animal

Review: the cellular mechanisms underlying mammary tissue plasticity during lactation in ruminants

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The mammary tissue is characterized by its capacity to adapt in response to a wide variety of changing conditions. This adaptation capacity is referred to as the plasticity of mammary tissue. In dairy ruminants, lactation is challenged by modifications that can either be induced on purpose, such as by modifying management practices, or occur involuntarily, when adverse environmental constraints arise. These modifications can elicit both immediate changes in milk yield and composition and carryover effects that persist after the end of the challenge. This review focuses on the current knowledge concerning the cellular mechanisms underlying mammary tissue plasticity. The main mechanisms contributing to this phenomenon are changes in the activity and number of mammary epithelial cells (MECs). Changes in the number of these cells result from variations in the rates of cell proliferation and death as well as changes in the rate MEC exfoliation. The number of MECs also depends on the number of resident adult mammary stem cells and their progenitors, which can regenerate the pools of the various mammary cells. Several challenges, including changes in milking frequency, changes in level of feed supply and hormonal manipulations, have been shown to modulate milk yield together with changes in mammary cell activity, turnover and exfoliation. Epigenetic changes may be an additional mechanism of adaptation. Indeed, changes in DNA methylation and reductions in milk yield have been observed during once-daily milking and during mastitis in dairy cows and may affect cell activity persistently. In contrast to what has been assumed for a long time, no carryover effect on milk yield were observed after feed supply challenges in dairy cows and modification of milking frequency in dairy goats, even though the number of mammary cells was affected. In addition, mammary tissue plasticity has been shown to be influenced by the stage of lactation, health status and genetic factors. In conclusion, the cellular mechanisms underlying mammary tissue plasticity are diverse, and the mammary tissue either does or does not show elastic properties (with no permanent deformation), in response to environmental changes.

Keywords: dairy ruminant, mammary gland, review, adaptation, milk production

Implications

Lactation in dairy ruminants is challenged by perturbations that can either be induced on purpose, such as by modifying management practices, or occur involuntarily, when adverse environmental constraints arise. These challenges can have immediate effects on milk yield and could be followed or not by carryover effects. A series of studies have investigated the cellular mechanisms involved in the process of adaptation of the mammary tissue to these challenges. An improved understanding of these mechanisms may help predict irreversible effects on the mammary tissue.

Introduction

During a female mammal's life, the mammary gland undergoes many changes in size, structure, composition and activity. In dairy ruminants, females are subjected to successive reproductive cycles, and the mammary tissue adapts as it goes through the gestation, lactation and involution phases. During a normal lactation cycle, milk production follows a lactation curve (a strong increase up to a peak and then a gradual decrease) resulting from modulations of both the activity and the number of mammary secretory cells (Capuco *et al.*, 2001). Meanwhile, the mammary gland responds to perturbations that the animal must cope with. This organ is characterized by its capacity to adapt in response to changes to a wide variety of conditions. This adaptation capacity is referred to mammary tissue plasticity.

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The mammary tissue plasticity is a component of the robustness of the animal that can be defined as the ability of an animal to adapt to environmental constraints (Friggens *et al.*, 2010).

Several transient external perturbations can challenge the lactating mammary gland. The word 'challenge' is used in order to conceptualize the action of an external perturbation that exercises a constraint to the 'mammary tissue' system and can stimulate or impair lactation. In sciences other than biology, scientists have questioned how a system can adapt to changing conditions (Sauvant and Martin, 2010). The concepts developed in economics can be transposed to conceptualize how a biological system can adapt to challenges (Sauvant and Martin, 2010). When an animal is subjected to a challenge, the mammary tissue sets up an adaptive response that involves biological processes, and the production of milk can be affected. The mammary tissue is thus a deformable system, characterized by its resistance and its resilience (Figure 1). Resistance could be defined as its ability to tolerate a disruption induced by a challenge and can be illustrated by the difference in milk yield before and during the application of the challenge. Resilience could be defined as the ability of a system to return to its initial state when a challenge is finished. The resilience of the mammary tissue can thus be characterized by the carryover effects on milk yield (Figure 1). If no carryover effect is observed (the system goes back to its initial state), then the mammary tissue shows elastic properties. In contrast, if some carryover effects are observed (milk yield remains different from before the challenge), then the mammary tissue shows flexible properties. Our assumption is that understanding the cellular mechanisms underlying the adaptation of the mammary tissue during the application of challenges could make it possible to understand why there are carryover effects on milk yield or not. The objective of this review is, therefore, to contribute to a better understanding of the cellular and molecular mechanisms involved in mammary tissue plasticity. We will limit the review to the mammary tissue and, particularly, to the mammary epithelial cells (MECs) to simplify the system, even though it is obvious that the challenges also lead to wide physiological modifications at the whole-body level (effects on blood flow, nutrient and hormone concentrations etc.). In this review, we will first describe the indicators of the potential mechanisms involved in mammary tissue plasticity. We will, then, describe how these mechanisms could be affected during the application of diverse challenges. And, finally, we will address the question of the carryover effects when the challenge is finished.

What are the indicators of the potential molecular and cellular mechanisms underlying mammary tissue plasticity?

