrix metalloproteinase 9 activity also increased as involution advanced and CNH treatment hastened this rise on d 1 and 3. This increase in MMP-9 activity could be related to the faster increase in SCC in CNH-treated quarters. Indeed, it has been shown that PMNL represent the main source of MMP during mastitis (Merzhad et al., 2005) and that MMP-9 activity is strongly associated with SCC (Miller et al., 2006). Previous studies have reported that activities of PA and plasmin were increased by CNH intra-mammary infusions in goats (Shamay et al., 2002) and in cows (Shamay et al., 2003). Plasmin is a proteolytic enzyme naturally present in milk and is predominantly found in its inactive form, plasminogen (Politis, 1996). The conversion of plasminogen into plasmin is regulated by PA and both plasmin and PA activities are increased during milk stasis (Politis, 1996). Moreover, plasmin may activate different pro-MMP to activate MMP such as MMP-9 (Lijnen et al., 1998). Therefore, the increase in MMP activities in CNH-treated quarters in the current study could be related to an increase in plasmin and PA activities.

Globally, the results from this study suggest that CNH infusion can hasten mammary gland involution, but the mechanism is still elusive. Observations from experiments where differential milking frequency (Sorensen et al., 2001; Bernier-Dodier et al., 2010) or unilateral milk stasis (Tremblay et al., 2009) were applied indicate the presence of a local mechanism of regulation for mammary gland involution. Peaker and Wilde (1996) have postulated the existence in milk of a low-molecular-weight protein with an autocrine inhibitory activity on mammary epithelial cells. The increase in proteolytic activity observed during milk stasis

(Talhouk et al., 1992; Politis, 1996) could generate casein breakdown products that are able to trigger a local inhibition of milk synthesis. It has been shown that the casein breakdown products released by the hydrolysis of β-casein by plasmin and containing O-phospho-L-serine residues could block the activity of K+ channels located at the apical side of the mammary epithelial cells (Silanikove et al., 2000). The blockade of K^+ channels by CNH could affect mammary epithelial cell secretory activity. Another possible explanation for CNH-induced impairment of mammary tight junctions could be reduced extracellular calcium levels. It has been shown in dairy goats that intra-mammary infusions of CNH led to reduction in milk calcium concentrations (Shamay et al., 2002). In mouse mammary epithelial cells in which extracellular calcium was depleted, junction organization was destabilized and junction continuity was disrupted (Pitelka et al., 1983). Intra-mammary infusion of EGTA, a chelator of calcium, led to a transient disruption of tight junction integrity in goats (Stelwagen et al., 1995). Therefore, CNH could impair the integrity of the mammary epithelium by acting on both K+ channels and calcium availability.

Intra-mammary infusion of EGTA appears to weakly stimulate mammary gland involution. In goat, intra-mammary infusion of EGTA solution led to a decrease in milk secretion and to an impairment of tight junction integrity (Stelwagen et al., 1995; Ben Chedly et al., 2010). The absence of effect of EGTA in the present study on parameters related to tight junction status, such as Na^+, K^+, or BSA concentrations was probably due to the smaller amount of the substance infused. Stelwagen et al. (1995) have observed that an infusion of EGTA reaching a final concentration of 68 m*M* when diluted with residual milk was able to decrease milk secretion and to cause tight junction disruption. However, these authors reported little effects at a final EGTA concentration of 16 m*M*. In the present study, 50 mL of a 300 m*M* EGTA solution was infused per quarter. Assuming that approximately12% of milk remains in the gland after milking (approximately, volume of 0.36 L per quarter; Knight et al., 1994), a concentration of 42 m*M* was reached in our experiment. Moreover, as the experiment was carried out in dairy cows, the minimal effective EGTA dose to cause mammary tight junction disruption could have been higher than in goats. However, EGTA intra-mammary infusions led to a faster increase in SCC on d 1 and 3 compared with the control quarters. Moreover, EGTA infusions increased MMP-9 activity on d 3 compared with the control quarters. These results suggest that the low EGTA dose, even if not high enough to impair tight junction integrity, could have increased the recruitment of somatic cells.

No effect of lactose intra-mammary infusions was observed in this study. This treatment was tested according to the works in the goat by Ben Chedly et al. (2010) who have noticed that 4 intra-mammary infusions of 32 mL lactose (300 m*M*) induced a decrease in milk production and milk lactose, milk fat and milk casein contents. Moreover, α-lactalbumin and κ-casein mRNA were less expressed, suggesting that the activity of mammary epithelial cells was reduced. In addition, mammary tight junction integrity seemed to be impaired, as milk K^+ concentration was decreased and milk BSA concentration tended to increase (Ben Chedly et al., 2010). The possible effects of lactose on tight junction integrity were reinforced by an *in vitro* experiment in which the transepithelial electrical resistance of cultured mammary epithelial cells was decreased by lactose. (Ben Chedly et al., 2010). In the present study, cows received a single 50 mL infusion of a 300 m*M* lactose solution, leading to a final concentration in the treated quarter of approximately 42 m*M*. The absence of effect on the parameters related to tight junction integrity or to mammary gland involution might be due to a lower dose of lactose. In the goat, 4 infusions of 32 mL of a 300 m*M* lactose solution have been used (Ben Chedly et al., 2010). Once again, the minimal effective dose for cows would be higher than for goats. However, it remains unclear how lactose, the main osmotic component in milk, could stimulate mammary gland involution.

In conclusion, intra-mammary infusion of CNH was the most efficient treatment to accelerate mammary gland involution in our study. This treatment could help the gland to be fully involuted more rapidly and, therefore, to be more resistant to intra-mammary infections. Further studies should be conducted to evaluate the effects of CNH intra-mammary infusion on the immune system of the dairy cow and on the health status of the mammary gland.

3.6 Aknowledgements

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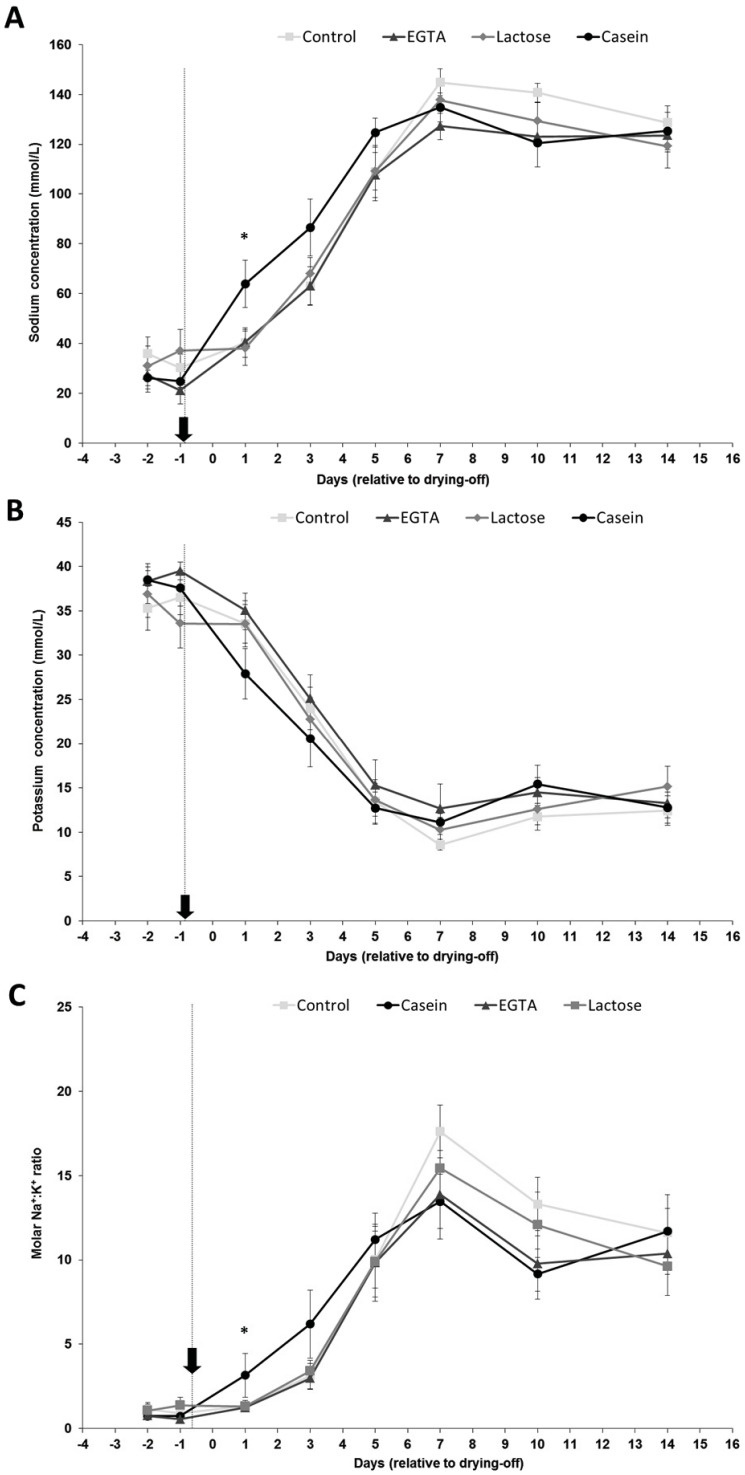
Supplemental figures



Sodium concentration (A), potassium concentration (B) and the molar Na^+:K^+ ratio (C) in milk and mammary secretions from quarters of dairy cows (n=8) infused with a casein hydrolysate (CNH), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraactic acid (EGTA), lactose or saline (Control) at drying-off. The time of the intra-mammary infusions is represented by the black arrow. Data are presented as least squares means ± SEM and significant differences relative to the control are indicated as follows: $*P < 0.01$.

Connective text

In Chapter 3, we investigated the effects of three different treatments that are known to alter tight junction permeability on the speed of the mammary gland involution process in dairy cows. Whereas EGTA and lactose intra-mammary infusions did not significantly affect the involution rate, intra-mammary infusions of casein hydrolysates at drying-off clearly hastened mammary gland involution. These results not only make casein hydrolysates a potential tool to facilitate drying-off but also make it a probable candidate as a milk component triggering mammary gland involution.

In Chapter 4, we would like to evaluate the effects of short day photoperiods and oral administration of melatonin from 2 weeks before to 2 weeks after the cessation of milking on mammary gland involution speed in dairy cows. Short day photoperiods are known to decrease both milk production and prolactin secretion in ruminants. Melatonin is a hormone secreted by the pineal gland in response to the perception of darkness and has previously been tested to mimic short day photoperiod in mammals. We would like to determine whether those treatments can depress milk yield prior cessation of milking and thus, facilitate drying-off, and whether they can accelerate mammary gland involution through an inhibition of prolactin secretion.

Chapter 4 Effects of photoperiod modulation and melatonin feeding around drying-off on bovine mammary gland involution

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4.1 Abstract

In modern dairy cows, the risk that the mammary gland will acquire new intramammary infections is high during the transition from lactation to the dry period, because of udder engorgement and altered immune functions. Once the gland is fully involuted, it becomes much more resistant to intramammary infections. Therefore, strategies that depress milk yield before drying-off and accelerate the involution process after drying-off could be beneficial for udder health. The objective of this study was to assess the effect of photoperiod manipulation and melatonin feeding from 14 d before to 14 d after drying-off on the speed of the involution process. Thirty Holstein cows in late lactation were allocated to one of the following treatments: (1) a long-day photoperiod (16 h of light:8 h of darkness); (2) a short-day photoperiod (8 h of light:16 h of darkness), and (3) a long-day photoperiod supplemented by melatonin feeding (4 mg/kg body weight). Milk and blood samples were collected on d −26, −19, −12, −5, −1, 1, 3, 5, 7, 10, and 14 relative to the last milking to determine concentrations of mammary gland involution markers and serum prolactin. Additional blood samples were taken around milking on d −15, before the start of the treatments, and on d −1, before drying-off, to evaluate the treatment effects on milking-induced prolactin release. The short-day photoperiod slightly decreased milk production and basal prolactin secretion during the dry period. The milking-induced prolactin surge was smaller on d −1 than on d −15 regardless of the treatment, although this effect was greater in the cows exposed to the short-day photoperiod than in the two other groups. Lactoferrin concentration, somatic cell count, and BSA concentration as well as matrix metalloproteinase-2 and -9 activities increased in mammary secretions during the first 2 wk of the dry period, whereas milk citrate concentration and the citrate:lactoferrin molar ratio decreased. The rates of change of these parameters were unaffected by the treatments, except for matrix metalloproteinase-9 activity, which tended to be greater in the cows exposed to the short-day photoperiod. The long-day photoperiod supplemented by melatonin feeding did not affect milk production, prolactin secretion, or mammary gland involution. Under the conditions in this study, photoperiod modulation and melatonin feeding did not appear to affect the rate of mammary gland involution.

4.2 Introduction

After cessation of milking, the bovine mammary gland continues to synthesize milk components during the first days of the dry period, and milk accumulates in the gland. In modern high-producing dairy cows, this accumulation may cause engorgement of the udder, leading to milk leakage and facilitating the entry of microorganisms. The first days of drying-off are therefore critical for dairy cows because of the high susceptibility to contract new IMI. It has been established that the risk of IMI at calving increases by 77% for every 5 kg of milk produced above 12.5 kg at the time when milking is stopped (Rajala-Schultz et al., 2005). Dingwell et al. (2002) estimated that 16.7% of quarters that are bacteriologically negative before the cessation of milking become infected during the dry period regardless of antibiotic treatments. The beginning of the dry period is also the time of active mammary gland involution. Active involution is the remodeling process that takes place at the end of the lactation period after the cessation of milking or suckling in female mammals and through which the gland returns to a nonlactating state. In cows, this process starts as soon as 2 d after the last milk removal (Holst et al., 1987) and seems to be complete after approximately 21 d (Hurley, 1989; Akers et al., 1990). When involution is advanced, the mammary gland becomes much more resistant to new IMI (Oliver and Smith, 1982). Consequently, a strategy that speeds up the involution process would be a valuable tool to improve mammary gland resistance and udder health around drying-off. Indeed, accelerating the involution rate by intramammary infusions of colchicine, endotoxin, or a combination of both was shown to reduce the frequency of pathogen isolation during the first 4 wk of involution and the rate of new IMI during the first week of involution (Oliver and Smith, 1982).

Prolactin plays a survival role during mammary gland involution by inhibiting the increase in metalloproteinase (**MMP**) expression and preventing mammary epithelial cell apoptosis (Accorsi et al., 2002; Flint et al., 2006). In rats, a deficiency in prolactin induced the involution process (Travers et al., 1996). Furthermore, there is compelling new evidence that prolactin plays a galactopoietic role during bovine lactation (Wall et al., 2006; Lacasse et al., 2011, 2012). Therefore, the inhibition of the prolactin signal around drying-off may help to decrease milk production before the start of the dry period and to hasten mammary gland involution by removing the inhibition exerted by prolactin. It was already shown that the inhibition of prolactin secretion by a dopaminergic agonist, quinagolide, successfully

decreased milk production in cows (Lacasse et al., 2011) and hastened bovine mammary gland involution (Ollier et al., 2013).

Modulating the photoperiod, which is the relative duration of light and dark exposure within a day, affects both prolactin secretion and milk production. Ewes (Morrissey et al., 2008), goats (Garcia-Hernandez et al., 2007), and cows (Peters et al., 1981) exposed to a long day photoperiod (**LDPP**; more than 16 h of light/d) during lactation produce more milk than those exposed to a short day photoperiod (**SDPP**; less than 12 h of light/d). When the photoperiod is modulated during the dry period, the effects on milk production during the subsequent lactation are different. Cows exposed to SDPP during the dry period produce more milk during the following lactation than cows exposed to LDPP do (Miller et al., 2000; Auchtung et al, 2005; Lacasse et al., 2014). During either lactation or the dry period, LDPP stimulated prolactin secretion whereas SDPP reduced it (Dahl et al., 1997; Miller et al., 2000; Auchtung et al., 2005). Moreover, treatment with melatonin, which is a hormone synthesized during darkness by the pineal gland, induced a reduction in both milk yield and circulating prolactin concentration (Auldist et al., 2007). Therefore, exposing cows to SDPP during late lactation or treating them with melatonin during late lactation could decrease milk production before drying-off and reduce the prolactin signal, thus facilitating mammary gland involution.

