Sixth International Workshop on the Biology of Lactation in Farm Animals

Sponsored by

American Society of Animal Science

European Association for Animal Production

American Dairy Science Association

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The financial assistance for the *Sixth Joint EAAP/ASAS International Workshop on the Biology of Lactation in Farm Animals* that was provided by the following sponsors is gratefully acknowledged.

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Acknowledgments

The organizers are very grateful to the following sponsors of this workshop, whose generous financial contributions allowed us to invite outstanding speakers from around the world:

DeLaval, Tumba, Sweden
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The organizers wish to thank the speakers, chairpersons, contributors of short communications, and reviewers of manuscripts for their diligent efforts. We also wish to thank Ellen Bergfeld and the ASAS team for their assistance in organizing the workshop and in the production of this publication.

Finally, we thank the boards of directors of the American Society of Animal Science and the council of the European Association for Animal Production for their support.

Preface

The Sixth International Workshop on the Biology of Lactation in Farm Animals was held in Quebec City, in July 2002, immediately before the joint American Society of Animal Science (ASAS), American Dairy Science Association (ADSA), and Canadian Society of Animal Science (CSAS) annual meetings. Since first held in 1992, these workshops have brought together scientists from all over the world to discuss fundamental and applied aspects of lactation. Accordingly, this workshop was attended by more 200 participants from 20 different countries and several continents. This biennial event results from the collaboration between ASAS and the European Association for Animal Production (EAAP) and is held in conjunction with each society's annual meeting in alternation.

The overall objective of this series of workshops is to create a forum at which animal scientists can present and discuss the latest concepts, discoveries, and technologies concerning the biology of lactation in farm animals. The main themes selected for the 2002 workshop, *alternative strategies in dairy cow management* and *lactation biology in the post-genomic era,* were in line with this overall goal.

The session on dairy cow management was chaired by Dr. Kerst Stelwagen, from the Ruakura Research Centre in New Zealand. Dr. Hogeveen presented some of the technical challenges and progress in the development of high-capacity milking parlors and automatic milking. Dr. Dahl summarized the knowledge acquired over the last 30 years on the photoperiodic control of milk production. He also presented previously unpublished results from Dr. Petitclerc and him on photoperiod manipulation during the dry period. Lactation persistency is an aspect of lactation that could improve productivity and animal well-being by extending the lactation cycle. Dr. Capuco reviewed some of the concepts and discoveries that could help in reaching that goal.

Dr. Walter Hurley, from the University of Illinois, chaired the session on lactation biology in the postgenomic era. The concept of using the mammary gland as a bioreactor has been around for some time now. Dr. Wheeler presented some of the successes that his team obtained at producing transgenic swine that secrete foreign proteins in their milk. Dr. Kerr continued in this concept by presenting some of his work that uses the transgenic approach to enhance mammary gland resistance to intramammary infection. Finally, Dr. Sheffield presented the proteome concept and how proteomics may enable breakthroughs in mammary gland biology.

In addition to the invited speakers, several participants have contributed to the success of the meeting by presenting short oral presentations or posters. Readers are invited to consult their abstracts at the end of this publication.

I hope that this publication will encourage researchers to appreciate the importance and potential of lactation biology as a research field. I wish to thank personally Kerst Stelwagen and Walter Hurley for their help in the organization of this workshop and in the preparation of this publication. I also wish to thank Rupert Bruckmaier for his support in approaching sponsors. I am also pleased that Dr. Bruckmaier has accepted the chair for the Seventh Workshop, which will be held in Bled, Slovenia, in September 2004. Let us all look forward to another exciting workshop, next time in Slovenia.

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Journal of
Animal Science

Volume 81
2003
Supplement 3

CONTENTS

Sensors and management support in high-technology milking

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ABSTRACT: Two directions can be distinguished in the development of high-tech milking equipment: 1) high-capacity milking parlors with a high throughput of cows per person per hour and 2) automatic milking systems in which manual labor is replaced by a milking robot.

High-capacity milking parlors are developed in such a way that one operator is able to milk many cows, partly by automation and partly by optimization of available labor. In such parlors, one operator can milk up to 125 cows per hour. This means that there are only a few seconds available for udder preparation. In an automatic milking system, a robot takes over all manual labor during milking. Currently available systems have one robot arm working with one milking stall (one-stall system) or one robot arm working with more milking stalls (multiple-stall systems). Cows have to go to the automatic milking system voluntarily. Therefore, there is a large variation in milking intervals. Moreover, a large variation between milkings and between cows was observed in milk flow rate, machine-on time and udder preparation time.

Both developments in high-tech milking have effects on the milk ejection. The small amount of time dedicated to udder preparation in high-capacity milking parlors has negative effects on the milk ejection, among others leading to more bimodal milk flow curves and longer machine-on time. In automatic milking systems, the variation in time between udder preparation and cluster attachment and in milking frequency might have an effect on milk ejection. Lactation physiology can play a role in solving the questions around milk ejection in high-tech milking systems.

The introduction of high-tech milking systems makes decision support systems using sensors necessary. These systems should assist in detection of abnormal milk and mastitis. To a lesser extent, diseased cows need to be brought to the attention of the dairy farmer. Some sensors are currently available for this purpose, but they do not fulfill all demands. In the near future other sensors might be developed. It is important that this development is demand driven and not technology driven. Lactation physiology can play an important role in the determination of milk components useful for automatic detection.

Key Words: Milking Parlor, Automatic Milking System, Milk Ejection, Sensors

 J. Anim. Sci. 2003. 81(Suppl. 3):1–10

Introduction

Milking cows is an important part of the activities of a dairy farmer. The first purpose is to harvest the milk, which is carried out 1 to 3 times per day, depending on the region and applied farm system. Since the first attempts to develop milking machines in the end of the 19th century, many changes have been made. An important improvement was the development of the milking parlor, which started in the 1930's. The introduction of milking parlors improved the productivity of the milker greatly (Dodd and Hall, 1992). The development of milking parlors has continued to the current day. A next big step was the introduction of electronics into milking technology (Ordolff, 2001), starting with automatic take-off of milking clusters, followed by the development of individual cow identification in the 1970's. Both facts were important to the automation of milking, and the availability of individual cow identification can be regarded as the key of further automation in the milking parlor. Based on individual cow identification, monitoring of the physiological status of a cow became possible (Eradus and Jansen, 1999). The latest step in the development of milking technology is the complete automation of the milking procedure, including teat cup attachment. First initiatives for automatic milking (**AM**) were made in Europe (Rossing et al., 1985; Grimm and Rabold, 1987; Marchant et al., 1987; Montalescot, 1987) in the middle of the 1980's. In 1992, the first AM systems were installed on commercial (Dutch) dairy farms (Bottema, 1992).

Received: August 8, 2002.
Accepted: December 18, 2002.
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Currently, two trends can be distinguished in the application of milking technologies on dairy farms: 1) high capacity (in terms of milkings per person per hour) milking parlors and 2) AM systems. The major change for management in high capacity milking parlors, is the decrease in available labor per cow. For instance, the cow throughput for a double 10 herringbone parlor has been estimated to range between 60 and 101 cows/h, depending on the efficiency of the milker (Armstrong et al., 1994). Efficiency of the milker can be defined as speed of working, but also in the number of tasks carried out. The time used for udder cleaning and preparation for certain circumstances have been estimated to be less than 9 s/cow (Armstrong et al., 1994). This decreased preparation time is less than the advised 15 s/cow. This might have effects on the milk let down. Although required because of legislation, many farmers do not check foremilk in order to increase the throughput of cows. This might affect the detection rate of abnormal milk and/or clinical mastitis.

Although the time that the milker spends at a cow decreases in a high-capacity milking parlor, there is still the presence of a person. Each and every cow will pass the milker every day. Problems with cows can still be detected. With an AM system, there is no milker present at milking and thus there is no obligatory contact between the herdsman and the cows. At the same time decision-making, which takes place during milking will not be possible anymore. This means that sensors will have to take over part of the monitoring work of the herdsman. Moreover, in an AM system, the way of working is completely changed. Cows can be milked 24 h per day and will visit the AM system more or less voluntarily. This means that the milking frequency varies between farms, cows, and even within cows between days. Moreover, the time between preparation and attachment of clusters might vary from milking to milking, depending on the process of teat detection. This may have effects on the milk let down of the cows.

Both directions of milking technology, high capacity milking parlors, and AM systems, indicate a need of management support to be able to allocate the attention of the herdsman to those cows that need it: management by exception. Therefore, sensors will be useful and necessary. Since the 1980's, work has been carried out on the development and application of in-line sensors. Most work was concentrated on electrical conductivity, which is currently still the most applied technique for in-line mastitis detection but is not suited for automatic separation. Sensor development up to now was technique-driven. A demand-driven approach will be better and the following questions need to be answered before further development of sensors: What information is necessary to optimize the basic process of milking, and what information is necessary to support the decision-making process around milking, e.g., detection of clinical mastitis. Because of the increased societal interest in animal health and welfare, these questions have to be answered within the constraints that milk must be a safe product, produced by healthy, well-managed animals in a hygienic and animal-friendly environment.

Besides the need for more management information, application of high-tech milking changes milking procedures. These may influence the milk let down. Although available data to date provide some information, the full short- and long-term consequences of the indicated changes in milking procedures on milk production are not fully known.

The goal of this paper is to describe the demands for management support and sensors and to describe the important aspects influencing the milk ejection in high-tech milking.

Milking in a High-Capacity Milking Parlor

Milking parlors have been in development since the 1930's. However, spread of this technology went slow, and only after the development of the herringbone parlor in New Zealand in 1952, the number of installations increased more rapidly (Dodd and Hall, 1992). In northwestern Europe, most dairy farms changed from bucket milking towards a milking parlor in the 1970's. The large-scale introduction of the bulk milk tank and free stalls helped this rapid change in those years. In general, two types of milking parlors can be distinguished: static and rotary parlors (Whipp, 1992). In a rotary parlor, each cow enters and leaves individually. Because of the movement of the cows in the rotor, the milker does not have to walk very much. The design of static parlors varies widely. The best-known types are the side-open (tandem), herringbone, and the parallel parlor. In side-open parlors, cows are let in one by one. In the herringbone and parallel parlor, cows are let in by groups. Last types of parlors can be installed with rapid exit gates, which means that all cows in a group can leave simultaneously, thus increasing the throughput of cows.

In a milking parlor, the milker has to perform a number of work routines. The time spent on these routines, to a large extent determines the capacity of a milking parlor in cows per hour (Table 1). An optimization of work routines increases the maximum number of cows per hour per man from 50 to 65. Automation of the cluster removal, teat disinfection and cow let-out, increases the maximum number of cows per hour to 100 (Table 1). These maximum numbers should be seen as a theoretical maximum, in which a milker is continuously working. Moreover, the capacity of cows per hour is also dependent on the milking time per cow (Whipp, 1992).

Milking with an Automatic Milking System

An AM system has to take over the "eyes and hands" of the milker and therefore these systems need to have electronic cow identification, cleaning, and milking devices and computer-controlled sensors to detect abnormalities. In this section, the various components of an AM system will be briefly described.

Table 1. Effects of different work routines in parlor performance[1]

Element	Type of milking parlor[2]		
	Not automated (min/cow)	Somewhat automated (min/cow)	Fully automated (min/cow)
Let in and feed	0.25	0.05	0.05
Foremilk	0.10	0.10	0.10
Wash and dry teats	0.20	0.20	0.20
Attach cluster	0.20	0.20	0.20
Remove cluster	0.10	0.10	Auto
Disinfect teats	0.10	0.10	Auto
Let out cow	0.20	0.10	Auto
Miscellaneous	0.05	0.05	0.05
Total	1.20	0.90	0.60
Max. cows/person hour	50	65	100

[1]Source: Whipp, 1992.
[2]Milking parlors range from non-automated, where everything has to be done by hand, to a fully automated milking parlor, where almost everything is automated.

All commercially available AM systems are based on milking stalls, in which concentrates can be supplied. Most AM-systems are provided with milking stalls that modify the posture of the cow to improve performance of the system (Mottram, 1992; Mottram et al., 1994; Devir et al., 1996). In some AM systems, the length of the milking stall can be adjusted to the length of the cow (Devir et al., 1996). There are one-stall systems, in which each milking stall is serviced by one robot and multi-stall systems, in which one to four milking stalls are serviced by one robot.

Each AM system has an active teat detection system to localize the teats. Construction of teat detection sensors proved to be technically quite difficult, and this problem has been solved in various ways. Manufacturers have used ultrasonic sound, laser techniques, and vision techniques (Artmann, 1997) to find the position and place of the teats in reference to a fixed point on the robot arm. In fact, the system creates a three-dimensional view, so the system knows where to attach the teat cup to the teat. Different types of robot arms are used in the various types of AM systems (Artmann, 1997; Rossing and Hogewerf, 1997), varying from grippers that pick up the teat cups one by one from a storage rack at the side of the stall, robot arms that pick a whole milk rack at the same time or robot arms integrated with the milk rack.

There are several principles of teat cleaning with AM systems: Sequential cleaning by brushes or rollers, simultaneous cleaning by a horizontal rotating brush, cleaning with water in the same teat-cup as used for milking or cleaning by a separate "teat cup like" device. Besides cleaning the teats, automatic cleaning devices also stimulate the milk letdown process. Stimulation of the milk ejection reflex is necessary for efficient milking. The most important stimuli are udder and teat cleaning and the action of the milking machine (liner wall movement). It is not known if there is a difference between the teat cleaning methods of the different AM systems with respect to the intensity of the milk ejection reflex. However, it is clear that an automated pretreatment is more repeatable than manual pretreatment. This might have a positive effect on the milk ejection.

High-Tech Milking and Milk Ejection

Important parameters in the evaluation of high-tech milking systems are milk yield and milk flow. More indirectly related to milking, effects on health are important.

High-Capacity Milking Parlors

Although machine milking has developed well over the years and cows are producing more milk due to breeding and management, the basic regulation of milk ejection has not changed (Bruckmaier, 2001). The milk, secreted by epithelial cells, accumulates in alveoli and cisterns. The cisternal milk (milk stored in large mammary ducts and cisternal cavities) is immediately available when the milking starts. But the alveolar milk (milk stored in alveoli and small milk ducts) needs to be actively expelled through the release of oxytocin. The cisternal milk fraction comprises not more than 20% of the total milk yield (Pfeilsticker et al., 1996). Therefore, the most important milk fraction is the alveolar milk. A rapid and complete ejection of this milk fraction is essential for a good result of the milking. To reach a good milk ejection, sufficient lag time between the onset of tactile teat stimulation and the actual start of milking is important. This time normally ranges from 1 to 2 min. (Bruckmaier et al., 1994; Bruckmaier and Hilger, 2001).

The most important change in a high-capacity milking parlor with a high throughput (cows/person per hour) compared with lower throughputs, is the amount of time that the milker can spend on each cow. In Table 1, effects of change in time for work routines on theoretical throughput of cows in terms of cows per hour are given (Whipp, 1992). There are not many field data on actual performance of various high-capacity milking parlors. The most complete review is summarized in Table 2 (Armstrong et al., 1994). These data are collected on commercial farms with large herds and high-capacity milking parlors. The maximum throughput measured, was 128 cows/h for a one-person operated milking parlor. The minimum throughput measured, was 49 cows/h. Recalculated in maximum amount of available time per cow, this varies from 1 min and 13 to 28 s per cow. This time includes all time per cow, including post milking teat disinfection, entrance and exit times. From the same study, standardized time spent on udder preparation was on average 10.5 and 8.7 s, respectively, for a double 20 herringbone parlor with two operators and a double 20 parallel parlor with two operators.

In a study carried out in the research milking parlor of the Research Institute of Animal Husbandry (Lely-

Table 2. Capacity (cows per hour) for high-capacity milking parlors (including power operated entrance and exit gates, crowd gates and detachers) under good management for one operator[1]

Parlor type	Slow operator	Fast operator
Rotary parlor		
8-stall Tandem	58	—[2]
Herringbone parlor (standard exit)		
Double 10	49	92
Herringbone parlor (rapid exit)		
Double 10	60	101
Double 12	88	110
Double 16	123	128
Parallel parlor		
Double 10	84	91
Double 12	72	106
Double 14	110	121
Double 20	122	128

[1]Source: Armstrong et al. (1994).
[2]No minimum and maximum figures were available.

stad, The Netherlands), four different methods of udder preparation were applied to four groups of 11 cows with an equal distribution of parities. There were four procedures for udder preparation: 1) cleaning and attachment (8 s), 2) cleaning, stripping and attachment (8 s plus stripping), 3) cleaning, manual stimulation, and stripping (25 s plus stripping) and 4) cleaning and attachment (8 s) and automatic stimulation (30 s with a pulsation rate of 250 cycles min^{-1} and a pulsation ratio of 50%). Cows were milked twice per day with intervals of 13 and 11 h. There was no difference in milk yield between the four treatments (Table 3). The extended manual preparation (including stripping) showed the best results in this study. The machine-on time was 29 s lower than the machine-on time for only cleaning. Moreover, the percentage of milkings without a bimodal milk flow curve was 60%, compared with 20% for the standard cleaning and 34% for the cleaning and stripping. A bimodal milk flow curve shows at the beginning of the milking a fast increase in milk flow when the cisternal milk is released. Then the milk flow decreases sharply because the alveolar milk is not yet released. When the milk ejection starts the alveolar milk (the largest milk fraction) is released and the milk flow increases again. Also, the average milking time in phase one (the start of milk ejection) is shorter with the extended udder preparation. These results show that a short udder preparation time delays the start of the milk ejection, since the percentage of bimodal milkings increased. Moreover, it has negative effects on the machine-on time.

The data above show that the preparation time in high capacity milking parlors is much lower than the 1 min necessary for an optimal release of oxytocin. Short udder preparation times gave a delayed milk ejection and longer machine-on times. A longer preparation gave shorter milkings and is less inefficient than thought on forehand. Mechanical stimulation or a change in milking method can help to overcome the inefficiencies. Since continuous tactile stimulation during the udder preparation is not necessary, it is possible to first clean the udders of three cows and then attach the milking clusters of these three cows.

Automatic Milking System

To be milked in an AM system, cows have to more or less voluntarily move to the AM system themselves (Rossing et al., 1997). This means that there are no equal milking intervals anymore, which was illustrated in a study of Hogeveen et al. (2001), using data from 66 cows on an experimental farm, milked with an AM

Table 3. Effects of different udder preparation procedures on duration of milking, milk yield, and milk flow of evening milkings[1]

	Standard	Standard + stripping	Extended + stripping	Standard + automatic
Duration (s)	10	16	31	40[2]
Machine-on time (min)	5.20	5.12	4.91	5.21[2]
Milk yield (kg)	11.5	11.5	11.4	11.6
Average flow (kg/min)	2.28	2.33	2.43	2.32
Max. flow	4.02	4.08	4.17	4.04
Milk flow profile[3]				
Phase 1	75	70	56	73
Phase 2	98	93	101	109
Phase 3	113	119	113	107
Phase 4	25	26	25	23
Milk in phase 2 (%)	31	30	33	34
Milkings not bimodal (%)	20	34	60	35

[1]Source: Research Institute for Animal Husbandry, Lelystad, The Netherlands.
[2]Time is including the 30 s automatic stimulation time.
[3]The milk profile curve is divided into 4 phases: phase 1 is the start of the milk ejection, phase 2 is the period of a high, constant milk flow, phase three is the decline in milk flow, and phase 4 is the low milk flow at the end of milking.

Table 4. Milk yield and milking intervals divided over the lactation stage[1]

		Milk yield (g/h)			Milking interval (h*100)		
Lactation stage (days)	#Milkings	Avg	Min	Max	Avg	Min	Max
1–30	9152	1505	243	3000	899	107	2400
31–60	9352	1690	435	3375	844	168	2351
61–90	8367	1589	317	3016	881	130	2397
91–120	8929	1503	341	3081	875	120	2399
121–150	8600	1424	307	2868	873	103	2400
151–180	8299	1373	422	2789	872	172	2395
181–210	8043	1307	261	2878	886	162	2398
211–240	7839	1222	271	2254	902	245	2387
241–270	7220	1111	329	2353	962	182	2385
271–300	6399	1007	235	2266	1022	122	2400
301–330	4044	947	176	2328	1066	207	2399
331–360	2387	946	159	2056	1105	250	2394
>360	3989	853	159	2000	1108	138	2388

[1]Data previously unpublished.

system (Lely Astronaut). The average milking interval was 9.2 h (2.6 milkings per day). However, very short and very long milking intervals were observed. Of all milkings, 17.6 and 4.2 percent had a preceding milking interval of, respectively, more than 12 and 16 h. In addition to long intervals, short intervals occurred also. For 9.7 and 0.5% of all milkings a preceding milking interval shorter than 6, respectively, 4 h occurred. The effects of milking interval on milk flow rate and milk yield were also studied. A longer milking interval was associated with an increase in milk flow rate, and this was not dependent on production level. Statistical models indicated that shorter milking intervals gave an increase in milk production (in terms of kg/h). However, the level of this effect was found to be dependent on the level of milk production.

Using the same dataset, milk yield and milking interval throughout the lactation were calculated and summarized in Table 4. It can be seen that the milk yield is highest from d 30 to 90 in postpartum. The minimum milking interval is found in the second period postpartum. Later in lactation the milking interval increases.

With an average milk yield of 11.8 kg/milking and an average milk flow rate of 2.5 kg/min, the average machine-on time was 5 min. Although most milkings had a machine-on time of approximately 4 min. (the median), a considerable number of milkings were much longer. Respectively, 8.6 and 2.7% had a machine-on time longer than 8 and 10 min. However, there was a large difference in machine-on time between the quarter with the shortest machine-on time and the quarter with the longest machine-on time.

For the same dataset, for each milking entrance time in the milking unit and time of removal of the last teat cup were recorded in hours and minutes. From these two figures the total duration of the visit of the milking unit was calculated. Furthermore milking speed was recorded in kilograms per minute and milk yield was recorded in units of 100 g. From these two figures, duration of milk flow was calculated in seconds. Preparation time per cow was estimated as total duration of visit minus duration of milk flow. Most milkings have a preparation time of approximately 60 s (Figure 1), but there is considerable variation. Very long preparation times, up to 300 s, are possible.

When automatic stimulation is used, it might be good to adjust the tactile stimulation to the cow. It has been shown that the necessary udder preparation time is dependent on the milking interval. Moreover, in that specific study it has been made plausible that the necessary udder preparation time is in fact dependent on the degree of udder filling (Bruckmaier and Hilger, 2001). A higher degree of udder filling (in terms of expected milk yield divided by the maximum storage capacity) makes a longer udder preparation time necessary. Given the large variation in milking frequencies and the effect of these on milk yield, a cow and milking specific adjustment of mechanical stimulation is even more important in AM systems. It has been shown that most milkings in an AM system have an udder preparation time of 1 min or more. With these preparation times, no additional measures are necessary. The very long attachment times do not have a negative effect on

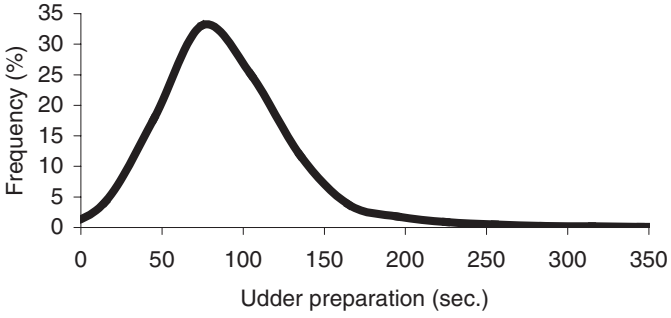

Figure 1. Frequency distribution of time between entrance of milking stall and milking (udder preparation time) in automatic milking (data previously unpublished).

Table 5. Demands for sensors to detect abnormal milk, mastitis cows and diseased cows to be used in high-capacity milking parlors or automatic milking (AM) systems

	High-capacity milking parlor		AM System	
Task	Sensitivity	Specificity	Sensitivity	Specificity
Separation of abnormal milk	+ +[1]	+/–	+ +	+ + +
Detection of mastitis	+	+/–	+ +	+
Detection of diseased cows	+/–	+/–	+ +	+/–

[1]The more + signs, the larger the necessity of a high sensitivity or specificity.

milk ejection, so there should not be a problem there (Bruckmaier et al., 2001). On the other hand, another study found lower levels of oxytocin release in automatic milking compared with "classical" milking (Marnet et al., 2001). In the latter study cows were brought to the automatic milking system twice a day to prevent an effect of changed milking intervals in the measurements of oxytocin. This way of automatic milking cannot be directly compared to automatic milking in practice, where cows have to move to the system voluntarily. Therefore, more research is necessary in lactation biology with respect to high-tech milking. This research should be directed to the development of optimal (cow and milking specific) automatic stimulation in high-capacity milking parlors and towards the effects of the variation in milking procedures of AM systems on milk ejection and milk synthesis.

Demands for Sensors in High-Tech Milking

With AM systems, because there is no milker present during milking, sensors have to take over detection of clinical mastitis and abnormal milk. Moreover, through general examination of the entering dairy cows, first signs of disease might be detected in conventional parlors. In milking hygiene regulations, the detection and separation of abnormal milk is mandatory (i.e., EU directive 89/362 and Pasteurized Milk Ordinance). The same holds for separation of not obviously abnormal milk from visibly sick cows. Because of the lack of time in high-capacity milking parlors, there is pressure on observation tasks, such as foremilk inspection. Therefore, in both AM systems and high-capacity milking parlors, sensors and the additional management software can support the herdsperson in taking correct decisions. The tasks for which sensors are necessary or can be useful are: detection of abnormal milk, clinical mastitis, and diseased cows.

In Table 5, the demands for sensors for management support in high-tech milking systems are given. These sensors can be seen as diagnostic tests, which can be characterized by epidemiological parameters such as sensitivity (the probability that a cow with a certain condition will be classified as having this condition) and specificity (the probability that a cow without a certain condition will be classified as such). Sensitivity and specificity are interdependent. If the threshold of a test is increased, the number of positive outcomes and thus the sensitivity will decrease. On the other hand, the specificity will increase. Therefore, thresholds have to be set in such a way that an optimal sensitivity and specificity is reached. However, for practical use the predictive values (positive and negative) of a test are more important. The predictive value is dependent on prevalence of the condition of interest. When discussing methods to detect clinical mastitis and abnormal milk the test characteristics described above should be taken into account.

As mentioned earlier, detection of abnormal milk is very important because it is mandatory in almost all dairy-producing countries. Under the current legislation, abnormal milk is almost equal to milk from cows with clinical mastitis. Besides, for food safety, the prevention of abnormal milk in the food chain is also important for the image of the milk products. The sensitivity for abnormal milk needs to be high under all circumstances. However, because a milker can check the milk before separation, the needed specificity for a high-capacity milking parlor is not necessarily high. On the other hand, since an AM system needs to separate milk automatically, the specificity for abnormal milk needs to be high in order to prevent false positive results and thus unnecessarily removed milk.

For detection of clinical mastitis, sensors and the attached management information system should assist the herdsperson in such a way that this person is able to treat cows with clinical mastitis easily and effectively. It is known that treating as early as possible gives higher cure rates (Milner et al., 1996). Detection of mastitis will in practice work with attention lists. The management information system can produce lists with cows for which abnormal sensor readings were given. These lists will be used to check individual cows at a later stage. It is important that as many cows with clinical mastitis as possible (preferably all) will be identified (high sensitivity). At least, cows with severe clinical mastitis (grave systemic and local symptoms) must be detected. However, if a cow with a mild clinical mastitis (mild local symptoms such as some flocks) is not immediately detected, from a veterinary point of view this will not be a large problem. The number of cows that are on the attention list and do not have clinical mastitis or other abnormalities should not be too high (low number of false-positive outcomes) which

means a relatively high specificity. Because it is more difficult to search and control cows that are on an attention list, the specificity needs to be higher for AM systems than for high-capacity milking parlors. Moreover, although in high-capacity milking parlors foremilk of cows is not checked usually, an attentive milker can detect part of the mastitis cases without help. Therefore, the needed sensitivity for high-capacity milking parlors is a little lower than for AM systems.

An attentive milker can easily detect diseased cows. Therefore, in a high-capacity milking parlor, there is not much need for sensors. However, sensors may help the herdsperson, especially when employees who do not pay much attention to the cows, a situation that can occur on large farms, carry out the milking. With AM systems, the herdsperson has to check the cows in the barn. Attention lists may help in these tasks.

In the next sections, current sensors are discussed.

Electrical Conductivity

Electrical conductivity (**EC**) is a measure of the resistance of a particular material to an electric current. Because of increased blood capillary permeability, mastitis causes a change in ion concentrations and thus in EC. Typical EC of normal milk appears to be around 4.6 mS/cm (Neville and Jensen, 1995). When the EC of milk (at quarter level or at cow level) is increased this is an indication for mastitis (clinical as well as subclinical). However, the correlation between EC and SCC is not very high (Hogeveen et al., 1998), indicating that there are more factors influencing EC. Because EC is relatively easy to measure, it has for a number of years been commercially available. There are many studies carried out on the use of EC for mastitis detection (Hamann and Zecconi, 1998). Nielen et al. (1992) carried out a meta analysis and found varying results of 77 known studies up to 1992. The overall sensitivity was 66% with an overall specificity of 94%. Recent experiments with conditioned infections show that EC could very well be used (100% sensitivity) to detect clinical mastitis before clinical signs appear (Milner et al., 1996). However, these mastitis cases were artificially induced. Moreover, the measurements were done using a hand-held apparatus and not by in-line equipment. De Mol et al. (2001) described the most recent extensive field study. Commercially available in-line mastitis detection systems were used at 4 farms for several years. Test results of the software of the manufacturer gave sensitivity, varying from 18 to 36% and a specificity varying from 98.1 to 99.4%. On the same data, a special algorithm using not only EC data but also milk production and temperature data was applied. This model has the possibility to give data with various confidence levels (varying from 95 to 99.9%). At a confidence level of 95%, overall sensitivity and specificity were 80 and 98.6%, respectively. At a confidence level of 99.9%, these figures were respectively 55 and 99.3%, respectively, for sensitivity and specificity. With such characteristics, EC combined with other available sources of information, might well be used to generate attention lists for clinical mastitis.

The only known study on detection of abnormal milk reported a sensitivity of 39% (Rasmussen, 2000). When decreasing the detection threshold, the sensitivity would improve greatly, but also the number of false positive results would increase to unacceptable levels. Although this study was rather limited (138 cows were followed for 48 h), results are not unexpected. After all, there is a large correlation between abnormal milk and clinical mastitis, and results for detection of abnormal milk will probably not deviate very much from the results presented above. With those results, EC is not sufficient to withhold abnormal milk automatically. Too many milkings with abnormal milk will still be delivered and too much milk will be discarded unnecessarily, resulting in economic losses.

In the past, the development of sensors was merely technology driven rather than demand driven. Because of the existing relation between mastitis and EC, and the availability of EC sensors, this was the method that was developed and marketed. However, there was no clear demand for these sensors. The use of these sensors hardly had any added value to the current milking practice at that time. Partly this was due to insufficient performance of those sensors and the connected computer software, partly it was also due to the lack of a clear goal for usage. Detection of clinical mastitis in a milking parlor was not a large problem for dairy farmers. To make detection of subclinical mastitis useful, the farmer needs to know what to do when a case of subclinical mastitis is detected. Moreover, the performance of EC sensors to detect subclinical mastitis was bad (Nielen et al., 1995). It is important that proper algorithms for specific tasks will be developed. Proceeding work on algorithms (e.g. De Mol and Woldt (2001) can improve the sensitivity and especially the specificity of current and future sensors.

Color Measurement

Color measurement has shown to be a promising method to detect abnormal milk under laboratory circumstances (Ouweltjes and Hogeveen, 2001). Quarter milk samples taken from eight cows with clinical mastitis and dilution strings made by gradually diluting a mix of cow blood and consumption milk were used to evaluate the color measurement sensor. Both abnormalities due to clinical mastitis and blood in milk resulted in color values clearly different from those of normal milk. First results of a field study on two farms also showed promising results under practical circumstances (Espada and Vijverberg, 2002). More extensive field studies have to provide information on sensitivity and specificity of color measurement in practice.

Other Available Parameters

There are many parameters that are available and that can be used to distinguish cows, such as milk yield,

number of visits to the AM system, concentrates intake and so on. These parameters were not directly meant for detection of mastitis or sick cows, but if used in a good decision support system, they can be useful. Many diseases influence the milk yield of cow, the activity (and thus the willingness to visit the AM system) and the feed intake on a short notice and often in a subclinical stage. Moreover, there are also temperature sensors developed and commercially available. Although milk yield and temperature are used by De Mol et al. (1999) to optimize the EC performance, these parameters are difficult to interpret automatically and are not very specific. Also pedometers, which are developed for estrus detection (Thompson et al., 1995), can be used to monitor the activity of a cow. Algorithms have been developed to use a combination of activity measurement, temperature, and milk yield for illness detection (De Mol et al., 2001). However, no results for illness detection are known. The BW of dairy cows (Maltz, 1997; Maltz et al., 1997) might also be used to detect general illness.

There are also some other developments that might be used in the future to further improve the detection of clinical mastitis and abnormal milk. Especially for the detection of abnormal milk new technologies should be used. Di-electrical constant, near infra-red technology (Tsenkova et al., 2001), image processing and diffusing wave spectroscopy (Dalgleish and Hallett, 1995; Elofsson et al., 1996) might be used. Many milk components are described to change due to mastitis. Under these are milk components such as somatic cells and lactose, but also enzymic changes (Kitchen, 1981). Lately, new research has been done in this direction (Eckersall et al., 2001). These changes might be useful for automatic monitoring of mastitis (Hamann and Kromker, 1997; Mottram, 1997). Given all these changes, biosensors might in the near future also be used to support the management in high-tech milking systems. The possibilities of using bio-sensors for NAGase to detect mastitis on-line are described (Mottram et al., 2000).