Indicators of the secretory activity of the MECs
One of the main cellular mechanisms that may contribute to mammary tissue plasticity is the changes in the secretory
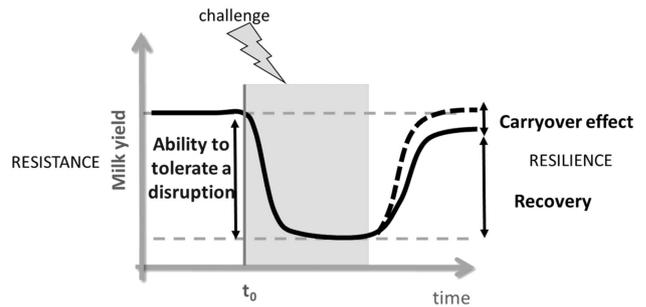
Figure 1 Schematic representation of the effect of a challenge (represented here with a negative effect on milk yield) on mammary tissue plasticity during lactation in ruminants as indicated by the variation in milk yield. The grey box indicates the phase when the challenge is applied, and t_0 corresponds to the time zero of the application of the challenge. The adaptation of the mammary tissue when animals are challenged during lactation can be characterized by an initial resistance phase followed by a resilience phase (Adapted from Sauvant and Martin, 2010).

activity of the MEC. The secretory activity of MEC can be analyzed in terms of enzyme activity and target-gene expression and using global transcriptomic or proteomic approaches. MECs express messenger RNA (mRNA) coding for milk-specific proteins and proteins necessary for the synthesis of milk constituents (enzymes, transporters, receptors, intra-cellular messengers etc.). Microarray and KEGG pathway analyses were used to provide a picture of the transcriptomic adaptations in the mammary tissue between the end of pregnancy and the onset of lactation, showing an overall induction of the metabolism categories, with the 'lipid metabolism,' 'glycan biosynthesis and metabolism' and 'carbohydrate metabolism' pathways being the most impacted (Bionaz *et al.*, 2012). Another way to study the secretory activity of MEC is to target genes of interest. The main target genes studied are the ones coding for milk components such as milk proteins like casein αs1 type 1 (*CSN1S1*), αs1 type 2 (*CSN1S2*), β- (*CSN2*), or κ (*CSN3*) or α-lactalbumin (*LALBA*). This last gene is particularly interesting because α-lactalbumin is the co-factor of the enzyme galactosyltransferase (GT), which is involved in the last step of lactose synthesis. Since lactose is the main osmotic agent in most mammalian species, it has a prominent role in the determination of the volume of milk produced. Changes in mammary cell activity can also be shown through the activity of several key mammary enzymes, such as GT, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN); the last two being involved in the synthesis of milk fat. It has been shown that the increase in milk production at the onset of lactation partly results from an increase in the activity of ACC, FASN and GT (Knight and Peaker, 1984). In addition, the proteome of milk or mammary tissue can also reveal changes in secretory cell activity.

Indicators of epigenetic modifications that may impact secretory cell activity
Epigenetic changes may participate in regulating mammary tissue plasticity. Indeed, epigenetic regulations may be partly

responsible for the molecular regulation of milk constituent production. Studies in rodents or lagomorphs have shown that a combination of epigenetic events (chromatin opening, chromatin loop formation, histone acetylation and DNA methylation), on a global scale or targeted to specific genes, occurs during the development of the mammary gland and alters gene expression (Rijnkels *et al.*, 2010). In cattle, DNA is hypomethylated in the 5′ region of the αs1 casein gene when this gene is expressed (Platenburg *et al.*, 1996). It has been shown recently that the stage of mammary development modulates the degree of DNA methylation in the distal regulatory region upstream of this gene. This area of DNA contains three CpG islands near the binding sites of signal transducer and activator of transcription 5 (STAT5), a transcription factor involved in signal transduction of the hormone prolactin (Vanselow *et al.*, 2006). In the mammary gland, these CpG sites are less methylated (<30%) during lactation than during puberty, gestation or involution (between 35% and 60%), while being hypermethylated in the liver (>65%; Vanselow *et al.*, 2006; Singh *et al.*, 2012; Nguyen *et al.*, 2014). The high level of DNA methylation at these three CpG sites during mammary involution was associated with a low expression of the *CSN1S1* gene (Singh *et al.*, 2012). Epigenetic changes, such as DNA methylation, may occur during lactation and may result in long-term modulation of gene expression, thus affecting cell activity for a long period.

Indicators of the number of MECs

Another cellular mechanism that may contribute to mammary tissue plasticity is changes in the number of MEC in the mammary tissue. This number results from changes in the rates of cell proliferation and cell loss. The loss of MEC in the mammary tissue has been first considered to be due to cell death. However, MEC can be lost from the mammary tissue through exfoliation of MEC from the mammary epithelium into milk during lactation (Herve *et al.*, 2016). The number of MEC in the goat mammary gland increases slightly during early lactation up until the peak of lactation (Knight and Peaker, 1984). After this, the gradual decrease in milk yield is associated with a decrease in MEC number in the mammary gland, due to a greater cell apoptosis rate than cell proliferation rate (Knight and Peaker, 1984; Capuco *et al.*, 2001) and a gradual increase in the MEC exfoliation rate (Herve *et al.*, 2016). Standard indicators of variations in MEC number in the mammary tissue correspond to histological analyses that allow the rates of cell proliferation or cell death to be measured (Capuco *et al.*, 2001). Moreover, DNA concentration or overall DNA content in the mammary tissue (Capuco *et al.*, 2001) and the expression of genes involved in cell proliferation such as ATP-binding cassette super-family G member 2 (*ABCG2*), CCAAT enhancer binding protein delta (*CEBPD*), clusterin (*CLU*) and integrin subunit beta 6 (*ITGB6*) and cell death such as Bcl-2-modifying factor (*BMF*), insulin-like growth factor binding protein 5 (*IGFBP5*), Cathepsin B (*CTSB*), B-cell lymphoma 2 (*BCL2*) and BCL2 associated X (*BAX*) and *caspases* can also be indicators of variations in the number of MEC in the mammary gland (Boutinaud *et al.*, 2013).