The objective of this study was to evaluate the effect of photoperiod manipulation, and particularly SDPP, and of melatonin feeding on the speed of the involution process. Different involution markers, such as SCC and lactoferrin, BSA, and citrate concentrations in milk and mammary secretions, as well as matrix metalloproteinase-2 (**MMP-2**) and -9 (**MMP-9**) activities, were determined in order to assess the extent of the involution process.

4.3 Materials and methods

4.3.1 Animals and experimental design

The experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993) and approved by the Animal Ethics Committee of McGill University. A total of 30 first- to third-parity Holstein dairy cows in late lactation (327 ± 10 DIM at the start of the treatments) were used in this experiment. The cows were

assigned to 1 of 3 groups of 10 animals according to their milk production, parity, and SCC. Before the treatments, the cows were exposed to LDPP (16 h of light:8 h of darkness). Each group received 1 of the following treatments: (1) LDPP; (2) SDPP (12 h of darkness:1 h of light:4 h of darkness:7 h of light); (3) LDPP supplemented with melatonin feeding (**LDPP+MEL**; 4 mg/kg BW). In the SDPP group, 1 h of light had been inserted during the 16 h of darkness schedule in order to allow milking. The cows exposed to SDPP were housed in a room separate from the rest of the herd. The lights in that room were controlled by an automatic timer and were on from 0700 to 0800 h and from 1200 to 1900 h. Each treatment started 14 d before drying-off and lasted 14 d after drying-off, for a total duration of 28 d. Melatonin (Sigma-Aldrich Canada Co., Oakville, ON) was dissolved in sterile distilled water (1 g/L), and a sufficient volume of the melatonin solution to reach a concentration of 4 mg/kg BW was mixed into the diet of the LDPP+MEL cows 1 h after the morning milking.

The cows were milked twice daily at around 0700 and 1800 h, and milk yield was recorded during the last 4 wk before drying off. The cows exposed to SDPP were moved to the regular barn for milking only. However, in order to avoid intra-mammary infections and to maintain health of the cows engaged in the experiment, an extra milking was performed on d 2-3 of the dry period to release udder pressure and to reduce mammary gland engorgement. The cows were fed ad libitum a late-lactation diet containing (on a DM basis) 59.0% corn silage, 3.1% dry hay, 26.5% corn grain, 6.0% soybean meal, 3.1% nonmineral supplement, and 2.3% mineral supplement. After drying-off, the cows were fed only dry hay until d 14.

4.3.2 Sample collections

Milk samples (50 mL) were manually collected before drying-off on d −26 and −19 (before treatment) and d −12, −5, and −1 (after the start of treatments) from the 4 quarters before the morning milking. Mammary secretion samples (between 25 and 50 mL depending on the volume of harvestable milk) were also collected in the same way on d 1, 3, 5, 7, 10, and 14 after drying-off. After collection, the milk and mammary secretion samples from the quarters were centrifuged (1,900 × *g*, 4°C, 15 min), defatted, and stored independently at −20°C until determination of lactoferrin, citrate, and BSA concentrations and MMP-2 and MMP-9 activities. After the last mammary secretion sampling, on d 14, the cows were infused

with antibiotics (Novodry Plus; Zoetis Canada Inc., Kirkland, QC) and an internal teat sealant (OrbeSeal; Zoetis Canada Inc.).

On the same days as the milk and mammary secretion samples were collected, caudal blood samples (10 mL) were taken 30 min before the morning milking. The blood samples were left at room temperature for 2 h before being centrifuged (1,900 × *g*, 4°C, 15 min). Serum was harvested and stored at −20°C until determination of prolactin concentration.

On d −15, just before the beginning of the treatments, and on d −1, just before drying-off, a series of blood samples were collected before, during, and after the evening milking (−30, −20, −10, 0, 3, 5, 7, 10, 15, 20, 25, 30, 40, and 60 min relative to the start of milking) through a catheter (Angiocath intravenous catheter; BD, Mississauga, ON) inserted into the jugular vein. After collection, the serum samples were prepared as described above and stored at −20°C until determination of prolactin concentration. Milk and mammary secretions of the healthiest quarter were then analyzed for each cow.

4.3.3 Sample analyses

Somatic cell counts were measured using an automatic cell counter (DeLaval International AB, Tumba, Sweden). After drying-off, the mammary secretion samples were diluted with commercial microfiltered skim milk until the SCC reached a value between 100 and 200 cells/µL.

Concentrations of BSA, lactoferrin, and citrate in milk and mammary secretions were measured as previously described (Ponchon et al., 2014). Before each analysis, milk and mammary secretion samples (2 mL) were centrifuged (1,900 × *g*, 4°C, 15 min). The concentration of BSA in milk and mammary secretions was analyzed by a colorimetric assay. The intra- and interassay coefficients of variation (**CV**) were 3.82% and 7.74%, respectively. The concentration of lactoferrin in milk and mammary secretions was measured by ELISA using a commercial bovine lactoferrin ELISA quantitation set (Bethyl Laboratories Inc., Montgomery, TX). The intra- and interassay CV were 2.08% and 8.95%, respectively. The concentration of citrate in milk and mammary secretions was measured by an enzymatic assay

using a commercial citrate assay kit (Megaenzyme International Ireland, Bray, Ireland). The intra- and interassay CV were 1.87% and 6.46%, respectively.

As an indicator of the progress of involution, the citrate:lactoferrin molar ratio was calculated for each sample by dividing the molar concentration of citrate (citrate concentration/citrate molecular weight [192.1 g/mol]) by the molar concentration of lactoferrin (lactoferrin concentration/lactoferrin molecular weight [78,056 g/mol]).

Gelatinase (MMP-2 and MMP-9) activities in milk and mammary secretions were analyzed by gelatin zymography as previously described by Ponchon et al. (2014).

The concentration of prolactin in serum was determined by a radioimmunoassay as described by Bernier-Dodier et al. (2011). Bovine prolactin, rabbit antiserum specific for bovine prolactin, and goat anti-rabbit gamma globulin were purchased from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). The intra- and interassay CV were 4.20% and 9.47%, respectively.

4.3.4 Statistical analyses

Data were analyzed separately for the pretreatment period (d −26 and −19), the pre-drying-off treatment period (d −12, d −5, and −1), and the post-drying-off treatment period (from d1 to d14) by ANOVA using the MIXED procedure of the SAS package (SAS Institute Inc., Cary, NC). Time was used as a repeated effect, and cow(treatment) was used as the subject. The following model was used in the analysis of variance:

$$Y_{ijk} = \mu + trt_i + day_k + trt_i*day_k + e_{ijk}$$

where Y_{ijk} is the measured value of the dependent variable of the j^{th} cow on the i^{th} treatment on the k^{th} day, μ is the overall mean of the dependent variable, trt_i is the fixed effect of the i^{th} treatment on the dependent variable, day_k is the fixed effect of the k^{th} day on the dependent variable, trt*day is the fixed effect of the interaction of trt_i and day_k on the dependent variable, and e_{ij} is the random residual effect associated with the j^{th} cow on the i^{th} treatment on the k^{th} day.

The amount of prolactin released during milking was calculated by determining the area under the curve between 0 and 40 min relative to the start of milking. Volumes of milk

measured during the pretreatment period between d −26 and −15 were averaged and used as a covariate for milk production data analysis between d −14 and −1. All data except milk production and prolactin concentration were $\log_{10}$-transformed before analysis. Orthogonal contrasts were used to compare least squares means between the SDPP group and the LDPP and LDPP+MEL groups, and between the LDPP and the LDPP+MEL groups. The least squares means of the SDPP and LDPP+MEL groups were compared by using the Tukey-Kramer adjustment. Differences were considered statistically significant at $P \leq 0.05$. During the experiment, two cows (one from the SDPP group and one from the LDPP+MEL group) displayed clinical signs of mastitis in 3 quarters, and those cows' values of involution markers were removed from the statistical analysis.

4.4 Results

4.4.1 Prolactin concentration

Before the start of the treatments, even though basal serum prolactin concentration in SDPP cows tended to be greater than that of the other 2 groups, we did not observe any significant difference in basal serum prolactin concentration between the 3 groups of cows (Figure 4.1A). Before drying-off, we observed a treatment × day interaction ($P = 0.001$), with a faster decline in prolactin concentration in the SDPP-exposed cows. After drying-off, the basal serum prolactin concentration declined ($P < 0.05$) and was lower in the SDPP cows than in the cows in the other 2 groups ($P = 0.03$). The basal serum prolactin concentration in LDPP+MEL cows was not significantly different from that of LDPP cows.

Before the start of the treatments, the milking-induced prolactin release was not different between the 3 groups of cows on d-15 (Figure 4.1B). The day before drying-off, basal prolactin concentration and the amount (area under the curve) of prolactin released at milking tended to be lower in the cows exposed to SDPP than in the cows in the other 2 groups ($P = 0.1$ and $P = 0.07$, respectively; Figure 4.1C). Regardless of treatments, basal prolactin concentration and the amount of prolactin released at milking were lower on d −1 than on d −15 ($P = 0.01$ and $P = 0.001$, respectively).

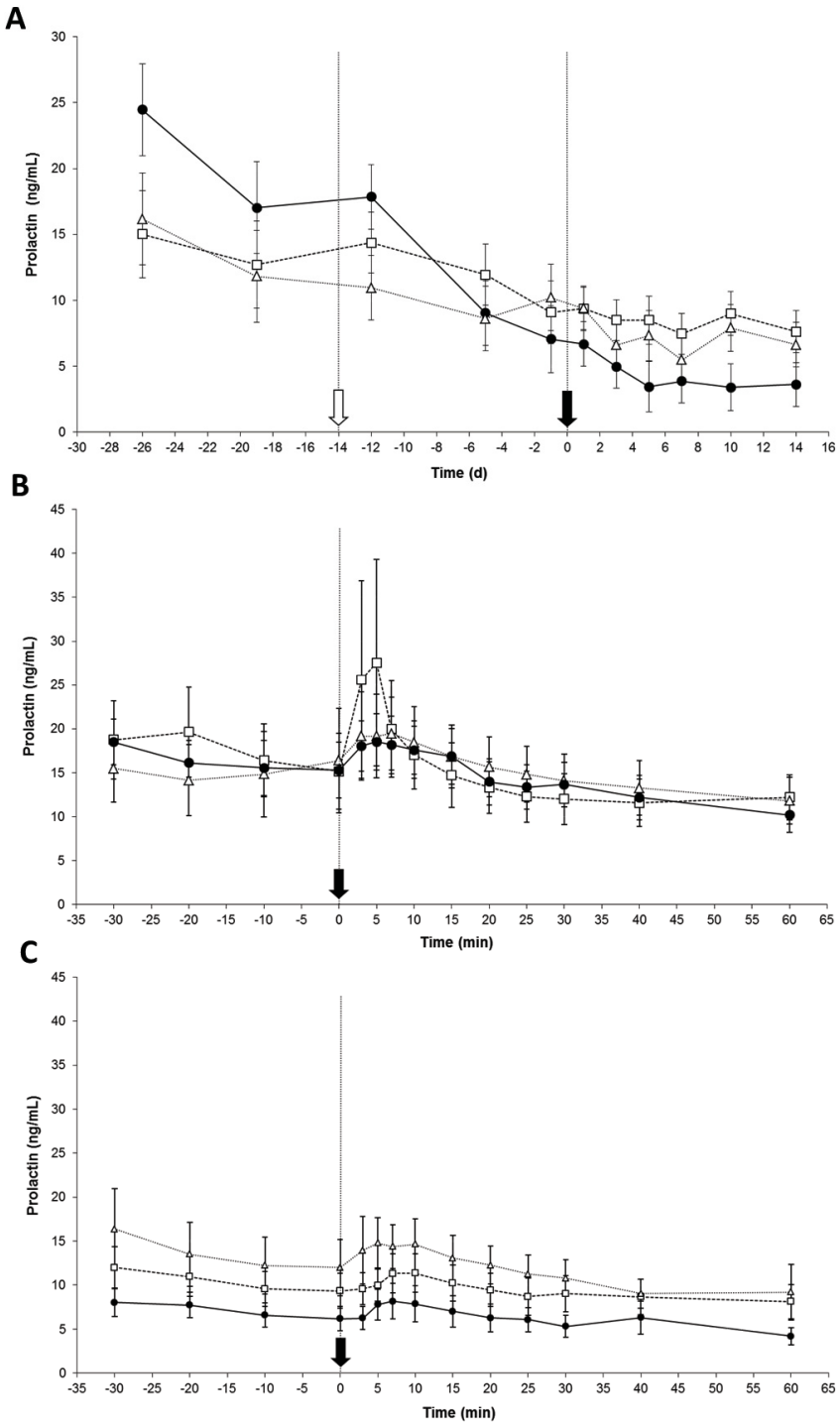

Figure 4.1. Basal concentrations of prolactin (A), prolactin concentration released during the evening milking on d -15 (B), and prolactin concentration released during milking on d -1 (C) in cows exposed to a short day photoperiod (SDPP; ●; n=10), to a long day photoperiod (LDPP; □; n=10), or to LDPP and treated with oral administration of melatonin (LDPP+MEL; △; n=10) from 14 days before to 14 days after drying-off.
In panel A, the white arrow indicates the beginning of the treatments, and the black arrow indicates the start of drying-off. In panels B and C, the black arrow indicates the start of milking. Data are presented as least squares means ± SEM.

4.4.2 Milk production

Before the start of the treatments (d −26 and −19), milk production was similar in the 3 groups of animals, averaging 23.8, 23.3, and 24.0 ± 1.7 kg/d for the SDPP, LDPP, and LDPP+MEL groups, respectively. After the treatments began, milk production declined progressively until the cessation of milking in all 3 groups ($P < 0.001$; Figure 4.2) and was lower in the SDPP cows ($P < 0.01$) than in the LDPP and LDPP+MEL cows. No significant difference in milk production was observed between LDPP and LDPP+MEL cows from d −14 and −1.

