ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΕΠΙΔΡΑΣΗ ΤΗΣ ΔΙΑΔΙΚΑΣΙΑΣ ΞΗΡΑΝΣΗΣ ΣΤΟ ΤΕΛΟΣ ΤΗΣ ΓΑΛΑΚΤΙΚΗΣ ΠΕΡΙΟΔΟΥ, ΣΤΗΝ ΥΓΕΙΑ ΤΟΥ ΜΑΣΤΟΥ ΤΩΝ ΠΡΟΒΑΤΙΝΩΝ

ΙΩΑΝΝΗΣ Γ. ΠΕΤΡΙΔΗΣ

Κτηνίατρος

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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ΠΕΡΙΛΗΨΗ

Η παρούσα διατριβή εστιάζεται στη σημασία της διαδικασίας ξήρανσης στο τέλος της γαλακτικής περιόδου στην υγεία του μαστού των προβατίνων και αποσκοπεί: (α) στην αξιολόγηση της επίδρασης της εφαρμοζόμενης διαδικασίας για την ξήρανση του μαστού (με απότομη ή προοδευτική παλινδρόμηση) σε επακόλουθη μόλυνση των μαστικών αδένων και εκδήλωση μαστίτιδας και (β) στην καταγραφή, με υπερηχοτομογραφική εξέταση, μεταβολών πού λαμβάνουν χώρα στο μαστό των προβατίνων κατά τη διάρκεια της παλινδρόμησης.

Η διατριβή χωρίζεται σε τρία κεφάλαια και ακολουθεί η Γενική Συζήτηση.

Στο Κεφάλαιο Ι ανασκοπείται η σχετική βιβλιογραφία. Το Κεφάλαιο υποδιαιρείται σε δύο τμήματα. Στο τμήμα Α ανασκοπείται η βιβλιογραφία που αφορά στην παλινδρόμηση του μαστού των προβατίνων. Στο τμήμα Β ανασκοπείται η βιβλιογραφία που αφορά στη χορήγηση αντιβιοτικών σε προβατίνες στην έναρξη της 'ξηρής περιόδου'.

Στο Κεφάλαιο ΙΙ, μετά από μία σύντομη εισαγωγή (τμήμα Α), αξιολογείται η επίδραση της εφαρμοζόμενης διαδικασίας για την ξήρανση του μαστού στην επακόλουθη μόλυνσή του και στην εκδήλωση μαστίτιδας, σε δύο πειραματισμούς, με ή χωρίς την ενδομαστική χορήγηση αντιβιοτικών στο τέλος της γαλακτικής περιόδου (τμήμα Β).

Περιγράφονται δύο πειραματισμοί. Στον πρώτο πειραματισμό, στα ζώα της ομάδας Α (n=19), η ξήρανση του μαστού πραγματοποιήθηκε προοδευτικά, σε διάστημα 22 ημερών. Οι προβατίνες αρμέχτηκαν δυο φορές καθημερινά για τελευταία φορά την ημέρα D0, στη συνέχεια αρμέγονταν μια φορά καθημερινά επί μία εβδομάδα (D1-D7), στη συνέχεια αρμέγονταν μία φορά κάθε δύο ημέρες (D9, D11, D13) και στη συνέχεια μία φορά κάθε τρεις ημέρες (D16, D19, D22). Στα ζώα της ομάδας Β (n=12), η ξήρανση του μαστού πραγματοποιήθηκε απότομα. Στον δεύτερο πειραματισμό, στις προβατίνες της ομάδας C (n=6), η ξήρανση του μαστού πραγματοποιήθηκε προοδευτικά, σε διάστημα 22 ημερών ως παραπάνω, ενώ στα ζώα της ομάδας D (n=6) η ξήρανση του μαστού πραγματοποιήθηκε απότομα. Σε αυτόν τον πειραματισμό, στο τέλος της γαλακτικής περιόδου, πραγματοποιήθηκε ενδομαστική χορήγηση συνδυασμού προκαϊνικής πενικιλλίνης και νεομυκίνης στον δεξιό μαστικό αδένα όλων των ζώων (ομάδες C και D). Συλλέχθηκαν δείγματα υλικού θηλαίου πόρου και γάλακτος για βακτηριολογική και κυτταρολογική εξέταση πριν από την έναρξη της διαδικασίας ξήρανσης, καθώς και δύο φορές μετά τον τοκετό και, τέλος, έγινε κατάλληλη επεξεργασία και ανάλυση των δεδομένων.

Στον πρώτο πειραματισμό, η διάμεση τιμή του διαστήματος για την πρώτη μόλυνση μετά τον τοκετό ήταν 0 ημέρες για τους θηλαίους πόρους και τους μαστικούς αδένες για την ομάδα Α. Για την ομάδα Β, τα αντίστοιχα διαστήματα ήταν 2,25 και 0 ημέρες για τους θηλαίους πόρους και τους μαστικούς αδένες ($P > 0,38$). Από τα 33 βακτηριακά στελέχη που απομονώθηκαν, 79% ήταν πηκτάση-αρνητικοί σταφυλόκοκκοι. Μετά τον τοκετό, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στη συχνότητα μόλυνσης του θηλαίου πόρου ($P > 0,13$), στη συχνότητα μόλυνσης του μαστικού αδένα ($P > 0,8$), στη συχνότητα εκδήλωσης υποκλινικής μαστίτιδας ($P > 0,78$) ή στη συχνότητα εκδήλωσης παθολογικών καταστάσεων στο μαστό ($P > 0,11$). Επίσης, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στο ποσοστό προσβολής για καμία παράμετρο που μελετήθηκε: μόλυνση του θηλαίου πόρου ($P = 0,545$), μόλυνση του μαστικού αδένα ($P = 0,647$), εκδήλωση υποκλινικής μαστίτιδας ($P = 0,476$) ή εκδήλωση παθολογικών καταστάσεων στο μαστό ($P = 0,259$). Τέλος, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στο ποσοστό αποκατάστασης των παθολογικών καταστάσεων στο μαστό ($P = 0,847$).

Στον δεύτερο πειραματισμό, η διάμεση τιμή του διαστήματος για την πρώτη μόλυνση μετά τον τοκετό ήταν 2 και 4,5 ημέρες για τους θηλαίους πόρους (αριστερούς και δεξιούς, αντίστοιχα) και 4,5 και 7 ημέρες για τους μαστικούς αδένες (αριστερούς και δεξιούς, αντίστοιχα) για την ομάδα C. Για την ομάδα D, τα αντίστοιχα διαστήματα ήταν 6,5 και 3,5 ημέρες για τους θηλαίους πόρους και τους μαστικούς αδένες (αριστερούς και δεξιούς, αντίστοιχα) (για όλες τις συγκρίσεις, $P > 0,22$). Από τα 38 βακτηριακά στελέχη που απομονώθηκαν, 74% ήταν πηκτάση-αρνητικοί σταφυλόκοκκοι. Μετά τον τοκετό, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στη συχνότητα μόλυνσης του θηλαίου πόρου ($P > 0,17$), στη συχνότητα μόλυνσης του μαστικού αδένα ($P > 0,36$), στη συχνότητα εκδήλωσης υποκλινικής μαστίτιδας ($P > 0,36$) ή στη συχνότητα εκδήλωσης παθολογικών καταστάσεων στον μαστό ($P > 0,17$). Επίσης, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στο ποσοστό προσβολής για καμία παράμετρο που μελετήθηκε: μόλυνση του θηλαίου πόρου ($P > 0,75$), μόλυνση του μαστικού αδένα ($P > 0,42$), εκδήλωση υποκλινικής μαστίτιδας ($P > 0,39$) ή εκδήλωση παθολογικών καταστάσεων στο μαστό ($P > 0,85$). Τέλος, δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των δύο ομάδων στο ποσοστό αποκατάστασης των παθολογικών καταστάσεων στο μαστό ($P > 0,89$), παρατηρήθηκε όμως σημαντική διαφορά μεταξύ αριστερών και δεξιών μαστικών αδένων και στις δύο ομάδες ($P < 0,045$).

Στο Κεφάλαιο ΙΙΙ, μετά από μία σύντομη εισαγωγή (τμήμα Α), περιγράφονται τα ευρήματα της υπερηχοτομογραφικής εξέτασης του μαστού των προβάτων κατά τη διάρκεια της παλινδρόμησης (τμήμα Β).

Περιγράφεται ένας πειραματισμός, στον οποίο στα ζώα της ομάδας Ε (n=7), η ξήρανση του μαστού πραγματοποιήθηκε προοδευτικά, σε διάστημα 22 ημερών ως παραπάνω, ενώ στα ζώα της ομάδας F (n=7), η διαδικασία ξήρανσης του μαστού πραγματοποιήθηκε απότομα. Οι προβατίνες αρμέχτηκαν για τελευταία φορά την ημέρα D0, χωρίς να ακολουθήσει άλλο άρμεγμα. Πραγματοποιήθηκαν υπερηχοτομογραφικές απεικονίσεις B-mode και Doppler του μαστού όλων των ζώων κατά τη διάρκεια της διαδικασίας ξήρανσης και υπολογίστηκαν οι εξής παράμετροι: ένταση της φωτεινότητας του γκρι στο μαστικό παρέγχυμα, επιφάνεια του γαλακτοφόρου κόλπου του μαστικού αδένα σε οβελιαία τομή, επιφάνεια του γαλακτοφόρου κόλπου του μαστικού αδένα σε ραχιαία τομή, μέγιστη απόσταση τοιχώματος (διάμετρος της τομής) της έξω αιδοιικής αρτηρίας, δείκτης αντίστασης της έξω αιδοιικής αρτηρίας, δείκτης παλμικότητας της έξω αιδοιικής αρτηρίας. Τέλος, έγινε κατάλληλη επεξεργασία και ανάλυση των δεδομένων.

Παρατηρήθηκαν προοδευτικές μεταβολές στις παραμέτρους που εξετάστηκαν κατά τη διάρκεια του πειραματισμού, οι οποίες ήταν σημαντικές και στις δύο ομάδες ($P < 0,01$). Ο μέσος όρος (±τυπικό σφάλμα του μέσου όρου) της έντασης της φωτεινότητας του γκρι στην ομάδα Ε ήταν 78,0±2,6 την ημέρα D0, 78,5±2,8 την D14 και 72,9±2,1 την D37, ενώ οι αντίστοιχες τιμές στην ομάδα F ήταν 76,6±4,1, 68,6±2,6 και 67,8±2,5 ($P = 0,049$ μεταξύ των ομάδων). Αντίθετα, δεν υπήρξε σημαντική διαφορά στην επιφάνεια του γαλακτοφόρου κόλπου μεταξύ των ζώων των δύο ομάδων ($P > 0,3$), αλλά μόνο μια παροδική αύξηση αυτής αμέσως μετά τη διακοπή της κένωσης του μαστού. Η μέγιστη απόσταση τοιχώματος (διάμετρος της τομής) της έξω αιδοιικής αρτηρίας προοδευτικά μειώθηκε κατά τη διάρκεια της μελέτης και διέφερε σημαντικά μεταξύ των δύο ομάδων ($P = 0,007$). Ο δείκτης αντίστασης και ο δείκτης παλμικότητας αυξήθηκαν προοδευτικά στη διάρκεια του πειραματισμού στα ζώα και των δύο ομάδων, οι δε διαφορές μεταξύ των ζώων των δύο ομάδων ήταν σημαντικές και για τις δύο παραμέτρους ($P < 0,001$).

Τα συμπεράσματα που προκύπτουν από τα αποτελέσματα της παρούσας διατριβής είναι τα παρακάτω.

(α) Τα ευρήματα υποστηρίζουν την υπόθεση ότι η διαδικασία ξήρανσης του μαστού των προβατίνων (δηλαδή, προοδευτική ή απότομη ξήρανση) δεν επηρεάζει την πιθανότητα μόλυνσης αυτού και την εκδήλωση μαστίτιδας. Η ενδομαστική χορήγηση αντιβιοτικών στο τέλος της γαλακτικής περιόδου βελτίωσε το ποσοστό θεραπείας των παθολογικών καταστάσεων στο μαστό, ανεξάρτητα από τη διαδικασία ξήρανσης που έλαβε χώρα.

(β) Τα αποτελέσματα της υπερηχοτομογραφικής απεικόνισης B-mode έδειξαν ότι υπήρχαν διαφορές στις μεταβολές που λάμβαναν χώρα στο μαστικό παρέγχυμα, ανάλογα με τη διαδικασία ξήρανσης του μαστικού αδένα. Αντίθετα, το μέγεθος του γαλακτοφόρου κόλπου του

μαστικού αδένα δεν επηρεάστηκε από τη διαδικασία ξήρανσης. Τέλος, η υπερηχοτομογραφική απεικόνιση Doppler έδειξε ότι η ροή του αίματος κατά τη διάρκεια της απότομης ξήρανσης του μαστού ήταν μικρότερη απ' ό,τι κατά την προοδευτική ξήρανση αυτού.

Με βάση την κείμενη νομοθεσία και μετά από σχετική απόφαση στη με αριθμό 8/12.11.2013 συνεδρίαση της Γενικής Συνέλευσης Ειδικής Σύνθεσης του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, η συγγραφή της διατριβής έγινε στην αγγλική γλώσσα.

Δημοσιεύσεις σχετιζόμενες με την παρούσα διατριβή

Στις παρακάτω επιστημονικές δημοσιεύσεις παρουσιάζονται τμήματα της παρούσας διατριβής:

I. I.G. Petridis, V.S. Mavrogianni, D.A. Gougoulis, G.S. Amiridis, C. Brozos, G.C. Fthenakis (2012). "Effects of drying-off procedure of ewes' udder, with intramammary antibiotic administration, in subsequent mammary infection and development of mastitis" *Journal of the hellenic veterinary medical Society*, 63:273-282.

II. I.G. Petridis, G.C. Fthenakis (2013). "Mammary involution in sheep" *Proceedings of the 11th World Conference in Animal Production (Beijing, China)*, p. 300.

III. I.G. Petridis, V.S. Mavrogianni, I.A. Fragkou, D.A. Gougoulis, A. Tzora, K. Fotou, I. Skoufos, G.S. Amiridis, C. Brozos, G.C. Fthenakis (2013). "Effects of drying-off procedure of ewes' udder in subsequent mammary infection and development of mastitis" *Small Ruminant Research*, 110:128-132.

IV. I.G. Petridis, G.C. Fthenakis (2014). "Administration of antibiotics to ewes at the beginning of the dry-period" *Journal of Dairy Research*, 81:9-15.

V. I.G. Petridis, P.G. Gouletsou, M.S. Barbagianni, G.S. Amiridis, C. Brozos, I. Valasi, G.C. Fthenakis (2014). "Ultrasonographic findings in the ovine udder during involution" *Journal of Dairy Research*, υπό εκτύπωση.

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

Γ.Χ. Φθενάκης, Καθηγητής — Επιβλέπων
Κλινική Μαιευτικής και Αναπαραγωγής, Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Γ.Σ. Αμοιρίδης, Καθηγητής — Μέλος Συμβουλευτικής Επιτροπής
Κλινική Μαιευτικής και Αναπαραγωγής, Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Χ. Μπρόζος, Επίκουρος καθηγητής — Μέλος Συμβουλευτικής Επιτροπής
Κλινική Παραγωγικών Ζώων, Τμήμα Κτηνιατρικής, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

ΕΠΤΑΜΕΛΗΣ ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

Γ.Σ. Αμοιρίδης, Καθηγητής	Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας
Γ.Χ. Φθενάκης, Καθηγητής	Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας
M. Caroprese, Επίκουρη καθηγήτρια	Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università degli Studi di Foggia
Λ.Β. Αθανασίου, Επίκουρη καθηγήτρια	Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας
Ε. Βαλάση, Επίκουρη καθηγήτρια	Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας
Χ. Μπρόζος, Επίκουρος καθηγητής	Τμήμα Κτηνιατρικής, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης
D. Lacasta, Λέκτορας	Facultad de Veterinaria, Universidad de Zaragoza

UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

EFFECTS OF THE DRYING-OFF PROCEDURE AT THE END OF A LACTATION PERIOD, IN THE HEALTH OF THE UDDER OF EWES

IOANNIS G. PETRIDIS

DVM (Thessaloniki)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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of the Faculty of Veterinary Medicine of the University of Thessaly

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ABSTRACT

The objectives of the present thesis were: (i) the evaluation of the effects of the procedure followed for drying-off of ewes' udder (i.e., initiated or progressive involution) in subsequent mammary infection and development of mastitis and (ii) the exploration, by means of ultrasonographic examination, of changes occurring in the udder of ewes during involution.

The thesis is divided into three chapters followed by the General Discussion.

In the first Chapter, the relevant literature is reviewed. The Chapter is subdivided into two Parts. In Part A, the literature on mammary involution of ewes is reviewed. In Part B, the literature on administration of antibiotics to ewes at the beginning of the 'dry-period' is reviewed.

In the second Chapter, after a brief introduction (Part A), the effects of udder drying-off procedure in ewes in subsequent mammary infection and development of mastitis, with or without intramammary antibiotic administration at the end of the lactation period, are described (Part B).

Two experiments are described. In the first experiment, in ewes of group A (n=19), udder drying-off procedure took place progressively during a period of 22 days ewes; animals were milked twice daily for the last time on D0; then, they were milked once daily for a week (D1-D7), followed by another week during which they were milked once every two days (D9, D11, D13), followed by a third week during which ewes were hand-milked once every three days (D16, D19, D22). In ewes of group B (n=12), the procedure took place abruptly. In the second experiment, in ewes of group C (n=6), udder drying-off procedure took place progressively during a period of 22 days as above; in ewes of group D (n=6), it took place abruptly; intramammary administration of a combination of procaine penicillin and neomycin was carried out into the right mammary gland of all animals in that experiment (C or D). Samples of teat duct material and of milk for bacteriological and cytological examination were collected before start of the drying-off procedure, as well as on two occasions after the subsequent lambing. In both experiments, appropriate data management and analysis were performed.

In the first experiment, median time to first teat duct or mammary infection *post-partum* was 0 days (teat ducts and mammary glands) for group A and 2.25 and 0 days (teat ducts and mammary glands, respectively) for group B ($P > 0.38$). Of the 33 bacterial isolates obtained, 79% were coagulase-negative staphylococci. No significant differences were observed between the two groups in the *post-partum* frequency of teat duct infection ($P > 0.13$), of mammary

infection ($P > 0.8$), of subclinical mastitis ($P > 0.78$) or of abnormal findings in a mammary gland ($P > 0.11$). Moreover, no significant differences were seen between the two groups in the *post-partum* incidence risk of any of the above outcomes: teat duct infection ($P = 0.545$), mammary infection ($P = 0.647$), subclinical mastitis ($P = 0.476$) or abnormal findings in a mammary gland ($P = 0.259$). No significant differences were evident between the two groups in cure rate of abnormal findings in a mammary gland ($P = 0.847$).

In the second experiment, median time to first teat duct infection *post-partum* was 2 and 4.5 days (left and right, respectively) for group C and 6.5 and 3.5 days for group D ($P > 0.38$); median time to first mammary infection *post-partum* was 4.5 and 7 days (left and right, respectively) for group C and 6.5 and 3.5 days for group D (for all comparisons, $P > 0.22$). Of the 38 bacterial isolates obtained, 74% were coagulase-negative staphylococci. No significant differences were observed between the two groups in *post-partum* frequency of: teat duct infection ($P > 0.17$), mammary infection ($P > 0.36$), subclinical mastitis ($P > 0.36$), abnormal findings in a mammary gland ($P > 0.17$). Moreover, no significant differences were seen between the two groups in *post-partum* incidence risk of any of the above outcomes: teat duct infection ($P > 0.75$), mammary infection ($P > 0.42$), subclinical mastitis ($P > 0.39$), abnormal findings in a mammary gland ($P > 0.85$). No significant differences were evident between the two groups in cure rate of abnormal findings in a mammary gland ($P > 0.89$), although a significant difference was evident between left and right mammary glands in both groups ($P < 0.045$).

In the third Chapter, after a brief introduction (Part A), the findings of ultrasonographic imaging of the ovine udder during involution are described (Part B).

One experiment is described. In ewes of group E (n=7), udder drying-off procedure took place progressively during a period of 22 days as above; in ewes of group F (n=7), it took place abruptly; ewes were milked twice daily for the last time on D0 and no milking was carried out after that. B-mode and Doppler ultrasonographic examinations of the udder of all ewes were performed throughout the drying-off procedure and the following parametres were computed: mammary parenchyma gray-scale, mammary gland cistern area at sagittal plane, mammary gland cistern area at dorsal plane, external pudendal artery diametre, external pudendal artery resistance index, external pudendal artery pulsatility index. Appropriate data management and analysis were performed.

There was clear evidence that progressive changes of the parametres evaluated throughout the study period were significant in both groups ($P < 0.01$). Mean (±standard error of the mean) gray-scale results in group E were 78.0±2.6 on D0, 78.5±2.8 on D14 and 72.9±2.1 on D37;

respective values in group F were 76.6±4.1, 68.6±2.6 and 67.8±2.5 (P = 0.049 between groups). A temporary increase in cistern volume was evident after cessation of lactation, but differences were not significant between the two groups (P > 0.3). Diametre of the external pudendal artery progressively decreased during the study and differed significantly between the two groups (P = 0.007). Both resistance index and pulsatility index progressively increased throughout the study period in both groups; there was evidence that, for both parametres, differences between the two groups were significant (P < 0.001).

Conclusions derived from the results of the present thesis are as follows.

(a) The results support a hypothesis that the procedure for udder drying-off (i.e., initiated or progressive involution) does not affect the risk of subsequent mammary infection and development of mastitis. Intramammary administration of antibiotics improved cure rates of mammary abnormalities, independently of the procedure followed for udder drying-off.

(b) Results of B-mode ultrasonographic examination suggested that there were differences in remodelling of the extracellular matrix in relation to the procedure for udder drying-off. Volume of the gland cistern did not appear to be affected by the procedure for udder drying-off. Results of Doppler ultrasonographic examination indicated that blood flow during initiated drying-off procedure was smaller than during a procedure with progressive drying-off.