In this paper, sensors have been described to support the dairy farmer in milking related management, i.e., detection of mastitis and abnormal milk. However, there are also other abnormalities that can be detected with sensors. There are various types of sensors available for estrus detection (Firk et al., 2002) and recently a biosensor to predict estrus based on components in milk has been described (Velasco-Garcia and Mottram, 2001). Also, an optical instrument to detect metabolic changes due to microbial activity has been described (Firstenberg-Eden et al., 2002). The possibilities of using milk components to detect disorders have been reviewed by (Hamann and Kromker, 1997; Mottram, 1997).

Although there are many technological possibilities to support the dairy farmer in a high-tech milking system, it is important that before development and marketing of a technology, it is clear what the demands for sensors are. Table 5 in this paper describes roughly these demands for tasks around milking. However, the information in this table needs to be specified further. For instance, under the current regulations, abnormal milk can be regarded as milk with visible abnormalities (not smooth, not white). With the introduction of AM systems, discussions were initiated on definitions of abnormal milk (Rasmussen, 2001). These definitions must be clear before development of sensors can be started. Moreover, demands for sensitivity and specificity need also to be clear. Because there is so much to measure in milk, lactation physiology can assist in the determination of milk components useful for management support. When such components are described, engineers can use this information to start development. This is a demand-driven approach with a much higher probability of successful introduction than the technology-driven approach applied up to now.

Conclusions

Two directions of development of milking equipment have been described. The first, which is going on for a large number of years, is the development and installation of high-capacity milking parlors in terms of throughput of cows per hour per person. The second direction is the development of AM systems. Especially in large milking parlors, robots might be introduced to take over the task of one or more employees. These robots might be supervised by a controller. There will be a moment in the future when milking robots have taken over the manual labor on most farms. However, this moment might still be far away. As a matter of fact, it took also almost 70 yr after the introduction of the milking machine before nearly all farmers milked with a milking machine.

Both developments have effects on the milk ejection. The small amount of time dedicated to udder preparation in high-capacity milking parlors especially has negative effects on the milk ejection. In AM systems, the variation in interval between udder preparation and cluster attachment and in milking frequency might have an effect on milk ejection. Lactation physiology can play a role in solving the questions around milk ejection in high-tech milking systems.

The introduction of high-tech milking systems makes decision support systems using sensors necessary. In this paper, the future demands for sensors in high-technology milking systems are described. Electrical conducivity might still be a useful parameter to meet these demands. The other currently commercially available sensor for on-line detection of mastitis and abnormal milk is color measurement. No descriptions of algorithms and reports of overall performance are yet available. The current and future sensors and associated software should assist in detection of abnormal milk and mastitis. To a lesser extent diseased cows need to be brought to the attention of the dairy farmer. Although some sensors are available, in the near future

other sensors might be developed. It is important that this development is demand driven and not technology driven. Lactation physiology can play an important role in the determination of milk components useful for automatic detection.

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Management of photoperiod in the dairy herd for improved production and health

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ABSTRACT: Environmental influences on lactation efficiency are frequently associated with reductions in milk output. Heat stress, for example, leads to depressed feed intake and, subsequently, losses in production. Conversely, cold stress may limit nutrients available for milk synthesis. Fortunately, one environmental factor, photoperiod, can exert a positive effect on dairy performance when managed properly. Long days have consistently been shown to improve milk yield during established lactation. In addition, photoperiod management can be used to improve heifer growth and maximize accretion of lean tissue, including mammary parenchyma. There is, however, evidence of refractoriness to long day stimulation. Recent work has focused on the dry period as a time when photoperiod manipulation can influence subsequent milk production. In contrast to lactating cows, multiparous cows benefit from exposure to short days when the dry period is followed by long days or natural photoperiod after calving. Similarly, primiparous animals also respond positively to short days late in pregnancy when subsequently exposed to long days during lactation. Furthermore, emerging evidence suggests that short days positively influence immune function in cattle. Mechanistically, it appears that prolactin has a causal relationship with the observed dairy performance effects during the dry period and on immune function, via altered sensitivity to prolactin through differential expression of prolactin receptor in multiple tissues. The objectives of this paper include a review of fundamental aspects of photoperiod physiology, integration of applied and basic research findings, and development of management recommendations for the entire life cycle of the dairy cow to optimize performance.

Key Words: Photoperiod, Management, Immune Function

 J. Anim. Sci. 2003. 81(Suppl. 3):11–17

Introduction

The response to photoperiod or the relative duration of light and dark exposure within a day is the most commonly adapted environmental cue used by animals to predict changes in and alter physiological responses to shifts in their physical environment (Gwinner, 1986). Most often the physiological consequence associated with photoperiod is the influence on seasonal reproductive status, although other processes affected by photoperiod include body growth, composition, and pelage changes. Most seasonal impacts on reproductive competence have been selected against in breeding the modern dairy cow, although heat stress remains a major negative influence. Yet seasonal effects on lactation persist and likely result in large measure from the influence of photoperiod (reviewed in Dahl et al., 2000). Although photoperiodic effects on lactation have been studied most frequently in dairy cows, similar responses are observed in other domestic species, including sheep (Bocquier et al., 1990), goats (Terqui et al., 1984), and pigs (Stevenson et al., 1983). Emerging evidence suggests that photoperiod also controls seasonal shifts in immune function and thus animal health (Dowell, 2001).

The first objective of this review is to consider the physiological basis of photoperiodic effects on lactation and immune function and integrate those data to propose a model of how day length alters the response of mammary epithelial cells and leukocytes to result in photoperiodic responses in cell function and metabolism. In addition, recommendations for the application of photoperiod treatment at the production level will be made with emphasis on the dry and lactating state in the mature cow.

Lactating Cows

As summarized in Figure 1, relative to a natural or short day photoperiod (**SDPP**), exposure of lactating

Received August 16, 2002.
Accepted December 13, 2002.
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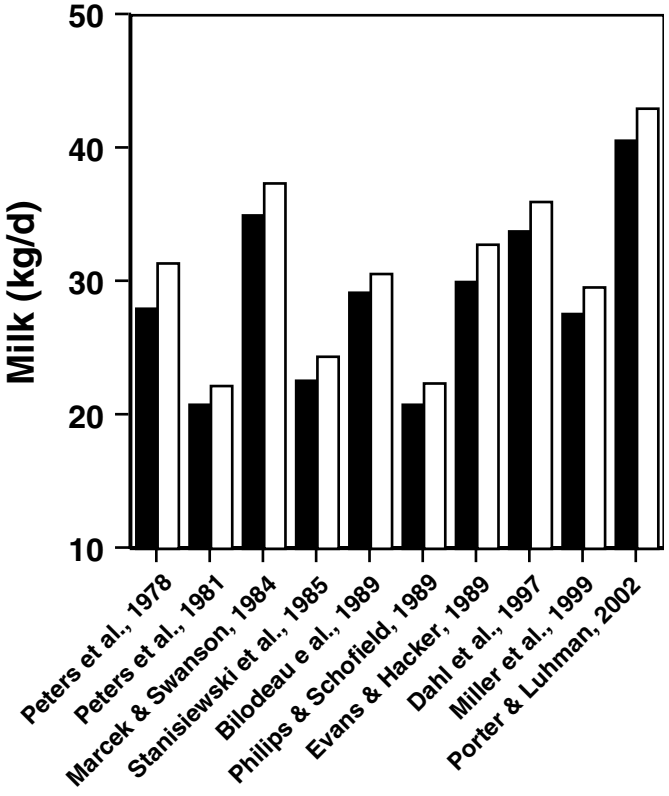

Figure 1. Summary of 10 published studies examining the effect of increased photoperiod on milk yield in lactating cows. Full citations appear in the references section. Solid bars indicate the average daily milk yield (kg/d) of cows on natural photoperiod (range of 8 to 13.5 hr light/d; control), whereas open bars indicate milk yield (kg/d) of cows exposed to extended photoperiod of 16 to 18 hr of light/d.

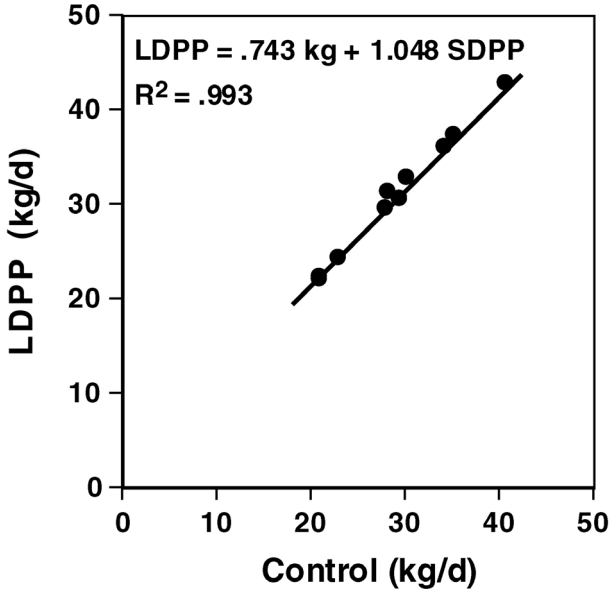

Figure 2. Scatter plot of the response of milk yield to long day photoperiod (LDPP; kg/d) compared with natural photoperiod (control; kg/d) that have been reported in the literature (Peters et al., 1978; Peters et al., 1981; Marcek and Swanson, 1984; Stanisiewski et al., 1985; Bilodeau et al., 1989; Phillips and Schofield, 1989; Evans and Hacker, 1989; Dahl et al., 1997; Miller et al., 1999; and Porter and Luhman, 2002). Regression analysis yields the equation for predicting milk response to long days.

cows to long days is consistently linked to increases in milk production (referenced in Figure 2). Indeed, Reksen et al. (1999) analyzed records from 1538 herds in Norway and observed that across a spectrum of light exposure from 11.7 to 21.5 h duration, cows with more light produced more milk. However, continuous lighting is not associated with greater milk yield, and, in fact, production between cows on natural photoperiod and those under 24 h of light did not differ (Marcek and Swanson, 1984). This is not surprising as photoperiodic responses occur within a range of entrainment, and continuous lighting is likely outside this range. Milk composition in cows is generally unaffected by photoperiod, although slight depressions of milk fat percentage have been observed (reviewed in Dahl et al., 2000).

Cows respond to long days at any stage of lactation and across a range of production levels. Regression analysis of published data from lactating cows reveals that the response exhibits a slight positive relationship with production level (Figure 2). The response to long days develops gradually but is typically significant after 3 to 4 wk of exposure. The range of duration of long-day exposure in published studies is 8 to 43 wk. Whether or not cows become refractory to long days from the standpoint of milk yield is unknown, though there is evidence that a break in exposure to long days during the dry period can enhance subsequent production (see below). We have treated cows with long days for up to 20 wk without loss of production increment relative to natural photoperiod, which suggests that treatment responses can persist through an entire lactation, although this has not been confirmed experimentally.

It is now generally accepted that long day responses are mediated via endocrine changes that accompany changes in light exposure. The initial step in the endocrine cascade in response to variable light duration is in the circadian pattern of melatonin secretion. Melatonin release from the pineal is inhibited by light; thus, under natural conditions circulating concentrations are high at night and undetectable during the day (Hedlund et al., 1977). Melatonin secretory patterns are responsive to illumination provided from external sources as well. The relative concentration of melatonin at critical times in the endogenous circadian rhythm then influences physiological interpretation of day length and modulates secretion of other hormones to express shifts in lactation, growth, and health.

Across ages and gender, the most consistent secondary endocrine response to photoperiod is a direct relationship between photophase duration and circulating prolactin (**PRL**; reviewed in Dahl et al., 2000). That is,

long-day exposure increases PRL relative to shorter photoperiods. Evidence argues against a direct role for PRL in the response of lactating cows to long days, as exogenous PRL does not increase (Plaut et al., 1987), and bromocriptine does not depress (Schams et al., 1972) milk yield in lactating cows. Furthermore, long-day responses are observed at cold temperatures, which suppress the PRL response (Peters et al., 1981). Long days are associated with an increase in circulating IGF-I, an increase that is independent of changes in growth hormone (**GH**) secretion (Dahl et al., 1997; Spicer et al., 1994). The increase in IGF-I observed under long days is also independent of any shift in hepatic response to GH, as expression of GH-receptor 1A is unaffected by photoperiod (Kendall et al., 2003). Yet, the potential galactopioietic action of IGF-I is the subject of some controversy, as milk yield responses to mammary infusion of IGF-I have been inconsistent (reviewed in Tucker, 2000). Recent work in rodents and goats suggests that an interaction between PRL and the IGF system may play a role in lactational persistency, and this is a possible mechanistic explanation for the galactopoietic impact of long days (Flint et al., 2001). Specifically, PRL is inversely related to expression of IGF-binding protein-5 (**IGFBP-5**), which is considered an apoptotic factor in the mammary gland. The elevated PRL observed under long days would thus be expected to hold IGFBP-5 expression in check and reduce cell loss in the mammary gland. Such an outcome would be consistent with higher persistency and greater overall milk yield. Whereas circulating IGFBP-2 and -3 do not appear to be altered by photoperiod in lactating cows (Dahl et al., 1997), recent evidence using a more sensitive approach to detect IGFBP in steers indicates that IGFBP-3 is elevated under long days relative to short days (Kendall and Dahl, unpublished). If those findings are confirmed in lactating cows, reduced IGF-I clearance may provide a mechanistic explanation for the increases observed under long days. There are no reports that have examined IGFBP-5 directly in cows exposed to different photoperiods.

Dry Cows—Milk Yield Response

In contrast to the lack of effect of exogenous PRL during an established lactation in cattle (Plaut et al., 1987), a robust periparturient PRL surge is essential to complete lactogenesis at calving (Akers et al., 1981). Newbold et al. (1991) observed that long days during the final trimester increased the magnitude of the periparturient PRL surge in heifers. Based on those results, it was hypothesized that an enhanced PRL surge would increase production in the next lactation. Three experiments were completed to test that hypothesis in our laboratories. In the first, 34 multiparous cows were exposed to either 8L:16D or 16L:8D during the dry period to determine the effects on subsequent milk yield (Miller et al., 2000). At calving, all cows returned to the ambient photoperiodic conditions of the herd. As in Newbold et al. (1991), long days during the dry period caused a larger periparturient PRL surge relative to SDPP. Surprisingly, however, cows exposed to short days during the dry period produced an average of 3.1 kg/d more milk than cows on long days. In the second study, cows were exposed to SDPP or long-day photoperiod (**LDPP**) during the dry period and then exposed to LDPP after calving (Petitclerc et al., 1998). Again, animals on SDPP during the dry period and transferred to LDPP at parturition produced significantly more milk relative to those exposed to LDPP while dry and after calving (Figure 3). In a third study, pregnant heifers were placed on LDPP or SDPP for the final 60 d before calving (Petitclerc et al., 1990). Similar to the multiparous cows, exposure to LDPP increased concentrations of PRL, but SDPP cows produced more milk in the first lactation. Clearly, a transfer from a shorter photoperiod when dry to a longer photoperiod when lactating maximizes milk yield, but what is the physiological basis for that response?

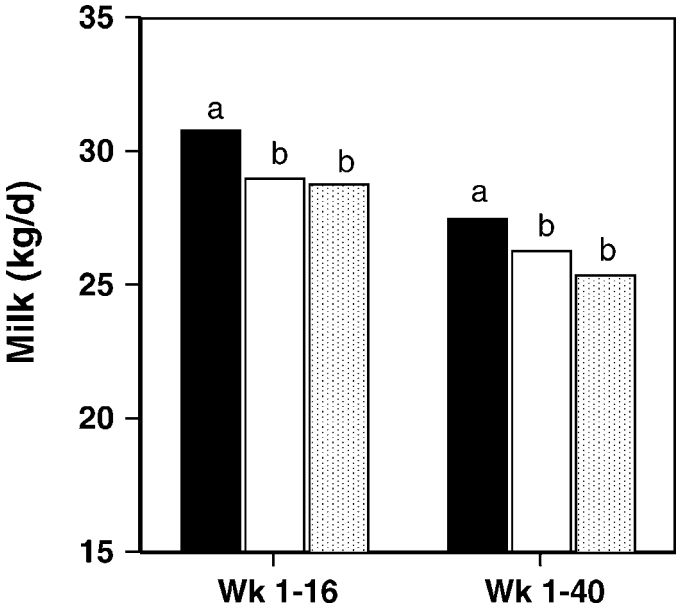

Figure 3. Milk yield response of cows exposed to short days (8L:16D, solid bars), long days (16L:8D, open bars), or long days and fed melatonin (25 mg/d, stippled bars) during the dry period. Bars represent average daily production for wk 1–16 and wk 1–40 of the lactation immediately following treatment. Letters indicate significant differences between treatments (wk 1 to 16, $P < 0.03$; wk 1 to 40, $P < 0.07$). All cows were exposed to long days following parturition. Data adapted from Petitclerc et al., 1998.

Cows on SDPP had lower circulating PRL at calving relative to the LDPP cows (7.7 vs. 15.2 ng/ml). Considered with the PRL results, these data suggest that increasing the PRL surge via LDPP is without effect on subsequent yield of milk, but other factors associated with SDPP were stimulatory. However, a third group of cows in the second study treated with long days and

fed melatonin (25 mg/d) in the middle of the photophase to mimic a short day pattern did not yield more milk relative to the LDPP group (Figure 3), despite a reduction in circulating PRL relative to the LDPP cows (9.7 vs. 15.2 ng/ml). Yet previous use of melatonin feeding to mimic a SDPP in lactating cows has not been successful in altering the milk yield response; thus, melatonin replacement may not be an appropriate model for a short day in mature cows with regard to milk production (Dahl et al., 2000).

The observed effect of SDPP during the dry period (and late pregnancy in primiparous animals) may be due to the fact that animals become refractory to a constant light pattern and exposure to SDPP resets a cow's responsiveness to the stimulatory signal. An alternative interpretation of the data from the three studies presented above is that LDPP during the dry period suppressed milk yield in the subsequent lactation. Such an interpretation would be consistent with previous studies in sheep and quail that examined the influence of direction of photoperiodic change (i.e., exposure to increasing vs. decreasing photoperiod) on responses of the reproductive systems (Robinson and Follett, 1982; Robinson and Karsch, 1987). However, comparison of 305-d mature equivalent data between the lactation preceding the LDPP treatment and the lactation following (i.e., the full record of the data reported in Miller et al., 2000) revealed no difference between years. This suggests that the LDPP treatment during the dry period was not detrimental to the subsequent lactation. Rather, SDPP during the dry period stimulated subsequent production.

Further confirmation of the importance of dry period light exposure on subsequent milk yield has emerged from examination of seasonal effects of parturition on milk production. It has long been known that cows that calve in July and August in the Northern Hemisphere, when day length and temperature are near maximum, produce less milk than contemporaries that calve in November and December (Wunder and McGilliard, 1971; Barash et al., 1996). This influence of season of calving on milk yield has traditionally been attributed to heat induced depression of intake and subsequent milk production (Wunder and McGilliard, 1971; Wolfenson et al., 1988). However, recent work suggests that a majority of the seasonal effect can be accounted for by environmental factors during the dry period, specifically photoperiod. Aharoni et al. (2000) examined the records of more than 2000 cows and found that photoperiod exposure during the final 21 d of the dry period was inversely related to milk yield in the subsequent lactation. That is, exposure to SDPP meant that cows produced 2.1 kg more milk per day than exposure to a LDPP. Extrapolated out for an entire lactation, this would be an increase of 640 kg. Putting that into perspective, that would be about 67% of the expected increase in response to bST treatment according to label instructions (Bauman, 1999).

PRL Sensitivity in Peripartum Cows

The apparent paradox of photoperiodic effects on lactating vs. dry cows may be due to the different impacts of PRL and IGF-I during each physiological state. Specifically, we believe that PRL is exerting a developmental effect in the dry/transition period, and this response is not present once lactation is fully established. In contrast, the impact of elevated IGF-I during lactation is a metabolic action at the mammary gland, which would be absent in the nonlactating state. With regard to PRL, our theory is that elevated PRL during the dry period produces a "PRL resistance" at the mammary gland. At the time of parturition, the periparturient PRL surge is critical to the secondary stage of lactogenesis (Akers et al., 1981), and early in lactation the interplay of PRL and PRL-receptor (**PRL-r**) may be important to maximize mammary epithelial cell recruitment and differentiation. Cows on a SDPP during the dry period and transferred to a longer photoperiod in early lactation would therefore be expected to have greater PRL-r expression and an increasing concentration of circulating PRL. Cows on long days in the dry period would have reduced PRL-r expression and a depressed PRL secretory stimulus due to negative feedback. Therefore, an increase in circulating PRL in the periparturient stage layered over a greater number of PRL-r should maximize cell numbers and hence lactation. Direction of change in photoperiod is therefore as important as absolute duration with regard to the dry cow response (Robinson and Follett, 1982; Robinson and Karsch, 1987).

A number of observations support this hypothesis regarding PRL resistance. Indeed, chronic elevation of PRL suppresses PRL-r expression (Barash et al., 1983; Kazmer et al., 1986; Smith et al., 1989). Other physiological perturbations that elevate PRL, such as high temperature, also have a negative impact on milk yield in the next lactation (Barash et al., 1996). In contrast to the previously mentioned lack of effect of PRL on milk yield in cows once lactation is established, several reports suggest that PRL elevations may be important early postpartum. These include the stimulatory effect of PRL immediately postmilking in goats (Jacquemet and Prigge, 1991) and the stimulatory effect of multiple daily milking (e.g., 6 times a day) early in lactation on milk yield for the remainder of the lactation (Bar-Peled et al., 1995; Henshaw et al., 2000). The same multiple daily milking scheme did not induce permanent effects when imposed on midlactation cows (Bar-Peled et al., 1995). We have recently observed that frequent milking in early lactation (i.e., four vs. two milkings per day) induces a significant, though transient, increase in PRL-r mRNA using lymphocytes as a proxy for mammary cells (Dahl et al., 2002). Though circumstantial, these observations all support the concept that increasing PRL with a higher relative PRL-r expression produces maximal, permanent increases in milk secretory capacity for that lactation when imposed during the

immediate postpartum period. But what is the effect of photoperiod or PRL itself on PRL-r expression in cattle? We have used PRL-r mRNA as an index of PRL-r expression in liver, lymphocytes, and mammary parenchyma in steer calves (Auchtung et al., 2002a). Expression of PRL-r mRNA was inversely related to plasma PRL concentrations; thus, SDPP increased PRL-r mRNA relative to LDPP. More importantly, when the same calves had photoperiodic treatments reversed, the effects on PRL and PRL-r reversed also, suggesting that photoperiodic shifts in PRL secretion mediated these shifts in PRL-r expression. In addition, relative expression of PRL-r moved in parallel in the three tissues, indicating that lymphocytes can be used to track shifts in PRL-r expression in other tissues without the invasive disruption of a biopsy.

Photoperiod and Mammogenesis

In addition to the impact in the mature cow, photoperiod manipulation has effects on mammary development. Relative to short days, parenchymal cell number was greater in heifers exposed to long days during the prepubertal period (Petitclerc et al., 1985). Because long days hasten puberty (reviewed by Hansen, 1985), a portion of that growth may be associated with the acceleration in gonadal steroid secretion associated with long day treatment. However, long days also increase parenchymal cell number after puberty, suggesting that other factors in addition to gonadal steroids play a role (Petitclerc et al., 1985). Certainly the effects of photoperiod on PRL and IGF-I would be consistent with greater mammary growth, though definitive experiments have not been reported.

Photoperiodic Effects on Health

In the wild, animals must adjust their growth, reproduction, and lactational priorities to meet the energetic demands and resource limitations that occur on a seasonal basis, so to they are likely to face seasonal shifts in pathogens. Thus, annual patterns of variation in immune function have been observed in many species, yet the physiological foundation for those patterns is unknown (Dowell, 2001). Because of the linkage to other seasonal responses, a role for photoperiod has been investigated. In hamsters, photoperiod alters immune function (Yellon et al., 1999), with short days reducing the severity of infectious responses (Bilbo et al., 2002a, 2002b). Photoperiod induced shifts in cortisol and PRL have been implicated in the development of this altered immune function in rodents, and preliminary evidence from one of our laboratories suggests that PRL serves a similar role in the bovine.

Because PRL is considered a cytokine and PRL-r is a member of the cytokine receptor superfamily (Kelly et al., 1991), our initial inquiry related to identification of PRL-r on lymphocytes. In steer calves, quantification of PRL-r mRNA using real-time PCR led to the observations that bovine lymphocytes express short and long forms of PRL-r (Auchtung et al., 2001, 2002b), and expression of both forms of PRL-r increases under SDPP, inverse to the shift in PRL secretion observed with that photoperiod (Auchtung et al., 2001). Further, SDPP was associated with greater lymphocyte proliferation in vitro, and this was reversed with reversal of photoperiod treatment (Auchtung et al., 2002a). These observations support the hypothesis that short days enhance immune function in calves, yet questions remain regarding application to mature cows.

The transition period from the dry to lactating state is the acme of immunosuppression in the lactation cycle. Because cows exposed to SDPP when dry produce more milk in the next lactation, it became critical to evaluate the effect of SDPP on immune function to ensure that further immunosuppression did not occur and negate the production effect. Preliminary results suggest that cows on SDPP when dry had greater PRL-r expression and lower circulating PRL relative to LDPP cows. In addition, SDPP dry cows had increased lymphocyte proliferation and chemotaxis response to interleukin-8 (Auchtung and Dahl, unpublished). Cows on SDPP also subsequently produced more milk. These results suggest that exposure to SDPP during the dry period not only improve production in the subsequent lactation but also potentially improve animal health and well-being.

With regard to health, it is important to consider the potential influence then of LDPP in lactating cows. That is, will cows exposed to LDPP to increase production have higher incidence of disease? The photoperiod literature does not support this speculation, as there were no reports of increased mastitis or other infectious disease in cows on LDPP relative to natural photoperiod. It is most likely that LDPP exposure would not heighten the incidence of disease, as the risk is greatest during the transition phase, and once lactation is fully established, disease incidence is dramatically reduced. In addition, the increment from a natural photoperiod to LDPP may be insufficient to significantly influence immune function. These questions remain, however, as areas requiring further investigation.

Summary and Recommendations

Some 25 yr after the initial report of galactopoietic effects of long days in cattle, new findings continue to increase our understanding of the role of photoperiod in production and health. Considering the research to date, we can make the following recommendations regarding light exposure during the life cycle of the cow. First, heifers should be exposed to LDPP during the postweaning phase until puberty to maximize mammary parenchymal growth. Data for yearling heifers is lacking at present. However, during the final 60 d of pregnancy, primiparous heifers and dry cows should be under SDPP to maximize production in the next lactation and enhance immune function in the transition period. During lactation, exposure to LDPP is rec-

ommended to increase milk yield, particularly in cases where dry period exposure to SDPP is not possible.

Acknowledgments

Studies described in this paper have been supported by the Agricultural Experiment Stations of Maryland and Illinois, Agriculture and Agri-Food Canada, Pharmacia and Upjohn Animal Health, USDA-Binational Agricultural Research and Development Fund (01-3201), and the Illinois Council for Food and Agricultural Research. The authors express appreciation to Tera Auchtung, Paul Kendall, Allison Miller, Thomas McFadden, Janeen Salak-Johnson, Pierre Lacasse, C. M. Vinet, and G. Roy for their assistance in the conduct of these studies. Dairy and Swine R&D Centre contribution No. 789.

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Lactation persistency: Insights from mammary cell proliferation studies

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ABSTRACT: A persistent lactation is dependent on maintaining the number and activity of milk secreting cells with advancing lactation. When dairy cows are milked twice daily, the increase in milk yield from parturition to peak lactation is due to increased secretory activity per cell rather than to accretion of additional epithelial cells. After peak lactation, declining milk yield is due to loss of mammary epithelial cells by apoptosis. During lactation, only 0.3% of mammary cells proliferate in a 24-h period. Yet this proliferative rate is sufficient to replace most mammary epithelial cells by the end of lactation. Management practices can influence lactation persistency. Administration of bovine somatotropin may enhance persistency by increasing cell proliferation and turnover, or by reducing the rate of apoptosis. Increased photoperiod may also increase persistency of lactation by mechanisms that are as yet undefined. Increased milking frequency during the first weeks of lactation increases milk yield, even after return to less frequent milking, with increases of approximately 8% over the entire lactation. A mammary cell proliferation response to frequent milking during early lactation appears to be involved. Conversely, advanced pregnancy, infrequent milking, and mastitis increase death of epithelial cells by apoptosis. Regulation of mammary cell renewal provides a key to increasing persistency. Investigations to characterize epithelial cells that serve as the proliferative population in the bovine mammary gland have been initiated. Epithelial cells that stain lightly in histological sections are evident through all phases of mammary development and secretion and account for nearly all proliferation in the prepubertal gland. Characterization of these cells may provide a means to regulate mammary cell proliferation and thus to enhance persistency, reduce the effects of mastitis, and decrease the necessity for a dry period.

Key Words: Apoptosis, Cell proliferation, Cell renewal, Lactation Efficiency, Ruminants

 J. Anim. Sci. 2003. 81(Suppl. 3):18–31

Introduction

The number of mammary epithelial cells and their secretory activity determine the shape of the lactation curve. Depending on the species, increases in the number or activity of mammary secretory cells account for increased milk yield to peak lactation. Conversely, declining mammary cell number or secretory activity accounts for declining milk yield after peak. Management and disease can potentially impact the proliferation or loss of cells during lactation. Knowledge about mammary cells that are capable of cell proliferation and of pathways that regulate proliferation cell death may provide means to alter persistency of lactation.

If persistency of lactation could be increased, considerable benefits would accrue to the dairyman. Flattening the declining portion of the lactation curve promotes a more efficient lactation. By lengthening lactation, a smaller portion of a dairy animal's life would be spent during the periparturient period with its increased health risks and associated costs. Delayed breeding could increase reproductive efficiency. Lengthening lactation and use of sexed semen for artificial insemination may serve to more efficiently match a decreased need for replacement heifers, thus reducing the need to slaughter bobby calves. Management schemes that improve maintenance of mammary cell numbers will almost certainly increase persistency. Another management goal might be elimination of a dry period between successive lactations. During this pe-

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Received August 8, 2002.
Accepted January 16, 2003.

riod, accelerated renewal of mammary cells, perhaps progenitor cells, may be of particular importance. Although important for dairy cows, the impact of a dry period on lactation in dairy goats has not been established.

This review will discuss changes in mammary cell number, proliferation, and death during lactation, factors that may impact these processes, and putative cellular targets for strategies to impact mammary cell number. Hypotheses pertaining to the value of a dry period between lactations will also be discussed. Although this review will focus on dairy cattle and goats, rodents will be discussed for comparative purposes and when there are limited data pertaining to ruminants.

Cell Number and Cellular Secretory Activity During Lactation

Knight and Peaker estimated mammary cell number and secretory activity during lactation of goats (Knight and Peaker, 1984b). This was accomplished by using multiple biopsies to evaluate changes in nucleic acid concentrations and enzymatic activity. When coupled with measures of udder volume, the data were extrapolated to whole udder measures. This study demonstrated that increases in milk production during early lactation were first the result of an increase in mammary cell number followed by an increase in secretory activity per cell. After peak lactation, decreased milk yield with advancing lactation was primarily the result of declining cell number. However during late lactation, when goats were concomitantly pregnant, the secretory activity per cell also declined.

Recently, a comparable analysis of changes in mammary cell number and secretory activity during a bovine lactation was performed (Capuco et al., 2001a). This study was based on the slaughter of nonpregnant Holstein cows at four time points during lactation with quantification of total mammary DNA. Increased milk yield during early lactation appeared solely due to increased secretory activity per cell because no increase in mammary cell number was detected prior to peak lactation. The decline in milk yield with advancing lactation was solely due to decreased cell number (total mammary DNA). The secretory activity per cell, estimated as milk yield per unit of mammary DNA, increased prior to peak lactation but did not change significantly with advancing lactation. It is important to realize that these data (Capuco et al., 2001a) pertain to nonpregnant multiparous cows. When cows are concomitantly lactating and pregnant, it is likely that a decline in secretory capacity per mammary cell accompanies advanced pregnancy due to conflicting metabolic demands of gestation and lactation. Indeed, this is readily apparent during late pregnancy, when the number of mammary epithelial cells increases simultaneous with a rapid decline in milk production (Capuco et al., 1997).

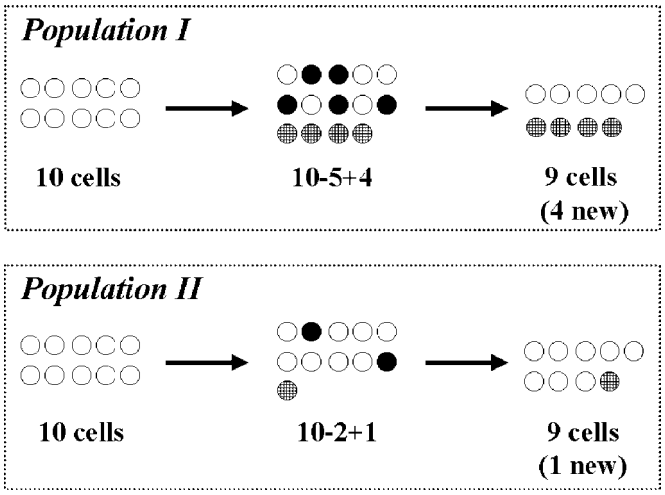

Figure 1. Rates of cell proliferation and cell death determine the net gain or loss of cells and extent of cell renewal within the population. Depicted is a population of cells undergoing net regression. Open circles represent the initial populations of cells; cross-hatched circles depict new cells formed by cell proliferation; and cells that die during this period are depicted by black circles. In both panels, the net loss is identical because the difference between rates of cell death and proliferation are one cell. However, cell renewal differs markedly between panels. In the upper panel, the population of nine cells contains four new cells. In the lower panel, the population of nine cells contains one new cell. (Capuco et al., 2001a)

In addition to evaluating the relationship between the lactation curve and the number and activity of mammary cells, we used a quantitative approach to evaluate mammary cell renewal during lactation (Capuco et al., 2001a). This was accomplished by separately evaluating rates of cell proliferation and cell death. Cell number reflects the sum of relative rates of cell proliferation and cell death. The mammary gland grows when the rate of proliferation exceeds the rate of cell death, regresses when the rate of cell death exceeds the rate of cell proliferation, and maintains constant cell number when rates of proliferation and death are equal. Regardless of the net change in cell number, a population may undergo varying degrees of cell replacement or turnover determined by the absolute rates of proliferation and cell death (Figure 1).