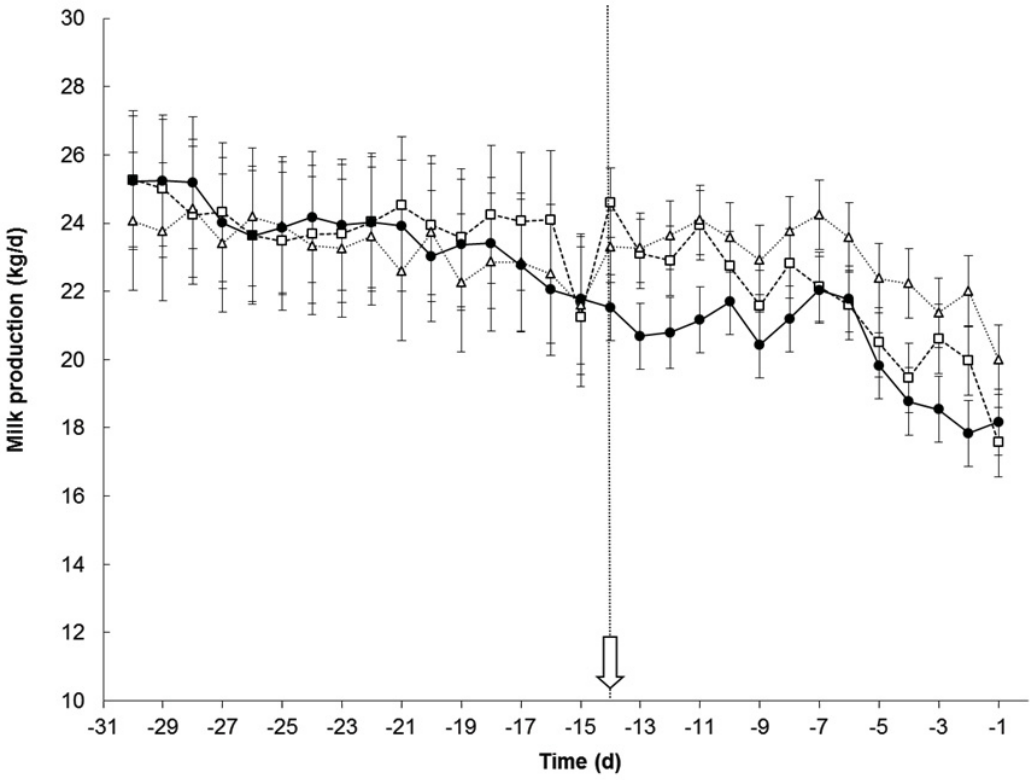

Figure 4.2. Milk production during the last month of lactation from cows exposed to a short day photoperiod (SDPP; ●; n=10), to a long day photoperiod (LDPP; □; n=10), or to LDPP and treated with oral administration of melatonin (LDPP+MEL; △; n=10) from 14 days prior drying-off to 14 days after.
The white arrow indicates the beginning of the treatments. Data are presented as least square means ± SEM.

4.4.3 Mammary gland involution markers

Before the start of the treatments, SCC, the concentrations of lactoferrin, citrate, and BSA, the citrate:lactoferrin molar ratio, and MMP-2 and MMP-9 activities in milk were not different among the 3 groups of animals.

From the beginning of the treatments until drying-off, BSA concentration (Figure 4.3A), SCC (Figure 4.3B), citrate concentration (Figure 4.4A), and lactoferrin concentration (Figure 4.4B) in milk did not differ among the treatments. The citrate:lactoferrin ratio (Figure 4.4C) was also unaffected by the treatments. Milk MMP-2 and MMP-9 activities (Figure 4.5) increased during this period ($P < 0.001$ and $P < 0.05$, respectively) but were not affected by the treatments.

After drying-off, as expected, BSA concentration (Figure 4.3A), SCC (Figure 4.3B), and lactoferrin concentration (Figure 4.4B) in mammary secretions increased ($P < 0.001$). However, none of them were affected by the treatments. Similarly, the citrate:lactoferrin molar ratio declined after drying-off ($P < 0.001$) but was not affected by treatments. The activities of MMP-2 and MMP-9 increased ($P < 0.001$) during involution (Figure 4.5). The activity of MMP-9 tended to be greater (P=0.07) in the SDPP cows than in the LDPP and LDPP+MEL cows. The activity of MMP-2 was not affected by treatments.

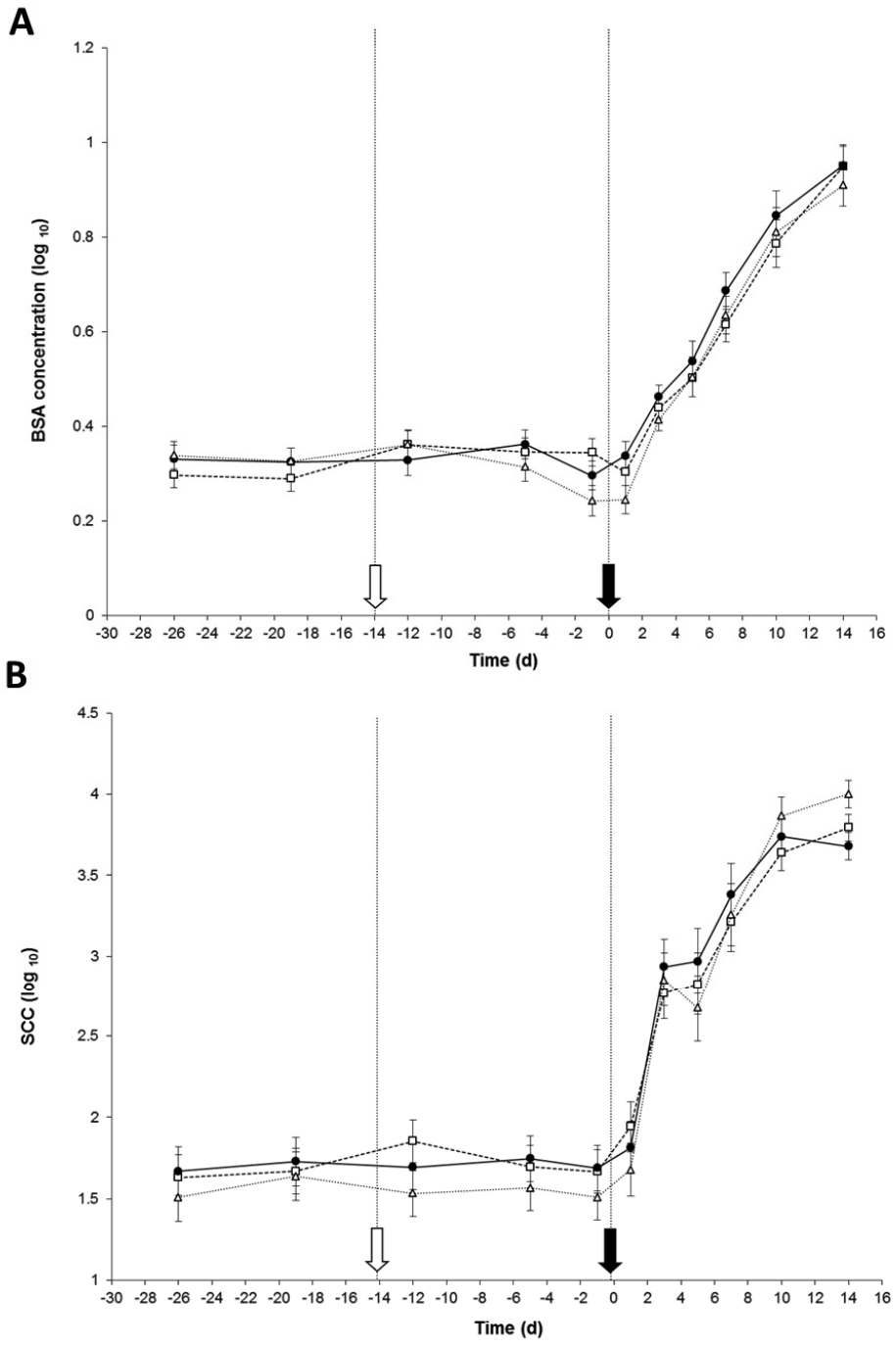

Figure 4.3. Concentration of BSA (A) and SCC (B) in milk and mammary secretions from cows exposed to a short day photoperiod (SDPP; ●; n=9), to a long day photoperiod (LDPP; □; n=10), or to LDPP and treated with oral administration of melatonin (LDPP+MEL; △; n=9) from 14 days before to 14 days after drying-off.
The white arrow indicates the beginning of the treatments, and the black arrow indicates the start of drying-off. Data are presented as least squares means ± SEM of log_{10}-transformed values.

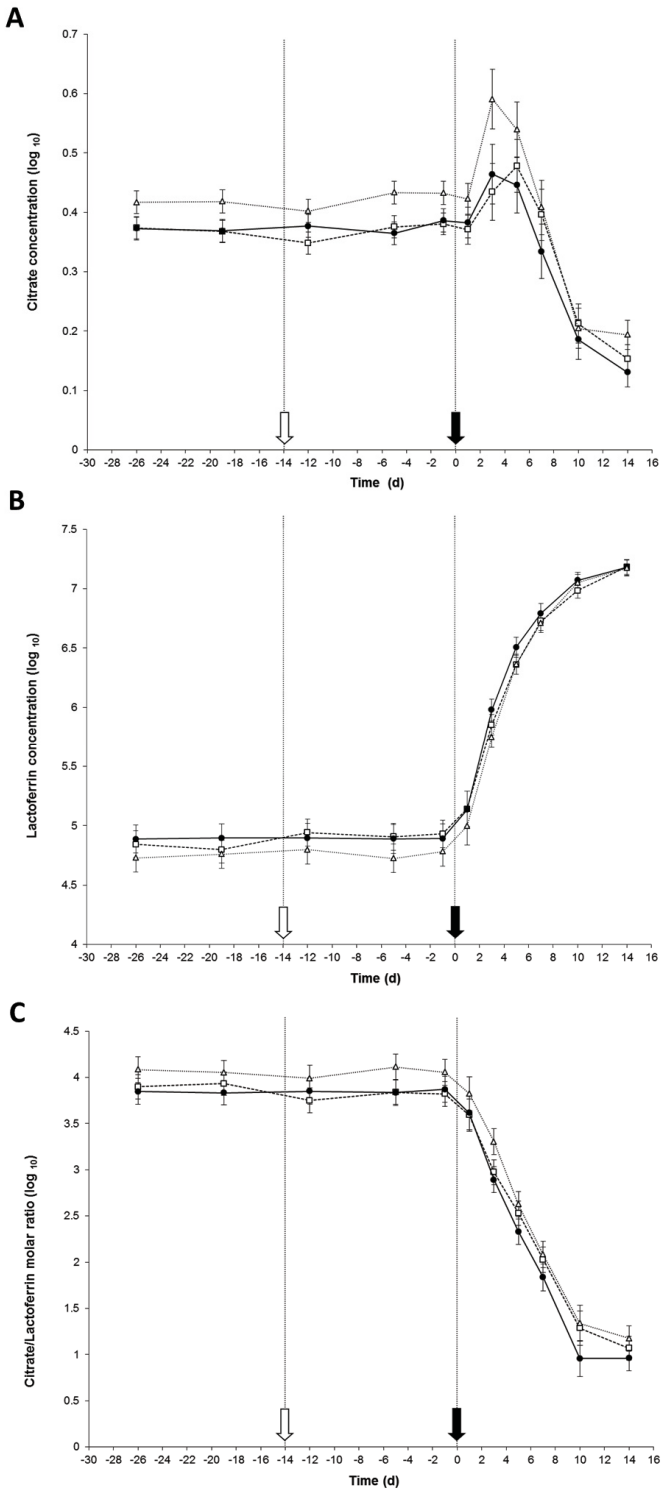

Figure 4.4. Citrate concentration (A), lactoferrin concentration (B) and citrate:lactoferrin ratio (C) in milk and mammary secretions from cows exposed to a short day photoperiod (SDPP; ●; n=9), to a long day photoperiod (LDPP; □; n=10), or to LDPP and treated with oral administration of melatonin (LDPP+MEL; △; n=9) from 14 days before to 14 days after drying-off.

The white arrow indicates the beginning of the treatments, and the black arrow indicates the start of drying-off. Data are presented as least squares means ± SEM of log_{10}-transformed values.

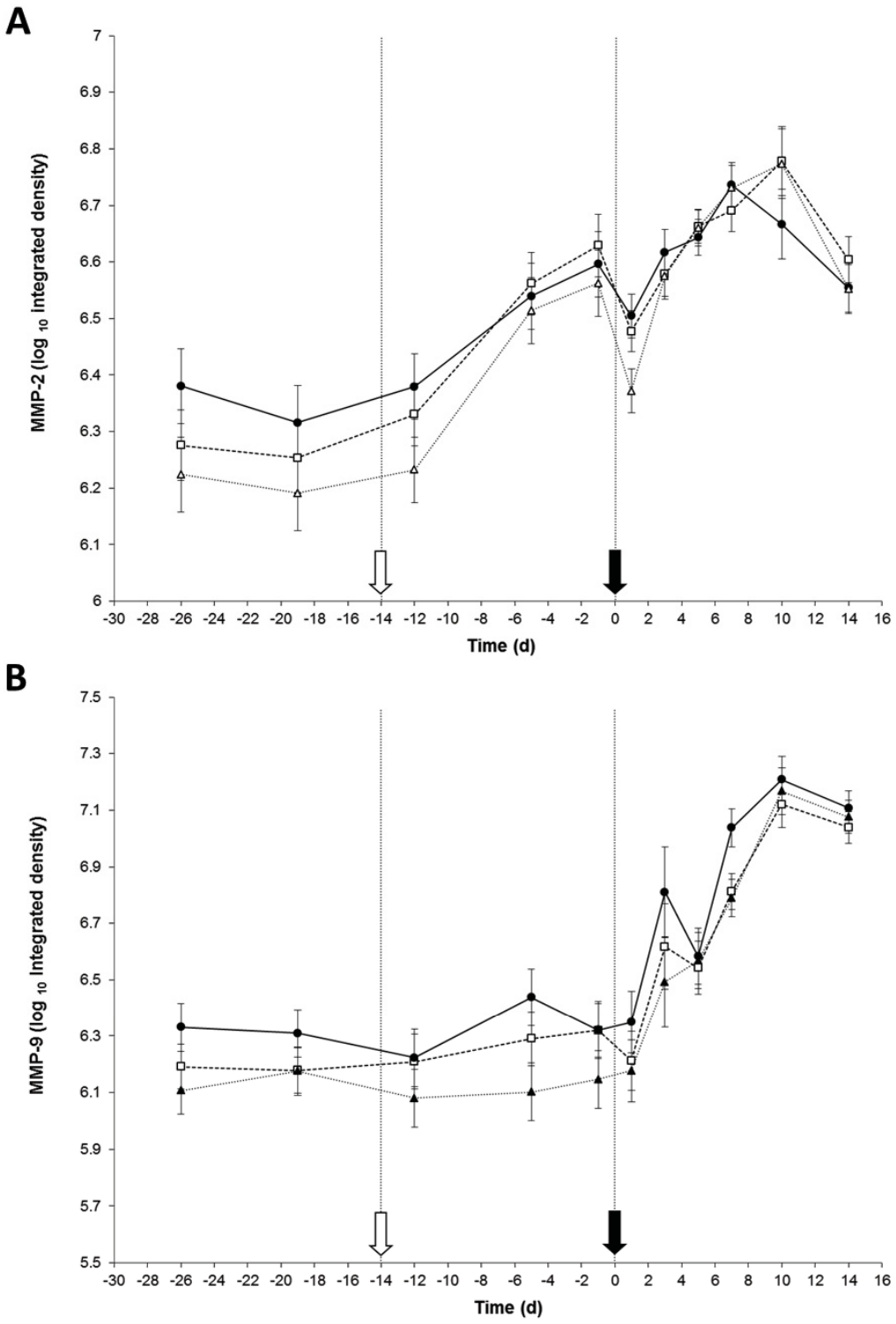

Figure 4.5. Matrix metalloproteinase-2 (MMP-2) (A) and (MMP-9) (B) activities in milk and mammary secretions from cows exposed to a short day photoperiod (SDPP; ●; n=9), to a long day photoperiod (LDPP; □; n=10), or to LDPP and treated with oral administration of melatonin (LDPP+MEL; △; n=9) from 14 days prior to 14 days after drying-off.
The white arrow indicates the beginning of the treatments, and the black arrow indicates the start of drying-off. Data are presented as least squares means ± SEM of log_{10}-transformed integrated density values.

4.5 Discussion

Basal prolactin concentration declined faster before drying-off and was lower after drying-off in blood of the cows exposed to SDPP than in blood of the LDPP and LDPP+MEL cows. In addition, these cows tended to have less prolactin released during milking than the cows exposed to LDPP and LDPP+MEL did. The effects of SDPP on prolactin secretion in this study are in accordance with previous studies (Dahl et al., 1997; Miller et al., 2000; Lacasse et al., 2014). Nevertheless, the effect before drying-off was lower than expected, because the SDPP cows tended to have greater prolactin concentrations before the start of the treatments. Moreover, cows were in late lactation, a period when prolactin release naturally decreases (Miller et al., 2006), whereas previous works were performed either on early or mid-lactation cows. The relative short exposure to the treatments before drying-off may also have contributed to the small differences noted before drying-off.