Publications associated with the present thesis

The following scientific papers presenting facets of the present thesis, are available:

I. I.G. Petridis, V.S. Mavrogianni, D.A. Gougoulis, G.S. Amiridis, C. Brozos, G.C. Fthenakis (2012). "Effects of drying-off procedure of ewes' udder, with intramammary antibiotic administration, in subsequent mammary infection and development of mastitis" *Journal of the hellenic veterinary medical Society*, 63:273-282.

II. I.G. Petridis, G.C. Fthenakis (2013). "Mammary involution in sheep" *Proceedings of the 11th World Conference in Animal Production (Beijing, China)*, p. 300.

III. I.G. Petridis, V.S. Mavrogianni, I.A. Fragkou, D.A. Gougoulis, A. Tzora, K. Fotou, I. Skoufos, G.S. Amiridis, C. Brozos, G.C. Fthenakis (2013). "Effects of drying-off procedure of ewes' udder in subsequent mammary infection and development of mastitis" *Small Ruminant Research*, 110:128-132.

IV. I.G. Petridis, G.C. Fthenakis (2014). "Administration of antibiotics to ewes at the beginning of the dry-period" *Journal of Dairy Research*, 81:9-15.

V. I.G. Petridis, P.G. Gouletsou, M.S. Barbagianni, G.S. Amiridis, C. Brozos, I. Valasi, G.C. Fthenakis (2014). "Ultrasonographic findings in the ovine udder during involution" *Journal of Dairy Research*, in press.

ADVISORY COMMITTEE

Professor G.C. Fthenakis — Supervisor
Department of Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Thessaly

Professor G.S. Amiridis — Member of the advisory committee
Department of Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Thessaly

Assistant professor C. Brozos — Member of the advisory committee
Department of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki

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TABLE OF CONTENTS

GENERAL INTRODUCTION

Preface - Objectives of the thesis

The mammary gland fulfils a variety of functions (Oliver and Sordillo 1989). As part of the reproductive system of mammals, it undergoes repeated cycles of structural development, functional differentiation and regression (Hurley and Loor 2011). During these cycles, the mammary gland of adult animals undergoes three distinct functional transitions: (i) from end of lactation to lactogenesis which is termed 'involution', (ii) from lactogenesis (including the colostrogenesis) to lactation and (iii) from lactation to beginning of involution. During each of these phases, marked changes occur in the size, the structure and the function of the organ; moreover, distinct changes also occur in the mammary secretion during each of the above physiological transitions (Oliver and Sordillo 1989).

Mammary involution is the regression of mammary tissue to a non-secreting state. Three procedures for mammary involution have been described: initiated or induced involution (occurring after abrupt cessation of milk removal), progressive or gradual involution (occurring during the declining phase of lactation) and senile involution (occurring at the end of the reproductive life of the animal) (Hurley 1989). In farm animals, initiated involution and progressive involution are, of course, those with highest importance. In these species, mammary involution occurs at the end of each lactation period and is characterized by the reduction of numbers of mammary epithelial cells coupled with the extensive proteolytic degradation of the extracellular matrix (Quarrie et al. 1996, Flint et al. 2005).

In dairy-type sheep production systems, mammary involution is either initiated (i.e., milking is stopped at once) or progressive (i.e., milking frequency is gradually decreased over a period of several days or weeks) (Gelasakis et al. 2010). In mutton-production systems, mammary involution is initiated, as cessation of lactation takes place when lambs are removed from their dam (Sargison 2008).

The period from complete cessation of milk removal until the beginning of the subsequent lactation period is termed 'dry-period'. The 'dry-period' is distinguished in three distinct stages: (i) stage of active involution, (ii) stage of the 'steady-state' involution and (iii) stage of redevelopment and lactogenesis (including colostrogenesis). The 'dry-period' is important in the health management of sheep for achieving optimum milk production during the subsequent lactation period (Contreras et al. 2007, Fthenakis et al. 2012).

The 'dry-period' is necessary for the renewal of damaged or senescent mammary epithelial cells (Capuco et al. 1997). The remodelling of the mammary gland during involution,

characterized by the renewal of mammary epithelial cells, is essential for optimum milk production during the subsequent lactation period (Capuco et al. 1997).

'Dry-period' duration of at least 60 days is recommended for optimum involution and subsequent lactogenesis (Lee and Lascelles 1969, Tatarczuch et al. 1997, 2000). A shorter 'dry-period' has been associated with incomplete renewal of mammary cells (Capuco et al. 1997) and decreased subsequent milk yield (Pinedo et al. 2011, Hernandez et al. 2012), because, perhaps, a hormonal environment suitable for mammary cell renewal cannot be reached at short periods (Bernier-Dodier et al. 2011).

Increased milk production in the subsequent lactation period is indicated by the number of mammary epithelial cells, which depends on their proliferation and apoptosis rates (Knight 2000, Capuco et al. 2003), and the secretory activity of mammary epithelial cells, which depends on their differentiation (Akers et al. 2006). With no sufficient cell renewal after involution, milk yield produced during the subsequent lactation period would be declining fast as a result of the decrease in both cell numbers and cellular secretory activity (Capuco and Akers 1999). Moreover, intramammary infections during that period affect normal *pre-partum* development of mammary epithelial cells and subsequently lactogenesis (Oliver and Sordillo 1989), hence, quality and quantity of milk to be produced (Sordillo and Nickerson 1988).

Little work has been performed specifically in mammary involution in sheep; most relevant references address the condition in cows and may be of some relevance in ewes. Moreover, no studies are available to compare effects of the type of involution process (i.e., initiated or progressive involution) and to identify potential differences between the two procedures.

The present thesis focusses in the study of the involuting mammary gland of dairy ewes, with the general objective to increase available knowledge regarding the normal involution process in that species. Specific objectives of the thesis were as follows.

- The evaluation of the effects of the procedure followed for drying-off of ewes' udder (i.e., initiated or progressive involution) in subsequent mammary infection and development of mastitis, tested in two experiments, with or without intramammary antibiotic administration at the end of a lactation period.
- The recording, by means of ultrasonographic examination, of changes occurring in the udder of ewes during initiated or progressive involution.

The present thesis has been carried out at the Department of Obstetrics and Reproduction of the Veterinary Faculty of the University of Thessaly. Research work started in

2009 and was carried out until the end of 2012; it was followed by analysis of results and writing up of the thesis. The thesis was financially supported by departmental funds.

Parts of the work described in the thesis were carried out at the Department of Animal Production of the Technological Educational Institution of Epirus.

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CHAPTER I

REVIEW OF THE LITERATURE

A. MAMMARY INVOLUTION IN EWES

Physiological mechanisms involved in mammary involution

Active involution takes place with declining frequency or cessation of milk removal and continues for three to four weeks. It is followed by the 'steady-state' involution, at which stage the mammary gland is fully involuted. After involution, three to four weeks before the expected lambing date starts the redevelopment of the mammary gland, with formation of the colostrum (colostrogenesis) (Hurley and Loor 2011). In general, mammary involution is not regulated by the decreased activity of galactopoietic hormones (e.g., prolactin, growth hormone), but by the interruption of release of these hormones that follows cessation of milk withdrawal from the mammary gland.

Moreover, local mechanisms, occurring in response to milk accumulation in the mammary gland, also play a significant role in the process of involution (Wilde et al. 1999). The volume of mammary secretion is greatly reduced during involution, with concurrent changes in concentrations of its constituents; for example, fat, protein and lactose concentrations decrease rapidly, whilst concentration of lactoferrin increases. Moreover, during active involution, there is increased leucocytic infiltration of the mammary parenchyma; the leucocytes participate in the involution process, specifically in the removal of fat globules, casein micelles and cellular debris.

Mechanisms involved in involution of mammary epithelial cells

Milk accumulation plays a major role in triggering apoptosis and, thus, involution of mammary epithelial cells (Green and Streuli 2004). Among the various mechanisms proposed, one is the accumulation of factors (e.g., α-lactalbumin), which promote mammary epithelial cell apoptosis, but during lactation are continuously removed at milking (Hakansson et al. 1995, 1999, Green and Streuli 2004). Another factor may be the change in the shape of mammary epithelial cells by stretching, when alveolar lumen is engorged with milk accumulation after cessation of lactation. Preservation of the structure of mammary epithelial cells is important for their proper functioning; alteration of their shape may lead to damage in the tight junctions, resulting in the leakage of pro-apoptotic factors from the apical to the basal surface, where they

would trigger apoptosis, either directly or by antagonizing survival signals (Stelwagen et al. 1994, 1995, 1997, Singh et al. 2005). Moreover, stretch receptors could be activated (e.g., adhesion receptors linking the basal epithelial surface to the basement membrane) or cell to cell adhesion junctions may be damaged; both pathways lead to production of pro-apoptotic signals (e.g., E-cadherin) (Boussadia et al. 2002, Wernig et al. 2003, Green and Streuli 2004). It has been shown that, following induction of involution, there is a decline in gene expression of various integrins, which mediate survival signals from the extracellular matrix to the mammary epithelial cells (Singh et al. 2005). Thus, it has been concluded that communications between mammary epithelial cells and the extracellular matrix would be compromised during the involution process and that there may be an interaction between integrins and growth factor receptors, since the integrins mediate the survival signals via the PI3-K/Akt survival pathway (Farrelly et al. 1999, Singh et al. 2005).

Another regulator of apoptosis is the feedback inhibitor of lactation (FIL), a milk-whey protein with molecular mass of 7,600 Da, which can reduce the rate of milk secretion, by inhibiting transfer of proteins through the Golgi apparatus within the mammary epithelial cells (Peaker and Wilde 1996, Knight et al. 1998). Its concentration increases with longer periods of milk accumulation, that way down-regulating milk production in a chemical feedback loop (Van Veldhuizen-Staas 2007). FIL acts within the alveolar tissue, more precisely, in the apical surface of the secretory cells (Peaker and Wilde 1996, Knight et al. 1998). The final consequences of blockage of the secretory pathway within the epithelial cells are the inhibition of protein synthesis and the down-regulation of the mammary prolactin receptors. The latter may render FIL a possible regulator of apoptosis, although this regulation would be more pronounced in progressive involution, with long intervals between milk removals, as induction of apoptosis seems to be a rather long-term and indirect effect of the hormone's action (Peaker and Wilde 1996, Knight et al. 1998, Wilde et al. 1999).

Mammary cell proliferation, differentiation and apoptosis are controlled by hormones and factors acting systemically (growth hormone, prolactin) or at mammary gland level (FIL, insulin-like growth factor 1 [IGF-1], insulin-like growth factor-binding protein [IGFBP]). The balance between cell proliferation and apoptosis defines the cell turnover in the mammary gland. Mammary epithelial cells are removed through increased apoptosis during active involution in a period of three to four weeks, whilst increased cell proliferation takes place at the stage of redevelopment and lactogenesis, continuing during the early stage of a lactation period.

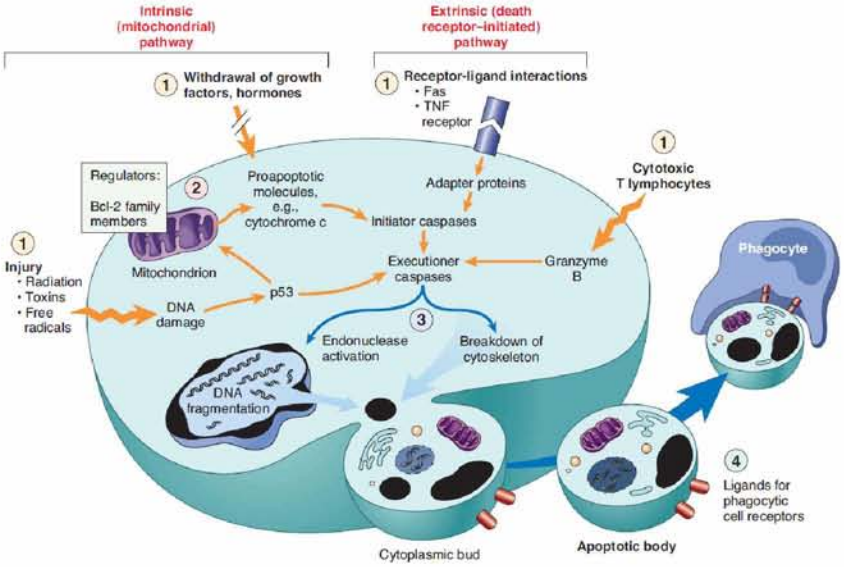
Survival of mammary epithelial cells is primarily dependent on the activity of IGF-1, which, in turn, is modulated by the production, locally, of IGFBPs from the stromal cells that surround them. Increased concentration of specific IGFBPs produced at the early stages of involution inhibit activity of IGF-1 and prevent it from binding to its receptors, which would suppress apoptosis and would delay involution (Wilde et al. 1999, Colitti and Farinacci 2009, Hurley and Loor 2011).

Pathways to apoptosis of mammary epithelial cells

There are two major pathways leading to apoptosis, an intrinsic and an extrinsic. The intrinsic (or mitochondrial) pathway involves the mitochondria and can be triggered by various intracellular stressors, which change the integrity of the outer mitochondrial membrane. The extrinsic (or death receptor-initiated) pathway involves death receptors of the cell surface and their activation by their ligands. These two mechanisms occur independently of each other and involve distinct molecular interactions (Green and Streuli 2004, Myers and McGavin 2007) (Fig. I.1.). Although they may interconnect and overlap at numerous steps (Green and Streuli 2004), no shift between the two pathways during a mammary gland cycle has been found in cows (Norgaard et al. 2008).

Induction of apoptosis is a multi-step process, where several pathways are involved. Some of these are pro-apoptotic, whilst others inhibit existing cellular survival pathways (Green and Streuli 2004).

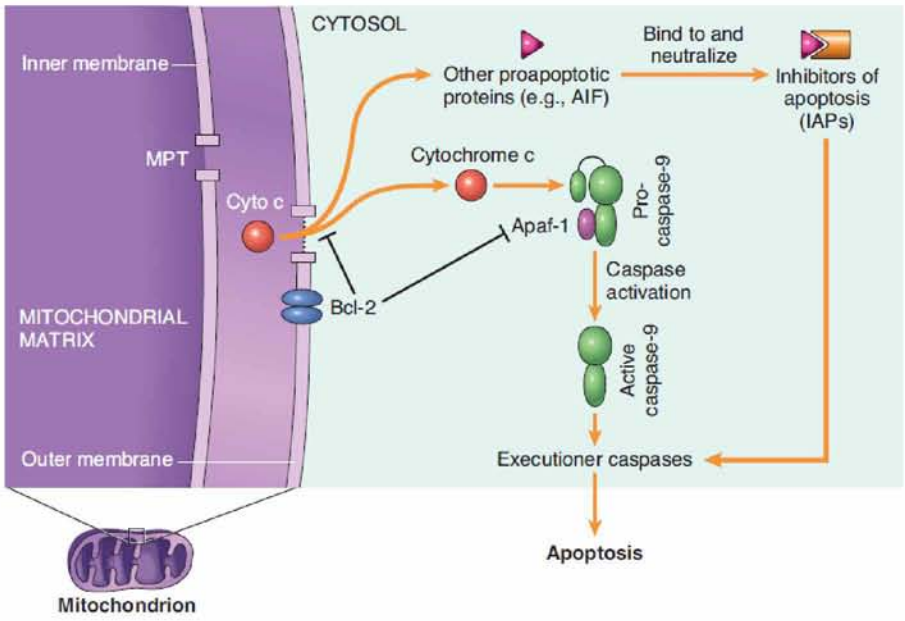
Figure I.1. Mechanisms of apoptosis. Labeled (1) are some of the major inducers of apoptosis. These include specific death ligands (tumor necrosis factor [TNF] and Fas ligand), withdrawal of growth factors or hormones and injurious agents (e.g., radiation). Some stimuli (e.g., cytotoxic cells) directly activate execution caspases (right) and some others act by way of adapter proteins and initiator caspases or by mitochondrial events involving cytochrome c (2). Control and regulation are influenced by members of the Bcl-2 family of proteins, which can either inhibit or promote the cell's death (3). Executioner caspases activate latent cytoplasmic endonucleases and proteases that degrade nuclear and cytoskeletal proteins, which results in a cascade of intracellular degradation, including fragmentation of nuclear chromatin and breakdown of the cytoskeleton (4). The end result is formation of apoptotic bodies containing intracellular organelles and other cytosolic components; these bodies also express new ligands for binding and uptake by phagocytic cells (Myers and McGavin 2007).



Intrinsic pathway

In the intrinsic pathway, increase in the permeability of the outer membrane of mitochondria and the release of pro-apoptotic molecules (e.g., cytochrome c) in the cytosol trigger the beginning of apoptosis. Mitochondria act as stress sensors, which detect cell damage and changes in the environment, like withdrawal of survival signals. These survival signals and growth factors stimulate production of anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2, Bcl-x), which play a key role in this pathway (Myers and McGavin 2007) (Fig. I.2.).

Figure I.2. The intrinsic (mitochondrial) pathway of apoptosis. Death agonists cause changes in the inner mitochondrial membrane, resulting in the mitochondrial permeability transition (MPT) and release of cytochrome c and other pro-apoptotic proteins (e.g., apoptosis inducing factor, AIF) into the cytosol, which activate caspases (Myers and Gavin 2007).



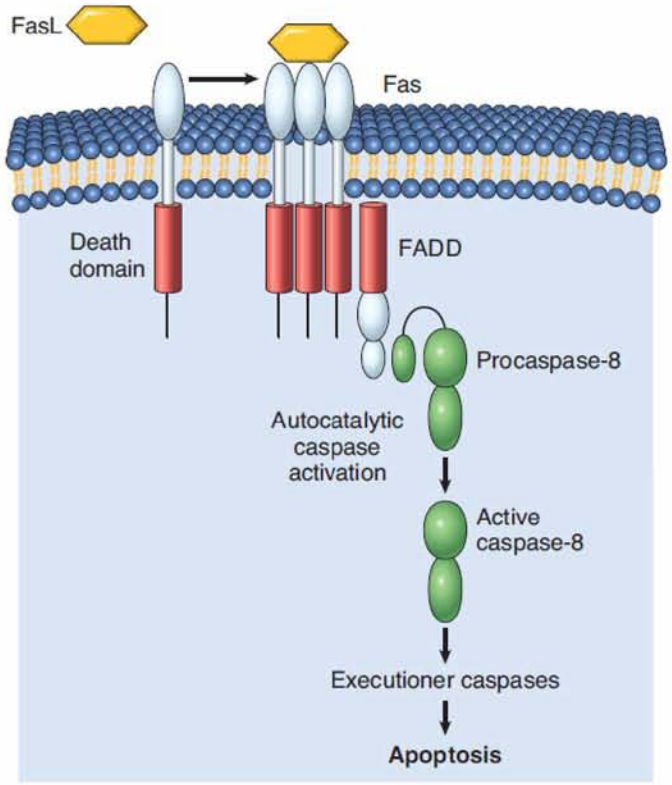
A growth factor with significant importance in survival signaling in the mammary gland is IGF-1. This is secreted primarily in the liver as an endocrine hormone and at various other tissues (among them, the mammary gland stroma) in a paracrine/autocrine way, stimulated by the growth hormone (Flint et al. 2005). IGF-1 stimulates mammary epithelial cell proliferation and inhibits apoptosis (Flint et al. 2005). Moreover, prolactin inhibits the secretion of IGF-1 binding protein-5 (IGFBP-5) from mammary epithelial cells, enhancing that way the action of growth hormone, since IGFBP-5 inhibits the action of IGF-1. Hence, growth hormone is considered to be a factor promoting milk secretion indirectly, through stimulation of IGF-1 production, while prolactin acts directly in mammary epithelial cells (Flint et al. 2005, Colitti and Farinacci 2009). In the mammary glands of ewes, expression level of IGFBP-5 is up-regulated during involution (Colitti and Farinacci 2009), perhaps stimulated by milk accumulation therein (Green and Streuli 2004).

Extrinsic pathway

As observed in cows, the extrinsic pathway is initiated when death receptors are cross-linked by their ligands. The most commonly described death receptor present in the mammary gland is Fas. The interaction of FasL (Fas ligand) with Fas brings together three or more

molecules of Fas (trimerisation), this way creating in the cytoplasm a binding site for an adapter protein called Fas associated death domain (FADD). Binded FADD results in activation of caspase-8 and consequent downstream activation of execution caspases (Green and Streuli 2004, Myers and McGavin 2007) (Fig. I.3). However, no relevant data are available regarding the involution process specifically in ewes.

Figure I.3. The extrinsic (death receptor-initiated) pathway of apoptosis, illustrated by the events following Fas engagement (FADD: Fas-associated death domain, FasL: Fas ligand) (Myers and McGavin 2007).



Mechanisms involved in extracellular matrix modelling

In general, during involution, the stromal area increases, whilst during redevelopment of the mammary gland it decreases (Capuco et al. 1997, De Vries et al. 2010). This remodelling is partly regulated by the matrix metalloproteinases. The action of matrix metalloproteinases is controlled, in order to prevent inappropriate degradation or proliferation of mammary tissue, by gene transcription, the aforementioned enzyme activation system and/or the balance between them and their inhibitors (tissue inhibitors of metalloproteinases), which bind to matrix

metalloproteinases or their inactive pro-enzymes (Rabot et al. 2007). The increase in mammary stromal area at the stage of active involution may result from increased production of stromal proteins, while the decrease at the stage of lactogenesis may be associated with the decreasing number of fibroblasts, which produce stromal proteins (De Vries et al. 2010). Moreover, IGFBP-5 interacts with proteins of the extracellular matrix (e.g., components of the plasminogen system or matrix metallo-proteinases) involved in tissue remodelling during mammary involution (Flint et al. 2005).

Histological changes in the structure of the involuting mammary gland

The initial change in involuting mammary glands is the formation of large stasis vacuoles in the epithelial cells, as a result of accumulation of secretory vesicles and milk fat globules in the cytoplasm of the alveolar cells. This accumulation is the result of reduction in fusion of secretory vesicles with the apical membrane and indicates that milk secretion ceases prior to milk synthesis inhibition (Hurley 1989). As a consequence of decreased synthetic activity, a marked reduction in cytoplasmic organelles of epithelial cells involved in synthesis of milk components occurs already on the 2nd day of involution (Holst et al. 1987). Their number becomes minimal after four weeks subsequently to the cessation of milk removal (Holst et al. 1987, Sordillo and Nickerson 1988).

