ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΜΕΛΕΤΗ ΤΗΣ ΠΑΛΙΝΔΡΟΜΗΣΗΣ ΤΗΣ ΜΗΤΡΑΣ ΣΕ ΠΡΟΒΑΤΙΝΕΣ

ΑΙΚΑΤΕΡΙΝΗ ΣΑΡ. ΙΩΑΝΝΙΔΗ

Κτηνίατρος

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

που εκπονήθηκε

στην Κλινική Μαιευτικής και Αναπαραγωγής

Καρδίτσα, 2020

And however difficult life may seem,
there is always something you can do,
and succeed at.
It matters that you don't just give up.
Stephen Hawking

Η διατριβή είναι αφιερωμένη στην οικογένειά μου

ΠΕΡΙΛΗΨΗ

Η παρούσα διατριβή αποσκοπούσε:

- (α) Στη μελέτη της παλινδρόμησης της μήτρας σε προβατίνες με αναπαραγωγικά προβλήματα – ειδικότερα, χρησιμοποιήθηκαν η επιλόχεια λοίμωξη της μήτρας και η τοξιναιμία της εγκυμοσύνης ως πρότυπα για λεπτομερή μελέτη.
- (β) Στην αξιολόγηση της χρήσης της υπερηχογραφικής εξέτασης για τη μελέτη της παλινδρόμησης της μήτρας σε προβατίνες μετά τον τοκετό.
- (γ) Στην αξιολόγηση ενδεχόμενων επιπτώσεων της επιλόχειας λοίμωξης της μήτρας και της τοξιναιμίας της εγκυμοσύνης στην αναπαραγωγική απόδοση των προσβεβλημένων προβατίνων.

Στο Κεφάλαιο Ι, ανασκοπούνται η βιβλιογραφία σχετικά με το γεννητικό σύστημα των προβατίνων κατά την επιλόχεια περίοδο και η βιβλιογραφία σχετικά με τις παθολογικές κατάστασεις της μήτρας κατά την επιλόχεια περίοδο.

Στο Κεφάλαιο ΙΙ, περιγράφονται τα κλινικά, υπερηχογραφικά, βακτηριολογικά, κυτταρολογικά και ιστοπαθολογικά ευρήματα της παλινδρόμησης της μήτρας σε προβατίνες με επιλόχεια λοίμωξη της μήτρας, καθώς και οι επιπτώσεις στην αναπαραγωγική απόδοση των προσβεβλημένων ζώων. Αμέσως μετά τον τοκετό, προκλήθηκε λοίμωξη της μήτρας σε προβατίνες (ομάδα Ι, n = 10) με ενδομητριαίο ενοφθαλμισμό *Escherichia coli* – στον πειραματισμό περιλήφθηκαν και μη ενοφθαλμισμένα ζώα ως μάρτυρες (ομάδα C, n = 12). Οι προβατίνες εξετάζονταν επί 60 ημέρες μετά τον τοκετό, σε τακτικά χρονικά διαστήματα, πριν από και μετά τον ενοφθαλμισμό. Πραγματοποιήθηκαν κλινικές και υπερηχογραφικές εξετάσεις. Συλλέχθηκαν κολπικά επιχρίσματα με βαμβακοφόρους στυλεούς και δείγματα ιστών από τη μήτρα με βιοψία για βακτηριολογικές, κυτταρολογικές και ιστολογικές εξετάσεις. Στη συνέχεια, οι προβατίνες τοποθετήθηκαν με κριούς και αξιολογήθηκε η αναπαραγωγική απόδοσή τους. Μετά τον ενοφθαλμισμό, κατά την υπερηχογραφική εξέταση βρέθηκε ότι οι διαστάσεις των φυμάτων της μήτρας, το πάχος του μυομητρίου και η διάμετρος της κοιλότητας της μήτρας ήταν μεγαλύτερες στις ενοφθαλμισμένες προβατίνες. Στα ενοφθαλμισμένα ζώα η μεγαλύτερη μείωση των διαστάσεων των ανωτέρω έλαβε χώρα τη δεύτερη εβδομάδα μετά τον τοκετό, ενώ στα ζώα-μάρτυρες

έλαβε χώρα την πρώτη εβδομάδα μετά τον τοκετό. Η διάμετρος της μητριαίας αρτηρίας και ο όγκος της αιματικής ροής στη μήτρα είχαν επίσης μεγαλύτερες τιμές στα ενοφθαλμισμένα ζώα από τα ζώα-μάρτυρες. Η απομόνωση *E. coli* ήταν πιο συχνή και διήρκεσε για μεγαλύτερο χρονικό διάστημα στα ενοφθαλμισμένα ζώα από τα ζώα-μάρτυρες: το βακτήριο απομονώθηκε από 68,1% και 50,0% των προβατίνων, με διάμεση διάρκεια απομόνωσης 19,5 και 14,0 ημέρες, αντίστοιχα. Σε κολπικά επιχρίσματα, υπήρχε μικρότερη αναλογία ουδετερόφιλων λευκοκυττάρων και μεγαλύτερη αναλογία λεμφοκυττάρων στα ενοφθαλμισμένα ζώα από τα ζώα-μάρτυρες. Στα ενοφθαλμισμένα ζώα, κατά την ιστοπαθολογική εξέταση, βρέθηκαν καταστροφή του επιθηλίου της μήτρας, αυξημένη λευκοκυτταρική διήθηση, υπεραιμία και εξαγγείωση, για περίοδο έως 42 ημερών μετά τον τοκετό. Κατά την επόμενη αναπαραγωγική περίοδο, όλες οι ενοφθαλμισμένες προβατίνες γέννησαν με φυσιολογικό τοκετό υγιή νεογέννητα αρνιά. Τελικά, δεν υπήρχε διαφορά στην αναπαραγωγική απόδοση μεταξύ των ενοφθαλμισμένων ζώων και των ζώων-μαρτύρων.

Στο Κεφάλαιο ΙΙΙ, περιγράφονται τα κλινικά, υπερηχογραφικά, βακτηριολογικά, κυτταρολογικά και ιστοπαθολογικά ευρήματα της παλινδρόμησης της μήτρας σε προβατίνες, οι οποίες είχαν εκδηλώσει τοξιναιμία της εγκυμοσύνης στην αμέσως προηγούμενη κύηση, καθώς και οι επιπτώσεις στην αναπαραγωγική απόδοση των προσβεβλημένων ζώων. Χορηγήθηκε σιτηρέσιο ελλειμματικό σε ενέργεια σε προβατίνες (ομάδα Α, n = 12), με αποτέλεσμα την ανεύρεση αυξημένων συγκεντρώσεων β-υδροξυβουτυρικού οξέως στο αίμα τους. Στον πειραματισμό περιλήφθηκαν και ζώα στα οποία χορηγήθηκε κανονικό σιτηρέσιο (μάρτυρες, ομάδα C, n = 9). Οι προβατίνες εξετάζονταν επί 60 ημέρες μετά τον τοκετό σε τακτικά χρονικά διαστήματα. Πραγματοποιήθηκαν κλινικές και υπερηχογραφικές εξετάσεις. Συλλέχθηκαν κολπικά επιχρίσματα με βαμβακοφόρους στυλεούς και δείγματα ιστών από τη μήτρα με βιοψία για βακτηριολογικές, κυτταρολογικές και ιστολογικές εξετάσεις. Στη συνέχεια, οι προβατίνες τοποθετήθηκαν με κριούς και αξιολογήθηκε η αναπαραγωγική απόδοσή τους. Μετά τον τοκετό, κατά την υπερηχογραφική εξέταση βρέθηκε ότι οι διαστάσεις της μήτρας ήταν μεγαλύτερες στα ζώα της ομάδας Α. Σε όλα τα ζώα, η μεγαλύτερη μείωση των διαστάσεων της μήτρας έλαβε χώρα την πρώτη εβδομάδα μετά τον τοκετό. Ο όγκος της αιματικής ροής στη μήτρα ήταν μεγαλύτερος στα ζώα της ομάδας Α. Η

συχνότητα απομόνωσης βακτηρίων δεν διέφερε μεταξύ των δύο ομάδων, ενώ η διάμεση διάρκεια απομόνωσης βακτηρίων ήταν μεγαλύτερη στα ζώα της ομάδας Α. Στα δείγματα από τον κόλπο όλων των ζώων κυριαρχούσαν τα ουδετερόφιλα λευκοκύτταρα, όμως στα ζώα της ομάδας Α υπήρχε μικρότερη αναλογία ουδετερόφιλων λευκοκυττάρων από τα ζώα-μάρτυρες. Δεν βρέθηκαν διαφορές στα ιστολογικά ευρήματα μεταξύ των δύο ομάδων. Κατά την επόμενη αναπαραγωγική περίοδο όλες οι προβατίνες της ομάδας Α γέννησαν με φυσιολογικό τοκετό υγιή νεογέννητα αρνιά. Τελικά, δεν υπήρχε διαφορά στην αναπαραγωγική απόδοση μεταξύ των ενοφθαλμισμένων ζώων και των ζώων-μαρτύρων.

Συμπερασματικά, η μελέτη αξιολόγησε τις μακροπρόθεσμες επιπτώσεις των λοιμώξεων της μήτρας και της τοξιναιμίας της εγκυμοσύνης στην παλινδρόμηση της μήτρας και στην αναπαραγωγική απόδοση των προσβεβλημένων προβατίνων. Σε ζώα με λοίμωξη της μήτρας, η βακτηριακή εισβολή αντιμετωπίστηκε με επιτυχία – μολαταύτα η διαδικασία παλινδρόμησης διήρκεσε μεγαλύτερο χρόνο στα προσβεβλημένα από τα υγιή ζώα. Σε ζώα τα οποία είχαν εκδηλώσει τοξιναιμία της εγκυμοσύνης στην προηγούμενη κύηση, δεν υπήρχαν διαφορές στη διαδικασία παλινδρόμησης από τα υγιή ζώα. Η υπερηχογραφική εξέταση βρέθηκε ότι ήταν χρήσιμη για την αξιολόγηση του γεννητικού συστήματος των προβατίνων μετά τον τοκετό. Εφόσον εφαρμοζόταν η σωστή διαχείριση υγείας των προσβεβλημένων προβατίνων κατά την επόμενη κύηση, δεν παρατηρούνταν προβλήματα στην αναπαραγωγική απόδοση των προβατίνων, οι οποίες είχαν προηγουμένως εκδηλώσει λοίμωξη της μήτρας ή τοξιναιμία της εγκυμοσύνης.

Με βάση την κείμενη νομοθεσία και μετά από σχετική απόφαση στη με αριθμό 54/20.11.2019 συνεδρίαση της Συνέλευσης του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, η συγγραφή της διατριβής έγινε στην αγγλική γλώσσα.

Δημοσιεύσεις σχετιζόμενες με την παρούσα διατριβή

- K.S. Ioannidi, V.S. Mavrogianni, I. Valasi, M.S. Barbagianni, N.G.C. Vasileiou, G.S. Amiridis, G.C. Fthenakis, D.C. Orfanou (2017). Ultrasonographic examination of the uterus of ewes during the post-partum period. *Small Ruminant Research* 152, 74-85.
- K.S. Ioannidi, N.G.C. Vasileiou, M.S. Barbagianni, D.C. Orfanou, G. Mantziaras, T.M. Chouzouris, E. Dovolou, D.C. Chatzopoulos, E. Karavanis, N. Papadopoulos, A.I. Katsafadou, I.A. Fragkou, N.G. Kordalis, G.S. Amiridis, G.C. Fthenakis, V.S. Mavrogianni (2020). Clinical, ultrasonographic, bacteriological, cytological and histopathological findings of uterine involution in ewes with uterine infection. *Pathogens* 9, 54.
- K.S. Ioannidi, N.G.C. Vasileiou, M.S. Barbagianni, D.C. Orfanou, T.M. Chouzouris, E. Dovolou, D.C. Chatzopoulos, E. Karavanis, N. Papadopoulos, G.S. Amiridis, G.C. Fthenakis, V.S. Mavrogianni (2020). Clinical, ultrasonographic, bacteriological, cytological and histological findings of uterine involution in ewes with pregnancy toxaemia and subsequent reproductive efficiency. *Animal Reproduction Science* υπό δημοσίευση.

ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

Αναπληρώτρια καθηγήτρια Β.Σ. Μαυρογιάννη, Επιβλέπουσα

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Καθηγητής Γ.Σ. Αμοιρίδης, Μέλος τριμελούς συμβουλευτικής επιτροπής

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Καθηγητής Γ.Χ. Φθενάκης, Μέλος τριμελούς συμβουλευτικής επιτροπής

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

Καθηγητής Γ.Σ. Αμοιρίδης

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Καθηγητής Ε. Κιόσης

Τμήμα Κτηνιατρικής, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

Καθηγητής Χ. Μπρόζος

Τμήμα Κτηνιατρικής, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

Καθηγητής Γ.Χ. Φθενάκης

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Αναπληρώτρια καθηγήτρια Π.Γ. Γκουλέτσου

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Αναπληρώτρια καθηγήτρια Β.Σ. Μαυρογιάννη

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

Επίκουρη καθηγήτρια Ε. Βαλάση

Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας

UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE

UTERINE INVOLUTION IN EWES

KATERINA S. IOANNIDI

Veterinarian

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

work carried out at

the Department of Obstetrics and Reproduction

Karditsa, Greece, 2020

ABSTRACT

The general objectives of this thesis were as below.

- (a) The study of the uterine involution in ewes with reproductive disorders; post-partum uterine infection and pregnancy toxaemia were used as models for detailed study in the work.
- (b) The evaluation of the use of ultrasonographic examination for the post-partum study of uterine involution in ewes.
- (c) The assessment of any potential adverse effects of post-partum uterine infection and pregnancy toxaemia in the subsequent reproductive performance of the affected ewes.

In Chapter I, the literature regarding the genital tract of ewes during the puerperium and the disorders of the uterus of ewes during the puerperium is reviewed.

In Chapter II, the clinical, ultrasonographic, bacteriological, cytological and histopathological findings of uterine involution in ewes with post-partum uterine infection and the subsequent effects in the reproductive performance of affected ewes are described. Uterine infection was induced immediately post-partum in ewes (group I, n = 10) by intrauterine inoculation of *Escherichia coli*; uninoculated controls were included (group C, n = 12). The ewes were studied up to 60th day post-partum by examinations at regular intervals before and post-inoculation. Clinical and ultrasonographic examinations were performed. Vaginal swab samples and biopsy uterine tissue samples were collected for bacteriological, cytological and histological examination. Finally, the ewes were put to rams and their reproductive performance was monitored. After challenge, it was ultrasonographically found that caruncular dimensions, myometrial thickness and diametre of uterine lumen were greater in I ewes. In these ewes, particular reduction of dimensions occurred during the second week post-partum, whilst in C ewes during the first week. The uterine artery diametre and the blood flow into the uterus were also greater in I than in C ewes. *E. coli* infection was more frequent and of longer duration in I than in C ewes: in 68.1% and 50.0% of ewes, with a median duration of 19.5 and 14.0 days, respectively. In vaginal smears, there was lower proportion of neutrophils and higher of lymphocytes in group I than in C. In inoculated ewes, there was histological evidence of uterine epithelial destruction, increased leucocytic infiltration, hyperaemia and extravasation, which persisted

up to 42 days post-partum. During the subsequent reproductive season, all ewes in group I lambed normally and produced healthy and viable lambs. Finally, no significant difference in reproductive performance parametres were seen in I in comparison to C ewes.

In Chapter III, the clinical, ultrasonographic, bacteriological, cytological and histopathological findings of uterine involution in ewes that had pregnancy toxaemia during the preceding gestation and the subsequent effects in the reproductive performance of affected ewes are described. Ewes (group A, n = 12) were provided an energy-deficient feed during the last stage of gestation; consequently, increased β-hydroxybutyrate concentrations in their blood were detected. Control animals provided with an appropriate ration, were also included (group C, n = 9). The ewes were studied up to 60th day post-partum by examinations at regular intervals. Clinical and ultrasonographic examinations were performed. Vaginal swab samples and biopsy uterine tissue samples were collected for bacteriological, cytological and histological examination. Finally, the ewes were put to rams and their reproductive performance was monitored. After lambing, it was ultrasonographically found that the uterine structures of A ewes were greater than those of C ewes. Particular reduction occurred during the first week after lambing in all ewes into the study. Post-partum uterine blood flow volume was greater in A than C ewes. Frequency of bacterial isolation did not differ between groups; period from lambing to first infection and duration of infection was longer in A than C ewes. Neutrophils predominated in vaginal samples from all ewes, but the neutrophil proportion in A was smaller than in C ewes. Histological findings did not indicate major differences in involution process between groups. During the subsequent reproductive season, all ewes in group A lambed normally and produced healthy and viable lambs. Finally, no significant difference in reproductive performance parametres were seen in A in comparison to C ewes.

In conclusion, the study has investigated the long-term effects of uterine infection and pregnancy toxaemia in uterine involution and in the subsequent reproductive performance of affected ewes. In ewes with uterine infection, the uterus was able to counteract the bacterial invasion; nevertheless, the process of involution took longer in affected ewes than in healthy animals. In ewes that had developed pregnancy toxaemia during the preceding gestation, no overall differences were evident in the involution process compared to healthy animals. The ultrasonographic examination was found to be a useful means for the

assessment of the genital tract of ewes post-partum. After undertaking correct health management in all affected ewes during the subsequent gestation, no adverse effects were noted in the reproductive performance of ewes previously with uterine infection or pregnancy toxaemia.