Exposing cows to SDPP hastened the decline of milk production before drying-off. The effects of photoperiod on milk production in dairy cows are well known (for a review, see Dahl and Petitclerc, 2003). The parallel decline between prolactin concentration and milk production suggests that prolactin is the main driver of the effect of photoperiod on milk production. Auldist et al. (2007) reported that melatonin administered as slow-release s.c. implants for 12 wk reduced plasma prolactin concentration by 4 wk of treatment and decreased milk production by 6 wk of treatment. Considering that SDPP decreased basal prolactin concentrations faster before drying-off and after drying-off and tended to decrease milking-induced prolactin release, it appears that prolactin played a pivotal role in the effect of photoperiod on milk production.

In the present study, feeding melatonin 14 d before and 14 d after drying-off did not affect blood prolactin concentration and milk production. Previous works reported that feeding melatonin was able in some cases to decrease blood prolactin levels in cattle. For instance, feeding melatonin (4 mg/kg BW) in the middle of a long day (16 h of light:8 h of darkness) reduced prolactin concentration and mammary parenchymal growth in prepubertal heifers (Sanchez-Barcelo et al., 1991). In late lactation cows, feeding melatonin for 8 weeks was able to suppress prolactin secretion (Dahl et al., 2000). Again, the relative short treatment duration may have contributed to the absence of effect of melatonin on prolactin secretion in the present study. However, compared with SDPP, the effects of melatonin administration are

less pronounced and are not necessarily associated with a decrease in milk production. The administration of 12 implants of melatonin (18 mg/implant) to dairy cows at drying-off moderately suppressed prepartum prolactin concentration but did not affect milk production postpartum (Garcia-Ispierto et al., 2013). Melatonin feeding did not affect milk yield in late lactation cows (Dahl et al., 2000). In prepartum cows and heifers, prolactin concentration was reduced by melatonin feeding, but not as much as by SDPP (Lacasse et al., 2014). The results of the present experiment do not support the hypothesis that feeding melatonin can mimic SDPP in cattle.

After the cessation of milking, milk composition changed dramatically because of a decrease in the synthesis and the secretion of milk components by mammary epithelial cells and an increase in tight junction permeability (Nguyen and Neville, 1998; Capuco and Akers, 1999). Consequently, lactose, citrate, and milk fat contents decrease after the cessation of milking, whereas total protein content increases, mainly because of the increases in lactoferrin and blood-originated proteins, such as immunoglobulins and BSA (Nonnecke and Smith, 1984; Hurley, 1989). Mammary gland involution also involves the recruitment of immune cells (Monks et al., 2002), leading to an increase in SCC (Ollier et al., 2013; Ponchon et al., 2014), as well as tissue restructuring due to the action of several proteases, notably plasminogen activators, plasmin, and MMP (Talhouk et al., 1992; Politis, 1996). These changes in the composition of mammary secretions can be used as indicators of mammary gland involution. In the present study, all the indicators used (BSA, citrate, and lactoferrin concentrations; lactoferrin/citrate ratio; SCC; and MMP-2 and MMP-9 activities) followed the expected changes (Oliver and Sordillo, 1989; Ollier et al., 2014). However, except for MMP-9 activity, the pattern of these changes was not affected by the photoperiod and melatonin treatments. The remodeling process that takes place during mammary gland involution involves extracellular matrix degradation through the activation of different proteases, including MMP-2 and MMP-9, and the activities of these enzymes increase during mammary gland involution in rodents (Talhouk et al., 1992; Lund et al., 2000) and cows (Tremblay et al., 2009). Therefore, the trend for a faster increase suggests a positive effect of SDPP on mammary gland remodeling. Nevertheless, none of the results show clearly that involution was affected by the photoperiod or melatonin treatments.

In a recent experiment, Ollier et al. (2014) compared the changes in mammary involution markers in cows in which milk production at drying-off was reduced by prolactin inhibition or feed restriction. Both treatments clearly hastened mammary gland involution, indicating that milk production at drying-off is an important factor modulating mammary gland involution. In the present experiment, although milk production was reduced by SDPP, the magnitude of the decrease was lower than that of the decreases observed by Ollier et al. (2014), and this difference probably explains the lack of effect observed on most indicators of mammary gland involution.

In conclusion, photoperiod modulation and melatonin feeding did not affect the speed of mammary gland involution under the conditions in this study. Exposure to SDPP during late lactation slightly reduced milk production and tended to decrease milking-induced prolactin release, whereas there were no effects of the LDPP and LDPP+MEL treatments. Mean basal prolactin secretion was lower during the first 2 wk of the dry period when the cows were exposed to SDPP. The effects of SDPP on prolactin secretion and mammary gland involution may need to be reevaluated with a longer exposure before drying-off.

4.6 Aknowledgements

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Connective text

In Chapters 3 and 4, we evaluated the effects of intra-mammary infusions of substances known to affect tight junction permeability and of a modification of the photoperiod around the cessation of milking in dairy cows. Among those treatments, casein hydrolysates intra-mammary infusion was the only strategy that efficiently hastened mammary gland involution and that appears useful in order to facilitate drying-off. An indirect approach to decrease the pathological risks associated with the arrest of lactation would be to improve lactation persistency. From this perspective, it seems relevant to investigate what could maintain mammary cell number and activity and particularly to study what are the factors that regulate the secretion of prolactin, which has recently been reported to be galactopoietic in ruminants. In Chapter 5, we would like thus to focus on the regulation of prolactin release, and more precisely, on what could be the molecular events driving the decrease in prolactin release after multiple mammary gland stimulation. Our hypothesis is that glucocorticoids may play a role in prolactin release regulation. One trial will consist to study the effect of a glucocorticoid analogue, dexamethasone and an inhibitor of glucocorticoid synthesis, metyrapone, on the secretion of prolactin induced by mammary gland stimulation. A second trial will consist to determine whether the milk depression induced by glucocorticoid administration is due to a decrease in prolactin secretion or not. Dexamethasone will be used in combination with domperidone, a dopaminergic antagonist, in order to alleviate the dopaminergic inhibition of prolactin secretion at the pituitary level.

Chapter 5 Evaluation of the relationship between glucocorticoids and prolactin during mammary gland stimulation in dairy cows

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5.1 Abstract

The objectives of this study were to determine the role of glucocorticoids in the regulation of the prolactin release induced by mammary gland stimulation and to investigate whether the milk depression induced by glucocorticoids in dairy cows is due to a decrease in prolactin release. In Experiment 1, 8 dairy cows were used in a 4 × 4 Latin square design. Four hours after the morning milking, the cows received one of the following treatments: (1) a 5-min manual stimulation of the mammary gland; (2) an i.v. injection of 1 mg of dexamethasone; (3) a 5-min manual stimulation after 2 infusions of 2.5 g of metyrapone (an inhibitor of cortisol biosynthesis) in the omasum (4 and 2 h before the stimulation); or (4) no treatment. Sixty minutes later, the mammary gland of each cow was stimulated for 5 min. Blood samples were collected from 20 min before to 120 min after the start of the treatment. When the mammary gland was stimulated twice in 60 min, less prolactin and cortisol were released during the second stimulation than during the first one. Metyrapone did not affect prolactin or cortisol release. Dexamethasone decreased serum cortisol concentration but did not affect prolactin concentration. In Experiment 2, 16 cows were used in a crossover experimental design consisting of 2 experimental weeks separated by 1 resting week. During the first week, the cows were treated as follows: (1) 4 cows were injected with 0.5 g of domperidone (a prolactin secretagogue) in canola oil on d 1 and 2 and with 20 mg of dexamethasone on d 1; (2) 4 cows were injected with 0.5 g of domperidone on d 1 and 2; (3) 4 cows were injected with canola oil on d 1 and 2 and with 20 mg of dexamethasone on d 1; and (4) 4 cows were injected with canola oil on d 1 and 2. During the second experimental week, the same 4 treatments were repeated except that cows that did not receive dexamethasone on the first week, were injected on d 1 of the second week, and the converse. On d 1 and 2 of each week, blood samples were collected during the morning milking for prolactin determination. Dexamethasone caused a reduction in milk production and a decrease in both basal and milking-induced prolactin release. Dexamethasone increased milk fat and protein percentages and decreased milk lactose content. Domperidone increased basal prolactin levels in serum and milk but did not affect milk yield. Glucocorticoids affect both basal and milking-induced PRL release, and that effect may contribute to the inhibitory effect of glucocorticoids on milk production.

5.2 Introduction

Prolactin is one of the major hormones involved in the control of mammary gland functions, including galactopoiesis (Lacasse et al., 2016). This hormone is released during milking and nursing in response to mammary gland stimulation. The regulation of prolactin secretion is not completely understood, however. As lactation advances, basal prolactin concentration and the amount of prolactin released during milking are both reduced (Koprowski and Tucker, 1973; Miller et al., 2000; Bernier-Dodier et al., 2011). As well, milking-induced PRL release in cows was shown to decrease as the interval between milkings or manual stimulations decreased (Lacasse and Ollier, 2014). The reason for such a reduction in the prolactin release during lactation or after multiple mammary gland stimulations is not known. The reduction could be due to the decreased sensitivity of mammary skin to the tactile stimulus or to reduced responsiveness at the level of the hypothalamus or the pituitary gland.

Besides prolactin, milking also induces the release of glucocorticoids, which are steroid hormones synthesized by the adrenal glands. Interestingly, adrenalectomy was found to prevent the usual decrease in prolactin levels during the second half of lactation in rats (van der Schoot and de Greef, 1983). Furthermore, female rats treated with dexamethasone, a glucocorticoid analogue, exhibited a lower suckling-induced prolactin response and lower milk secretion in comparison with control animals (Horváth et al., 2001). Glucocorticoid administration was also reported to inhibit milk production in both rodents and cows (Shamay et al., 2000; Vilela and Giusti-Paiva, 2011). The mechanism by which glucocorticoids inhibit milk secretion is not known. Given the recent body of evidence supporting the galactopoietic role of prolactin in ruminants (Lacasse et al., 2016), the negative effect of glucocorticoids on milk secretion may be mediated in part by the inhibition of prolactin release or the reduction of mammary gland responsiveness to this hormone. However, dexamethasone was also shown to decrease plasma IGF-1 concentrations in cows (Maciel et al., 2001). Given that IGF-1 stimulated milk yield in goats (Prosser et al., 1990), glucocorticoid-induced milk inhibition may also involve an action on IGF-1 secretion or clearance.

The objectives of this study were to evaluate the effects of glucocorticoids on milking-induced prolactin release and to test whether the glucocorticoid-induced inhibition of milk production is due to a reduction in prolactin secretion.

5.3 Material and methods

5.3.1 Animals and experimental design

The experiments were conducted at Agriculture and Agri-Food Canada's Sherbrooke Research and Development Centre (Sherbrooke, QC, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. The animals were housed in a tie-stall barn and were milked twice daily with a 12-h milking interval.

5.3.1.1 Experiment 1

Eight multiparous fistulated Holstein dairy cows in mid-lactation (196 ± 11 DIM) were used in a 4 × 4 Latin square design for this experiment. Cows were allocated to 1 of 4 groups that were balanced in terms of the cows' parity, milk production, and DIM. Three days before the start of the treatments, a Silastic catheter (i.d. 1.02 mm, o.d. 2.16 mm; Dow Corning Corp., Midland, MI) was inserted into each cow's jugular vein. Each experimental day was separated by at least 2 resting days.

On each experimental day, the cows received one of the following treatments: (1) a 5-min manual stimulation of the mammary gland 4 h after the morning milking (**STIM**); (2) an i.v. injection of 1 mg of the glucocorticoid analogue dexamethasone (Vetoquinol, Lavaltrie, QC, Canada) 4 h after the morning milking (**DEXA**); (3) infusions of 2.5 g of metyrapone (Sigma-Aldrich, Oakville, ON, Canada), an inhibitor of cortisol biosynthesis, in the omasum 4 and 2 h before a 5-min manual stimulation of the mammary gland 4 h after the morning milking (**METY**); or (4) no treatment 4 h after the morning milking (**CTL**). Sixty minutes later, the mammary gland of each cow was manually stimulated for 5 min.

Blood samples were collected before, during, and after the first manual stimulation or injection (−20, −10, 0, 3, 5, 7, 10, 15, 20, 25, 30, 40, 60 [start of the second stimulation], 63, 65, 67, 70, 75, 80, 85, 90, 100, and 120 min relative to the start of the first manual stimulation or injection) in Vacutainer tubes without additives (BD, Mississauga, ON, Canada). These blood tubes were left overnight at 4°Cto allow clotting before centrifugation (1,900 × *g*, 4°C, 15 min). The serum samples were then kept at −20°C until determination of prolactin and cortisol concentrations. Additional blood samples were collected at 60, 63, 65, and 67 relative

to the start of the first manual stimulation or injection in EDTA-coated Vacutainer tubes. These tubes were immediately placed on ice and centrifuged (1,900 × *g*, 4°C, 15 min). The plasma samples were then stored at −80°C until determination of ACTH.

5.3.1.2 Experiment 2

Sixteen multiparous Holstein cows in mid-lactation (162 ± 9 DIM) were used in a crossover experimental design. Cows were allocated to 1 of 2 groups (n = 8) balanced according to their parity, milk production and DIM. The experiment consisted of 2 experimental periods of 1 wk separated by 1 wk of rest. During the first experimental period, the cows were treated 2 h after the morning milking as follows: (1) 4 cows received a s.c. injection of 0.5 g of domperidone (10 mL of a 50-g/L suspension of domperidone in canola oil; Equi-Tox Pharma Inc., Central, SC) on d 1 and 2 and an i.m. injection of 20 mg of dexamethasone on d 1; (2) 4 cows only received a s.c. injection of 0.5 g of domperidone on d 1 and 2; (3) 4 cows received a s.c. injection of canola oil (10 mL) on d 1 and 2 and an i.m. injection of 20 mg of dexamethasone on d 1; and (4) 4 cows only received a s.c. injection of canola oil on d 1 and 2. During the second experimental period, the same 4 treatments were repeated except that the cows that had been treated with dexamethasone during the first period did not receive the injection during the second period, and those that did not receive dexamethasone during the first period were injected during the second period. The domperidone and canola oil treatments were not alternated.

Three days before the start of the treatments, a Silastic catheter was inserted into each cow's jugular vein. On d 1 and 2, blood samples were collected before, during, and after the morning milking (−20, −10, 0, 3, 5, 7, 10, 15, 20, 25, 30, 40, and 60 min relative to the start of milking) in Vacutainer tubes without additives. These blood samples were left at room temperature for approximately 2 h to allow clotting before centrifugation (1,900 × *g*, 4°C, 15 min). The serum samples were then kept at −20°C until determination of prolactin concentration.

On d −3, 1, 2, 3, 4, 5, and 7 of each experimental period, blood samples were collected from the coccygeal vein before the morning milking in Vacutainer tubes without additives. These blood samples were left at room temperature for 2 h to allow clotting before

centrifugation (1,900 × *g*, 4°C, 15 min) and then stored at −20°C until determination of serum PRL and cortisol concentrations. Additional blood samples were collected on the same days in EDTA-coated Vacutainer tubes and placed immediately on ice before centrifugation (1,900 × *g*, 4°C, 15 min) and storage at −80°C until determination of plasma IGF-1 concentration.