Mammary stem cells may impact the number of MECs in the mammary tissue

The ability of the mammary gland to undergo many cycles of cell proliferation and tissue involution through successive gestations and lactations highlights the existence of resident cells able to regenerate the entire epithelial tissue. As in other somatic tissues, stem cells are believed to exist during the entire lifecycle of the mammary gland, thus representing a key mechanism in the plasticity of this organ. The mammary stem cells (MaSC) are required to generate the various cell populations in different lineages (epithelial and myoepithelial) during pregnancy and might sustain cell turnover to replace cells during lactation. Although research on MaSC has focused mainly on the murine and human models, pioneer studies in bovine identified a population of lightly stained cells with high proliferative capacity within the mammary parenchyma of the heifer mammary gland that may be the putative MaSC (Ellis and Capuco, 2002). More recently, when flow cytometry was used to define the MaSC by the co-expression of cell surface proteins CD49f and CD24, it was found that the proportion of MaSC was stable during a cow lactation cycle (Perruchot *et al.*, 2016) but was markedly decreased at drying-off (Finot *et al.*, 2018). These studies agree with the notion that the MaSC and their progeny may play a key role in mammary tissue regeneration. The regulation of the number of MaSC and their involvement in mammary tissue plasticity during cow lactation remain to be clarified.

What are the adaptation processes of the mammary tissue when lactation is challenged?

The challenges, including changes in milking frequency and level of feed supply and hormonal and heath manipulations, have been shown to modulate milk yield in association with changes in the mammary tissue functioning. To identify the mechanisms involved in the plasticity of the ruminant mammary gland, we analyzed mammary tissue responses to challenges that were experimentally applied during lactation.

Effect of challenges on secretory cell activity

The adaptation of the mammary tissue to milking frequency has been widely studied (Table 1). The decrease in milk yield observed after a reduction in milking frequency from twice daily (2×) to once daily (1×) was accompanied by a reduction of ACC and FASN enzyme activity (Farr *et al.*, 1995). Conversely, the increase in milk yield in response to a high milking frequency per day (3× or 4×) was associated with an increase of mammary enzyme activity in the first days after the milking frequency was modified (Wilde *et al.*, 1987; Travers and Barber, 1993). After several weeks of 3× milking, FASN, GT and ACC activities were no longer

different than during 2× milking (Wilde *et al.*, 1987; Nørgaard *et al.*, 2005), whereas greater glucose-6-phosphate dehydrogenase, lactate dehydrogenase and hexokinase activities were reported (Wilde *et al.*, 1987). Regardless of whether a target-gene or transcriptomic approach was used (Table 1), most studies showed an increase in the expression of the genes involved in the synthesis of milk when milking frequency was increased and, accordingly, a decrease when milking frequency was reduced.

The effect of feeding level manipulation on mammary secretory activity has not been as extensively investigated (Table 2). Feed or energy restriction has been found to induce a loss of milk yield. One study showed that the reduction of milk yield with energy restriction in mid-lactation dairy cows was associated with a tendency for

lower GT enzyme activity (Nørgaard *et al.*, 2005). Moderate feed restriction (30% and 20% less than the control diet) increased milk protein transcripts in early lactation (Sigl *et al.*, 2014) but did not affect them in a later stage of lactation (Boutinaud *et al.*, 2008; Herve *et al.*, 2018). In contrast, a severe feed restriction in early lactation that reduced milk production by 38% was accompanied by decreases in *LALBA* and *CSN3* gene expression (Dessauge *et al.*, 2011), with no effect on *CSN1S1* mRNA level. In another study, a severe feed restriction (40% less than the control diet) in mid-lactation dairy cows reduced the expression of genes involved in lipid metabolism (Abdelatty *et al.*, 2017). Thus, the effect of feed restriction on secretory cell activity likely depends on the severity of the challenge and the timing of its application.

Table 1 *Effects of the modification of milking frequency on indicators of secretory cell activity in the mammary tissue of dairy cows and goats)*

| Treatment[1] | Species | Milk yield | Indicators of secretory cell activity modifications[2] | | Reference |
			Enzyme activity	mRNA	
4× *v.* 2× for 4 weeks	Cow	↑	Tendency for ↑ ACC, FASN, GT and G6PH		Hillerton *et al.*, 1990
3× *v.* 2× for 7 days and 3 weeks	Cow	↑	↑ ACC and FASN at 7 d but no variation at 3 weeks		Wilde and Henderson, 1985
3× *v.* 2× for 37 weeks	Cow	↑	↑ Enzyme activity (G6PD, hexokinase, LDH)		Wilde *et al.*, 1987
3× *v.* 2× for 3 or 6 weeks	Cow	↑		No variation in mRNA involved in milk lipid and protein[3]	Grala *et al.*, 2014
3× *v.* 2× for 8 weeks	Cow	↑	No difference		Nørgaard et al., 2005
3× *v.* 2× for 3 weeks	Goat	↑	↑ ACC and FASN	↑ ACC and FASN[3]	Travers and Barber, 1993
4× *v.* 2× for 7 days	Cow	↑		↑ *CSN1S1*, *CSN2* and *LALBA*[3]	Murney *et al.*, 2015
4× *v.* 2× for 7 or 14 days	Cow	↑		↑ *CSN1S1*, *CSN2*, *LALBA*, β-lactoglobulin and glycam 1[4]	Connor *et al.*, 2008
4× *v.* 1× over 230 days	Cow	↑		↑ *LALBA*[3]	Alex *et al.*, 2015
3× *v.* 1× for 3 weeks	Cow	↑		No variation in *CSN3*[3]	Boutinaud *et al.*, 2003
3× *v.* 1× for 4 and 8 weeks	Cow	↑		↑ *CSN2*[3]	Bernier-Dodier *et al.*, 2010
1× *v.* 2× for 7 or 20 days	Cow	↓	↓ ACC and FASN		Farr *et al.*, 1995
1× *v.* 2× for 1 week	Cow	↓		↓ *CSN3* and *LALBA*[3]	Boutinaud *et al.*, 2012
1× *v.* 2× for 3 or 6 weeks	Cow	↓		↓ *LALBA*, *CSN2*, *CSN1S1*, *B4GALT1*, *FASN* and *ACC*[3]	Grala *et al.*, 2014
1× *v.* 2× for 5 days	Cow	↓		↓ *LALBA*, *B4GALT1*, *UGP2*, *CSN2*, *GPAM*, *LPL*[4]	Littlejohn *et al.*, 2010
1× *v.* 2× for 1 week	Cow	↓		↓ *PAH*, *PTGES*, *RBP1* (*small molecule biochemistry*), *SCD*, *CD36*, *FABP3*, *LPL* (*lipid*), *GPAM*, *Chi3L1* (*carbohydrate*) and *SLC25A24* (*amino acid*)[4]	Boutinaud *et al.*, 2013a
1× *v.* 2× for 1 and 5 weeks	Goat	↓		↓ *LALBA* and *SLC2A1*[3]	Ben Chedly *et al.*, 2011
1× *v.* 2× for 2 weeks	Goat	↓		↓ *LALBA*[3]	Ben Chedly *et al.*, 2013