In sheep, the epithelial cells become flattened two days after cessation of milk removal, with simultaneous formation of large stasis vacuoles. As apoptosis advances, their cytoplasm fills with vacuoles with clusters of dense material and fragmented nucleus. By the 4th day, they fill with large empty vacuoles and autophagosomes in their cytoplasm and later, by the 7th day, they become highly vacuolated with small dense mitochondria, some rough endoplasmic reticulum and numerous free ribosomes. On the 30th and 60th days after cessation of milk removal, the epithelial cells have been found to be cuboidal with numerous ribosomes, mitochondria, a small Golgi apparatus and a small amount of rough endoplasmic reticulum, i.e., a characteristic image of a resting gland (Tatarczuch et al. 1997).

The alveolar lumen initially becomes distended, but, from the 4th day after cessation of lactation, progressively, its size is reduced; 30 days after that, the alveoli appear to have little or no lumen. Ultimately, alveoli appear to be irregularly-shaped and collapsing, shrunken or fully collapsed. The few epithelial cells in each alveolus are flattened and slender (Tatarczuch et al. 1997, Colitti et al. 2009). No lacteal content is present in the alveolar lumen. Nevertheless, the

alveolar structure is maintained throughout the duration of involution (Capuco et al. 1997), as destruction of the mammary alveoli is not complete (Norgaard et al. 2008).

Thirty days after initiation of the process, there is a marked reduction of numbers of the organelles involved in milk synthesis (rough endoplasmic reticulum, Golgi system), although sufficient numbers of mitochondria and ribosomes are still present in the cytoplasm of epithelial cells (Tatarczuch et al. 1997). During active involution, the alveolar area of the gland decreases, whilst its stromal area increases. Progressively, the amount of between-lobules connective tissue becomes abundant and dense (like a wide band); increased numbers of fibroblasts are present therein. The stroma of connective tissue within the mammary lobules contains large numbers of inflammatory cells (primarily macrophages and lymphocytes) (Lee and Lascelles 1969, Tatarczuch et al. 2000).

The proportion of epithelial area in the mammary gland of ewes is minimal on the 8th day after cessation of lactation and on the 30th day of lactation (Colitti and Farinacci 2009). It is noteworthy that its maximum proportion is reached on the 60th and the 150th day of a lactation period, at which time-points the stromal area reaches its lowest (Colitti and Farinacci 2009). The lumen area decreases eight days after cessation of lactation. The cell proliferation index, which has its maximum during lactation (>40% during lactation), is reduced to its lowest (<10%) during involution. Rate of apoptosis is highest at the early stages of the process, but thereafter stabilizes to a rate similar to that occurring at early lactation (Colitti and Farinacci 2009).

Initially, the outline of myoepithelial cells becomes irregular and distinct cytoplasmic processes enveloped by the basement membrane can be seen. Progressively, they become irregular in form and their cytoplasmic processes protrude deep into the glandular stroma. Finally, they decrease in size and their cytoplasm is condensed around the nucleus (Tatarczuch et al. 1997).

During involution, numbers of leucocytes (initially neutrophils, subsequently lymphocytes and macrophages) in the mammary gland increase sharply (Lee and Lascelles 1969, Tatarczuch et al. 2000). On the 15th day of involution, there is a marked presence of many, highly-vacuolated macrophages in the alveolar lumens and in the ductal system, which are termed ‘cells of Donné’ (Tatarczuch et al. 1997). As involution progresses, the stroma of connective tissue within the lobules contains large numbers of these cells. The leucocytes participate in the involution process, specifically in the removal of fat globules, casein micelles and cellular debris, which may lead to decreased efficiency of phagocytosis and intracellular killing of bacteria during the procedure (Sordillo and Nickerson 1988).

Defences of the mammary gland during involution

An important defence structure during the 'dry-period' is the keratin plug, which is formed at the teat orifice and seals the teat duct, thus preventing invasion of microorganisms into the mammary parenchyma. The plug is formed by continuous cornification of the teat duct epithelium, which is not being removed as milking or suckling has ceased. The plug plays a role of mechanical barrier and, moreover, inhibits growth of microorganisms due to the antibacterial properties of the fatty acids, which are major constituents of the plug (Hogan et al. 1986, 1987, Capuco et al. 1992). In any case, the keratin plug takes some time to be established. Until then, milk accumulation into the mammary gland leads to increased pressure to the teat, which results to dilatation of the teat orifice and teat duct, thus facilitating potential invasion of pathogens. Characteristically, formation of the plug takes longer in animals with increased milk yield, i.e., in those with larger milk accumulation and wider teat dilatation, which are thus at higher risk of infection (Dingwell et al. 2004). Moreover, lack of regular milking or suckling, which contributes to removal of invading microorganisms, and discontinuation of teat sanitation practices may further contribute to increased risk of infection during the stage of active involution.

As discussed above, there is a marked increase of leucocytes in the mammary gland during involution. During the first week of involution, neutrophils are the main type of leucocytes therein, whilst later macrophages and lymphocytes become the predominant cells in the mammary tissue (Lee and Outteridge 1981, Tatarczuch et al. 2000, 2002). Nevertheless, during the stage of active involution, leucocytes in the mammary gland may have a decreased phagocytic ability, because they are heavily laden with ingested material (fat globules, casein micelles, cellular debris) (Sordillo and Nickerson 1988, Tatarczuch et al. 2000, 2002). Ingestion of fat globules or casein micelles through internalization of the cell membrane results in cell rounding and loss of pseudopodia, which are necessary for trapping and engulfing bacteria, as neutrophils lack the ability to regenerate plasma membrane (Paape et al. 2003). Moreover, there is a loss of lysosomes, which fuse to vacuoles containing fat globules or casein micelles instead of those containing bacteria (Paape et al. 2003).

Concentration of immunoglobulins is low during the stage of active involution, hence not supporting effective bacterial opsonisation and phagocytosis. Later, however, during the stage of 'steady-state' involution, that concentration increases. Thus, at that stage, opsonisation of invading bacteria increases leading to effective phagocytosis (Sordillo et al. 1987).

Nevertheless, progressively, the number and the efficiency of leucocytes increase, hence, at the state of 'steady-state' involution the mammary gland is well protected against potential invading pathogens. One should also refer to the sub-epithelial lymphoid nodules located at the border between the teat duct and the teat cistern (Mavrogianni et al. 2005, Fragkou et al. 2010). These structures regulate the early defence response of the mammary gland through lymphocytes; hence, as during involution there is an influx of lymphocytes into the mammary gland, one may postulate that their defence ability would increase at that period.

Another defensive factor, the concentration of which in mammary secretion increases at the involuting mammary gland, is lactoferrin. Lactoferrin acts by sequestering iron, which is necessary for growth of Gram-negative bacteria, some of which are confirmed mammary pathogens (e.g., *Escherichia coli*) (Bishop et al. 1976, Nickerson 1989, Oliver and Sordillo 1989). Citrates compete with lactoferrin for iron binding, but, when iron is bound by citrates, it becomes available to bacteria. Immediately after cessation of lactation, concentration of lactoferin in mammary secretion is minimal, which increases susceptibility of the mammary gland to infections. Subsequently, during the stage of 'steady-state' involution, concentration of lactoferrin is increased, whilst citrates have been resorbed, which further enhances the potential anti-bacterial action of lactoferrin (Smith and Oliver 1981, Nickerson 1989, Oliver and Sordillo 1989).

Finally, at the stage of redevelopment and lactogenesis, i.e. before the expected lambing, as the peri-parturient relaxation of immunity is established (Coop and Kyriazakis 1999), numbers and phagocytic efficiency of leucocytes in the mammary gland decrease, as do amounts of lactoferrin and cytokines. Moreover, decreased cytokine production at that stage also leads to reduced chemotaxis of neutrophils to the mammary gland. Specifically, $CD4^+$ cells produce small quantities of interleucin-2 and IFN-g, but larger ones of interleucin-4 and interleucin-10 (Shafer-Weaver et al. 1999). That pattern has been correlated with increased susceptibility to mastitis, as decreased levels of those immunoregulatory cytokines had been correlated with impaired immune cell function and increased susceptibility to mastitis (Sordillo et al. 1991); for example, interleucin-2 activity has been found to be significantly smaller in mammary secretion samples collected immediately *pre-partum* than in samples collected 14 days prior to that (Sordillo et al. 1991). Although that way susceptibility of the mammary gland may increase, presence of keratin plug at the teat orifice minimizes invasion of pathogens, whilst persistent efficacy of antibiotics that had been already administered intramammarily can contribute to counteract potential infections (Hurley 1989, Bradley and Green 2004).

B. ADMINISTRATION OF ANTIBIOTICS TO EWES AT THE BEGINNING OF THE 'DRY-PERIOD'

Principles of udder health management of ewes at the beginning of the 'dry-period'

As discussed above, during the stage of active involution, there is (i) easier invasion of pathogens, due to dilatation of the teat orifice and duct as a result of milk accumulation into the mammary gland and consequent increased pressure to the teat, whilst the protective keratin plug which seals the teat canal is not yet formed (Dingwell et al. 2004), and (ii) impaired defensive ability of the mammary gland as a result of decreased concentration of lactoferrin and lactoferrin:citrate ratio (Smith and Oliver 1981, Nickerson 1989, Oliver and Sordillo 1989), decreased phagocytic ability of leucocytes (Sordillo and Nickerson 1988, Tatarczuch et al. 2000, 2002) and reduced amount of immunoglobulins in lacteal secretions (Sordillo et al. 1987). The above predispose the mammary gland to new mastitis cases during involution and support recrudescence of subclinical infections, which had occurred during the previous lactation period (Orphanou 1987, Barkema et al. 1998, Saratsis et al. 1998).

Udder health management at the end of a lactation period aims (i) to cure infections which have occurred during the previous lactation period and (ii) to prevent development of new intramammary infections during the 'dry-period' (Fthenakis et al. 2012). Initially, clinical examination of the mammary glands of ewes in the flock should be carried out; that way, ewes with mammary abnormalities can be identified. The udder of all ewes in the flock is examined by palpation, whilst the animals are run through a race (Orphanou 1987, Saratsis et al. 1998). If mammary abnormalities are suspected, animals should be individually examined. Diffuse hardness, abscesses and nodules in the mammary glands are the most common clinical findings during the examination (Saratsis et al. 1998). Samples (e.g., mammary secretion, abscess material) should also be collected for bacteriological examination (Fthenakis 1994, Saratsis et al. 1998, Mavrogianni et al. 2005).

Based on the results of the clinical examination of the udder and the ancillary tests performed (e.g., bacteriological examination), the following categories of ewes should be considered for culling: (i) animals with at least one mammary gland permanently damaged, (ii) animals chronically affected and (iii) animals, which had showed incidents of relapsing mastitis

or had not fully responded to mastitis treatment during the preceding lactation period. The benefits of culling such animals include: (i) decrease of veterinary expenses for mastitis control in the flock, (ii) elimination of sources of potential infection for other animals in the flock and, specifically in dairy flocks, (iii) decrease of flock bulk somatic cell counts in the subsequent lactation period (Mavrogianni et al. 2011). Moreover, lambs (especially in large litters) from ewes with extensive mammary lesions do not thrive well and may require additional feeding (Fthenakis and Jones 1990a), which increases expenses and labour in the flock.

The above procedures should be complemented by administration (preferably by the intramammary route) of antibiotics, which is an integral part of udder health management (Fthenakis et al. 2012).

Pathogens involved in 'dry-period' mastitis

The main pathogens involved in the so-called 'dry-period mastitis' are primarily coagulase-negative staphylococci (Saratsis et al. 1998, Croft et al. 2000, Chaffer et al. 2003, Gonzalo et al. 2004, Shwimmer et al. 2008, Spanu et al. 2011). Reservoirs of these microorganisms are the subclinically infected mammary glands, although the bacteria are also part of the normal flora of the udder skin, which renders their control difficult. In sheep, these organisms are frequent aetiological agents of clinical or subclinical mastitis and elicit a strong host response (Fthenakis and Jones 1990b, Pengov 2001). Various coagulase-negative staphylococcal species are involved in the aetiology of the disease. Phenotypic tests (e.g., API Staph ID 32) are used often for speciation of these organisms. Genotypic methods can provide information (Sampimon et al. 2009) for use in epidemiological studies in sheep flocks, e.g., regarding increased prevalence and persistence of infection by some staphylococcal species.

Other organisms involved in 'dry-period mastitis' are streptococci (Chaffer et al. 2003, Linage and Gonzalo 2008) and *Trueperella pyogenes* (Saratsis et al. 1998). As these bacteria are not frequent aetiological agents of mastitis in lactating mammary glands, the findings raise a question regarding a possible increased susceptibility of involuting ovine mammary glands to those organisms.

Benefits of administration of antibiotics at the end of a lactation period

Intramammary administration of antibiotics

The principle of antibiotic administration at the end of a lactation period involves the intramammary infusion of a pharmaceutical preparation to both mammary glands of ewes in the flock. In all published works carried out in dairy flocks, a significantly greater cure rate of mammary abnormalities recorded at the end of lactation has been achieved after administration of the antibiotics. Pharmaceutical preparations used in the studies include combinations of antibiotics, which afford a broader spectrum of antibacterial activity. Preferably, the product for administration should be selected on the results of susceptibility testing of bacteria (Constable and Morin 2003, Mavrogianni et al. 2011) to be isolated from samples (e.g., mammary secretion, abscess material) from ewes individually examined, as detailed above. Moreover, antibiotic administration has led to a smaller incidence of new infections during the 'dry-period', which indicates the preventive role of the procedure during a period of high risk for intramammary infections and underlines the usefulness of strategic administration. The beneficial effects of the administration are clearly shown in the subsequent lactation, in which increased milk yields of treated ewes have been recorded.

Chaffer et al. (2003) found that prevalence of intramammary infections in ewes at drying-off was 45%; cure rate after intramammary administration of a combination of benzylpenicillin, nafcilin and dihydrostreptomycin at the end of lactation period was found to be 65% 15 to 20 days after the subsequent lambing, compared to 6.5% cure rate in control animals. In a similar study, Shwimmer et al. (2008) reported that prevalence of intramammary infections in ewes at drying-off was 47.5%; cure rate after intramammary administration of the above combination at the end of lactation period was found to be 71% two to three weeks after the subsequent lambing, compared to 8% cure rate in control animals. These authors also documented that mean milk yield throughout the lactation period subsequent to antibiotic administration increased by 19% and flock bulk milk mean somatic cell counts during the same period decreased to 1.0×10^6 cells mL^{-1} from 2.5×10^6 cells mL^{-1} in the previous lactation period (Shwimmer et al. 2008). De Santis et al. (2001) reported that cure rate of intramammary infections in ewes after intramammary administration of cloxacillin at the end of lactation period was found to be >60% 4 to 59 days after the subsequent lambing, compared to <50% cure rate in control animals. De Santis et al. (2001) and Spanu et al. (2011) also documented that treated ewes had significantly smaller somatic cell counts in the subsequent lactation compared to

controls. However, Gonzalo et al. (2009) found beneficial results in decreasing flock bulk milk somatic cell counts only in machine-milked ewes and not in hand-milked animals. Linage and Gonzalo (2008) reported that prevalence of intramammary infections in ewes at drying-off was 49%; cure rate after intramammary administration of a combination of penethemate and framycetin at end of lactation period was found to be 82% five days after the subsequent lambing, compared to 13% cure rate in control animals. Moreover, these authors found that incidence risk of new intramammary infections, during the 'dry-period', was 8% in treated and 23% in control ewes. Gonzalo et al. (2004) found that prevalence of intramammary infections in ewes at drying-off was 54.5%; cure rate after intramammary administration of a combination of penicillin and novobiocin at the end of a lactation period was found to be 61.5% at the subsequent lambing. Moreover, these authors indicated that milk yield of ewes increased by 7% in the lactation period subsequent to antibiotic administration, as a result of decreased incidence of new intramammary infections during the 'dry-period' (Gonzalo et al. 2004).

Thus, it becomes evident that in dairy ewes intramammary administration of antimicrobial agents at the end of a lactation period is effective in curing intramammary infections present at cessation of a lactation period, as well as in minimising the risk for intramammary infections during the 'dry-period'. Moreover, there are further benefits from a potential increase in milk yield and a decrease in flock bulk milk somatic cell counts during the subsequent lactation period.

The procedure has been found to be just as beneficial in ewes in mutton-production systems (Hendy et al. 1981, Watson and Buswell 1984, Hueston et al. 1989). In mutton-production systems, healthy mammary glands of the dam and increased milk yield during the first month of a lactation period are important for optimum growth rate of lambs (Fthenakis and Jones 1990a).

One can suggest that differences in the length of the 'dry-period', which reflect differences in production systems, may potentially affect the efficacy of intramammary administration of antibiotics at the end of a lactation period. However, Linage and Gonzalo (2008), who studied this particular factor in their work, did not find any significant differences in the efficacy of administration between groups of ewes with varying length of the 'dry-period'.

The potential beneficial effects of intramammary administration of antibiotics at the end of a lactation period in relation to the procedure followed for mammary involution (i.e., initiated or progressive) have not been studied.

Injectable administration of antibiotics

McCarthy et al. (1988) proposed the intramuscular administration of procaine penicillin to ewes at the end of a lactation period, after administration of which prevalence of subclinical mastitis at lambing was found to be 27% in treated animals and 31% in controls. Croft et al. (2000) indicated that the subcutaneous injection of tilmicosin one month prior to the expected start of the lambing period, concurrently with the anti-clostridial vaccination, led to a 43% decrease in prevalence of mammary abnormalities at the subsequent lambing. Moreover, the latter authors documented that mean bodyweight of 50-day-old lambs of treated ewes was greater by 520 g to that of lambs of control (untreated) ewes and attributed the benefit to the cure of pre-existing intramammary infections in treated ewes, which led to increased milk yield by the animals (Croft et al. 2000).

In comparison to the intramammary administration, it is noteworthy that an advantage of the injectable administration is the minimal risk of potential iatrogenic contamination of the mammary glands, as handling of the udder is avoided.

Publications regarding administration of antibiotics to ewes at the beginning of the 'dry-period' are summarised in Table I.i.

Table I.i. Summary presentations of publications regarding administration of antibiotics to ewes at the beginning of the 'dry-period'.

Reference	Route of administration/ Antibiotics used	Summary of findings
Hendy et al. (1981)	Intramammary/ Procaine penicillin, dihydrostreptomycin	Prevalence of mammary abnormalities at mating 1.5% in treated ewes and 4.5% in controls
Watson and Buswell (1984)	Intramammary/ Cloxacillin	Prevalence of intramammary infections at lambing 1% in treated ewes and 3.5% in controls; increased (up to 7%) bodyweight gain in lambs of treated ewes
McCarthy et al. (1988)	Intramuscular/ Procaine penicillin	Prevalence of subclinical mastitis at lambing was 27% in treated ewes and 31% in controls
Hueston et al. (1989)	Intramammary/ Cephapirin	Untreated ewes had a 2.6 times higher risk to developing intramammary infections at the early stage of the subsequent lactation period
Croft et al. (2000)	Subcutaneous/ tilmicosin	Cure rate of mammary abnormalities at the subsequent lambing was 43%; bodyweight of 50-day-old lambs of treated ewes was greater by 520 g to that of lambs of controls
De Santis et al. (2001)	Intramammary/ Cloxacillin	Cure rate of intramammary infections 4-59 after the subsequent lambing was >60%, compared to <50% in controls; treated ewes had significantly smaller milk somatic cell counts in the subsequent lactation compared to controls
Chaffer et al. (2003)	Intramammary/ Benzylpenicillin, nafcilin, dihydrostreptomycin	Cure rate of intramammary infections 15-20 days after the subsequent lambing was 65%, compared to 6.5% in controls
Gonzalo et al. (2004)	Intramammary/ Penicillin, novobiocin	Cure rate of intramammary infections at the subsequent lambing was 61.5%; milk yield in the subsequent lactation period increased by 7% compared to controls; no difference between 'complete' and 'selective' treatment in cure of intramammary infections
Bogolin and Vasiu (2008)	Intramammary/ Cloxacillin	'Selective' administration in ewes with subclinical mastitis; cure rate at lambing was 78.5%, compared to 23% in controls
Linage and Gonzalo (2008)	Intramammary/ Penethemate, framycetin	Cure rate of intramammary infections 5 days after the subsequent lambing was 82%, compared to 13% in controls; incidence risk of new intramammary infections during the 'dry-period' was 8% in treated ewes and 23% in controls; no differences in efficacy of administration with regard to length of the 'dry-period'
Shwimmer et al. (2008)	Intramammary/ Benzylpenicillin, nafcilin, dihydrostreptomycin	Cure rate of mammary abnormalities 2-4 weeks after the subsequent lambing was 71%, compared to 8% in controls; milk yield increased by 19% and bulk milk somatic cell counts decreased by 60% compared to ones in the previous lactation period
Gonzalo et al. (2009)	Intramammary/ Penethamate, benethamine penicillin, framycetin	Treated machine-milked, but not hand-milked, ewes had significantly smaller milk somatic cell counts in the subsequent lactation compared to controls
Spanu et al. (2011)	Intramammary/ Cephapirin	Treated ewes had significantly smaller milk somatic cell counts in the subsequent lactation compared to controls

‘Complete’ or ‘selective’ administration of antibiotics?

Administration of antibiotics to ewes at drying-off may be performed to all animals in a flock (‘complete’) or only to those considered to be infected (‘selective’). The need for selective administration was developed, due to public concerns regarding (i) potential antibiotic residues in the food chain and (ii) increased incidence of antibiotic-resistant bacterial strains in animals. Moreover, selective administration has a smaller cost to the farmer and a decreased risk of potential iatrogenic contamination of the mammary glands (Mavrogianni et al. 2011).