Milk production was recorded daily from 3 d before to 7 d after the start of the treatments for each experimental period. Milk samples (50 mL) were collected at the morning milking on d −3, 1, 2, 3, 4, 5, and 7, centrifuged (1,900 × *g*, 4°C, 15 min), skimmed, and stored at −20°C until determination of milk PRL concentration. Additional milk samples were harvested on the same days for milk composition analysis. Milk fat, protein, and lactose concentrations were measured in a commercial laboratory (Valacta inc., Sainte-Anne-de-Bellevue, QC, Canada).

5.3.2 Sample analyses

Serum cortisol concentration was determined by ELISA using the Cortisol Parameter Assay Kit (R&D Systems Inc., Minneapolis, MN) following the manufacturer's recommendations with some modifications. Briefly, samples were centrifuged (12°C, 5 min), and 100 µL of the serum was diluted in 300 µL of Calibrator diluent before being added to a 96-well goat anti-mouse microplate. After the addition of cortisol coupled with horseradish peroxidase, the samples were incubated with primary anti-mouse antibody at room temperature under gentle agitation for 2 h. After washing, the plates were incubated with the enzyme substrate at room temperature in dark conditions for 30 min. The reaction was then stopped with 2 *N* H_2SO_4, and the optical density was read at 450 nm using a SpectraMax 250 microplate reader (Molecular Devices LLC, Sunnyvale, CA). The intra- and inter-assay coefficients of variation were 1.7% and 15.6%, respectively.

Serum and milk prolactin concentrations were measured by RIA as described by Bernier-Dodier et al. (2011). Bovine PRL, rabbit antiserum specific for bovine PRL, and goat anti-rabbit gamma globulin were purchased from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). The intra- and inter-assay coefficients of variation were 4.8% and 8.9%, respectively.

Plasma IGF-1 concentration was measured by ELISA using the Human IGF-I Quantikine ELISA Kit (R&D Systems) following the manufacturer's recommendations with some modifications. Briefly, plasma samples were pretreated by adding 360 µL of pretreatment A solution to 40 µL of sample. After 10 min of incubation at room temperature, 50 µL of the previous mix was added to 200 µL of pretreatment B solution. Then, 100 µL of the pretreated plasma sample was diluted with 50 µL of Assay Diluent RD1-53. In a 96-well polystyrene microplate, 50 µL of the plasma sample was mixed with 150 µL of Assay Diluent RD1-53 and incubated at 4°C for 2 h. After being washed and incubated at 4°C for 1 h with 200 µL of unlabeled IGF-1 conjugate, the wells were washed and incubated with 200 µL of substrate solution at room temperature for 30 min. The reaction was stopped by the addition of 2 *N* sulfuric acid, and the optical density was read at 450 nm using a SpectraMax 250 microplate reader. The intra- and inter-assay coefficients of variation were and 5.2% and 4.1%, respectively.

Plasma ACTH concentration was determined by ELISA using the Bovine Adrenocorticotropic Hormone (ACTH) ELISA Kit (Cusabio, Wuhan, China) following the manufacturer's recommendations. The optical density was read at 450 nm using a SpectraMax 250 microplate reader. The intra- and inter-assay coefficients of variation were and 7.4% and 18.4%, respectively.

5.3.3 Statistical analyses

For Experiment 1, the amounts of PRL and cortisol released into blood during manual stimulation were calculated by determining the areas under the curves. The basal and peak concentrations were also calculated. Data were analyzed as Latin squares by ANOVA using the MIXED procedure of SAS software (SAS Institute Inc., Cary, NC). The following model was used in the analysis of variance:

$$Y_{ijk} = \mu + trt_i + day_k + trt_i*day_k + e_{ijk}$$

where Y_{ijk} is the measured value of the dependent variable of the j^{th} cow on the i^{th} treatment on the k^{th} day, μ is the overall mean of the dependent variable, trt_i is the fixed effect of the i^{th} treatment on the dependent variable, day_k is the fixed effect of the k^{th} day on the dependent variable, trt*day is the fixed effect of the interaction of trt_i and day_k on the dependent variable,

and e_{ijk} is the random residual effect associated with the j^{th} cow on the i^{th} treatment on the k^{th} day.

For Experiment 2, daily data were analyzed by ANOVA using the MIXED procedure of SAS software. Time was used as a repeated effect, and cow(treatment) was used as the subject. The following model was used in the analysis of variance:

$$Y_{ijk} = \mu + dex + domp + dex*domp + day_i + week_k + dex*day_i + domp*day_i + dex*domp*day_i + domp*week_k + e_{ijk}$$

where Y_{ijk} is the measured value of the dependent variable of the j^{th} cow on the i^{th} day of the k^{th} week, μ is the overall mean of the dependent variable, dex is the fixed effect of dexamethasone on the dependent variable, domp is the fixed effect of dexamethasone on the dependent variable, dex*domp is the fixed effect of the interaction of dex and dom on the dependent variable, day_i is the fixed effect of the i^{th} day on the dependent variable, $week_k$ is the fixed effect of the k^{th} week on the dependent variable, dex*day_i is the fixed effect of the interaction between dex and day_i on the dependent variable, domp*day_i day_i is the fixed effect of the interaction of domp and day_i on the dependent variable, dex*domp*day_i is the fixed effect of the interaction between dex, domp and day_i on the dependent variable, domp*$week_k$ is the fixed effect of the interaction of domp and $week_k$ on the dependent variable, and e_{ijk} is the random residual effect associated with the j^{th} cow on the i^{th} day of the k^{th} week.

Data were considered statistically significant when $P < 0.05$. The basal and peak concentrations of PRL and the amount of PRL released during milking (corresponding to the areas under the curves) were all calculated.

5.4 Results

5.4.1 Experiment 1

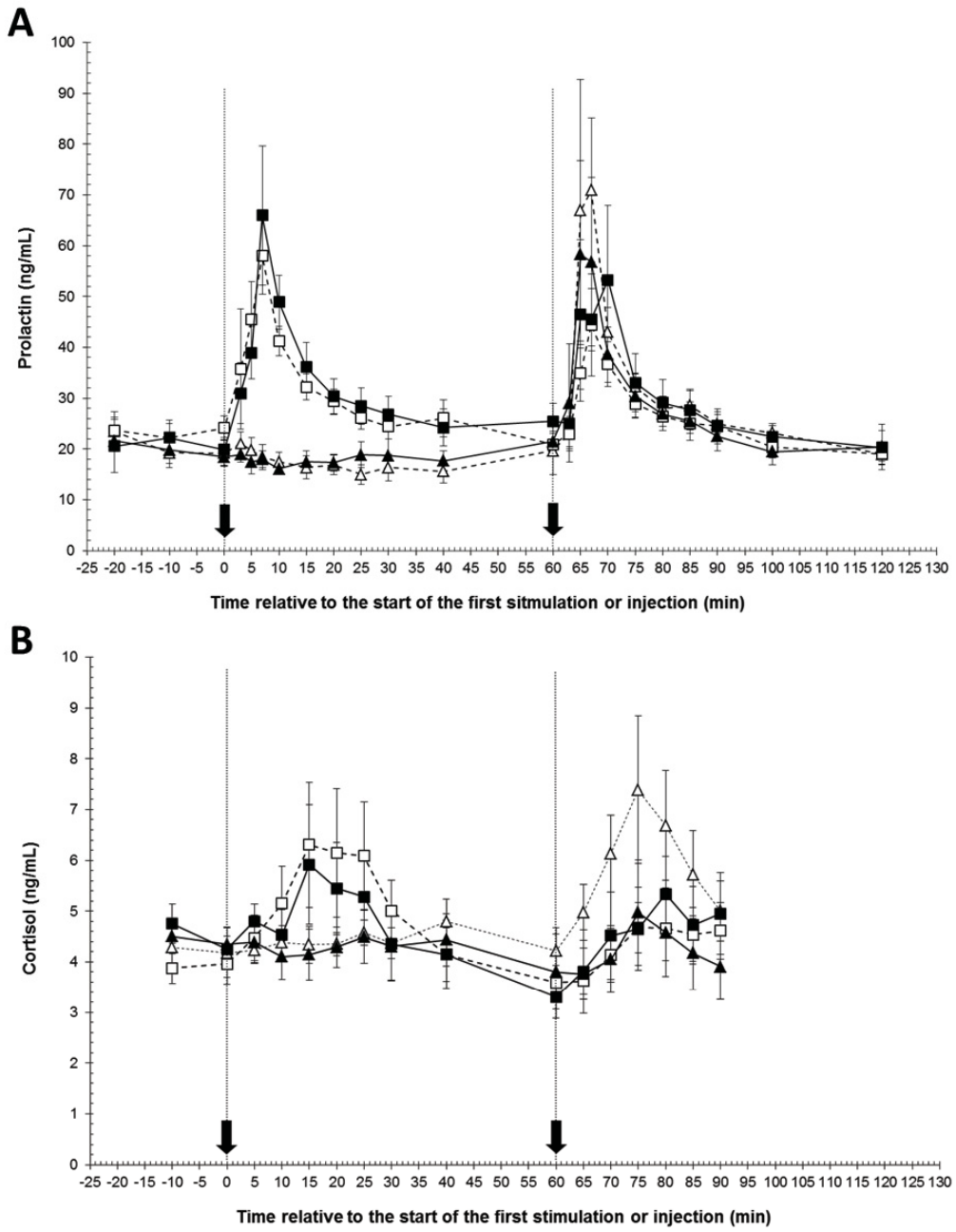

Figure 5.1. Prolactin (A) and cortisol (B) concentrations around and during mammary gland 5-min manual stimulation in the blood of cows stimulated once during the second sampling period beginning at 60 min (CTRL, △, dotted line; n=8), stimulated twice (STIM, □, dashed line; n=8), treated with 1 mg of dexamethasone and stimulated once (DEXA, ▲, solid line; n=8) and treated twice with 2.5 g of metyrapone and stimulated twice (METY; ■, solid line n=8).
The times of the administration of the treatments or manual stimulation are represented by the black arrows. Data are presented as arithmetic means ± SEM.

During the first phase of the sampling period (from −20 to 60 min), the basal prolactin concentration was similar among all treatments ($P > 0.1$; Figure 5.1A). As expected, the first mammary gland stimulation (STIM and METY treatments) induced a surge in prolactin release ($P < 0.01$). The amount of prolactin (area under the curve) and peak prolactin concentration during the stimulation were similar in both treatments. In the treatments where the mammary gland was not stimulated during the first phase (CTL and DEXA treatments), there was no significant release of prolactin. During the second phase of the sampling period (from 60 to 120 min), the peak concentration of prolactin was lower ($P = 0.03$) and the total amount of PRL released during the stimulation tended to be lower ($P = 0.09$) in the cows that had been stimulated during the first phase (STIM and METY treatments; Figure 1A and Table 1). The prolactin release induced by the second mammary gland stimulation was not affected by dexamethasone or metyrapone (Table 5.1).

Table 5.1. Effect of a 5-min manual mammary gland stimulation on milking-induced prolactin and cortisol releases.
Sixty minutes before this stimulation, the cows received 1 of the following treatments: (1) a 5-min manual stimulation of the mammary gland 4 h after the morning milking (STIM); (2) an i.v. injection of 1 mg of dexamethasone 4 h after the morning milking (DEXA); (3) infusions of 2.5 g of metyrapone in the omasum 4 and 2 h before a 5-min manual stimulation of the mammary gland 4 h after the morning milking (METY); or (4) no treatment 4 h after the morning milking (CTL). Data are presented as least squares means.

	Treatment				SEM	*P* value		
Treatments	METY	STIM	CTRL	DEXA		METY vs STIM	DEXA vs CTRL	METY+STIM vs DEXA+CTRL
Prolactin base, ng/mL	24.5	20.9	19.6	21.4	3.2	0.44	0.70	0.51
Prolactin peak, ng/mL	59.1	51.1	94.1	77.9	12.7	0.66	0.38	0.026
Prolactin AUC, ng.min/mL	1,191	1,104	1,317	1,198	129.6	0.64	0.52	0.41
Prolactin RELA, ng.min/mL	211.8	267.9	530.9	340.1	111.4	0.73	0.24	0.096
Cortisol base, ng/mL	3.9	4.3	4.5	4.2	0.3	0.40	0.52	0.40
Cortisol peak, ng/mL	6.2	5.8	7.9	5.2	0.7	0.70	0.01	0.41
Cortisol AUC, ng.min/mL	139.6	128.7	177.6	127	11.6	0.52	0.006	0.14
Cortisol RELA, ng.min/mL	4.8	11.1	50.4	-5.8	11.3	0.70	0.003	0.22

Abbreviations: AUC, area under the curve; RELA, AUC minus the baseline of prolactin or cortisol concentration.

During the first phase of the sampling period, the basal cortisol concentration was similar among all treatments ($P > 0.1$; Figure 5.1B). As was also the case for prolactin, the first mammary gland stimulation (STIM and METY treatments) induced a surge in cortisol release ($P < 0.01$), whereas no significant release of cortisol was observed in the treatments where the mammary gland was not stimulated manually (CTL and DEXA treatments). Metyrapone did not significantly affect the peak concentration or the total amount of cortisol released but tended ($P = 0.09$) to decrease the amount of cortisol released above the basal level compared with STIM treatment. During the second phase of the sampling period, dexamethasone decreased the peak concentration ($P = 0.01$) and the amount of cortisol released during mammary gland stimulation ($P < 0.01$; Table 1). Mammary gland stimulation in the first period reduced the amount of cortisol released in the second period in the STIM treatment ($P = 0.04$) but not in the METY treatment.

Manual stimulation in the second sampling period did not affect plasma ACTH concentration (data not shown).

5.4.2 Experiment 2

5.4.2.1 Milk yield and composition

Before the start of the treatments, milk production was similar among the treatment groups (Figure 5.2). There was a Dexamethasone*Day interaction ($P < 0.001$) during the treatment period. Milk production was reduced in the dexamethasone-treated cows ($P < 0.001$) in the 2 d that followed injection. However, domperidone did not affect milk yield, regardless of whether the cows were injected with dexamethasone or not.

Milk fat content was similar among the treatment groups before the start of the treatments (Figure 5.3A). There was a Dexamethasone*Day interaction ($P < 0.001$) during the treatment period. Milk fat percentage was greater ($P < 0.05$) in the dexamethasone-treated cows in the 3 d that followed injection. Domperidone did not affect milk fat response to dexamethasone (Dexamethasone*Domperidone, $P = 0.4$) but tended to increase milk fat percentage ($P = 0.07$). Milk fat yield was similar among the treatment groups in the pretreatment period and was not affected by dexamethasone injection during the treatment

period (data not shown). Domperidone injection significantly increased milk fat yield ($P = 0.035$), which was greater on d 4 and 5 ($P < 0.05$) in the domperidone-treated cows than in the oil-treated cows.