[1]1×, 2×, 3× or 4× for milking one, two, three or four times daily, respectively.
[2]Abbreviations are listed in the Supplementary material (S1).
[3]Target-gene approach was taken.
[4]Transcriptomic approach was taken.

Table 2 *Effects of the modification of feeding level on indicators of secretory cell activity in the mammary tissue of dairy cows*

| Treatment[2] | Milk yield | Indicators of secretory cell activity modifications[1] | | Reference |
		Enzyme activity	mRNA	
Energy restriction for 8 weeks (early lactation)	↓	Tendency for ↓ GT		Nørgaard *et al.*, 2005
FR[2] for 11 weeks (early lactation)	↓		↓ *LALBA* and *CSN3*, no variation for *CSN1S1*	Dessauge *et al.*, 2011
FR for 2 days (early lactation)	↓		↓ *LALBA* and *CSN3*, no variation for *CSN2*	Sigl *et al.*, 2014
FR for 2 days (mid-lactation)	↓		no variation for *CSN1S1*, *CSN1S2*, *CSN2*, *CSN3* and *LALBA*	Sigl *et al.*, 2014
FR for 1 week (mid-lactation)	↓		↓ *SLC2A1*, no effect on *LALBA* and *CSN2*	Boutinaud *et al.*, 2008
FR for 15 and 18 days (mid-lactation)	↓		↓ *ACC*, *GPAM*, *SCD*, *FABP3*, *LPL* and *SREBP1*	Abdelatty *et al.*, 2017
FR for 4 weeks (mid-lactation)	↓		Tendency for ↓ *LALBA* in milk purified MEC but not in mammary tissue	Herve *et al.*, 2018

[1]Abbreviations are listed in the Supplementary material S1.
[2]FR, feed restriction.

Mastitis is also a challenge that has a temporary negative effect on milk production and may, therefore, affect secretory cell activity (Table 3). Lipopolysaccharide-induced mastitis downregulated *LALBA* gene expression but not *CSN1S1* gene expression in bovine mammary tissue (Gross *et al.*, 2015). A downregulation of genes involved in lipid synthesis was also reported after challenges with lipopolysaccharide (Gross *et al.*, 2015) or *Streptococcus uberis* (Moyes *et al.*, 2009). In addition, a clear reduction in the content of milk proteins involved in the synthesis of milk (casein, α-lactalbumin, GT and β-lactoglobulin) was observed by means of proteomic approaches (Table 3). These results suggest that mammary tissue inflammation affects the activity of the MEC.

To understand the role of hormones in the adaptation process of the mammary tissue, milk production was modulated by hormonal manipulations (Table 4). The withdrawal of ovarian steroid secretion following ovariectomy limited the rate of decrease in milk yield after the peak of lactation (Yart *et al.*, 2012). The reduction of milk yield decline was not accompanied by the modification of milk protein gene expression Boutinaud *et al.*, 2013b. In contrast, long-term inhibition of prolactin secretion using quinagolide, a dopamine agonist, induced a reduction in milk yield (Lacasse *et al.*, 2011) accompanied by decreases in *LALBA* and *CSN3* mRNA levels (Boutinaud *et al.*, 2012).

For most of the challenges, the impact on milk yield (either positive or negative) was associated with a parallel variation of the secretory cell activity. This suggests that the change in cell activity, regulated through the modification of gene expression, enzyme activity and protein synthesis, is generally involved in the adaptation process of the mammary tissue.

Effect of challenges on epigenetic marks
The first study showing the effects of a challenge on epigenetic marks during lactation involved manipulation of the health status. An experimentally induced infection with *Escherichia coli* induced an increase in the level of methylation of the three CpG sites upstream of the *CSN1S1* gene and a condensation of the chromatin associated with a decrease in the *CSN1S1* RNA level (Vanselow *et al.*, 2006). Similar effects were observed following infection with *S. uberis* (Singh *et al.*, 2012). Milking frequency has also been shown to modify the epigenetic marks on the *CSN1S1* locus. During differential milking, the milk yield of the half-udder milked 1× was lower than its contralateral half-udder milked 2×. This reduction in milk yield was associated with greater CpG methylation levels upstream of the *CSN1S1* gene and a lower RNA level of this gene in the 1×-milked half-udder than in the 2×-milked half-udder (Nguyen *et al.*, 2014). In contrast, a severe and long-term feed restriction during early lactation in dairy cows did not affect the levels of *CSN1S1* mRNA and CpG methylation upstream of this gene (Boutinaud *et al.*, 2016). Although still scarce, studies evaluating epigenetic modulation in the mammary tissue suggest that epigenetic modifications are involved in the adaptive response of the mammary tissue to a challenge and modulate the secretory activity of MEC.