Publications associated with the present thesis

- K.S. Ioannidi, V.S. Mavrogianni, I. Valasi, M.S. Barbagianni, N.G.C. Vasileiou, G.S. Amiridis, G.C. Fthenakis, D.C. Orfanou (2017). Ultrasonographic examination of the uterus of ewes during the post-partum period. *Small Ruminant Research* 152, 74-85.
- K.S. Ioannidi, N.G.C. Vasileiou, M.S. Barbagianni, D.C. Orfanou, G. Mantziaras, T.M. Chouzouris, E. Dovolou, D.C. Chatzopoulos, E. Karavanis, N. Papadopoulos, A.I. Katsafadou, I.A. Fragkou, N.G. Kordalis, G.S. Amiridis, G.C. Fthenakis, V.S. Mavrogianni (2020). Clinical, ultrasonographic, bacteriological, cytological and histopathological findings of uterine involution in ewes with uterine infection. *Pathogens* 9, 54.
- K.S. Ioannidi, N.G.C. Vasileiou, M.S. Barbagianni, D.C. Orfanou, T.M. Chouzouris, E. Dovolou, D.C. Chatzopoulos, E. Karavanis, N. Papadopoulos, G.S. Amiridis, G.C. Fthenakis, V.S. Mavrogianni (2020). Clinical, ultrasonographic, bacteriological, cytological and histological findings of uterine involution in ewes with pregnancy toxaemia and subsequent reproductive efficiency. *Animal Reproduction Science* in press.

ADVISORY COMMITTEE

Associate professor V.S. Mavrogianni, Supervisor

Faculty of Veterinary Science, University of Thessaly

Professor G.S. Amiridis, Member of the advisory committee

Faculty of Veterinary Science, University of Thessaly

Professor G.C. Fthenakis, Member of the advisory committee

Faculty of Veterinary Science, University of Thessaly

EXAMINATION COMMITTEE

Professor G.S. Amiridis

Faculty of Veterinary Science, University of Thessaly

Professor C. Brozos

Faculty of Veterinary Medicine, Aristotle University of Thessaloniki

Professor G.C. Fthenakis

Faculty of Veterinary Science, University of Thessaly

Professor E. Kiossis

Faculty of Veterinary Medicine, Aristotle University of Thessaloniki

Associate professor P.G. Gouletsou

Faculty of Veterinary Science, University of Thessaly

Associate professor V.S. Mavrogianni

Faculty of Veterinary Science, University of Thessaly

Assistant professor I. Valasi

Faculty of Veterinary Science, University of Thessaly

TABLE OF CONTENTS

INTRODUCTION

General objectives of the thesis

The general objectives of this thesis were as below.

- (a) The study of the uterine involution in ewes with reproductive disorders; uterine infection and pregnancy toxaemia were used as models for detailed study in the work.

Uterine infection, although an occasional problem in ewes, is the main post-partum disease of the uterus in that species. Uterine infections in ewes have not been studied as extensively as the respective problems in cows. Although they occur less frequently than in cows, they can still be a cause of periparturient death.

Pregnancy toxaemia is the most frequent and significant metabolic disease of pregnant ewes. It has severe consequences for the affected ewes, which may die even if treatment would be initiated. Moreover, pregnancy toxaemia can predispose ewes to increased incidence of peri-parturient problems (Barbagianni et al. 2015c), as well as to mastitis (Barbagianni et al. 2015b) and reduced milk production (Karagiannis et al. 2018) during the subsequent lactation period.

- (b) The use of ultrasonographic examination for the evaluation of uterine involution in ewes post-partum.

Ultrasonographic examination is a non-invasive method, by means of which the genital tract of ewes can be easily imaged and monitored (Barbagianni et al. 2017, Valasi et al. 2017).

- (c) The assessment of any potential adverse effects of uterine infection and pregnancy toxaemia in the subsequent reproductive performance of the affected ewes.

Any potential adverse effects of these two disorders have not been reported. Knowledge of these will be of help in the subsequent management of ewes with either of these two diseases.

Work for the present thesis has been performed at the Department of Obstetrics and Reproduction of the Faculty of Veterinary Science of the University of Thessaly. Research work was carried out until 2018; this was followed by analysis of results and writing up of the thesis.

Ευχαριστίες

Ένα μεγάλο ευχαριστώ στην επιβλέπουσά μου, αναπληρώτρια καθηγήτρια του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, κ. Βασιλική Σ. Μαυρογιάννη, που μου έδωσε την ευκαιρία να εκπονήσω αυτή τη διατριβή και την επέβλεψε, υποστηρίζοντάς με καθ'όλη τη διάρκειά της. Της εκφράζω την ευγνωμοσύνη μου για την επιστημονική καθοδήγησή της, τις εύστοχες συμβουλές της, την αντιμετώπιση οποιασδήποτε δυσκολίας προέκυπτε και τις καίριες παρεμβάσεις της, καθώς και την ανεύρεση προγράμματος χρηματοδότησης της έρευνας, τα οποία συνέβαλαν στην απρόσκοπτη ολοκλήρωση της διατριβής.

Ευχαριστώ θερμά τον καθηγητή και πρόεδρο του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, κ. Γιώργο Χ. Φθενάκη, πρωτίστως για την αρχική σύλληψη της ερευνητικής ιδέας, για την αμέριστη βοήθειά του στον πειραματικό σχεδιασμό της όλης μελέτης, την ανεύρεση εξοπλισμού και υλικών πόρων, την επίλυση πρακτικών προβλημάτων, την αμέριστη στήριξή του σε όλη τη διάρκεια της μελέτης, τις εύστοχες παρατηρήσεις στο κείμενο της διατριβής και τον πολύτιμο χρόνο του που αφιέρωνε όποτε είχα ανάγκη υποστήριξης.

Ευχαριστώ τον καθηγητή και διευθυντή της Μαιευτικής και Αναπαραγωγής Κλινικής του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, κ. Γεώργιο Σ. Αμοιρίδη, για την πολύτιμη συμβολή του κατά τη διάρκεια της όλης μελέτης, την πρόθυμη και ευγενική παραχώρηση χρήσης εξοπλισμού και που δεν δίστασε ούτε στιγμή να αφιερώσει τον πολύτιμο χρόνο και την εμπειρία του για την πραγματοποίηση των λαπαροσκοπικών εξετάσεων, οι οποίες χωρίς αυτόν δεν θα είχαν υλοποιηθεί.

Τις θερμές ευχαριστίες μου για τη συμβολή τους στη διεκπεραίωση της διατριβής απευθύνω επίσης:

Στην αγαπητή συνεργάτιδα Ναταλία-Γαβριέλα Χ. Βασιλείου, που ήταν πάντα δίπλα μου σε όλη τη διάρκεια διεξαγωγής των πειραματισμών. Η βοήθεια της στην οργάνωση και παρακολούθηση της διαχείρισης των πειραματοζώων και στη διενέργεια των δειγματοληψιών ήταν ανεκτίμητη. Επιπλέον, η αγαστή συνεργασία μας και η αμφίδρομη ψυχολογική στήριξη συνέβαλαν στην ανάπτυξη μίας φιλίας.

Στη Μαριάνα Σ. Μπαρμπαγιάννη, που έπαιξε καίριο ρόλο στην εκπαίδευσή μου στην υπερηχογραφική εξέταση του γεννητικού συστήματος των προβάτων. Με την υπομονή της και την αστείρευτη μεταδοτικότητά της μου μεταλαμπάδευσε την τεχνογνωσία όσον αφορά το χειρισμό της συσκευής υπερηχογραφίας (B-mode και Doppler-mode), η οποία αποτέλεσε σημαντικό κομμάτι της διατριβής.

Στην επίκουρη καθηγήτρια του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας κ. Ειρήνη Βαλάση, η οποία συνέβαλε επίσης σημαντικά στην εκπαίδευσή μου στην υπερηχογραφία.

Στον υπεύθυνο τεχνικό της εταιρείας Esaote κ. Ευάγγελο Σαμαρά, για την προθυμία του για αντιμετώπιση όλων των προβλημάτων στη χρήση του υπερηχογράφου και για υπόδειξη τρόπων, ώστε, μέσω της συσκευής, να εφαρμόζονται οι θεωρητικές προσεγγίσεις.

Στη Διονυσία Κ. Ορφανού, για την υπομονή που επέδειξε στην εκπαίδευσή μου στην υπερηχογραφική εξέταση, στην αναγνώριση των στιβάδων της μήτρας και τις σχετικές μετρήσεις αυτών, καθώς επίσης και για την ουσιαστική βοήθεια και καθοδήγησή της στις μεθόδους ιστολογικής εξέτασης και για τη σημαντική συμβολή της στην αξιολόγηση των σχετικών ευρημάτων.

Στον αναπληρωτή καθηγητή της Κτηνιατρικής Σχολής του Πανεπιστημίου του Liverpool κ. Peter J. Cripps, για τις πολύτιμες ιδέες του στη διαχείριση και σωστή ανάλυση των αποτελεσμάτων.

Στον κτηνίατρο κ. Γιώργο Μαντζιάρα, για την παραχώρηση χρήσης εξοπλισμού και την εκπαίδευση στη σκιαγραφική υπερηχογραφία.

Στην κτηνίατρο κ Ελένη Ντόβολου, για τη συμμετοχή της κατά την πραγματοποίηση των βιοψιών της μήτρας και τη βοήθειά της στην ανεύρεση αναλώσιμων υλικών.

Στον κτηνίατρο κ. Θωμά Χουζούρη, που επίσης συνέβαλε την πραγματοποίηση των βιοψιών της μήτρας.

Στον Δημήτρη Χ. Χατζόπουλο, για την υποστήριξη σε θέματα μικροβιολογικών εξετάσεων.

Στους κτηνιάτρους κκ. Μανώλη Καραβάνη και Νίκο Παπαδόπουλο, για την ετοιμασία των ιστοπαθολογικών παρασκευασμάτων.

Στην κτηνίατρο κ. Ηλέκτρα Κατέρη, για την ανιδιοτελή προσφορά της κατά τη διάρκεια των πειραματισμών.

Στον κ. Θωμά Παπαδημητρίου, μέλος του προσωπικού της Κλινικής, για την φροντίδα των ζώων, τη βοήθεια του κατά τη διάρκεια των δειγματοληψιών και την πολύπλευρη συμπαράστασή του.

Ευχαριστίες απευθύνονται επίσης στο Ίδρυμα Κρατικών Υποτροφιών, καθώς κατά τη διάρκεια της εκπόνησης της διατριβής ήμουν κάτοχος σχετικής υποτροφίας.

Ευχαριστίες απευθύνονται επίσης στη Φωτεινή Κόρμπου, που, με το αμέριστο ενδιαφέρον της, αποτέλεσε τον συνδετικό κρίκο για την έναρξη της εκπόνησης αυτής της διατριβής.

Τέλος, ένα μεγάλο ευχαριστώ στους γονείς μου Βασιλική και Σαράντο, καθώς και στον αδερφό μου Σπύρο, που, με την αγάπη τους, την πίστη τους στις δυνατότητες μου, τη συνεχή εμψύχωση και τις συμβουλές τους, μου έδιναν συνεχώς δύναμη να συνεχίσω και να καταφέρω να ολοκληρώσω την όλη μελέτη. Επίσης, ευχαριστώ ιδιαίτερα τους φίλους μου Νίκο Κορδαλή, Ευαγγελία Καραβασιά, Αθηνά Βενιανάκη, Μάγδα Κοσμίδου και Έλενα Καρλαύτη, που ήταν δίπλα μου σε αυτό το «ταξίδι» και με στήριξαν με κάθε δυνατό τρόπο.

Χρηματοδότηση

«Το έργο συγχρηματοδοτείται από την Ελλάδα και την Ευρωπαϊκή Ένωση (Ευρωπαϊκό Κοινωνικό Ταμείο) μέσω του Επιχειρησιακού Προγράμματος «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Διά Βίου Μάθηση», στο πλαίσιο της Πράξης «Ενίσχυση του ανθρώπινου ερευνητικού δυναμικού μέσω της υλοποίησης διδακτορικής έρευνας» (MIS-5000432), που υλοποιεί το Ίδρυμα Κρατικών Υποτροφιών (ΙΚΥ).»

Ευρωπαϊκή Ένωση
Ευρωπαϊκό Κοινωνικό Ταμείο

Επιχειρησιακό Πρόγραμμα
Ανάπτυξη Ανθρώπινου Δυναμικού,
Εκπαίδευση και Διά Βίου Μάθηση
Με τη συγχρηματοδότηση της Ελλάδας και της Ευρωπαϊκής Ένωσης

ΕΣΠΑ 2014-2020
ανάπτυξη - εργασία - αλληλεγγύη

«This research is co-financed by Greece and the European Union (European Social Fund- ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (ΙΚΥ).»

CHAPTER I

REVIEW OF THE LITERATURE

The puerperium

The puerperium is the period after completion of parturition. The period includes many progressive changes in the genital tract for returning to the normal pre-gravid state. Thus, when the subsequent breeding season starts, the ovarian cyclic activity would resume. The most significant of these changes is the uterine involution. In any case, during the puerperium, the ewes' genital system would not return exactly to its non-gravid state, given that some changes taking place during pregnancy are not fully reversible.

In ewes, the puerperium most often coincides with the anoestrous (non-breeding) period. Even in geographic locations where the reproductive season has a long duration (e.g., in para-Mediterranean countries), the post-partum period would coincide with the anoestrous season. Hence, in most cases, ewes only occasionally would show reproductive activity after lambing (Noakes 2009).

The puerperium also includes the production of milk by the ewes, for consumption by lambs, as well as, in dairy production systems, for milking.

Stages of the puerperium

The puerperium can be divided in three stages.

The first stage starts with the expulsion of the foetal membranes and lasts until the end of the first week after lambing. During this period, most of the content of the genital tract is expelled; also, the most significant rate of uterine involution takes place. According to Noakes (2009), during the first week after lambing, the reduction of the size of the uterus occurs at a logarithmic scale. With regard to milk production, the early part of this stage coincides with initiation of the lactation period and colostrum production.

The second stage is the main stage of the puerperium and lasts until the end of the third week post-partum. During this stage, the uterine involution continues at a slower rate. With regard to milk production, there is a progressive increase of milk yield, in order to cover the increasing requirements of the growing lambs.

The third stage occurs subsequently to the third week post-partum. During that stage, the uterine involution is completed. Moreover, a further increase in milk production takes

place to cover the requirements of lambs, whilst in dairy production systems milking of ewes usually starts during that stage.

Uterine involution

Uterine involution refers to the simultaneous (a) reduction in the size (length and diametre) of the uterus, (b) reduction in its volume, (c) expulsion of its content and (d) endometrial 're-epitheliazation' (Gray et al. 2003).

The first notable change in the uterus is the closure of the cervix. Cervical closure occurs initially at the internal orifice (internal os) and then at the external orifice (external os). Within 18 to 24 hours after lambing, it is practically impossible to insert a hand into the uterine cavity (Noakes 2009).

After detachment of the cotyledons from the placentome, a constriction of the vessels in the caruncles takes place. This leads in caruncular necrosis, apoptosis of the necrotic plaques into the uterine lumen and exposure of the vessels on the surface of the caruncles (Gray et al. 2003). Eventually, after shedding of all necrotic tissues, the regeneration of the caruncular surface and the inter-caruncular area starts (Noakes 2009). Further, after the expulsion of all the uterine content, a mucus is produced, which closes the cervical orifice, thus inhibiting bacterial entrance into the uterus (Said et al. 1974).

At that time, the content of the uterus, termed 'lochia', consists of remains of the foetal fluids, placental debris and shreds of foetal membranes, uterine epithelial cells and endometrial tissue and leucocytes and erythrocytes from the ruptured umbilical vessels. The lochia is expelled through the vagina as the result of uterine contractions. The greatest outflow of lochia takes place during the first two to three days post-partum, although discharge can be present for up to six days (Valasi et al. 2011). The discharge is usually yellowish to reddish brown during the initial days post-partum, progressively becoming lighter in colour and finally clear. Normal lochia does not have an unpleasant odour (Noakes 2009).

During the first days post-partum, myometrial contractions occur every three to four minutes. From the third to the tenth day post-partum, a rapid reduction in the size of the uterus occurs, which coincides with a decrease in the frequency and duration of uterine

contractions (Noakes 2009). By the end of that period, the weight of the uterus would have decreased by as much as 70% of its weight on the day of lambing. The reduction in the size of the uterus is inverse to the process of increase of tissue collagen concentration in the organ (in the endometrium: 7- to 8-fold).

Thereafter, contractions continue with a reduced intensity and no particular pattern. The contractions aim to (a) completely expel the lochia from the uterus, (b) reduce the vascularization of the uterus and thus a possibility of haemorrhage and (c) reduce the size (length and diametre) and volume of the uterus (Valasi et al. 2011). Uterine contractions are upheld throughout the puerperium by oxytocin. Suckling or milking of ewes stimulates the milk ejection reflex. The efferent part of this neuroendocrine reflex arc is oxytocin, released from the posterior pituitary. Oxytocin enhances the uterine contractions, contributing to the full expulsion of the lochia, the uterine involution and the cessation of uterine haemorrhage. As the puerperium advances and uterine involution progresses, the number of oxytocin receptors in the uterus decreases and consequently uterine contractions also progressively decrease (Perumamthadathil et al. 2014, Prevost et al. 2014).