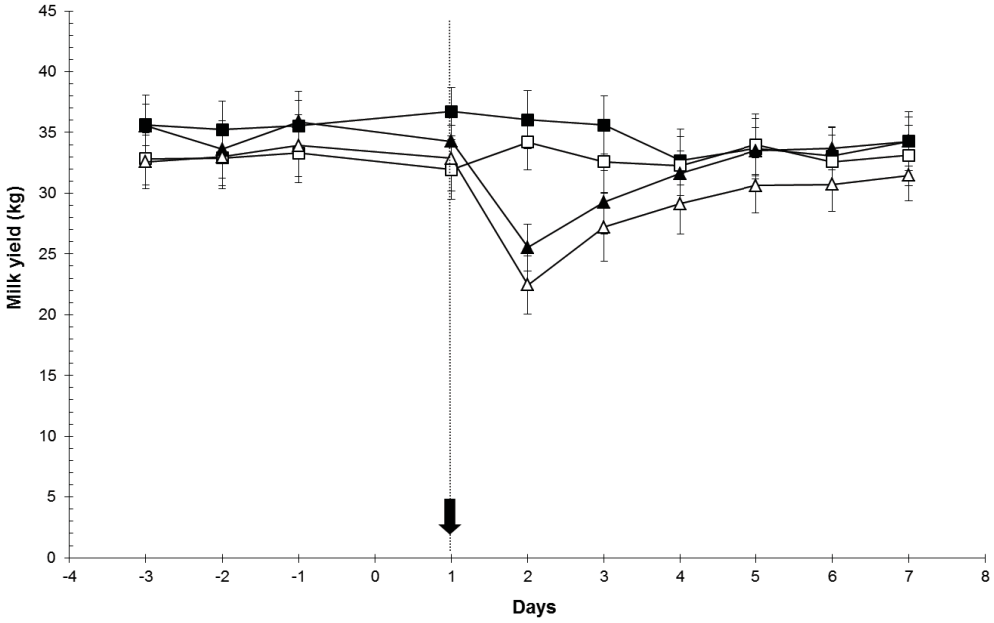

Figure 5.2. Milk production of cows injected with 0.5 g of domperidone on d 1 and 2 and with 20 mg of dexamethasone on d 1 (▲; n=8); only injected with domperidone on d 1 and 2 (■; n=8); injected with canola oil on d 1 and 2 and with dexamethasone on d 1(△; n=8); or only injected with canola oil on d 1 and 2 (□; n=8).
Data are presented as least square means ± SEM. The significant differences between dexamethasone-treated cows and non-treated cows are indicated as follows: $^{**}P < 0.01$.

Milk protein content was similar among the treatment groups before the start of the treatments (Figure 5.3B). There was a Dexamethasone*Day interaction ($P < 0.001$) during the treatment period. Milk protein percentage was greater ($P < 0.05$) in the dexamethasone-treated cows in the 2 d that followed injection. Domperidone did not affect milk protein response to dexamethasone (Dexamethasone*Domperidone, $P = 0.98$) or milk protein percentage. Milk protein yield was similar among the treatment groups in the pretreatment period (data not shown). There was a Dexamethasone*Domperidone*Day interaction ($P < 0.01$). Dexamethasone injection decreased milk protein yield ($P < 0.05$) on only the following day in the domperidone-treated cows and on the 2 following days in the oil-treated cows. Domperidone injection did not affect milk protein yield.

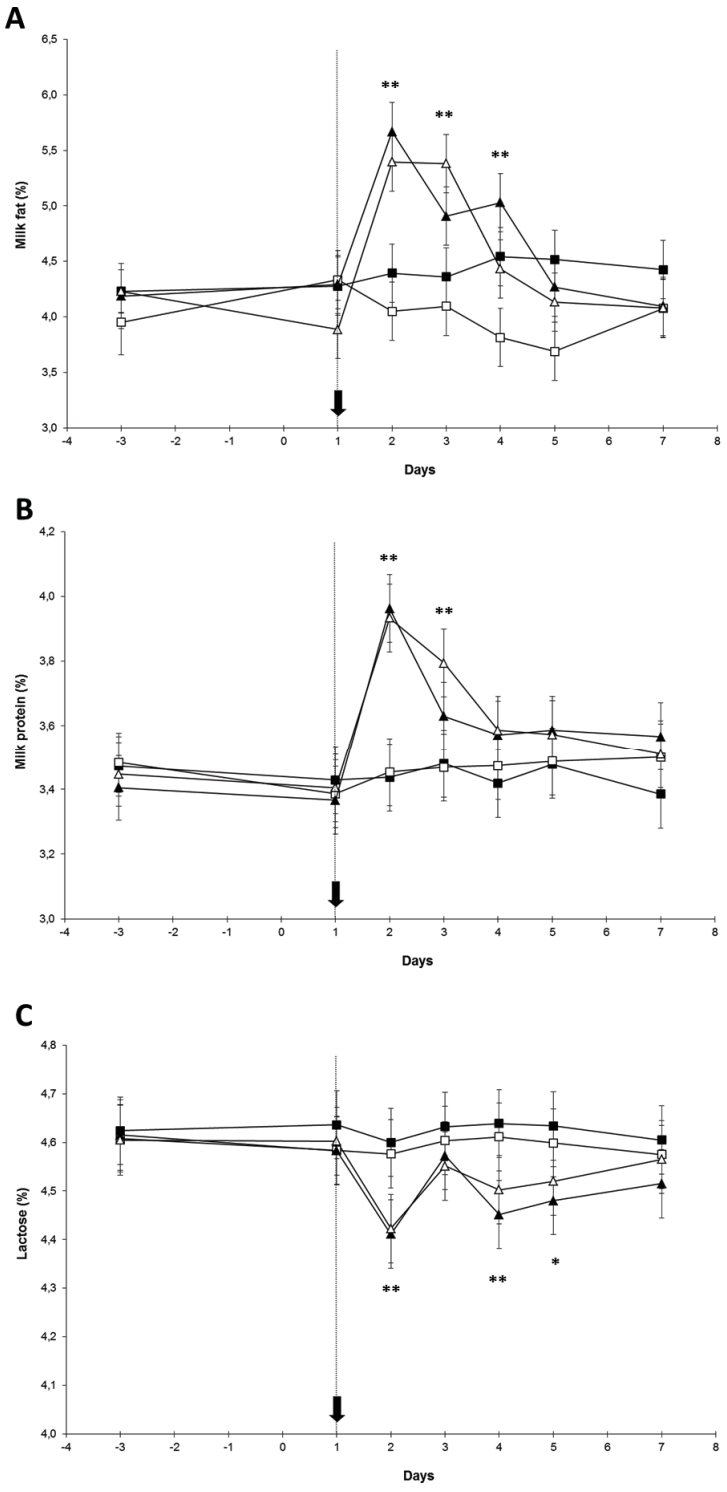

Figure 5.3. Milk fat (A), milk protein (B) and lactose contents (C) of cows injected with 0.5 g of domperidone on d 1 and 2 and with 20 mg of dexamethasone on d 1 (▲; n=8); only injected with domperidone on d 1 and 2 (■; n=8); injected with canola oil on d 1 and 2 and with dexamethasone on d 1(△; n=8); or injected with canola oil on d 1 and 2 and saline on d 1 (□; n=8).

Data are presented as least square means ± SEM and significant differences between dexamethasone-treated cows and non-treated cows are indicated as follows: **P* < 0.05, ***P* < 0.01.

Milk lactose content was similar among the treatment groups before the start of the treatments (Figure 5.3C). There was a Dexamethasone*Day interaction ($P < 0.01$) during the treatment period. Milk lactose content was lower ($P < 0.01$) in the dexamethasone-treated cows on d 2, 4, and 5. Domperidone did not affect milk lactose response to dexamethasone (Dexamethasone*Domperidone, $P = 0.6$) or milk lactose percentage. Milk lactose yield was similar among the treatment groups in the pretreatment period (data not shown). There was a Dexamethasone*Day interaction ($P < 0.001$) on lactose yield during the treatment period. Milk lactose yield was lower ($P < 0.05$) in the dexamethasone-treated cows from d 2 to 4 and tended to be lower ($P = 0.08$) on d 5. Domperidone did not affect lactose yield.

5.4.2.2 Prolactin, cortisol and IGF-1 concentrations

There was an interaction between domperidone and experimental period ($P = 0.03$) for serum prolactin concentration. In the first period, domperidone induced a gradual increase (Domperidone*Day, $P = 0.05$; Figure 5.4A) in serum prolactin, which was greater ($P < 0.05$) in the domperidone-treated cows than in the oil-injected cows on d 3, 4, 5, and 7. There was a carryover effect of domperidone from the first experimental period to the second. The serum prolactin concentration of the domperidone-treated cows during the first experimental period was still greater than that of the oil-treated cows before the start of the second period ($P < 0.001$; Figure 5.4B). Serum prolactin concentration remained elevated in the domperidone-treated cows during the second treatment period ($P < 0.01$). There was a Dexamethasone*Day interaction ($P < 0.01$) during both treatment periods. In comparison with the non-injected cows, serum prolactin concentration was lower ($P < 0.001$) in the dexamethasone-treated cows on d 2 (Figure 5.4A and 5.4B).

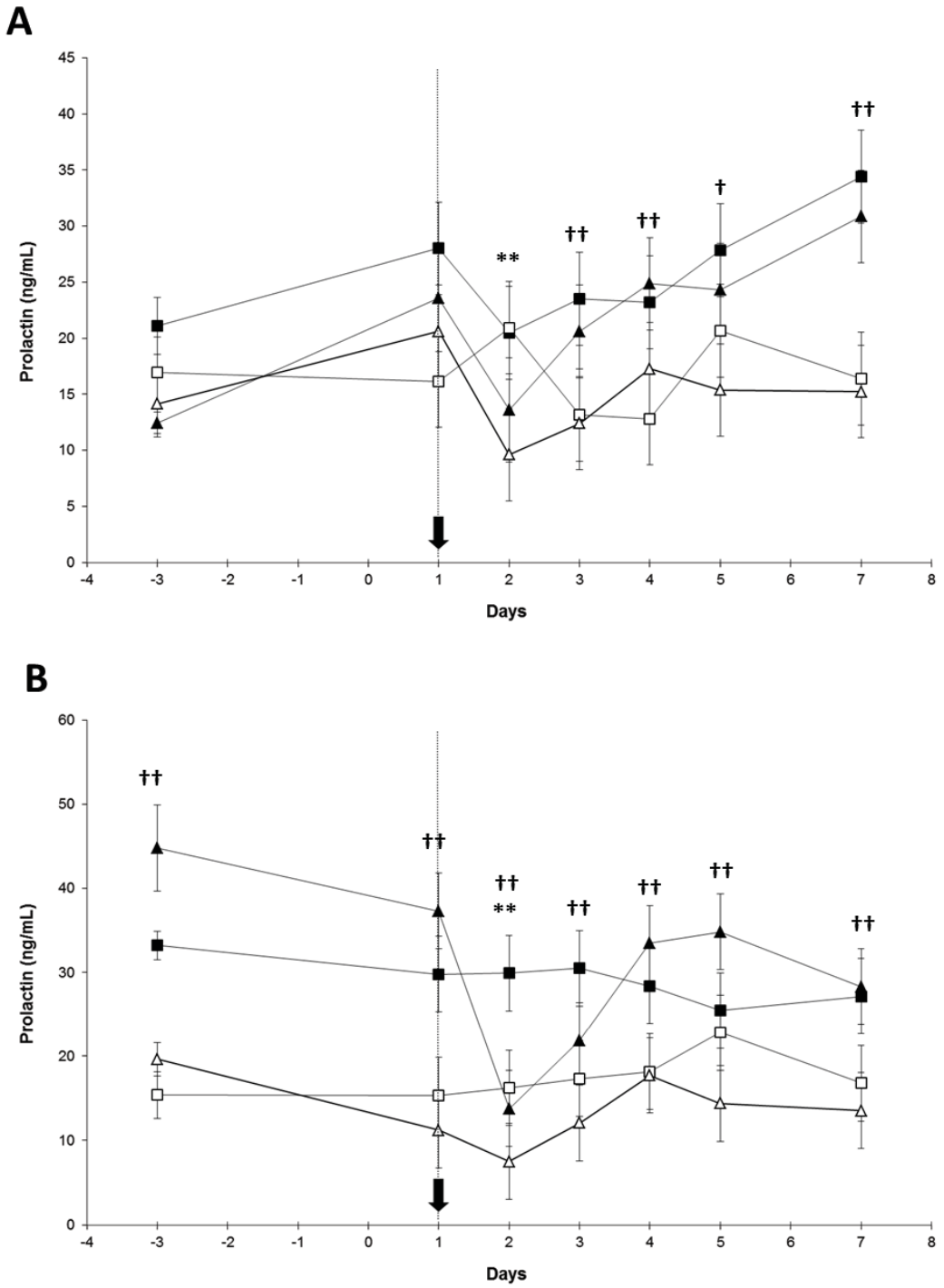

Figure 5.4. Prolactin concentration measured in serum of cows injected with 0.5 g of domperidone on d 1 and 2 and with 20 mg of dexamethasone on d 1 (▲; n=8); only injected with domperidone on d 1 and 2 (■; n=8); injected with canola oil on d 1 and 2 and with dexamethasone on d 1(△; n=8); or only injected with canola oil on d 1 and 2 (□; n=8) during the first experimental period (A) and the second experimental period (B).
Data are presented as least square means ± SEM. The significant differences between dexamethasone-treated cows and non-treated cows are indicated as follows: $^{**}P < 0.01$. The significant differences between domperidone-treated cows and oil-treated cows are indicated as follows: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$.

As was also the case for serum prolactin concentration, there was an interaction between domperidone and experimental period ($P = 0.04$) for milk prolactin concentration. In the first period, domperidone induced a gradual increase (Domperidone*Day, $P = 0.03$; Figure 5.5A) in milk prolactin concentration, which was greater ($P < 0.01$) in the domperidone-treated cows than in the oil-injected cows from d 3 to 7. There was a carryover effect of domperidone from the first experimental period to the second. The milk prolactin concentration of the domperidone-treated cows during the first experimental period was still greater than that of the oil-treated cows before the start of the second period ($P < 0.01$; Figure 5.5B). Milk PRL concentration remained greater in the domperidone-treated cows during the second treatment period ($P < 0.03$). There was a Dexamethasone*Day interaction ($P < 0.001$) during both treatment periods. In comparison with the non-injected cows, milk PRL was lower ($P < 0.001$) in the dexamethasone-treated cows on d 2 but was greater ($P < 0.03$) from d 4 to 7 during the first period, and milk prolactin concentration was lower ($P < 0.001$) in the dexamethasone-treated cows on d 2 but was greater ($P = 0.03$) on d 5 during the second period (Figure 5A and B).