Effect of challenges on mammary cell number
The effect of milking frequency on mammary cell number is not always consistent from one study to another (Table 5). Nevertheless, when milking frequency is increased, it generally induced an increase in milk yield associated with an increase in the number of MEC in the tissue, and the contrary is observed when the milking frequency is reduced. Increasing milking frequency generally increases the rate of cell proliferation and reduces that of apoptosis (Table 5). In some studies, however, cell proliferation or cell apoptosis was not affected with either an increase or a decrease in milking frequency (Nørgaard *et al.*, 2005; Wall and McFadden, 2012; Ben Chedly *et al.*, 2013). Furthermore, cell proliferation was surprisingly reduced (Bernier-Dodier

Table 3 *Effects of inflammation induced by intramammary infection or infusion of LPS[2] on indicators of secretory cell activity in the mammary tissue of dairy ruminant*

| Treatment | Species | Indicators of secretory cell activity modifications[1] | | Reference |
		mRNA	Protein	
LPS[2]	Cow	↓ *LALBA*, but not *CSN1S1* or *CSN2*		Schmitz *et al.*, 2004
LPS	Cow	↓ *FASN*, *ACC*, and *LALBA* but not *CSN1S1*		Gross *et al.*, 2015
Streptococcus uberis	Cow	↓ *GPAM*, *SCD*, *FABP4*, *CD36* and *LPL*		Moyes *et al.*, 2009
S. uberis	Cow		↓ β-1,4-galactosyltransferase, α-lactalbumin	Mudaliar *et al.*, 2016
Escherichia coli	Cow		↓ Casein, α-lactalbumin, β-lactoglobulin	Boehmer *et al.*, 2008
Clinical mastitis	Cow		↓ Casein, α-lactalbumin, β-lactoglobulin	Hogarth *et al.*, 2004
Clinical mastitis	Yak		↓ Casein, α-lactalbumin, β-lactoglobulin	Li *et al.*, 2014

[1]Abbreviations are listed in the Supplementary material S1.
[2]LPS = Lipopolysaccharides

Table 4 *Effects of hormonal manipulations on indicators of secretory cell activity in the mammary tissue of dairy cows*

| Treatment | Milk yield | Indicators of secretory cell activity modifications[1] | | Reference |
		Enzyme activity	mRNA	
Prolactin inhibition	↓	−	↓ *LALBA* and *CSN3*	Boutinaud *et al.*, 2012
Prolactin inhibition	↓	−	↓ *LALBA* and *CSN3*	Lollivier *et al.*, 2015
Ovariectomy	↑	−	No variation in milk protein mRNA levels	Boutinaud *et al.*, 2013b

[1]Abbreviations are listed in the Supplementary material S1.

et al., 2010), and cell apoptosis surprisingly increased by an increased milking frequency (Connor *et al.*, 2008). In most studies, milking frequency affected the expression of genes involved in cell proliferation and apoptosis, regardless of whether the analysis used the target-gene or transcriptomic approach (Table 5). Other indicators also suggest variations in cell number in the mammary tissue following modification of milking frequency. For instance, mean alveolar area, or the number of MEC per alveolus, were increased by greater milking frequency (Hillerton *et al.*, 1990; Boutinaud *et al.*, 2003). In line with this, total DNA content, or DNA concentration, was also shown to increase in the mammary gland of dairy goats when milking frequency was increased (Boutinaud *et al.*, 2003). However, this was not observed by Nørgaard *et al.* (2005) in a study performed in dairy cows. For MEC exfoliation, 7 days of 1× milking in comparison with 2× milking did not affect MEC exfoliation in dairy goats and cows, whereas several weeks of 1× milking induced a marked increase in MEC exfoliation in goats (Herve *et al.*, 2016). Thus, mammary cell number appears to be generally affected by milking frequency.

Similarly to milking frequency, feed restriction affected the indicators of variation of cell number differently depending on the study (Table 6). Indeed, the rate of mammary cell proliferation was reported to be decreased by a restriction of the energy in the diet (Nørgaard *et al.*, 2005) but not by a feed restriction (Dessauge *et al.*, 2011; Herve *et al.*, 2018). In addition, the rate of cell apoptosis was enhanced by feed restriction during early lactation (Dessauge *et al.*, 2011) but not when applied in a later stage of lactation (Herve *et al.*, 2018) or by energy restriction (Nørgaard *et al.*,

2005). The effects of feed restriction on MEC exfoliation are also not consistent. The exfoliation of MEC was enhanced during a 4 weeks feed restriction (Herve *et al.*, 2018) but not after 2 weeks of feed restriction of similar intensity (Herve *et al.*, 2016). The finding that cell apoptosis and proliferation and MEC exfoliation are most often affected by feed restriction suggests that the number of mammary cells decreases with milk yield during feed restriction, as also suggested by the decrease in total DNA content (Dessauge *et al.*, 2011).

Mastitis may also affect MEC number in the mammary tissue. The reduced ability of cows with mastitis to synthesize and secrete milk has been related to an increased apoptosis and sloughing of MEC in the mammary tissue (Akers and Nickerson, 2011). In addition, endotoxin administration strongly increased the percentage of milk MEC in the total somatic cell population (Wagner *et al.*, 2009). This finding also suggests that mastitis affects the number of mammary cells by influencing MEC exfoliation.