Rates of proliferation and apoptosis were estimated at four stages of lactation by bromodeoxyuridine **(BrdU)** and terminal deoxyuridine triphosphate nick end labeling **(TUNEL)** respectively. Rates of mammary cell proliferation were determined by using multiple injections of BrdU (a thymidine analog) to label all cells that synthesize DNA within a 24-h labeling period and then quantifying these cells by immunohistochemistry. The 24-h labeling period should provide a reliable measure of average proliferation rate even in the presence of an underlying diurnal pattern of DNA synthesis,

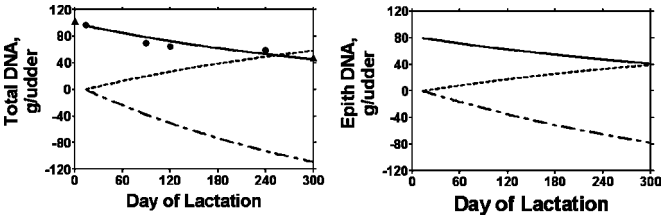

Figure 2. Components of mammary DNA (cell) turnover during lactation. The proliferation rate was 0.003/d. The apoptotic index averaged 0.0007; assuming a 3-h duration of apoptosis, the apoptotic rate is 0.0056/d. Shown is the accumulated synthesis of new DNA (formation of new cells) (------); the accumulated loss of DNA by apoptosis (- — - — -); and the net loss of DNA (difference between synthesis and loss) (——). Left panel is total mammary DNA; right panel is epithelial DNA. Experimental data points from two experiments are indicated by the symbols in the left panel. Adapted from Capuco et al. (2001a).

such as that reported for rodent mammary glands (Borst and Mahoney, 1980). The in situ labeling of apoptotic cells in histological sections by TUNEL detects cells with fragmented DNA, a hallmark of later stages of apoptosis. Because apoptotic cells appear to persist for about 3 h (Bursch et al., 1990), we multiplied the labeling index by eight (24/3) to obtain an estimate for a 24-h apoptotic rate (Capuco et al., 2001a).

Measures of proliferation rate and apoptotic rate were consistent with the observed decline in mammary cell number. Although the BrdU-labeling index did not vary significantly during lactation, there was a tendency for reduced rates of cell proliferation during early lactation (14 d). This suggested that the increased mammary cell proliferation evident during gestation and the dry period did not continue during early lactation. Lack of increased proliferation rate or decreased apoptotic index during early lactation precludes significant mammary growth during early lactation in cows. However, the latter conclusion is somewhat tentative because mammary growth during lactation may have occurred prior to the first sampling time, 14 d of lactation. A decline in cell number after peak lactation was predicted by the estimates of proliferation and apoptotic rate. Across lactation, 0.3% of mammary cells proliferated per day. After peak lactation, the apoptotic index averaged 0.07%. Although seemingly very low, this apoptotic index was extrapolated to an apoptotic rate of 0.56% per day—a value that exceeded the rate of cell proliferation. Using initial mammary DNA measures, the experimentally estimated rates of cell proliferation and death were used to predict mammary cell number or DNA with advancing lactation. The predicted decline in cell number closely approximated the decline in mammary DNA observed (Figure 2; Capuco et al. 2001a). In addition to the predicted curve for net mammary DNA, curves were generated for the accumulative cell proliferation and accumulative cell loss. The number of cells generated during lactation is predicted to equal the number that are present in the gland at 252 d of lactation (Figure 2, intersection of curves). If none of the cells that died by apoptosis were those that proliferated during lactation, then 100% of the mammary cells remaining after 252 d of lactation were formed during lactation. If apoptosis is random, equal death rates for preexisting cells and cells that have formed during lactation can only occur after considerable proliferation has occurred during lactation so that these two populations are equal in number. If apoptotic cell death is randomly distributed among cells of different ages, turnover of mammary cells will be less than 100%, but certainly greater than 50%.

The extent of mammary cell turnover during a bovine lactation differs markedly from that occurring during lactation in rats. In contrast to the extensive cell turnover during a bovine lactation, approximately 75% of mammary cells are maintained throughout an entire lactation in rats (Pitkow et al., 1972). This lack of extensive cell turnover may be due to the shorter length of lactation in rodents compared with cows. Furthermore, this low degree of cell renewal may be consistent with the decline in activity per mammary secretory cell that occurs during lactation in rats, but not in nonpregnant dairy cows.

After peak lactation, there was a decline in the number of mammary epithelial cells (Capuco et al., 2001a). Expressed as a percentage of total cells, the epithelial compartment declined from 79% at 90 d to 73% at 240 d. This was consistent with previous data indicating that around the time of calving, 83% of mammary cells were epithelial, but during late lactation, the percentage had decreased to 74% (Capuco et al., 1997). Because of the error inherent in quantifying very small percentages of proliferating and apoptotic cells, we were unable to subdivide the predictive equations in Figure 2 to adequately describe changes in the relative proportions of epithelial and stromal cells. However, the data clearly model a reduction in total cells and epithelial cells of the mammary gland during lactation.

In addition to apoptotic death, another potential mechanism for declining cell numbers during lactation is the continuous loss of mammary epithelial cells in milk. In the present experiment, milk somatic cell count **(SCC)** averaged 52,000 cells/mL, and milk production averaged 9,920 kg for 240 d. Because epithelial cells account for less than 20% of milk SCC (Miller et al., 1991), and DNA content is 6 pg/cell, cumulative loss of mammary DNA is less than 0.62 g by 240 d of lactation. This accounts for only 1.6% of the net loss of mammary DNA (38.3 g) by 240 d of lactation. Although clearance of apoptotic cells from the mammary gland may involve passage of phagocytes into the milk, removal of intact viable mammary cells via the milk does not significantly contribute to the declining number of mammary secretory cells during lactation.

These data (Capuco et al., 2001a) provided the first quantitative demonstration that apoptotic death of mammary epithelial cells accounts for gradual regression of the bovine mammary gland during lactation and the proportional decline in milk yield. Indeed, this was the first demonstration that apoptosis can fully account for cell loss during lactation of any species.

Impact of Pregnancy on Lactation

The persistency of lactation is decreased when cows are concomitantly pregnant. A negative effect of pregnancy on milk yield has been detected as early as d 100 of gestation (Bachman et al., 1988). Because this coincided with the onset of estrogen secretion by the fetal-placental unit, estrogens were implicated as mediators of the inhibitory effects of pregnancy on lactation (Bachman et al., 1988). Others had demonstrated that estrogens administered at supraphysiological doses inhibited milk production (Folley et al., 1941; Hutton, 1958; Cowie, 1969). The time course of the inhibition and subsequent return of milk yield, along with accompanying changes in milk composition, suggested to some that high concentrations of estrogen induced an initial phase of mammary involution followed by epithelial regrowth and differentiation (Mollett et al., 1976). Thus, Mollett et al. (1976) investigated the feasibility of treating with estrogen and progesterone during lactation to determine if production of additional secretory cells could be induced. The approach was unproductive since the combined effect of estrogen and progesterone was similar to the inhibitory effect of estrogen alone. However, there was an exception. Estrogen and progesterone treatment increased milk production in cows that had been hormonally induced into lactation. This suggested that additional steroid treatment during lactation might compensate for incomplete mammary development prior to lactation.

The impact of pregnancy on apoptosis of mammary epithelial cells has been investigated in mice (Quarrie et al., 1996). When mice were remated at postpartum estrus, concomitant pregnancy accelerated the gradual mammary involution occurring during lactation due to increased levels of apoptosis. These data suggest that in addition to decreasing the secretory activity per cell, concomitant pregnancy accelerates the gradual regression of the mammary gland as lactation advances. Similarly, preliminary data for lactating dairy cows (A. V. Capuco, unpublished data) indicate that concomitant pregnancy increases the apoptotic index (TUNEL-positive cells) several times over, but is accompanied by a substantial increase in cell proliferation (BrdU labeling index). This suggests that the rate of mammary cell turnover during lactation is increased by concomitant pregnancy. Whether apoptosis or proliferation dominates might depend on the stage of gestation and the net effect of steroids and other hormones of pregnancy.

Peaker and Linzell investigated effects of estrogen on caprine lactation (Peaker and Linzell, 1974). They found that administration of estrogen at doses designed to match the secretion rate in late pregnancy caused a significant decline in milk yield. Additionally, they found that changes in milk secretion occurred during the estrous cycle. Several days preceding estrus, there was a clear change in milk composition characterized by increases in milk sodium and chloride concentrations and decreases in potassium and lactose. Milk composition returned to normal by the time estrous behavior was observed. Milk yield often declined during estrus, but was associated with a decline in feed intake. Whether increased secretion of estrogens during the normal ovarian cycle directly impact milk yield and, ultimately, persistency of lactation is not clear. However, data indicate that estrogens at concentrations observed during pregnancy can reduce milk yield.

It is clear that estrogen can inhibit milk secretion. The site of action appears to be the mammary gland, as implantation of estrogen pellets in rat mammary gland inhibited milk secretion, whereas implantation at the pituitary enhanced milk secretion (Bruce and Ramirez, 1970). However, estradiol does not appear to serve as a regulator of the IGFBP-5-regulated apoptosis described subsequently (Tonner et al., 1997; 2000).

Impact of Bovine Somatotropin, Milking Frequency, and Photoperiod on Cell Proliferation During Lactation

Small decreases in the rate of apoptosis or increases in the rate of cell proliferation will alter mammary regression during lactation. The slow rate of apoptotic cell death during lactation indicates that there are clear limitations on the capacity to globally decrease this rate in nonpregnant cows. But the opportunity to effect substantial change by decreasing apoptosis in pregnant lactating cows may exist. In all cases, a fruitful approach will likely be to increase cell proliferation during lactation so as to increase the maintenance of epithelial cell number and lactational persistency. Three potential means to alter the lactation curve have been investigated in recent years: bovine somatotropin **(bST)** administration, alterations in milking frequency, and photoperiodic manipulation.

The apparent lack of mammary growth during early lactation in cows (Capuco et al., 2001a) does not preclude the ability of mammary epithelial cells to proliferate in response to management conditions. For example, milking 6× daily during early lactation caused an increase in milk yield that persisted after less frequent milking (3×) was resumed (Bar-Peled et al., 1995). Although mammary growth was not assessed, this carryover effect is consistent with increased mammary growth during early lactation. Finally, net mammary growth can occur during lactation, as demonstrated by the compensatory growth response to various stimuli (Knight and Peaker, 1984a; Capuco and Akers, 1990). Suckling and milk removal during early lactation are major controlling factors of mammary cell number in

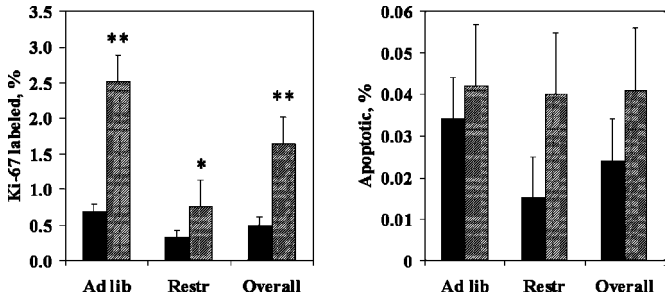

Figure 3. Effect of bovine somatotropin **(bST)** on mammary cell proliferation and apoptosis. Six cows were fed ad libitum (Ad lib) and six cows were restricted to 80% of their ad libitum intake (Restr). Values shown are for the control period (solid bar) and bST treatment period (hatched bar) for each dietary group and the overall effect (12 cows). Left panel: Ki-67 labeling index, expressed as a percentage of total cells. Right panel: apoptotic index, expressed as a percentage of total cells. Each bar represents the mean ± SE for six cows per dietary group and 12 cows for overall effect. * = $P < 0.05$, ** = $P < 0.01$ (Capuco et al., 2001a).

rodents. Current data suggest that similar mechanisms may be operative in dairy cows and goats.

The influence of bST on the proliferative and apoptotic status of cells within the lactating bovine mammary gland was recently evaluated (Capuco et al., 2001a). Previous experiments had indicated that bST increased the persistency of lactation (Phipps et al., 1991; Van Amburgh et al., 1997; Bauman et al., 1999). In light of the demonstration that decreasing milk yield with advancing lactation is due to a decline in mammary cell number and not cellular activity, it follows that the effect of bST on persistency is due to maintenance of the mammary cell population rather than maintenance of cellular secretory rate. Indeed, bST maintained cell number in lactating caprine mammary glands (Knight et al., 1990) and data suggest that bST increases mammary cell proliferation during late gestation in sheep (Stelwagen et al., 1993) and perhaps in heifers (Stelwagen et al., 1992). The nuclear proliferation antigen Ki-67 was used as an index for the relative proliferation state of mammary tissue. The Ki-67 protein serves as a marker for cells that are engaged in cell cycle progression due to its presence during all phases of the cell cycle, except the quiescent G_0 phase (Gerdes et al., 1984). This protein correlates with cellular proliferation under a variety of physiological conditions in human, rodent, and bovine tissues (Capuco et al., 2001a; Hardville and Henderson, 1966; Shayan et al., 1999). Treatment of first-lactation dairy cows with bST for 7 d increased the proportion of cells expressing the nuclear proliferation antigen by threefold (Figure 3). The effect of bST was evident when cows were given ad libitum access to feed (2.5 vs. 0.7%) or when they were restricted to 80% of ad libitum intake (0.8 vs. 0.3%) to induce a negative energy balance (Capuco et al., 2001b). Overall, cows in negative energy balance had a lower percentage of mammary cells expressing Ki-67 than did comparable cows in positive energy balance. These data strongly support the hypothesis that bST increases cellular proliferation in the bovine mammary gland and that mammary cell proliferation is blunted by reduced energy balance. Additionally, these data suggest that a proliferative response to bST would be blunted during early lactation (Capuco et al., 2001a) due to the negative energy balance cows are in during early lactation.

Although treatment of lactating cows with bST apparently increased mammary cell proliferation (Capuco et al., 2001a), earlier investigations had indicated that bST treatment did not cause net growth of the lactating bovine mammary gland (Capuco et al., 1989; Binelli et al., 1995). The earlier results do not infer that bST does not increase the rate of cell proliferation, but that the rate of cell proliferation did not exceed the rate of cell death. We suggest that treatment with bST increased the rate of mammary cell proliferation so as to reduce the rate of mammary regression during lactation. Additional study will be necessary to determine if extended treatment with bST increases the number of mammary cells relative to that of cohort controls. Previous slaughter studies may not have been sufficiently sensitive to detect small (e.g., 10%) differences in cell number. Alternatively, increased cell renewal may enable milk synthesis in bST-treated cows to continue at high capacity by enhanced replacement of senescent secretory cells.

Increased milking frequency **(IMF)** at the beginning of lactation has been shown to increase milk yield not only during IMF, but also after its cessation (Bar-Peled et al., 1995). Using a within-udder experimental design, increased milking frequency (4×) during mid-lactation was shown to increase cell proliferation when assessed 4 wk after initiation of treatment (Hillerton et al., 1990). We have recently evaluated the immediate effects of increased milking frequency initiated during early lactation on mammary growth and the long-term effects on milk yield (Hale et al., 2002). A novel approach of using four very uneven milking intervals was employed to obtain increased frequency without the added labor and other inherent costs involved in establishing a traditional pattern of increased milking frequency with fairly even milking intervals. Cows were divided into three treatment groups: 1) controls were cows milked twice daily (2×) beginning at parturition, 2) IMF1: cows milked four times daily (4×) from d 1 to 21 postpartum 3) IMF4: cows milked 2× from d 1 to 3 and 4× d 4 to 21 postpartum. The 4× cows were milked immediately before 2× cows and again (approximately 3 h later) after the 2× cows at both morning and evening milkings. All cows were milked 2× from d 21 to 305 postpartum. Milking 4× during early lactation increased milk yield not only during the treatment period, but it also elicited an increase in milk yield for the entire lactation. Milk yields were 33.5, 42.3, and 38.3 kg/d during wk 1 to 3

($P = 0.018$) and 34.8, 37.4, and 37.5 kg/d during wk 4 to 40 ($P = 0.058$) for control, IMF1, and IMF4, respectively. Mammary biopsies from four cows per treatment were obtained on d 7 and 14 postpartum to assess mammary cell proliferation. Tritiated-thymidine incorporation by tissue slices was increased ($P = 0.09$) on d 7 in IMF1 cows, and arithmetic means for the percentage of cells expressing Ki-67 proliferation antigen were consistent with a proliferative response to IMF. Increased milking frequency during early lactation may increase mammary growth and thus produce a carryover effect on milk production for the majority of lactation. A carryover effect was observed with a minimal increase in labor and operating costs and was effective when increased milking was initiated on the first day of lactation or on d 4 after the routine interval for discarding milk colostrum. Whether this protocol significantly increases milk component secretion remains to be demonstrated.

Other investigations had shown that increased milking frequency enhanced mammary cell proliferation. Increased milking frequency increased milk production of goats due to a rapid increase in the activity of mammary secretory cells, often followed by proliferation of secretory tissue (Wilde et al., 1987; Knight et al., 1990). Increased milking frequency is hypothesized to increase milk production by lessening the accumulation of a feedback inhibition of milk secretion (Henderson and Peaker, 1984). Because frequent milking of one mammary gland has no effect on milk secretion by the opposing gland, it is clear that milk removal, and not systemic effects of milking, plays an important role in establishing secretion rate (Linzell and Peaker, 1971). Whether removal of inhibitors of lactation can adequately explain the impact of supplemental milkings with very short intermilking intervals is uncertain.

Lactating dairy cows exposed to long-day photoperiods (16 to 18 h of light) produce more milk than cows exposed to short-day (<12 h light) photoperiods (reviewed in Dahl et al., 2000). The endocrine mediators of this response appear elusive. Because long-day photoperiods increase systemic concentrations of prolactin, and because prolactin is known to be galactopoietic in rodents, it was hypothesized that this hormone may mediate the galactopoietic effects of long days in cattle. However, prolactin administration does not increase milk yield of lactating dairy cows (Plaut et al., 1987). Furthermore, milk production responses to long-day photoperiods are observed in the absence of photoperiod-induced changes in prolactin concentrations, such as during freezing temperatures and melatonin feeding (Dahl et al., 2000). The galactopoietic effects of long-day photoperiod are associated with increases in IGF-I secretion that precede the milk production response (Dahl et al., 1997), and IGF-I appears the most likely mediator of the galactopoietic effects of increased photoperiod (Dahl et al., 2000).

In addition to uncertainty regarding the hormonal mediator of the lactational response to increased photoperiod, the cellular mechanisms responsible are uncertain. The lactation of cows on long-day photoperiods appears more persistent than that of cows on short days. Consistent with the delay (approximately 4 wk) in onset of response, effects on persistency may be a contributing factor to the galactopoietic response to photoperiod.

Galactopoietic effects of bST, IMF, and long-day photoperiod are additive. It is reasonable to assume that effects on persistency will similarly be additive, and that the greatest persistency will be achieved by a combination of the three treatments. For the producer, there may be considerable flexibility in incorporating these treatments since IMF may be effective when restricted to the immediate postpartum period and when 4× milking is imposed with a very uneven milking intervals as previously described. This reduces problems associated with incorporating a regulated lighting regime with frequent milking. Flexibility is also increased by the recent demonstration that galactopoietic effects may be realized by restricting photoperiodic manipulation to the dry period (Miller et al., 2000).

Impact of Insulin-Like Growth Factor Survival Factors on Lactation Persistency

Insulin-like growth factors appear to be galactopoietic and to serve as participants in the galactopoietic response to exogenous bST. When the production of IGF-I is uncoupled from somatotropin regulation, such as occurs during negative energy balance, then a milk production response to bST is abrogated (Gluckman et al., 1987; McGuire et al., 1992). Infusion of IGF-I into the local arterial supply to the mammary gland of goats rapidly increased milk synthesis (Prosser et al., 1990; Prosser and Davis, 1992), whereas infusion of bST was ineffective (McDowell et al., 1987), supporting a galactopoietic action of IGF-I. Furthermore, treatment of lactating cows with bST altered the distribution pattern of IGF-I within mammary tissue from a predominantly stromal localization to a prominent epithelial localization (Glimm et al., 1988), and altered the nature of IGF-I receptor transcripts in mammary tissue (Glimm et al., 1992), suggestive of IGF-I mediation of bST action in mammary tissue. The milk yield response to local mammary infusion of IGF-I is considerably less than that obtained with systemic administration of bST; however, this is consistent with known direct effects of bST on lipid metabolism of adipose tissue (Bauman, 1999) and does not necessarily weaken the somatomedin hypothesis. Although a degree of uncertainty may remain when ascribing IGF-I as a mediator of the lactational effects of bST (Tucker, 2000), the above data and previously discussed data regarding the galactopoietic effects of photoperiod (Dahl et al., 2000) support a galactopoietic role for IGF-I.

Several levels of complexity are involved in modulating IGF-regulated functions: the local concentration of IGF, expression of IGF receptors and their downstream

signaling pathways, and the types and quantities of IGF binding proteins (Peaker and Linzell, 1974; Cohick, 1998; Hadsell et al., 2002). To date, six high-affinity IGFBP (1 to 6) and nine low-affinity IGFBP, also known as IGFBP-related proteins, have been identified. Mammary epithelial cells synthesize a number of IGFBP. Depending on the specific IGFBP, the binding proteins may reduce IGF activity by competing with IGF-receptors for ligand, increase IGF-activity by serving as delivery vehicles to the target cell, or serve as a reservoir for IGF, causing their slow release and reducing IGF turnover. Furthermore, the IGFBP may have activities that are independent of their interaction with IGF, and they are subject to enzymatic modifications that may alter their various activities. The IGF system is highly complex with multiple levels of regulation, making the specific actions of IGF during mammary development and lactation difficult to resolve.

IGF-I is a mammary mitogen and survival factor. The ability of IGF-I to induce cell proliferation has been demonstrated in several in vitro and in vivo mammary model systems (Peaker and Linzell, 1974; Baumrucker and Erondu, 2000; Hadsell et al., 2002). Recently, administration of bST was shown to increase the percentage of mammary epithelial cells expressing Ki-67, a nuclear antigen marker for cell proliferation, by approximately threefold (Capuco et al., 2001a). It was proposed that this apparent proliferation response to bST was mediated by IGF-I. However, bST may directly influence mammary function without invoking mediation by IGF-I (Glimm et al., 1990; Hauser et al., 1990; Lincoln et al., 1995).

The concept that IGF-I regulates mammary gland apoptosis has evolved in recent years. In vitro studies with MCF-7 (human mammary adenocarcinoma cell line) cells demonstrated that IGF-I could serve as an inhibitor of mammary cell apoptosis (Geier et al., 1992). Subsequent studies in rats demonstrated that mammary apoptosis was correlated with enhanced expression of IGFBP-5, which serves as a negative regulator of IGF-I action by virtue of its ability to bind IGF-I and reduce its cell survival promoting activity (Tonner et al., 1995). Mammary-specific expression of IGF-I in transgenic mice demonstrated that IGF-I promoted cell survival and delayed mammary gland involution (Hadsell et al., 1996; Neuenschwander et al., 1996). Prolactin appeared to be a key factor in regulation of IGFBP-5 (Tonner et al., 1997), so that the outcome of prolactin insufficiency is increased mammary apoptosis. Recent investigations utilizing bovine mammary explants demonstrated the same relationship between level of apoptosis and expression of IGFBP-5 (Accorsi et al., 2002). Similar to the situation in rodents, apoptosis and IGFBP-5 expression were facilitated by the absence of the lactogenic hormones: prolactin, IGF-I and, in this case, somatotropin. Consistent with these findings, bST increases lactational persistency and maintains mammary cell number as lactation advances in ruminants by increasing cell renewal or survival (Knight et al., 1990; Capuco et al., 2001a).

Impact of Mastitis on Death of Mammary Epithelial Cells

A significant negative correlation exists between somatic cell count in the milk and milk yield (Raubertas and Shook, 1982). Infection is the primary reason for increased SCC. During experimentally induced *Staphylococcus aureus* mastitis, extensive tissue damage is evident in regions where neutrophils appear to traverse the epithelium (Harmon and Heald, 1982). The tissue damage and the decline in milk production are associated with the immunological defense mechanisms. Processes related to the bacterial infection as well as normal neutrophil function induce cell death and detrimentally affect milk secretion (Capuco et al., 1986; Long et al., 2001).

Two forms of cell death predominate: death by necrosis and by apoptosis. The relationship between mastitis and mammary cell apoptosis was evaluated in vivo after injection of *Escherichia coli* into mammary glands of lactating Holstein cows (Long et al., 2001). The apoptotic index was significantly increased in mastitic tissue compared to uninfected control. Infection elicited increases in expression of proapoptotic genes (Bax and interleukin-1β converting enzyme), whereas expression of the antiapoptotic gene (Bcl-2) was decreased. Induction of matrix metalloproteinase-9, stromelysin-1, and urokinase-type plasminogen activator were also increased, consistent with degradation of the extracellular matrix and cell loss during mastitis. The Ki-67 labeling index suggested that mastitis also increased cell proliferation, perhaps as a tissue repair mechanism after mastitis. This response may be consistent with an increase in local (milk) concentrations of IGF-I in the mammary glands of cows infected with *E. coli* (Shuster et al., 1995). The signaling events involved in mammary cell apoptosis and proliferations induced by *E. coli* infection are not fully understood. The ligands, receptors, and genes participating in transmitting the death and proliferating signals have not been clarified and require further investigation.

Infection with Gram-positive bacteria similarly appears to induce apoptosis. *Streptococcus agalactiae*-induced bovine mastitis increased the expression of an apoptosis marker, RTPM-2 (Sheffield, 1997), and *S. aureus*-induced apoptosis of MAC-T cells, a bovine mammary epithelial cell line (Bayles et al., 1998; Wesson et al., 1998).

A mammary explant model has been used to dissociate tissue damage resulting from neutrophil diapadesis from effects of neutrophil function and bacterial toxins (Capuco et al., 1986). Mammary tissue from lactating Holsteins was cultured in the presence of intact or lysed neutrophils or neutrophils that were allowed to phagocytose opsonized zymosan. Phagocytosing neutrophils inflicted the greatest damage on epithelial cells. Cyto-

logical damage observed included cell sloughing from the basement membrane, nuclear condensation, cell debris in luminal areas, and epithelial vacuolation. During routine immune surveillance, neutrophils migrate through the epithelial layer and into alveolar and ductal lumena, where they phagocytose fat globules, casein micelles, and bacteria when they are present. Thus, phagocytosis is the consequence of routine immune surveillance and has the potential to damage the mammary epithelium. Such effects may account for the negative correlation between SCC and milk yield, even in the absence of infection.

Using an in vitro co-culture of MAC-T cells and bovine neutrophils, the efficacy of antioxidants as protectants against neutrophil-induced epithelial damage was investigated (Boulanger et al., 2002). Data implicated hydroxyl radicals released by activated neutrophils as agents that could damage the secretory epithelium, and that antioxidants such as melatonin may be useful for protecting mammary tissue during mastitis.

Significance of a Dry Period and the Concept of Regenerative Involution

In contrast to other species, normal management of dairy cows and goats results in an overlap of lactation and pregnancy, such that these animals are typically pregnant when milking is terminated during late lactation. Thus, when milk stasis occurs, the mammogenic and lactogenic stimulation of pregnancy opposes stimuli for mammary involution. Processes of mammary growth and involution both occur during this nonlactating or "dry period" between successive lactations. Milk production efficiency can be increased by development and utilization of schemes, which increase persistency of lactation and which minimize the duration of the dry period.

In cows, a dry period of at least 40 d has been recommended to maximize milk production in the following lactation (Swanson, 1965; Coppock et al., 1974; Sorensen and Enevoldsen, 1991). This may be to permit restoration of body reserves prior to the next lactation or to permit necessary growth and differentiation events within the mammary gland during this period. Although the data are not definitive, they strongly suggest that a dry period is necessary for reasons that center on the mammary gland rather than on the nutritional status of the animal (Swanson, 1965; Smith et al., 1966; Smith et al., 1967). Interestingly, a dry period between successive lactations was also found to be necessary for optimal lactation in rats (Paape and Tucker, 1969).

Aspects of mammary growth during the dry period have been investigated (Capuco et al., 1997). Multiparous Holstein cows were dried off 60 d prior to expected parturition or were milked twice daily during this prepartum period. Cows were slaughtered at 7, 25, 40, and 53 d into the dry period (53, 35, 20, and 7 d prepartum), and total mammary DNA and thymidine incorporation into mammary tissue slices was determined. There was no significant loss of mammary cells (DNA) during the dry period, and total number of mammary cells increased with advancing stages of the dry period. Total DNA did not differ between mammary glands of dry and lactating cows; however, increased DNA synthesis in dry cows indicated that replacement of mammary cells increased during the dry period. Autoradiographic localization of incorporated ^{3}H-thymidine indicated that the replicating cells were primarily (>90%) epithelial. Thus, in cows, the dry period may be important for replacing senescent cells prior to the next lactation. Furthermore, although cows appeared to enter the next lactation with the same number of mammary cells regardless of whether they had a dry period, a greater percentage of those cells were epithelial in cows that had a dry period.

It is important to realize that events occurring during the dry period differ significantly from mammary involution as frequently studied in rodent models. In the absence of concomitant pregnancy mammary involution is rapid and extensive. In contrast, the mammogenic effects of pregnancy ameliorate the involution process and promote mammary cell turnover. When mice are pregnant at the time of weaning, mammary apoptosis is reduced and cell proliferation enhanced relative to their nonpregnant counterparts (Capuco et al., 2002). Consequently, we propose the term "regenerative involution" to more fully describe the processes of cell renewal and tissue remodeling that occur during involution with concomitant pregnancy.

We have hypothesized that the dry period may be critical for replacing progenitor cells that are responsible for expanding and maintaining the number of mammary secretory cells (Capuco and Akers, 1999). Indeed the mammary glands of rats that were not permitted a dry period had fewer cells at midlactation than glands of rats that were permitted a dry period of optimal length, although cell number did not differ at onset of the lactation (Paape and Tucker, 1969). If replacement of senescent cells is a critical event during the dry period, one would hypothesize that without a dry period of sufficient length to replace senescent cells, persistency of the ensuing lactation will be decreased.

In contrast to cows, a dry period may not be necessary for optimal milk production in dairy goats. Two investigations have addressed the importance of a dry period in goats. In the first (Knight and Wilde, 1988), lactating goats were induced into ovulation and mated out of season. The goats entered the next lactation without a dry period, and milk production was found to be 12% less than in the previous lactation. However, seasonal effects on lactation may have confounded the results. Subsequently, Fowler et al. (1991) investigated the necessity for a dry period using a within-animal, half-udder design. One gland was milked during the prepartum period, whereas the other was dried off 24 wk (170 d) prior to parturition. In this experiment, magnetic resonance imaging was used to monitor parenchymal

volume as an index of mammary growth and involution. There was no difference in milk production between glands. Indeed, at no stage of lactation was milk yield of glands that had experienced a dry period numerically greater than that of continuously milked glands, even though those glands were larger than continuously milked glands during the first few weeks after parturition. These data suggest that a dry period is not necessary for optimizing milk production in the next lactation in goats. Issues of half-udder design may confound interpretation (Capuco and Akers, 1999); however, similar studies in cattle demonstrate a beneficial effect of a dry period. Because significant mammary growth occurs during early lactation of goats, and milking induces the release of somatotropin in goats, but not cows, the mammary gland of goats may demonstrate a greater capacity to continue cell-renewal processes, such as those detected during a bovine dry period, into early stages of lactation, thus largely negating the importance of a dry period in goats.

Bachman and colleagues have evaluated the feasibility of accelerating mammary involution during the dry period by administration of estradiol-17β at dry off (Athie et al., 1996; 1997). By accelerating the early stages of involution, it was hoped that dry periods <40 d could be employed without a loss of milk production in the subsequent lactation. The most recent report indicated that in the absence of treatment, no milk deficit was incurred by utilizing a dry period of 34 vs. 59 d (Bachman et al., 1988), as well as the balance of other management and health costs. Management decisions regarding the length of a dry period are based on the balance of forfeited milk during the dry period and enhanced milk production in the ensuing lactation. The quantitative impact of dry period length on milk production should be reevaluated. The dairy cow population today differs significantly from that used in the classical investigations upon which current management decisions are based.

Identification of Putative Progenitor Cells

Evidence for the existence of mammary stem cells is available from a variety of sources. Numerous transplantation experiments have shown that isolated segments from any portion of the developing or even lactating gland are capable of regenerating a complete mammary ductal and alveolar network (DeOme et al., 1959; Hogg et al., 1983; Smith and Medina, 1988). Perhaps most convincingly, Kordon and Smith showed that an entire mammary gland could be regenerated with the progeny of a single cell following transplantation into cleared mammary fat pads (Kordon and Smith, 1998). Additional evidence for the existence of mammary stem cells may be derived from observations that entire mammary lobules are often comprised of cells showing identical X-inactivation patterns, and from cancer studies where mammary tumors comprised of a variety of cell types are frequently found to be of clonal origin (Tsai et al., 1996).