At that time, the restoration of the endometrium takes place in a sequel of procedures. Noakes (2009) has reported that 16 days were needed for degeneration of the surface of the caruncles and removal of the surface of the uterus.

Methods for studying uterine involution

Various techniques have been proposed for the study of uterine involution in ewes, e.g., hormone measurement (Ishwar 1995, Degefa 2003), radiographic imaging (Tian and Noakes 1991, Goddard 1995), laparoscopic investigation (Ishwar 1995) or post-mortem examination (Rubianes and Ungerfeld 1993, Ababneh and Degefa 2005, Degefa et al. 2006). Some of the disadvantages of the above techniques include invasiveness, reduced accuracy, difficulty to apply in clinical conditions and need to sacrifice the experimental animals.

Ultrasonographic examination is a technique that lacks those limiting factors. Actually, it is the only non-invasive technique that may reveal details of the progressive changes in the uterus of ewes. Various authors have reported extensive studies of the genital system during the post-partum period in other animal species: cows (e.g., Okano and Tomizuka

1987), mares (e.g., Griffin and Ginther 1991), sows (e.g., Irie 1987), bitches (e.g., Yeager and Concannon 1990, Pharr and Post 1992, Orfanou 2012) and queen cats (e.g., Ferretti et al. 2000).

Ultrasonographic monitoring of uterine involution

Methodology of ultrasonographic examination of the uterus post-partum

Equipment

A real-time ultrasound, with various multi-frequency transducers is best for use.

Convex transducers with 2.5 to 7.5 MHz frequency and linear transducers with 7.5 to 12.0 MHz frequency for examination by the transcutaneous technique, as well as transducers for examination by the transrectal technique (frequency: 5.0 - 7.5 MHz) would cover most requirements.

A higher frequency during imaging would provide improved image quality, but would reduce scanning depth of the procedure. Reduced frequency (3.0 MHz) can be used for deep, intra-uterine imaging, mid-range frequency (5.0 MHz) for general use and increased frequency (≥ 7.5 MHz) for detailed scanning structures in short distance from the transducer (Buckrell 1988). A standard frequency (6.6 MHz) can be used for Doppler imaging (Petridis et al. 2017).

Examination technique

B-mode examination

Examination of the genital system can be performed by the transcutaneous or the transrectal technique. During the examination, longitudinal or transverse images of the uterus can be taken.

For examination by the transcutaneous technique, hair in the abdomen should be clipped and coupling gel should be applied thereupon. The ewe can be examined in the standing position, inside a crate or a box, which would reduce her ability to move and increase comfort of the examiner, or in the cast position with the forelimbs held by an assistant. During the examination, the transducer is placed on the abdominal wall,

immediately in front of the udder, with caudal direction. Imaging starts by locating the bladder (*vesica urinaria*), which can be used as an acoustic window, to facilitate examination at longitudinal or transverse planes, especially after the first week post-partum. Alternatively, the transducer can be placed on the inguinal area and moved from lateral to ventral and from caudal to cranial direction. The uterus (*uterus*) is located ventrally to the rectum (*rectum*) and laterally or dorsally to the bladder. The probe is moved cranially, in order to image the uterine horn bifurcation; then, it is moved laterally to ventrally and caudally to cranially, in order to image the uterine horns (*cornu uteri*). Images are obtained on the longitudinal and the transverse ultrasonographic planes. Ideally, a 90 to 100 mm scanning depth is used. Immediately after parturition, especially up to the second day after lambing, the mass of the uterus can be particularly pronounced, hence a scanning depth of 120 to 150 mm might be necessary during that period for optimal imaging. During the examination, echogenicity of the uterus, number of layers in the uterine wall: perimetrium (*tunica serosa*, *perimetrium*), myometrium (*tunica muscularis*, *myometrium*) and endometrium (*tunica mucosa*, *endometrium*), presence of content in the uterine lumen (*cavum uteri*) and width of uterine body (*corpus uteri)* and horns are evaluated; the various layers of the uterine wall can be observed and their thickness can be measured.

During examination by the transrectal technique, the probe can be fixed to an extension rod. The rectum is emptied of faeces. The transducer is inserted into the rectum within a plastic glove full of coupling gel (this also protects the transducer) or, alternatively, gel (> 20 mL) can be injected into the rectum. The transducer is moved medially until the cranial pole of the urinary bladder is imaged. Following that, the probe is moved laterally, left and right, up to 45 ^{o} of the vertical axis to image the uterus.

According to Hauser and Bostedt (2002), the transcutaneous technique allowed thorough assessment of the uterus during the first week post-partum, due to the size and the position of the uterus at the ventral abdomen; subsequently (after the 11th day post-partum), it was not possible to image the uterus, due to a significant decrease of its size and increase of its distance from the abdominal wall. Similar results, i.e., difficulty to image the uterus after the 13th day post-partum, have been reported by Ababneh and Degefa (2005). Examination by the transrectal technique can provide better results during that period. The technique does not provide similarly good results during the first 10 days after lambing, as

neither good images, nor relevant information could be produced for involution of the body of the uterus, due to the increased size of the organ and its cranioventral abdominal position (Hauser and Bostedt 2002). Improvement of images obtained by the transrectal technique, immediately after lambing, may be achieved by placing the ewe in dorsal recumbency or by lifting the caudal abdominal wall in standing animals (Ababneh and Degefa 2005). Hence, it is advisable that transducers for transrectal examination would be better used subsequently to the first week after lambing and even better after the 11th day (Hauser and Bostedt 2002, Ababneh and Degefa 2005).

Measurements of dimensions of the uterus (body and horns) can be done in longitudinal or transverse section images taken by the transcutaneous technique, by using, preferably, convex transducers; these provide a greater spectrum of imaging. Uterine length is measured in longitudinal sections from bifurcation of horns to cranial part of the cervix; uterine widths are measured in longitudinal or transverse sections from one edge of uterine body to the precise opposite; endometrial thickness is measured in transverse sections.

Doppler examination

Doppler ultrasonographic examination (Petridis et al. 2017) by means of the transcutaneous technique has been used for the evaluation of blood flow into the uterus of ewes with the animal in the standing position. The technique is applied in the uterine artery (*arteria uterina*), which is the main artery of the uterus.

At the start, a cross-section of the vessel is taken, to measure its diametre. The Doppler gate is positioned inside the vessel and a spectral mode waveform is taken to obtain a more objective estimation. A Doppler angle of < 60 ° is set and uniform spectral waveforms of at least three consecutive cardiac cycles are taken and stored for subsequent detailed examination (Petridis et al. 2017).

Findings in healthy ewes during B-mode ultrasonographic examination of the uterus post-partum

In general, a progressive, significant reduction in the size of the uterus, especially during the first week after lambing, in the size of the uterine lumen and in the size of the caruncles (*carunculae*) is observed (Hauser and Bostedt 2002, Ababneh and Degefa 2005,

Fernandes et al. 2013). Degeneration and necrosis of the surface of the caruncles and removal of the surface of the endometrium lead to presence of echogenic content within the anechoic lumen. The uterine lumen and its content can be easily imaged during the early stage of the puerperium; however, the significant contractions of the genital tract and the rapid involution process (at that stage) reduce sensitivity of the technique.

The first week post-partum, the uterus appears enlarged, with a heterogeneous echopattern, due to the observation of the layers of the organ (Hauser and Bostedt 2002). The organ can be easily visualised, as its diametre can be up to 10 cm (Fernandes et al. 2013). Subsequently, as its size decreases progressively, it may be more difficult to image it. In most cases, a rapid decrease in uterine diametre takes place during the first week post-partum (Hauser and Bostedt 2002, Ababneh and Degefa 2005), although other researchers (Hayder and Ali 2008, Badawi et al. 2014) have indicated that 50% of uterine reduction had been achieved within two weeks after lambing. According to Fernandes et al. (2013), the reduction in uterine size continued until the 21st day post-partum, but at a slower rate; up to 28th day post-partum, uterine body diametre is approximately 2 cm, considered normal for a lactating ewe, as compared to previous results by Hauser and Bostedt (2002). Later after lambing, the uterine bifurcation and the uterine horns can be imaged intermittently up to approximately 25th and 28th day post-partum, respectively. After that, and up to the 60th day post-partum, the uterus is imaged as an entire, round entity near the bladder, with its other parts not viewed separately. A summary of the results of various studies regarding dimensions of uterus of ewes during involution, as measured ultrasonographically, is presented in Table 1.

Table 1. Summary of findings of uterine dimensions (cm) in ewes during involution, as measured ultrasonographically.

Day post-partum	Uterine body (Ali et al. 2001)	Uterine body (Hauser and Bostedt 2002)	Uterine horns (Ababneh and Degefa 2005)	Uterine horns (Medan and El-Daek 2015)
1		4.9 / 5.9 / 6.4[a]		
1			7.8 / 9.0[b]	
7	5.0			
7			4.8 / 5.6[b]	
7				5.6 / 5.3 / 5.2[c]
25			2.3 / 2.3[b]	
28	2.6			
28				2.0[d]
30		1.8 / 1.9 / 1.9[a]		
35				2.0[d]

a: ewes that had lambed with no obstetrical intervention / ewes in which conservative obstetrical intervention had been applied / ewes in which caesarean section had been applied.
b: primiparous / multiparous ewes.
c: ewes that had lambed in January / February / March (study performed in north Africa).
d: ewes that had lambed in spring (28th day post-partum) or winter (35th day post-partum).

During examination by the transcutaneous technique, one can image the uterine caruncles, measure their dimensions and recognise their regression. After birth, that would be the most obvious ultrasonographic feature within the uterine lumen (Kähn 1992, 2004). These are located on the endometrium and in cases of presence of hypoechoic fluid within the lumen, they may be imaged to protrude as 'mushrooms' (Kähn 1992, 2004). In general, placentomes are echogenically similar to the endometrium and its folds; however, some differences in their echogenity are evident: the most superficial of its layer is hyperechoic and the deeper tissue of the placentome is less echoic, according to presence of loose tissue (Kähn 1992, 2004). They change their appearance from concave to convex during regression (Hauser and Bostedt 2002). Their size has been estimated at 2.02 cm on the first day post-partum, subsequently becoming 1.24 cm on the eighth day (Hauser and Bostedt 2002). Fasulkov (2014) measured the diametre of caruncles and found a significant progressive decrease of their size until the ninth day post-partum. Most authors have reported that imaging of caruncles after the tenth day post-partum is not possible or, at least, extremely difficult and rare (Hauser and Bostedt 2002, Ababneh and Degefa 2005), whilst others (Kähn 1992, 2004) mentioned that caruncles were visible during the second week after lambing despite their decreased size. Hauser and Bostedt (2002) also mentioned that often it was

difficult to differentiate caruncles from uterine endometrial folds, which were also only occasionally imaged subsequently to the 13th day post-partum (Ababneh and Degefa 2005).

The uterine wall can be imaged initially (during the early post-partum period) as a multi-layer structure. The perimetrium and the outer layer of the myometrium are highly echogenic structures. The middle layer of the myometrium, the 'vascular stratum', is anechoic to hypoechoic, due to the increased vascularity of the area and looks like a dark rim. The inner layer of the myometrium and the endometrium are also echogenic structures. The uterine lumen can be imaged as echogenic or anechoic and characterised by irregular shape, because of the uterine folds and the caruncles (Hauser and Bostedt 2002). In some images, the anechoic lumen is barely visible, because of the more echogenic content (lochia), due to the presence of cellular components, whilst in others, it is observed greatly contrasting to the hyperechoic endometrium (Ababneh and Degefa 2005). Badawi et al. (2014) have reported that increased uterine content was noted during the first week post-partum. Hauser and Bostedt (2002) have reported that the diametre of the lumen on the first day post-partum was 2.24 cm for ewes that had lambed with no obstetrical intervention, 3.12 cm for ewes in which conservative obstetrical intervention had been applied and 3.47 cm for ewes in which caesarean section had been applied; the diametre was reduced to 0.5 cm on the 11th, 14th or 17th day post-partum, for the above categories of ewes, respectively.

Factors that may affect normal uterine involution

Uterine involution may be affected by various factors, as detailed below (Noakes 2009, Valasi et al. 2011).

- Age of ewes. Complete uterine involution is more rapid in primiparae than in multiparae ewes.
- Season of the year. Uterine involution is completed sooner in ewes that lambed in the summer.
- Environmental conditions. Uterine involution may be delayed in hight-temperature environments.
- Number of foetuses borne. In ewes that lambed twins, uterine involution took longer than in ewes that bore a single foetus.

Reproductive activity post-partum

The process of uterine involution results in anatomic and functional restoration of the genital system to its non-gravid state. Thus, a new breeding period can ensue, with initiation of new oestrous cycles, mating and finally conception. The resumption of reproductive activity depends on the time within a year that ewes would lamb and the length of the anoestrus in each particular breed.

In animals that lamb outside the breeding period (which is what takes place most often), reproductive activity would start at the subsequent breeding period. Occasionally, ovarian activity with follicular growth may occur after lambing, but ovulation would be unusual or associated with a silent oestrus. It is considered that this would occur as the result of failure of follicular maturation and ovulation, possibly because of inadequate release of luteinizing hormone as a result of deficiency in GnRH synthesis and secretion in these animals (Wright et al. 1981, Noakes 2009).

Ewes that lambed within the breeding season are considered to have increased chances for normal ovarian rebound and reproductive activity (Noakes 2009, Valasi et al. 2011). No lactational anoestrus occurs in ewes. However, in dairy ewes, a silent oestrus and delayed oestrous cycles may occur at the start of a breeding period (Valasi et al. 2011).

Microbial populations in the genital system post-partum

Lambing takes place in a non-sterile environment, predisposing to bacterial entry into the uterus. Thus, inevitably, during and immediately after lambing, bacterial invasion occurs into the vagina and the uterus, as the genital tract opens widely and a negative pressure occurs at the time of embryo expulsion (Noakes 2009).

The bacteria that are most frequently isolated from the genital tract of ewes post-partum include *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp., *Trueperella pyogenes*, and other Gram-negative bacteria (e.g., *Klebsiella* spp., *Proteus* spp.), as well as the anaerobes *Bacteroides* spp. and *Fusobacterium necrophorum* (Tzora et al. 2002, Hussain et al. 2013, Manes et al. 2018). Various factors may influence the bacterial species isolated from the vagina of

ewes after lambing, as well as their numbers. Among others, these include the intravaginal insertion of progestagen sponges (Manes et al. 2010, 2018, Gatti et al. 2011) and the length of the animal's tail (Orihuela et al. 2019).

Within the uterus, lochia is an ideal substrate for bacterial growth. In most cases however, bacterial intrauterine invasion would not lead to disease; the defenses of the uterus can gradually limit bacterial numbers, thereby with no development of clinical disease is presented (Lewis and Wulster-Radcliffe 2013, Shao et al. 2017). The main defense mechanisms of the uterus are (a) the presence of neutrophils within the uterine cavity and of the antibacterial agents produced by the uterine glands and (b) the progressive expulsion of the lochia through the uterine contractions and the uterine involution. Indeed, the removal of the uterine content through the contractions post-lambing contributes significantly to the bacterial elimination. Thus, after the fifth to ninth day post-lambing, the uterine content would be mostly free of bacteria.

Disorders of the uterus of ewes during the puerperium

Uterine infections

Aetiology and course of the disorder

In ewes, post-partum uterine infection (metritis) usually occur immediately after lambing or abortion. In this species, the disorder has not been studied as extensively as in cows (Palmieri et al. 2011). Nevertheless, the disorder can be a cause of periparturient death in ewes (Mavrogianni and Brozos 2008).

The circumstances leading to bacterial invasion of the uterus post-partum have been described above. Metritis is an opportunistic infection of the uterus, most often caused by contaminant environmental bacteria (Sargison 2008). It usually follows an abnormal first or second stage of labour, especially when there has been severe dystocia that had required prolonged traction or resulted in damage to the genital tract (Smith 2009). Uterine infections can usually develop as the consequence of obstetrical manipulations (commonly unskilled or unhygienic), retention of foetal membranes, delivery of dead lambs and uterine prolapse (Sargison 2008).

Various bacteria may act as causal agents. *E. coli* and *T. pyogenes* are the ones most frequently involved; however, all bacteria that can invade into the uterus post-partum (e.g., staphylococci, streptococci, Gram-negative rods, anaerobic bacteria) can also be implicated in the aetiology of the disease (Tzora et al. 2002, Hussain et al. 2013). In animals that had aborted, metritis can also be the caused by the abortifacient organism.

In general, the frequency of the pathological condition is low. Further, the prevalence of clinically healthy animals with histopathological findings in the uterus is also low (< 1%). This is likely due to (a) the relatively low incidence of dystocia in ewes and (b) continuous sucking by lambs, which results in secretion of oxytocin, contributing to the expulsion of the uterine content.

Various factors that can lead to compromise of the relevant defences or to entry of an increased number of pathogenic bacteria into the uterus, may create conditions for clinical disease. In fact, immediately post-partum, the uterine environment is a particularly suitable means for bacterial multiplication: the temperature therein supports bacterial growth and the lochia provides relevant nutrients. The main factors predisposing to development of clinical metritis are the following.