Hormonal manipulations also affected indicators of cell number variations in the mammary tissue (Table 7). Greater milk yield in ovariectomized cows than in control cows was associated with a decrease in the expression of the enzyme poly ADP ribose polymerase (PARP) protein (Yart *et al.*, 2012), which intervenes in DNA fragmentation during one of the final stages of cell death. This effect was accompanied by lower MEC exfoliation (Boutinaud *et al.*, 2013b). Both indicators suggest lower losses of MEC in the mammary tissue in ovariectomized cows. The lower milk yield in cows treated with a prolactin inhibitor in comparison with untreated cows was associated with either a decrease (Boutinaud *et al.*,

Table 5 *Effects of the modification of milking frequency on indicators of cell number variation in the mammary tissue of dairy cows and dairy goats (1×, 2×, 3× or 4× for milking one, two, three or four times daily, respectively)*

Treatment[1]	Milk yield	Indicators of cell number modifications[1,2]			Reference
		Cell proliferation	Cell apoptosis	Other indicator	
4× v. 2× for 4 weeks	↑	↑ Thymidine incorporation		↑ Mean alveolar area	Hillerton et al., 1990
4× v. 2× for 7 days	↑	↑ Cell proliferation rate	no variation in BAX:BCLX_L mRNA ratio[3]		Murney et al., 2015
4× v. 2× for 3 weeks	↑	Tendency for ↑ thymidine incorporation, no variation in KI67 staining			Hale et al., 2003
4× v. 2× for 3 weeks	↑	No variation in cell proliferation rate			Soberon et al., 2010
4× v. 2× for 5 days	↑	No variation in thymidine incorporation, but changes in mRNA involved in cell proliferation	No variation in rate of cell apoptosis but changes in mRNA involved in cell death[3]		Wall and McFadden, 2010
4× v. 2× for 7 or 14 days	↑	Changes in mRNA involved in cell proliferation	↑ Cell apoptosis rate changes in mRNA involved in cell death[3]		Connor et al., 2008
3× v. 2× for 8 weeks	↑	No variation in cell proliferation rate	No variation in cell apoptosis rate	No difference in DNA concentration	Norgaard et al., 2005
3× v. 2× for 3 or 6 weeks	↑		No variation in FAS and BAX:BCL2 mRNA ratio[2]		Grala et al., 2014
4× v. 1× over 230 days	↑		No variation in caspase-3 staining		Alex et al., 2015
3× v. 1× for 3 weeks	↑		↓ DNA laddering		Li et al., 1999
3× v. 1× for 3 weeks	↑			↑ Total DNA content, ↑ DNA concentration, ↑ alveolar area, ↑ MEC number per alveolus	Boutinaud et al., 2003
3× v. 1× for 4 or 8 weeks	↑	↓ Cell proliferation rate after 4 and 8 weeks	↓ Cell apoptosis rate after 4 weeks, ↓ BCL-2 mRNA level[3]		Bernier-Dodier et al., 2010
1× v. 2× for 1 week	↓		No variation in BAX mRNA[3]		Boutinaud et al., 2012
1× v. 2× for 3 or 6 weeks	↓		↑ FAS and BAX:BCL2 mRNA ratio[3]		Grala et al., 2014
1× v. 2× for 5 days	↓		Pathways related to apoptotic signaling[4]		Littlejohn et al., 2009
1× v. 2× for 1 week	↓	↓ Cell proliferation rate and changes in mRNA involved in cell proliferation	↑ Cell apoptosis rate, and changes in mRNA involved in cell death[4]		Boutinaud et al., 2013a
1× v. 2× for 1 and 5 weeks	↓		↑ BAX mRNA level[3]		Ben Chedly et al., 2011

Table 5 (*Continued*)

Treatment[1]	Milk yield	Cell proliferation	Cell apoptosis	Other indicator	Reference
1 × v. 2 × for 2 weeks	↓	No variation in cell proliferation rate	No variation in cell apoptosis rate but ↑ BAX mRNA level[3]		Ben Chedly et al., 2013
1 × v. 2 × for 1 or 3 weeks	↓	No variation in cell proliferation rate	↑ Cell apoptosis rate and changes in mRNA involved in cell death[4]		Boutinaud et al., 2017

Indicators of cell number modifications[1,2]

[1] 1×, 2×, 3× or 4× for milking one, two, three or four times daily, respectively.
[2] Abbreviations are listed in the Supplementary material S1.
[3] Target-gene approach was taken.
[4] Transcriptomic approach was taken.

2012) or no variation (Lollivier *et al.*, 2015) in the rate of cell proliferation, an increase in the rate in cell apoptosis (Boutinaud *et al.*, 2012) and an increase (Lollivier *et al.*, 2015) in MEC exfoliation. Taking together, these effects suggest a reduction in mammary cell number with the prolactin inhibitor.

The adaptation of the mammary tissue during a challenge does not necessarily involve all processes related to modifications in cell number (proliferation, death and MEC exfoliation), but most of the indicators show a change in the number of cells in the same direction as the change in milk production. The fact that variations in gene expression involved in cell turnover were always observed, whereas the rates of cell proliferation and apoptosis were not always affected, may be due to methodological limits of the immunohistological analyses or due to the lack of representativeness of the mammary tissue samples collected. Indeed, many of the studies were conducted using mammary biopsy. This would not have captured changes in total cell number or in rates of quiescence and senescence within the whole gland (Wall and McFadden, 2008).