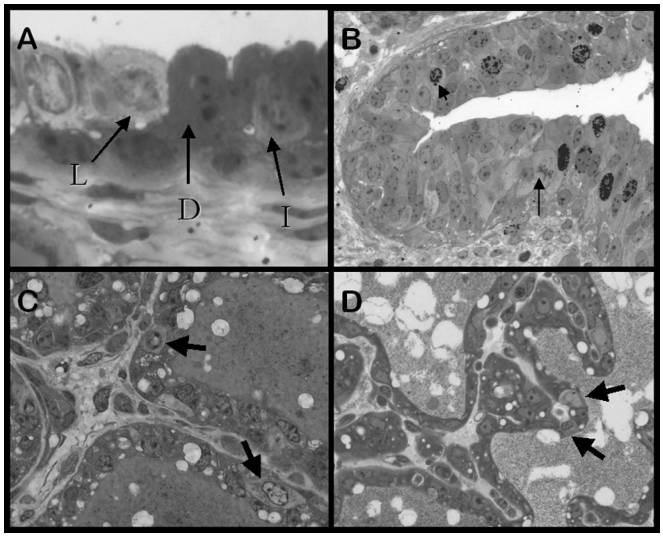

Figure 4. Lightly staining cells in the mammary epithelium. Panel A: light (L), intermediate (I), and dark (D) cells of a 2-mo-old calf. Panel B: Mammary epithelium of a 2-mo-old calf with numerous lightly staining epithelial cells. A mitotic cell is indicated (long arrow) and one of 10 cells labeled with bromodeoxyuridine is indicated (short arrow). Panel C: Mammary epithelium of a dry cow, approximately 1 wk prepartum, with lightly staining cells indicated by arrows. Panel D: Lactating mammary epithelium with lightly staining cell indicated by arrows.

No genetic marker has yet been found to identify mammary stem cells in situ. However, histological analyses have indicated that a pale staining cell population present during all stages of mammary development and differentiation in mice and rats may serve as mammary stem cells (Chepko and Smith, 1997). Such "pale cells" have been described in mammary tissue from all species so far examined, including humans (Ferguson, 1985), mice (Smith and Medina, 1988), rats (Chepko and Smith, 1997), goats (Li et al., 1999), and cattle (Ellis et al., 2000; Ellis and Capuco, 2002).

To test the hypothesis that lightly staining mammary epithelial cells represent a stem cell population, we evaluated the proliferative capacity of these cells in mammary glands of prepubertal heifers (Ellis and Capuco, 2002). Prepubertal heifers were used because of the active proliferative state of the mammary gland during this stage of mammary development (Hardville and Henderson, 1966). Heifers were injected with BrdU to label cells in S-phase of the cell cycle, and the labeling index of mammary epithelium, obtained at slaughter 2 h post injection, was evaluated relative to histological appearance of the cells. We observed light, dark, and intermediate staining cells in histological sections (Figure 4A, B). Light cells comprised 10% of the total parenchymal cell population, but accounted for the majority of epithelial cell proliferation. Light and intermediate

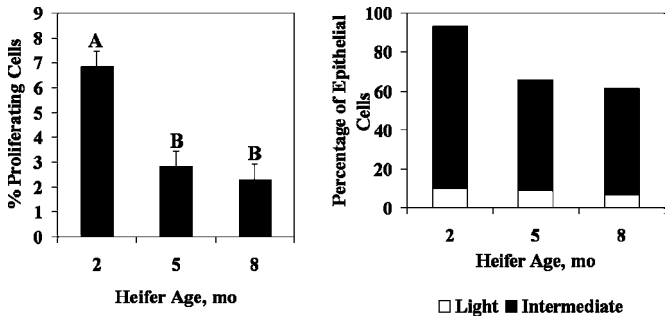

Figure 5. Proliferation of mammary epithelial cells of Holstein heifers (left panel) and percentage of light and intermediately staining epithelium. The percentage of proliferating cells represents those cells that were labeled with in vivo injection of bromodeoxyuridine plus those cells that were mitotic. The percentage of light and intermediate staining cells is depicted in the right panel. Bars without common superscripts differ ($P < 0.05$). Adapted from Ellis and Capuco (2002).

cells together accounted for more than 90% of the proliferating cells. The proportion of light cells was relatively constant across the stages of development that were evaluated (2, 5, and 8 mo). However, the proportion of light plus intermediate staining cells correlated with the tissue's proliferative rate (Figure 5). These data strongly support the hypothesis that lightly staining mammary epithelial cells function as the primary proliferative population and provide solid justification for further studies into the light staining cell phenotype. We hypothesize (Figure 6) that the lightly staining cells provide the basal population of mammary epithelial progenitor or stem cells and that the intermediately staining cells represent a daughter population that serves to amplify the progenitor population and may have a more restricted developmental potential. The dark cells represent more differentiated mammary epithelial cells.

In addition to prepubertal mammary gland, lightly staining epithelial cells are evident during lactation and the dry period (Figure 4). However, their identification is often difficult because of the presence of fat globules in epithelial cells that tend to obscure their cytoplasmic staining character. Future studies will employ a variety of approaches to identify differences in gene expression that are related to the mammary epithelial cell's staining characteristics. Development of markers for these cells would facilitate kinetic analyses of the relationship between the different staining phenotypes and permit identification of factors that regulate the proliferation of these cells. Such knowledge should provide a key for regulating mammary development and persistency of lactation.

Summary

During a bovine lactation, milk yield increases during early lactation as a consequence of increased activity per cell, whereas this increase is supplemented by mammary growth in goats and rodents. Following peak lactation, milk yield declines due to gradual regression of the epithelium by apoptotic cell death. During late

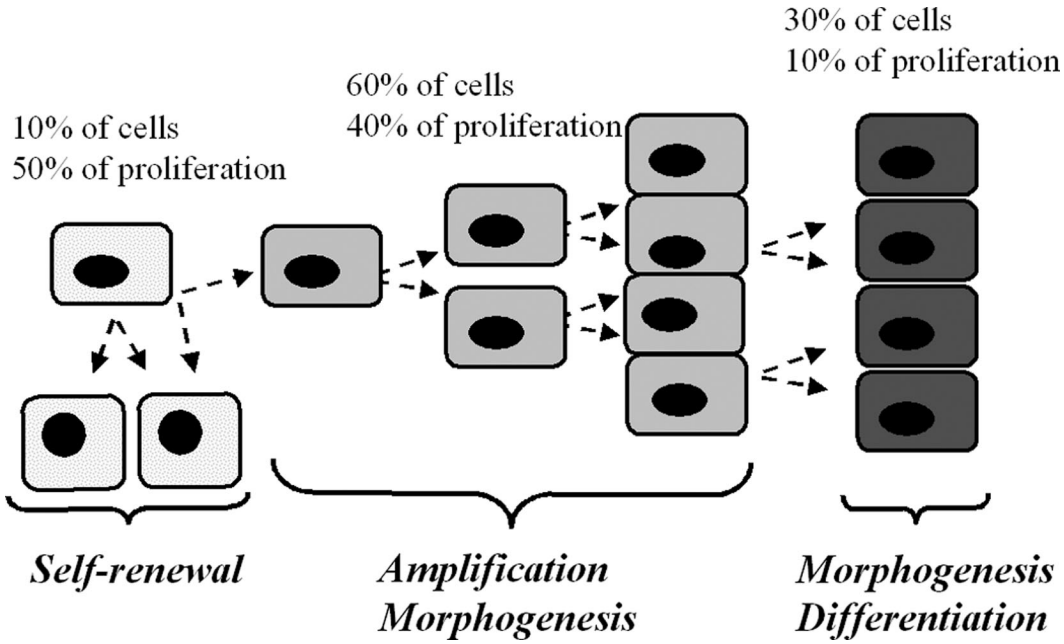

Figure 6. Proposed relationship between mammary epithelial cells displaying differing staining intensity. The light staining cells are hypothesized to represent mammary stem cells, intermediate staining cells are hypothesized to represent a secondary level of progenitor cells with more restricted lineage potential that serve to amplify the growth state of the tissue, and the dark cells are hypothesized to represent the most differentiated at the stage of development.

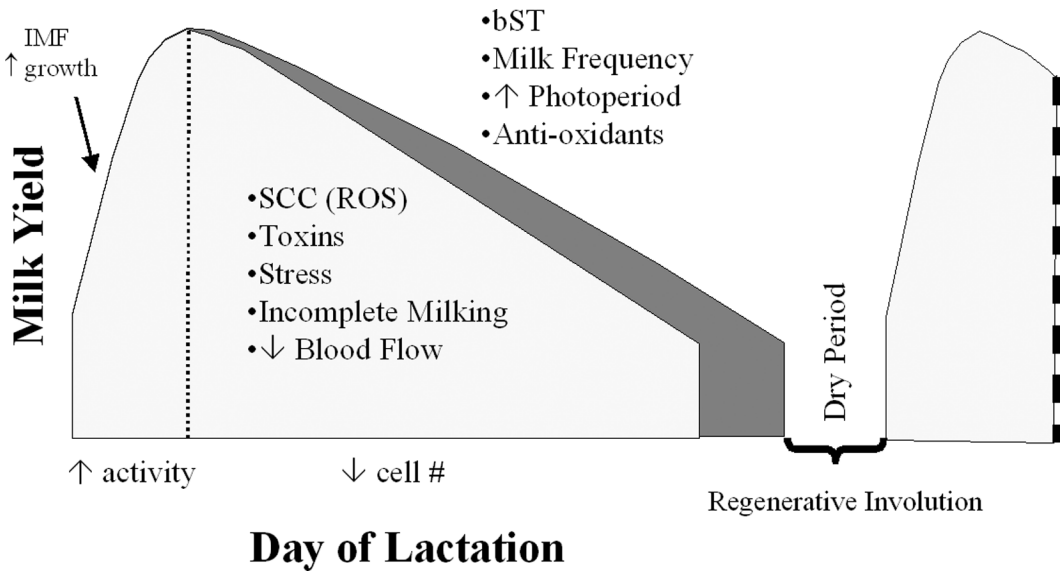

Figure 7. Representation of the relationship between milk yield, changes in the mammary epithelial cell population, and events that alter cell number. Events that hypothetically decrease persistency are tabulated within the lactation curve; events that positively enhance persistency are depicted above the lactation curves.

lactation, the decline in milk yield due to declining cell numbers is supplemented by decreased secretory activity by cell. During a bovine lactation, considerable cell renewal occurs during lactation, such that by the conclusion of lactation, most of the cells present in the mammary gland were formed during that lactation. Factors that may contribute to the loss of cells during lactation include mastitis, the normal immunological surveillance activity of neutrophils, and other stresses of lactation (Figure 7). With incomplete milking, regions of the mammary gland likely undergo reductions in blood flow and oxygen tension, which can initiate apoptosis. Also, with incomplete milking, mammary tight junctions are compromised and resulting changes in cytoskeletal organization lead to decreased milk synthesis and secretion (Stelwagen et al., 1997). If not remedied, these changes will ultimately induce apoptosis (Kulms et al., 2002).

Positive regulators of lactation include increased milking frequency and bovine somatotropin administration. Increased milking frequency during a brief period during early lactation causes a long-lived increase in milk yield, seemingly through the stimulation of cell proliferation. Administration of bovine somatotropin increases mammary cell proliferation, but because there is no net gain of cell number, it is assumed that this represents enhanced cell renewal. Photoperiodic manipulation can be used to increase milk production. During lactation, effects may be primarily on galactopoiesis and mammary regression. During the dry period, manipulation of photoperiod may influence mammary growth or differentiation. Effects of bovine somatotropin, milking frequency, and photoperiod are additive. The ability to elicit long-term milk production effects by abbreviated increased milking frequency during early lactation or photoperiodic manipulation during the dry period makes combining these treatments more flexible and feasible.

A reevaluation of the optimal dry period length is in order. During the dry period there is extensive cell renewal, which may reflect a critical need to replace senescent mammary epithelial cells. Because dairy cows are typically pregnant at the time of dry-off, there is extensive remodeling of the mammary gland during this preparturient, nonlactating period. A descriptive term for the nature of the opposing events of involution and mammogenesis is "regenerative involution." This contrasts with the often-studied involution in rodents that occurs after forced weaning, during which the mammary gland involutes to a state that resembles that of a virgin animal. Schemes to increase the rapidity of regenerative involution will permit shorter dry periods. However, the plasticity of the mammary gland may provide opportunities to utilize dry periods of short duration.

Implications

Increasing the mammary gland's ability to replace those cells that die during lactation is key to enhancing persistency. The proliferative population of mammary epithelial cells has been identified in histological sections as lightly staining cells. Epithelial cells with this staining characteristic are evident during all stages of mammary development and function. Development of markers for these cells and their further characterization may provide important keys to manipulating cell proliferation to enhance mammary development and lactational persistency. Because apoptosis is typically very low in the lactating gland, mechanisms to globally

reduce mammary apoptosis will be difficult to evaluate and perhaps of minimal value. However, treatments to minimize apoptosis during mastitis may be of particular importance. The complexity of pathways for regulating mammary cell proliferation and apoptosis provides a challenge to comprehensive understanding, but also provides opportunities for developing novel regulatory strategies to reduce the length of the dry period and increase lactational persistency. Manipulation of these processes can be achieved through transgenic approaches, manipulation of hormonal and paracrine factors, and selection for naturally occurring genetic polymorphisms.

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Production of transgenic livestock: Promise fulfilled[1]

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ABSTRACT: The introduction of specific genes into the genome of farm animals and its stable incorporation into the germ line has been a major technological advance in agriculture. Transgenic technology provides a method to rapidly introduce "new" genes into cattle, swine, sheep, and goats without crossbreeding. It is a more extreme methodology, but in essence, not really different from crossbreeding or genetic selection in its result. Methods to produce transgenic animals have been available for more than 20 yr, yet recently lines of transgenic livestock have been developed that have the potential to improve animal agriculture and benefit producers and/or consumers. There are a number of methods that can be used to produce transgenic animals. However, the primary method to date has been the microinjection of genes into the pronuclei of zygotes. This method is one of an array of rapidly developing transgenic methodologies. Another method that has enjoyed recent success is that of nuclear transfer or "cloning." The use of this technique to produce transgenic livestock will profoundly affect the use of transgenic technology in livestock production. Cell-based, nuclear transfer or cloning strategies have several distinct advantages for use in the production of transgenic livestock that cannot be attained using pronuclear injection of DNA. Practical applications of transgenesis in livestock production include enhanced prolificacy and reproductive performance, increased feed utilization and growth rate, improved carcass composition, improved milk production and/or composition, and increased disease resistance. One practical application of transgenics in swine production is to improve milk production and/or composition. To address the problem of low milk production, transgenic swine over-expressing the milk protein bovine α-lactalbumin were developed and characterized. The outcomes assessed were milk composition, milk yield, and piglet growth. Our results indicate that transgenic overexpression of milk proteins may provide a means to improve swine lactation performance.

Key Words: α-Lactalbumin, Mammary Glands, Milk, Transgenic Animals

 J. Anim. Sci. 2003. 81(Suppl. 3):32–37

Introduction

The ability to insert genes into livestock embryos, the incorporation of those genes and their stable transmission into the genome of the resultant offspring will enable major genetic advances to be realized in animal agriculture. Production of transgenic livestock provides a method to rapidly introduce "new" genes into cattle, swine, sheep, and goats without crossbreeding (Pursel and Rexroad, 1993). It is a more abrupt methodology, but in practicality, not really different from crossbreeding or genetic selection in its result. There are two basic strategies used when producing transgenic animals. These are the so-called "gain of function" or "loss of function" transgenics. The basic idea behind the gain of function paradigm is that by the addition of a cloned fragment of DNA to an animal's genome, one can accomplish several objectives. One objective is to obtain new expression of a gene product that did not previously exist in that cell or tissue type. This is the type of strategy that was used for the animals discussed in this paper.

Some of the other methods that have been used to produce transgenic animals include: 1) DNA transfer by retroviruses; 2) microinjection of genes into pronuclei of fertilized ova; 3) injection of embryonic stem (**ES**) cells and/or embryonic germ (**EG**) cells, previously exposed to foreign DNA, into the cavity of blastocysts; 4) sperm-mediated exogenous DNA transfer during in vitro fertilization; 5) liposome-mediated DNA transfer into cells

[1]The author would like to thank his colleagues who have contributed to the work described herein: M. S. Noble, G. T. Bleck, J. B. Cook, W. L. Hurley, D. Bidner, and S. Hughes. This material is based on work partially supported by the Illinois Council for Food and Agricultural Research (C-FAR) Project No. 97I-136-3, the C-FAR Sentinel Program, and the Cooperative State Research, Education and Extension Service, USDA National Research Initiative under Project No. NRI-960-3240.

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Received August 8, 2002.

Accepted April 10, 2003.

and embryos; 6) electroporation of DNA into sperm, ova or embryos; 7) biolistics; and 8) nuclear transfer with somatic or embryonic cells (see review by Wheeler and Walters, 2001).

Historical Overview of Transgenic Technology

The foundation for the production of transgenic animals was provided by the pioneering experiments of Brackett et al. (1971) using sperm-mediated gene transfer in rabbits. Although gene transfer was accomplished in that study, germ-line transmission of the transgene was not successfully reported until 1976 using retroviral gene transfer vectors in mice (Jaenisch, 1976). In 1980, Gordon et al. showed that pronuclear microinjection of DNA into one-cell mouse zygotes was a relatively efficient method to accomplish germ line gene transfer. Microinjection of cloned DNA into the pronucleus of a fertilized ovum has been and continues to be the most widely used and most successful method for producing transgenic mice and livestock (Hammer et al., 1985; Wall, 2002).

There have been a number of methods reported in the past 15 yr, which have successfully produced germ line transgenic animals from various species (see review by Wheeler and Walters, 2001). Since the famous cloned sheep "Dolly" was born (Wilmut et al., 1997), nuclear transfer technology has become another methodology available for the production of transgenic animals. These new methods for the production of genetically identical individuals from embryonic (Campbell et al., 1996; Wilmut et al., 1997) and somatic (Wilmut et al., 1997; Polejaeva et al., 2000) cells, via nuclear transfer, should allow the rapid development of genetically identical animals with a targeted gene insertion. These developments will enhance our ability to produce transgenic animals with genes inserted into specific sites in the genome. This method will most likely become the most useful method for future production of transgenic livestock. These nuclear transfer strategies have several distinct advantages for use in the production of transgenic livestock that cannot be attained using pronuclear injection of DNA.

Applications for Trangenics in Livestock Production

Practical applications of transgenics in livestock production include improved milk production and composition, increased growth rate, improved feed utilization, improved carcass composition, increased disease resistance, enhanced reproductive performance, increased prolificacy, and altered cell and tissue characteristics for biomedical research (Wheeler and Choi, 1997) and manufacturing. The production of transgenic swine with growth hormone serves as an example of the value of this technology (Brem et al., 1985, Hammer et al., 1985).

Transgenic alteration of milk composition has the potential to enhance the production of certain proteins and/or growth factors that are deficient in milk (Bremel et al., 1989). The improvement of the nutrient or therapeutic value of milk may have a profound impact on survival and growth of newborns in both humans and animals. The enhancement of the nutrient or therapeutic value of milk may have profound impact on survival and growth of newborn pigs.

In many production species such as cattle, sheep, and goats, the nutrients available to the young may not be limiting. However, milk production in sows limits piglet growth and therefore pig production (Hartmann et al., 1984). In swine, 44% of the growth rate of the developing piglets can be attributed to yield and composition of the sow milk (Lewis et al., 1978). Methods that increase the growth of piglets during suckling result in an increase in weaning weights, a decrease in the number of days required to reach market weight, and thus a decrease in the amount of feed needed for the animals to reach market weight.

The high percentage in growth rate attributed to milk indicates the potential usefulness of this technology to the developing piglet. An approach to increase milk production in pigs may be accomplished by alteration of milk components such as lactose, a major osmole of milk in mammary gland cells. The overexpression of lactose in the milk of pigs will increase the carbohydrate intake by the developing young, resulting in improvement of piglet growth.

The overall result of the transgenic modification of milk will be the creation of more uses of milk and milk products in both agriculture and medicine. Increasing the concentrations of existing proteins or producing entirely new proteins in milk is truly a "value-added" opportunity for animal agriculture.

Transgenic Animals with Specific Gene Expression in the Mammary Gland

Numerous laboratories have studied the expression of transgenes specifically in mammary tissue (Simons et al., 1987; Vilotte et al., 1989; Clark et al., 1989; Bleck and Bremel, 1994; Bleck et al., 1995, 1996; Bleck et al., 1998). The 5′ flanking regions of many milk protein genes, which have a regulatory function, have been used to drive expression of foreign proteins in mammary epithelial cells of transgenic animals (Simons et al., 1987; Vilotte et al., 1989). Regulatory regions of milk proteins have been linked to genes that have been expressed as transgenes in a variety of animals (pigs, sheep, and goats; Clark et al., 1989; Ebert et al., 1991; Wall et al., 1991). Levels and patterns of expression have been very similar to those observed in numerous transgenic mouse experiments. These regulatory regions have shown little or no species specificity and have even been regulated properly in species that do not express those proteins (Wall et al., 1991; Simons et al., 1987).

Something as complex as milk production is controlled in part by counterbalancing factors (homeostatic control), where altering expression of one gene may be counterbalanced by endogenous expression of another. This means that identifying single-gene transgenic approaches to enhancing production characteristics is a difficult challenge. Understanding the biological control of a production characteristic before making the tremendous investment in developing transgenic animals is of great importance.

Previous work has suggested that the volume of milk produced is directly dependent upon the amount of lactose synthesized. Lactose is synthesized in the Golgi apparatus of mammary secretory cells by the lactose synthase complex. This complex is composed of the mammary-specific protein α-lactalbumin and the enzyme β1,4 galactosyltransferase. The reaction catalyzed by lactose synthase is UDP-Galactose + Glucose $\rightarrow$ Galactose-Glucose (Lactose) + UDP.

Lactose is formed inside the secretory vesicles of the mammary Golgi (Brew and Grobler, 1992). These vesicles are budded off from the Golgi complex, transported to the apical membrane of the epithelial cell, and secreted into the lumen. Because lactose cannot diffuse out of the vesicles, it acts to draw water by osmosis into the vesicle. Since lactose synthase is necessary for the production of lactose and the movement of water into the mammary secretory vesicles and then into the lumen of the gland, it is critical in the control of milk secretion (Hayssen and Blackburn, 1985). There is evidence that suggests that milk volume is directly related to the expression of the α-lactalbumin gene. α-Lactalbumin is a normal constituent of milk, and its expression correlates with the induction of copious milk secretion at the onset of lactation (Goodman and Schanbacher, 1991).

Of all the bovine milk protein genes, the expression of bovine α-lactalbumin is the most lactation-specific and strictly controlled (Goodman and Schanbacher, 1991). The unique expression pattern of the bovine α-lactalbumin gene makes its promoter and regulatory elements an attractive choice for a mammary expression system in transgenic animals.

Improvement of the Lactation Performance in Sows

Large increases in average milk production of dairy cattle have been realized over the past several decades because of intense selection for milk yield, which is a trait that is easy to objectively measure. Similarly, milk production by sows has increased over the past three decades (King, 2000). However, the fact remains that milk production remains a significant limiting factor in determining piglet growth. With the emphasis on increasing litter size, high milk production is particularly important. Although research has provided more insight into the process of milk secretion, we have only a limited understanding of the physiological factors that control the amount of milk a mammal produces. Previous work has suggested that the volume of milk produced is directly dependent upon the amount of lactose synthesized. There is evidence that suggests that milk volume is directly related to the expression of the α-lactalbumin gene (Goodman and Schanbacher, 1991).

High milk production is vital for growth of the offspring. Low milk production is manifested not only by slow growth before weaning but also by slow growth later in life, since animal performance also suffers through the grower and finishing stages (Mahan and Lepine, 1991; Boyd and Kensinger, 1998; Miller et al., 1999). Current swine production management schemes attempt to maximize the number of piglets born per litter and piglet survival (Hartmann et al., 1984). In addition, pork producers have continuously reduced the duration of lactation to maximize the number of piglets born per sow per year. Currently, in the swine industry approximately 14-d lactation periods are common. In order to get maximum growth from larger litter sizes and shorter lactation, increased milk production in early lactation must be obtained. Early weaning has decreased neonatal mortality and increased litter sizes from selected high genetic merit sows but has also make milk production one of the most important limiting factors in piglet growth (Miller et al., 1999).

The effect of increased sow milk production on U.S. pork production is dramatic. Using current milk production values (Auldist et al., 1998), we estimate that increasing milk production by 10% would result in an additional \$2.46 per litter which would be worth \$28.4 million/year in the U.S. due to increased weight gains prior to weaning using a typical hog price of \$50/cwt. Modern sows are able to produce about 1 kg of milk per piglet per day for litter sizes up to 14 pigs (Auldist et al., 1998). This calculation does not consider the potential for decreased feed and labor costs, which would be associated with the higher postweaning weight gains and the shorter time to achieve market weight.

Production of Transgenic Swine Expressing Bovine α-Lactalbumin

We have previously produced two lines of transgenic pigs containing the bovine α-lactalbumin gene. This transgene has been inherited in a normal Mendelian fashion in F_1 crosses. These animals have been extensively characterized and reported elsewhere (Bleck et al., 1996, 1998; Noble et al., 2002).

The bovine α-lactalbumin gene was chosen for some very specific reasons. First, the expression of bovine α-lactalbumin is the most tightly regulated and lactation specific of all the bovine milk protein genes (Goodman and Schanbacher, 1991; Mao et al., 1991). The unique expression of the bovine α-lactalbumin gene makes its promoter and regulatory elements a useful mammary expression system in transgenic animals. In contrast to the caseins and β-lactoglobulin, the production of α-lactalbumin mRNA and protein shows a dramatic rise

at parturition, remains elevated during lactation, and drops sharply during lactational cessation and involution. Next, the bovine, murine, and porcine α-lactalbumin genes have all been sequenced and their proteins have molecular weights of about 14 kD (Brew and Grobler, 1992; Vilotte et al., 1992; Das Gupta et al., 1992). Thus the α-lactalbumin gene and gene product have been quite well characterized, which was an important factor in its selection for our studies. Physiologically, α-lactalbumin is produced at a concentration of approximately 0.2 to 1.8 mg/mL in the milk of most mammals, which makes it an excellent choice for a variety of species. Further, unlike β1,4 galactosyltransferase, α-lactalbumin is a mammary-specific protein whose expression is regulated by numerous hormones and growth factors (reviewed by Tucker, 1981; Forsyth, 1983; Kuhn, 1983; Vonderhaar, 1987; Ziska et al., 1988; Brew and Grobler, 1992). Among the proteins found in milk, it is unique in that its expression is tightly coupled to the onset of lactation after the gland is fully differentiated, suggesting that the regulation of expression of the α-lactalbumin gene is fundamentally distinct from that of other milk proteins (Goodman and Schanbacher, 1991). Most milk proteins (i.e., caseins, β-lactoglobulin) are found in the mammary glands of pregnant animals as soon as secretory cells begin to differentiate in early to mid-pregnancy.

Because α-lactalbumin is tightly correlated to milk production, it may be limiting for lactose synthesis. Several studies have shown that reducing α-lactalbumin reduces milk production. Rats fed low-protein diets produced less milk and lactose, but β1,4 galactosyltransferase content of the glands remained constant (Grimble and Mansaray, 1987). The lactose synthase activity of the glands isolated from protein-limited rats was half that found in control rats. The addition of bovine α-lactalbumin to the gland homogenates of the protein-limited animals stimulated lactose synthase activity by 60%, compared with 10% in control animals (Grimble and Mansaray, 1987). These data indicate that α-lactalbumin can be a limiting component in the lactose synthase complex and may be involved in control of milk production. Further, we selected a form of bovine α-lactalbumin gene containing a specific polymorphism. This polymorphism is a single base variation located 15 basepairs 3′ of the bovine α-lactalbumin transcription start point (Bleck and Bremel, 1993). This is referred to as the +15 polymorphism. This allele has previously been associated with increased milk production in Holstein cattle (Bleck and Bremel, 1993) and shown to increase milk volume in transgenic mice (Bleck and Bremel, 1994).

The integrated information above, point to α-lactalbumin as being an ideal candidate for overexpression in the mammary gland to study milk production and composition. Additionally, to address the question of whether α-lactalbumin is limiting for milk production most thoroughly, an in vivo approach must be used. The production of α-lactalbumin-deficient mice has allowed the lactose synthase complex to be analyzed under conditions when α-lactalbumin is greatly reduced or totally removed from the mammary gland (Stinnakre et al., 1994). As expected, mice with one remaining copy of the gene produce milk with lower lactose levels and mice without the gene produce no lactose. The milk from these mice is also higher in total solids, caused by a reduction in water content due to the lack of lactose, the osmoregulator. However, all knockout experiments only show the effect of removing the protein and yield no information about whether the protein is actually rate-limiting in the normal state. This can only be tested directly by increasing expression of specific genes. One group has replaced mouse α-lactalbumin with human α-lactalbumin, which is expressed at higher levels (Stacey et al., 1995). They show results suggesting there may be an increase in milk production in mice producing human α-lactalbumin. However, those studies used only four animals and were preliminary. To obtain a definitive answer in swine, α-lactalbumin must be overexpressed in the mammary gland and more careful studies of the phenotype must be performed. Even though a number of α-lactalbumin expressing transgenic animals have been produced, our studies are presently the only experiments examining lactose and milk production in transgenic swine overexpressing α-lactalbumin.

Results

Our results have shown that the bovine α-lactalbumin protein was expressed in the milk of our transgenic but not our control full-sibling gilts (Bleck et al., 1998; and Noble et al., 2002). Further, we have shown that milk composition is altered in the transgenics as compared to control gilts (Noble et al., 2002). Mean lactose concentrations in milk from transgenic sows was greater ($P < 0.01$) than that for controls sows over the entire lactation period (Noble et al., 2002). There was no difference in mean protein concentrations in transgenic and control sows over the lactation period. However, the mean total solids concentrations were lower ($P < 0.002$) in transgenic sows, up to 12 h postpartum but not thereafter, than control sows. This work (Noble et al., 2002) has also shown that milk production increased ($P < 0.001$) an average of 0.98 kg/day during d 3 to 9 of lactation in transgenic sows compared with control sows and that piglet growth rate significantly increased ($P < 0.05$) in the transgenic gilts compared with the control sows (192 ± 4 g/d and 168 ± 4 g/d, respectively). Please refer to Bleck et al., 1998 and Noble et al., 2002 for the specific details of these studies.

One of the curious results from the Noble et al. (2002) study is the apparent discontinuity of the enhanced milk production by transgenic sows and the increased growth rate of piglets. In this study, we saw a continued increase in growth in the absence of increased milk production. Although we are continuing to study this, the most plausible explanation is that there are some

changes that occur early in lactation in the piglets that allow this continued growth enhancement. The other explanation is that there continues to be additional bovine α-lactalbumin protein in the milk of these gilts and that provides additional amino acids for piglet growth. Similarly, the milk composition, data presented in Noble et al. (2002) showed significant differences in milk composition, which may enhance piglet growth without increased milk yield in later lactation. For example, this may be an effect of greater consumption of colostrum and transition by the neonate piglet causing enhanced protein deposition later on. There is growing evidence that the nutritional environment of the newborn metabolically imprints the animal for future growth and development (Burrin et al., 1994; Burrin et al., 1997; Lucas, 1998; Waterland and Garza 1999).

Another open question in the α-lactalbumin study is whether α-lactalbumin is indeed limiting. The results of this transgenic work lend themselves to addressing that unresolved issue. We are continuing to work on this question; however, a substantial amount of work still needs to be done to address these many interesting questions.

Summary

Bovine α-lactalbumin transgenic swine have been produced with the purpose of studying the role of this protein on milk production and lactation. We have observed enhanced lactation performance and consequently enhanced litter growth performance (Noble et al., 2002). Our results have demonstrated that the bovine α-lactalbumin gene can be expressed in the pig and the protein can be secreted into milk. To date, the animals containing the bovine α-lactalbumin transgene have shown no obvious abnormal phenotype. Both the transgenic and control animals grew at the same rate, reached puberty at similar ages, gestated and farrowed normally, lactated normally, and their litters grew at rates consistent with or faster than controls.

Efficient and optimal pork production is reliant upon the production of healthy, fast growing piglets. Milk production in the first few weeks after birth is critical to modern swine production (Boyd and Kensinger, 1998). Our recent studies have demonstrated that overexpression of α-lactalbumin in first parity gilts during lactation enhances lactational performance and enhances preweaning piglet growth rates (Noble et al., 2002). This is an important example of the application of transgenic technology to a livestock species with the expected outcome of subsequent enhancement of growth characteristics of the young. The advent of transgenic animal technology nearly 20 yr ago carried with it promise for biomedical research, pharmaceutical production in animal systems, and enhancement of food animal production. This promise has been realized in many areas, with the exception of production of transgenic livestock with rigorously demonstrated superior production characteristics. We demonstrate for the first time based upon extensive production data, that transgenic technology can be used to enhance production characteristics of a farm species (Noble et al., 2002).

Implications

Transgenic technology is a powerful tool for improving the production characteristics of livestock. One important application is enhancement of the growth of offspring. The use of the bovine α-lactalbumin gene promoter and regulatory regions has great potential for studying the basic biology of milk secretion as well as for many additional applications in agriculture and biomedicine.

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Mammary expression of new genes to combat mastitis

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ABSTRACT: Continual advances in the ability to produce transgenic animals make it likely that such animals will become important components of animal agriculture. The full benefit of the technology, and justification of its initial cost outlay, will be dependent on the establishment within these animals of new traits not easily achievable by other means. Potential applications include enhanced nutrient digestibility with reduced fecal losses, significantly altered milk composition with superior nutritional properties, and enhanced disease resistance. Our goal is to enhance mastitis resistance of dairy cows by enabling the cells of the mammary gland to secrete additional antibacterial proteins. Proof of concept has been obtained through experimentation with a transgenic mouse model. Three lines of mice were developed that produce varying levels of lysostaphin in their milk. This protein has potent antistaphylococcal activity and its secretion into milk confers substantial resistance to infection caused by intramammary challenge with *Staphylococcus aureus*, a major mastitis pathogen. Additional antibacterial proteins are being sought that will complement lysostaphin. A potential benefit of transgenic application of antibacterial proteins is the concomitant sparing in the agricultural use of antibiotics currently used as human therapeutics. Antibacterial proteins, such as lysostaphin, are not typically used as injectable or oral therapeutics because of immune-mediated or digestive destruction of their activity. In contrast, the immune system of transgenic animals will not consider the transgenic protein as being foreign. In addition we are exploring the potential of involution or mastitis responsive promoter elements for use in subsequent transgenic experiments designed to restrict lysostaphin production to these important time points. It is anticipated that genomics will play a role in unveiling candidate genes whose promoter elements will enable desired temporal expression patterns. The transgenic approach to insertion of new genetic material into agriculturally important animals is feasible but requires extensive prior evaluation of the transgene and transgene product in model systems.