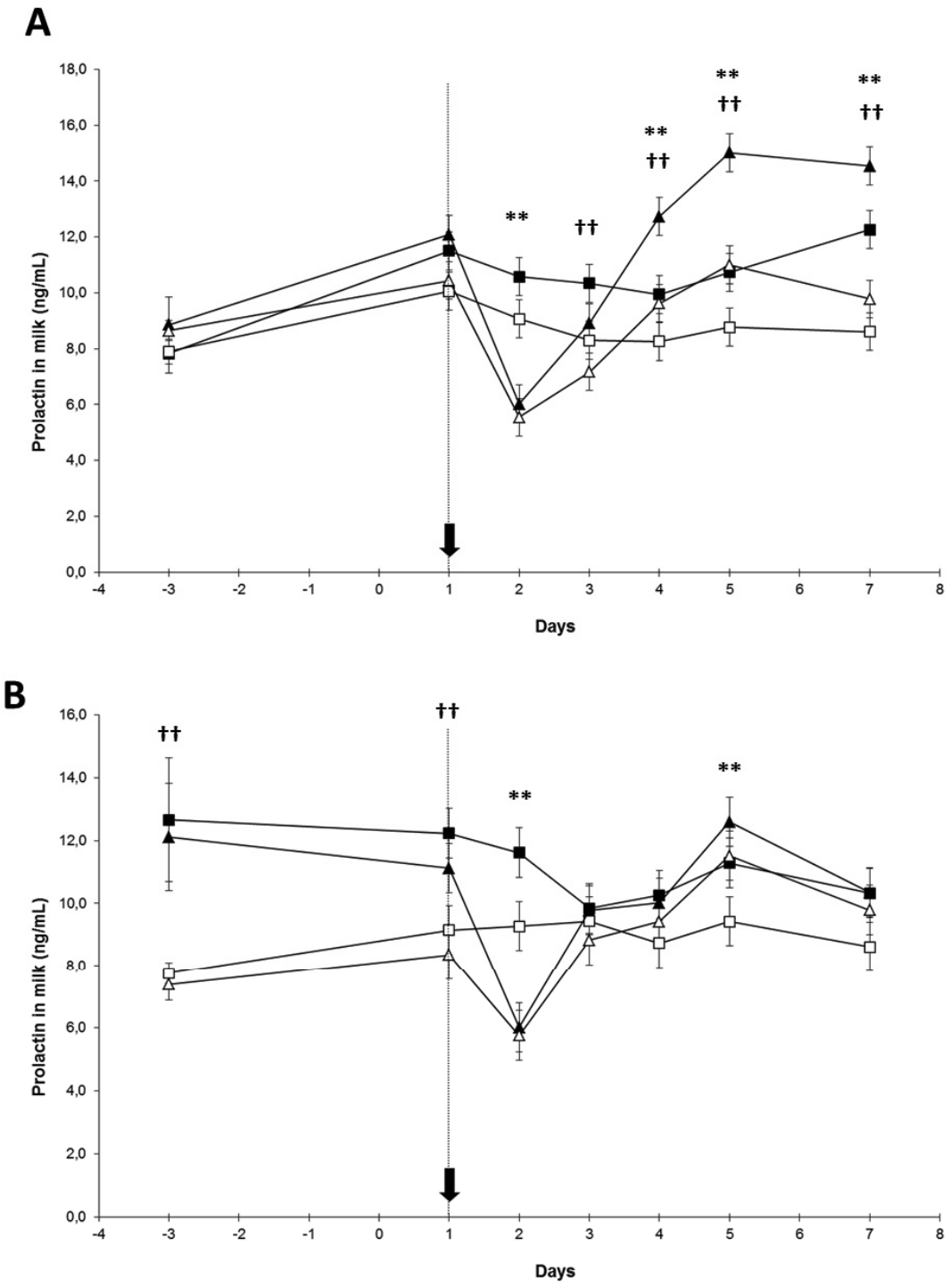

Figure 5.5. Prolactin concentration measured in milk of cows injected with 0.5 g of domperidone on d 1 and 2 and with 20 mg of dexamethasone on d 1 (▲; n=8); only injected with domperidone on d 1 and 2 (■; n=8); injected with canola oil on d 1 and 2 and with dexamethasone on d 1(△; n=8); or only injected with canola oil on d 1 and 2 (□; n=8) during the first experimental period (A) and the second experimental period (B).
Data are presented as least square means ± SEM. The significant differences between dexamethasone-treated cows and non-treated cows are indicated as follows: $^{**}P < 0.01$. The significant differences between domperidone-treated cows and oil-treated cows are indicated as follows: ††$P < 0.01$.

Blood samples were collected around the time of the morning milking before and after the start of treatments in the cows treated with dexamethasone. As was also the case for daily prolactin concentration, there were significant ($P < 0.03$) interactions between experimental period and domperidone on milking-induced parameters. During the first experimental period, domperidone did not affect basal and peak prolactin concentrations or total amount of prolactin released (Figure 5.6A). However, on the day following dexamethasone injection, basal prolactin concentration ($P = 0.02$) and total amount of prolactin released ($P < 0.01$) were reduced and peak prolactin concentration tended to be reduced ($P = 0.1$). During the second experimental period, domperidone increased ($P = 0.01$) basal PRL concentration and tended ($P = 0.06$) to increase total amount of prolactin released (Figure 5.6B). As was also the case for the first experimental period, dexamethasone reduced basal prolactin concentration ($P = 0.02$), peak prolactin concentration ($P = 0.02$) and total amount of prolactin released ($P < 0.03$).

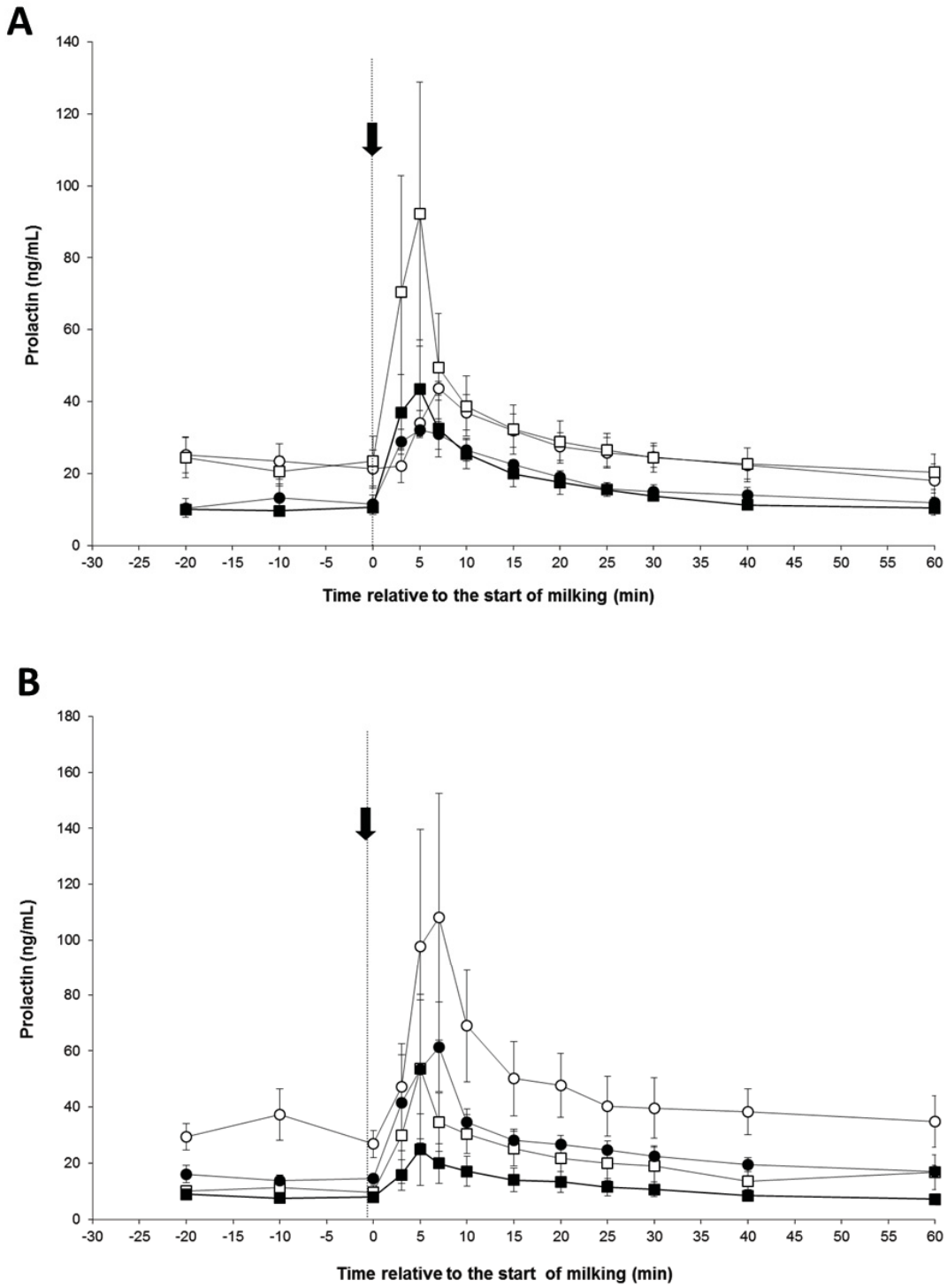

Figure 5.6. Serum concentrations of prolactin released during d 1 (prior to injection) and d 2 milkings in cows injected on d 1 with 20 mg of dexamethasone and on d 1 and 2 and with 0.5 g of domperidone (○ and ●, for d 1 and d 2 milkings, respectively; n=4) or canola oil (□ and ■, for d 1 and d 2 milkings, respectively; n=4) during the first experimental period (A) and the second experimental period (B).
The start of milking is represented by the black arrow. Data are presented as arithmetic means ± SEM.

Serum cortisol concentration was similar among the treatment groups before the start of the treatments (Figure 5.7A). There was a significant interaction between dexamethasone and day of sampling ($P = 0.01$) during the treatment period. In comparison with the non-injected cows, cortisol concentration were lower ($P \leq 0.05$) on d 2, 3, 4, and 5 in the dexamethasone-treated cows. Domperidone did not affect serum cortisol concentration.

The plasma concentration of IGF-1 was similar among the treatment groups before the start of the treatments (Figure 5.7B). There was a significant interaction between dexamethasone and day of sampling ($P < 0.001$) during the treatment period. Plasma IGF-1 concentration in the dexamethasone-treated cows was lower on d 2 and 3 ($P < 0.04$) but was greater on d 4 ($P = 0.03$) and tended to be greater on d 5 ($P = 0.06$) than in the non-injected cows. Domperidone did not affect plasma IGF-1 concentration.

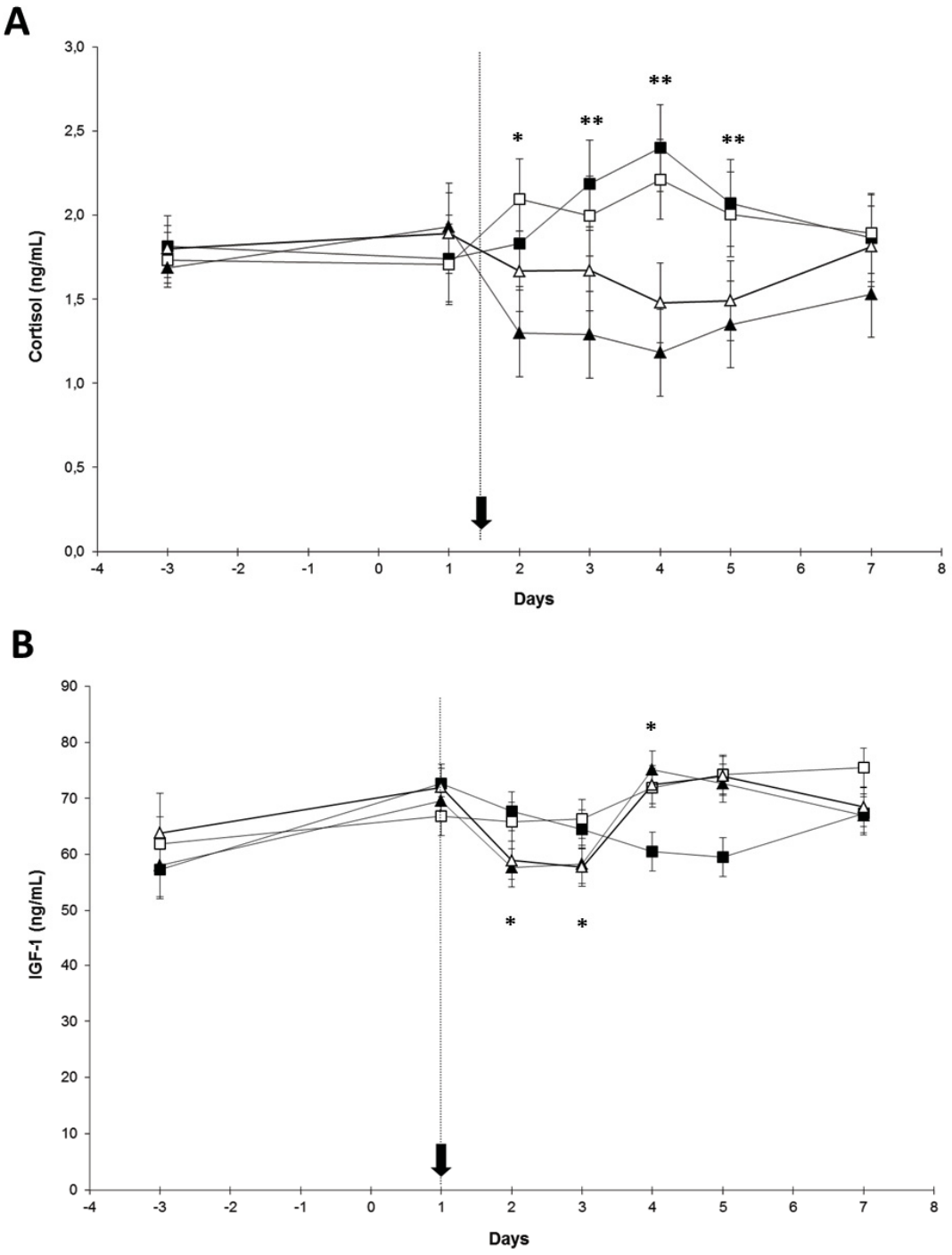

Figure 5.7. Basal cortisol (A) and IGF-I (B) concentrations measured in serum of cows injected with 0.5 g of domperidone on d 1 and 2 and with 20 mg of dexamethasone on d 1 (▲; n=8); only injected with domperidone on d 1 and 2 (■; n=8); injected with canola oil on d 1 and 2 and with dexamethasone on d 1(△; n=8) or only injected with canola oil on d 1 and 2 (□; n=8).

Data are presented as least square means ± SEM and significant differences between dexamethasone-treated cows and non-treated cows are indicated as follows: **P* < 0.05, ***P* < 0.01.

5.5 Discussion

The aim of Experiment 1 was to test whether glucocorticoids are responsible for the decrease in prolactin release associated with multiple mammary stimulations. Peak prolactin concentration was decreased during the second mammary gland stimulation, occurring one hour after the first one. Moreover, the total amount of prolactin released during the second stimulation tended to be reduced. This result is in accordance with previous results showing that the amount of prolactin released at milking decreased as the number of mammary gland stimulations increased (Lacasse and Ollier, 2014). However, this inhibition could not be reproduced by an injection of 1 mg of dexamethasone. Therefore, in our experimental conditions, the reduction in prolactin concentration caused by multiple stimulations of the mammary gland does not seem to be due to milking-induced glucocorticoid release.

Previous mammary gland stimulation reduced the amount of cortisol released during a second stimulation in the STIM treatment but not in the METY treatment. A previous study in calves found that administering 750 mg of metyrapone orally 6 times every 4 h, for a total dose of 4.5 g, reduced the increase in cortisol release induced by transport stress without suppressing that release (Agnes et al., 1990). In another study in calves, the oral administration of 3 g of metyrapone every 4 h for 48 h decreased the cortisol surge due to castration without suppressing it (Fisher et al., 1997). In the present study, 5 g of metyrapone was infused in the omasum to avoid ruminal degradation. Despite this method, the dose was probably not large enough to significantly depress basal cortisol levels. Nevertheless, the amount of cortisol released in response to the first manual stimulation was numerically reduced, and that result probably explains why cortisol release during the second stimulation was not significantly reduced in the metyrapone-treated cows.

Dexamethasone induced a decrease in the amount of cortisol released during mammary gland stimulation. This inhibitory effect of dexamethasone on cortisol release has been reported in humans (Weiner, 1989; Koopmans et al., 1992; Huizenga et al., 1998), dogs (Pessina et al., 2009), horses (Soma et al., 2005; Abraham et al., 2009), and cattle (Toutain et al., 1982). Dexamethasone inhibits cortisol concentration because glucocorticoids exert negative regulatory feedback on the hypothalamo-pituitary-adrenal axis by inhibiting ACTH secretion (Keller-Wood and Dallman, 1984; Feldman and Weidenfeld, 1995). In response to the injection of the synthetic glucocorticoid into the blood stream, the synthesis and release of

cortisol by the adrenal glands were therefore downregulated. Even though ACTH concentration in plasma was not affected by the treatments, a decrease in ACTH release cannot be ruled out, given that measurements were taken at only a few time points after the second manual stimulation of the mammary gland. A previous study also showed that the intramuscular administration of dexamethasone in bovines suppressed the response of the adrenal cortex to ACTH (Toutain et al., 1982). Therefore, it is possible that dexamethasone injections inhibited cortisol synthesis by decreasing the sensitivity of the adrenal gland to direct effect of ACTH.