- Abortion of bacterial aetiology, which had resulted in the presence of pathogens within the uterus.
- Particularly long labour, with the cervix remaining open for a longer than normal period (which facilitates entry of microorganisms) and compromise of relevant defences.
- Obstetrical manipulations, particularly if they had been performed without following the aseptic procedures, thus increasing the number of bacteria within the uterus.
- Retention of lochia and of foetal membranes, providing a medium for bacterial multiplication within the uterus.
- Metabolic disorders, e.g., hypocalcaemia, reducing post-partum uterine contractions and thus expulsion of uterine content.
- Prolapse of the genital tract, thus exposing it to the environment and increasing chance of infection.

Clinical signs

Clinical signs of metritis usually develop within three days after lambing. In mild metritis, clinical signs associated with the genital tract predominate (e.g., oedema of the vulva and the vagina, discharge of varying amounts of dark-coloured, thick, malodorous, mucous or mucosal discharge). The colour of the discharge is initially reddish brown, over time turning whitish. The excretion of discharge is accompanied by tenesmus, which, occasionally, can result to uterine prolapse (Smith 2009, Tsousis et al. 2011).

In severe cases, metritis is accompanied by systemic signs. Fever (> 40.5 ^{o}C), recumbence, dehydration, anorexia, tachypnea and tachycardia can be present. The affected animal ignores the newborn lambs, does not suckle and has a decreased milk yield. In some cases, death of the affected ewe may occur (Smith 2009, Tsousis et al. 2011).

Diagnosis

Cinical diagnosis is based on history and clinical findings. Metritis should be included in the differential diagnosis in all cases of illness, fever or even reduction in appetite and milk yield during the first week post-partum. Use of vaginoscopy would increase the number of animals that are clinically diagnosed with the disease (Tsousis et al. 2011).

Microbiological examination of vaginal discharge is necessary for the isolation - identification of the causal agent and for their susceptibility testing to antimicrobial agents. Microbiological examination is paramount in cases of increased incidence of the disease in a flock. Cytological examination of vaginal discharge should also be performed to confirm the presence of inflammation by detection of neutrophils therein. Ultrasonographic examination of the uterus can also be used.

Other disorders

Uterine prolapse

Prolapse of the uterus most often occurs immediately (within 12 hours) after lambing. It can be the consequence of tenesmus in cases of metritis, although it can also be the effect of (among others) metabolic disorders (e.g., hypocalcaemia), a protracted lambing, overdose

of oxytocin and retention of foetal membranes. The disorder can also predispose ewes to metritis.

Retention of foetal membranes

The disorder occurs infrequently in sheep (incidence risk: 1% of lambings) (Fthenakis et al. 2000).

CHAPTER II

UTERINE INVOLUTION IN EWES WITH UTERINE INFECTION AND CONSEQUENCES IN SUBSEQUENT REPRODUCTIVE PERFORMANCE

OBJECTIVES

The objectives of this work were:

- (a) the study of the characteristics of uterine involution in ewes that had developed uterine infection in the immediately post-partum period and
- (b) the evaluation of the subsequent reproductive performance of such ewes.

MATERIALS AND METHODS

Experimental design

In total, 24 Lacaune-cross ewes (age 3 – 5 years) were included in the study. Before enrolment, a standardised detailed clinical examination was performed to assess the general health of the ewes. After enrolment in the study, ewes were identified using neck straps and plastic tags with unique serial numbers. Reproductive control (latitude of location: N 39.37 °, month of application: September) was performed by intravaginal insertion of progestogen sponges. Ewes were mated by rams of known fertility and repeatedly examined ultrasonographically to confirm pregnancy and its normal progress (Valasi et al. 2017). Standard health management procedures were performed in the animals during gestation (Fthenakis et al. 2012).

Two weeks before the expected lambing date, animals were allocated in two equal groups (I and C) by using complete randomisation, through random number generator. After allocation into groups, all animals in the same group were penned together and separately from the other group.

All ewes lambed normally (L0 = day of lambing) and produced twin lambs. A detailed clinical examination was performed immediately post-partum. Vaginal-uterine swab samples were collected as described below; two animals in group I that yielded *E. coli* in those samples were excluded from the study. The remaining 10 ewes in group I were challenged on the first day post-partum (L1, D0 = day of inoculation) with approximately 3.5 × 10^5 colony-forming-units of an *E. coli* isolate originally from a field case of uterine infection, inoculated into the uterus of each of these ewes. In ewes in group C, sterile phosphate buffer saline pH 7.3 (PBS) was injected into the uterus.

The challenge procedure was as follows (Amiridis et al. 2003, Mavrogianni et al. 2007a). Two colonies of the isolate obtained from a fresh culture, were inoculated into 10 mL of Soy-broth (BioMerieux, Marcy-l' Etoile, France) and incubated aerobically at 37 °C. After 6 h incubation, serial dilutions of the broth into PBS were carried out and 1.0 mL of the desired dilution was centrifuged for 10 min. at 1,750 *g*. The supernatant was discarded and the bacteria resuspended in approximately 2.0 mL sterile PBS and transferred into 5 mL syringes. The number of viable bacteria in the suspension was counted by the method of Miles and Misra (1938). Each syringe was attached to the end of a sterile catheter, which was

inserted into the uterus of the ewe, through the cervix, under strict aseptic conditions. The inoculum was deposited at the body of the uterus.

Conditions prescribed by legislation of the European Union in relation to animal experimentation procedures (Council Directive 86/809/EEC) were met during this work.

Post-partum examinations and sample collections

Examination and sampling points

Lambings took place in February to March, which was outside the annual breeding season in the area (latitude: N 39.37 °), where the study was conducted. All ewes produced twin lambs.

Detailed examinations of all ewes and sample collections were performed at regular intervals throughout the study. Before challenge, two samples were collected, one immediately after lambing (L0) and the second on the following day (L1). After inoculation, samples were collected initially 6 h and 12 h post-challenge (D0 + 6 h and D0 + 12 h, respectively) and then on D1, D2, D3, D4, D6, D9, D12, D17, D22, D27, D32, D42, D52, D62.

Clinical examination

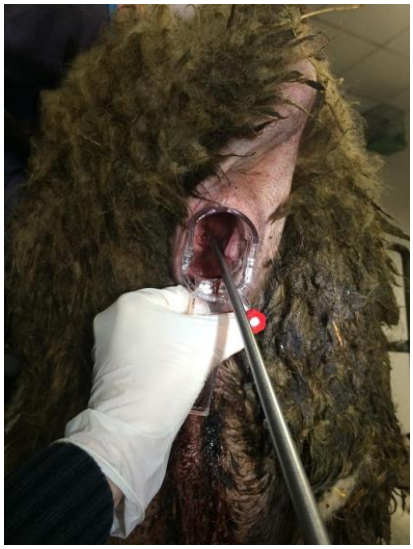
On each of the above sampling points, initially, a detailed clinical examination of the genital tract was performed (Figure 1). This included the examination of the vulva, the vestibule, the walls of the vagina and the cervix by using a speculum and the examination of the external os and the area around the opening of the cervix (Dawson 2007).

Figure 1. Examination of the vagina of a ewe by using a speculum.

Collection of samples from the vagina

Subsequently, samples of vaginal discharge were also collected. Initially, thorough cleansing of the external genitalia was performed with povidone iodine scrub solution (Betadine®; Mundipharma Medical Company, Basel, Switzerland). Then, a sterile swab was introduced into the vagina, through a plastic, single-use speculum and a long, lubricated, sterile protective sheath (Figure 2), in order to sample any discharge present at the outer entrance of the cervix and the anterior part of the vagina; the wall of the genital tract was gently swabbed and the swab was withdrawn. Thereafter, a cell collector with a gentle-touch tip designed to minimise trauma to the genital tract (Cytobrush Plus; Cooper Surgical, Trumbull, USA) was inserted into the genital tract for cell collection.

Figure 2. Collection of material from the vagina of a ewe by means of a sterile swab introduced into the vagina, through a plastic, single-use speculum and a long, lubricated, sterile protective sheath.



Collection of blood samples

Blood samples were collected on these occasions for a standard haematological examination.

Ultrasonographic examination

Ultrasonographic examination of the genital tract was performed on the above occasions using an ultrasound scanner (MyLab® 30; ESAOTE SpA, Genova, Italy). For B-mode ultrasonography, linear (7.5 - 12.0 MHz) and convex (2.5 - 7.5 MHz) transducers were used; the methodology and technicalities described in detail in chapter I were followed; longitudinal and transverse sections were taken by means of the transcutaneous technique performed at the inguinal area (Figure 3).

Figure 3. The ultrasound scanner (MyLab® 30) with the linear (7.5 - 12.0 MHz) transducer (left picture) and the convex (2.5 - 7.5 MHz) transducer (right picture) employed in for ultrasonographic examination of ewes in the study.

For Doppler ultrasonography, a linear (6.6 MHz) transducer with the transcutaneous technique was employed. The Doppler angle was set at 0 – 60 °. During the examination, by using as reference point the external iliac artery (*arteria iliaca externa*), the uterine artery (*a. uterina*) was located craniolaterally to the bladder (*vesica urinaria*). The methodology and technicalities described in detail by Petridis et al. (2017) were followed.

Contrast-enhanced ultrasonographic (CEUS) examination was performed on D1 in two group I and two group C ewes, using an ultrasound scanner (Vivid®-I; General Electric, Tirat Carmel, Israel), with a convex transducer (4C RS) of varying frequencies (1.8 - 6.0 MHz); initially, B-mode sections were taken using a frequency of 5.0 MHz and a scanning depth of 120 mm, eventually switching the imaging settings to a preset coded phase inversion mode; frequency, mechanical index and power were automatically set to lower values (i.e., 2.0 / 4.0 MHz, 0.09 and 22 dB, respectively); one focal zone was used at a scanning depth of 70 mm. A volume of 5.0 mL of the contrast agent (40 μL of sulphur hexafluoride in microbubbles, equivalent to 112.5 mg; excipients: macrogol 4000, distearoylphosphatidylcholine, dipalmitoylphosphatidylglycerol sodium, palmitic acid;

solvent: sodium chloride 9 mg mL^{-1}) was injected into the jugular vein, followed by intravenous injection of 10 mL of normal saline. This is a second generation contrast agent consisting of microbubbles, containing sulphur hexafluoride, which is an inert and hydrophobic gas, stabilised by a thin and flexible monolayer shell of phospholipids (SonoVue®, Bracco, Milano, Italy), and is licenced for use (Schneider 1999).

Uterine tissue biopsies

Uterine tissue biopsies were performed laparoscopically (250.2 H; Schoelly Fiberoptic, Denzlingen, Germany) for tissue sample collection from the uterus of the experimental ewes. These were performed on D7, D14, D24, D42 (left horn) and D27, D34, D44, D62 (right horn) (2 animals from group I and 1 animal from group C on each sampling point; biopsy was performed in each animal twice, once in the left and once in the right horn). The animal was placed in dorsal recumbency. Analgesic procedures, with administration of lidocaine 2% (dose rate: 4 mg / kg bodyweight) (Xylocaine 2%, Astra Zeneca, Cambridge, United Kingdom) were applied (Galatos 2011) and a strict aseptic technique was used.

Two 5.5 mm trocars were introduced into the abdominal cavity positioned 8 to 10 cm across the mid line and 5 to 7 cm cranially to the front attachment of the udder. The light source (Flexilux 250.2 H; Schoelly Fiberoptic, Denzlingen Germany) was connected to the endoscope (Schoelly Fiberoptic 5.0, 30o), which was then introduced into the abdominal cavity through the trocar placed on the left side. Through the trocar on the right side, a disposable atraumatic endoscopic forceps was introduced for the manipulation of the uterine horn. The horn was fixed and moved slightly upwards and close to the abdominal wall. A third 3.5 mm trocar was then inserted, close to the midline of the abdomen, through which a biopsy forceps (Wolf 8380.01, 3.0 mm; Richard Wolf, Knittlingen, Germany) was inserted. The uterine horn was carefully perforated with the forceps and a small tissue sample (endometrium and myometrium) was collected from the uterine horn. After sampling, the uterus was observed for excessive bleeding or possible leakage of uterine contents. In one occasion that minor bleeding occurred, this ceased by applying slight pressure with the atraumatic forceps; the uterus was then rinsed with sterile physiological

saline. In no case, leakage of uterine content was observed. Finally, after collection of the tissue sample, the abdominal wall of the ewes was sutured.

Laboratory examinations

Bacteriological and cytological examination of vaginal swab samples

Vaginal swab samples were processed soon (< 10 min.) after collection. Swabs were cultured on 5% sheep blood agar and McConkey plates. Media were incubated aerobically and anaerobically at 37 ^{o}C for up to 48 hours; if no bacterial growth was evident, they were reincubated for another 24 hours. Bacterial identifications were performed by using standard methods (Barrow and Feltham 1993, Euzeby 1997).

Cells from cell collectors were transferred on glass slides and stained with the Giemsa technique.

Haematological examination of blood samples

Samples for haematological examination were mixed by gentle repeated inversions for several seconds to avoid coagulation and were processed within 30 min. after collection. Initially, blood smears were prepared and kept dry at room temperature. A complete blood count was performed by an automated haematological analyser (ADVIA 2120i, Siemens Healthineers, Erlangen, Germany). The following parametres were determined: erythrocyte count, haematocrit, haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin concentration, total leucocyte count and thrombocyte count. Blood smears were evaluated for detection of morphological abnormalities and leucocyte type differentiation. From the proportion of the various leucocyte types, the respective counts were then calculated.

Bacteriological and histopathological examination of uterine tissue samples

Uterine tissue samples were initially cultured on 5% sheep blood agar and McConkey plates, which were incubated as above.

Then, the uterine tissue samples were fixed in 10% neutral-buffered formalin; finally, haematoxylin and eosin (HE) standard staining procedures were used for processing.

Evaluation of subsequent reproductive performance

Two months after the end of the monitoring period and after the reproductive season had started (i.e., in June), ewes were put with rams of known fertility (n = 2) for mating. Rams were left with ewes for 60 days. Ultrasonographic examinations for pregnancy diagnosis (Barbagianni et al. 2017) were performed twice, 50 and 100 days after ram introduction. Throughout that period, during the pre-conception period and gestation, appropriate health management of ewes had been performed as recommended (Fthenakis et al. 2012); all ewes were maintained under the same conditions. Finally, lambings and number of lambs born from each ewe were recorded.

Data management and analysis

Post-partum stages

The post-partum period was divided into four stages: S_1 included samples collected before inoculation, on L0 and L1 (2 sampling points), S_2 included samples collected after challenge up to and including D4 (L5) (6 sampling points), S_3 included samples collected from D6 to D12 (3 points) and S_4 included samples collected from D17 to D62 (7 points).

B-mode ultrasonographic measurements

Initially, stored ultrasonographic images were viewed for description. Then, images of (a) caruncular tissue and (b) inter-caruncular tissue obtained from each ewe on each

occasion, were processed by means of ImageJ software (National Institutes of Health, Rockville Pike, USA), which can edit, process and analyse grey-scale images, by calculating area and pixel value statistics to produce intensity values (National Institutes of Health 2013); in an image processing context, grey-scale analysis refers to the image's overall pixel grey intensity values (Ojala et al. 2002), with results expressed on a 0 (black) to 255 (white) scale.

For analysis of results of grey-scale measurements, data were normalised by calculating the ratio GS_{CT} / GS_{IT}, where GS_{CT}: Grey-scale of caruncular tissue and GS_{IT}: Grey-scale of inter-caruncular tissue.

Measurements of dimensions of uterine structures were performed in images taken by the linear transducer and were calculated by the equipment's software. For calculation of the diametre of caruncles and the uterine lumen, a measurement of opposing points of the section of the structure was taken, followed by another one at an angle of 90 ^{o} to the first and the mean value of the two was calculated. The thickness of the myometrium and the endometrium was also measured.

Reduction of dimensions of uterine structures (R_s) within a stage (S_n) compared to those in the preceding stage (S_{n-1}) was calculated as follows for each animal into the study: R_s = 1 - (average of measurements taken within S_n / average of measurements taken within S_{n-1}). Daily reduction of dimensions (dR_s) within a stage (S_n) was calculated as dR_s = [R_s / ('mid time-point' of S_n – 'mid time-point' of S_{n-1})] (expressed in days), where 'mid time point' of S_i = [(last day of samplings made during S_i – first day of samplings made during S_i) / 2] + first day of samplings made during S_i (last day of samplings: L1, D4, D12, D62 - first day of samplings: L0, D 0 + 6 h, D6, D17, for S_1, S_2, S_3, S_4, respectively). Overall (throughout the study period) reduction of dimensions (R_o) was calculated as follows for each animal into the study: R_o = 1 - (average of measurements taken within S_4 / average of measurements taken within S_1).

Doppler mode ultrasonographic measurements

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

Spectral waveforms of the uterine artery were processed by means of MyLab® software (ESAOTE SpA). On each occasion, waveforms from three consecutive cardiac cycles of the animal under examination were considered for calculations. The software, based on the outline of the waveform, calculated directly the below haemodynamic parametres in that vessel (Maulik 2005, Ginther 2007, Wood et al. 2010, Petridis et al. 2017).