In the selective administration approach, there may be a query regarding criteria to be used in the selection of animals which will receive the antibiotic preparation. Clinical examination of all animals in the flock, as discussed above, has a small cost and can be used to identify animals in need of antibiotic administration. The procedure can be complemented with bacteriological examination of samples collected from clinically affected udders, which will support the decision for selection of the most appropriate antibiotic (Orphanou 1987, Saratsis et al. 1998). Milk somatic cell counting alone, as a means of identifying ewes in need of antibiotic administration, may not be a useful method, because somatic cell counts have been found to increase physiologically at the end of a lactation period, i.e., even in healthy ewes (Fthenakis 1995b), hence are not indicative of intramammary infections. Gonzalo et al. (2004) have not found any significant differences at flock level between the two methods (i.e., ‘complete’ or ‘selective’) in the cure of pre-existing intramammary infections. Bogolin and Vasiu (2008) used the approach of ‘selective’ treatment in ewes with subclinical mastitis and reported a cure rate of 78.5% in the treated animals.

A disadvantage of the ‘selective’ administration is the incomplete protection of the untreated ewes in the flock against new intramammary infections during the ‘dry-period’ (Berry and Hillerton 2002, Bergonier and Berthelot 2003), especially during the stage of active involution when there is an increased risk of mastitis (Orphanou 1987, Barkema et al. 1998, Saratsis et al. 1998).

Potential concerns regarding intramammary administration of antibiotics at the end of a lactation period

Administration of antibiotics should be performed under hygienic conditions and disinfection of the teat, to prevent introduction of pathogens, which may subsequently cause mastitis. Organisms that may be introduced into the teat at that point, include *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (Las Heras et al. 2000, Bergonier and Berthelot 2003, Spanu et al. 2011), for which the antibiotics usually administered are not effective.

It has been proposed, during intramammary antibiotic administration, the partial insertion of the tip of the tube or the use of short-tipped tubes, with the aim to avoid excessive dilatation of the teat canal and destruction of its protective lining (Bergonier and Berthelot 2003, Bergonier et al. 2003). The results of Gonzalo et al. (2004) indicated that the procedure was as effective as complete insertion of the tip of the tube into the teat. In view of previous findings, which pointed out to the significance of damaged teats as a risk factor for development of mastitis (Mavrogianni et al. 2006b, Fragkou et al. 2007), one may find the suggestion useful. Moreover, this method allows some antibiotic to be left inside the teat canal, that way preventing bacterial invasions into the mammary parenchyma.

After administration of the antibiotic, definite and complete cessation of the lactation period is essential for success of the procedure (Fthenakis et al. 2012). This implies that ewes should not be milked again after administration of the antibiotic, whilst, in mutton-production systems, lambs should have been removed from their dams before that.

Finally, some concerns have been voiced regarding potential problems with residues of the antibiotics in milk of the subsequent lactation (Chaffer et al. 2003, Linage and Gonzalo 2008, Shwimmer et al. 2008). Nevertheless, it should be noted that, at least in the European Union, veterinary pharmaceutical products are only licenced if adequate scientific evidence can be presented about residues in the animal products (e.g., meat, milk) and if appropriate withdrawal periods have been calculated (Athanasiou et al. 2009). Calculation of withdrawal periods for intramammary veterinary pharmaceutical products at the beginning of the 'dry-period' takes into account minimum length of the 'dry-period', as well as minimum time after parturition that milk cannot be given for human consumption. For example, in the study by Linage and Gonzalo (2008), no antibiotic residues in milk have been detected as early as 54 hours after the lambing subsequent to antibiotic administration. Therefore, maintenance of the prescribed withdrawal periods is essential to safeguard public health.

CHAPTER II

EFFECTS OF DRYING-OFF PROCEDURE IN EWES, IN SUBSEQUENT MAMMARY INFECTION AND DEVELOPMENT OF MASTITIS

A. INTRODUCTION

As discussed above, in dairy-type sheep production systems, mammary involution is either initiated (i.e., milking is stopped at once) or progressive (i.e., milking frequency is gradually decreased over a period of several days or weeks) (Gelasakis et al. 2010). In mutton-production systems, mammary involution is initiated, as cessation of lactation takes place when lambs are removed from their dam (Sargison 2008).

Currently, no studies are available to compare effects of the type of involution process (i.e., initiated or progressive involution) on incidence risk of subsequent intramammary infections and mastitis development and identify potential differences between the two procedures.

Anecdotal claims made in Greece have suggested that abrupt cessation of lactation with no milk removal during the process of involution would lead to mastitis. In contrast, respective claims made in Great Britain have suggested that milk removal during the process of initiated involution would lead to mastitis.

Objective of this study was to evaluate effects of the procedure followed for drying-off of ewes' udder (i.e., initiated or progressive involution) in subsequent mammary infection and development of mastitis. Two relevant experiments have been carried out; in these, intramammary antibiotic administration was or was not practiced.

B. EFFECTS OF THE DRYING-OFF PROCEDURE

Materials and methods

Study design

The work was carried out in a semi-intensive dairy flock in Central Greece. Lambs were removed from their dams at the age of 45 to 60 days and, then, ewes in the flock were hand-milked twice daily. After a total lactation period 6 to 8 month-long, multiparous ewes were included in the study.

In experiment I, 31 Lacaune-cross ewes were allocated at random in one of two groups (A or B). In experiment II, 12 Lacaune-cross ewes were allocated at random in one of two groups (C or D).

In ewes of group A (n=19) or C (n=6), udder drying-off procedure took place progressively during a period of 22 days; ewes were milked twice daily for the last time on D0; then, they were hand-milked once daily for a week (D1-D7), which was followed by another week during which ewes were hand-milked once every two days (D9, D11, D13), followed by a third week during which ewes were hand-milked once every three days (D16, D19, D22); in total, during the whole process, ewes were hand-milked on 13 occasions, always by the same milker. In ewes of group B (n=12) or D (n=6), udder drying-off took place abruptly; ewes were milked twice daily for the last time on D0 and no milking was carried out after that.

In Experiment II only, on D22 for group C animals and on D0 for group D animals (i.e., at the end of the lactation period), intramammary administration of a combination of 500 mg procaine penicillin and 300 mg neomycin sulphate (Neo-Mastitar®; MSD Animal Health, Boxmeer, The Netherlands) was carried out into the right mammary gland of all animals (C or D).

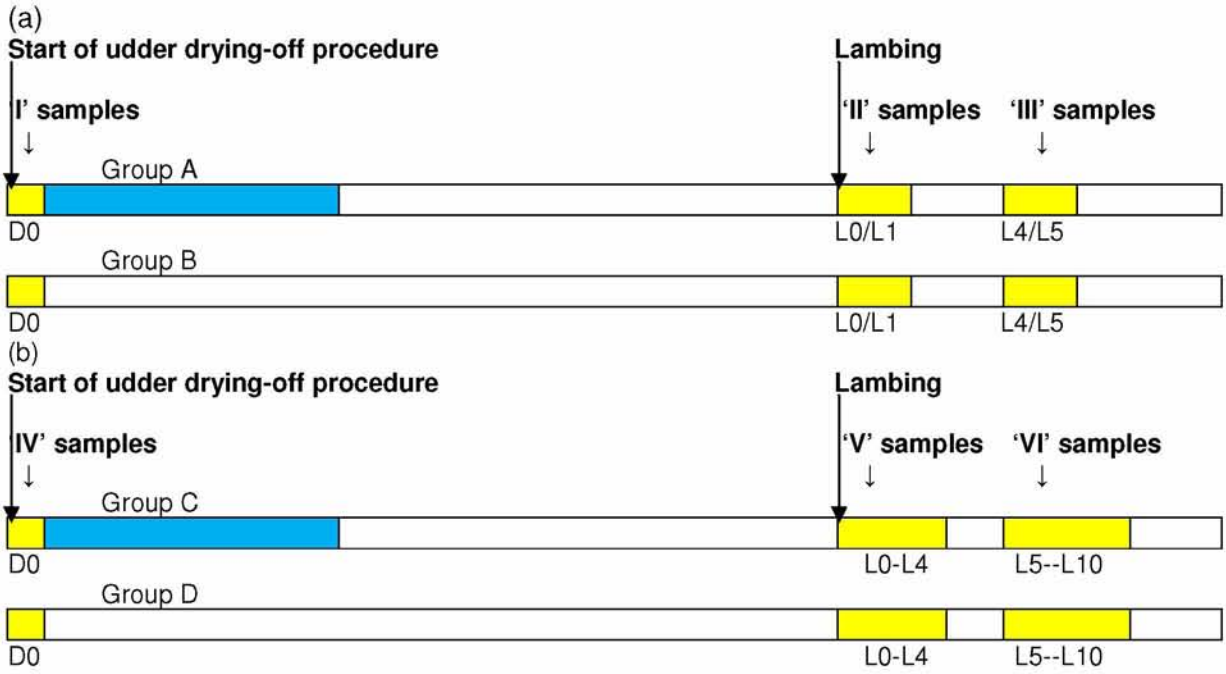
Teat duct material and milk samples were collected on D0 for bacteriological and cytological examinations. Subsequently to the above procedures, animals were mated. During the study and throughout pregnancy, no animal received any antimicrobial agents. At the end of pregnancy, ewes were moved to individual pens, where they lambed normally 180 to 211 days (Experiment I) or 183 to 209 days (Experiment II) after D0 (day of lambing: L0). Teat duct material and milk samples were collected again, after the subsequent lambing of the ewes.

Samplings and examinations performed

At the start of the drying-off procedure (on D0), all ewes were clinically examined, with special attention paid to their mammary glands and teats (Fthenakis 1994, Saratsis et al. 1998, Mavrogianni et al. 2005). A thorough disinfection was carried out using povidone iodine scrub solution on the teat apex and the lower (1 cm) part of the teat skin. A fine (20 G), plastic, sterile catheter (Abbocath®; Abbott, Abbott Park, IL, USA) was used for sampling the teat duct and collecting teat duct material. The stylet was taken out and the catheter was cut with a sterile blade to a length of 2 mm. In order to ensure accurate and consistent cutting of the catheter at the desired length, a sterilized ruler was always placed beside the catheter. The whole procedure was carried out under aseptic conditions. The catheter was held from the cannula hub and was inserted into the teat, rolled around the internal teat wall, in order to sample the mucosa, and then withdrawn. Description and validation details of the method have been presented previously (Mavrogianni et al. 2006a). Milk samples were then obtained. The first two squirts of secretion were drawn onto the palm of the gloved hand of the investigator and examined for the presence of abnormal signs; then, 10 to 15 ml of secretion were carefully collected into a sterile container.

In both Experiments, in all cases, samples were collected from both teats and both mammary glands of each ewe. In Experiment I, initially, samples were obtained on D0 before start of the drying-off procedure (all ewes) ['I' samples]. Subsequently to lambing (L0), further samples were collected. The first sample was collected on L0 before the lambs sucked their dam for the first time (15 ewes: 9 group A and 6 group B) or on L0 or L1 soon thereafter (16 ewes: 10 group A and 6 group B) ['II' samples]. The second sample was collected on L4 or L5 (all ewes) ['III' samples]; in all cases, an interval of ≥3 days elapsed between the two *post-partum* sampling occasions. In Experiment II, initially, samples were obtained on D0 before start of the drying-off procedure ['IV' samples]. Subsequently to lambing (L0), further samples were collected. The first sample was collected on L0 to L4 ['V' samples] and the second sample on L5 to L10 ['VI' samples]; in all cases, an interval of ≥5 days elapsed between the two *post-partum* sampling occasions (Fig. II.1.).

Figure II.1. Graphical presentation of design and samplings in: (a) Experiment I, (b) Experiment II.



Group A/C: progressive drying-off (blue coloured areas denote hand-milking period), group B/D: abrupt drying-off.
D0: day of start of udder drying-off, L0: day of lambing (yellow coloured areas denote sampling time-ponts [not at scale]).

Samples of material collected on the tip of the catheter ('teat duct material') and milk samples were plated onto Columbia 5% sheep blood agar; the media were incubated aerobically at 37 °C for up to 72 h. Throughout this study, all bacteria isolated were identified by using conventional techniques (Barrow and Feltham 1993, Euzeby 1997).

The California Mastitis Test (CMT) was carried out in milk samples, as described by Fthenakis (1995a) for ewes' milk, by using a reagent (Jorgen Kruuse A/S, Marslev, Denmark). Five degrees of reaction scores ('negative', 'trace', '1', '2', '3'), were recognized, according to the standards of Schalm et al. (1971) and Fthenakis (1995a) for ewes' milk. Reactions scored ≥'1' were considered to be indicative of increased cellular content in milk. Finally, leucocyte subpopulations were identified by direct microscopy after Giemsa stain of milk films; in each case 100 cells were observed and counted.

Data management and analysis

In the present study, there was a difficulty in estimating incidence rate (new 'infection' per individual at risk for each time point at risk). In many cases, an individual teat duct / mammary gland might change from being 'infected' to being 'uninfected' and *vice-versa*; therefore, when there was a long time-interval between sampling occasions, it was not possible to know what happened between the two sampling occasions, i.e. how many infections and 'cures' there might have been. Therefore, the following definitions were initially made: (i) 'isolation of bacteria' was equivalent to 'infection with'; 'isolation of bacteria from the teat duct material' was equivalent to 'infection of the teat duct' and 'isolation of bacteria from the milk' was equivalent to 'infection of the mammary gland'; (ii) on a particular sampling point, a teat duct / mammary gland was defined as being 'at risk of becoming infected' (i.e., yielding bacteria during the microbiological examination) if it had been uninfected (i.e., did not yield any bacteria during the microbiological examination) on the previous sampling point; (iii) on the subsequent sampling point, this teat duct / mammary gland could be either 'infected' (in which case it was not at risk) or 'uninfected' (in which case it was still at risk); (iv) on subsequent sampling occasions, if this teat duct / mammary gland was 'uninfected', then it was again 'at risk'; (v) if a teat duct / mammary gland was infected on one sampling occasion but not on the next one, then the infection was deemed to have been eliminated half-way between the two sampling occasions; conversely, if a teat duct / mammary gland was uninfected on one sampling occasion and infected on the next one, then the infection was considered to have taken place half way between the two occasions; (vi) if a teat duct / mammary gland was infected with the same organism on two consecutive sampling occasions, then it was infected throughout the time between those two sampling occasions; conversely, if a teat duct / mammary gland was uninfected on two consecutive sampling occasions then it was considered to be uninfected throughout the time between those two sampling occasions.

Based on the above, it was possible to calculate an estimate of the length of time a teat or a mammary gland was at risk before it became infected; teats ducts / mammary glands contributed more than one value if they became uninfected and then were re-infected. These results were modelled by using survival analysis.

In Experiment I, analysis of results was carried out by comparing changes in status between 'I' *versus* 'II' and 'III' samples. In Experiment II, analysis of results was carried out by comparing changes in status between 'IV' *versus* 'V' and 'VI' samples.

Incidence rate was calculated from the formula: M/[A-(V/2), where M: number of new cases during the *post-partum* period (i.e.: for Experiment I, either on L0/L1 or on L4/L5 – for Experiment II, either on L0-L4 or on L5-L10), A: animals at risk at lambing and V: number of animals withdrawn from the study during the internal time period evaluated (Martin et al. 1987). Cure rate was calculated from the formula: N/[B-(W/2), where N: number of cases that had shown the outcome of interest on D0, but did not show it again during the *post-partum* period (i.e., neither on L0/L1, nor on L4/L5), B: number of cases that had shown the outcome of interest on D0 and W: number of animals withdrawn from the study during the internal time period evaluated (Martin et al. 1987).

During the analysis, the following outcomes were evaluated: 'teat duct infection' (i.e., isolation of bacteria from teat duct material samples), 'mammary infection' (i.e., isolation of bacteria from milk samples), 'subclinical mastitis' (i.e., isolation of bacteria from a milk sample coupled with increased CMT score in the same sample, but with no clinically detectable abnormalities in that mammary gland) and 'abnormal findings in a mammary gland' (i.e., presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings, including clinical mastitis, in a mammary gland). Specifically for cure rate, the only outcome evaluated was 'abnormal findings in a mammary gland'.

In all cases, teat duct material and milk samples were considered separately. Statistical significance was assessed by the Sign Test, which allowed for the readings to be paired. Data were modelled in Minitab 14 (Minitab Inc., State College, PA, USA).

Significance level was set at $P = 0.05$, on a 2-sided null hypothesis of no difference.

Results

Experiment I

All animals lambed normally. No lamb deaths were recorded during the study.

Median time to first teat duct or mammary infection *post-partum* was 0 days (teat ducts and mammary glands) for group A and 2.25 and 0 days (teat ducts and mammary glands, respectively) for group B ($P > 0.38$). Bacteria were isolated always in pure culture. Of the 33 bacterial isolates obtained, 79% were coagulase-negative *Staphylococcus* spp.; moreover, *Bacillus* spp., *Staphylococcus aureus* and *Streptococcus* spp. were also isolated. Detailed results are in Table II.i.

No significant differences were observed between the two groups in the *post-partum* frequency of teat duct infection ($P > 0.13$), of mammary infection ($P > 0.8$), of subclinical mastitis ($P > 0.78$) or of abnormal findings in a mammary gland ($P > 0.11$). Detailed results are in Table II.ii.

No significant differences were seen between the two groups in the *post-partum* incidence risk of any of the outcomes studied: teat duct infection ($P = 0.545$), mammary infection ($P = 0.647$), subclinical mastitis ($P = 0.476$) or abnormal findings in a mammary gland ($P = 0.259$). Detailed results are in Table II.iii.

Finally, no significant differences were evident between the two groups in the *post-partum* cure rate of abnormal findings in a mammary gland ($P = 0.847$). Detailed results are in Table II.iv.

No significant differences were evident in leucocyte subpopulations between groups A and B after lambing. Detailed results are in Table II.v.

Table II.i. Frequency of bacterial isolates obtained from teat duct material and milk samples from ewes, in which udder drying-off procedure took place progressively (group A) or abruptly (group B).

	Group A	Group B
Isolates from teat duct material samples		
Samples 'I' (collected on D0)	*Bacillus* spp.: 1 *S. chromogenes*: 1 *S. epidermidis*: 2	*S. simulans*: 4 *Streptococcus* spp.: 1
Samples 'II' (collected on L0-L1)	*S. chromogenes*: 1 *S. simulans*: 2	*S. aureus*: 1
Samples 'III' (collected on L4-L5)		*S. aureus*: 1 *S. xylosus*: 3
Isolates from milk samples		
Samples 'I' (collected on D0)	*S. chromogenes*: 2 *S. epidermidis*: 1 *Streptococcus* spp.: 1	*S. epidermidis*: 1 *S. simulans*: 1
Samples 'II' (collected on L0-L1)	*S. chromogenes*: 1 *S. simulans*: 3	*S. aureus*: 1 *S. simulans*: 2
Samples 'III' (collected on L4-L5)	*S. epidermidis*: 2	*S. aureus*: 1

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
L: left, R: right.

Table II.ii. Frequency of teat duct infection, mammary infection, subclinical mastitis and abnormal findings in the mammary gland in ewes, in which udder drying-off procedure took place progressively (group A) or abruptly (group B).

	Group A (n=19)	Group B (n=12)
Teat duct infection		
Samples 'I' (collected on D0)	0.105	0.208
Samples 'II' (collected on L0-L1)	0.079	0.042
Samples 'III' (collected on L4-L5)	0.000[a]	0.200[a]
Mammary infection		
Samples 'I' (collected on D0)	0.105	0.083
Samples 'II' (collected on L0-L1)	0.105	0.125
Samples 'III' (collected on L4-L5)	0.067	0.050
Subclinical mastitis		
Samples 'I' (collected on D0)	0.211	0.167
Samples 'II' (collected on L0-L1)	0.105	0.083
Samples 'III' (collected on L4-L5)	0.067	0.100
Abnormal findings in a mammary gland		
Samples 'I' (collected on D0)	0.211	0.583
Samples 'II' (collected on L0-L1)	0.316	0.333
Samples 'III' (collected on L4-L5)	0.267	0.600

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
'Teat duct infection' = isolation of bacteria from teat duct material samples; 'mammary infection' = isolation of bacteria from milk samples; 'subclinical mastitis' = isolation of bacteria from a milk sample coupled with increased CMT score in the same sample; 'abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row indicates statistically significant difference at $P < 0.05$.

Table II.iii. Incidence rate (during the first 5 days *post-partum*) of teat duct infections, mammary infections, subclinical mastitis and abnormal findings in the mammary gland in ewes, in which udder drying-off procedure took place progressively (group A) or abruptly (group B).

	Group A (n=19)	Group B (n=12)
Teat duct infection		
	0.033	0.176
Mammary infection		
	0.167	0.118
Subclinical mastitis		
	0.231	0.111
Abnormal findings in a mammary gland		
	0.385	0.750

'Mammary infection' = isolation of bacteria from milk samples; 'subclinical mastitis' = isolation of bacteria from a milk sample coupled with increased CMT score in the same sample; 'abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row indicates statistically significant difference at $P < 0.05$.

Table II.iv. Cure rate (during mammary involution and the first 5 days *post-partum*) of abnormal findings in the mammary gland in ewes, in which udder drying-off procedure took place progressively (group A) or abruptly (group B).

Group A (n=19)	Group B (n=12)
0.500	0.429

'Abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row indicates statistically significant difference at $P < 0.05$.

Table II.v. Mean proportion of leucocytes in milk samples from ewes, in which udder drying-off procedure took place progressively (group A) or abruptly (group B).

	Group A	Group B
Abnormal findings in a mammary gland		
Samples 'I' (collected on D0)	Lym: 14% Mac: 43% Neu: 43%	Lym: 16% Mac: 37% Neu: 47%
Samples 'II' (collected on L0-L1)	Lym: 11% Mac: 30% Neu: 59%	Lym: 15% Mac: 44% Neu: 41%
Samples 'III' (collected on L4-L5)	Lym: 10% Mac: 31% Neu: 59%	Lym: 6% Mac: 36% Neu: 58%
No abnormal findings in a mammary gland		
Samples 'I' (collected on D0)	Lym: 7%, Mac: 65% Neu: 28%	Lym: 11% Mac: 63% Neu: 26%
Samples 'II' (collected on L0-L1)	Lym: 7% Mac: 38% Neu: 55%	Lym: 7% Mac: 31% Neu: 61%
Samples 'III' (collected on L4-L5)	Lym: 6% Mac: 33% Neu: 62%	Lym: 7% Mac: 40% Neu: 53%

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
L: left, R: right.
Lym: lymphocytes, Mac: macrophages: Neu: neutrophils.