Key Words: Mastitis, *Staphylococcus aureus*, Transgenic, Mice

 J. Anim. Sci. 2003. 81(Suppl. 3):38–47

Introduction

The ability to produce transgenic dairy cows opens the door to countless new strategies aimed at enhancing the efficiency of dairy production. Goals of projects include increasing milk production efficiency, enhancing feedstuff nutrient availability to the cow with reduced fecal losses, increasing milk protein content, and improving animal health through enhanced disease resistance. Advances in the ability to generate dairy cows containing new genes are continuing, and are the basis for continued optimism of this strategy. However, in addition to the substantial cost associated with the generation of founder animals, the cost of the technology in terms of time to implementation will remain enormous (Wall et al., 1997). A 5 to 10-yr time frame between initial embryo manipulations to generation of transgenic herds of lactating cows is likely. This time does not include the effort involved in the design, assembly, and testing of appropriate gene constructs in model systems that must be performed to ensure success of the technology. Strategies for introgression of trangenes into a dairy cattle breeding population have previously been reviewed and appear economically viable for traits with major effects on net merit (Cundiff et al., 1993). This review will focus on the use of transgenic technology to enhance mastitis resistance. This is currently the most economically important disease of dairy cattle. Effects of mastitis go beyond treatment and prevention costs to include issues of animal welfare, and the impact of agriculture use of antibiotics on the development of antibiotic resistant human pathogens (Smith et al., 2002). In addition, as mastitis is clearly associated with causing apoptosis of mammary epithelial cells there is likely a substantial impact of this disease in preventing the realization of an animal's full genetic potential to produce milk.

Received August 8, 2002.
Accepted December 18, 2002.
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Mastitis

Mastitis is an inflammatory reaction of the mammary gland, usually to a microbial infection, and its prevalence is alarming. Wilson et al. (1997) recently published the results of a retrospective study of milk samples collected from more than 100,000 cows in New York and northern Pennsylvania between 1991 and 1995. They found that IMI were present in 36% of cows enrolled in the Dairy Herd Improvement Association. This disease, in addition to causing distress for the cow, is estimated to cost the producer approximately $200/cow per year, which corresponds to a US total of $1.7 billion. The dairy processor also incurs losses from the detrimental changes in milk composition that occur during mammary inflammation. These changes are associated with reduced cheese yields and reductions in the shelf-life of dairy products (Barbano et al., 1991; Auldist et al., 1995; Auldist et al., 1996; Klei et al., 1998).

Mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils (**PMN**), into the mammary gland and by an increase in milk protease content (Verdi et al., 1987). Clinical infections are diagnosed by red, swollen appearance of the gland and flakes or clots (protein aggregates) in the milk. Subclinical infections, by definition, show no obvious signs of disease. Five bacterial species—*Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Escherichia coli*—are responsible for the bulk of bovine mastitis cases. *Staphylococcus aureus*, *S. dysgalactiae*, and *S. agalactiae* exhibit a contagious route of transmission, whereas *S. uberis* and *E. coli* are considered to be environmental agents. The incidence of contagious mastitis has greatly declined over the last 30 yr with implementation of a five-point control plan (Bramley and Dodd, 1984). The plan recommends use of correctly maintained milking equipment, postmilking teat disinfection, both therapeutic and prophylactic use of antibiotics, and culling of persistently infected animals. With this plan, the once-common mastitis pathogens—*S. agalactiae* and *S. dysgalactiae*—have been eliminated from many herds. However, *S. aureus*, which accounts for 15 to 30% of infections, has proven more difficult to control (Sutra and Poutrel, 1994). The cure rate for treatment of *S. aureus* infections with antibiotics is often less than 15%. This is attributed to incomplete penetration of the antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of disease once treatment has ended (Craven and Anderson, 1984; Yancey et al., 1991). Because *S. aureus* mastitis can be induced experimentally with as few as 100 organisms, a few chronic infections within a herd can maintain a persistent bacterial reservoir. Antibiotic treatment of mastitis caused by environmental pathogens is also practiced, but recurrence of infection from environmental reservoirs is a continuing problem.

Susceptibility of the mammary gland to new IMI is markedly increased during early involution and during the periparturient period (Nickerson 1989; Oliver and Sordillo, 1988). These infections are often associated with clinical mastitis during early lactation and can have a marked detrimental effect on subsequent milk yield and quality. Susceptibility to mastitis can also be quite high during the prepartum period of first-lactation heifers (Nickerson et al., 1995). These infections are associated with a decrease in alveolar epithelial and luminal area and an increase in connective tissue, which could potentially lead to a life-long reduction in milk yield.

Current therapies for mastitis rely heavily on the use of β-lactam antibiotics such as penicillins and cephalosporins. The parent compounds of these orally active agents are naturally produced by *Penicillium* spp. or *Cephalosporium* spp., respectively. They are bicyclic ring structures that contain a 4-membered-lactam ring fused to a five-membered thiazolidine ring (penicillin) or a six-membered dihydrothiazine ring (cephalosporin). A number of enzymatic steps are required for the synthesis of the β-lactam ring structure and although this has been accomplished chemically, these antibiotics are currently produced more economically through biosynthesis or by semisynthesis starting from fermentation intermediates (Andersson et al., 2001). These agents have had an enormously beneficial impact on dairy-animal health and milk production. However, concern that accidental exposure of susceptible consumers may produce drug-induced anaphylaxis has necessitated the imposition of a posttreatment milk discard period and strict industry surveillance of all milk shipments. Further, there is growing concern that the agricultural use of antibiotics contributes to the emergence of antibiotic resistance in human pathogens (Smith et al., 2002).

A transgenic approach to enhance mastitis resistance would enable mammary epithelial cells to produce antibacterial enzymes that, in contrast to β-lactam antibiotics, would be degraded along with other milk proteins during the digestion process and not pose a health risk to the consumer. Furthermore, as antibacterial proteins are not orally active, and are likely to be immunogenic if given by an intravenous route, they are not being widely used in human or veterinary medicine and thus pose a reduced threat for the development of organisms resistant to currently used therapeutics. There are currently a large number of bacterially derived enzymes and products approved by the FDA for use in food production (US FDA, 2001). The dairy products industry makes extensive use of these enzymes for yogurt and cheese manufacture and agents such as nisin and natamycin are used extensively in food manufacture for antibacterial and antifungal purposes, respectively.

Transgenic Approach to Enhance Mastitis Resistance

The use of transgenesis to direct expression of a foreign protein into mouse milk was first reported in 1987

(Gordon et al., 1987). Shortly thereafter, it was proposed that mammary production of trout lysozyme II (Grinde 1989) or bacterial lysostaphin (Bramley and Foster, 1990) would be an effective means to enhance mastitis resistance. Both of these proteins have considerable antistaphylococcal activity. However, the initial applications of the technology were the generation of transgenic mice producing human lysozyme (Maga et al., 1994) or human lactoferrin (Platenburg et al., 1994) in milk.

Milk from various lines of the lysozyme-transgenic mice contained approximately 0.5 mg/ml of human lysozyme (Maga et al., 1995). Bioactivity of the lysozyme, as detected by its ability to degrade *Micrococcus lysodeikticus* cell walls, was retained. Limited, but significant bacteriostatic activity of the milk against *S. aureus*, but not a mastitis-causing clinical isolate of *E. coli*, was also found (Maga et al., 1998). Further, the lysozyme-containing milk also slowed the growth of the cold spoilage organisms *Pseudomonas fragi* and *Lactobacillus viscous*, indicating the potential of this strategy to also address issues of product shelf life and food safety. Interestingly, the lysozyme also altered the physical and functional properties of the milk. Rennet clotting time was reduced and rennet-induced casein gel strength was increased. Clearly, the potential exists that the production of foreign proteins in milk of dairy cows could positively or negatively affect its antibacterial or functional characteristics. Unfortunately, human lysozyme has very limited potency against *S. aureus*. We find no indication of activity when up to 1 mg/ml aliquots (15 μl) of human lysozyme in PBS are spotted onto bacterial plates that have just previously been streaked with *S. aureus*. After an overnight incubation, a fully confluent lawn of bacteria develops. In contrast, similarly applied lysostaphin at a concentration of 1 μg/ml results in a completely clear zone. Both lysostaphin and human lysozyme are ineffective against *E. coli* and *S. uberis* isolates obtained from mastitis milk. Lysozyme from hen egg white also has very limited antistaphylococcal activity but has been shown to substantially enhance the activity of lysostaphin (Cisani et al., 1982).

Transgenic technology has resulted in the production of mice and cows that secrete human lactoferrin into their milk (Platenburg et al., 1994; van Berkel et al., 2002). Lactoferrin is an iron-binding glycoprotein found in most exocrine secretions including tears, saliva, and milk, and there are numerous reports of its antibacterial activity in vitro and in vivo (Nuijens et al., 1996; Nibbering et al., 2001). Although bovine milk normally contains low levels of lactoferrin, the transgenic cows were generated as potential bioreactors to produce large quantities of recombinant hLF (**rhLF**) for applications in human health care. The human form of the protein was chosen to potentially limit immunogenic reactions in humans treated via an intravenous route with human lactoferrin purified from bovine milk. The transgenic cows, which produce approximately 1 g of rhLF per liter of milk, have now supplied sufficient quantities of rhLF for thorough evaluation (van Berkel et al., 2002). The rhLF from cows was essentially identical to natural hLF except for a different glycosylation pattern. Importantly, both natural hLF and bovine-derived rhLF were equally effective in three different mouse infection models. The models involved infection of mice with *K. pneumoniae* or a multidrug-resistant stain of *S. aureus* followed by intravenous administration of 50 μg of rhLF or natural hLF. In all cases, a substantial reduction in recovery of viable organisms was observed as compared to control animals. In one model, a substantial dose of *S. aureus* (10^6 cfu) was injected i.m. into the hip muscle with rhLF (50 μg) given i.v. 24 h later. Up to 99% reduction in recovery of *S. aureus* from the injected muscle was found 24 to 48 h following rhLF injection. The cows that produce the rhLF are reported to have normal milk production, composition, and SCC. The ability of these animals to resist mastitis has not been reported. Presumably, the pharmaceutical company backing the production of these animals has not wanted to jeopardize their lactational capabilities with a deliberate challenge of mastitis causing pathogens. With three fertile transgenic bulls now available it will likely not be long before mastitis challenge studies are conducted on their daughters.

The potency of bovine lactoferrin (**bLF**) against mastitis causing organisms is not very great, and is clearly not sufficient to prevent mastitis. Purified bLF has very limited antistaphylococal activity even at concentrations of 1 mg/ml, although there is indication that it enables the activity of β-lactam antibiotics, such as penicillin G (Diarra et al., 2002). The mechanism of lactoferrin activity has not been clearly defined but appears to stem primarily through iron sequestration or through direct interaction of its cationic *N*-terminal region with bacterial components. The contribution of bLF to protection of the mammary gland is compromised by its low concentration in milk and the presence of other milk constituents. Thus, milk citrate can effectively compete with lactoferrin for iron binding and the resulting iron-citrate complex can be utilized by bacteria (Schanbacher et al., 1993). Action of the cationic *N*-terminal region of lactoferrin in milk may be inhibited in much the same manner as we observe inhibition of a potent, cationic antimicrobial peptide, tachyplesin, when diluted in milk (unpublished observation). The inhibitory effect may be related to the abundance of anionic, phosphorylated casein molecules binding the cationic peptide, or insertion of the peptide into milk fat globule membranes. Interestingly, bovine lactoferrin, but not hLF has been shown to inhibit the growth of bovine mammary epithelial cells in vitro (Hurley et al., 1994). Thus, overexpression of bovine lactoferrin does not seem to be a candidate for enhancing mastitis resistance, although its gene regulatory region may be suitable to direct expression of new antibacterial proteins (see below).

In addition to the antibacterial proteins previously mentioned, there is a class of compounds—the defensins—that are produced in neutrophils, macrophages, and epithelial cells lining mucosal surfaces [for review see (Kaiser and Diamond, 2000)]. Defensins are relatively small, cationic peptides with an amphiphilic charge distribution that enables them to interact with, and disrupt, bacterial cell membranes. Their antibacterial action appears to result from their ability to form pores in target membranes leading to cell lysis. Analogous antibacterial peptides have been isolated from a diverse array of multicellular organisms including insects, amphibians, and plants and are currently being evaluated as topical antibiotics for human medicine (Zasloff 2002).

Transgenic mice have been generated that produce small quantities (5 μg/ml) of a mammalian defensin—bovine tracheal antimicrobial peptide (**bTAP**)—in their milk (Yarus et al., 1996). The bTAP purified from milk was bioactive in vitro, but no challenge studies were reported. Transgenic mice that secrete lysostaphin into their milk provide a clear demonstration of the transgenic approach to enhancing mastitis resistance (Kerr et al., 2001). Lysostaphin is a potent peptidoglycan hydrolase naturally secreted by *Staphylococus simulans*. The lysostaphin gene is contained on a large plasmid and encodes a preproenzyme of 493 amino acids that is processed extracellularly to a 246 amino acid mature form (Recsei et al., 1987). The activity of the enzyme is specific to hydrolysis of the polyglycine interpeptide bridges of the staphylococcal cell wall (Schindler and Schuhardt, 1964). This specificity restricts its antibacterial activity to staphylococcal species, having little effect on other mastitis-causing organisms. However, the enzyme's specificity for pentaglycine peptides also makes it an ideal candidate for use as an antibacterial in milk as it does not appear to degrade milk proteins even after 4 d at 37°C (Kerr, unpublished). Furthermore, we have found that bacterial lysostaphin, at concentrations up to 100 μg/ml, has no effect on yogurt production using bovine milk and a commercial, freeze-dried starter culture.

The potential of the lysostaphin protein for the therapeutic or prophylactic control of staphylococcal mastitis was demonstrated initially in a mouse model (Bramley and Foster, 1990) and subsequently in dairy cattle (Oldham and Daley, 1991). The application of lysostaphin, a prokaryotic protein, to a transgenic animal program first required that it be successfully produced and secreted by eukaryotic cells (Kerr et al., 2001). Transfection studies were conducted with the lysostaphin gene and the COS-7 monkey kidney fibroblast-like cell line (ATCC #CRL1651). These studies revealed that the cells could produce lysostaphin, but that it was secreted in an inactive, glycosylated form. Two potential N-linked glycosylation sites (Asn-Xxx-Ser/Thr) exist within the native lysostaphin enzyme and apparently these had become targets of the eukaryotic glycosylation machinery. We modified these sites by a PCR technique that generated asparagine to glutamine codon substitutions within the glycosylation motifs. The resulting $Gln^{125,232}$-lysostaphin variant produced by the COS-7 cells retained approximately 20% of the activity of the native form. Given that lysostaphin exhibits substantial bioactivity at concentrations of less than 1 μg/ml, we decided to pursue the transgenic mouse experiment knowing that production of transgenic proteins in milk at 0.1 to 1.0 mg/ml can be typically obtained.

Three lines of mice were produced that secrete the bioactive variant of lysostaphin into milk (Kerr et al., 2001). Production is under the control of the 5′-regulatory region of the ovine β-lactoglobulin (**BLG**) gene (Whitelaw et al., 1991). The concentration of lysostaphin in the transgenic mouse milk was approximately 100 μg/ml in two of the lines and 1 mg/ml in the third line. Mice from the transgenic lines, and nontransgenic controls, were challenged on d 10 of lactation with an intramammary infusion of a strain of *S. aureus* (M60) that had been isolated from a case of bovine mastitis. Two glands per mouse were infused with a substantial dose of bacteria (10^4 cfu/50 μl). Pups were then removed and dams returned to their cages. Twenty-four hours later the mice were euthanized, and infection status of the glands was determined by visible inspection and enumeration of viable *S. aureus* in mammary homogenates. Approximately 80% of the challenged, nontransgenic glands became heavily infected (>10^8 cfu/gland) and showed visible signs of hemorrhagic inflammation. The remaining, challenged, nontransgenic glands were infected but to a lesser extent. In marked contrast, none of the glands from the transgenic mice were visibly infected. No bacteria survived in mammary glands from the highest expressing line and approximately 50% of glands from the other two lines were also free of infection. The infected transgenic glands in these lines contained less than 10% of the bacterial load observed in the heavily infected controls, and it is possible that these infections would have cleared given additional time. Clearly these transgenic mice will be protected from *S. aureus* mastitis when the oBLG regulatory region is active.

Another transgenic approach to enhance disease resistance involves the production of pathogen-specific antibodies into milk (Castilla et al., 1998; Sola et al., 1998; Kolb et al., 2001). To date, the primary goal of these projects has been to supply antibodies to offspring and thus provide enhanced passive immunity. However, with these projects as proof of concept it is not inconceivable that antibodies to mastitis causing pathogens could enhance protection of the mammary gland. Two of the reports indicate the potential of producing coronavirus neutralizing antibodies into milk under the control of the 5′-regulatory regions of the murine whey acidic protein (WAP) gene (Castilla et al., 1998) or the oBLG gene (Sola et al., 1998). In both instances transgenic mice secreted functional antibody into milk as determined by an in vitro neutralization assay. The production of functional antibodies was made possible

by the coinjection of two constructs encoding light and heavy chain variable regions, respectively, cloned from a specific monoclonal antibody producing hybridoma cell line. The beauty of the technique is that a specific high titer antibody is first produced and characterized by hybridoma technology, and then the appropriate gene is cloned for the production of transgenic animals. A recent report describes the production of an encephalitis-neutralizing antibody using similar technology (Kolb et al., 2001). In this case the antibody was transferred to offspring via the milk and conferred full protection to an experimental challenge with an otherwise lethal dose of the virus. Application of this technology to enhance mastitis resistance awaits generation of the appropriate hybridoma cell line.

One other reported transgenic model that may find application in mastitis resistance is the over-expression of the polymeric immunoglobulin receptor (**pIgR**) gene in mammary epithelial cells (de Groot et al., 2000). This receptor transports immunoglobulin A (IgA) across epithelial layers and into the secretions of various mucosal tissues including the mammary gland. The receptor binds circulating IgA at the basolateral side of the cell then transports it via transcytosis secretory component into the secretion. Secretory IgA is not naturally a major component of bovine milk perhaps due to a deficit of the pIgR. In transgenic mice, expression of 60- up to 270-fold above normal levels of pIgR resulted in only a modest 1.5- to 2-fold higher levels of total IgA in milk. The discrepancy suggests a shortage of circulating IgA available for transport. An interesting finding from this experiment was that milk from the highest pIgR expressing line of mice was significantly altered (de Groot et al., 2001). This milk contained little if any κ-casein and contained substantial quantities of a non milk protein identified as serum amyloid A-1. These mice were unable to support the growth of suckling offspring. The mechanism of the transgene effect on the endogenous proteins is unknown. It could result from over expression of the transmembrane receptor protein or be a function of the integration site within the genome. The lack of effect on other milk proteins suggest that it is in some fashion specific to κ-casein rather than a general effect on transcriptional ability of the mammary gland. Clearly milk from transgenic animals will have to be extensively characterized prior to its approval for human consumption.

Candidate Antibacterial Proteins for Mastitis Resistance

Our goal is to enhance mastitis resistance of dairy cows by enabling the cells of the mammary gland to secrete additional antibacterial proteins. The proteins that we are focusing on are enzymes—such as lysostaphin—that are able to cleave the peptidoglycan of the bacterial cell wall. The peptidoglycan is the structural framework of the cell wall, and its degradation exposes the bacteria to mechanical damage, and osmotic lysis. A complete description of peptidoglycan hydrolases is beyond the scope of this review. However, two classes of these enzymes that may be particularly relevant are the bacteriophage lysins required to complete the phage lytic cycle and the bacterial autolysins involved in bacterial cell division. As seen with lysostaphin, the transgenic animal strategy opens the door to utilization of these nonmammalian enzymes with the possibility that a combination of enzymes may be effective against all species of mastitis pathogens, and that the combination approach against any particular species may limit the development of resistant bacteria.

The potential for development of resistance to a peptidoglycan hydrolase does exist. However, it is difficult to predict how this will affect the virulence of the pathogen. Resistance to lysostaphin is property of *S. simulans*, its natural producer organism, but most other staphylococcal species are very sensitive to its lytic activity (Cisani et al., 1982). A lysostaphin immunity factor (*lif*) gene has been located within *S. simulans* and is thought to enable the substitution of serine for glycine residues in the cross-bridge of the peptidoglycan (Thumm and Gotz, 1997). The catalytic action of lysostaphin, a glycyl-glycyl endopeptidase, is thus prevented. A second resistance mechanism may be the inability of lysostaphin to bind to the *S. simulans* peptidoglycan. The binding domain of lysostaphin has been identified, and reporter proteins containing this region are able to bind to *S. aureus*, but not to *S. simulans* (Baba and Schneewind, 1996). Further, removal of the binding domain from lysostaphin disables its ability to kill *S. aureus*. A third mechanism of resistance has been identified in lysostaphin-resistant *S. aureus* mutants that developed following low dose administration of lysostaphin for 3 d in a rabbit model of endocarditis (Climo et al., 2001). The mutation resulted in a monoglycine, rather than the usual penta-glycine cross-bridge. Interestingly, these mutants were more susceptible to β-lactam antibiotics, and furthermore, the development of the lysostaphin mutants was largely prevented by coadministration of β-lactam antibiotics. Thus, bacterial resistance to peptidoglycan hydrolases may develop along with some cost to the pathogen's fitness or virulence. It is our strategy to develop additional antistaphylococcal proteins such that a combination will reduce the development of resistant organisms. Proteins such as *S. aureus* autolysins, and bacteriophage lysins (see below) have evolved over time to bind to, and catalyze degradation of peptidoglycan. These evolutionarily selected binding/catalytic sites are likely to be critical to the organism, and not amenable to mutation (Schuch et al., 2002).

Bacteriophage lysis is generally mediated by the production of two proteins, a holin and a lysin (Wang et al., 2000). The holin creates a hole in the cell membrane that enables the lysin to access the peptidoglycan. The lysin then degrades the peptidoglycan resulting in lysis of the bacteria. This phenomenon is known as "lysis from within," and in contrast, external addition of the

lysin to a gram-negative bacteria such as *E. coli* does not result in lysis presumably because the lysin can not access the peptidoglycan through the outer cell membrane. However, the application of phage lysins to treat infections caused by gram-positive bacteria, which lack an outer membrane, is seeing a resurgence. Recently, streptococcal phage lysins have been shown to be very effective in the treatment of experimentally induced nasopharyngeal, streptococcal infections. Mice that had been inoculated with a strain of group A streptococci—and subsequently demonstrated significant oral colonization—were cleared of infection within 2 h of lysin application (Nelson et al., 2001). In a similar study, mice previously colonized with *Streptococcus pneumoniae* were cleared of infection by a single dose of a specific lysin protein (Loeffler et al., 2001). Finally, a third report from this group demonstrates the ability of PlyG lysin, isolated from the γ phage of *Bacillus anthracis*, to kill *B. anthracis* (Schuch et al., 2002). In this study repeated exposure of a susceptible organism to PlyG did not result in the generation of spontaneously resistant mutants suggesting that the lysin targets essential cell-wall molecules. Given that bacteriophage have been described for nearly all bacteria, it is likely that appropriate lysins will be found that can kill mastitis-causing pathogens.

Peptidoglycan degradation is also required for bacterial growth and division. In fact, the peptidoglycan layer is a highly dynamic structure undergoing continual synthesis and degradation. Endogenous hydrolases are referred to as autolysins. The major autolysin of *S. aureus* is encoded by the *Atl* gene (Oshida et al., 1995; Foster 1995). This bifunctional gene product contains both N-acetylglucosaminidase (**GL**) and N-acetylmuramoyl-L-alanine amidase (**AM**) activities. The relative bacteriolytic activities of GL and AM were found to be 250-fold and 25-fold less than lysostaphin (Sugai et al., 1997). These enzymes are promising candidates but it remains to be determined if they will be potent enough to confer mastitis resistance.

Candidate Gene Regulatory Regions

The production of novel proteins into milk of transgenic animals has relied on the incorporation of milk protein regulatory regions into the transgene constructs. This ensures production of the protein in lactating mammary epithelium. However, mastitis susceptibility is not confined to the lactating state. In fact, transition periods, either from the dry to lactating state or vice versa, are periods of enhanced susceptibility to mastitis. This has led us to explore genes known to be active in these states as candidate regulatory regions for a transgenic approach to enhance mastitis resistance.

Lactoferrin

The concentration of lactoferrin in normal bovine or murine milk is reported to be between 20 and 200 μg/ml (Neville et al., 1998), approximately one-hundredth that of human milk. However, upon cessation of bovine lactation, the lactoferrin concentration in mammary secretion begins to increase after 2 to 4 d, reaching peak levels 14 to 21 d later that are 100-fold greater than during lactation (Welty et al., 1976). These protein levels are a reflection of mammary gene expression. In both mouse and cattle, lactoferrin mRNA is barely detectable by northern blot analysis of RNA from lactating tissue, but is dramatically induced upon cessation of lactation (Goodman and Schanbacher, 1991; Lee et al., 1996). In the mouse, this induction can easily be detected within 1 d of involution, while the first time point in the bovine report was 3 d. The intense signal on Northern blots of involuting bovine mammary tissue indicates that lactoferrin production is a major function of the tissue.

Perhaps related to its antibacterial properties, lactoferrin concentrations in bovine milk are increased by mastitis (Harmon et al., 1975). Furthermore, in acute experimentally induced infection, lactoferrin concentrations can increase 30-fold within 90 h of inoculation (Harmon et al., 1976). The major source of this appears to be the mammary epithelium and the additional PMNs found in milk from inflamed glands (Harmon and Newbould, 1980). These authors estimated that PMN contributed only about 5% of peak lactoferrin levels in endotoxin-induced inflammation of the bovine mammary gland. Changes in lactoferrin gene expression by mammary epithelial cells in response to mastitis have not been specifically investigated. However, Molenaar et al. (1996) reported some interesting observations in this regard during a survey of abattoir-derived mammary tissue. The glands surveyed were from virgin, pregnant, lactating, and dry cows. Using in situ hybridization, these authors found that lactoferrin gene expression varied inversely with the lactational state of the secretory cells, being generally restricted to developing alveoli in pregnant animals, generally low in actively lactating alveoli, and generally high during involution. Importantly, they also noted that lactoferrin expression was high at any lactational stage in those alveoli that contained somatic cells in their lumen, indicative of mastitis. Thus, a strong body of evidence exists suggesting that inflammation and involution induces mammary expression of lactoferrin. For our goals of strengthening the mammary gland's repertoire of antibacterial proteins it appears that the regulatory region of the lactoferrin gene would provide appropriate tissue and developmental expression and the ability to respond to inflammation.

Genes Induced by Mastitis or Involution

Appropriate regulatory regions may also be found through studies of mammary tissues either responding to infection or undergoing involution. One similarity between these states is a marked increase in epithelial cells undergoing apoptosis. Perhaps gene regulatory re-

gions upregulated within apoptotic cells or within the neighboring surviving cells would be effective candidates for transgene regulation.

It is now well documented that bacterial infection of epithelial cells stimulates apoptosis (for review see (Weinrauch and Zychlinsky, 1999). The relationship of this to lost milk production is difficult to measure but potentially has a substantial negative effect. Experimental challenge of lactating cows with *E. coli* has clearly documented the resulting stimulation of apoptosis (Long et al., 2001). In that study, infected mammary glands were biopsied 24 h postinfection with the resulting tissues processed for RNA, protein, and histological examination. Both mRNA and protein analysis indicated a substantial up-regulation of pro-apoptotic factors—Bax and interlukin-1β converting enzyme—and a down-regulation of the antiapoptotic factor Bcl-2. Further, induction of a 92-kDa gelatinase—presumably MMP-9—was clearly observed by gelatin zymography. Finally, the number of apoptotic epithelial cells/10 microscopic sections, as determined by TUNEL assay, increased from 1.8 ± 0.5 to 8.8 ± 2.8 cells. Interestingly, an increase in epithelial cell proliferation was also observed that might be a restorative mechanism to maintain alveolar integrity. Whether these cells represent scar tissue formation or do in fact go on to produce milk proteins is unknown. Evidence for induction of apoptosis by other mastitis pathogens has been provided by experimental infection of the bovine mammary gland with *Streptococcus agalactiae* (Sheffield 1997). In this study a fivefold induction of a putative marker of apoptosis—testosterone-repressed prostate mucin-2 (TRPM-2) mRNA was observed. Finally, in vitro studies indicate that *S. aureus* causes apoptosis in a bovine mammary cell line (Bayles et al., 1998).

An increase in apoptotic epithelial cells is also a characteristic of mammary gland involution in the cow (Wilde et al., 1997; Capuco and Akers, 1999), and the mouse model (Jerry et al., 2002). In the mouse model, genes associated with cell cycle progression and arrest are rapidly induced along with pro-apoptotic genes. The activation of the tumor repressor protein, p53, appears to play a central role in modulating the expression of various involution responsive genes. Activation of nuclear factor-κB (**NF-κB**) is one of the most rapid events, clearly visible 24 h postweaning (Brantley et al., 2000). In fact, the activation of NF-κB can be observed within 30 min in a mammary cell culture model system (Clarkson et al., 2000). In this system, KIM-2 mammary cells can be induced to differentiate in the presence of prolactin and dexamethazone with complete suppression of NF-κB after 11 d. Abrupt withdrawal of the lactogenic hormones by media replacement induces apoptosis and NF-κB activity. However, the role of NF-κB in meditating the apoptosis is not clear. Rather, it appears that only about one third of the cells become apoptotic after 17 h of hormone depletion, and it seems that it is the surviving cells that produce the active NF-κB. Examining KIM-2 cells that had been stably transfected with an NF-κB-responsive green fluorescent protein (**GFP**) reporter construct further substantiated this finding. Under apoptotic conditions a large number of cells expressed GFP; however, in an analysis of over 1000 cells positive for annexin V (a marker of apoptosis) not one instance of GFP co-localization was observed. Thus, NF-κB appears to have been selectively activated in surviving rather than dying cells. A gene regulatory region responsive to NF-κB may be a good candidate to drive mastitis resistance genes.

Microarray-based experiments are now being employed to evaluate changes in murine mammary gene expression that occur during pregnancy, lactation, and involution (Lemkin et al., 2000). Similar experiments are also shedding new light on the response of cultured cells to infection (Rosenberger et al., 2000; Coussens et al., 2002). The application of these high throughput techniques to bovine mammary cells undergoing involution or responding to infection will reveal genes, whose promoter regions may be ideal for directing the expression of antibacterial proteins during these critical periods. We are pursuing antibacterial proteins that function in mammary secretions to kill mastitis causing pathogens. The location and secretory capability of the mammary epithelial cells appear to make them the best cell type to produce these proteins in a localized fashion. However, locating epithelial specific responses with microarray experiments based on mammary tissue samples will be challenging given that the mammary gland also contains numerous other cell types such as fibroblastic, endothelial, adipose, myoepithelial, and infiltrating lymphoid cells. Further, the relative proportions of these cell types changes drastically with the physiological state of the tissue. Techniques such as laser dissection microscopy, or mammary cell culture may be more appropriate sources to identify candidate genes that can then be verified histologically on tissue sections.

Transgenic Technology to Enable Mammary Production of New Proteins

The use of transgenesis to direct mammary gland expression of foreign protein in the mouse was first reported by Gordon et al. (1987). This technology has now progressed to transgenic pigs, sheep, goats, and cattle, powered primarily by pharmaceutical interests seeking to generate animal bioreactors (Wall, 1996). Human clinical trials using livestock-milk-derived human α1-antitrypsin and antithrombin III are now in progress. However, due primarily to costs and the time frame involved, this technology has yet to be used for strictly dairy purposes. The cost of producing transgenic founder animals which, using standard microinjection techniques, may approach $60,000 for a sheep or goat and $300,000 for a cow, with approximately 7 yr required for generation of a herd of production of milk (Wall et al., 1997). Progress in techniques for nuclear transfer from transgenic cells to enucleated embryos

should reduce this cost considerably as the number of recipient animals required will be greatly reduced (Schnieke et al., 1997). Recent advances in production of transgenic ruminants include targeted, nonrandom, insertion of transgenes into the genome (McCreath et al., 2000), and insertions of very large DNA segments containing multiple genes (Kuroiwa et al., 2002). These and other advances will aid in the realization of transgenic, agriculturally important livestock. However, the cost, and the 5 to 10-yr time frame are still daunting.

Conclusion

The amazing advances in agriculture that have already been produced by transgenic plant technologies foretells advances in animal agricultural to be delivered by application of transgenic animal technologies. Lysostaphin is but one of a host of bacterial proteins that could have transgenic application in the prevention of mastitis. The development of an inflammation- and involution-inducible expression construct may be superior to constitutive expression of antibacterial proteins during lactation, delivering antibacterial proteins only when needed. New technologies often raise public concerns. These concerns must be addressed through education on the scientific basis of transgenic animal technology and through demonstration of the safety of the food produced and the well-being of the animals. The need for new technologies to meet future demands for food production must also be explained. The goal is not only to satisfy future needs, but also to do so with far less use of resources and production of waste than would be required through simple expansion of current agricultural practices. In addition, if the goal of enhanced mastitis resistance can be realized, it will positively affect animal welfare and lessen the need for antibiotics to treat this disease.

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Proteomics methods for probing molecular mechanisms in signal transduction[1]

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ABSTRACT: mRNA splicing and various posttranslational modifications to proteins result in a larger number of proteins than genes. Assessing the dynamic nature of this proteome is the challenge of modern proteomics. Recent advances in high throughput methods greatly facilitate the analysis of proteins involved in signal transduction, their production, posttranslational modifications and interactions. Highly reproducible two dimensional polyacrylamide gel electrophoresis (2D-PAGE) methods, coupled with matrix assisted laser desorption-time of flight-mass spectrometry (MALDI-TOF-MS) allow rapid separation and identification of proteins. These methods, alone or in conjunction with other techniques such as immunoprecipitation, allow identification of various critical posttranslational modifications, such as phosphorylation. High throughput identification of important protein-protein interactions is accomplished by yeast two hybrid approaches. In vitro and in vivo pulldown assays, coupled with MALDI-TOF-MS, provide an important alternative to two hybrid approaches. Emerging advances in production of protein-based arrays promise to further increase throughput of proteomics-based approaches to signal transduction.