- Resistance index: [(PSV - EDV) / PSV] (PSV: peak systolic velocity, EDV: end diastolic velocity) indicating the effect of the vessel resisting blood flow.
- Pulsatility index: [(PSV - EDV) / TAMV] (TAMV: time-averaged maximum velocity) measuring the systolic-diastolic differential of the velocity pulse.
- Systolic : diastolic velocity ratio: [ASF / ADF] (ASF: average diastolic flow, ADF: average systolic flow) delineating systolic and diastolic phases of a flow waveform.
- General pressure: [$P_{syst} - P_{diast}$] ([P_{syst}: systolic pressure, P_{diast}: diastolic pressure) measuring the change in pressure from the diastolic level to the systolic level (mm Hg).
- Mean pressure: [(⅓ × P_{syst}) + (⅔ × P_{diast})] measuring the average blood pressure over time by proprietary pulse dynamics pattern-recognition algorithms (mm Hg).
- Mean velocity: indicating blood speed across the vascular lumen at a given instance (m s^{-1}).
- Systolic acceleration: indicating blood acceleration across the vascular lumen (m s^{-2}).
- Blood flow volume: indicating the volume of blood entering the uterus per unit of time (mL $min.^{-1}$).

Contrast-enhanced ultrasonographic measurements

Video images were analysed in sequence of frames (JPG format; first frame at time 0 and then one frame every 2 s) using the Free Studio (v. 6.6.35.323) multimedia software developed by DVDVideoSoft (Digital Wave Ltd, London, United Kingdom). The frames

were opened as a stack with ImageJ software. Two regions of interest were used in the evaluation: caruncular tissue and inter-caruncular area for calculation of intensity of signals. Image enhancement in each region was measured in linear arbitrary enhancement units (AEU). A time–intensity curve was generated for each region of interest and for each examination the below parametres were calculated (Mantziaras et al. 2018).

- Peak enhancement (expressed in AEU): enhancement curves were produced after measurement of intensity by means of Vivid-I software (General Electric) and dividing by the maximum value of intensity.
- Time to peak (s): calculated from injection of contrast agent to peak intensity.
- Time to wash-out (s): calculated from injection of contrast agent to return to baseline.
- Total enhancement time (s): calculated from beginning of enhancement to return to baseline.
- Wash-in time (s): calculated from beginning of enhancement to peak intensity.
- Wash-out time (s): calculated from peak intensity to return to baseline.

Modelling for analysis of infection results

In the study, there was a difficulty with attempts to estimating incidence rate (new 'infection' per animal at risk for each time point at risk), because, in many cases, the site under study might change from being 'infected' to being 'uninfected' and *vice-versa*; therefore, when there was a long time-interval between sampling points, it was not possible to know what happened between the two sampling points (i.e., how many infections and 'cures' there might have occurred). Therefore, the following definitions were initially made (Mavrogianni et al. 2007b).

- 'Isolation of bacteria' was equivalent to 'infection with'; 'isolation of bacteria from the swab' was equivalent to 'infection of the anterior part of the vagina' and 'isolation of bacteria from the uterine tissue sample' was equivalent to 'infection of the uterus'.
- On a particular sampling point, a sampling site (anterior part of the vagina, uterus) was defined as being 'at risk of becoming infected' (i.e., becoming bacteriologically positive) if it had been uninfected (i.e., bacteriologically negative) on the previous sampling point. On the subsequent sampling point, this sampling site (anterior part of the vagina, uterus) could

be either 'infected' (in which case it was not at risk) or 'uninfected' (in which case it was still at risk). On subsequent sampling points, if this site was 'uninfected', then it was again 'at risk'.

- If a sampling site was infected on one sampling point but not on the next one, then the infection was deemed to have been eliminated half-way between the two sampling points; conversely, if a sampling site was uninfected on one sampling point and infected on the next one, then the infection was deemed to have taken place half way between the two sampling occasions.
- If a sampling site was infected with the same organism on two consecutive samplings, then it was considered to have been infected throughout the period between those two sampling points; conversely, if a sampling site was uninfected on two consecutive samplings, then it was uninfected throughout the time between those two sampling points.
- Recurrence of infection was defined as re-isolation of an organism from a previously infected sampling site, after a sampling, in which no isolation of any organism took place in-between two samplings.

Based on the above, it was possible to calculate an estimate of the length of time a sampling site was at risk before it became infected, as well as the length of time of each infection. Sampling sites contributed more than one value, if recurrence of infection had occurred.

Evaluation of cellular infiltration

Smears from vaginal swab samples were evaluated to assess leucocyte subpopulations, by means of semi-quantitative observational method using the 40 × objective lens of a Zeiss-Axiostar Microscope (Carl Zeiss, Göttingen, Germany) with a 10 × eyepiece lens. In each slide, at least 50 fields were observed and at least 100 leucocytes were counted.

Uterine tissue samples were evaluated as above and 50 fields were observed on each slide. An arbitrary score was assigned for average number ($\bar{x}$) of leucocytes per field therein as follows; 0: $\bar{x} < 1$ leucocyte, 1: $1 \leq \bar{x} < 5$ leucocytes, 2: $5 \leq \bar{x} < 10$ leucocytes, 3: $10 \leq \bar{x} < 15$ leucocytes, 4: $\bar{x} \geq 15$ leucocytes per field.

Measures for reproductive performance

The following measures of reproductive performance were calculated (Fthenakis 2004).

- Mating rate: number of ewes mated by rams during the whole reproductive period / number of ewes exposed to the ram × 100.
- Pregnancy rate: number of ewes that were found pregnant at ultrasonographic examination 50 and 100 days after ram introduction / number of ewes exposed to the ram × 100.
- Abortion rate: number of ewes that aborted before the 140th day of gestation / number of ewes exposed to the ram × 100.
- Lambing rate: number of ewes that lambed / number of ewes exposed to rams × 100.
- Total lambs per ewe: number of liveborn and stillborn lambs / number of ewes that lambed.
- Stillbirth rate: number of stillborn lambs / number of liveborn and stillborn lambs × 100.

Statistical analysis

Data were entered into Microsoft Excel for analysis. Basic descriptive analysis was performed. The outcomes of interest were considered.

Comparisons of frequencies of clinical signs and bacterial isolations for I *versus* C group were performed in a table of cross-categorised frequency data by use of Pearson chi-square test or Fisher-exact test, as appropriate. Time of first appearance of an outcome and duration of outcome under evaluation were compared between groups as above by means of the Mann-Whitney test. The Kruskal-Wallis test and the Mann-Whitney tests were used to evaluate differences in bacterial isolations between post-partum stages.

For ultrasonographic measurements (B-mode, Doppler mode), repeated measures mixed effect linear regression models were used to determine whether outcomes changed over the course of the study period. Fixed effect was the time-point of the study (i.e., L0, L1,

etc.). Effect of experimental subjects (animals) was included as random effect in the model. Models were adjusted for repeated measures within animals. Initially, separate analyses were carried out for each stage post-lambing (S_1 – S_4), which were followed by an analysis that took into account all measurements performed (18 time-points). The same method was also used for analysing results of haematological examinations.

For ultrasonographic (B-mode, Doppler mode) and haematological parametres, as well for proportions of types of leucocytes in vaginal swab samples, analysis of covariance was performed between ewes in the two groups (I and C). In this analysis, measurements obtained after challenge were compared between groups after taking into account and eliminating a possible effect of measurements made before challenge.

For CEUS results, repeated measures mixed effect linear regression models were used to study outcomes over the measurement period. The effect of animals was included as random effect in the model, which was adjusted for repeated measures within animals and comparisons were made between inoculated and control ewes.

The Mann-Whitney test was used to evaluate differences between groups in scores for average leucocyte numbers in uterine tissue samples.

Results of reproductive performance were evaluated by comparison of proportions or the Mann-Whitney t-test, as appropriate per type of result.

In all cases, level of significance was set at $P = 0.05$.

RESULTS

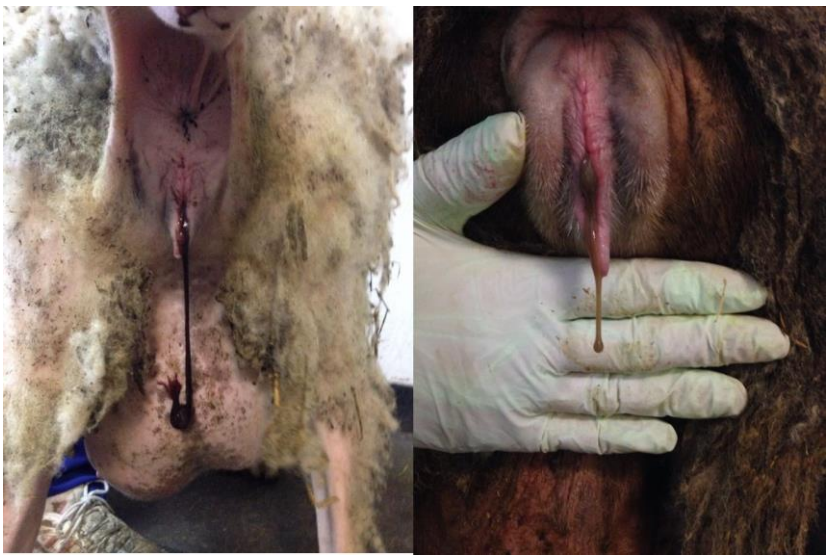
Clinical findings

In group I, all 10 ewes developed uterine infection after challenge. One ewe (0.100, 95% confidence interval [CI]: 0.018 – 0.404) developed transiently increased rectal temperature (40.9 °C for 1 day). All ewes (1.000, 95% CI: 0.723 – 1.000) developed genital clinical signs (presence of malodorous, thick, purulent, yellow- to brown- to black-coloured vaginal discharge [n = 7] and vaginal hyperaemia and vulval oedema [n = 7]) within one day post-challenge (Figure 4). Median duration of genital clinical signs was 29.5 (19.5 – 61) days. Recurrence of clinical signs was recorded in three ewes (0.300, 95% CI: 0.108 – 0.603).

In group C, systemic signs were not observed in any ewe. In 2 ewes (0.167, 95% CI: 0.047 – 0.448), there was transient presence of vaginal discharge; median duration of its presence was 11 (9 – 13) days. No recurrence occurred in these ewes.

Frequency of uterine infection was significantly higher in group I ($P < 0.001$). Inoculation increased the risk of development of uterine infection; odds ratio was 88.200 (95% CI: 3.762 – 2067.754) ($P = 0.005$). Median duration of clinical signs was significantly longer in group I ($P = 0.025$).

Figure 4. Presence of thick brown- to black-coloured vaginal discharge in two inoculated ewes 2 days after challenge.

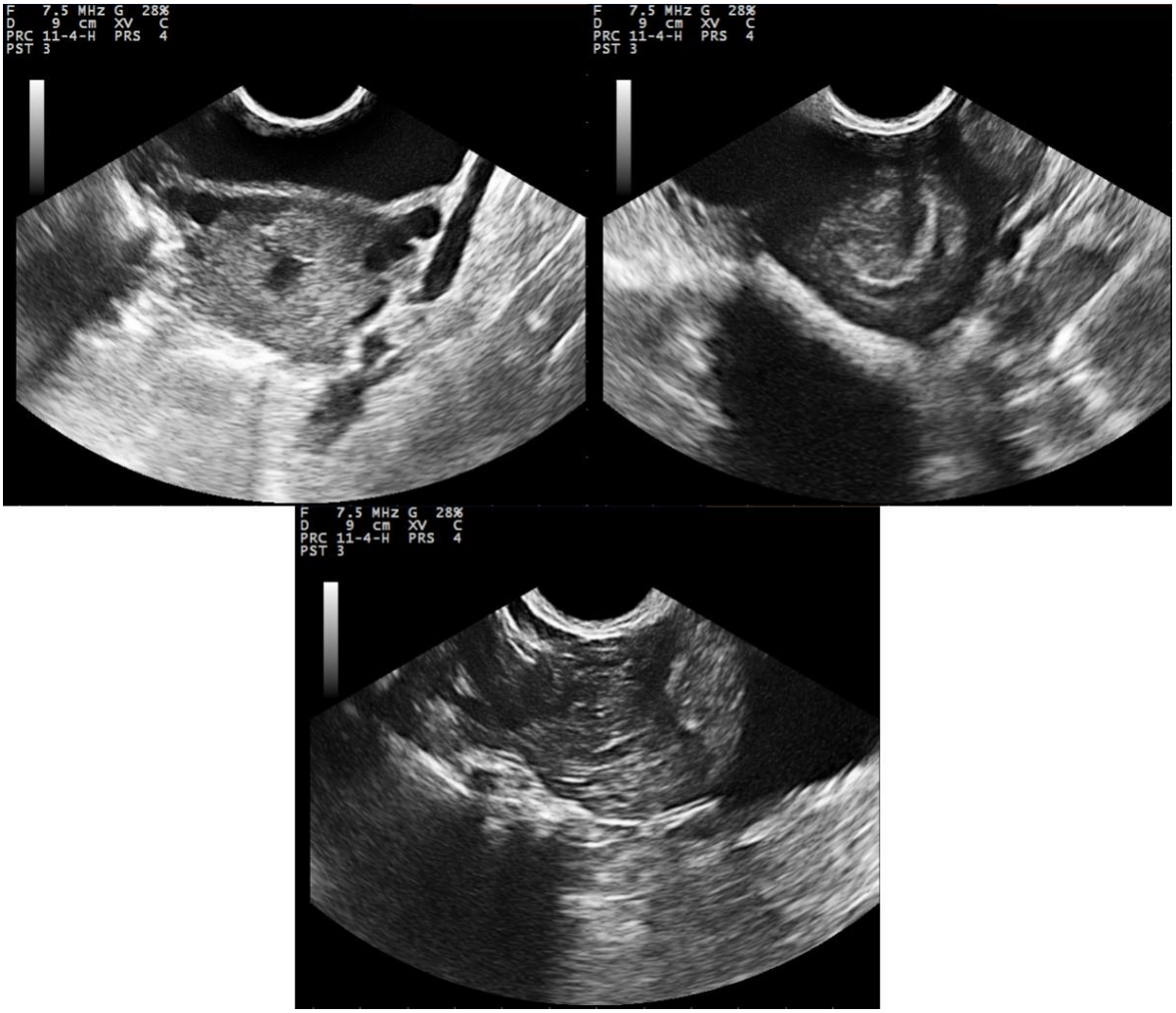


Ultrasonographic findings

B-mode examination

In I group ewes, the uterus could be easily imaged during the first week post-challenge, with the endometrium appearing more hyperechoic than in healthy control ewes. The layers of the uterine wall appeared thickened and particularly echogenic until D12. Content of the uterine lumen was imaged with hyperechoic foci, due to the presence of fluid and cellular components in most ewes until D9 (until D17 in two ewes). Progressively, the uterine content was imaged containing inconsistent and blurred structures of increased or mixed echogenicity; the last day that uterine content was imaged was D17. Post-challenge, the caruncular tissue was echogenically similar to the inter-caruncular tissue and its folds, but briefly, on the second week post-challenge, it became more echogenic than that (Figure 5). Indeed, during S_3, GS_{CT} / GS_{IT} ratio was greater in group I than in group C ewes ($P < 0.001$) (Table 2). For all measurements after challenge, GS_{CT} / GS_{IT} ratio was 1.05 ± 0.03 in group I and 0.96 ± 0.03 in group C ewes ($P = 0.057$).

Figure 5. Sequential post-partum B-mode ultrasonographic presentation of the uterus of ewes with experimentally induced uterine infection (group I).



Top left: immediately after lambing, top right: 2 days, bottom centre: 12 days after inoculation; the uterine body and lumen are imaged (transverse sections obtained by transcutaneous examination at the inguinal area, image taken and processed on a MyLab® 30 ultrasonography system with convex transducer, imaging frequency: 7.5 MHz - scanning depth: 90 mm).

Table 2. Post-partum echogenicity: ratio GS_{CT} / GS_{IT}[1] (mean ± standard error of the mean) in the uterus of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

		Stage post-partum[2]			
		S_1	S_2	S_3	S_4
Group	I	$0.94 \pm 0.06^{a,m}$	1.00 ± 0.04^{n}	$1.22 \pm 0.05^{b,m,n}$	0.88 ± 0.04
	C	$0.84 \pm 0.04^{a,m,n}$	0.97 ± 0.04^{m}	$0.97 \pm 0.04^{b,n}$	0.93 ± 0.02

a, b: same superscripts within a column indicate significant difference between I and C groups (a: $0.05 < P \leq 0.01$, b: $P < 0.01$) – m, n: same superscripts within a row indicate significant difference between stages ($P < 0.01$).