Experiment II

All animals lambed normally. No lamb deaths were recorded during the study.

Median time to first teat duct infection *post-partum* was 2 and 4.5 days (left and right, respectively) for group C and 6.5 and 3.5 (left and right, respectively) for group D ($P > 0.38$). Median time to first mammary infection *post-partum* was 4.5 and 7 days (left and right, respectively) for group C and 6.5 and 3.5 (left and right, respectively) for group D (for all comparisons, $P > 0.22$). Bacteria were isolated always in pure culture. Of the 38 bacterial isolates obtained, 74% were coagulase-negative *Staphylococcus* spp.; moreover, *Bacillus* spp., *Escherichia coli*, *Mannhemia haemolytica* and *S. aureus* were also isolated. Detailed results are in Table II.vi.

No significant differences were observed between the two groups in the *post-partum* frequency of teat duct infection ($P > 0.17$), of mammary infection ($P > 0.36$ for both left and right mammary glands), of subclinical mastitis ($P = 1.00$ for left and $P > 0.36$ for right mammary glands) or of abnormal findings in a mammary gland ($P > 0.36$ for left and $P > 0.17$ for right mammary glands). Moreover, no significant differences were observed between left and right

side of the udder in frequency of teat duct infection ($P > 0.2$), of mammary infection ($P > 0.36$), of subclinical mastitis ($P > 0.2$) and of abnormal findings in a mammary gland ($P > 0.17$) for both groups. Detailed results are in Table II.vii.

No significant differences were recorded between the two groups in the *post-partum* incidence risk of any of the outcomes studied: teat duct infection ($P = 0.754$ for left and $P = 1.00$ for right mammary glands), mammary infection ($P = 0.753$ for left and $P = 0.423$ for right mammary glands), subclinical mastitis ($P = 0.623$ for left and $P = 0.391$ for right mammary glands) or abnormal findings in a mammary gland ($P = 0.851$ for left and $P = 0.852$ for right mammary glands). Moreover, no significant differences were seen between left and right side of the udder in any of the outcomes studied: teat duct infection ($P > 0.67$), mammary infection ($P > 0.75$), subclinical mastitis ($P > 0.79$) and abnormal findings in a mammary gland ($P > 0.39$), for both groups. Detailed results are in Table II.viii.

Finally, no significant differences were evident between the two groups in the *post-partum* cure rate of abnormal findings in a mammary gland ($P = 0.956$ for left and $P = 0.887$ for right mammary glands). A significant difference was evident between left and right mammary glands ($P = 0.027$ and $P = 0.043$ for group C and D, respectively). Detailed results are in Table II.ix.

No significant differences were evident in leucocyte subpopulations between groups C and D after lambing. Detailed results are in Table II.x.

Table II.vi. Frequency of bacterial isolates obtained from teat duct material and milk samples from ewes, in which udder drying-off procedure took place progressively (group C) or abruptly (group D), with intramammary antibiotic administration in their right mammary gland at cessation of lactation.

	Group C (n=6)	Group D (n=6)
Isolates from teat duct material samples		
Samples 'IV'-L (collected on D0)	*S. caprae*: 1 *S. epidermidis*: 1 *S. simulans*: 1	*S. xylosus*: 1
Samples 'IV'-R (collected on D0)	*Bacillus* spp.: 1 *Staphylococcus caprae*: 1 *S. epidermidis*: 1 *S. simulans*: 1	*S. chromogenes*: 1 *S. epidermidis*: 1 *S. xylosus*: 1
Samples 'V'-L (collected on L0-L4)	*M. haemolytica*: 1 *S. chromogenes*: 1	
Samples 'V'-R (collected on L0-L4)	*S. aureus*: 1	*S. epidermidis*: 1
Samples 'VI'-L (collected on L5-L10)	*M. haemolytica*: 1 *S. epidermidis*: 1	*E. coli*: 1
Samples 'VI'-R (collected on L5-L10)	*M. haemolytica*: 1	*S. epidermidis*: 2
Isolates from milk samples		
Samples 'IV'-L (collected on D0)	*S. caprae*: 1 *S. epidermidis*: 1 *S. simulans*: 1	*S. xylosus*: 1
Samples 'IV'-R (collected on D0)	*S. caprae*: 1 *S. epidermidis*: 1 *S. simulans*: 1	*S. chromogenes*: 1 *S. epidermidis*: 1
Samples 'V'-L (collected on L0-L4)	*M. haemolytica*: 1	
Samples 'V'-R (collected on L0-L4)		*S. epidermidis*: 1
Samples 'VI'-L (collected on L5-L10)	*M. haemolytica*: 1 *S. epidermidis*: 1	*E. coli*: 1
Samples 'VI'-R (collected on L5-L10)	*M. haemolytica*: 1	*S. epidermidis*: 2

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
L: left, R: right.

Table II.vii. Frequency of teat duct infection, mammary infection, subclinical mastitis and abnormal findings in a mammary gland in ewes, in which udder drying-off procedure took place progressively (group C) or abruptly (group D), with intramammary antibiotic administration in their right mammary gland at cessation of lactation, during the first 10 days *post-partum*.

	Group C (n=6)	Group D (n=6)
Teat duct infection		
Samples 'IV'-L (collected on D0)	0.500	0.167
Samples 'IV'-R (collected on D0)	0.667	0.600
Samples 'V'-L (collected on L0-L4)	0.333	0.000
Samples 'V'-R (collected on L0-L4)	0.167	0.167
Samples 'VI'-L (collected on L5-L10)	0.333	0.167
Samples 'VI'-R (collected on L5-L10)	0.167	0.333
Mammary infection		
Samples 'IV'-L (collected on D0)	0.500	0.167
Samples 'IV'-R (collected on D0)	0.500	0.400
Samples 'V'-L (collected on L0-L4)	0.167	0.000
Samples 'V'-R (collected on L0-L4)	0.000	0.167
Samples 'VI'-L (collected on L5-L10)	0.333	0.167
Samples 'VI'-R (collected on L5-L10)	0.167	0.333
Subclinical mastitis		
Samples 'IV'-L (collected on D0)	0.333	0.000
Samples 'IV'-R (collected on D0)	0.333	0.200
Samples 'V'-L (collected on L0-L4)	0.000	0.000
Samples 'V'-R (collected on L0-L4)	0.000	0.167
Samples 'VI'-L (collected on L5-L10)	0.167	0.167
Samples 'VI'-R (collected on L5-L10)	0.167	0.000
Abnormal findings in a mammary gland		
Samples 'IV'-L (collected on D0)	0.333	0.500
Samples 'IV'-R (collected on D0)	0.333	0.833
Samples 'V'-L (collected on L0-L4)	0.333	0.167
Samples 'V'-R (collected on L0-L4)	0.000	0.333
Samples 'VI'-L (collected on L5-L10)	0.500	0.500
Samples 'VI'-R (collected on L5-L10)	0.167	0.333

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
L: left, R: right.
'Teat duct infection' = isolation of bacteria from teat duct material samples; 'mammary infection' = isolation of bacteria from milk samples; 'subclinical mastitis' = isolation of bacteria from a milk sample coupled with increased CMT score in the same sample; 'abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row or in the same column indicates statistically significant difference at $P < 0.05$.

Table II.viii. Incidence rate (during the first 10 days *post-partum*) of teat duct infections, mammary infections, subclinical mastitis and abnormal findings in a mammary gland in ewes, in which udder drying-off procedure took place progressively (group C) or abruptly (group D), with intramammary antibiotic administration in their right mammary gland at cessation of lactation.

	Group C (n=6)	Group D (n=6)
Teat duct infection		
L	0.333	0.200
R	0.500	0.500
Mammary infection		
L	0.000	0.200
R	0.000	0.333
Subclinical mastitis		
L	0.000	0.167
R	0.000	0.250
Abnormal findings in a mammary gland		
L	0.250	0.333
R	0.000	0.000

L: left, R: right.
'Mammary infection' = isolation of bacteria from milk samples; 'subclinical mastitis' = isolation of bacteria from a milk sample coupled with increased CMT score in the same sample; 'abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row or in the same column indicates statistically significant difference at $P < 0.05$.

Table II.ix. Cure rate (during mammary involution and the first 10 days *post-partum*) of abnormal findings in a mammary gland in ewes, in which udder drying-off procedure took place progressively (group C) or abruptly (group D), with intramammary antibiotic administration in their right mammary gland at cessation of lactation.

	Group C (n=6)	Group D (n=6)
L	0.000^{a}	0.333^{b}
R	0.500^{a}	0.600^{b}

L: left, R: right.
'Abnormal findings in a mammary gland' = presence of mammary abnormalities, defined as any case of subclinical mastitis or of clinically detectable abnormal findings in a mammary gland, including clinical mastitis.
Same superscript in the same row or in the same column indicates statistically significant difference at $P < 0.05$.

Table II.x. Mean proportion of leucocytes in milk samples from ewes, in which udder drying-off procedure took place progressively (group C) or abruptly (group D).

	Group C (n=6)	Group D (n=6)
Abnormal findings in a mammary gland		
Samples 'III'-L (collected on D0)	Lym: 11% Mac: 9% Neu: 80%	Lym: 26% Mac: 25% Neu: 49%
Samples 'III'-R (collected on D0)	Lym: 9% Mac: 10% Neu: 81%	Lym: 33% Mac: 45% Neu: 22%
Samples 'IV'-L (collected on L0-L4)	Lym: 8% Mac: 17% Neu: 75%	(2)
Samples 'IV'-R (collected on L0-L4)	(1)	Lym: 5% Mac: 11% Neu: 84%
Samples 'V'-L (collected on L5-L10)	Lym: 10% Mac: 20% Neu: 70%	Lym: 6% Mac: 20% Neu: 74%
Samples 'V'-R (collected on L5-L10)	Lym: 3% Mac: 7% Neu: 90%	Lym: 6% Mac: 20% Neu: 74%
No abnormal findings in a mammary gland		
Samples 'III'-L (collected on D0)	Lym: 20% Mac: 29% Neu: 51%	Lym: 7% Mac: 34% Neu: 59%
Samples 'III'-R (collected on D0)	Lym: 21% Mac: 13% Neu: 66%	Lym: 9% Mac: 10% Neu: 81%
Samples 'IV'-L (collected on L0-L4)	Lym: 5% Mac: 19% Neu: 76%	Lym: 7% Mac: 27% Neu: 66%
Samples 'IV'-R (collected on L0-L4)	Lym: 10% Mac: 25% Neu: 65%	Lym: 8% Mac: 27% Neu: 65%
Samples 'V'-L (collected on L5-L10)	Lym: 8% Mac: 27% Neu: 65%	Lym: 10% Mac: 19% Neu: 71%
Samples 'V'-R (collected on L5-L10)	Lym: 10% Mac: 33% Neu: 57%	Lym: 10% Mac: 27% Neu: 63%

D0: day of start of the drying-off procedure, L0: day of subsequent lambing.
L: left, R: right.
Lym: lymphocytes, Mac: macrophages, Neu: neutrophils.
(1): no samples into that category on that sampling point, (2): sample not possible to process.

CHAPTER III

ULTRASONOGRAPHIC FINDINGS IN THE OVINE UDDER, IN RELATION TO THE DRYING-OFF PROCEDURE

A. INTRODUCTION

The previous two experiments have indicated that there were no effects of the drying-off procedure (i.e., progressive or abrupt) in subsequent mammary infection and development of mastitis. Currently, no studies are available to compare structural changes in the udder according to the type of involution process, which may potentially play a role in subsequent intramammary infections and mastitis development, and to identify differences between the two procedures. Nevertheless, structural changes during involution (e.g., distension of teat cistern due to milk accumulation, changes in blood flow etc.) may be responsible for increased incidence risk of infection during the procedure.

Ultrasonographic examination can be useful in monitoring the reproductive organs of domestic animals (Lazaridis et al. 2012). The technique can provide information about the structure of the organs not available by any other means, whilst being non-invasive, non-ionizing, rapid and painless (Gouletsou et al. 2004, Mendelson 2007). In studies where time-long examination of animals is required, ultrasonographic imaging has the advantage over histological examination after biopsy tissue sampling, that animals can continue to be in the study for its entire duration.

In B-mode ultrasonography, an image is the result of ultrasound waves being reflected into tissues of varying density, then returning in different degrees (Goddard 1995).

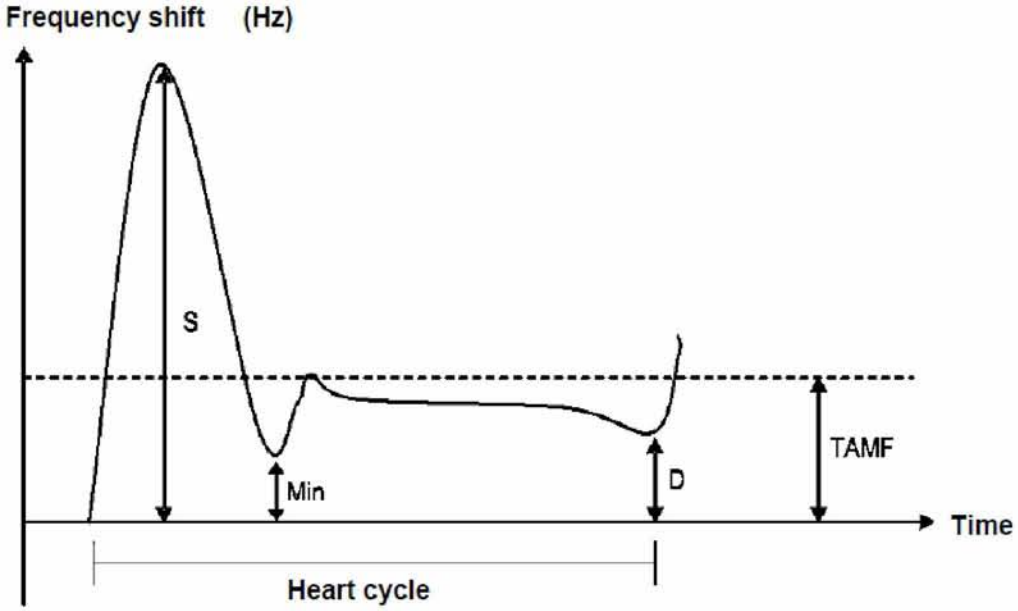
In Doppler ultrasonography, waves emitted by the transducer are reflected by erythrocytes moving within the blood vessels (Brant and Dougherty 2012, Pellerito and Polak 2012). When these move towards the transducer, frequency of returning ultrasound waves is greater than that of emitted waves; in the image seen, blood flow is depicted in red colour. When erythrocytes move away from the transducer, frequency of reflected waves is smaller than that of emitted ones; in the image seen, blood flow is depicted in blue colour. When movement of erythrocytes is perpendicular to the ultrasound beam, there is no difference between the two ultrasound frequencies (emitting and returning) and no colouration can be observed in the image taken (Brant and Dougherty 2012). The angle at which the ultrasound beams intersect the path of flowing blood, is termed ‘Doppler angle’ or ‘angle of insonation’ (Ginther 2007).

Doppler ultrasonography can be appled for measuring blood flow in various tissues, which helps to determine the function of these tissues. Velocity of blood flow measured by

means of Doppler ultrasonography is proportional to the difference between frequency of returning wanes and frequency of emitted waves and inversely proportional to the cosine of the Doppler angle. Specifically, the equation is as follows: $V_{bf} = (Freq_{rw} - Freq_{ew}) / cos\ ANG_D$, where V_{bf}: blood flow velocity, $Freq_{rw}$: frequency of returning waves, $Freq_{ew}$: frequency of emitted waves, ANG_D: Doppler angle. Hence, these measurements are more accurate when taken with an angle of 45 ° to 60 ° (Arning 2002, Mendelson 2007, Pellerito and Polak 2012).

Doppler ultrasonography allows an immediate qualitative evaluation of the vascular perfusion of a tissue or organ, by looking into the existence of a diastolic blood flow, its direction and continuity during one heart cycle (Goswamy and Steptoe 1988, Tekay et al. 1996). For quantitative evaluation, a Doppler spectral analysis is necessary. A Doppler graphical spectrum can be produced based in the changing velocity of the blood flow over time during the examination (Rohren et al. 2003, Scheinfeld et al. 2009, Brant and Dougherty 2012). When blood flow of an artery is measured, in each cardiac cycle there is a systolic and a diastolic peak, hence spectral curve has a characteristic form (Potapow 2010) (Fig. III.1). Equipment and software currently available can provide results of blood volume and blood velocity by taking into account measurements taken during the examination.

Figure III.1. Schematic illustration of a Doppler wave with the maximal systolic (S), the minimal diastolic (Min), the end-diastolic (D) and the time averaged maximum (TAMF) frequency shift during one heart cycle (Potapow 2010).



For obtaining a Doppler graphical spectrum, the Doppler gate must be placed within the lumen of the vessel and positioned inside the vessel under examination; the direction of the blood flow should be defined, so that the Doppler angle can be calculated. Finally, when optimal colour flow is achieved, a spectral mode waveform can be taken. The Doppler gate must not be wider than the diametre of the vessel examined; otherwise, possible movements of the vessel wall are taken into account, which would result in artifacts in the spectral graph obtained. In general, it might be technically demanding to examine for a long time period in order to obtain a spectrum (Mendelson 2007), especially with animals moving during the examination. Size of the cursor may also have an impact on the variation of velocity of erythrocytes shown in the spectral graph. A wide gate would lead to increased variation of velocities, as erythrocytes move at varying speeds within a blood vessel, ones at the centre of the vessel moving faster than those nearer the vessel wall. Hence, a vessel length of >2 cm is necessary for placing the angle cursor and correct measurement of the velocities (Ginther 2007, Brant and Dougherty 2012).

By using the 'peak systolic velocity' (i.e., maximum blood velocity during a systole) and the 'end diastolic velocity' (i.e., blood velocity at the end of the cardiac cycle immediately before the new systole), it is possible to calculate the Doppler resistance index and pulsatility index (Ginther 2007, Mendelson 2007). Both indices increase when blood perfusion is decreasing (Brant and Dougherty 2012).

Objective of the present study was to record by means of ultrasonographic examination (B-mode and Doppler), changes occurring in the udder of ewes during involution and to compare differences between the two procedures followed (i.e., progressive or abrupt) for udder drying-off.

B. ULTRASONOGRAPHIC FINDINGS

Materials and methods

Study design

The work was carried out in multiparous Lacaune-cross ewes housed at the Department of Obstetrics and Reproduction of the University of Thessaly and monitored throughout the study. Lambs had been removed from their dams at the age of 45 to 60 days and, then, ewes were hand-milked twice daily. After a total lactation period 6 to 8 month-long, animals were included into the experiment.

Initially, 20 ewes were allocated at random in one of two groups (E or F). Inclusion criteria for animals into the study were: (a) no abnormal findings in the clinical examination before inclusion, (b) bacteriologically negative results in teat duct material and milk samples, (c) California Mastitis Test scores in milk samples <1 and (d) daily milk yield 350 to 400 mL per ewe.

In ewes of group E (n=10), udder drying-off procedure took place progressively during a period of 22 days; ewes were milked twice daily for the last time on D0; then, they were hand-milked once daily for a week (D1-D7), which was followed by another week during which ewes were hand-milked once every two days (D9, D11, D13), followed by a third week during which ewes were hand-milked once every three days (D16, D19, D22); in total, during the whole process, ewes were hand-milked on 13 occasions, always by the same milker. In ewes of group F (n=10), udder drying-off procedure took place abruptly; ewes were milked twice daily for the last time on D0 and no milking was carried out after that.

Throughout the involution process, ultrasonographic examinations (B-mode and Doppler) were performed in the udder of the experimental animals, on the following days after start of the study: D0 (last day when ewes were milked twice daily), D2, D4, D7, D10, D14, D18, D23, D30 and D37. Clinical examinations were performed on each occasion when ultrasonographic examination of the udder was carried out.

The subsequent lambing of the ewes took place 72 to 76 days after D37. Then, 4 to 5 days after lambing, teat duct material and milk samples were collected again for bacteriological examination and California Mastitis Test scoring.

Samplings and examinations performed

Clinical examination, with special attention to the mammary glands and teats, was performed as described before by Fthenakis (1994), Saratsis et al. (1998) and Mavrogianni et al. (2005). Teat duct material and milk samples were collected by following the procedures previously described in detail (Chapter II). In all cases, samples were collected from both teats and both mammary glands of each ewe. All samples were processed bacteriologically and cytologically as previously described (Chapter II).

Ultrasonographic examination

Ultrasonographic examination was performed with the animal on the standing position and restrained inside a crate, using the support of an assistant. For examination, hair on the udder had been fully clipped.

Ultrasonographic examination of the udder was performed with an ultrasound scanner (MyLab® 30; ESAOTE SpA, Genova, Italy) fitted with a linear transducer with 4.0-13.0 MHz imaging frequency and a microconvex transducer with 2.0-8.0 MHz imaging frequency. Coupling gel was applied. The transducer was placed on the caudal surface of the udder and moved around it.

Initially, the left side of the udder was imaged. The linear transducer was placed in a position perpendicular to the long axis and dorsal sections of the mammary parenchyma were taken, starting from the upper part downwards. In each mammary gland, three images were saved for further processing; first image was taken before the branching of the external pudendal artery (*arteria pudenda externa*), second when distance between branches of the external pudendal artery was ~1 cm and third image was taken immediately before the gland cistern (*sinus lactiferous*) became visible; 60 mm scanning depth and 10 MHz frequency were used for this procedure.

Subsequently, the gland cistern (*sinus lactiferous*) was imaged by using the microconvex transducer. At first, an inclined sagittal imaging plane was used, from the upper part of the intermammary groove (*sulcus intermammarius*) towards the teat; the teat was used as the scanning axis. A second image was taken, by rotating the microconvex transducer at 90 °, at the same examination point as previously, i.e., from the upper part of the intermammary

groove towards the teat but at the dorsal plane; 120 mm scanning depth and 3.3 MHz frequency were used for this procedure.