Initial conclusion about the adaptation processes of the mammary tissue when lactation is challenged

The cellular mechanisms underlying mammary tissue plasticity in response to challenges are diverse. The mammary tissue adapts to the challenges by modifying its secretory activity and/or number of cells. Part of the mechanisms responsible for the change in secretory activity involves epigenetic modifications. In most studies, not all processes are studied at once, and there is no clear description of each potential process of adaptation. Moreover, the lack of variation of some indicators in some studies suggests that not all mechanisms are involved in the milk yield response at the same time. The response of the mammary tissue could depend on the type of challenge (energy or global feed restriction for cell proliferation and cell death), the intensity of the challenge (as observed for feed restriction), the length of the challenge (number of weeks of modified milking frequency for enzyme activity or number of weeks of feed restriction for MEC exfoliation), and the stage of lactation when the challenge is applied (observed for both feed restriction and changes in milking frequency). For some challenges, the adaptation of the mammary tissue also differs according to species, as seems to be the case for the effect of milking frequency on total DNA content. Since the adaptation of the mammary tissue is time-dependent, it is important to develop experimental designs that provide the possibility of performing dynamic studies. This would permit catching the window of time when the adaptive phenomenon occurs. In most of the studies, the analysis of mammary functions was performed at steady state, when the adaptation had already occurred, which most likely explains why no effects were observed. The most appropriate timing for these analyses may be when milk yield begins to be affected.

Table 6 *Effects of the modification of the feeding level on indicators of cell number variation in the mammary tissue of dairy cows*

| Treatment | Milk yield | Indicators of cell number modifications[1] | | | Reference |
		Cell proliferation	Cell apoptosis	Other indicators	
Energy restriction for 8 weeks (early lactation)	↓	↓ Cell proliferation rate	No variation in cell apoptosis rate		Nørgaard *et al.*, 2005
FR[2] for 11 weeks (early lactation)	↓	No variation in cell proliferation rate	↑ Cell apoptosis rate	↓ DNA total content, ↑ DNA concentration	Dessauge *et al.*, 2011
FR for 2 weeks (mid-lactation)	↓			No variation in MEC exfoliation	Herve *et al.*, 2016
FR for 4 weeks (mid-lactation)	↓	No variation in cell proliferation rate	No variation in cell apoptosis rate	↑ MEC exfoliation, ↑ DNA concentration	Herve *et al.*, 2018

[1]Abbreviations are listed in the Supplementary material S1.
[2]FR, feed restriction.

Table 7 *Effects of hormonal manipulations on indicators of cell number variation in the mammary tissue of dairy cows*

| Treatment | Milk yield | Indicators of cell number modifications[1] | | | Reference |
		Cell proliferation	Cell apoptosis	MEC exfoliation	
Ovariectomy	↑		↓ PARP protein		Yart *et al.*, 2012
Ovariectomy	↑			↓	Boutinaud *et al.*, 2013b
Prolactin inhibition	↓	↓ Cell proliferation rate	↑ Cell apoptosis rate	No variation	Boutinaud *et al.*, 2012
Prolactin inhibition	↓	No difference (PCNA)		↑	Lollivier *et al.*, 2015

[1]Abbreviations are listed in the Supplementary material S1.

Do the mechanisms underlying mammary tissue plasticity participate to the carryover effects?

Theoretical impact of challenges on the resilience of the mammary tissue

The first two parts of this review focused on the first step of the process of adaptation, that is resistance, to explain how the mammary tissue adapts to a challenge. It has been shown that the mammary tissue is a deformable system and that its adaptation to new conditions is accompanied by a variation in the amount of milk produced. The next part of this review focuses on the resilience of the mammary tissue and discusses whether the adaptation of the tissue is reversible or not. Irreversible processes could explain the carryover effects on milk yield when the challenge is terminated. The effects may become irreversible when the intensity of the challenge (length or strength) reaches a threshold that could lead to homeostatic ruptures. Most of the works dedicated to the effects of challenges have analyzed the effects on mammary functions only during the application of the challenge. It is, therefore, not possible to deduce the permanency of the effects on mammary functioning from those experiments. For a long time, it has been assumed that short-term modulations of milk production without carryover effects were due to changes in cell activity, while modulations of lactation that extended beyond the end of the challenge were due to changes in the number of MEC. However, a recent discovery has shown that environmental events can lead to stable changes in gene expression through modification of DNA methylation. This suggests that carryover effects might not only due to changes in the number of MEC. Therefore, it is necessary to not only determine if a challenge has carryover effects but also the mechanisms involved. We will focus on the effect of milking frequency and feed restriction, which are the most investigated challenges.

Changing milking frequency: an interesting model to investigate mammary tissue resilience

The carryover effects of changing milking frequency on milk yield have been described in the literature (Stelwagen, 2001; Rémond and Pomiès, 2005; Wall and McFadden, 2012; Charton, 2017). Such effects clearly depend on the duration of the treatment. Indeed, reducing milking frequency from 2× to 1× for 1 week has no persistent effect on milk yield, whereas a residual milk loss of 10% was observed after 7–10 weeks of 1× milking (Rémond and Pomiès, 2005). The impact of the modification of milking frequency for a short period is also modulated by the lactation stage during which the challenge is applied. The deleterious impact of a short-term application of 1× milking was greater during early lactation than at a later stage of lactation (Rémond and Pomiès, 2005). With respect to a persistent increase in milk yield with increased milking frequency, Wall and McFadden (2012) suggested that the timing (between day 7 and 21 of lactation) may be more critical than the duration. The

mammary gland may be especially responsive to the stimulus of milk removal for a window of time at the beginning of lactation. Frequent milking during this time can, indeed, elicit a persistent increase in milk production for the remainder of the lactation (Wall and McFadden, 2008). However, one study reported that increased milking frequency for the first 3 weeks induced only a transient carryover effect (over less than 2 weeks) (Nørgaard et al., 2005).