1. GS_{CT}: Grey-scale of caruncular tissue, GS_{IT}: Grey-scale of endometrium.

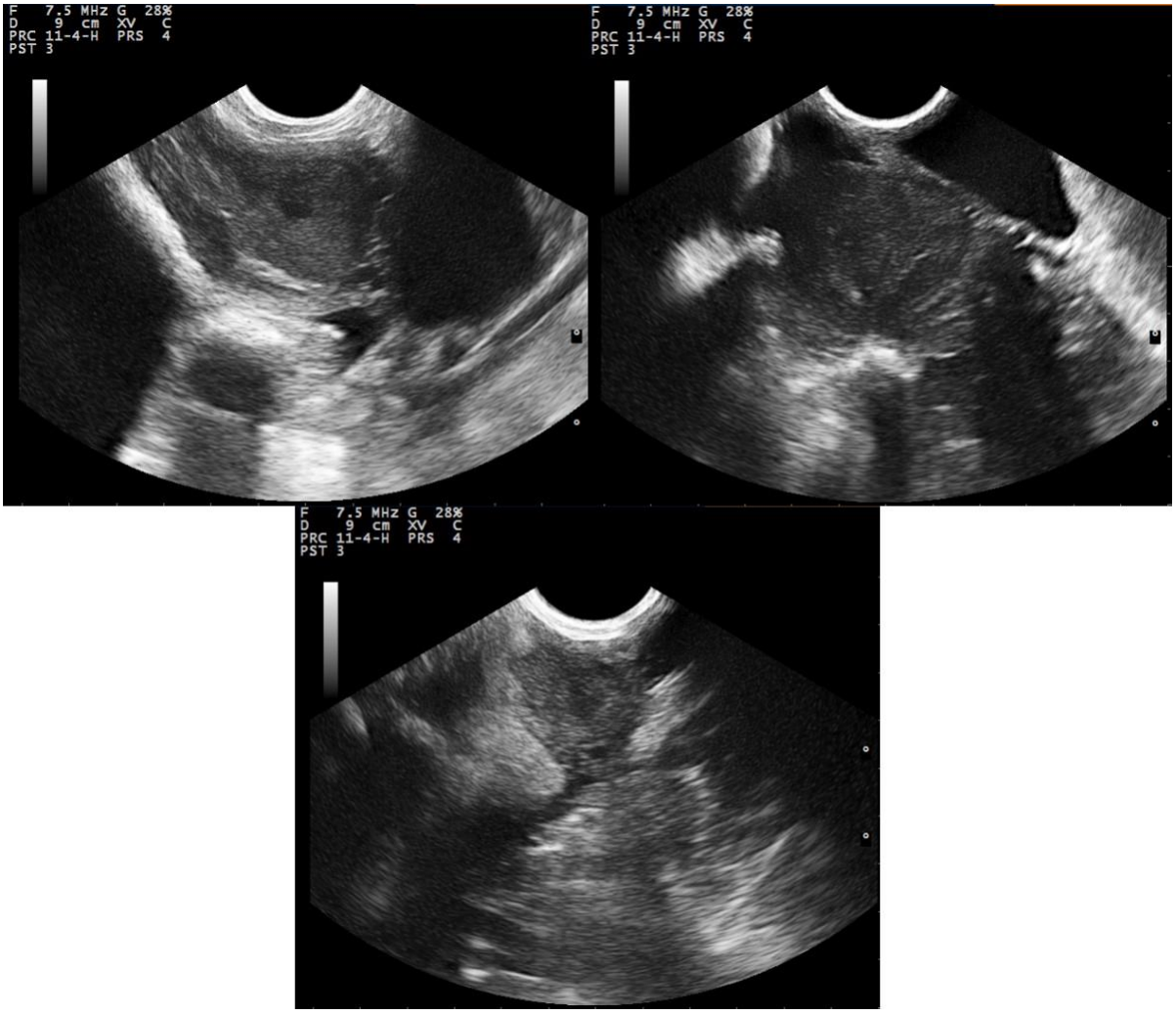
2. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and inclu ding D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

In group C, layers in the uterine wall were clearly imaged: the perimetrium and the outer layer of the myometrium were highly echogenic, the middle layer of the myometrium was anechoic to hypoechoic, like a dark rim, the inner layer of myometrium and the endometrium appeared as echogenic structures. The uterine lumen was seen irregularly shaped. After the tenth day post-partum, the organ appeared circular or elliptical (longitudinal sections) or polygonal to elliptical, progressively changing to compressed circular to circular (transverse sections). Caruncles were seen on the endometrium, often appearing in mushroom-like shape. In general, they were echogenically similar to the endometrium and its folds (Table 2); differences in their echogenity were evident: the most superficial layer was hyperechoic, with the deeper tissue being less echoic; caruncles were last observed on the 23rd day post-partum. Progressive degeneration of caruncles and removal of endometrium surface led to presence of echogenic content within the anechoic lumen. The uterine body bifurcation and the horns could be imaged up to 33rd day post-partum. Until then, the myometrium and the endometrium were imaged as clearly separate layers. Subsequently, the organ was imaged as a round entity near the bladder, as its size decreased. The echopattern of the uterine wall was finely textured, homogeneous and hypoechoic to moderately echogenic (Figure 6).

After challenge, caruncular dimensions were greater in inoculated ewes (group I) than in controls (group C) (Table 3). Myometrial thickness was also greater in I than in C ewes:

0.318 ± 0.007 cm *versus* 0.297 ± 0.008 cm (for all measurements taken, P = 0.016), as was diametre of uterine lumen: 0.368 ± 0.027 cm *versus* 0.339 ± 0.013 cm (for all measurements taken, P = 0.11).

Figure 6. Sequential post-partum B-mode ultrasonographic presentation of the uterus of normally involuting ewes (group C).



Top left: immediately after lambing, top right: 2 days, bottom centre: 12 days after inoculation; the uterine body and lumen are imaged (transverse sections obtained by transcutaneous examination at the inguinal area, image taken and processed on a MyLab® 30 ultrasonography system with convex transducer, imaging frequency: 7.5 MHz - scanning depth: 90 mm).

In all ewes, there was a reduction of dimensions of the uterine structures progressively, as the post-partum period advanced. In group I ewes, particular reduction of the dimensions occurred during S_3, when the mean daily reduction of the various structures was calculated up to 15.5%. In contrast, in group C ewes, the greatest reduction of the

dimensions occurred during S_2 (i.e., during the first week after lambing), when the mean daily reduction of dimensions of the uterine structures varied from 4.2% to 9.2% (Table 4). Overall, throughout the monitoring period, there were no significant differences in the daily reduction of the various structures between the two groups; for group I, it was 41.2% for the caruncular diametre, 50.5% for the myometrial thickness, 63.2% for the endometrial thickness and 63.3% for the uterine lumen diametre, whilst for group C, it was 45.0%, 52.4%, 54.8% and 61.9%, respectively (P = 0.39, 0.28, 0.11, 0.40, respectively).

Table 3. Post-partum ultrasonographically measured dimensions (mean ± standard error of the mean) of the uterine structures of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Uterine dimensions	diametre of caruncles (cm)	I	$1.44 \pm 0.03^{m,n,o}$	$1.32 \pm 0.02^{b,m,p,q}$	$1.15 \pm 0.05^{b,n,p}$	$0.94 \pm 0.14^{o,q}$
		C	$1.37 \pm 0.03^{m,n,o}$	$1.23 \pm 0.02^{b,m,p,q}$	$0.99 \pm 0.04^{b,n,p,r}$	$0.77 \pm 0.02^{o,q,r}$
	myometrial thickness (cm)	I	$0.48 \pm 0.01^{m,n,o,n}$	$0.40 \pm 0.01^{m,p,q}$	$0.31 \pm 0.01^{a,n,p,r}$	$0.23 \pm 0.01^{a,o,q,r}$
		C	$0.47 \pm 0.01^{m,n,o}$	$0.39 \pm 0.01^{m,p,q}$	$0.29 \pm 0.01^{a,n,p,r}$	$0.22 \pm 0.00^{a,o,q,r}$
	endometrium thickness (cm)	I	$0.72 \pm 0.06^{m,n,o}$	$0.58 \pm 0.03^{m,p,q}$	$0.44 \pm 0.05^{n,p,r}$	$0.25 \pm 0.02^{o,q,r}$
		C	$0.64 \pm 0.03^{m,n,o}$	$0.54 \pm 0.02^{m,p,q}$	$0.44 \pm 0.03^{n,p,r}$	$0.28 \pm 0.01^{o,q,r}$
	diametre of uterine lumen (cm)	I	$0.62 \pm 0.06^{m,n,o}$	$0.50 \pm 0.02^{m,p,q}$	$0.36 \pm 0.01^{n,p,r}$	$0.24 \pm 0.01^{o,q,r}$
		C	$0.63 \pm 0.05^{m,n,o}$	$0.46 \pm 0.02^{m,p,q}$	$0.35 \pm 0.01^{n,p,r}$	$0.23 \pm 0.01^{o,q,r}$

Group

a, b: with reference to the same parametre, same superscripts within a column indicate significant difference between I and C groups (a: $0.01 \leq P < 0.05$, b: $P < 0.01$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.035$).

1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

Table 4. Mean daily reduction of dimensions of the uterine structures, as calculated based on ultrasonographic measurements, of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

			Stage post-partum[1]		
			S_2	S_3	S_4
Uterine dimensions	diametre of caruncles (cm)	Group I	0.034	0.017[a]	0.009
		Group C	0.042	0.027[a]	0.008
	myometrial thickness (cm)	Group I	0.068	0.030	0.007
		Group C	0.067	0.033	0.007
	endometrium thickness (cm)	Group I	0.101	0.155[b]	0.138[b]
		Group C	0.092	0.018[b]	0.007[b]
	diametre of uterine lumen (cm)	Group I	0.127[a]	0.142[b]	0.130[b]
		Group C	0.076[a]	0.044[b]	0.012[b]

a, b: with reference to the same parametre, same superscripts within a column indicate significant difference between I and C groups (a: $0.01 \leq P < 0.05$, b: $P < 0.01$).

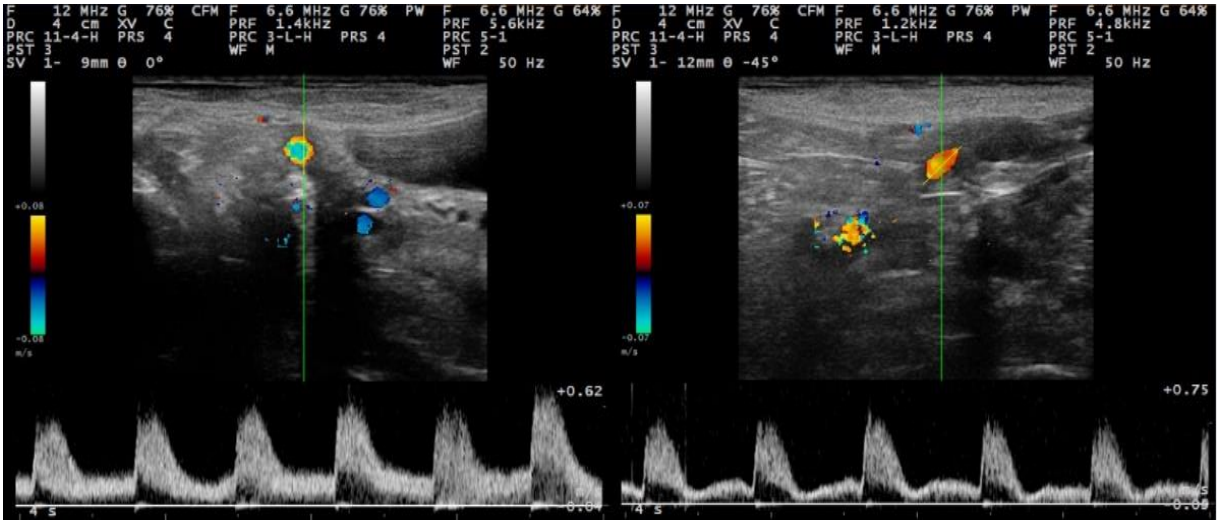
1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

Doppler mode examination

The spectral display in Doppler mode was observed as a broad band structure (Figures 7 and 8). The diametre of the uterine artery was similar in the two groups on S_1, but thereafter remained significantly larger in group I ewes ($P < 0.02$). The resistance and pulsatility indexes remained smaller in the group I ewes throughout the study, whilst the blood pressure was higher in these ewes. Finally, the blood flow volume into the uterus was significantly higher in group I ewes after challenge ($P < 0.02$) (Table 5). Moreover, for both groups, the progressive changes in the haemodynamic parametres (except for the mean velocity) were significant (Table 5).

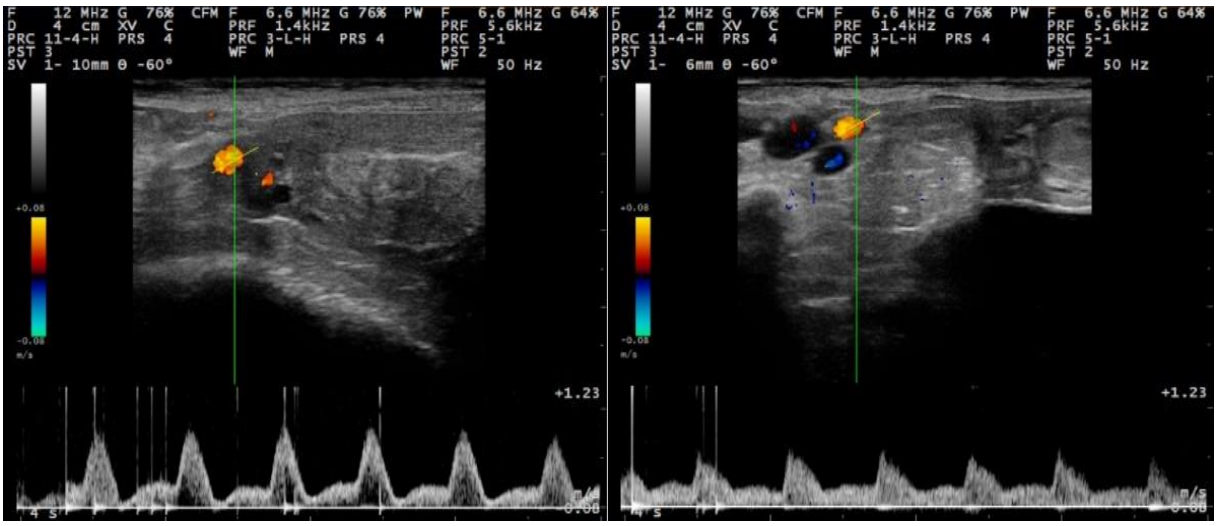
For all measurements taken after challenge, the uterine artery diametre and the blood flow volume into the uterus were significantly greater in I than in C ewes: 0.31 ± 0.01 cm *versus* 0.27 ± 0.01 cm ($P = 0.008$) and 92.7 ± 7.2 mL min.$^{-1}$ *versus* 62.0 ± 5.3 mL min.$^{-1}$ ($P = 0.032$), respectively (Figure 9). For the other haemodynamic parametres evaluated, no significant differences were evident ($P > 0.20$).

Figure 7. Sequential post-partum spectral waveforms of the uterine artery (Doppler ultrasonography) of ewes with experimentally induced uterine infection (group I).



Left: 2 days, right: 12 days after inoculation (images taken and processed on a MyLab® 30 ultrasonography system with linear transducer, Doppler imaging frequency: 6.6 MHz - scanning depth: 40 mm).

Figure 8. Sequential post-partum spectral waveforms of the uterine artery (Doppler ultrasonography) of normally involuting ewes (group C).



Left: 2 days, right: 12 days after inoculation (images taken and processed on a MyLab® 30 ultrasonography system with linear transducer, Doppler imaging frequency: 6.6 MHz - scanning depth: 40 mm).

Table 5. Post-partum Doppler ultrasonographic measurements (mean ± standard error of the mean) in the uterine artery of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

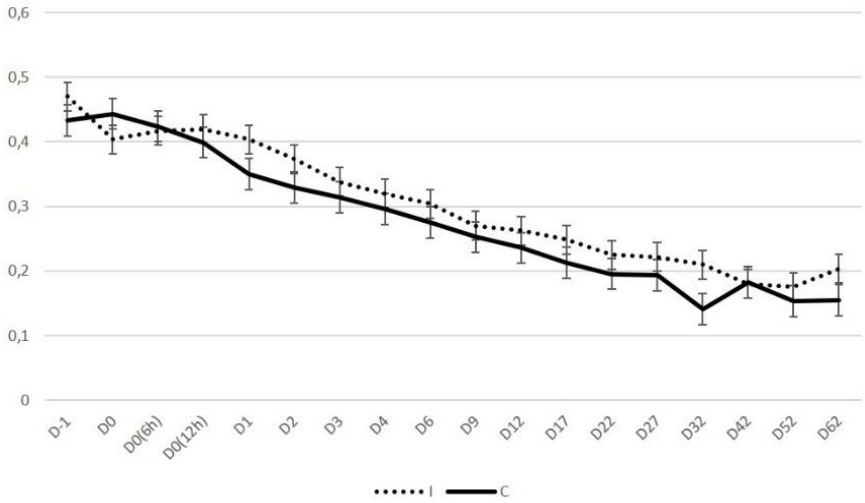
			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Doppler ultrasonographic parametre	uterine artery diametre (cm)	I	$0.44 \pm 0.02^{m,n,o}$	$0.38 \pm 0.01^{b,m,p,q}$	$0.28 \pm 0.01^{b,n,p,r}$	$0.22 \pm 0.01^{c,o,q,r}$
		C	$0.44 \pm 0.02^{m,n,o}$	$0.35 \pm 0.01^{b,m,p,q}$	$0.25 \pm 0.01^{b,n,p,r}$	$0.18 \pm 0.01^{c,o,q,r}$
	resistance index	I	$0.70 \pm 0.02^{m,n,o}$	$0.77 \pm 0.01^{c,m,p}$	$0.81 \pm 0.02^{n,q}$	$0.86 \pm 0.02^{o,p,q}$
		C	$0.74 \pm 0.02^{m,n,o}$	$0.81 \pm 0.01^{c,m,p}$	0.84 ± 0.02^{n}	$0.85 \pm 0.02^{o,p}$
	pulsatility index	I	$1.50 \pm 0.12^{a,m,n,o}$	$1.98 \pm 0.08^{c,m,p}$	$2.18 \pm 0.11^{b,n,q}$	$2.81 \pm 0.17^{o,p,q}$
		C	$1.79 \pm 0.10^{a,m,n,o}$	$2.45 \pm 0.09^{c,m,p}$	$2.58 \pm 0.15^{b,n,q}$	$2.99 \pm 0.13^{o,p,q}$
	systolic : diastolic velocity ratio	I	$4.27 \pm 0.74^{m,n}$	5.88 ± 0.86^{o}	$6.93 \pm 0.73^{m,p}$	$10.70 \pm 1.96^{n,o,p}$
		C	$4.27 \pm 0.30^{m,n,o}$	$6.84 \pm 0.45^{m,p}$	$7.44 \pm 0.69^{n,q}$	$12.91 \pm 1.57^{o,p,q}$
	general pressure (mm Hg)	I	$1.22 \pm 0.24^{m,n}$	0.87 ± 0.06^{o}	$0.72 \pm 0.07^{m,p}$	$0.53 \pm 0.07^{n,o,p}$
		C	$1.09 \pm 0.19^{m,n}$	0.82 ± 0.07^{o}	0.71 ± 0.08^{p}	$0.43 \pm 0.02^{n,o,p}$
	mean pressure (mm Hg)	I	$0.40 \pm 0.08^{m,n,o}$	$0.22 \pm 0.02^{b,m,p}$	$0.20 \pm 0.03^{n,q}$	$0.10 \pm 0.02^{o,p,q}$
		C	$0.26 \pm 0.03^{m,n,o}$	$0.16 \pm 0.02^{b,m,p}$	$0.14 \pm 0.02^{n,q}$	$0.07 \pm 0.01^{o,p,q}$
	mean velocity ($m\ s^{-1}$)	I	0.73 ± 0.05	0.69 ± 0.02	0.71 ± 0.03	0.73 ± 0.03
		C	0.67 ± 0.03	0.69 ± 0.02	0.74 ± 0.04	0.69 ± 0.02
	systolic acceleration ($m\ s^{-1}$)	I	6.60 ± 1.26	6.02 ± 0.38^{m}	8.95 ± 1.15^{m}	6.68 ± 0.72
		C	6.89 ± 0.75^{m}	$6.67 \pm 0.30^{n,o,p}$	$8.89 \pm 0.69^{m,o}$	$7.78 \pm 0.45^{n,p}$
	blood flow volume ($mL\ min.^{-1}$)	I	$242.1 \pm 31.4^{m,n,o}$	$144.7 \pm 10.6^{c,m,p,q}$	$68.9 \pm 8.3^{c,n,p,r}$	$28.2 \pm 3.3^{b,o,q,r}$
		C	$209.1 \pm 25.2^{m,n,o}$	$105.5 \pm 9.6^{c,m,p,q}$	$43.9 \pm 3.8^{c,n,p,r}$	$17.0 \pm 1.3^{b,o,q,r}$