The final measurements were taken at the external pudendal artery, which was used for Doppler measurements in the mammary gland by means of the linear transducer; 60 mm scanning depth and 10 MHz frequency were used for this procedure. At the start, a cross-section of the vessel was taken, with the objective to measure the diametre of the vessel. Then, the colour Doppler gate was positioned inside the vessel examined, but not touching the vessel's wall; when optimal colour flow was achieved, a spectral mode waveform was taken. A skilled assistant was helping when measuring the blood flow velocity, in order to make adjustments necessary for optimal quality of colour-flow images. Animals were stressed as little as possible during the examination, with the objective to have them examined remaining still for the period of time necessary to apply correctly the Doppler gate and to take the spectral display after localising the vessel to be studied. Initially, the vessel to be examined with the colour-flow imaging, was localized. Then, adjustments necessary for optimal colour-flow image quality were made. Spectral mode was switched on and the Doppler gate was positioned inside the vessel, whilst taking care not to include the vessel wall and to avoid artifacts; this was followed by setting a Doppler angle of 50 ° to 60 ° for the examination.

All the imaging procedures described above were subsequently repeated for the right mammary gland. In all cases, images (B-mode or Doppler) were frozen and saved on the equipment hard-disk for performing subsequently appropriate measurements and data analysis.

Data management and analysis

Results taken into account

In the analysis, ultrasonographic results of only 14 ewes (group E n=7, group F n=7) were taken into account. These animals were the ones, which met cumulatively the following criteria: (a) no clinical abnormalities detected throughout the study and after lambing, (b) bacteriologically negative results in teat duct material and milk samples collected after lambing, (c) California Mastitis Test scores <1 in milk samples collected after lambing. This approach supported use of results only from animals with no abnormalities in the udder throughout the study and guaranteed that ultrasonographic findings were those of the udder of healthy ewes.

Quantitative evaluations of ultrasonographic images

During the ultrasonographic examination four sets of images were recorded: (a) images of mammary parenchyma, (b) images of mammary gland cistern, (c) images of cross-sections of external pudendal artery and (d) spectral waveforms of external pudendal artery. All images were initially evaluated visually for presence of abnormal structures therein. Data management was performed on images that had been saved at the time of examination.

Stored images of mammary parenchyma were processed by means of ImageJ software (National Institutes of Health, Rockville Pike, MD, USA), which can edit, process and analyse gray-scale images, by calculating area and pixel value statistics (National Institutes of Health 2013). In an image processing context, gray-scale analysis refers to the image's pixel intensity values (Ojala et al. 2002). For analysis of gray-scale, results of each of the three images stored from each mammary gland on each occasion were considered together; standard descriptive statistics were applied in each case. Areas with vessels or ductal formations were not taken into account for the gray-scale analysis. Results were expressed on a 0 (black) to 255 (white) scale.

Stored images of mammary gland cistern were processed by means of ImageJ software, which, after appropriate scale setting, was able to measure on the image the selected area within the boundaries of the cistern (National Institutes of Health 2013). Two different measurements were taken, one from the image taken at the sagittal plane and another from the image taken at the dorsal plane; data for left and right mammary gland of the same animal were considered separately. Results were expressed as mm^2.

Stored images of cross-sections of external pudendal artery were processed by means of MyLab software (ESAOTE SpA, Genova, Italy), which, after pointing out the internal boundaries of the vessel, calculated the internal diametre of the vessel. Results were expressed as mm.

Finally, spectral waveforms of the external pudendal artery were processed by means of MyLab software (ESAOTE SpA, Genova, Italy), which, based on the outline of the waveform, calculated directly the resistance index (RI) and the pulsatility index (PI) of the vessel, based on the following equations RI = (PSV-EDV)/PSV and PI = (PSV-EDV)/TAMV, where PSV: peak systolic velocity, EDV: end diastolic velocity and TAMV: time-averaged maximum velocity (Ginther 2007); data for left and right mammary gland of the same animal were considered separately. Results were expressed as ratios.

Statistical computations

All data were entered into Excel spreadsheets (Microsoft Corporation; Redmond, WA, USA). Initially, descriptive statistics for all parametres and all groups (mean±standard error of the mean) were performed.

Initially, repeated measurements mixed effect linear regression models were used to determine whether outcomes in the two mammary glands of each animal changed over the course of the study period. Models were adjusted for repeated measurements within animals. Independent variables (fixed effects) included mammary gland (i.e., left or right) and time-point of the study (i.e., D0, D2, D4, etc.). In all cases, no significant differences were evident between the left and the right glands of the experimental animals within each group (for all parametres, $P > 0.56$ or $P > 0.68$, for group E or F, respectively).

In view of that, the time-series results of all measurements (left and right glands) obtained from animals of the same group were considered together for between group comparisons, i.e., n=14 subjects within each group. Then, repeated measurements mixed effect linear regression models were used to determine whether outcomes in the two groups of the study changed over the course of the study period. Models were adjusted for repeated measurements within animals. Independent variables (fixed effects) included experimental group (i.e., progressive or abrupt cessation of lactation), time of the study (i.e., D0, D2, D4, etc.) and a time of the study by experimental group interaction.

Finally, another analysis was performed, which took into account results of only three time-points as follows: D23, D30, D37 for group E versus D2, D10, D18 for group F. Objective of this analysis was to compare findings at the early stages after cessation of lactation in both groups. The above model was also used for this analysis.

All analyses were performed by a commercial statistical program (SPSS, v. 15 for Windows; SPSS Inc., Chicago, IL, USA). Significance level was set at $P = 0.05$.

Results

In general, anatomic structures in the mammary gland had a medium echogenicity to hyperechogenicity. Blood vessels and lactiferous ducts were imaged therein. No abnormal structures were observed. On D0, the mammary parenchyma was seen with a lobular structure (consisting of the alveolar areas with reduced echogenicity), the connective tissue (with increased echogenicity) and the ductal part of the gland and the blood vessels therein (imaged

as anechoic antra). As involution progressed, the mammary parenchyma was imaged to have homogeneity, with the disappearance of the lobular picture. Moreover, an overall reduced (compared to D0) echogenicity was recorded (Figs III.2 and III.3). Structures with medium echogenicity could include fat, ductal and lobular epithelial tissues and loose, intralobular and periductal stromal fibrous tissue. In contrast, hyperechoic structures could include the compact interlobular stromal fibrous tissue, the mammary fasciae, the ligaments and the skin. During scanning, other findings, e.g., the external pudendal artery and its branches, could also be imaged as spontaneous observations during the various procedures (Fig. III.4).

Mean (±standard error of the mean) gray-scale results in group E were 78.0±2.6 on D0, 78.5±2.8 on D14 and 72.9±2.1 on D37; respective values in group F were 76.6±4.1, 68.6±2.6 and 67.8±2.5 (for differences between groups, $P = 0.049$). In all cases, there was clear evidence that the progressive decrease of gray-scale results throughout the study period was significant in both groups, E or F ($P = 0.004$), as were interactions between time and group ($P < 0.0005$).

Milk clots were imaged into the gland cistern the earliest on D30 in group E ewes (i.e., 8 days after cessation of milking) and on D7 in group F ewes (i.e., 7 days after cessation of milking). Initially, these appeared as small flakes that 'flew' into the gland cistern, later aggregating to form large clots (1-2 cm in diametre), which remained visible ultrasonographically up to one 1 month after milk removal had stopped (Fig. III.5). A temporary increase in cistern area was evident on D2, D4 and D7 in group F animals, which, was followed by a subsequent decrease thereafter (Fig. III.6). Cistern area results did not differ between animals in group E or F ($P = 0.349$ for sagittal plane images, $P = 0.573$ for dorsal plane images). However, differences throughout the study period were significant ($P < 0.001$), as were interactions between time and group ($P < 0.001$).

Diametre of the external pudendal artery (Fig. III.7) differed significantly between the two groups ($P = 0.007$). Mean (±standard error of the mean) diametres in group A were 2.85±0.12 mm on D0, 2.59±0.19 mm on D14 and 2.44±0.08 mm on D37; respective values in group B were 2.84±0.08 mm, 2.32±0.08 mm and 2.18±0.08 mm. Differences throughout the study period were also significant ($P < 0.001$), but interactions between time and group were not ($P = 0.071$).

The spectral display in Doppler mode was observed as a broad band structure. Both resistance index and pulsatility index progressively increased throughout the study period in both groups. There was clear evidence that differences between animals in group E or in group F were significant for both parametres studied ($P < 0.001$) for pulsatility index and resistance

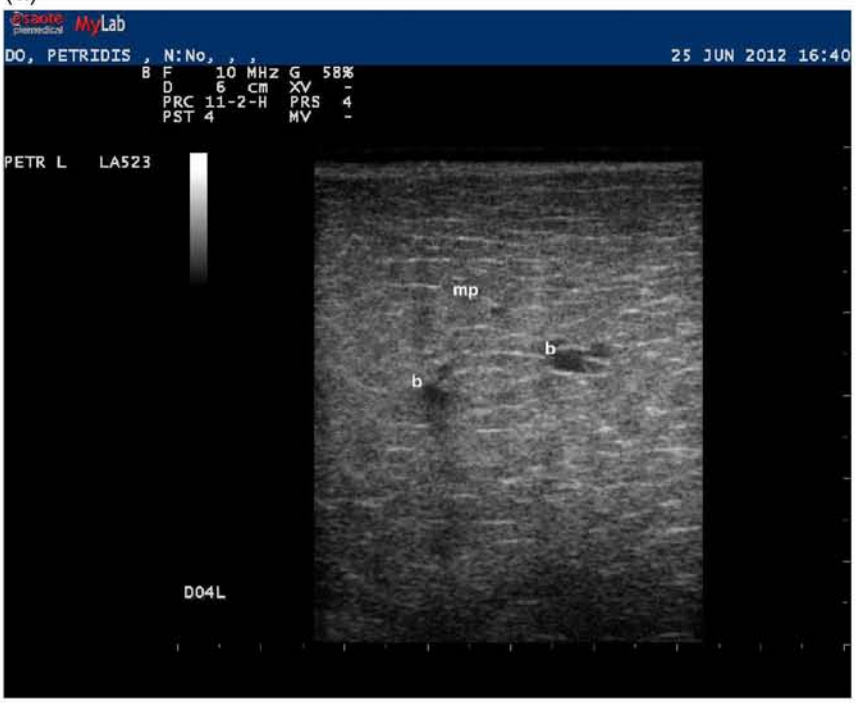
index) (Fig. III.8). Also, progressive changes in blood flow results throughout the study period were significant in both groups, A or B ($P < 0.001$), as were interactions between time and group ($P < 0.001$).

Finally, when data at the early stages after cessation of lactation (i.e., D23, D30, D37 in group E and D2, D10, D18 in group F) were used for comparisons between the two groups, there was no evidence that differences in gray-scale results ($P = 0.425$) or cistern area ($P = 0.537$ for sagittal plane images and $P = 0.163$ for dorsal plane images) were significant. Progressive changes throughout the analysis period were significant in cistern area results ($P < 0.001$), but not in gray-scale ($P = 0.425$). Interactions between time and group were not significant for any of the above parametres ($P > 0.3$). In contrast, differences in resistance index and pulsatility index were significant between the two groups of ewes ($P = 0.021$ for pulsatility index and $P = 0.015$ for resistance index); progressive changes throughout the analysis period were significant in both groups, A or B ($P < 0.0005$), as were interactions between time and group ($P < 0.015$).

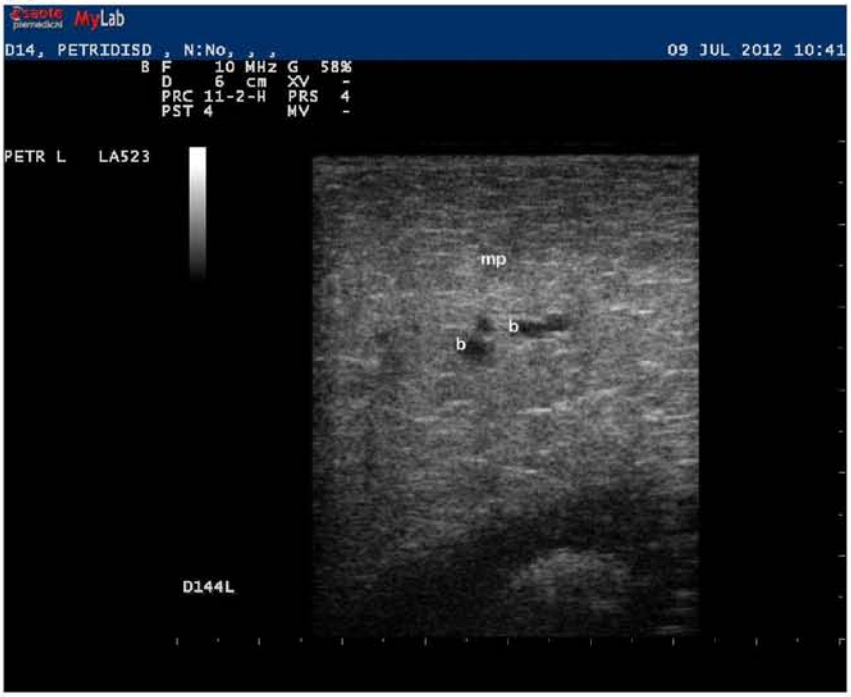
Detailed results are in Table III.i.

Figure III.2. Serial ultrasonographic appearance of mammary parenchyma [*mp*] of ewes during progressive udder drying-off procedure: (a) D0 (before start of the drying-off procedure), (b) D14, (c) D30, (d) D37. Images taken at a level after the branching of the external pudendal artery (when distance between the two branches [*b*] was ~1 cm) on a MyLab® 30 ultrasonography system with linear transducer 10.0 MHz and scanning depth 60 mm.

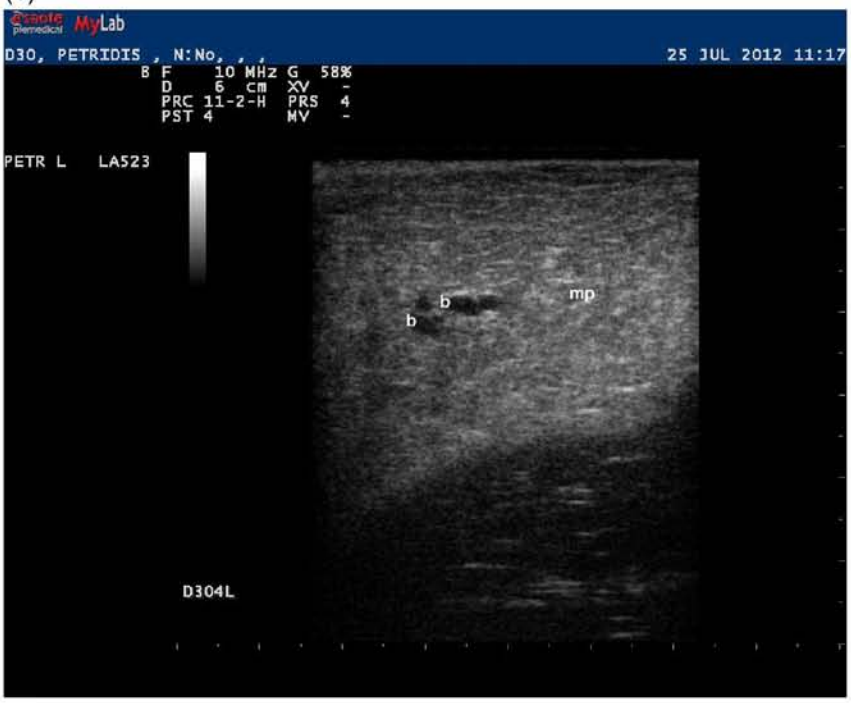
(a)



(b)



(c)



(d)

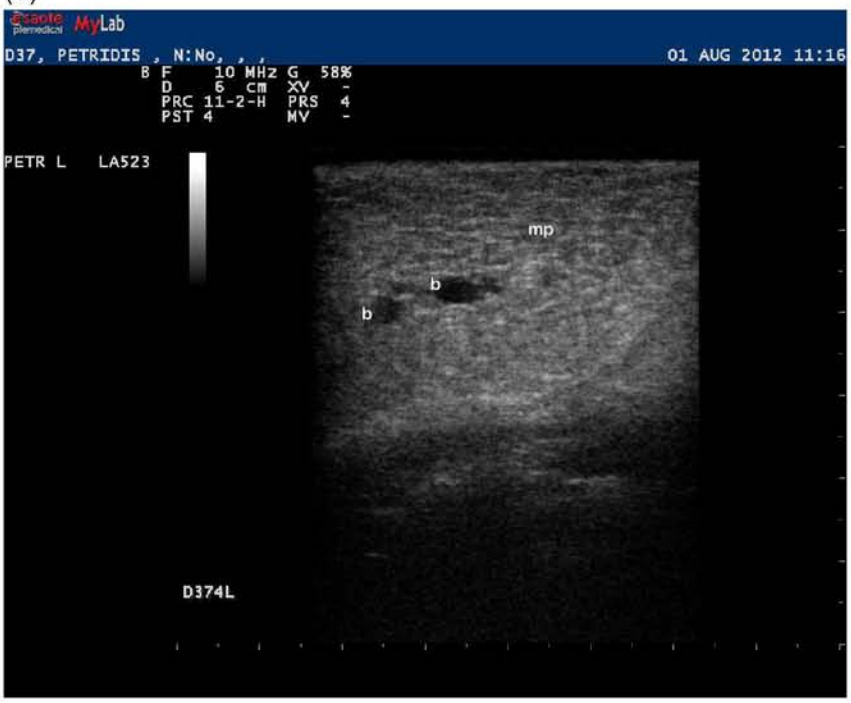
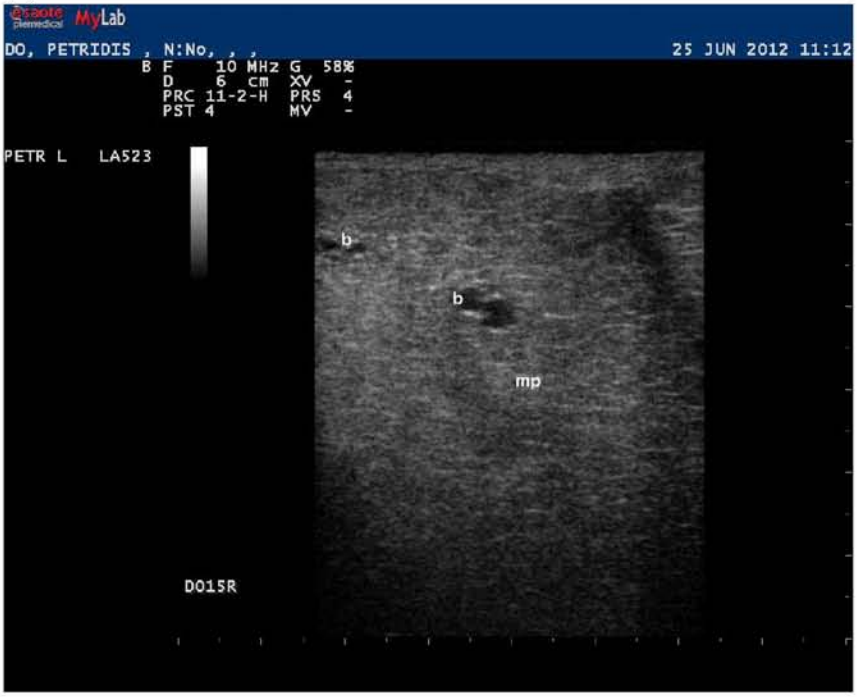
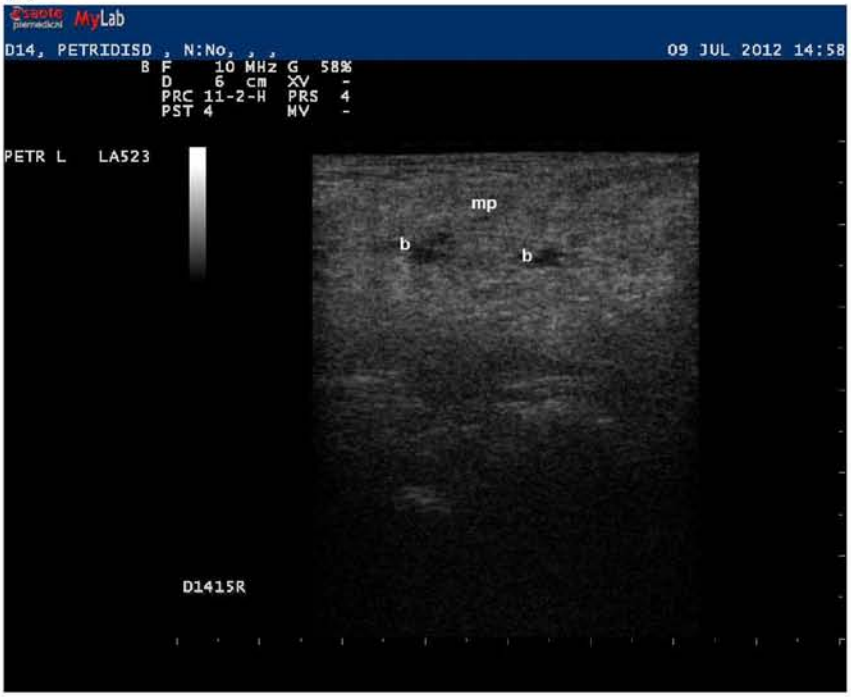


Figure III.3. Serial ultrasonographic appearance of mammary parenchyma [*mp*] of ewes during abrupt udder drying-off procedure: (a) D0 (before start of the drying-off procedure), (b) D14, (c) D30, (d) D37. Images taken at a level after the branching of the external pudendal artery (when distance between the two branches [*b*] was ~1 cm) on a MyLab® 30 ultrasonography system with linear transducer 10.0 MHz and scanning depth 60 mm.