Key Words: Proteomics, Signal Transduction, Protein, Mammary Gland

 J. Anim. Sci. 2003. 81(Suppl. 3):48–56

Introduction

In the broadest sense, signal transduction can be considered the mechanisms by which a cell responds to its environment. Most often, signal transduction reflects responses to hormones, growth factors, neurotransmitters, and other such molecules. However, similar pathways also mediate responses to extracellular matrix, inorganic ions and various other factors. Often, distinctions are made among endocrine, neuronal, paracrine, autocrine, and juxtacrine signaling. Such distinctions are useful in the context of organ or animal physiology, but the signaling pathways activated are often very similar.

Activation of a signal transduction pathway requires a sensing molecule, such as a hormone receptor. Activation of this receptor elicits one or more of a wide variety of pathways that lead to alteration of the transcription/translation process, alteration of the structure of existing proteins or both. These pathways are activated in the context of a specific genome. Obviously, transcription of a particular set of genes requires the presence of appropriate genes and regulatory elements. Additionally, genotype can affect nontranscriptional pathways by its impact on the specific signaling molecules present.

Although genomics has proven to be a powerful field, and the sequencing of several important organisms, as well as the amount of information available in others is impressive and useful, genome organization alone is insufficient to explain physiological processes. Critical questions emerging in the postgenome era involve how genetic information is expressed as a phenotype and how that expression is altered in response to environment. Emerging methods place considerable emphasis on high throughput techniques. DNA arrays, capable of simultaneous analysis of the expression of thousands of genes, are the current archetype of high throughput analysis in biology (Cheung et al., 1999). A frequent use of these arrays is to assess the cell or tissue content of several thousand mRNA simultaneously (Duggan et al., 1999). As useful as this information is, it still provides an incomplete picture of cell biology (as described below). More complete understanding of how genotype and its interactions with the environment result in the expressed phenotype requires additional information

Received July 8, 2002.
Accepted April 7, 2003.
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[1]Supported by University of Wisconsin College of Agriculture and Life Sciences and USDA Project WIS 3108 and WIS 4665.

on protein production and posttranslational modifications. Unfortunately, protein-based methods cannot yet achieve the throughput of DNA-based techniques. However, rapid progress in proteomics promises to greatly improve the throughput of protein-based analytical procedures.

The objective of the present review is to describe some of the issues and techniques involved in improving our understanding of the broad field of signal transduction, with an emphasis on protein-based approaches. Toward this end, this review will focus on proteomics issues, including identification of specific proteins, posttranslational modification and protein-protein interaction, including examples from mammary gland biology.

Proteome Concept

If the genome is defined as the collection of all genes in an organism, one could define the collection of all expressed genes (at the mRNA level) and their translated proteins as the transcriptome. Furthermore, the proteome can be defined as all proteins present in a cell or tissue at any given time. This includes all posttranslational modifications that occur. Thus, unlike the genome that is stable, the transcriptome and proteome are highly dynamic. The genome of mammals is generally considered to consist of about 30,000 genes. Surprisingly, this number is only about twofold greater than that found in what are generally considered less complex organisms, including insects and the nematode *Caenorhabditis elegans* (Rubin et al., 2000). However, mammals generally produce a considerably more complex array of proteins from their genome. This additional complexity in the mammalian proteome arises from a number of sources, including alternative splicing of mRNA, multiple pathways of proteolytic processing of proteins and various other posttranslational modifications, such as glycosylation, perenylation, and phosphorylation. Furthermore, subcellular localization of a protein can affect its function (Petsko, 2001). Although these mechanisms exist in all eucaryotes, they are employed to a greater extent in some phyla, such as mammals, giving rise to more complex proteomes.

mRNA Splice Variants

The presence of multiple mRNA species corresponding to a single transcribed region of DNA is well known and exist for 40 to 60% of expressed genes (Modrek and Lee, 2002). In some cases, this represents the presence of multiple states of processing (mature mRNA vs. unspliced pre-mRNA). However, in many cases, multiple splice variants exist. In the case of prolactin receptor, all species examined to date have multiple isoforms of the prolactin receptor (Bole-Feysot et al., 1998; Horseman, 2002). These prolactin receptor isoforms are often termed long and short isoforms, although the so-called short isoform is a variety of specific isoforms. In addition, an intermediate isoform has been identified in rat and human. The long isoform of the receptor is capable of both mitogenic and lactogenic signaling, whereas the short isoforms are generally considered incapable of supporting these functions (Chang and Clevenger et al., 1996; Chang et al., 1998). The rat intermediate isoform of prolactin receptor has been reported to be mitogenic and activate JAK2, while a human intermediate isoform has minimal mitogenic activity, although it activates JAK2 (Kline et al., 1999). Short isoforms of the prolactin receptor have been shown to inhibit lactogenic signaling by long isoforms, probably because of the formation of inactive heterodimers of long and short isoform receptors (Berlanga et al., 1997). However, in the rat corpus luteum (in which prolactin is luteotropic), short isoforms of prolactin receptor appear to be involved in activating specific pathways, including binding of a specific prolactin receptor associated protein (Duan et al., 1997).

In addition to transmembrane isoforms of prolactin, a splice variant that gives rise to a soluble form of the prolactin receptor lacking transmembrane and intracellular domains has recently been identified (Trott et al., 2003). Similar findings have also been observed for growth hormone receptor in rodents (Postel-Vinay and Finidori, 1995). Interestingly, while the soluble forms of growth hormone receptor in rodents arises from alternative splicing, a similar protein in humans appears to arise from proteolytic processing of the growth hormone receptor (Postel-Vinay and Finidori, 1995).

Several splice variants of epidermal growth factor (**EGF**) receptor have also been reported (Reiter and Maihle, 1996; Reiter et al., 2001). A soluble 110-kDa EGF receptor, consisting of the extracellular domain, is present in human serum, appears to be regulated by reproductive hormones (Baron et al., 2001) and is elevated in certain cancers, most notably ovarian (Baron et al., 1999). Recently, we have also detected a similar protein in bovine serum (Figure 1).

At least three splice variants of transforming growth factor alpha (**TGFα**), an EGF receptor agonist, are known to exist. Although they interact with members of the ErbB family, they do so with varying specificities. The so-called wild-type variant coimmunoprecipitated with ErbB4, whereas variants I and II coimmunoprecipitated with ErbB2. All of these variants activated ErbB2, but the biological consequences differed, with Variants I and II, but not wild type, producing autonomous growth (Xu et al., 2000).

As a final example of splice variants, Yu et al. (1992) observed a splice variant of acidic fibroblast growth factor. This variant produces a frameshift mutation, resulting in a different C-terminal sequence. Unlike acidic fibroblast growth factor, which is mitogenic to a number of cells, including fibroblasts, this variant appears to be an acidic fibroblast growth factor antagonist. Unfortunately, its physiological significance is unclear.

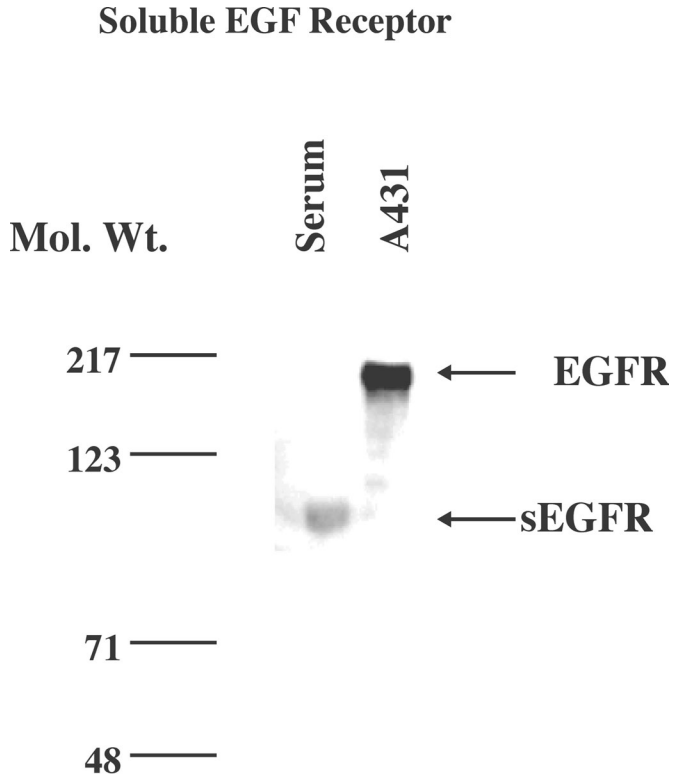

Figure 1. Soluble epidermal growth factor (EGF) receptor (sEGFR) in bovine serum. Bovine serum or A431 cell membranes (a human epidermoid carcinoma that expresses high levels of EGF receptor and is used as a positive control) were separated by SDS-PAGE (10% gel), transferred to polyvinylidene fluoride (PVDF) membranes and probed with anti-EGF receptor as previously described (Hendrix et al., 1996). Note the presence of a 110-kDa protein immunologically reactive with anti-EGF receptor in bovine serum.

Protease Processing

Signal peptides are generally removed from proteins during processing. The identity of consensus signal peptides is known, and can be readily predicted from DNA sequence information (Baldi et al., 2000). Furthermore, protein sequence is also capable of predicting a number of other processing features, including likely subcellular localization (Emanuelsson and von Heijne, 2001). However, other processing events are more difficult to predict, and many proteins are processed by multiple pathways, giving rise to multiple processing variants.

Transforming growth factor alpha is one important regulator of mammary development that is processed by multiple pathways. Transforming growth factor alpha is produced as a 160-amino acid transmembrane precursor. In most cases, this precursor is processed to a 50-amino acid mature peptide that is biologically active. However, transmembrane forms of TGFα have been identified (Luetteki and Lee, 1990). Juxtacrine actions of these forms of TGFα have been suggested (Bush et al., 1998; Xiao and Majumder, 2001). In addition, dimerization of transmembrane TGFα molecules with anti-TGFα induces protein phosphorylation reminiscent of receptor activation (Shum et al., 1994). Because the intracellular domain of the transmembrane form of TGFα does not appear to contain kinase activity, this is most likely due to association of transmembrane TGFα with other signaling molecules. This appears to require palmitoylation of Cys 153 and 154 in the intracellular domain of transmembrane TGFα (Shum et al., 1996). Recently, Shi et al. (2000) determined that CD9 physically associates with transmembrane forms of TGFα, while Kuo et al. (Kuo et al., 2000) found that the Golgi associated protein GRASP55 was also associated with transmembrane TGFα. These results indicate that transmembrane forms of growth factors may have functions beyond serving as juxtacrine ligands, although this possibility has received little attention.

Epidermal growth factor is also produced as a large (>1200 amino acid) glycoprotein precursor that contains a transmembrane domain and a short intracellular domain (Mroczkowski and Carpenter, 1988). In some tissues, preproEGF is almost completely processed to mature peptides, including the 53-amino acid mature EGF. However, in others, such as the kidney, very little is processed in this way, and most remains as a transmembrane protein. In the mammary gland, some EGF does appear to be processed and secreted into milk (Grosvenor et al., 1993), although a substantial amount of EGF remains as partly processed preEGF (Mroczowski and Reich, 1993). Recently, we have observed that bovine mammary epithelial cells also produce pre-EGF like molecules, although they do not appear to produce mature EGF in detectable amounts (Figure 2). The biological role of these forms of EGF remains unclear. Mroczkowski et al. (1989) suggested that they might give rise to juxtacrine signaling, by activating EGF receptors in adjacent cells. Maheshwari et al. (2001) has also observed that expression of EGF in transmembrane forms results in directional migration of cells, whereas soluble EGF stimulates random migration.

Phosphorylation

A major modification important in signal transduction is protein phosphorylation. This can arise from receptor kinases, receptor-associated kinases or kinases downstream of the initial signal. In addition, cells contain a diverse array of phosphatases that dephosphorylate proteins. Although one often thinks of hormone action as stimulating protein phosphorylation and phosphatase activity reversing those actions, this is not always the case. For example, the kinase src is actually inhibited by a specific tyrosine phosphorylation (Courtneidge et al., 1993). Whereas the details of kinase regulation are beyond the scope of this review, the ability to detect this modification is critical to studying signal transduction.

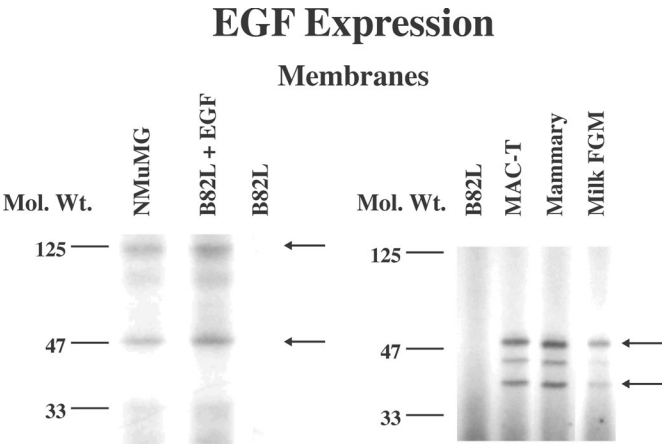

Figure 2. Forms of epidermal growth factor (EGF) in mammary tissue. Left: Cell membranes from mouse mammary epithelial cell line (NMuMG), mouse fibroblasts lacking EGF expression (B82L), or B82L cells transfected with murine EGF were separated by SDS-PAGE on a 10% gel, transferred to polyvinylidene fluoride (PVDF) membranes and probed with anti-EGF essentially as previously described (Hendrix et al., 1996), except that anti-EGF was used instead of other antibodies. Right: Cell membranes were isolated from B82L cells (negative control), a cultured bovine mammary epithelial cell line (MAC-T), lactating bovine mammary tissue (Mammary) or milk fat globule membranes. Proteins were separated by SDS-PAGE on a 10% gel, transferred to PVDF membranes and probed with anti-EGF essentially as previously described (Hendrix et al., 1996), except that anti-EGF was used instead of other antibodies. Note the presence of two major partly processed forms of EGF (arrows) and other less intense bands. B82L cells are a negative control, lacking EGF.

Studying the Proteome

Protein Separation and Identification

A major advance in the study of protein expression has been in two-dimensional PAGE. Initial methods (O'Farrell, 1975) involved initial separation by isoelectric focusing (usually in a small tube gel) followed by transferring this gel to an SDS-PAGE gel for the second dimension separation. The initial isoelectric focusing gel was usually generated in situ by the use of carrier ampholytes added to samples, gels and/or running buffers. Such gels provided high resolution, but were difficult to reproduce, in large part because of inconsistencies in reproducing the pH gradient of the first dimension gel. More recently, the availability of immobilized pH gradients has greatly facilitated two-dimensional PAGE (Righetti et al., 1983; Fichmann, 1999; Gianazza, 1999; Matsui et al., 1999; Gorg et al., 2000). Immobilized pH gradient gels of various lengths and pH ranges are available from a number of commercial suppliers. Further advances in computer assisted image analysis methods has improved the ability to accurately compare specific spot intensities.

For two-dimensional PAGE to be particularly useful, methods of identifying specific proteins are necessary. Some clues may be obtained from molecular weight and isoelectric point estimations, but identification by these methods is not particularly reliable. If antibodies to a suspected protein are available, immunological detection (Western blot) is useful. However, this method is not particularly useful for the identification of a large number of proteins or for novel proteins.

Historically, protein sequencing, either of the N-terminus or of tryptic fragments, is a useful method of protein identification. Even without a full sequence, fragments can often be matched to a specific DNA sequence for identification. More recently, developments in mass spectrometry provide a rapid method of protein identification. Entire proteins are often not particularly useful for mass spectrometry, but protease digestion products (such as tryptic fragments) are readily separated by modern methods. In particular, matrix assisted laser desorption time of flight mass spectrometry (**MALDI-TOF-MS**) techniques are emerging as the method of choice for identifying tryptic fragments (Hamdan et al., 2001; Leushner, 2001; Mann et al., 2001; Zaluzec et al., 1995).

Modern mass spectrometry instruments have a resolution well below 1 atomic mass unit. This allows ready matching of a particular tryptic fragment to a specific amino acid composition, since only a single combination of amino acids will provide an appropriate match in almost all cases. However, this does not generally provide an unambiguous determination of the specific sequence of amino acids, since any sequence combination of the same amino acids match the molecular weight estimates. Therefore, automated search routines that compare the tryptic map with that of other proteins are necessary. Importantly, these maps can be theoretical maps, based on DNA sequence alone.

In addition to appropriate algorithms for analyzing mass spectra, the quality of the underlying database is also critical to the success of proteomics efforts (Stupka, 2002). In the case of researchers using human, mouse, or other widely used models, very complete and high-quality databases are available and readily accessible. In other cases, such as most livestock and many less studied species, database completeness can be a major limitation. In the case of genomics or gene expression profiling, identifying a sequence with 80 or 90% identity to a human sequence is a reasonable prospect. In the case of tryptic mapping by mass spectrometry, this is likely to prove difficult or impossible.

Statistical approaches allow calculation of the probability that a particular match of a peptide map with a protein in a database is due to chance (Berndt et al., 1999; Eriksson et al., 2000; Eriksson and Fenyo, 2002). However, complete match is rarely obtained. In some cases, predicted fragments are absent from the mass spectra, whereas in others, some mass spectra peaks

are not predicted for the particular protein. Often, these extraneous peaks are caused by known contaminants, such as trypsin, or to acrylamide contaminants. Regardless, verification by other methods, such as microsequencing or immunological approaches is desirable for proteins identified for further study.

Previously, Beaton et al. (1997) used a proteomics approach to identify proteins, including glucose-regulated protein 78 and protein disulphide isomerase, associated with lactation and prolactin action in mammary glands. In other studies, proteomics approaches have been used to map the proteome of the endoplasmic reticulum and milk fat globule membrane, and to examine the changes in the golgi associated with transition from a basal state to maximum secretion (Wu et al., 2000a, 2000b).

As an additional example of the applications of proteomics methods to mammary gland biology, we have investigated the possibility that the previously described soluble variants of EGF receptor induce signaling by associating with transmembrane forms of EGF. Such reverse signaling has been demonstrated for transmembrane forms of TGFα (using antibodies, rather than soluble receptors, to induce signaling by the transmembrane growth factor) (Shum et al., 1994). For these studies, the extracellular domain of mouse EGF receptor was cloned into pIZ/V5-His in frame with a polyhistidine tag. Cabbage looper embryo cells (High-Five, Invitrogen, Carlsbad, CA) were transfected, and stable lines were generated that were resistant to Zeocin. Soluble EGF receptor was found to be secreted into culture media in these cells. The soluble EGF receptor was purified from culture media by wheat germ agglutinin chromatography (Quijano and Sheffield, 1998) followed by Ni-affinity chromatography (Lilius et al., 1991). Mammary epithelial cells previously shown to express transmembrane forms of EGF (NMuMG line, American Type Culture Collection, Rockville, MD) were treated with soluble EGF receptor for various times, proteins were extracted with 8 *M* urea, 4% (3-[(3-cholamidopropyl)dimethylamino]-1-propane sulfonate, 40 m*M* Tris base, 1 m*M* phenylmethanesulfonyl fluoride, 100 μg of aprotonin/ml and 100 μg of leupeptin/ml. Proteins were separated by two-dimensional PAGE using immobilized pH gradient strips for the first dimension and a 10% PAGE gel for the second (Amersham Pharmacia Biotech, Piscataway, NJ). After 24 h of treatment with EGF receptors, substantial differences were apparent in proteins resolved by two-dimensional PAGE (Figure 3). Protein spots were chosen for subsequent identification by MALDI-TOF-MS based on their change in spot intensity and resolution in the area of the spot. Among the proteins increased are cytokeratin 8, phospholipase C alpha, and tubulin α6. Cytokeratin 8 resolved as three independent spots, probably because of different phosphorylation states of the protein.

An important consideration in interpreting these results, as in other analyses of protein content by two-dimensional PAGE is the mechanism by which a decrease in spot intensity is achieved. Several possibilities exist in addition to the most obvious alteration in protein production. Protein stability could be altered. Alternatively, the solubility of the protein in the lysis buffer used could be altered. This could be due to either posttranslational modifications to the protein itself or alterations in its association with other cell components, such as caveoli or the cytoskeleton (Prusheik et al., 1997; Schlegel and Lisanti, 2001; Schroeder et al., 2001). These alternatives are currently under investigation for the impacts of EGF receptors on cellular protein content.

Assessing Phosphorylation Status

Classically, protein phosphorylation was detected by incubating cells with ^{32}P and assessing incorporation into proteins. In addition, immunological approaches are also available, particularly for assessing tyrosine phosphorylation. Immunological approaches to determining phosphorylation of a specific protein formerly involved immunoprecipitation of the protein and probing with anti-phosphotyrosine or immunoprecipitation from ^{32}P-labeled cells and autoradiography. More recently, antibodies that recognize specific phosphopeptides have been developed and used to differentiate phosphorylated and nonphosphorylated states of proteins. These have been widely used to assess tyrosine phosphorylation of a number of proteins, including mitogen-activated protein kinase, c-src, focal adhesion kinase, various members of the STAT (signal transducers and activators of transcription) family, and others (Kaufmann et al., 2001; Nagata et al., 2001). However, one should note that these antibodies recognize only a short peptide sequence. If that sequence is present in other proteins, this approach cannot distinguish between them. For example, the tyrosine phosphorylation of STAT5a and 5b are identical, and so STAT5a tyrosine phosphorylation cannot be resolved from STAT5b tyrosine phosphorylation by using phosphorylation state-specific antibodies alone (although prior immunoprecipitation with specific antibodies allows separation of STAT5a and STAT5b).

Another approach to protein phosphorylation is the use of mass spectra. Because the addition of phosphate to a peptide results in a distinctive shift in mass spectra, this can be used as a signal for the presence of phosphate in a protein (Wilkins et al., 1999). Often, the specific phosphorylation site can be mapped using this technique. However, the method is technically demanding, in that very high sensitivity or enrichment of samples is usually required (Sickmann and Meyer, 2001).

Recently, considerable interest has arisen on the use of protein array-based approaches to study protein phosphorylation. Conceptually, antibody arrays would be very useful in identifying proteins present in a cell extract as well as their post-translational modifications (Borrebaeck, 2000; Srinivas et al., 2001; Yarmush and

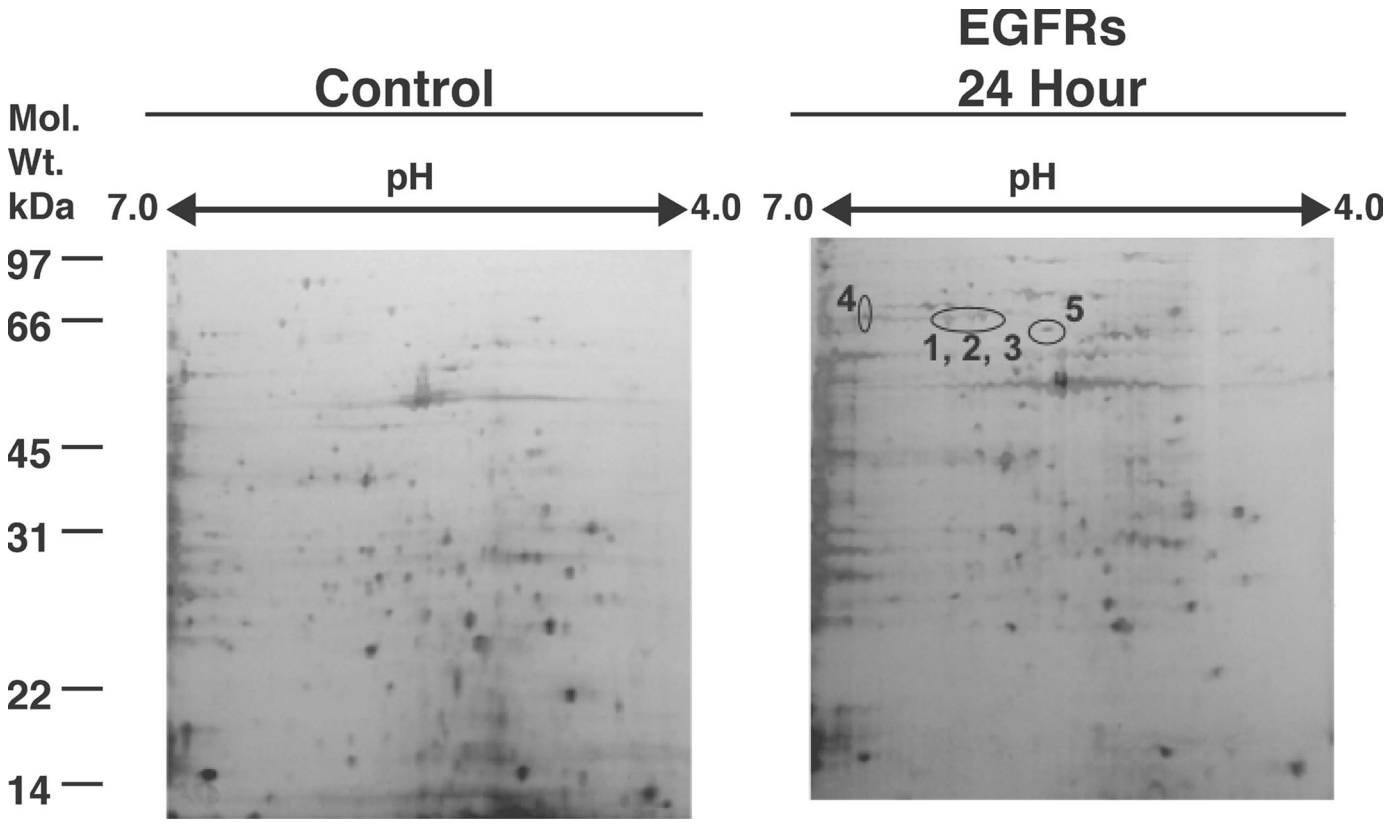

Figure 3. Two-dimensional PAGE analysis of soluble epidermal growth factor (EGF) receptor effects on mammary epithelial cells. NMuMG mouse mammary epithelial cells were treated as controls or for 24 h with 500 ng/ml of recombinant soluble EGF receptor (EGFRs). Proteins were extracted and separated by two dimensional PAGE on a 4.0–7.0 pH isoelectric focusing gel, followed by a 12% SDS-PAGE gel. A number of proteins spots were seen to increase and decrease upon treatment. 1, 2, 3 = cytokeratin 8, 4 = phospholipase Cα, 5 = tubulin α6.

Jayaraman, 2002). Antibodies attached to a solid surface could be designed to capture a wide variety of proteins. Anti-phosphotyrosine, serine, or threonine could then be used to probe the array to determine phosphorylation status of specific proteins. Alternatively, a large number of phosphorylation state-specific antibodies could be arrayed. A major disadvantage of this approach is that production of antibodies is considerably more time consuming than production of DNA fragments. In addition, choosing DNA fragments that require similar hybridization conditions is relatively straightforward, particularly when compared to developing a large number of antibodies that have similar optimal conditions. As a result, few such arrays are currently available.

To investigate the possible signaling by soluble forms of EGF receptor, we also used standard phosphotyrosine western blots to determine whether EGF receptors induced changes in cellular phosphotyrosine. These studies determined that EGF receptors increased protein tyrosine phosphorylation in Triton X-100-insoluble proteins, but not in soluble proteins (Figure 4), suggesting an increased tyrosine phosphorylation of cytoskeletally associated proteins (Prusheik et al., 1997). Immunoprecipitation followed by SDS-PAGE and MALDI-MS has identified some of the tyrosine phosphorylated proteins. Interestingly, these appear to be members of the cytokeratin family.

Cytokeratins are tyrosine phosphorylated in responses to various cell signals, including EGF and the phosphotyrosine phosphatase inhibitor pervanadate (Aoyagi et al., 1985; Feng et al., 1999). Because phosphorylation changes are often associated with changes in molecular structure and function, and because cytokeratins are major components of intermediate filaments, these results suggest possible roles for growth factor signaling in modifying cytoskeleton and intermediate filament organization. Interestingly, such organizational changes have been associated with normal mammary development, differentiation, and tumorigenesis (Ben-Ze'ev, 1987; Taylor-Papadimitriou et al., 1992; Hendrix et al., 1996; Lelievre et al., 1996; Rudland et al., 1998).

Intermolecular Interactions

In addition to identifying proteins directly modified by a signaling pathway, identifying interactions among proteins is critical to understanding signal transduction (Legrain et al., 2001; Tucker et al., 2001). Currently, major efforts are under way to map all possible protein-

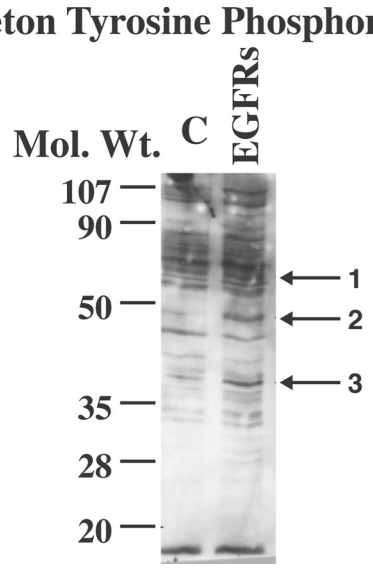

Figure 4. Tyrosine phosphorylation in response to soluble epidermal growth factor (EGF) receptor. NMuMG mouse mammary epithelial cells were treated as controls or for 10 min with 500 ng/ml of recombinant soluble EGF receptor (EGFRs). Triton X-100 soluble proteins (cytosol) and insoluble (cytoskeleton) proteins were separated on a 10% SDS-PAGE gel, transferred to polyvinylidene fluoride (PVDF) membranes and probed with anti-phosphotyrosine as previously described (Hendrix et al., 1996). Tyrosine phosphorylation of several proteins was modified by treatment. 1 = cytokeratin 8, 2 = cytokeratin 18, 3 = cytokeratin 19.

protein interactions in simple models, such as *Saccharomyces cerevisiae*. In more complex mammalian systems, this is currently an intractable problem, but some specific subsets of the problem are being investigated. These efforts contain a number of major challenges, including not only the complexity of the mammalian proteome itself, but also the fact that intermolecular interactions are often highly dynamic and respond rapidly to changes in cellular environment (Auerbach et al., 2002; Figeys, 2002).

Classically, ligand binding methods, such as radioreceptor assays, were standard methods of determining protein interactions. These same methods could be used to assess interactions among nonreceptor proteins. Additionally, coimmunoprecipitation studies are commonly used to assess protein-protein interactions (for example, Johnson et al., 1996). Pulldown assays (in vitro and in vivo) are extensions of ligand binding methods. In these methods, a target is expressed in a cell (in vitro) or added to a cell lysate (in vitro), usually fused with a tag, such as glutathione S transferase (Smirnova et al., 2001) or polyhistidine (Lu et al., 1993). The glutathione S transferase tag is then immunoprecipitated, and associating proteins are identified by immunological methods, sequencing, or mass spectrometry.

A major advance in high-throughput analysis of protein-protein interactions is the Yeast two hybrid screen and its various relatives (Gietz and Woods, 2002). The basic principal of this assay is that a number of transcription factor DNA-binding domains are capable of activating transcription only when associated with a transcription activating domain. This activation domain do not need to be the same protein provided it is in appropriate proximity to the DNA binding domain. If the DNA binding domain and transcription activation domain are expressed separately but fused with proteins that interact, transcription can be activated via the interaction of the fusion proteins. In practice, one usually expresses a known protein (often called "bait") fused with a DNA binding domain. A cDNA library is then fused with the transcription activation domain, such that transcription of a reporter gene (often β-galactosidase or green fluorescent protein driven by an appropriate promoter) is activated if the known protein and the protein coded by a cDNA fragment interact. In this way, thousands of possible interacting proteins can be screened simultaneously, and only positive clones need be sequenced and identified.

Although useful, two hybrid approaches are subject to a number of artifacts, including relatively large numbers of false positives and false negatives. As a result, additional methods to verify a specific interaction are generally considered necessary. A large number of methods are available (Lakey and Raggett, 1998). In addition to coimmunoprecipitation, ligand binding and affinity purification methods discussed above, such methods as microcalorimetry or plasmon resonance spectrometry can provide information on binding kinetics and thermodynamics, although their use requires specialized equipment (Phipps and Mackin, 2000; Cooper, 2002).

Array approaches similar to DNA arrays have recently begun to emerge for identifying protein-protein interactions (Emili and Cagney, 2000; Figeys and Pinto, 2001; Li, 2000;Yarmush and Jayaraman, 2002). A major limitation is the difficulty of producing large protein fragments in substantial numbers. As a result, most applications to date have focused on arraying relatively small peptide fragments that are readily synthesized by chemical methods. For example, these can be useful for mapping phosphotyrosine sites interacting with a particular src homology 2 (SH2) domain (Yeh et al., 2001). However, a number of protein-protein interactions may require structures larger than those represented by short peptides, or may be modified by the global protein structure, such that binding assays based on peptide fragments may be misleading. Nonetheless, the potential throughput for these methods is considerable.

Conclusions

Developments in protein chemistry, including reproducible two-dimensional PAGE and MALDI-TOF-MS,

coupled with database and statistical developments, allow rapid and high throughput identification of proteins and posttranslational modifications. Yeast two hybrid approaches and other high throughput methods allow analysis of a large number of possible protein-protein interactions of potential importance in signal transduction. Recent advances in antibody and protein arrays, analogous to DNA arrays, hold considerable promise for further increases in the throughput of proteomics approaches to signal transduction.

Acknowledgments

Authors would like to acknowledge the technical assistance of Kimberly Smuga-Otto. Research was supported by University of Wisconsin College of Agricultural and Life Sciences and USDA project WIS 3108 and WIS 4665.

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Short Oral and Poster Presentations

Modeling the interaction of milking frequency and nutrition in lactation. I. Vetharaniam, S. R. Davis. AgResearch Limited, Hamilton, New Zealand.