a - c: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.025 < P \leq 0.050$, b: $0.01 < P \leq 0.025$, c: $P \leq 0.01$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.045$).

1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

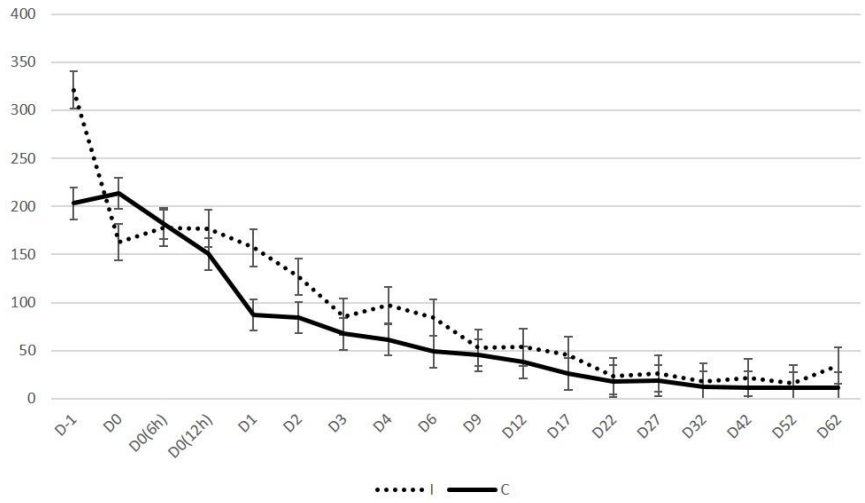
Figure 9. Time-series graphs (D0: day of inoculation) of Doppler ultrasonography results of the uterine artery of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C) (a) diametre of the uterine artety, (b) blood flow volume.

(a)



Vertical axis: diametre of the uterine artery (cm), horizontal axis: days post-inoculation (D0 = day of inoculation).

(b)



Vertical axis: blood flow volume (mL min.$^{-1}$), horizontal axis: days post-inoculation (D0 = day of inoculation).

Contrast-enhanced examination

No adverse effects were observed clinically in any animal after administration of the contrast agent.

The dose administered allowed clear imaging of the structures in all cases. In healthy ewes, CEUS examination revealed a steady biphasic pattern of contrast agent kinetics, characterised by initial uptake (wash-in phase) within 70 s post-injection, at which time intensity peaked with strong enhancement (80 - 100 AEU for caruncles and 100 - 120 AEU for inter-caruncular areas), followed by a gradual wash-out phase (Figure 10). In contrast, in the uterus of group I ewes, the pattern showed the enhancement being weak in the caruncles (< 50 AEU; $P < 0.001$), but similar to that in group C in the inter-caruncular area (100 - 120 AEU; $P = 0.47$) (Table 6).

Enhancement and clearance were evident initially in the caruncular tissue. Enhancement in the inter-caruncular area revealed an irregular pattern; it started with a delay, but lasted longer than in caruncles. Enhancement allowed clear visualisation of the entire caruncles in the control ewes; in inoculated ewes, caruncles could be visualised only partially (Figure 11).

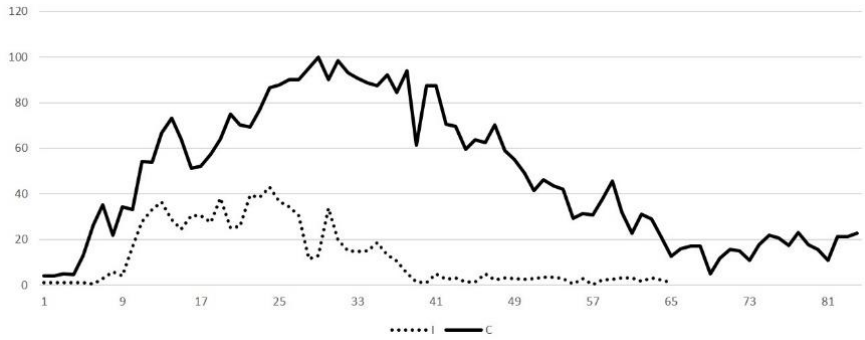
Table 6. Quantitative results (median) of contrast-enhanced ultrasonographic examination of the uterus of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

	Region of interest	
Ultrasonographic parametre	Caruncular tissue	Inter-caruncular area
Group I		
Peak enhancement (AEU)	42.934[a,b]	118.359[b]
Time to peak (s)	48	52
Time to wash-out (s)	78	128
Total enhancement time (s)	64	122
Wash-in time (s)	34	46
Wash-out time (s)	30	76
Group C		
Peak enhancement (AEU)	99.947[a,b]	118.565[b]
Time to peak (s)	58	70
Time to wash-out (s)	138	164
Total enhancement time (s)	130	150
Wash-in time (s)	50	56
Wash-out time (s)	80	94

a: same superscripts within a column indicate significant difference between I and C groups ($P < 0.001$) – b: same superscripts within a row indicate significant difference between the two tissues imaged ($P < 0.01$).

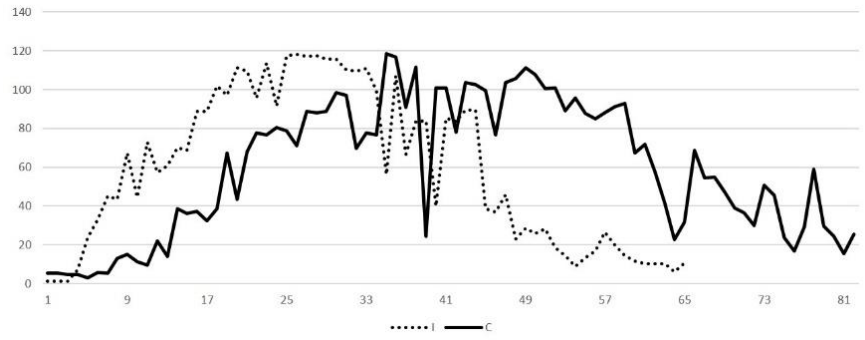
Figure 10. Patterns of image enhancement by contrast-enhanced ultrasonographic examination in the uterus of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C) on D1; (a) caruncular tissue, (b) inter-caruncular area.

(a)



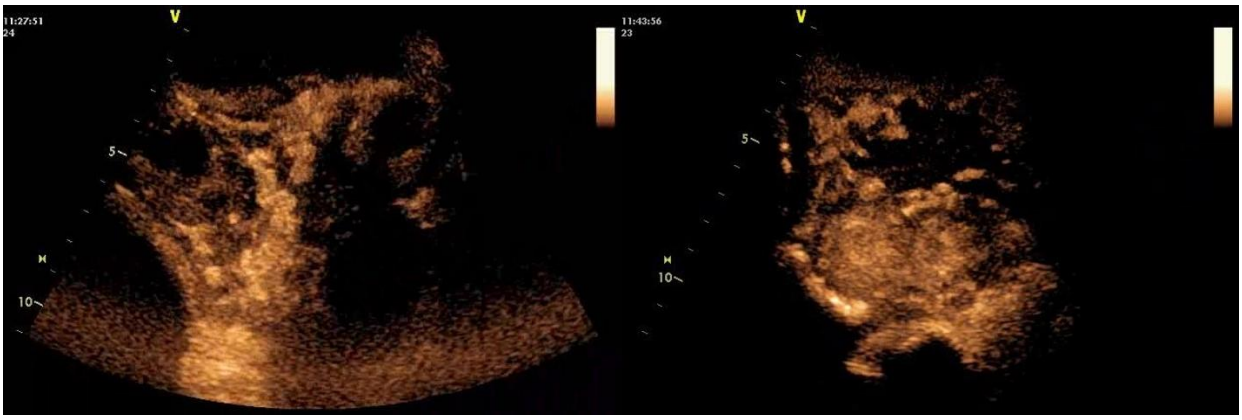
Vertical axis: image enhancement (AEU), horizontal axis: time after administration of the contrast agent.

(b)



Vertical axis: image enhancement (AEU), horizontal axis: time after administration of the contrast agent.

Figure 11. Contrast-enhanced ultrasonographic presentation of uterine caruncles; image taken 1 day after inoculation.



Left: weak and partial imaging of uterine caruncles of a ewe with experimentally induced uterine infection, with reduced enhancement in 48 s – right: full imaging of uterine caruncles of an uninoculated control ewe, with peak enhancement in 58 s (images taken and processed on a Vivid-I ultrasonography system with convex transducer, imaging frequency: 2.0 / 4.0 MHz - mechanical index: 0.09 - power: 22dB – scanning depth: 70 mm – contrast agent: 40 μL sulphur hexafluoride in microbubbles).

Bacteriological findings

Vaginal swab samples

In group I, *E. coli* was isolated from all ewes (1.000, 95% CI: 0.723 – 1.000) after challenge. Median duration of infection was 19.5 (1.5 – 62) days. Recurrence of infection was noted in 6 ewes (0.600, 95% CI: 0.313 – 0.832). *E. coli* was isolated in 109 samplings (0.681). In 29 samplings (0.181) other bacteria (staphylococci, streptococci, *T. pyogenes*, Enterobacteriaceae) were isolated, in 12 of these (0.414) in mixed culture with *E. coli*. In total, 142 bacterial isolates were recovered (0.888 isolates per sampling).

In C group, bacteria (*E. coli* or other organisms) were isolated from all ewes (1.000; 95% 0.758 – 1.000) at least once. Median duration of infection was 14 (1.25 – 62) days ($P < 0.001$ when compared with group I). Recurrence of infection was noted in 8 ewes (0.800, 95% CI: 0.391 – 0.862) ($P = 0.55$ when compared with group I). *E. coli* was isolated in 96 samplings (0.500), i.e. less frequently than from I ewes ($P < 0.001$). Other bacteria were isolated in 69 samplings (0.359), i.e. more frequently than from I ewes ($P < 0.001$), in 25 of these (0.362) in

mixed culture with *E. coli*. In total, 173 bacterial isolates were recovered (0.901 isolates per sampling) ($P = 0.34$ when compared with group I).

In group I, frequency of *E. coli* isolation was significantly higher in S_2 and S_3 than in S_4 ($P < 0.001$). In contrast, no such difference was seen between stages in group C ($P > 0.13$). Details are in Table 7.

Table 7. Frequency of isolation of bacteria from anterior vagina swab samples of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

	Stage post-partum[1]			
Group	S_1	S_2	S_3	S_4
Isolation of any bacterial species				
I	3 / 24[2]	50 / 60	28 / 30	48 / 70
C	3 / 24	56 / 72	28 / 36	56 / 84
Isolation of *E. coli*				
I	2 / 24	48 / 60	27 / 30	34 / 70
C	2 / 24	39 / 72	20 / 36	37 / 84

1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).
2. m / n = samples that yielded bacteria / total number of samples collected.

Uterine tissue samples

In group I, *E. coli* was recovered twice, on the first sampling of the respective animals (0.182), one on D7 and one on D14. In group C, no bacteria were recovered from any uterine tissue sample (0.000) ($P = 0.43$).

Cytological findings

In vaginal swab samples from all ewes, neutrophils were the predominant leucocyte type observed therein (Table 8). There was a significantly lower proportion of neutrophils in group I (80.5% ± 2.7%) than in group C (91.0% ± 1.8%) ewes; proportion of lymphocytes in group I (7.3% ± 1.3%) increased immediately after challenge and was higher than in group C

(3.8% ± 1.0%) (for all measurements taken after challenge, $P = 0.003$ and 0.034, respectively) (Table 8). Further, the progressive changes throughout the study in the proportions of leucocytes (neutrophil reduction, lymphocyte increase) were also significant in both groups ($P < 0.03$).

Haematological findings

Details of haematological findings are in Table 9. The only parametre in which there was a significant difference between group I and C ewes for all measurements after challenge, was eosinophil counts: 204 ± 10 cells mL^{-1} *versus* 429 ± 34 cells mL^{-1}, respectively ($P = 0.003$). For the other parametres measured, no significant differences were evident ($P > 0.06$).

Table 8. Proportions (%) of leucocyte types observed in vaginal swab samples of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Leucocyte types	Neutrophils	I	$98.7 \pm 0.9^{m,n,o}$	$94.0 \pm 1.5^{c,m,p}$	$87.0 \pm 4.6^{a,n,p,q}$	$66.7 \pm 5.0^{a,o,p,q}$
		C	$98.6 \pm 0.7^{m,n}$	$98.6 \pm 0.3^{c,o,p}$	$95.9 \pm 1.0^{a,m,o,q}$	$79.6 \pm 4.6^{a,n,p,q}$
	Lymphocytes	I	$1.3 \pm 1.0a^{m,n,o}$	$5.3 \pm 1.5^{c,m}$	9.1 ± 3.6^{n}	8.2 ± 2.1^{o}
		C	$0.7 \pm 0.4^{m,n}$	$1.0 \pm 0.2^{c,o,p}$	$3.5 \pm 1.0^{m,o}$	$7.2 \pm 2.6^{n,p}$
	Macrophages	I	$0.0 \pm 0.0^{a,m}$	0.8 ± 0.4^{m}	0.5 ± 0.3	0.8 ± 0.7
		C	0.7 ± 0.4^{a}	0.4 ± 0.1	0.6 ± 0.3	0.8 ± 0.4

Group

a, b: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.025 < P < 0.05$, b: $0.01 < P < 0.025$, c: $P \leq 0.01$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.03$).