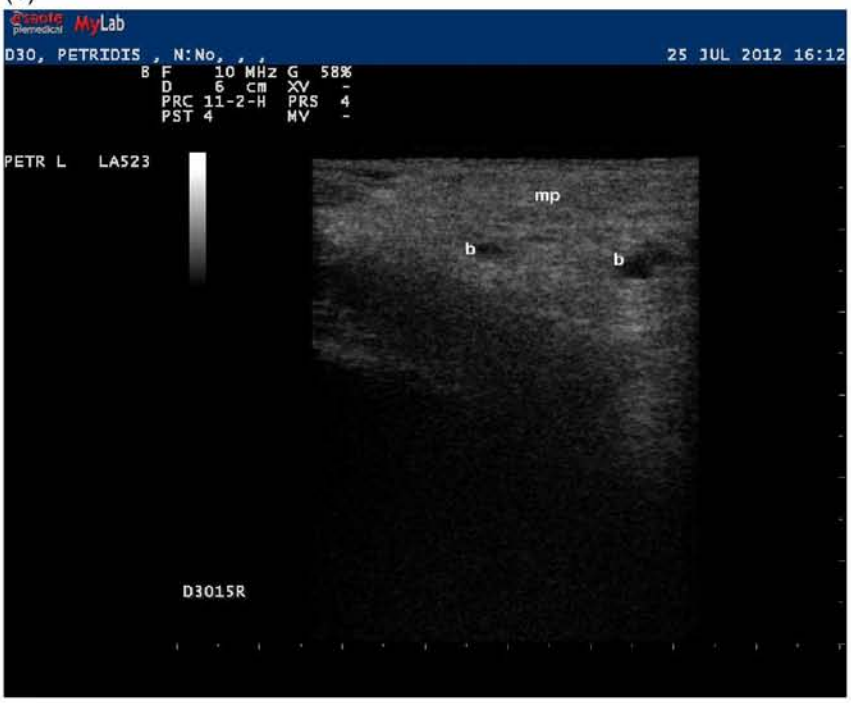
(a)



(b)



(c)



(d)

Figure III.4. Ultrasonographic appearance of the external pudendal artery [*p*] and its branches [*b*] at the mammary gland of ewes. Image taken at a sagittal imaging plane on a MyLab® 30 ultrasonography system with microconvex transducer 3.3 MHz and scanning depth 120 mm.

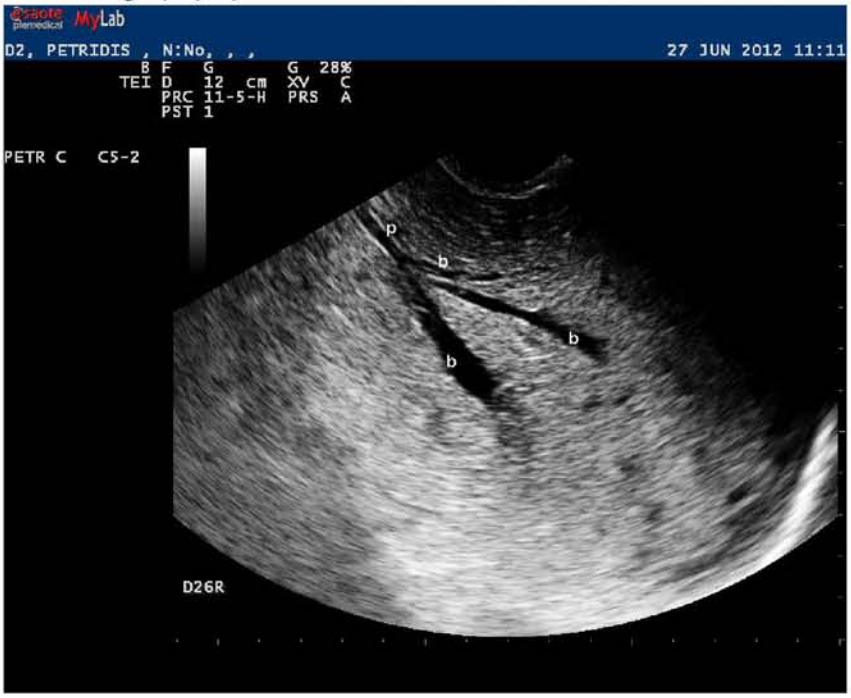
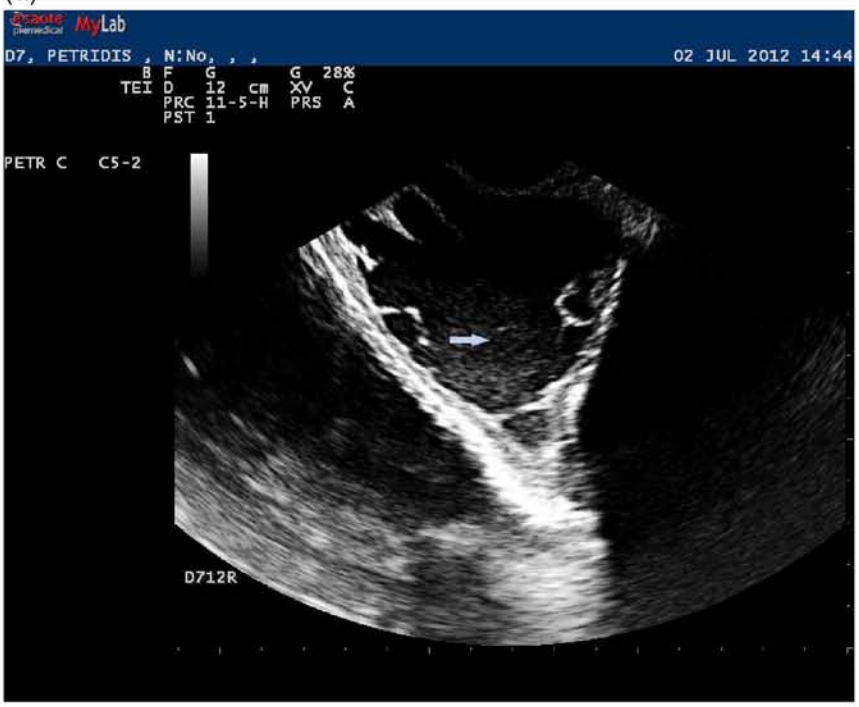


Figure III.5. Ultrasonographic appearance of milk clots [*arrow*] in the gland cistern of ewes during abrupt udder drying-off procedure: (a) D7 (7 days after cessation of milking), (b) D18. Images taken at an inclined sagittal imaging plane, from the upper part of the intermammary groove towards the teat, which was used as the scanning axis on a MyLab® 30 ultrasonography system with microconvex transducer 3.3 MHz and scanning depth 120 mm.

(a)



(b)

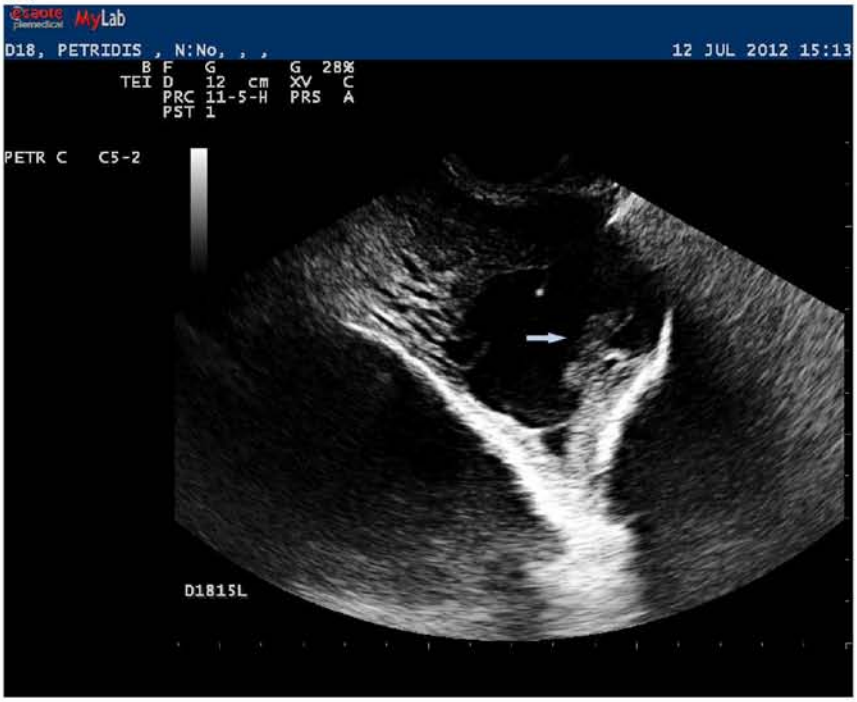
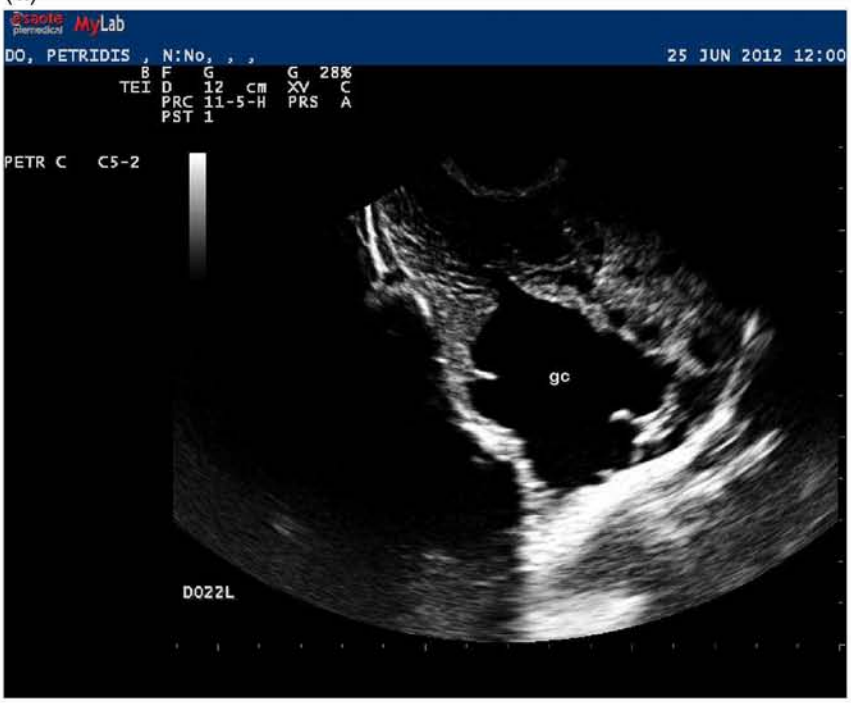
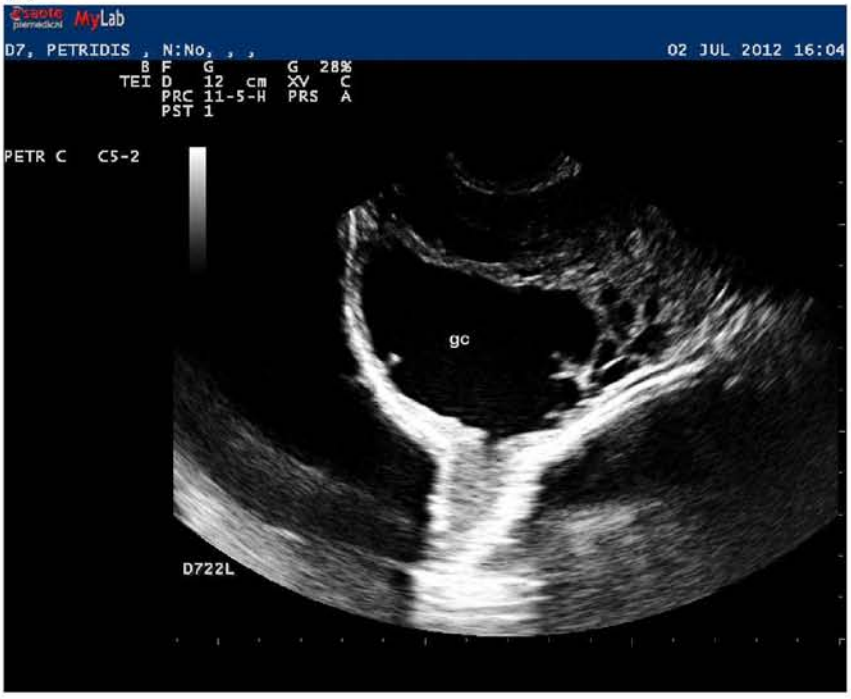


Figure III.6. Serial ultrasonographic appearance of gland cistern [*gc*] of ewes during abrupt udder drying-off procedure: (a) D0 (before start of the drying-off procedure), (b) D7, (c) D30. Images taken at an inclined dorsal imaging plane, from the upper part of the intermammary groove towards the teat, which was used as the scanning axis on a MyLab® 30 ultrasonography system with microconvex transducer 3.3 MHz and scanning depth 120 mm.

(a)



(b)



(c)

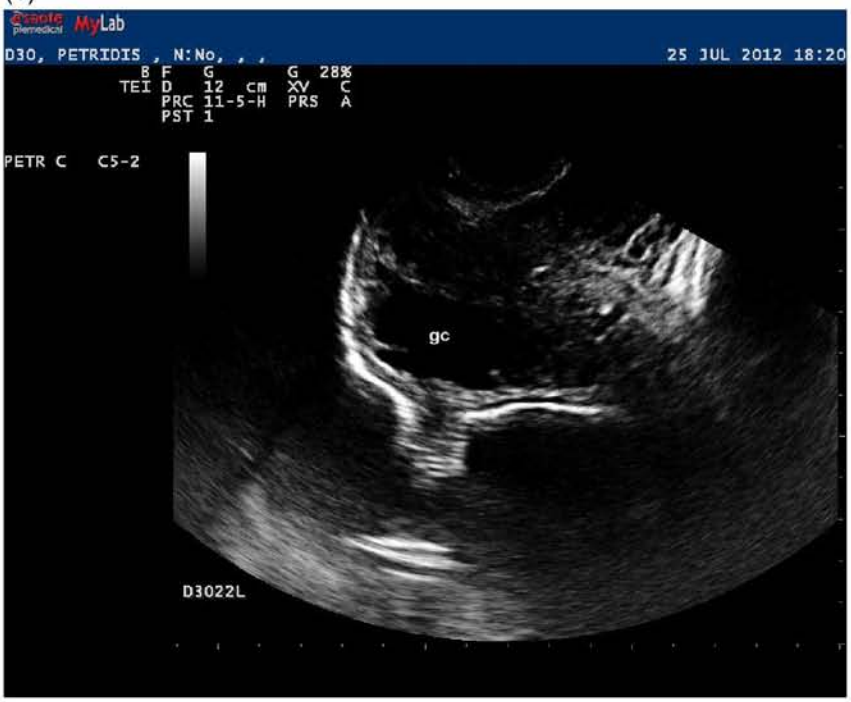


Figure III.7. Ultrasonographic appearance of cross-section of external pudendal artery [*p*] of ewes on D0 (before start of the drying-off procedure). Image taken on a MyLab® 30 ultrasonography system with with linear transducer 10 MHz and scanning depth 60 mm.

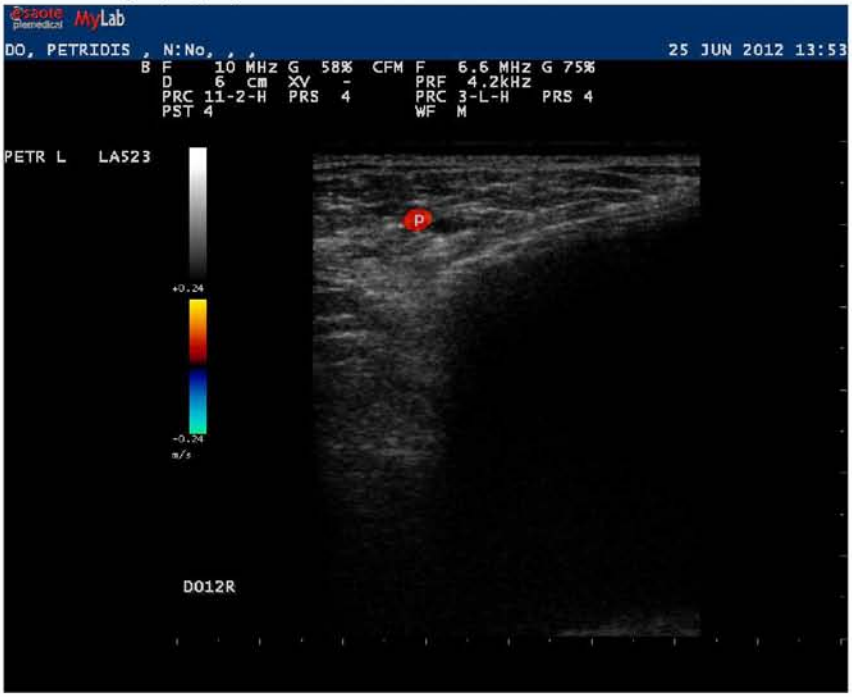
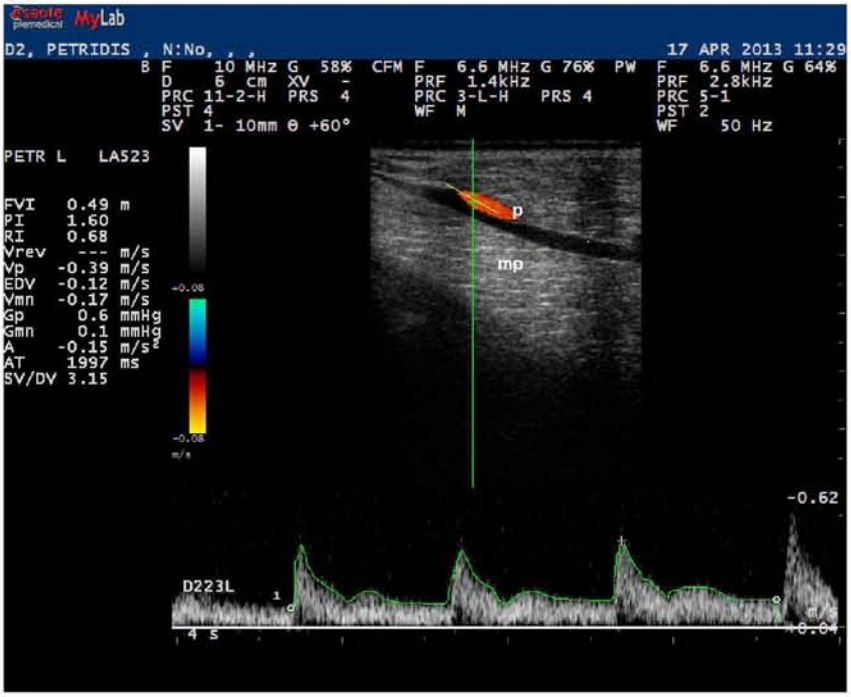
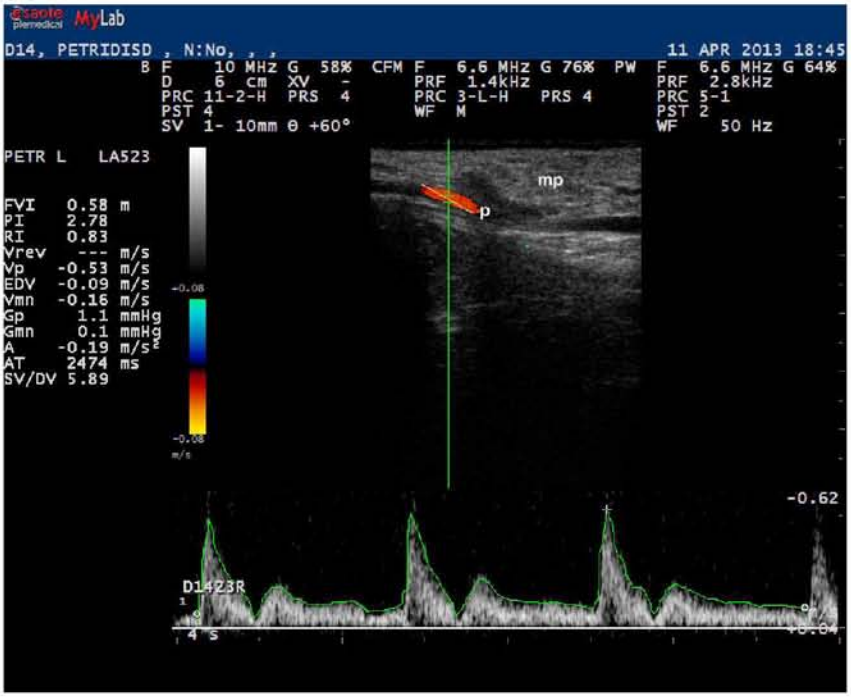


Figure III.8. Serial spectral waveforms of the external pudendal artery [*p*] at the mammary parenchyma [*mp*] of ewes: (a) D2 (2 days after start of the udder drying-off procedure), (b) D14, (c) D37. Images taken and processed on a MyLab® 30 ultrasonography system with linear transducer 10 MHz and scanning depth 60 mm.