A model of lactation, parameterized for primiparous New Zealand cows grazing pasture was used to understand and quantify how milking frequency interacts with nutrition. In a simulation, cows were given one of two intakes over a lactation of 270 days: a low allowance (LA) reflecting actual pasture intake patterns, and a higher (on average 20%) allowance (HA) designed to counter deficit periods. Milking frequencies were varied from 1 to 4 times per day. Once daily milking (1DM) compared with twice daily (2DM) resulted in a production losses of 29% on LA and 32% on HA. 3 and 4 milkings per day (3DM and 4DM) increased production compared with twice daily milking by 9% and 12% respectively on LA, and 11% and 17% on HA. At the end of the lactation, 1DM resulted in 44% less mammary tissue than with 2DM, while 3DM and 4DM respectively gave mammary tissue increases of 22% and 40% over 2DM. Increasing the solids' content of milk by 20% reduced the loss associated with 1DM by 4%. Increasing cistern capacity by 20% only reduced this loss by 1%. Temporary 1DM for the first 3 weeks of lactation resulted in a production loss of 19%, compared with 2DM, on HA. With LA, this effect was only 9%. After 3 weeks, there was a long term loss in mammary tissue of 4%, and a loss in production of the same amount for either allowance. A 20% increase in cistern capacity of the udder reduced production loss in the first three weeks by 3% for HA. The model shows that mammary gland size over time is modulated by milking frequency, and determined the production potential of the udder, but actual production is strongly influenced by nutrition affecting secretion rates of alveoli. The response to milking frequency varied considerably with nutrition. A significant portion of the loss associated with 1DM is due to udder fill effects inhibiting secretion as opposed to loss of mammary tissue. The model reflects the underlying biology and its behavior is in good agreement with experiment. It demonstrates that higher milking frequencies need to be coupled with higher nutrition to obtain the potential benefits, and thus would be a useful teaching or research tool.

Key Words: Milking frequency, Nutrition, Lactation

Changes in cisternal and alveolar milk throughout lactation in dairy sheep. M. Rovai*, X. Such, G. Caja, J. Piedrafita. Universitat Autonoma de Barcelona, Bellaterra, Spain.

With the aim to study the changes in the cisternal traits of the udder of dairy ewes, a total of 212 primiparous and multiparous dairy ewes (Manchega, **MN**; n= 133; and, Lacaune, **LC**; n= 79) were used during suckling (wk 0 to 5) and milking (wk 6 to 20) periods. Udder evaluation was done 8 h after the a.m. machine milking (0800 h) at 30, 60 and 105 d of lactation. Milk yield, machine fractioning and main udder traits (depth, length and teat distance) were also measured throughout the milking period. Cisternal scans were obtained by using a portable ultrasound scanner with a 5 MHz and 80° sectorial transducer and their area measured. Cisternal milk was measured after drainage by using a teat cannula, and alveolar milk was machine milked after an oxytocin i.v. injection (4 IU/ewe). Milk yield varied according to breed (MN, 0.86 l/d; and LC, 1.69 l/d; $P<0.001$) and lactation stage ($P<0.001$). Machine milking fractioning (machine milk: stripping and residual milk) was 62:38 and 79:21 for MN and LC ($P<0.001$), respectively. Cisternal area (MN, 14 cm^2; and LC, 24 cm^2) and cisternal milk (MN, 149 ml; and LC, 275 ml) varied according to breed ($P<0.001$) and tended to increase with parity ($P<0.10$). Moreover, both cisternal area and cisternal milk, decreased in both breeds through lactation ($P<0.001$). Values for cisternal area were: MN (15.9, 13.7 and 12.3 cm^2) and LC (24.6, 24.0 and 22.2 cm^2); and for cisternal milk were: MN (178, 161 and 109 ml) and LC (335, 263 and 228 ml), for 30, 60 and 105 d, respectively. Alveolar milk decreased with lactation stage in both breeds ($P<0.001$) but only showed a tendency between breeds (MN, 160 ml; and LC, 194 ml; $P= 0.060$). Cisternal area and cisternal milk were correlated ($r= 0.76$; $P<0.001$) in both breeds, as well as milk yield during the milking period ($r= 0.42$ to 0.60; $P<0.01$) and udder size traits ($r= 0.21$ to 0.51; $P<0.05$). Positive correlations of cisternal area with machine milking fractions ($r= 0.47$ to 0.55) were also observed. Results indicate that cisternal scanning is an efficient method to evaluate the cistern size and the productive capacity of the ovine udder.

Key Words: Cisternal Milk, Alveolar Milk, Udder Scans

Insulin response to amino acid infusions in Holstein cows. C. A. Toerien*, J. P. Cant. University of Guelph, Guelph, Canada.

Despite reported positive effects of insulin (INS) on milk precursor availability, milk protein production and milk yield, few studies have focussed on the effect of individual amino acids on insulin release in dairy cows. In Experiment 1, our objective was to investigate the insulin response in non-pregnant cows in early lactation (EL; mean ± SEM: 32 ± 1 kg milk/d) to pulse i.v. infusions of phenylalanine (Phe), arginine, glycine, histidine or lysine (at 14 mg/kg BW). Baseline INS values were similar across treatments, and all amino acids stimulated similar total INS release (as area under the curve; AUC). Peak response above baseline (ng/mL) was the highest for arginine (3.4 ± 0.6) and differed from that of glycine and histidine (1.6 ± 0.5 and 1.3 ± 0.6; $P < 0.05$). In Experiment 2, we compared INS release to various levels of Phe, between non-pregnant lactating (Lact; 16 ± 0.5 kg milk/d) and dry (Dry) cows. Treatments were pulse i.v. doses of Phe at 7, 14, 76, and 112 mg/kg BW. Baseline INS was higher in Dry cows across treatments. Contrary to responses in Dry cows, high levels of Phe failed to elicit an appreciable response in Lact cows in AUC or peak INS. Results indicate that physiological state plays an important role in regulation of INS release in dairy cows. Because INS stimulates protein synthesis in various tissues, caution should be used when applying the flooding dose Phe technique to measure protein synthesis in cows at different physiological stages.

		Treatments (mg Phe/kg BW)				
	Group	7	14	76	112	Trt*Gr
AUC[1] ($ng.mL^{-1}.min^{-1}$)	Dry	17.9[a]	9.2[a]	95.9[b]	103[b]	P<0.001
	Lact	-9.1[a]	-7.4[a]	1.21[a]	0.9[a]	
Peak INS[1] (ng/mL)	Dry	1.9[a]	1.8[a]	7.7[b]	6.2[c]	P<0.001
	Lact	0.8[d]	0.9[d]	1.7[a]	2.0[a]	

[1]Differences within and between groups in AUC and Peak INS reported at $P < 0.05$.

Key Words: Insulin response, Amino acids, Dairy cows

Involvement of Oct-1 in transcriptional regulation of beta-casein gene expression in mouse mammary gland. Feng-Qi Zhao*[1], Takami Oka[2]. [1]University of Vermont, Burlington, Vermont, [2]National Institute of Health, Bethesda, Maryland.

Mouse beta-Casein gene promoter contains a region termed block C which is crucial for its gene transcription induced by lactogenic hormones. Nuclear extracts from mouse mammary glands contain at least two binding complexes (DS1 and DS2) which specifically bind to double-stranded block C region DNA. The binding sequence of these complexes was identified to be 5'- AAATTAGCATGT -3' which contains a sequence element related to the consensus octamer motif's complement ATTTGCAT. In the present study, we demonstrate that this sequence element indeed is the binding site for octamer-binding transcription factor (Octs) and Octs represent the double-stranded DNA binding proteins specifically binding to the block C region. Formation of the specific double-stranded binding complexes can be completely blocked by Oct binding motif oligonucleotides and anti-rOct1 antiserum. We also show that Oct-1B represents at least partial, if not all, double-stranded binding protein, DS1, in mammary nuclear extract. Oct-1B may functions as a transcriptional activator on casein gene promoter. The Oct binding activity to beta-casein gene promoter in the mammary gland is affected under influence of hormones both in vitro and in vivo. The DS1 binding activity can be induced by the combination of insulin, hydrocortisone and prolactin in virgin mouse mammary gland organ culture and induced by injection of progesterone or the combination of progesterone and estradiol in virgin mice.

Key Words: Transcriptional regulation, beta-Casein gene, Oct-1

Synthesis of insulin-like growth factor binding proteins by a bovine mammary cell line. F. Cheli*[1], A. Baldi[1], L. Rossi[1], M. Vestergaard[2], S. Purup[2]. [1]Dept. VSA, University of Milan/I, [2]Danish Institute of Agricultural Sciences/DK.

The insulin-like growth factor binding proteins (IGFBPs) are a family of locally-produced growth regulators involved in mammary gland development. The production of IGFBPs within the mammary gland is species specific and depends on the stage of mammary gland differentiation. The aim of this work was to investigate whether bovine mammary epithelial cells (BME-UV1) produce IGFBPs and whether retinoic acid (RA) modulates the production in vitro. BME-UV1 cells were kept cultured in either control medium or in media supplemented with either, insulin (1µg/ml), all-trans-retinoic acid (1µM), or insulin+RA. Cell proliferation was evaluated at 48 and 72 h. At the same time samples of the medium were collected. Concentration of IGFBPs was evaluated by Western ligand blotting. Autoradiographs from the blots were exposed for 14 days and were evaluated by desktop scanning densitometry. RA inhibited ($P<0.05$) proliferation of both control and insulin-stimulated BME-UV1 cells by 30 and 26%, respectively. IGFBP-2 and IGFBP-3 were detected in BME-UV1 culture medium. RA affected the relative distribution of the two IGFBPs in the culture medium. RA significantly ($P<0.01$) increased IGFBP-2 content in both control and insulin-stimulated cells. In conclusion, BME-UV1 cells produce IGFBP-2 and IGFBP-3 and the production seems to be regulated by RA. Results also indicate that RA-induced inhibition of BME-UV1 cell proliferation is related to an increase in IGFBP-2 in the culture medium.

Key Words: bovine mammary cells, retinoic acid, IGFBP

Influence of dietary starch and of phase of lactation on haematological markers of oxidative stress in early lactation. G. Stradaioli[1], G. Gabai[2], B. Stefanon*[1]. [1]Dipartimento di Scienze della Produzione Animale - Universita' di Udine (Italy), [2]Dipartimento di Scienze Sperimentali Veterinarie - Universita' di Padova (Italy).

Ten Friesian heifers were randomly assigned to two groups and fed until 30 DIM a basal TMR. At 35 DIM the control group (CTR, 24% starch/DM) continued to receive the same ration and the experimental group (EXP) was allotted to the experimental diets, which consisted in a stair step compensated starch regimen. Experimental diets were designed isoenergetic in order to have a reduction of starch (LSD, 21% starch/DM) followed to an increase (HSD, 28% starch/DM) of starch contents, with a final return to the basal diet. Blood was sampled at 37, 50, 60, 70, 80 and 94 DIM and analysed for glutathione peroxidase activity (GPx), glutathione (GSH), malondialdheyde (MDA), glucose (GLU), beta-hydroxy butyrate (b-OHB) and free fatty acids (FFA) concentrations. The stair step compensated starch regimen did not significantly affected milk yield and FCM between the groups during the experiment, the average milk yield for the EXP group being 25.88 kg/d, lower than the 27.42 kg/d of the CTR group. Plasma GLU was significantly lower with LSD diet and higher with HSD compared to mean values at 37 DIM. Plasma b-OHB significantly decreased in the EXP group at 70 DIM, when the LSD was replaced by HSD, but no variations were observed for plasma FFA concentrations. GPx activity decreased considerably after HSD administration to the EXP group (DIM 70 and 80), and recovered to initial mean value after return to basal diet(94 DIM). GSH concentrations, a measure used to identified antioxidant pool depletion, were not statistically affected from dietary variations of starch, although numerically lower mean values were observed during the LSD and HSD administration to the EXP group. Plasma MDA was significantly higher ($P<0.001$) for CTR group compared to EXP group, as was the interaction "dietary treatment" X "DIM" at 60 and 80 DIM. The results indicated that a moderate starch variation in the diet can contribute to enhance specific scavenger enzymatic activity, i.e. GPx, but did not substantially cause a reduction of blood antioxidant pool or an enhancement of MDA. The variations of plasma MDA were positively related to milk yield, indicating that milk production per se is a factor potentially affecting the level of oxidative stress during the early phase of lactation in dairy cows.

Key Words: Oxidative stress, Milk yield, Dairy cows

Effect of milking and a suckling/milking combination on oxytocin and prolactin release and on milk yield in crossbred Gir x Holstein cows. J. A. Negrao*[1], P. G. Marnet[2]. [1]USP/FZEA, FAPESP, Pirassununga/SP, Brazil, [2]UMR INRA/ENSAR, Production de lait, Rennes, French.

Unspecialized cattle farmers in Brazil have used crossbred Gir x Holstein cows to produce both calves and milk during spring and summer in extensive systems. Traditionally, these crossbred cows are reputed not to be well-adapted to machine milking and are milked with their calves. However, this type of management increases the labour of milkers and also milking time. For these reasons, 10 Gir x Holstein cows (F2) were used to evaluate the effect of different milking methods on oxytocin (OT) and prolactin (PRL) release and on milk yield. All experimental cows were milked twice/day: 5 cows were suckled by their calves, immediately before and after milking (SM group) and the other 5 cows were separated from their calves and submitted to exclusive milking (M group). Milk yield was recorded throughout lactation. Blood samples were taken on days 60, 61, 62 and 63 of lactation, before and after udder stimulation. Plasma concentrations of OT and PRL were measured by EIA and RIA method, respectively. Highest OT levels were observed during suckling, however there were no significant differences between peak levels of OT measured during suckling and milking in the SM group. At the same time, both groups had similar levels of OT during machine milking, however, the hormone profiles were different because OT increased more rapidly in the SM group (2 min after the beginning of milking) than in the M group (5 min). In general, the SM group showed higher PRL levels than the M group and during suckling, PRL levels were significantly higher in the SM than in the M group. Despite these results, SM cows produced more milk (milking plus suckling, 18.4 ± 1.2 L/day) than M cows (15.0 ± 0.4 L/day). Our results indicate that both type of management were effective to induce OT and PRL release in crossbred Gir x Holstein cows.

Key Words: Milking/suckling, Oxytocin, Prolactin

Milk emission during machine milking in dairy sheep. M. Rovai*, X. Such, G. Caja, J. Piedrafita. Universitat Autonoma de Barcelona, Bellaterra, Spain.

The aim of this work was to compare the milkability of Manchega (MN, 1.03 L/d, n=133) and Lacaune (LC, 1.71 L/d, n=79) dairy ewes. The kinetic parameters of milk emission during machine milking were measured at 6, 10 and 14 weeks post-partum. Data were recorded by the manual method in two successive days for individual udder halves during the evening milking. The curves were classified into three groups: 1 peak (1P), 2 peaks (2P) and in plateau (IP). The last type refers to ewes with larger emission curves and did not show clear differences between peaks (1 and 2). The frequency of different curve types (1P:2P:IP) was 25:66:9 and 5:60:35 for MN and LC, respectively, which means a greater milk ejection reflex in LC ewes (95% vs 75%). Milk production varied according to curve type in both breeds (0.82,1.03,1.16 L/d in MN and 0.93,1.68,1.82 L/d in LC for 1P, 2P and IP, respectively). The IP as compared with 2P, showed greater total emission volume (0.30 vs 0.24 L) and total time of emission (49 vs 46 s). As compared to MN ewes, LC showed greater ($P<0.001$) flow rate (0.39 vs 0.29 L/min), milk volumes (0.28 vs 0.16 L) and emission time (44 vs 39 s) for all curve types. All parameters of milk emission kinetics increased with age in LC ewes ($P<0.01$), whereas in MN ewes the differences were observed only for some parameters which may be due to low variation in milk yield with age in MN ewes in this experiment. The frequency of milk emission type curves varied according to the parity number, showing an increase in the ejection reflex with age. Throughout lactation, all kinetic parameters decreased ($P<0.001$) except time of latency (28 s) and second peak flow (37 s) that remained constant in 2P'ewes, suggesting that the time of milk ejection reflex did not change throughout lactation. Moreover, the percentage of ewes presenting the ejection reflex decreased throughout lactation. In conclusion, herds selected for high milk production (e.g. LC ewes) tended to have a better milk ejection reflex (2P and IP) and milkability.

Key Words: Dairy Ewes, Milking Kinetics, Milk Ejection

Induction of milk yield decrease and mammary gland involution in lactating Holstein cows and female rats. L. Delbecchi, N. Miller, D. Petitclerc, P. Lacasse. AAFC-Dairy and Swine R&D Centre, Lennoxville, Quebec, Canada.

Increasing amounts of estrogens in the blood of pregnant lactating cows may be one factor inducing the progressive involution of the mammary gland after the peak of lactation. In a first experiment, non-pregnant, mid-late lactation cows received sc. injections of either 17ß-estradiol (15 mg/cow/day; treated group, n=4) or 95% ethanol (control group, n=4) from d 0 to d 8. Measurement of milk production (d -10 to d 20) and evaluation of milk composition (before, during and after estradiol injections) showed that treated cows presented signs of mammary

gland involution. Milk production was reduced ($P < 0.01$) in treated versus control cows by 14.8% on d 3, 37.2% on d 6, 76.5% on d 8, and 81.6% on d 11. Between d 0 and d 7, in treated cows, milk fat content and lactose concentration decreased ($P<0.05$) by 37.6% and 15.9%, respectively, while milk protein content increased by 61.9% ($P<0.05$). Control cows showed no significant variation in these parameters during the same period. Quantitative RT-PCR was performed on RNA extracted from mammary biopsies taken at d 0, d 1, d 2, and d 4. Levels of b-casein mRNA were reduced ($P<0.05$) by estradiol but those of bax and bcl-2 were not significantly affected, suggesting a lack of short-term effect of estradiol on regulation of apoptosis in the mammary gland. In a second experiment, three potential inhibitors of estradiol, i.e. trans-retinoic acid (tRA), melatonin, and mimosine were tested in lactating female rats. Milk production was reduced ($P<0.05$) in rats injected with estradiol as compared to controls. Melatonin and mimosine showed no significant effect ($P>0.1$) on this reduction, while tRA enhanced it ($P<0.01$). Analysis of gene expression is currently performed on mammary biopsies taken at the end of treatments. These results support the hypothesis that estrogens produced by the fetal-placental unit induce a gradual decline in milk production in pregnant lactating cows, but the molecular bases for this effect remain unclear. Work supported by Dairy Farmers of Ontario and Agriculture and Agri-food Canada.

Key Words: Involution, Mammary gland, Estrogens

Cloning lactoferrin gene in a novel expression vector and its expression/secretion in bovine mammary cells. N. Bissonnette*, P. Lacasse, D. Petitclerc. Agriculture and Agri-Food Canada, Dairy and Swine Development and Research Center.

Milk is a source of nutrients and a carrier of various forms of specific factors influencing bacterial growth, which may have significant benefit for the health of the suckling neonates. Milk proteins, such as lactoferrin (LF), are part of the innate immune system with antimicrobial properties and are an important component of this line of defence of the mammary gland. LF is a glycoprotein naturally produced by mammary cells and is found in milk of human (1-6 g/L) and cows (0.01-0.1 g/L). The aim of this study was to develop a molecular tool that permits the cloning and the expression of a bactericidal gene without killing the bacteria when the DNA vector is produced but allowing its full bactericidal expression when secreted by transfected eukaryotic cells. As conventional eukaryotic vectors with commonly used promoters (CMV, RSV or SV40) permit a basal expression in bacteria, others and we did not manage to clone lactoferrin gene in bacteria using such expression vectors. Expression systems reported so far to produce recombinant LF protein have used molds or yeasts. As glycans on mammalian glycoproteins influence their functions in many different ways, the major drawback of these lower eukaryotic systems is their inappropriate post-translational modifications. Hence, a method of inhibiting, in bacteria, LF gene expression by using a single eukaryotic expression vector without any repressor/activator molecule was developed. This system allowed the cloning of the bactericidal lactoferrin gene in an expression vector using bacteria as a host and permitted the production of high amount of vector by bacteria. Subsequently, this vector allowed the secretion of 200 ug/L of LF using our eukaryotic expression vector when transfected in bovine mammary cells. Therefore, DNA expression vector could be quantitatively produced by bacteria; thereby, large quantity of a bactericidal therapeutic gene, therein lactoferrin, could be used with the aim of being expressed as antimicrobial protein in transfected or transgenic eukaryotic cells.

Key Words: lactoferrin, expression vector, gene therapy

Effect of milking interval on milk yield and quality and the rate of recovery during subsequent frequent milking. K. Stelwagen*, V.C. Farr, S.R. Davis. AgResearch Ltd., Hamilton, New Zealand.

Forty multiparous cows (DIM: 118 ± 18) on an all-pasture diet were used in a completely randomised block design to examine the effects of milking interval (MI) on milk yield and quality, and its recovery during subsequent frequent milking. Following 2 d of normal twice-daily milking cows were not milked for either 6, 12, 18, 24 or 30 h, after which they were milked every 6 h for 24 h. Means shown are for, respectively, the 6, 12, 18, 24 and 30-h MI. Milk yield increased with increasing MI, but plateaued after 24 h of milk accumulation (4.7^{a} *vs.* 8.2^{b} *vs.* 11.5^{c} *vs.* 15.2^{d} *vs.* 16.4^{d} ± 0.6 kg, $^{abcd}P<0.01$). Although, yield recovered to at least pre-MI yields for all groups, the rate of recovery was slowest for the highest MI (regression coefficients: 0.72^{a} *vs.* 0.70^{ab} *vs.* 0.68^{b} *vs.* 0.67^{bc} *vs.* 0.64^{c} ± 0.02 kg/h, $^{abc}P<0.05$). The decrease in milk secretion after 18 h of milk accumulation coincided with an increase in

mammary tight junction permeability, based on plasma lactose levels (27^{a} *vs*. 29^{a} *vs*. 47^{a} *vs*. 255^{b} *vs*. 413^{c} ± 40 µM, $^{abc}P<0.001$). Increased permeability increased the concentration of serum albumin in milk (184^{ab} *vs*. 146^{b} *vs*. 211^{bc} *vs*. 235^{c} *vs*. 234^{c} ± 19 µg/ml, $^{abc}P<0.07$), and differences remained during the first three subsequent 6-hourly milkings. SCC(*1000/ml, ln-transformed) were not different at the end of each MI, but were significantly elevated in the milk from cows in the 24-h and 30-h groups during the subsequent frequent milking period (4th 6-hourly milking: 4.0^{a} *vs*. 4.1^{a} *vs*. 4.4^{a} *vs*. 5.0^{b} *vs*. 5.1^{b} ± 0.2, $^{ab}P<0.05$). This is consistent with earlier data on once-daily milking, showing an increase in SCC after a 24-h lag phase. In conclusion, the rate of milk secretion begins to decrease with MIs excess of 18 h, which may be, at least partly, related to increased mammary tight junction permeability, and leads to poorer milk quality. The adverse effects (except SCC) can be reversed if the long MIs are followed by a 24-h period of frequent milking.

Key Words: Milking interval, Milk quality, Tight junction

Leptin variations in dry and lactating periods of dairy cows with different genetic merit. R. Lombardelli[1], P. Bani[1], C. Delavaud[2], Y. Chilliard[2], G. Bertoni*[1]. [1]UCSC, Facolta di Agraria, Piacenza, Italy, [2]INRA-UHRH, Theix, France

Even though leptin is a quite recently discovered hormone, it has been intensively studied suggesting that it operates both directly and indirectly to orchestrate complex pathophysiological processes. In a previous paper we did demonstrate that, in the early lactation, dairy cows of different genetic merit mobilise their protein and fat reserves with a different intensity. To ascertain a possible role of leptin, 11 dairy cows of low (LG), medium (MG) and high (HG) genetic merit, 50 days before to 300 days after calving were checked: daily for milk yield and dry matter intake, twice a week for blood metabolites and hormones (including leptin) and fortnightly for live weight (LW) and BCS (on a 0-5 scale). The 300 days' mean milk yield was: 20.6 (LG), 27.2 (MG) and 32.8 kg/d (HG). Maximum post-partum LW loss was lower in LG (6%) than in MG (14.6%) and HG (13.8%). The level of leptin was not strictly related to the genetic merit, but 5 animals showed constantly low values (1.5 - 3.5 ng/ml) during dry and lactation stages; 5 more animals showed quite high values during dry period (7 - 11 ng/ml), a sharp decline after calving (1.5 - 3.5 ng/ml) and a partial recovery afterwards (2.5 - 4.0 ng/ml). The last one showed constantly high values during the whole experiment. Among the main data to be discussed there is the positive correlation between leptin and BCS found in LG cows only. Moreover, glucagon is related to leptin but in a positive way for LG cows whereas the reverse is true for the HG ones. Other important data are the higher live weight (but not BCS) and lower GH values recorded in the cows with higher leptin during late pregnancy. Also different, but not significantly, are milk yield, DMI and insulin, all higher in the latter cows. (Supported by RAISA- CNR).

Key Words: Leptin, Genetic merit, Dairy cow

Comparison of milk yield and of oxytocin and cortisol release during machine milking in Gir, Gir/Holstein and Holstein cows. J. A. Negrao*[1], P. G. Marnet[2]. [1]USP/FZEA, FAPESP, Pirassununga/SP, Brazil, [2]UMR INRA/ENSAR, Production de lait, Rennes, French.

Gir cows (adapted to tropical conditions) and Holstein bulls (more productive) have been crossbred in Brazil to improve dairy production. In general, this crossbreeding program was a success and at present, many specialized farmers have Holstein cows with varying percentages of Gir blood. Although Gir cows and Gir/Holstein cows are reputed to be easily stressed and not well-adapted to machine milking, adaptation to exclusive machine milking has not been described in the literature for these cows. Taking this into account, 6 Gir cows (group G), 6 Gir x Holstein cows (F3; group GH) and 6 Holstein cows (group H) were used to evaluate the effect of exclusive machine milking on oxytocin (OT) and cortisol (CORT) release and on milk yield. Milk yield was recorded throughout lactation. Blood samples were taken on days 45, 48, and 51 of lactation, before and after milking. Plasma concentration of OT was measured by EIA method and CORT was measured by commercial EIA Kit (dslab Inc). As expected, milk yield was significantly higher in the H group (25.6 ± 3.3 L/day) than in the GH group (20.2 ± 1.7 L/day), and the GH group produced more milk than the G group (13.1 ± 1.0 L/day). In contrast, all groups exhibited similar levels of OT, although OT increased more rapidly during milking of the H and GH groups (1 min) than of

the G group (3 min). Simultaneously, CORT levels were significantly different for groups, the G group presented highest levels of CORT than GH and H groups. Our results indicate that G, G/H and H groups presented similar release of OT during exclusive milking, however CORT levels were inversely related to milk production.

Key Words: Milking, Oxytocin, Cortisol

Mixed linear model analysis of factors affecting the evolution of milk electrical conductivity along lactation in dairy cattle. [1]N.P.P. Macciotta, *[2]M. Mele, [1]A. Cappio-Borlino, [2]P. Secchiari. [1]Dipartimento di Scienze Zootecniche - Università degli Studi di Sassari, Italy, [2]D.A.G.A. Settore Scienze Zootecniche - Università di Pisa, Italy.

Electrical conductivity (EC) of cow milk is affected by the health status of the mammary gland but also by other factors that usually affect milk yield such as variations among cows, Test date, stage of lactation and parity. In order to evaluate the effects of all these factors on EC, Test Day (TD) records of an index of EC obtained by means of a computerized milk meter and milk yields of 138 Holstein Friesian cattle affected by clinical mastitis were analysed by the following mixed linear model: Y= H + TD + PA + DIM + MAST + DIM(MAST) + LATT + E where H is the effect of the herd (2 levels), PA is the effect of parity (1, 2, 7), TD is the effect of test date (1020 levels), DIM indicates the effect of lactation stage (30 levels of 10d each), MAST is the effect of the period of mastitis occurrence (3 levels: MAST1=<100d, MAST2=99< and <200, MAST3=>199), LATT is the random effect of individual lactation, E is the random residual. EC and milk yield curves for different classes of period of mastitis occurrence were constricted by plotting DIM(MAST) estimates against days in milking. EC tends to increase along the lactation, with a different behaviour in the different classes of period of mastitis occurrence and with a variance among animals equal to about 50% of the total random variability. EC in MAST1 cows was higher than in MAST2 and MAST3 cows at the beginning of lactation (mS 12.07 vs. 11.43 and 11.55 respectively) but curves of MAST1 and MAST2 were quite similar from about 150 DIM. The EC curve of MAST3 cows showed an increasing rate markedly lower than the other two classes during the whole lactation. Parity affected EC, with first calving cows having lowest values. Milk yield was affected by all fixed factors considered in the analysis except from MAST class; however, lactation curves separated for this last effect show a low peak for cows having mastitis >100 DIM and a higher persistency for those that were affected by the disease in the last part of lactation.

Key Words: Electrical conductivity, mastitis, milk

Effect of stimulation intensity on oxytocin release before and after milking. D. Weiss*, A. Dzidic, R.M. Bruckmaier. Institute of Physiology, Techn. Univ. Munich-Weihenstephan.

Oxytocin (OT) released from the pituitary causes myoepithelial contraction and milk ejection. Elevated concentrations of OT are necessary throughout the whole milking process to ensure complete milk ejection. The objective of this study was to test the effect of teat stimulation intensity on the level of OT released. Six Brown Swiss cows were machine milked at 5 a.m. and 4 p.m. and blood samples were taken during milking at 1-min intervals for OT analysis. Milk flow was recordered on a quarter level. Control milking (CM) corresponded to daily milking routine and included forestripping, dry paper cleaning and a 1-min vibration stimulation before the start of milking. In addition, vacuum in liner closed position without pulsation was applied, for 5 min, either before the start of milking (LCBM) or after the end of milking (LCAM). In a third treatment a 1-min vibration stimulation was applied to one quarter before the end of milking (STIMO). Stripping was performed in all treatments if total milk flow declined below 0.3 kg/min. Milk yield, milking time and average milk flow rate did not differ between treatments. During liner closed phase before milking (LCBM) OT concentrations were significantly ($P = 0.05$) higher compared to the period before teat cleaning in CM. Area under curve (AUC)/min during this period was 7.5 ± 1.0 pg/ml and 5.2 ± 0.9 pg/ml, respectively. The slightly increased OT concentrations in LCBM were sufficient to induce the alveolar milk ejection as indicated by the absence of bimodal milk flow curves in LCBM. LCAM had no obvious stimulatory effect on OT release, the decline in OT concentrations after the end of milking was similar in LCAM and CM. In STIMO, no effect of the additional stimulation on OT levels could be observed as compared to CM. In conclusion, different degrees of stimulation by the milking machine release different amounts of OT. However, only slightly elevated OT levels induce milk ejection at the start of milking.

Key Words: oxytocin, cow, milking

Factors affecting level and post-feeding behaviour of insulin in dairy cows. G. Bertoni*, E. Trevisi, R. Lombardelli, F. Piccioli Cappelli. UCSC, Facolta di Agraria, Piacenza, Italy.

It is well-known that blood insulin level is reduced after calving and particularly in high yielding dairy cows although the data regarding the insulin release factors and/or its receptors sensitivity seem contradictory. To contribute to clarify some of these aspects we have studied the post-feeding behaviour of blood insulin in cows with different parity (trial 1) or genetic merit (trial 2) during the last month of pregnancy and the first 3 months of lactation. In the 1st trial 4 cows were considered in their 1st (L1) and 2nd lactation (L2); while 8 multiparous cows, 4 of high (HG) and 4 of average (AG) genetic merit, were used in the 2nd trial. Blood samples were taken every week before the morning meal and 1, 2, 3, 4, 5 and 6 hours after it, for metabolic profile and insulin determination. The environment conditions and diets were kept constant during the trials, while feed intake, milk yield and BCS were recorded. It is confirmed that insulin in dairy cows is strongly reduced before calving and rises again 1-2 weeks later, reaching the levels of dry period after 10 weeks. Furthermore, the after-meal insulin increase is stronger in dry period (+30-50%) than in the first 2 months of lactation (+10-30%). L1 and L2 of the same cows seem to have similar insulin values and behaviour after meal, particularly from 2 weeks before to 8 weeks after calving. HG showed lower levels of insulin before and after calving ($P<0.05$ vs AG). As regards the after-meal behaviour, HG had a similar but more prolonged rise in dry period, whereas the increase was less marked after calving. These variations were related to the glucose levels (positively), but particularly in AG cows and around calving; a negative correlation has been observed with ßOHB, NEFA and BCS, particularly for HG cows after calving. In general, insulin changes do not seem strictly related to the energy balance indices. It appears that insulin level and after-meal behaviour are strongly affected by stage of lactation and that genetic merit and/or parity could contribute to explain them.

Key Words: Insulin, Post-feeding behaviour, Dairy cow

Evidence for the presence of the cationic amino acid transporter CAT-1 in porcine mammary gland during lactation. J. Perez Laspiur*, J.L. Burton, P.S.D. Weber, N.L. Trottier. Michigan State University.

Biomedical literature suggests that mammary epithelial cell expression of the CAT-1 cationic amino acid transporter is important in determining cell activity. It is specific for transporting lysine, and thus may be involved in regulating milk protein synthesis. Manipulation of CAT-1 expression may help producers control the volume and quality of milk for neonatal animals. The objective of the current study was to determine if CAT-1 is expressed in porcine lactating mammary tissue. A multiparous sow was sacrificed on day 19 of lactation for this work, and the udder removed immediately following cessation of heartbeat. Four anterior mammary glands (two from each lateral side) were isolated, the parenchymal tissue collected and cut into approximately 1.0-g pieces and frozen in liquid nitrogen. Total RNA from mammary tissue was isolated using the TRIzol Reagent method. Samples (10-µg) of total RNA were run in duplicate on a Northern blot to assess CAT-1 gene expression. Expression of CAT-2 (another member of the CAT family of amino acid transporters) was used as a negative control and ß-actin expression as a RNA loading control. Duplicate liver RNA samples from a prepubertal gilt were used as the positive control for CAT-2 expression. Human CAT-1 and CAT-2 cDNA probes (donated by Dr. E. I. Closs, Johannes Gutenberg University, Germany) and a rat ß-actin cDNA were ^{32}P-labeled and hybridized sequentially to the Northern blot with complete stripping of the probes between hybridizations. Resulting autoradiographs revealed low level expression of CAT-1 in day 19 of lactation mammary tissue with no detectable expression in liver. As expected, CAT-2 was highly expressed in liver but not in mammary parenchyma. These preliminary results are the first to show that CAT-1 is expressed during lactation in porcine mammary tissue.