The effects of glucocorticoids on prolactin release were different between the two experiments. In Experiment 1, a dose of 1 mg of dexamethasone administered 60 min before blood sampling did not affect the amount of prolactin released during mammary gland stimulation. A previous study showed that in cattle, 2 to 3 h is necessary before the repressor effect of dexamethasone on cortisol release can be observed (Toutain et al., 1982). In contrast, in Experiment 2, a dose of 20 mg of dexamethasone was sufficient to cause a reduction in basal serum and milk prolactin concentrations on the day after the injection and to decrease the peak prolactin concentration and total amount of prolactin released during milking. The dose of glucocorticoids used (1 mg) during Experiment 1, the time of injection relative to the time of sampling (60 min), or both may have not been sufficient to allow us to detect any potential effect of dexamethasone on the PRL release induced by mammary gland stimulation. Nevertheless, the inhibitory effect of dexamethasone on basal and milking-induced prolactin secretions is consistent with the findings of previous rodent studies. In lactating rats, dexamethasone treatment inhibited the prolactin release induced by the suckling stimulus (Vilela and Giusti-Paiva, 2011), and Horváth et al. (2001) reported that prolactin release following domperidone injection is reduced by dexamethasone treatment and enhanced by adrenalectomy in rats. It is therefore tempting to state that glucocorticoids inhibit prolactin synthesis, prolactin secretion, or both.

The main physiological control of PRL secretion is exerted by the inhibitory action of dopamine on the lactotrophs of the anterior pituitary via dopamine D2 receptors (Torre and Falorni, 2007). Domperidone is a dopamine antagonist that enhances prolactin release by preventing the inhibitory action of dopamine secreted in the hypothalamus by the tuberoinfundibular neurons. Our results indeed show that prolactin concentrations in both serum and milk gradually increased in the cows treated with domperidone in comparison with

the cows treated with oil. The fact that dexamethasone inhibited prolactin levels in the domperidone-treated cows suggests that the inhibitory action of glucocorticoids is not mediated through the enhanced secretion of dopamine by the hypothalamus. As stated above, dexamethasone exerts its inhibitory action on cortisol and through the inhibition of ACTH release by the pituitary gland (Miller et al., 1992; Feldman and Weidenfeld, 1995; de Kloet et al., 1998). It is therefore possible that dexamethasone targeted the pituitary gland and affected the release of prolactin by lactotrophs. This possibility is supported by the results of a previous experiment conducted in rats: the release of immunoreactive prolactin from the pituitary gland was significantly reduced by dexamethasone (Taylor et al., 1995). The mechanism of such an inhibition has not yet been explained, but it does not involve a dopaminergic component. Glucocorticoids are known to bind and activate the glucocorticoid receptor, which will target and modulate the expression of certain genes (Burnstein and Cidlowski, 1992). Low doses of corticosterone were shown to decrease prolactin mRNA expression in vitro, whereas high doses of corticosterone were shown to enhance it (Yokoyama et al., 2008). Glucocorticoids may thus, at a certain concentration, reduce the expression of PRL by the lactotrophs of the pituitary gland.

In the present study, dexamethasone reduced milk yield for 2 d. Other studies already reported that i.m. injections of dexamethasone depressed milk yield in cows (Hartmann and Kronfeld, 1973; Shamay et al., 2000). Moreover, dexamethasone decreased milk lactose percentage and milk lactose yield. It was suggested that glucocorticoids alter the glucose partitioning between the mammary gland and other organs by disadvantaging the udder (Hartmann and Kronfeld, 1973). A recent study reported that dexamethasone induced transient hyperglycemia in cows (S. Ollier, F. Beaudoin, N. Vanacker, and P. Lacasse, unpublished data), a further indication that glucocorticoids decrease glucose uptake by the mammary gland. This reduced glucose uptake leads to a decrease in lactose synthesis. Given that lactose is the major osmotic component of milk, a reduction in glucose mammary uptake could explain the depression of milk production due to dexamethasone administration.

Despite the induction of an increase in serum and milk prolactin concentrations, domperidone did not affect milk production. In an earlier study, it has been reported that cows injected daily with 0.3g of domperidone for 5 wk displayed higher serum and milk prolactin concentrations from d 6 to d 28 and produced more milk during wk 3 and 4 of treatment compared with control cows (Lacasse and Ollier, 2015). Therefore, it seems there is delay

between the increase in prolactin secretion and the enhancement of milk production. The shorter duration of treatment (2 d versus 5 wk) may likely explain the absence of effect of domperidone on milk production in the present study. Moreover, basal concentrations of prolactin seemed to be greater than in the works of Lacasse and Ollier (2015); these levels might have already been sufficient for maximal milk production, preventing thus an effect of an increase in PRL release on milk yield.

Given that most recent studies highlight a galactopoietic role for prolactin (Lacasse et al., 2016), the fact that dexamethasone depressed both basal prolactin concentration and milking-induced prolactin release could have contributed to the decrease in milk production. Conversely, domperidone induced an increase in basal serum and milk prolactin concentrations from 2 d after injection. However, this stimulation of prolactin secretion was not able to prevent the dexamethasone-induced inhibition of milk production, although prolactin was transiently depressed by dexamethasone in these cows. A previous study reported that the inhibition of prolactin secretion depressed milk production and that this effect was associated with a decrease in milk lactose concentration (Lacasse et al., 2011). In the present study, the reduction in milk production induced by dexamethasone also caused a depression in milk lactose concentration. Therefore, it appears that prolactin inhibition could be one of the mechanisms by which glucocorticoids inhibit milk production. In addition, a decrease in mammary tissue sensitivity to prolactin induced indirectly by dexamethasone cannot be completely excluded. For instance, glucocorticoids were shown to modulate the number of prolactin receptors on the mammary cells of lactating mice (Sakai and Banerjee, 1979). A decrease in mammary cell sensitivity to prolactin could explain the negative effect of dexamethasone on milk yield even in the presence of domperidone.

In the present study, serum IGF-1 concentration was decreased during the 2 d following the injection of dexamethasone, a result that is consistent with those of previous studies (Maciel et al., 2001). This effect could come from the inhibitory role exerted by dexamethasone on prolactin release. Indeed, it has been suggested that prolactin helps maintain IGF 1 levels by repressing the inhibitory action of IGF-binding protein 5 on IGF-1 (Flint and Knight, 1997). However, Lacasse et al. (2011) did not observe any effect of prolactin inhibition on IGF-1 concentration. Nonetheless, a previous study already showed that IGF-1 can stimulate milk production in lactating goats (Prosser et al., 1990). It is

therefore possible that the reduction in IGF-1 levels contributed to the glucocorticoid-induced inhibition of milk yield.

In conclusion, the decrease in prolactin secretion after 2 mammary gland stimulations does not seem to involve glucocorticoids. The depression in milk yield induced by dexamethasone was associated with a decrease in basal prolactin concentrations in both serum and milk and with a reduction in milking-induced prolactin release. Although the enhancement of prolactin secretion by domperidone was not able to prevent the depression of milk yield, it cannot be ruled out that glucocorticoids reduce milk production partly through the inhibition of prolactin synthesis or the reduction of mammary gland responsiveness.

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Chapter 6 General discussion and conclusions

The transitions between the end of lactation and the dry period are very critical for dairy cows because of the high susceptibility of the mammary gland to contract new intra-mammary infections. Indeed, after cessation of milking, the gland continues to synthesize significant amounts of milk causing an engorgement of the udder and inducing milk leakage that facilitate the entry of micro-organisms. It has been estimated that between 11% and 16.7% of quarters which were bacteriologically negative before cessation of milking became infected during the dry period, regardless of antibiotic treatment (Dingwell et al, 2002; Dingwell et al., 2004), and that more than 50% of clinical mastitis observed during the subsequent lactation were due to enterobacterial intra-mammary infections contracted during the dry period (Bradley and Green, 2000). The risk for the gland to become infected increases as the volume of milk collected during the last milking is elevated (Rajala-Schultz et al., 2005. Newman et al., 2009). Due to the improvement in genetics and nutrition, modern dairy cows produce more milk than in the past decades and it is not rare to dry cows that produce around 28 kg of milk just before drying-off (Dingwell et al., 2001). Consequently, strategies that facilitate the cessation of lactation would improve udder health during this period. In order to reach this goal, one approach is to hasten the mammary gland involution process. Indeed, once milking is stopped and milk accumulates in the udder, some molecular and cellular mechanisms are put in place to decrease milk component synthesis and secretion, and the mammary gland undergoes a series of cytological and histological modifications during what is called involution. During this remodelling process, the permeability of tight junctions that link epithelial cells together is increased, a portion of the mammary epithelium die by apoptosis, and different proteases are expressed and activated. Once involution is achieved and mammary gland returned to a pre-lactating state, the gland is more resistant to new intra-mammary infections. In cows, mammary gland involution occurs approximately 21 days (Hurley, 1989). It seems therefore of interest to allow the gland to reach the involuted state earlier.

In our first study, we evaluated the effects of intra-mammary infusions of CNH, EGTA and lactose on the speed of involution because these compounds are known to affect tight junction permeability. Among those treatments, only CNH significantly increased the rate of the involution process. Casein hydrolysates are the breakdown products of the digestion of casein by plasmin in milk (Politis, 1996). This result makes CNH a good candidate for being one of the molecular signals triggering mammary gland involution in

cows. It has already been shown that intra-mammary infusions of CNH depressed milk synthesis in both goats and cows (Silanikove et al., 2000; Shamay et al., 2002) and our results confirmed that this treatment increased tight junction permeability in bovine (Shamay et al., 2003). Furthermore, we demonstrated that CNH enhanced MMP activity and decreased milk citrate concentrations, indicative of a reduction in mammary epithelial cell activity. Even though the exact mechanism of action of CNH on the mammary epithelium is not yet elucidated, CNH may alter ionic composition of mammary epithelial cells. As mentioned earlier, it has been suggested that CNH could block the activity of potassium channels located at the apical side of the mammary epithelial cells, therefore reducing their secretory activity (Silanikove et al., 2000). Moreover, CNH could decrease indirectly calcium availability. Indeed, CNH intra-mammary infusions depressed milk calcium concentrations in cows (Shamay et al., 2000) and extracellular calcium depletion has been shown to impair tight junction integrity in rodents and goats (Pitelka et al., 1983; Stelwagen et al., 1995). Therefore, CNH seemed to affect tight junction permeability through a reduction in the extracellular calcium available at the apical side of the mammary epithelium. Interestingly, the concentration of stanniocalcin in milk increases during milk stasis in bovine (Tremblay et al., 2009). Stanniocalcin is an anti-hypercalcemic glycoprotein hormone that decreases calcium absorption in mammals (Madsen et al., 1998). It is thus tempting to hypothesize that CNH signal could be mediated through an increase in stanniocalcin concentration in milk. Further researches are needed to elucidate the exact mode of action of CNH and to determine whether it implies stanniocalcin or not.

The fact that CNH treatment hastened mammary gland involution is promising from the perspective of improvement of udder health around drying-off. As a fully involuted mammary gland is more resistant against pathogens, we hypothesize that cows treated with CNH at drying-off would contract fewer infections than untreated cows. It has been shown that intra-mammary infusions of CNH in combination with an antibiotic treatment had the potential to cure coagulase negative staphylococci infections in dairy cows (Leitner et al., 2011). More experiments need to be performed to investigate the effects of CNH on the frequency of isolation of different pathogen strains, notably those responsible for mastitis, during dry period.

In our second experiment, we modified the photoperiod during the last 14 days of lactation and the first 14 days of the dry period in cows. We notably investigated the effects of SDPP and oral melatonin administration on milk production, serum prolactin concentrations

and mammary gland involution speed. Indeed, SDPP and melatonin treatment are known to be associated with low prolactin levels in dairy ruminants. As prolactin seems to prevent mammary gland involution (Ollier et al., 2013) and appears to be necessary for maintaining lactation (Lacasse et al., 2012) in dairy cows, its inhibition would help to decrease milk production before drying-off and to accelerate involution rate. In the research conditions of our study, SSPP did not affect milk yield and prolactin levels in the same extent as it has been reported in previous studies (Dahl et al., 1997; Miller et al., 2000; Auchtung et al., 2005). Indeed, SDPP slightly reduced milk production, tended to decrease the prolactin release associated with milking and decreased the mean basal prolactin secretion during the first 2 weeks of dry period. The fact that cows in late lactation display lower prolactin levels than cows in early or mid-lactation (Miller et al., 2006) might explain the lower response to SDPP compared with previous works. We can indeed speculate that prolactin-release inhibition would be less effective when prolactin levels are already low. However, it has already been reported in late lactation cows that quinagolide, a specific domapinergic agonist, was able to reduce markedly serum prolactin concentrations and milk production before drying-off (Ollier et al., 2013). In the latter study, basal prolactin levels before treatment were comparable to those measured in our experiment before photoperiodic manipulation. Therefore, it is more likely that the change in lighting regimen was not efficient enough to decrease prolactin secretion. This lack of effectiveness of SDPP can be attributed to a too short duration of treatments. According to previous experiments, at least 28 days are necessary to observe the effects of a photoperiodic manipulation on milk production during lactation (Dahl et al., 1997; Dahl, 2008). In dry cows, around 30 days during the dry period were necessary to observe a decrease in prolactin concentrations due to SDPP (Auchtung et al., 2005). In our case, the treatments were applied 14 days before and after drying-off which seems not long enough to elicit a depression in prolactin release and milk production. The effect of SDPP on milk production and prolactin secretion during late lactation should be investigated with a longer period of treatment, starting minimum one month before drying-off.

Short day photoperiod also did not affect the rate of mammary gland involution. Again, the duration of the treatment might be responsible for this result. However, even in case of SDPP success in hastening involution, this treatment is more difficult to apply in the dairy industry compared with CNH intra-mammary infusions. It implies that cows in late lactation need to be separated from the rest of the herd in an independent closed room with a

special lighting regimen. This would significantly impact the organization and management of a dairy farm.

The aim of the third experiment was to test the effects of glucocorticoids on the prolactin release induced by mammary gland stimulation and to test whether the inhibitory effect of glucocorticoids on milk production is due to a decrease in prolactin release. Compared with its own control, a dose of 20 mg of dexamethasone reduced both basal serum and milk prolactin concentrations and decreased the total amount of prolactin released during milking the day following the injection. This prolactinemic inhibition was associated with a significant depression in milk production for 2 days. In a previous study in cows, researchers injected 40 mg of dexamethasone; milk yield was reduced the day following the injection and this effect persisted during 4 more days (Shamay et al., 2000). Our results showed that the glucocorticoid-induced milk yield depression at least partly involved an inhibition of prolactin secretion at the pituitary level. A decrease in sensitivity to prolactin of the mammary tissue cannot be completely excluded knowing that glucocorticoids modulate the number of prolactin receptors on mammary cells of lactating mice (Sakai and Banerjee, 1979). The glucocorticoid receptor, after activation by glucocorticoid binding, can modulate the transcription of target genes by either DNA binding-dependent mechanisms (Burnstein and Cidlowski, 1992) or through interaction with transcription factors such as STAT5 (Reichardt et al., 2001). It would therefore be of interest to measure the expression of the 2 types of PRL-R in mammary cells, at the transcriptional and the protein levels as well as their concentration on the surface of these target cells. Glucocorticoid receptor possibly influencing its own transcription (Burnstein and Cidlowski, 1992), it might also affect that of PRL-R. Finally, a lot of questions concerning the role that glucocorticoids can have on milk production and on the regulation of prolactin secretion remain outstanding and it is possible that it involves factors that have not been elucidated yet. Certainly, prolactin and glucocorticoids are 2 key elements of the regulation of milk synthesis and secretion and more research is needed to study the possible inter-relation between them.

In conclusion, further studies are needed to study the efficacy of CNH to increase mammary gland resistance against bacterial infections, elucidate the mechanisms of prolactin secretion regulation and evaluate the effects of glucocorticoid inhibition on lactation persistency. Results from these studies would help to improve udder health at drying-off.

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