(a)



(b)



(c)

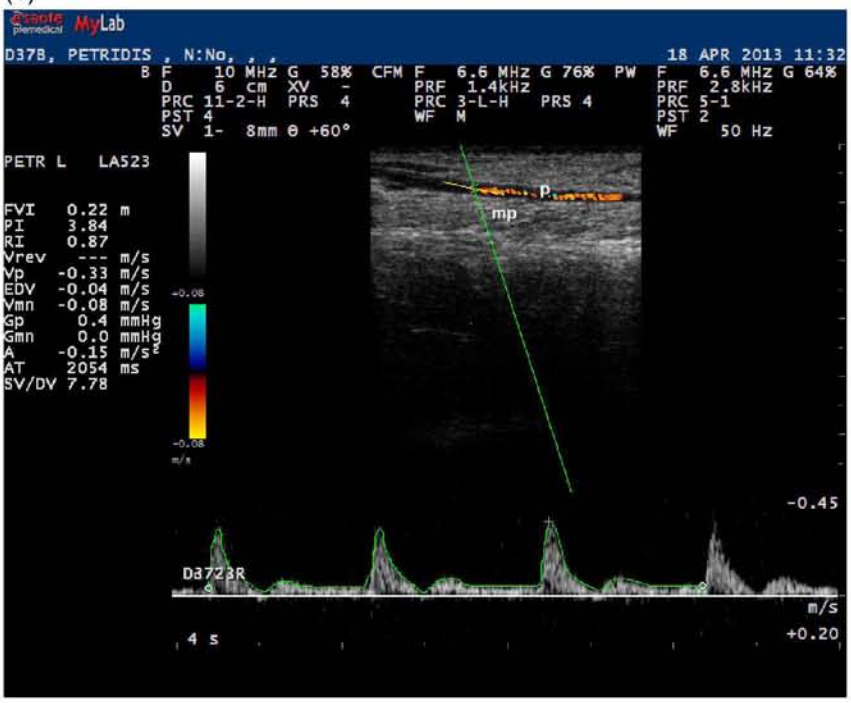


Table III.i. Quantitative results of ultrasonographic examination of udder in ewes, in which udder drying-off procedure took place progressively (group E) or abruptly (group F); for each parametre examined, mean±standard error of the mean (upper row) and median, distribution (lower row) are presented; results of both mammary glands of ewes are considered together, as no statistical difference ($P > 0.5$) was evident between left and right mammary glands.

	D0	D2	D4	D7	D10	D14	D18	D23	D30	D37
Group E										
Mammary parenchyma gray-scale	78.0±2.6	82.1±3.2	85.4±3.1	81.7±3.6	79.2±3.4	78.5±2.8	74.7±2.4	73.8±2.7	73.6±2.8	72.9±2.1
	77.3 (62.5-94.3)	82.2 (63.5-101.8)	88.8 (64.5-104.2)	82.1 (63.5-104.0)	79.7 (62.3-100.6)	80.9 (61.9-93.6)	75.9 (59.0-91.4)	75.6 (55.6-89.4)	75.9 (56.8-88.8)	73.2 (59.7-83.4)
Mammary gland cistern area at sagittal plane	580±47	519±48	495±48	495±48	528±46	551±52	605±65	576±69	464±64	361±49
	577.5 (259-850)	497 (227-787)	477.5 (226-760)	525.5 (217-775)	545.5 (257-824)	544 (254-840)	720.5 (221-874)	611 (187-932)	418 (160-925)	326.5 (145-665)
Mammary gland cistern area at dorsal plane	488±36	452±34	421±37	420±33	464±36	483±42	527±53	482±56	377±53	277±37
	462.5 (259-675)	448 (253-615)	382.5 (244-634)	399 (239-637)	488 (300-658)	508.5 (264-709)	538.5 (203-872)	506.5 (201-729)	348 (164-852)	229 (136-577)
External pudendal artery diametre (mm)	2.85±0.12	2.88±0.13	2.83±0.17	2.73±0.13	2.51±0.14	2.64±0.19	2.61±0.16	2.41±0.15	2.46±0.08	2.44±0.08
	2.8 (2.0-3.6)	2.7 (2.3-3.6)	2.75 (1.5-3.6)	2.65 (1.9-3.6)	2.45 (1.4-3.4)	2.45 (1.8-3.5)	2.65 (1.5-3.3)	2.40 (1.4-3.3)	2.55 (2.2-2.8)	2.45 (1.9-2.8)
External pudendal artery resistance index	0.58±0.02	0.55±0.02	0.59±0.01	0.62±0.01	0.62±0.03	0.64±0.02	0.66±0.02	0.69±0.02	0.69±0.02	0.70±0.02
	0.57 (0.47-0.69)	0.565 (0.40-0.65)	0.58 (0.50-0.69)	0.625 (0.55-0.72)	0.64 (0.47-0.82)	0.635 (0.56-0.74)	0.675 (0.56-0.76)	0.675 (0.60-0.80)	0.69 (0.59-0.82)	0.70 (0.57-0.81)

Table III.i (continued).

	D0	D2	D4	D7	D10	D14	D18	D23	D30	D37
Group E										
External pudendal artery pulsatility index	1.03±0.07 0.975 (0.71-1.65)	1.02±0.05 1.045 (0.71-1.42)	1.09±0.06 1.03 (0.84-1.58)	1.2±0.05 1.145 (0.88-1.53)	1.29±0.12 1.255 (0.76-2.30)	1.33±0.07 1.28 (1.01-1.80)	1.46±0.09 1.59 (0.96-2.04)	1.55±0.10 1.45 (1.12-2.40)	1.64±0.12 1.50 (1.17-2.79)	1.74±0.14 1.65 (1.00-2.74)
Group F										
Mammary parenchyma gray-scale	76.6±4.1 75.6 (54.4-104.0)	71.8±3.5 70.8 (51.3-97.9)	73.1±3.6 74.9 (47.3-97.4)	71.6±2.4 70.8 (55.5-89.8)	68.7±2.4 68.3 (54.5-84.4)	68.6±2.6 71.5 (45.1-78.7)	71.8±2.0 72.6 (57.0-80.3)	71.6±2.8 74.8 (50.0-85.2)	68.5±2.1 71.5 (57.0-79.9)	67.8±2.5 70.4 (52.1-85.6)
Mammary gland cistern area at sagittal plane	467±37 470.5 (234-731)	628±39 636 (311-845)	693±44 706.5 376-902)	651±65 646 (323-1070)	525±45 528 (295-762)	435±34 436.5 (276-636)	377±29 361.5 (239-584)	312±26 285 (181-499)	260±24 241.5 (156-468)	211±25 170 (121-412)
Mammary gland cistern area at dorsal plane	430±23 445.5 (306-569)	560±28 566.5 (369-740)	609±34 629 (383-836)	571±55 545 (340-1046)	463±37 440.5 (286-738)	400±34 366 (266-691)	339±24 304 (247-556)	281±26 242 (172-521)	239±23 206 (153-450)	204±24 171.5 (97-416)
External pudendal artery diametre (mm)	2.84±0.08 2.80 (2.5-3.2)	2.71±0.10 2.65 (2.4-3.6)	2.49±0.08 2.55 (2.0-3.2)	2.60±0.09 2.65 (1.9-3.1)	2.37±0.10 2.40 (1.6-2.8)	2.32±0.08 2.25 (1.8-2.7)	2.34±0.09 2.30 (1.7-3.0)	2.29±0.09 2.20 (1.7-3.0)	2.16±0.08 2.15 (1.8-2.6)	2.18±0.08 2.05 (1.7-2.6)
External pudendal artery resistance index	0.62±0.01 0.615 (0.52-0.68)	0.71±0.02 0.715 (0.60-0.81)	0.71±0.02 0.73 (0.60-0.79)	0.70±0.01 0.685 (0.65-0.79)	0.75±0.02 0.76 (0.62-0.81)	0.79±0.02 0.81 (0.65-0.88)	0.79±0.02 0.785 (0.69-0.89)	0.79±0.01 0.795 (0.72-0.88)	0.78±0.02 0.775 (0.69-0.89)	0.79±0.02 0.795 (0.68-0.90)

Table III.i (continued).

	D0	D2	D4	D7	D10	D14	D18	D23	D30	D37
Group F										
External pudendal artery pulsatility index	1.17±0.06 1.21 (0.84-1.45)	1.69±0.10 1.72 (1.20-2.48)	1.82±0.10 1.89 (1.10-2.28)	1.57±0.07 1.495 (1.29-2.18)	1.97±0.11 2.06 (1.22-2.41)	2.30±0.17 2.32 (1.23-3.25)	2.39±0.16 2.245 (1.48-3.53)	2.37±0.14 2.23 (1.7-3.69)	2.47±0.21 2.35 (1.57-3.81)	2.39±0.22 2.16 (1.30-3.87)

D1: day of start of the drying-off procedure.

GENERAL DISCUSSION

Introduction

In sheep, effective udder health management at the end of a lactation period is important for maintaining mammary health during the subsequent lactation period (Fthenakis et al. 2012). However, role of practices performed at that time, as well as potential interactions of mechanisms involved in mammary involution with mammary health have not been studied in ewes as extensively as in cows. Udder health management at that time aims (i) to cure infections which have occurred during the previous lactation period and (ii) to prevent development of new intramammary infections during the 'dry-period' (Fthenakis *et al.* 2012). Frequently, udder health management would include intramammary administration of antibiotics (Mavrogianni et al. 2011).

In the para-Mediterranean areas (and other areas of the world in same latitude), often the stage of active involution in sheep may coincide with the initial stage of pregnancy. In contrast, in North Europe (and other areas of the world in same latitude), usually mammary involution has been completed before mating and pregnancy of ewes.

Effects of drying-off procedure in subsequent mammary infection and development of mastitis

The procedure of drying-off is part of the udder health management of ewes. One may postulate that the method followed for drying-off the mammary glands could affect their health status (Newman et al. 2010). For example, one may suggest that accumulation of milk into the mammary gland cistern may predispose to bacterial multiplication and subsequent development of mastitis, as removal of milk (even at less frequent than usual intervals) serves to flush bacteria from the mammary gland; moreover, milk accumulation into the mammary cistern leads to increased intramammary pressure, which can cause leakage of milk from the teat and delayed formation of the protective keratin plug within the teat duct (Dingwell et al. 2004, Odensten et al. 2007). On the other hand, periodic (albeit infrequent) removal of milk may contribute to increasing the risk of infection of the mammary gland, by means of increased risk of mammary infection during milking, whilst, on the other hand, it may increase concentration of protective non-specific defence substances (e.g., lactoferrin, IgG) accumulated in the secretion of the involuting gland, thus making the mammary gland more resistant to infections (Newman

et al. 2010). Finally, during accumulation of milk into the mammary gland, the defence substances and the leucocytes can contribute to effective bacterial killing (Lee and Outteridge 1981).

Nevertheless, the results of the Experiments I and II allied together confirmed that the method of udder drying-off does not affect the development of the risk of subsequent mammary infection and development of mastitis subsequently to the next lambing. The conflicting anecdotal views of farmers in Greece or in Great Britain cited earlier likely originate from traditional opinions, which have been generated through spontaneous observations. One may also argue that in mutton-production flocks, there is anyway some degree of progressive involution, as frequency of sucking is reduced when lambs grow older and consume larger amounts of solid food, thus becoming less dependent on maternal milk. Potential effects of the procedure followed for udder drying-off on milk yield during the subsequent lactation period have not been investigated and this can be an area of further work.

The present results also indicated that, after intramammary administration of antibiotics at the end of a lactation period, improved cure rates of mammary abnormalities were recorded, independently of the procedure followed for udder drying-off. The beneficial effects of intramammary antibiotic administration confirm previous results in reducing the rate of infection (Fthenakis et al. 2012).

There is a paucity of pharmaceutical products licenced for intramammary administration to ewes. Hence, products licenced for use in cows are often administered. The present results indicate the efficacy of such a product for ewes. In all cases, however, care should be applied (i) to infuse a separate tube into each mammary gland of each ewe, as, often, one tube is divided among the two mammary glands of the same animal (a practice that may lead to inefficacy of the product and introduction of contaminants into the mammary gland) (Mavrogianni et al. 2011) and (ii) to maintain a withdrawal period of ≥7 days for the milk of these ewes after the subsequent lambing.

Isolation of bacteria immediately after lambing and before sucking by lambs (Experiment I), suggests that these organisms had invaded the mammary gland during the preceding lactation or during the dry period (Mavrogianni et al. 2007). One may therefore suggest that *post-partum* mastitis may not necessarily be caused by newly infecting organisms (Jones and Watkins 2001), but from pre-existing microorganisms as well. As a consequence of the peri-parturient relaxation of immunity (Walker 2000), these bacteria might have caused lesions not previously present in the gland (Kiossis et al. 2013).

In view of the findings, dairy sheep farmers may practice progressive udder drying-off, by reducing gradually the frequency of milking. This would provide an increased total milk yield throughout the lactation period. In contrast, in mutton-production flocks, removal of lambs from their dams leads to abrupt cessation of lactation, which appears not to increase risk of mammary infection and development of mastitis.

Ultrasonographic findings in the udder during involution

Specifically in the udder of ewes, ultrasonographic examination has been used to study the mammary parenchyma as a means of diagnosing mammary disorders (Bruckmaier and Blum 1992, Ruberte et al. 1994, Franz et al. 2001, 2003), usually by linear-type transducers by using a frequency of 5.0 to 8.5 MHz. Imaging of the teats of ewes required higher frequency transducers (6.0-12.0 MHz) (Franz et al. 2001, Mavrogianni et al. 2004). Finally, the size of the mammary cistern (*sinus lactiferous*) has been evaluated by using ultrasonographic examination; a close correlation between the ultrasonographically imaged area of the mammary cistern and its real volume has been reported by Ruberte et al. (1994), Nudda et al. (2000) and Castillo et al. (2008), with a view to estimate milk yield of the animal (Klein et al. 2005, Wójtowski et al. 2006, Castillo et al. 2008, Rovai et al. 2008). Doppler ultrasonographic examination of the udder of ewes has been reported by Piccione et al. (2004), whilst Christensen et al. (1989) and Nielsen et al. (1990) have described use of the technique in goats.

The present study described, to the best of our knowledge for the first time, the B-mode and Doppler ultrasonographic patterns of the involuting mammary gland of ewes.

Technical issues

The ultrasonographic examination of the udder (B-mode and Doppler) could be easily applied, with minimal restraint of the animals.

In B-mode ultrasonographic examination, three fields sufficed to receive a representative image of the entire mammary parenchyma; as it was forecast that mammary parenchyma would be reducing in size during involution, images were taken based on mammary anatomical structures (which would remain unchanged during involution) rather than dimensions (which would be modified during involution).

Doppler ultrasonographic examination has for long been confirmed to be useful in measuring mammary blood flow in small ruminants (Christensen et al. 1989, Nielsen et al. 1990, Piccione et al. 2004). During the examination, animals must remain still and actions should be taken quickly; that was why animals were restrained into a crate and a skilled assistant was required for help with using the equipment. It is noteworthy that even a slight move of the animal under examination might lead in taking an inappropriate spectral waveform.

It is also important to be careful in placing the sample gate fully inside the vessel, in order to avoid artifacts and false measurements. If a 'Doppler angle' of >60 ° would be used in the examination, measurement errors would occur and, consequently, the blood flow velocity would be overestimated (Arning 2002). Best estimation of velocity occurs at 45° to 60 ° (Ginther 2007), that is why an angle of 50 ° to 60 ° was used in this study. It was easy to distinguish arteries from veins by means of Doppler ultrasound, as in the latter vessels the spectral waveform was seen to be flat and broad with no appearance of cardiac cycles.

Significance of findings

Changes in ultrasonographic appearance of the mammary parenchyma can reflect to some extent histological changes, for example, 30 days after cessation of milk removal, mammary epithelial cells have been found to be greatly reduced in size and cuboidal in shape (Tatarczuch et al. 1997). Changes in gray-scale appearance (progressively decreasing echogenicity) of ultrasonographic images of mammary parenchyma during involution could be attributed to decreasing numbers of mammary epithelial cells and, consequently, to the increasing proportion of adipose tissue into the mammary gland. Transfer of milk fat from the alveoli to the cistern slows down with increased time of milk removal, leaving a backup of milk fat in the mammary alveoli (McKusick et al. 2002, Castillo et al. 2008), which, during involution, is removed by leucocytes (Lee and Lascelles 1969, Tatarczuch et al. 2000). In general, during involution, extensive remodelling of the extracellular matrix of the mammary gland takes place, which results in volume increase of glandular stroma (Sordillo and Nickerson 1988, Hurley 1989, Capuco et al. 1997, Stavros 2011).

The differences in gray-scale (i.e., in echogenicity) between the two groups reflect the differences in tissue remodelling and the speed by which they are taking place during the involution process of the mammary gland. Nevertheless, these changes did not appear to lead to differences in the risk of intramammary infection and of mastitis development.

Lack of differences in results of cistern area at the sagittal and the dorsal plane of ultrasonographic imaging between the two groups indicates that volume of the structure does not appear to be affected by the procedure followed for involution (initiated or progressive). After cessation of milking, an increase in cisternal volume is the result of accumulation of milk therein. Approximately one week after cessation of milk, milk therein was observed to have clotted and to start being reabsorbed, which led, thereafter, to decrease in volume of the cistern. If accumulation of milk into the gland cistern is a potential risk factor for development of mastitis, as discussed above, then obviously similar volume of the gland cistern between ewes of the two groups indicates that, risk of mammary infection and mastitis development is similar, independently of the procedure followed for drying-off. This finding can partly explain the lack of difference in mastitis development between the two procedures.

No significant difference was evident between left and right cistern area (and volume) in animals of both groups. In relation to that, it is noteworthy that Nudda et al. (2000) and Castillo et al. (2008) have mentioned that potential differences in cistern size between left and right size can reflect differences in the force applied during milking or suckling. The present results can be explained in view of those by Nudda et al. (2000), as lack of force for withdrawing milk at drying-off led to similar size of the two gland cisterns of the same animal.

Doppler ultrasonographic examination indices (resistance index and pulsatility index) may be used as alternatives to velocity Doppler measurements. They are useful, especially when setting of a 'Doppler angle' can be difficult, as in tortuous vessels, because they are independent of that, as they are ratios of velocity measurements. Increased resistance index indicates decreased mammary blood perfusion, whilst increased values of pulsatility index indicate decreased blood perfusion of distal tissues. In fact, the two indices are highly correlated. Resistance index is more suitable when blood flow in vessels under study persists during diastole and pulsatility index when flow is absent during diastole (Ginther 2007). The significant decrease in blood flow at the external pudendal artery indicates the importance of this vessel for the blood circulation in mammary glands.

In the present study, both indices (resistance index and pulsatility index) increased progressively during the involution procedure, in animals of both groups, obviously being the result of decreased blood perfusion as involution advanced. Reduction of blood flow in the mammary gland may be a consequence of reduced blood requirement due to the reduced milk secretory activity rather than of increased intramammary pressure, because of accumulation of milk after cessation of milk removal (Knight *et al.* 1998). Decrease of the diametre of the external pudendal artery further contributes to the reduced amount of blood transferred to the

mammary gland and that way contributes to the involution of the mammary tissue. Decreasing blood volumes provide weaker signals for Doppler spectrum analysis, because of the smaller number of moving erythrocytes (Widder and Goertler 2004).

During the stage of active involution, there is impaired defensive ability of the mammary gland as a result of decreased concentration of lactoferrin and lactoferrin:citrate ratio (Smith and Oliver 1981, Nickerson 1989, Oliver and Sordillo 1989), decreased phagocytic ability of leucocytes (Sordillo and Nickerson 1988, Tatarczuch et al. 2000, 2002) and reduced amount of immunoglobulins in lacteal secretions (Sordillo et al. 1987). The above predispose the mammary gland to new mastitis cases during involution and support recrudescence of subclinical infections, which had occurred during the previous lactation period (Orphanou 1987, Barkema et al. 1998, Saratsis et al. 1998). Reduced blood volumes during the involution process could further contribute to development of mammary disease, as smaller amounts of leucocytes would be transferred to the mammary gland.

The significant differences between the two groups reflect the differences in blood flow into the mammary gland after cessation of lactation. Initiated involution led to a more rapid decrease of blood flow, whilst progressive involution sustained milk production for a longer period. Nevertheless, these changes did not appear to lead to differences in the risk of intramammary infection and of mastitis development.

Lack of differences in blood flow between the two mammary glands was first recorded by Nielsen et al. (1990) in goats and is now confirmed by present results in ewes. This may be potentially used in identifying early stages of inflammation after mammary infection.

The above findings may be used to describe the normal ultrasonographic appearance of the udder of ewes during involution. A combination of clinical, bacteriological and cytological criteria was used to confirm udder health of the animals.

This way, reference standards would be available for interpreting abnormal features subsequently. Perhaps, ultrasonographic examination of ewes at the end of the first week after cessation of lactation can be of help in identifying early cases of ‘dry-period’ mastitis, which develop slowly and with no striking clinical features. That would be especially helpful in cases of selective administration of antibiotics to ewes at the beginning of the ‘dry-period’, in which case administration of antibiotics is performed only to animals found to be infected at the end of a lactation period. In that procedure, many ewes remain unprotected against new intramammary infections during the ‘dry-period’. In animals with abnormal ultrasonographic findings at that stage, perhaps antibiotic administration may be performed at that stage, although further studies would be required to assess potential value of such a procedure. As lactation would

have ceased, injectable administration of antibiotics would be the preferable route in such cases; the efficacy of injectable administration of antibiotics at various time-points during the dry-period has been described by Croft et al. (2000).

Increased blood demand in the udder during lactation (Braun and Forster 2012) puts a tremendous burden on and affects the entire circulatory system of the animal (Gürtler and Schweigert 2005), which may adversely affect various other systems and functions of the animal. In contrast, the present results indicate reduced blood requirements in the udder during pregnancy and the dry-period, hence easing burden on the circulatory system (Gürtler and Schweigert 2005). In view of that, one cannot underestimate the significance of correct health management of ewes during the dry-period (Fthenakis et al. 2012), which creates improved conditions for preventing subsequent microbial and parasitic infections of the ewes.

Epilogue

Conclusions

The conclusions from the results of the present thesis are summarised herebelow.

(a) The results support a hypothesis that the procedure for udder drying-off (i.e., initiated or progressive involution) does not affect the risk of subsequent mammary infection and development of mastitis. Intramammary administration of antibiotics improved cure rates of mammary abnormalities, independently of the procedure followed for udder drying-off.

(b) Results of B-mode ultrasonographic examination suggested that there were differences in remodelling of the extracellular matrix in relation to the procedure for udder drying-off. Volume of the gland cistern did not appear to be affected by the procedure for udder drying-off. Results of Doppler ultrasonographic examination indicated that blood flow during initiated drying-off procedure was smaller than during a procedure with progressive drying-off.

Prospects

Suggestions for further research, in continuation of the present work, are as below.

- Potential effects of the procedure followed for udder drying-off on milk yield during the subsequent lactation period.

- Use of other ultrasonographic techniques (e.g., the application of contrast media), in order to evaluate fine details of changes during involution, to assess the rate of involution and confirm complete mammary involution and, potentially, to associate these changes with histological features in the involuting mammary gland.

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