Key Words: cationic amino acid transporter, lactating mammary gland, porcine

Oxytocin release and milk ejection induced by teat cleaning in a single stall automatic milking system. A. Dzidic, D. Weiss, R.M. Bruckmaier*. Institute of Physiology, Techn. Univ. Munich - Weihenstephan, Freising, Germany.

Oxytocin (OT) is released in response to tactile teat stimulation and causes alveolar milk ejection. The objective of this study was to evaluate the effect of teat cleaning by two rolling brushes on OT release and milk ejection during milking in a single stall automatic milking system (AMS, Merlin, Lemmer-Fullwood). Forty-eight German Fleckvieh cows were investigated during their voluntary milkings. Five treatments B0 (no brushing), B1 (1 brushing cycle for 16 s, 4 s per teat), B2 (2 brushing cycles), B4 (4 brushing cycles) and B6 (6 brushing cycles) were performed for 2 days each and quarter milk flow was recorded. In addition blood samples were taken from 10 cows during milking at 1-min intervals for OT determination in treatments B0, B2, B4 and B6. Basal OT concentrations were similar (2.7 to 3.8 pg/ml) in all treatments. At the start of milking, OT concentration was lower ($P<0.05$) in B0 as compared to all other treatments. One min after the start of milking OT concentrations did not differ between treatments (20.8 to 26.8 pg/ml). OT concentrations throughout milking (AUC/min) were 26.5±4.1, 25.6±4.9, 20.2±2.7 and 19.8±3.1 pg/ml in B0, B2, B4 and B6 respectively. The portion of bimodal curves decreased ($P<0.01$) with increasing number of brushing cycles (46, 25, 11, 1 and 0 % in B0, B1, B2, B4 and B6 respectively). Time until milk ejection occurred, recorded in B0, decreased ($P<0.05$) with increasing degree of udder filling (defined as a percentage of actual milk yield from maximum milk yield in month two of the current lactation). At low degree of udder filling (i.e. after short interval from previous milking) cows needed a longer pre-stimulation for well-timed induction of milk ejection and to reduce number of bimodal curves. In conclusion, the teat cleaning device in the used AMS was suitable to induce milk ejection in cows before the start of milking. Duration of teat cleaning needs to be adapted to the actual degree of udder filling.

Key Words: Oxytocin release, Milk ejection, AMS

mRNA expression of immunologically important factors and milk proteins in mammary tissue of dairy cows during LPS-induced mastitis. S. Schmitz, M. W. Pfaffl, H. H. D. Meyer, R. M. Bruckmaier*. Institute of Physiology, Techn. Univ. Munich-Weihenstephan, Freising, Germany.

Inflammatory factors are known to increase during mastitis. This study was conducted to determine changes of mRNA expression of various immunologically important factors in mammary tissue during the first 12 h of lipopolysaccharide (LPS) induced mastitis. Five healthy lactating cows were injected in one quarter with 100 µg E.coli-LPS (O26:B6) and the contralateral quarter with saline (9 g/l) serving as control. mRNA expression in mammary biopsy samples of various factors at 0, 3, 6, 9 and 12 h after LPS administration was quantified by real-time RT-PCR. Blood samples were taken following the same time course and rectal temperature was measured at 1-h intervals. Temperature increased until 5 h ($P<0.05$) after LPS administration and decreased to pretreatment levels within 24 h after LPS-challenge. Blood leukocyte number decreased ($P<0.05$) from 0 to 3 h from 7.7±1.1 x 10^9/l to 5.7±1.0 x 10^9/l and thereafter recovered to pretreatment levels until 12 h after LPS-challenge. In LPS-challenged quarters tumor necrosis factor a and cyclooxygenase-2 mRNA expression increased to highest values ($P<0.05$) at 3 h after LPS-challenge. Lactoferrin, lysozyme, inducible nitric oxide synthase mRNA expression increased ($P<0.05$) and peaked at 6 h after challenge, while platelet-activating factor acetylhydrolase mRNA increased only numerically. mRNA expression of the investigated factors did not change in control quarters. mRNA expression of insulin-like growth factor-1, 5-lipoxygenase and of aS1-casein (CN), aS2-CN, ß-CN and ß-lactoglobulin did not change significantly, whereas mRNA expression of a-lactalbumin decreased ($P<0.05$) in LPS-treated and control quarters and that of ?-CN only in the LPS-treated quarters. In conclusion, mRNA expression of most inflammatory factors changed within hours, whereas that of most milk proteins remained unchanged.

Key Words: LPS-challenge, Mastitis, Inflammatory factors

Body lipid change in lactation: consequences for the prediction of energy requirements. N. C. Friggens*, K. L. Ingvartsen, G. C. Emmans. Danish Institute of Agricultural Sciences, Foulum, Denmark.

The size of the body lipid reserves (L) of the dam changes in a characteristic and repeatable manner through lactation in virtually all mammals. This is an evolutionary adaptation designed to support the changing reproductive priorities of the dam that is largely independent of current feed availability. Pregnancy leads to an increase in L to help meet the nutritional demands of the following lactation. Lactation is characterised by an initial decline in L followed by a return to the pre-pregnancy level. These patterns of changing L persist even under conditions that can reasonably be assumed to be nutritionally non-limiting. Thus there is a genetically driven, and therefore predictable, cycle of body energy mobilisation and deposition. Prediction of the cow's energy requirements can be substantially improved, particularly in early lactation, by incorporating genetically driven body energy mobilisation. With very few exceptions, existing prediction systems do not account for this. This paper presents in detail a method to quantify the genetically driven rate of change of L (dL/dt) at any given timepoint in lactation. The method requires assumptions about target levels of L at calving and in the pre-pregnant state, and about the time taken from calving to return to the pre-pregnant state. These assumptions are discussed and experimental results presented concerning the effects of breed and parity on the parameter estimates. The method requires input estimates of actual L at calving and time from calving to subsequent conception. A method to estimate L from body condition score and liveweight is described. In addition to being a practical means to improve prediction of energy requirements, this method provides a useful basis for exploring genetic variation in body lipid mobilisation and characterising the consequences of genetic selection on the lactational cycle in body lipid reserves. These issues are discussed.

Key Words: Lipid mobilisation, Lactation, Energy

Serum insulin-like growth factor 1 and placental lactogen profiles in Holstein nulliparous and multiparous cows in early gestation. W. J. Weber*[1], C. R. Wallace[2], H. Chester-Jones[1], B. A. Crooker[1]. [1]University of Minnesota, St. Paul, [2]University of Maine, Orono.

A positive relationship between serum insulin-like growth factor (IGF-1) and placental lactogen (bPL) in dairy cattle suggests that the increase in bPL may contribute to an increase in IGF-1 during gestation. However, this relationship is not well established. Objectives of this study were to examine the relationship between serum bPL and IGF-1 in nulliparous and multiparous Holstein cows in early gestation. Blood samples from nulliparous (n=17) and multiparous (n=15) cows were collected (± 3d) at 56, 70, 84, 98, 112, 140 and 168 d of gestation. Multiparous cows were less than 200 days in milk when sampling was initiated. Serum samples were analyzed for IGF-1 and bPL by RIA. Data were analyzed as repeated measures using PROC MIXED and results reported as least squares means. Means were considered different when $P < 0.05$. Serum bPL was less in nulliparous than multiparous cows (0.16, 0.31 ± 0.03 ng/ml) and increased from d 56 to 168 of gestation (0.11^{a}, 0.15^{b}, 0.19^{bc}, 0.20^{c}, 0.26^{d}, 0.31^{e}, 0.43^{f} ± 0.03 ng/ml). The rate (2.7 $\mu g \cdot ml^{-1} \cdot d^{-1}$) and overall (0.32 ng/ml) increase in bPL was similar for both parities from d 56 to 168 of gestation. Serum IGF-1 was greater in nulliparous than multiparous cows (231, 115 ± 5.0 ng/ml) and increased from d 56 to 168 of gestation (164^{a}, 167^{a}, 168^{a}, 165^{a}, 174^{ab}, 182^{bc}, 191^{c} ± 5.2 ng/ml). Although there was no interaction, the increase in IGF-1 in multiparous cows (35 ng/ml, 33%) was greater than in nulliparous cows (20 ng/ml, 9%) during this 122 d interval. From 56 to 98 d of gestation, serum bPL and IGF-1 in multiparous cows were relatively stable but both began to increase by d 112 and continued to increase through d 168 of gestation. The greater IGF-1 concentration in nulliparous cows made this relationship less apparent. The strong relationship between bPL and IGF-1 during early gestation supports the concept that bPL may play a role in regulating serum IGF-1.

Key Words: Gestation, IGF-1, bPL

Detecting beta-casein and beta-lactoglobulin variants using real-time PCR taking advantage of single nucleotide polymorphisms in milk cell DNA. Ralf Einspanier*[1], Andreas Klotz[1], Johann Buchberger[2], Ingolf Krause[2]. [1]Institute of Physiology TU Munich Germany, [2]Institute of Chemistry TU Munich Germany.

In cattle several genetic variants for the beta-casein and the beta-lactoglobulin locus have been described. With regards to a possible selection of genotypes being favorable to cheese making, we have applied a new technique (real-time-PCR) to determine the main genotypes of bovine beta-LG and beta-CN variants. The aim of this study was to rapidly detect genetic variants of beta-casein (beta-CN A1, A2, B) and beta-lactoglobulin (beta-LG A, B, C, D) directly from milk. Through introducing non-invasive and faster methods it appears advantageous to use milk cells instead of other DNA sources like blood. After the initial characterization of distinct mutations in the genome using PCR amplification, deduced proteins were verified by isoelectric focusing of corresponding milk samples. Furthermore, a partial nucleotide sequence of the beta-LG-gene D, containing allele-specific point mutations, could be determined. For beta-CN allel-specific mutations occur at amino acid residue 67 and 122, whereas for the beta-LG variants specific mutations occur at amino acid residues 45, 59, 64 + 118. Based on specific PCR fragments generated from milk cell DNA, genotyping of alleles of beta-CN and beta-LG or admixtures becomes efficient and simultaneous. Hence, a real-time PCR approach (LightCycler) was established specifically distinguishing three important beta-CN milk protein variants with remarkable benefits when compared to other DNA-based mutation detection systems. As a consequence, genotyping of cattle will become more easily and faster through introducing this new technique.

Effect of contact time between calves and cows on IgG transfer, cortisol release, milk yield and residual milk. F. A. Paiva, A. R. Bueno, A. Saran-Neto, M. S. Freiria, J. A. Negrao*. USP/FZEA, FAPESP, Pirassununga/SP, Brazil.

At parturition, cows and calves remain in contact for several hours and this period is essential for adequate absorption of IgG and for survival of neonates. During extended contact time, cows become selective and take longer to adapt to milking. Our objective was to verify if contact time between cows and calves could influence IgG transfer and milking adaptation. Thus, 18 Holstein cows and their calves were divided into 3 groups: SC group (short contact); cows and calves remained 6 h in contact, PC group (periodic contact); cows and calves were brought together twice/day for 30 min and LC group (long contact); cows and calves remained in contact for 3 days. Following, cows and calves of the SC and LC groups were definitively separated. After separation, all calves received 4L of colostrum/day. All groups were submitted to 2 milkings/day, without calves. Blood samples were taken once a day from cows and calves, from parturition to 4 days post-parturition. Milk yield and residual milk were measured on days 4, 15 and 30 of lactation. Cortisol (CORT) level was measured using EIA kit (dslabs, Inc) and IgG level was determined by radial immunodiffusion. After parturition, all cows had similar levels of IgG. Before separation, LC calves presented lower CORT levels than other calves but after separation, LC calves had a higher increase in CORT levels than PC calves. CORT profiles of cows were similar, however LC cows exhibited higher CORT levels after separation than PC and SC cows. During first milkings, SC and PC cows produced more milk (19.5 ± 1.1 L and 19.2 ± 0.5 L, respectively) than LC cows (16.5 ± 5.6 L). On day 4, residual milk was higher for LC and PC cows (6.53 ± 0.7 L and (5.6 ± 0.4 L, respectively) than for SC cows (2.0 ± 0.2 L). On days 15 and 30, milk yield and residual milk were similar for all groups. Our results indicate that IgG transfer was adequate to all calves, and that adaptation to milking was not influenced by contact time between cows and calves.

Key Words: Milk ejection, Milk residual, Cortisol

Prolactin receptor expression responds to photoperiod similarly in multiple tissues in dairy cattle. T. L. Auchtung*[1], B.C[1]. Pollard, P.E. Kendall[1], T.B. McFadden[2], [1]G.E. Dahl[1]. [1]University of Illinois, Urbana, IL, [2]University of Vermont, Burlington, VT.

Photoperiod (PP) influences circulating prolactin (PRL) concentrations in cattle. Prolactin exerts its effects through its receptor, which has two isoforms in the bovine. Therefore, it is likely that PP also has an effect on PRL receptors (PRLR), which are present in many tissues of the body. The objective of this experiment was to identify the effect of photoperiod on PRLR expression in multiple tissues of dairy cattle. Holstein steers (n = 10) were maintained on either long day photoperiod (LDPP; 16 h light: 8 h darkness) or short day photoperiod (SDPP; 8 h light: 16 h darkness) for 9 wk, then photoperiod treatments were reversed for four weeks. Liver tissue was obtained via biopsy at 2-wk intervals throughout the 13 wk experiment. Lymphocytes were isolated from blood collected on heparin at four times during the experiment. Density gradient centrifugation of the buffy coat through Histopaque-1077 was followed by washing of cells with RPMI-1640 cell growth media. Mammary tissue

was collected via biopsy at the end of the experiment. Isolation of RNA from all tissues was performed using Trizol reagent and RNA was converted to cDNA prior to real-time PCR. Concentrations of plasma PRL, measured by RIA, were greater ($P < 0.05$) in LDPP than SDPP animals. Compared to LDPP, SDPP increased ($P < 0.01$) expression of PRLR mRNA in liver at Week 5, and responses to photoperiod at Week 13, four weeks after the treatment reversal, were similar to those at Week 5 ($P < 0.05$). Lymphocyte responses were similar, with SDPP increasing PRLR mRNA expression significantly ($P < 0.05$) over LDPP, both before and after the treatment reversal. Expression of PRLR mRNA in mammary tissue was also increased ($P < 0.01$) in SDPP animals relative to LDPP. In summary, PRLR mRNA expression in liver, mammary, and lymphocytes is increased in animals on SDPP treatment as compared with LDPP. Expression of PRLR in lymphocytes provides a minimally invasive method to monitor PRLR expression in multiple tissues.

Key Words: Cattle, Prolactin Receptor, Photoperiod

Possible role of enterolactone on mammary development and lactation in cattle. S. Purup, M. Vestergaard, M.R. Weisbjerg, T. Hvelplund, K. Sejrsen. Danish Institute of Agricultural Sciences, Foulum.

The lignan enterolactone (Enl) is produced by microbial fermentation of the phyto-estrogens secoisolariciresinol (Seco) and matairesinol (Mata) in the gastro-intestinal tract. Seco and Mata occur as glycosides in wholegrain cereals, seeds, nuts, vegetables and berries. The objective of the present study was to measure the concentration and bioactivity of Enl in milk and blood and to investigate the effect of Enl on proliferation of mammary epithelial cells in culture. Blood and milk was collected from 35 dairy cows fed diets either with grass-clover silage or whole-crop barley silage as the main roughage. Concentrations of Enl in whey and serum was measured by TR-IFMA. Bioactivity of whey and serum was studied in mammary epithelial cells isolated from prepubertal heifers and cultured in collagen gels for 5d. Proliferation of epithelial cells was determined during the final 24 h of culture using [methyl-^{3}H]thymidine incorporation as a measure of DNA synthesis. The effect of Enl on mammary epithelial cell proliferation was investigated by addition of Enl in concentrations of 10-100,000 ng/ml. Concentrations of Enl were 1.84 and 2.40 ng/ml (P<0.10) in whey and 177 and 249 ng/ml (P<0.01) in serum from dairy cows fed diets based on grass-clover silage and whole-crop silage, respectively. Whey or serum added to mammary epithelial cells in concentrations of 0.5-10% in culture medium showed no significant differences in cell proliferation due to silage type. The effect of Enl added to cell culture medium on mammary epithelial proliferation was biphasic. Enl at low concentrations (10 and 100 ng/ml) stimulated proliferation slightly (approximately 15%; P<0.06 and P<0.05, respectively), whereas higher concentrations (>10,000 ng/ml) strongly inhibited (P<0.01) cell proliferation. Maximal inhibition at 100,000 ng/ml corresponded to a 97% inhibition (P<0.001) of mammary cell proliferation. It is suggested that phyto-estrogens such as Enl may have a role in mammary development and lactation in cattle.

Key Words: Enterolactone, Mammary Cells, Cattle

Effects of omitting one milking per week on milk yield, milk composition and udder health of dairy cows. M. Ayadi[1], G. Caja*[1], X. Such[1], E. Albanell[1], M. Ben M'Rad[2], R. Casals[1], [1]Universitat Autonoma de Barcelona, Spain, [2]Institut National Agronomique de Tunisie, Tunisia.

Five Holstein dairy cows (milk yield: 21.0 ± 3.4 l/d; 227 ± 67 DIM) were used for 10 weeks to study the effect of omitting one milking per week (Sunday afternoon) throughout lactation on milk yield, milk composition and udder health. Cows were milked twice a day (8.00 and 18.00 h) but on Sunday one milking only was performed at 12.00 h. Milk yield from each milking was recorded. Milk samples were taken individually from each milking to analyze milk composition and somatic cell count (SCC). Average milk yield and composition for Friday and Saturday were used as reference values to evaluate the effect of changing the milking frequency. Milk yield and milk composition did not vary (P > 0.14) during the experimental weeks, but SCC increased with lactation stage. On Sundays, milk yield (15.6 l/d), fat content (3.38%) and log SCC (2.59) decreased by 29, 21 and 27% (P < 0.05), respectively, as a result of omitting one milking. On Mondays, milk yield (23.9 l/d), fat content (4.84%) and log SCC (3.02) increased by 9, 14 and 100% (P < 0.05), respectively. The raise in SCC was dependent on the previous levels. All values reached the average level by Wednesday. Milk protein (3.47%) increased by 2% and lactose (4.37%) decreased by 2% (P < 0.05) by Saturday. Compared with estimated values for 14 milkings/week, omitting one

milking per week decreased the weekly yields of milk (3%), fat (4%), protein (5%) and lactose (5%), but milk SCC increased by 25%. Milk yield loss varied according to the cow's yield but not to lactation stage. Clinical mastitis was not observed in any cow at any time. We conclude that omitting one milking per week could be an adequate strategy to reduce farm labor (7%) without important losses in milk yield in farms with low milk SCC values. Official milk recording should be conducted in the middle of the week to avoid residual effects from the milking omission. An improvement in the farmer's quality of life is also expected.

Key Words: Milking Frequency, Milking Suppression, Milk Composition

Effects of conjugated linoleic acid (CLA) on milk fatty acid profiles and activities of lipogenic enzymes in the mammary gland, liver and adipose tissue of lactating rats. A. A. Hayashi*[1], S. R. Medeiros[2], D.P.D. Lanna[1], [1]ESALQ/ USP/ SP, Brazil, [2]Embrapa /Gado de Corte/ MS, Brazil.

The objective of the present study was to evaluate the effects of feeding a mixture of CLA isomers on milk fatty acid profiles and the activities of lipogenic enzymes in lactating rats. Dams were fed either a control diet or a diet supplemented with 2.5% of calcium salts of CLA-60 from parturition to the 15th day post-partum. The CLA-60, (Church & Dwight, Princeton, NJ) contained different isomers of CLA (24% c/t 9,11; 35% t,c 10,12; 15% c,t 8,10; 17% t,t 11,13 and 9% others). On the 15th day post-partum, the rats were anesthesized, milked and killed by exsanguination. Mammary gland, liver and adipose tissues were immediately freeze-clamped for subsequent assays of activities of enzymes involve in lipid synthesis. Pups growth were decreased by CLA ($P< 0.01$) and concentration of 12:0 to 16:0 fatty acids in the milk of CLA-fed rats were lower compared to the control. The Fatty acid synthase (FAS) activity was decreased by CLA in the mammary gland, adipose tissue and liver (by 43%, $P<0.01$, 56%, $P<0.01$ and 68%, $P<0.01$ respectively). The activities of Glucose-6 phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were decreased in all three tissues, by 35%, $P<0.01$; 36%, $P<0.05$ and 65%, $P<0.05$ for G6PDH, and by 28%, $P<0.01$, 22%, $P=0.10$ and 53%, $P<0.01$ for 6PGDH, for mammary, adipose and liver tissues respectively. In contrast, NADP malate dehydrogenase enzyme activities were unchanged by CLA supplementation to the diet in all tissues. Thus, CLA altered processes associated with de novo fatty acid synthesis. Furthermore, the reduction in the activities of these enzymes, with CLA treatment, was consistent with changes in milk fatty acid profiles, and similar to observations of feeding calcium salts of CLA-60 to lactating cows.

Key Words: Conjugated linoleic acid, Lactation, Lipogenesis

Mammary uptake of amino acids in response to dietary supplementation of rumen protected lysine and methionine in early and late lactating dairy goats. T. G. Madsen*, L. Nielsen, and M. O. Nielsen. Department of Anatomy and Physiology, The Royal Veterinary and Agricultural University, Grønnegaardsvej 7, DK-1870 Frederiksberg C, Denmark.

Dietary animo acid (AA) composition has in ruminants traditionally not been taken into consideration because of the microbial protein synthesis. However, supply of rumen bypass lysine and methionine have in some, but not all, studies been shown to increase milk protein production. These divergences could be due to problems with identifying the limiting amino acid in the diets because of limited knowledge about the actual post absorptive demand for AA's for milk synthesis. The objectives of the present experiment were to study how supplementation of lysine and methionine to a diet low in these two AA's wold affect 1) the arterial concentrations and thereby mammary supply and uptake of AA's, and 2) milk protein synthesis in early and late lactation. Four goats were fed a basal ration designed to be low in small intestine absorbable lysine and methionine. The diets were either I) not supplemented or supplemented with rumen protected II) lysine, III) methionine or IV) lysine+methionine in a 2 x 2 factorial design. By the end of each treatment period (14 days) blood samples were withdrawn simultaneously six times during a 24 h period from the carotid artery and one of the milk veins. The supplementation with rumen protected lysine and methionine increased arterial lysine and methionine concentrations significantly ($P=0.0009$ and $P=0.0045$, respectively). The increased arterial concentrations did not affect mammary arterio-venous differences, however, supplementation with lysine tended to and lysine+methionine in combination increased milk protein production in early lactation significantly ($P=0.0694$ and $P=0.0058$, respectively). The milk protein production in early lactation were 98, 96, 94, and 103 g/d for treatment I, II, III and IV, respectively. No effect of

supplementation on milk rotein production was observed in late lactation. In conclusion, the mammary gland seems to possess mechanisms enabling it to adjust the extraction of lysine and methionine according to its needs. However, supplementation with rumen protected lysine and methionine did affect milk protein production positively in early lactation, indicating that lysine and methionine could become limiting when production peaks and mammary demand and uptake efficiency are close to maximum.

Key Words: Ruminants, milk protein, lysine and methionine

Mammary development, growth and plasma levels of IGF-I and IGF-binding proteins in gilts provided different energy levels from weaning to puberty. Sørensen, M.T., Vestergaard, M., Purup, S. and Sejrsen, K. Danish Institute of Agricultural Sciences, Tjele, Denmark

We investigated the effect of feeding level from weaning (d 28) to slaughter at puberty (d 162) on growth rate, mammary development and plasma levels of IGF-I and IGF-binding proteins (IGFBP) in 10 litters of 4 female pigs. From d 28 to 90 (period 1) and from d 90 to 162 (period 2), pigs were fed either ad libitum (A) or restrictively (R; i.e. 30% lower feed intake in period 1 and 25% lower in period 2) in a 2x2 factorial design with treatments named AA, AR, RA and RR. In period 1, ADG of A-gilts was 622 g vs. 522 g for R-gilts (P<0.001). At the end of period 1, A- compared with R-gilts had higher plasma levels of IGF-I (303 vs. 220 ng/ml, P<0.01) and IGFBP-3 (770 vs. 564, arbitrary units, P<0.01), but lower IGFBP-2 (291 vs. 396 a.u., P<0.02) and 28 kDa IGFBP (P<0.06). In period 2, ADG of RA- and AA-gilts was 1012 g vs. 792 g for RR- and AR-gilts (P<0.001). Furthermore, RA-gilts showed compensatory growth compared with AA-gilts (1054 vs. 971 g/d, P<0.07) with no difference in feed intake. At the end of period 2, there was a tendency for higher plasma IGF-I (P<0.15) in AA- and RA-gilts compared with AR- and RR-gilts whereas IGFBP-2 and 28 kDa IGFBP were reduced (P<0.01). The amount of dissected mammary tissue was higher in AA- and RA-gilts compared with AR- and RR-gilts (86 vs. 59 g/gland, P<0.001), and although DNA concentration was lower in AA- and RA-gilts compared with AR- and RR-gilts (342 vs. 397 ug/g tissue, P<0.04), total amount of mammary DNA was highest in AA- and RA-gilts. The concentration of mammary RNA was not affected by treatment. Feeding level in period 1 did not affect the mammary measures. We conclude that to obtain high mammary growth, a period with ad libitum feeding before puberty is needed, however, this period does not have to commence at weaning. Furthermore, differences in growth rate are associated with differences in IGF-I and IGFBPs, and female pigs fed restrictively from weaning to d 90 and ad libitum until puberty grow as fast as do continuously ad libitum fed pigs.

Key words: Gilt, mammary, IGF

Effects of once-a-day vs twice-a-day milking throughout lactation in dairy goats. A.A.K. Salama, X. Such, G. Caja*, M. Rovai, R. Casals, E. Albanell, and A. Marti, Universitat Autonoma de Barcelona, Bellaterra, Spain.

The effects of once (**1x**) vs twice (**2x**) daily milking on milk yield, milk composition and udder health were studied in dairy goats throughout lactation. For two consecutive years, a total of 32 Murciano-Granadina dairy goats were assigned at wk 2 of lactation to two treatment groups, and were either milked 1x (0900; n= 17) or 2x (0900 and 1700; n= 15) daily until wk 28 of lactation. Goats were dried off at 300 DIM. Milk yield was recorded weekly, and milk composition, somatic cell count (SCC) and bacterial intramammary infections were evaluated for individual udder halves of each goat at each milking at wks 2 and 4 of lactation and then, monthly until the end of the experiment. Once-a-day milking resulted in 18\% reduction in the yield of energy corrected milk at 4% fat (FCM-4%) compared to 2x (1.6 vs 2.0 L/d; P<0.001). This reduction was more marked from wk 2 to 12 (19%; P<0.05) than in late lactation (14%; P<0.08). Response to milking frequency varied according to goat's parity number (P<0.01): =2nd parity (1.5 vs 2.4 L/d; P<0.01), 3rd parity (1.4 vs 1.8 L/d; P<0.05) and =4th parity (1.7 vs 1.9 L/d; P= 0.284) for 1x vs 2x, respectively. Milk of 1x goats contained more (P<0.05) total solids (13.6 vs. 12.9%), fat (5.4 vs. 4.6%) and casein (2.6 vs. 2.4%) than milk of 2x goats. However, yields of total solids, fat, protein and casein tended (P<0.10) to be higher for 2x than 1x. Udder health was not modified by the experimental treatments but one goat from each treatment suffered mastitis and their data were excluded from the analysis. Geometric mean of milk SCC did not differ between treatments (979 vs 917×10^3 cells/ml; P= 0.189) for 1x vs 2x, respectively. Total FCM-4% milk yield on 300 DIM was also lower for 1x vs 2x (504 vs 590 L; P<0.01) goats, respectively. We conclude that application of once-a-day milking in Murciano-Granadina dairy goats reduced moderately milk yield but did not have negative effects on milk composition and udder health. An increase in labor productivity is also expected.

Key words: Once-daily Milking, Milk Composition, Somatic Cell Count

Effects of chronic oxytocin administration on oxytocin release and milk ejection efficiency. J. Macuhova[1], V. Tancin[1,2], R. M. Bruckmaier[1], [1]Institute of Physiology, Techn. Univ. Munich-Weihenstephan, Freising, Germany, [2]Research Institute of Animal Production, Nitra, Slovakia.

The objective of this study was to test if reduced release of oxytocin (OT) from the pituitary or the sensitivity of OT receptors in the mammary gland are responsible for the reduced spontaneous milk ejection after long-term OT treatment. Fourteen healthy Brown Swiss dairy cows were used for the experiment. Cows were routinely milked twice daily at 5 a.m. and 4 p.m. in a 2x2 tandem milking parlour. They were randomly assigned to two treatment groups, seven animals in each group. During a period of 19 d they were i.m. injected with 5 ml NaCl solution (NaCl group) or 5 ml (50 IU) OT (OT group) 1 min before start of each milking. During evening milkings before and after chronic NaCl or OT treatment blood samples were collected at 1-min intervals for analysis of OT blood concentrations. At the end of these milkings OT (10 IU) was i.v. injected to remove residual milk. To detect changes in mammary gland sensitivity to OT, intramammary pressure (IMP) in the udder cistern was recorded during OT infusion before and after the chronic NaCl and OT treatment period. OT was infused at 0.15 IU/min, which caused a steady increase of OT blood concentration. The occurrence of milk ejection was visualized by an IMP rise in the cistern. Chronic NaCl treatment did not influence milk removal, OT release or IMP pattern. Chronic OT treatment reduced spontaneous milk removal by 15±5%. OT release during milking was not reduced after chronic OT treatment. During OT infusion and IMP recording, commencement of milk ejection was similar before and after chronic OT treatment. However, time to reach IMP maximum was prolonged after chronic OT treatment ($p<0.05$). In conclusion, chronic OT administration did not change OT release nor OT blood concentration required to commence myoepithelial contraction. However, the intensity of myoepithelial contraction was reduced thus causing incomplete udder emptying.

Key words: Oxytocin Treatment, Milk Ejection, Cow

Regulation of apoptosis in mammary gland of cows at early lactation. M. Colitti* and B. Stefanon, Dipartimento di Scienze della Produzione Animale - Universita' di Udine, Italy.

Apoptosis inducing factor (AIF) and bcl-2 proteins are involved in apoptosis control, but little is known about their interaction in lactation of cattle. In the present paper the onset of apoptosis and apoptosis-related signals in mammary gland at the beginning of lactation have been investigated. In addition a partial complementary DNA (cDNA) for bovine AIF has been identified and its expression evaluated. Mammary gland tissue was collected from 3 first-calving cows by biopsy at early lactation. The samples were processed for total RNA extraction and RT-PCR analysis were performed for bcl-2, bax, bcl-X and AIF genes. For AIF, ClustalX software was also utilised to align the coding sequences (cds) for rat (Genebank, accession AB04723), human (Genebank, accession XM010246) and mouse (Genebank, accession BC003292) AIF. Highly conserved regions of the AIF cds between the examined species were assessed with Genedoc software. Amplification and sequencing of AIF cDNA from bovine mammary tissue revealed a high degree of homology. In particular, the bovine AIF partial-cd was highly homologous (89%) between nucleotides 1584-1786 of the rat AIF sequence and nucleotides 1541-1743 that encode for the human PDCD8 (91%). The amino acid sequence of bovine AIF showed still higher similarity between species, with 96% homology for rat AIF (residues 496-562) and 93% with that of the human protein (residues 501-567). Within the time course of this experiment, we found a steady-state of bcl-2 and bcl-x expression and the up and down regulation of bax RNAs, which could indicate that in lactating cows these genes and related proteins are differently involved in apoptosis compare to mice. The *in situ* hybridisation data showed that the epithelial cells contained AIF expressed at a intracytoplasmatic level, but not into the ucleus. It was demonstrated that no AIF translocation was detectable in bcl-2 overexpressing cells and this could suggest that in mammary tissue during early lactation the protein was confined to the mitochondrial intermembrane space, in agreement with the low apoptotic index observed.

Key Words: Apoptosis Inducing Factor, Mammary gland, Dairy cows

Proliferation-associated gene expression in bovine mammary gland. T. B. McFadden*, University of Vermont

Mammary development is a crucial determinant of potential milk producing capacity in dairy cows. Fundamentally, milk production is a function of the number and synthetic activity of secretory cells in the udder. Optimal nutrition and management allow for full expression of lactational potential. Therefore, manipulation of mammary growth in developing heifers and dry cows offers an opportunity to increase the efficiency of milk production. However, realization of this opportunity will require substantial increases in understanding of the basic mechanisms that regulate mammary development. Currently, a wide variety of factors are known to influence mammary growth, including genetic merit, nutritional management, hormonal regulation, physiological state and photoperiod. Unfortunately, relatively little detail on underlying mechanisms is available. In recent years, rapid advances in genomic technology have made it possible to conduct high-throughput screening of tens of thousands of genes in an effort to determine relationships between levels of gene expression and physiological function. Such "functional genomics" experiments yield gene expression profiles that may confirm known roles of particular genes while illuminating associations with novel genes, or previously unsuspected involvement of known genes. Using such an approach, we recently identified ~200 candidate genes whose levels of mRNA expression were strongly associated with proliferation of mammary cells. Ongoing studies with a subset of these genes are aimed at confirming their relevance and further characterizing the regulation of their expression and their roles in control of mammary development. The objectives of this paper are to provide an overview of the factors that influence mammary development, to discuss fundamental concepts underlying genomic approaches, and to illustrate application of these techniques to studying regulation of mammary development and potential applications.

Key Words: Proliferation, Gene Expression, Mammary Development

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