In parallel with a more pronounced carryover effect when milking frequency challenge is longer (Rémond and Pomiès, 2005), it was suggested that milking frequency first induced changes in secretory activity followed by an effect on the number of mammary cells (Stelwagen, 2001). These observations argue in favor of the idea that the variation in cell number during a milking frequency challenge induces persistent effects. Accordingly, a persistent increase in milk yield occurred when cell proliferation tended to be increased by more frequent milking (Hale et al., 2003). In addition, no carryover effect was observed after increased milking frequency when a lack of effect on cell number had been observed (Nørgaard et al., 2005). However, a persistent effect on milk yield was also observed even though no changes in mammary proliferation and apoptosis rate was measured during the challenge period (Wall and McFadden, 2012). This contrasts with the idea that the change in MEC number is what causes the carryover effects. Moreover, the induction of MEC exfoliation during 1 × milking in dairy goats was not accompanied by a carryover effect on milk yield (Ben Chedly et al., 2013). The carryover effect of milking frequency could thus be explained by other mechanisms than change in cell number. Indeed, two studies performed in bovine showed that the carryover effect on milk yield induced by a 1 × milking period (Grala et al., 2011) or a 3 × milking period (Wall et al., 2013) were related to a persistent effect on gene expression in the mammary tissue. Grala et al. (2011) reported persistent reductions in CSN1S1, LALBA, FASN, ACACA mRNA levels after 1 ×, whereas Wall et al. (2013) observed a persistent modification of genes related to IGF-I signaling pathway after 3 ×. In goats, the absence of carryover effect when goats were switched back to 2 × milking after the application of 4 weeks of 1 × milking was associated with a recovery of LALBA gene expression (Ben Chedly et al., 2013). These results suggest that carryover effects on milk production can be due to a persistent regulation of transcription. The carryover effect of a period of 1 × milking has been related to an increase in DNA methylation in the distal region of the CSN1S1 gene (Nguyen et al., 2014). The persistent effect on gene expression in the mammary tissue would be the consequence of an imprinting process through epigenetic modifications. However, further studies are necessary to confirm the involvement of epigenetic modifications in persistent effects on milk yield.

Potential mechanisms underlying the resilience of the mammary tissue after modification of feed supply
The long-term effect of feed restriction on milk yield has been well documented. No carryover effect on milk yield has been observed regardless of the duration of the dietary challenge (from 4 days to 4 weeks; Friggens et al., 1998; Bjerre-Harpøth et al., 2012) and the stage of lactation (early, mid- or late lactation; Bjerre-Harpøth et al., 2012). Accordingly, a recent study showed no carryover effect of a 20% feed restriction of 4 weeks (Herve et al., 2018). This lack of persistent effect on milk yield was observed despite the negative effect of feed restriction on the number of MEC in the mammary tissue (as indicated by a higher rate of MEC exfoliation into milk; Herve et al., 2018). It is noteworthy that this was accompanied by an increase in the release of prolactin into blood at milking when cows were switched back to an ad libitum diet. Since prolactin is known to have galactopoietic effects, its enhanced release during the period following feed restriction may have counteracted the negative effect of the challenge on mammary cell number. This suggests that the mammary tissue, after a period of adaptation during the dietary challenge, enters a new process of adaptation when the animal is switched back to an ad libitum diet.

Some factors responsible for individual variability in the adaptation of the mammary gland to challenges
The study of individual response in the adaptation of the mammary tissue to reduced milking frequency such as during a 24-h milking interval revealed relationships between carryover effects on milk yield and some traits of the animal. Even if on average no carryover effect was observed after 1 day of 1 × milking, the recovery of milk yield when 2 × milking was resumed differed among cows. Cows were clustered according to their ability to recover milk yield after a 24-h milking interval. Cows that recovered poorly (on average 63.9% of the milk they had lost) exhibited a lower estimated milk yield potential or an initially inflamed udder (as indicated by a higher occurrence of initial milk interleukin-8; Charton et al., 2016). Therefore, several traits related to the physiological status of the mammary tissue (inflammation status, genetic parameters and stage of lactation) modulate the adaptation of the mammary tissue to a challenge and could result in carryover effects on milk yield. Thus, probably also for other challenges, the physiological status of the mammary tissue has to be taken into account to understand the mammary tissue plasticity.

Conclusion

The cellular mechanisms underlying mammary tissue plasticity during lactation may involve different cellular processes, such as modifications of secretory activity, epigenetic marks and mammary cell number. The processes will be different depending on the nature of the challenge (type, intensity and duration) and on animal-related factors (e.g., health, genetic background and stage of lactation). Depending on these conditions, the mammary tissue shows elastic properties, that is, non-permanent deformation or undergoes some

irreversible modifications of milk production. The non-permanent deformation is observed with dietary challenges, for instance. The irreversible modification can either be beneficial (e.g., after an increase in milking frequency during early lactation) or deleterious (e.g., after a reduction in milking frequency for a long period). However, the lack of suitable techniques for dynamic and complete analysis of the phenomena of adaptation has certainly limited a holistic and integral view of the physio-cellular adaptations in bovine mammary tissue. When considered alone, the cellular mechanisms underlying mammary tissue plasticity during challenges are not sufficient to understand the carryover effects on milk production. Epigenetic modifications may be an exception, since they could explain the residual effects of the modifications induced by changes in milking frequency. Special attention should thus be paid to epigenetic modifications because little is known about the persistency of these marks during lactation. Further investigations on the role of stem cells in mammary tissue plasticity need to be performed. Better knowledge of the mechanisms involved in that phenomenon will help us understand more about the high plasticity of mammary tissue, and especially the one that is observed during early lactation. The impact of that mammary tissue plasticity on animal productivity, longevity and robustness should also be addressed in the future.

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Declaration of interest

The authors declare that there is no conflict of interest for this manuscript.

Ethics statement
None.

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

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