1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

Table 9. Haematological results (mean ± standard error of the mean) in samples of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

	Stage post-partum[1]			
Parametre	S_1	S_2	S_3	S_4
Erythrocyte counts ($\times 10^6$ cells μL^{-1})				
I	9.02 ± 0.29	8.98 ± 0.18^{c}	9.00 ± 0.26	8.91 ± 0.16^{c}
C	8.58 ± 0.35	8.38 ± 0.18^{c}	8.62 ± 0.26	8.16 ± 0.14^{c}
Haematocrit (%)				
I	30.5 ± 1.0	30.2 ± 0.6	30.0 ± 0.9	29.1 ± 0.5^{c}
C	30.1 ± 1.2^{m}	29.5 ± 0.5^{n}	29.7 ± 0.7^{o}	27.5 ± 0.4^{c,m,n,o}
Haemoglobin concentration (g dL^{-1})				
I	9.94 ± 0.29	9.97 ± 0.16^{m}	9.85 ± 0.22	9.61 ± 0.14^{c,m}
C	9.73 ± 0.38^{m}	9.63 ± 0.18^{n}	9.61 ± 0.26^{o}	8.92 ± 0.13^{c,m,n,o}
Mean corpuscular volume (fL)				
I	34.0 ± 0.8	33.8 ± 0.4^{c,m}	33.4 ± 0.5	32.7 ± 0.3^{c,m}
C	35.2 ± 0.6^{m}	35.4 ± 0.3^{c,n}	34.6 ± 0.5	34.0 ± 0.3^{c,m,n}
Mean corpuscular haemoglobin concentration (g dL^{-1})				
I	32.9 ± 0.8	33.3 ± 0.4	33.2 ± 0.5	33.3 ± 0.3^{c}
C	32.4 ± 0.2	32.7 ± 0.1	32.4 ± 0.2	32.4 ± 0.2^{c}
Total leucocyte counts (cells μL^{-1})				
I	12696 ± 1126^{m,n,o}	10200 ± 421^{m}	10512 ± 445^{n}	10247 ± 279^{o}
C	12901 ± 856^{m,n,o}	10938 ± 411^{m,p}	10226 ± 404^{n}	9886 ± 288^{o,p}
Neutrophil counts (cells μL^{-1})				
I	7412 ± 1039^{m,n,o}	4648 ± 410^{c,m,p}	4596 ± 278^{n,q}	3760 ± 175^{o,p,q}
C	8275 ± 692^{m,n,o}	5867 ± 303^{c,m,p,q}	4649 ± 270^{n,p,r}	4031 ± 153^{o,q,r}
Lymphocyte counts (cells μL^{-1})				
I	4382 ± 361^{m,n}	4773 ± 194^{b,o,p}	5376 ± 269^{b,m,o,p}	5820 ± 169^{c,n,p,q}
C	3918 ± 224^{m,n}	4227 ± 136^{b,o,p}	4746 ± 244^{b,m,o}	4860 ± 146^{c,n,p}
Monocyte counts (cells μL^{-1})				
I	170 ± 31^{c,m,n}	237 ± 23^{c,m}	191 ± 26^{c}	240 ± 16^{a,n}
C	291 ± 38^{c}	354 ±21^{c,m}	374 ± 34^{c,n}	293 ± 22^{a,m,n}
Eosinophil counts (cells μL^{-1})				
I	271 ± 42^{m}	200 ± 13^{c}	188 ± 27^{c,m}	213 ± 17^{c}
C	270 ± 42^{m}	338 ± 22^{c,n}	301 ± 34^{c,o}	547 ± 68^{c,m,n,o}
Basophil counts (cells μL^{-1})				
I	77 ± 8^{m}	82 ± 4^{n}	90 ± 9^{o}	111 ± 6^{m,n,o}
C	76 ± 7^{m}	86 ± 3^{n}	83 ± 5^{o}	103 ± 7^{m,n,o}

Table 9 (continued).

	Stage post-partum[1]			
Parametre	S_1	S_2	S_3	S_4
Thrombocyte counts ($\times 10^3$ cells μL^{-1})				
I	$509 \pm 19^{c,m,n,o}$	$552 \pm 14^{c,m,p}$	$668 \pm 25^{c,n,p,q}$	$574 \pm 16^{c,o,q}$
C	$669 \pm 49^{c,m}$	747 ± 28^{c}	$826 \pm 47^{c,m,n}$	$727 \pm 31^{c,n}$

a - c: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.025 < P \leq 0.050$, b: $0.01 < P \leq 0.025$, c: $P \leq 0.01$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.045$).

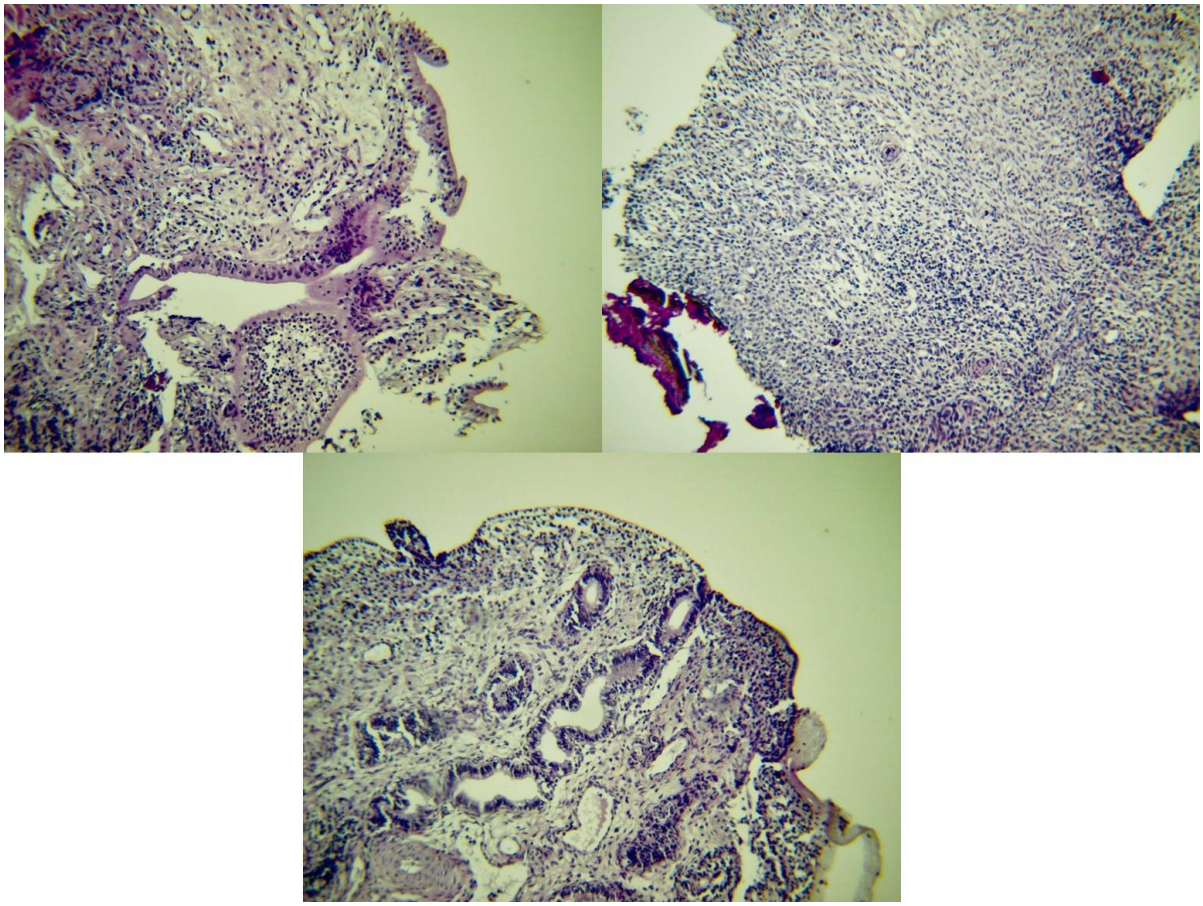
1. S_1: included samples collected before inoculation, on L0 and L1, S_2: included samples collected after challenge up to and including D4, S_3: included samples collected from D6 to D12 and S_4: included samples collected from D17 to D62 (D0: day of inoculation, which took place on the 1st day post-partum).

Histological findings

In group I ewes (Figure 12), in samples collected on D7, the epithelium of the endometrium had a loose structure, with high cylindrical-like cells, which contained vacuoles. There was intense vascularisation with lymphocytic infiltration, which occurred mainly subepithelially and occasionally around the uterine glands. These could be easily observed. The myometrium appeared to have lost its fine structrure; hyperaemia and extravasation were evident therein and it also had a loose appearance. On D14, the epithelium of the endometrium appeared destroyed with massive neutrophilic and lymphocytic infiltration; there was intense vascularisation and extravasation. There was also increased presence of lymphocytes in the myometrium. On D24 and D27, the epithelium of the endometrium included cylindrical cells, with several small glands with small diametre being clustered in groups; the epithelium of the glands was cuboidal. There was intense presence of lymphocytes in the endometrium, whilst no inflammatory cells were observed in the myometrium, which included intense vascularisation and extravasation. On D34, in the sample from one ewe the epithelium of the endometrium was characterised by single-layered cells, whilst in the sample from the second animal no epithelium could be observed. The uterine glands were collapsed and had fully regressed. The myometrium was thickened; no cellular infiltration could be observed therein in the sample from one ewe, whilst in the sample from the second animal there was evidence of massive cellular infiltration. On D42 and D44, there were single-layered cells on the epithelium of the

endometrium, but this was not absolutely intact, as there were areas in the samples that no epithelium could be seen. Cellular infiltration in the myometrium was still evident. On D62, the border between the endometrium and the myometrium could not be distinguished; there were leucocytes and mild vascularisation therein.

Figure 12. Post-partum histological pictures of the uterus of ewes with experimentally induced uterine infection (group I).



Top left: 7 days after inoculation (section of the endometrium, with cylindrical-like cells in the epithelium; occasionally, epithelium completely detached from the endometrium; presence of intense subepithelial infiltration primarily by lymphocytes with a few neutrophils as well), top right: 27 days after inoculation (section of the endometrium, with intense infiltration by lymphocytes), bottom centre: 42 days after inoculation (section of the endometrium, with cyboidal single-layered epithelium in almost the entire length of the endometrium; subepithelial infiltration primarily by lymphocytes; presence of vessels and uterine glands with cylindrical single-layered epithelium) (H & E, 200×).

In group C ewes, in samples collected on D7 and D14, the epithelium of the endometrium was intact, with cuboidal- to cylindrical-like cells; these contained vacuoles. There was clear hyperaemia with small-degree neutrophilic infiltration. The uterine glands

were observed easily and had also cuboidal- to cylindrical-like epithelial cells. The myometrium included trophoblast-like cells and vacuoles. On D24 and D27, the epithelium was still intact, but height of epithelial cells was decreased. The uterine glands had a smaller size compared to previous samples. Progressively, the thickness of the myometrium reduced. On D34, the epithelium of the endometrium was characterised by single-layered cells. The uterine glands were collapsed and had fully regressed. Leucocytic infiltration was characterised by presence of lymphocytes. On D42 and D44, there were single-layered cells on the epithelium of the endometrium, still with lymphocytic infiltration. Finally, on D62, the border between the endometrium and the myometrium could not be distinguished and the entire uterine wall was observed as one entity.

Median score for total number of leucocytes in samples from I ewes was higher than in C ewes: 4 *versus* 3 ($P = 0.014$); differences between I and C ewes in median scores for neutrophils and lymphocytes, when these were considered separately, were not significant ($P = 0.50$ and 0.38, respectively). Lymphocytes always predominated in uterine tissue samples, independently of sampling point or group.

Subsequent reproductive performance

During the subsequent reproductive (breeding) season, all ewes in group I and group C were mated and diagnosed to be pregnant by using ultrasonographic examination. Finally, all ewes lambed normally and produced healthy and viable lambs. There were no significant differences between groups I and C in any reproductive performance parametre ($P > 0.17$) (Table 10).

Table 10. Reproductive performance, during the subsequent reproductive season, of ewes with experimentally induced uterine infection (group I) or of uninoculated controls (group C).

	Group	
Measure of reproductive performance	I	C
Mating rate	100%	100%
Pregnancy rate	100%	100%
Abortion rate	0%	0%
Lambing rate	100%	100%
Total lambs per ewe	1.90	2.00
Stillbirth rate	0%	0%

CHAPTER III

UTERINE INVOLUTION IN EWES THAT HAD DEVELOPED PREGNANCY TOXAEMIA DURING THE PRECEDING GESTATION AND CONSEQUENCES IN SUBSEQUENT REPRODUCTIVE PERFORMANCE

OBJECTIVES

The objectives of this work were:

- (a) the study of the characteristics of uterine involution in ewes that had developed pregnancy toxaemia during the preceding gestation and
- (b) the evaluation of the subsequent reproductive performance of such ewes.

MATERIALS AND METHODS

Experimental design

In total, 21 Lacaune-cross ewes (age 3 – 5 years) were included in the study (group A, n = 12; group C, n = 9). In ewes in group A, an experimental model that has been described to lead in pregnancy toxaemia (Barbagianni et al. 2015a), was followed. The model was based in providing to animals a feed with reduced energy content during the last one-and-a-half months of pregnancy; the feed fulfilled satiation requirements of animals, hence not affecting their welfare status. Ewes in group C were provided a commercial concentrate feed and were used as controls. Detailed descriptions (composition and nutrient contents), as well as details of the administration regime of the feeds have been presented by Barbagianni et al. (2015a).

Among ewes in group A, those that developed pregnancy toxaemia were allocated in subgroup A1 (n = 7). Pregnancy toxaemia was diagnosed by detecting increased concentrations of β-hydroxybutyrate in blood samples, measured by the quick test Precision Xceed® (Abbott Laboratories, Abbott Park, Illinois, USA) (Panousis et al. 2012); the mean value of all measurements in these ewes after the 124th day of pregnancy was 2.36 ± 0.15 mmol L^{-1}, with range from 1.47 to 3.80 mmol L^{-1}. Ewes in group A in which no increased concentrations of β-hydroxybutyrate were detected, were allocated to subgroup A2 (n = 5); the mean value for all β-hydroxybutyrate measurements in these ewes was 0.89 ± 0.04 mmol L^{-1}, with range from 0.70 to 1.18 mmol L^{-1}. In the ewes of group C, the mean value for β-hydroxybutyrate measurements was 0.75 ± 0.02 mmol L^{-1}, with range from 0.65 to 0.95 mmol L^{-1} ($P < 0.002$ for all comparisons between groups).

Conditions prescribed by legislation of the European Union in relation to animal experimentation procedures (Council Directive 86/809/EEC) were met during this work.

Post-partum examinations and sample collections

Lambings took place in February to March, which was outside the annual breeding season in the area (latitude: N 39.37 °), where the study was conducted. All ewes produced twin lambs.

Detailed examinations of all ewes and sample collections were performed at regular intervals throughout the study. Samples were also collected 6 to 12 h after lambing (L0 = day of lambing) and at regular intervals thereafter (L1, L2, L4, L7, L10, L15, L20, L25, L30, L40, L50, L60).

On each of the above sampling points, initially, a detailed clinical examination of the genital tract was performed (details in Chapter II, Materials and Methods).

Samples of vaginal discharge were also collected (details in Chapter II, Materials and Methods).

Ultrasonographic examination of the genital tract was performed on the above occasions, bar the contrast-enhanced ultrasonographic examination (details in Chapter II, Materials and Methods).

Uterine biopsies were performed laparoscopically for tissue sample collection from the uterus of the experimental ewes (details in Chapter II, Materials and Methods). These were performed on L10 (left horn) and L30 (right horn) (no. of ewes from subgroup A1: n = 3, from subgroup A2: n = 3, from group C: n = 2) or on L20 (left horn) and L40 (right horn), respectively (no. of ewes from subgroup A1: n = 4, from subgroup A2: n = 2: from group C: n = 2).

Laboratory examinations

Vaginal swab samples were processed for bacteriological and cytological examination (details in Chapter II, Materials and Methods).

Uterine tissue samples were processed for bacteriological and histological examination (details in Chapter II, Materials and Methods)

Evaluation of subsequent reproductive performance

Two months after the end of the monitoring period and after the reproductive season had started (i.e., in June), ewes were put with rams of known fertility (n = 2) for mating. Rams were left with ewes for 60 days. Ultrasonographic examinations were performed for pregnancy diagnosis. Throughout that period, during the pre-conception period and

gestation, appropriate health management of ewes had been performed as recommended (Fthenakis et al. 2012); all ewes were maintained under the same conditions. Blood samples were periodically collected after the 124th day of pregnancy for measurement of β-hydroxybutyrate. Finally, lambings and number of lambs born from each ewe were recorded.

Data management and analysis

Post-partum stages

The post-partum period was divided in four stages: S_1 included samples collected on L0 and L1 (2 sampling points), S_2 included samples collected from L2 to L7 (3 sampling points), S_3 included samples collected from L10 to L25 (4 points) and S_4 included samples collected from L30 to L60 (4 points).

B-mode ultrasonographic measurements

Initially, stored ultrasonographic images were viewed for description. Then, images of (a) caruncular tissue and (b) inter-caruncular tissue obtained from each ewe on each occasion, were processed by means of ImageJ software. For analysis of results of grey-scale measurements, data were normalised by calculating the ratio GS_{CT} / GS_{IT}. Measurements of dimensions of uterine structures were performed in images taken by the linear transducer and were calculated by the equipment's software. The diameter of caruncles and of the uterine lumen, as well as the thickness of the myometrium and the endometrium were calculated. Reduction of dimensions of uterine structures (R_s) within a stage (S_n) compared to those in the preceding stage (S_{n-1}) was also calculated (for all the above, details in Chapter II, Materials and Methods).

Doppler mode ultrasonographic measurements

Stored images of cross-sections and spectral waveforms of the uterine artery were processed by means of MyLab® software (details in Chapter II, Materials and Methods). In total, eight haemodynamic parametres in that vessel were calculated (details in Chapter II, Materials and Methods).

Modelling for analysis of infection results

A model was employed for analysis of infection results (details in Chapter II, Materials and Methods).

Evaluation of cellular infiltration

Cellular infiltration in vaginal swab and uterine tissue samples was assessed by means of semi-quantititave methods (details in Chapter II, Materials and Methods).

Measures for reproductive performance

In total, six measures of reproductive performance were calculated (details in Chapter II, Materials and Methods).

Statistical analysis

Data were entered into Microsoft Excel for analysis. Basic descriptive analysis was performed. The outcomes of interest were considered.

Comparisons of frequencies of clinical signs and bacterial isolations for A1 *versus* A2 and A *versus* C were performed in a table of cross-categorised frequency data by use of Pearson chi-square test or Fisher-exact test, as appropriate. Time of first appearance of an outcome and duration of outcome under evaluation were compared between groups as

above by means of Mann-Whitney test. The Kruskal-Wallis and the Mann-Whitney tests were used to evaluate differences in bacterial isolations between post-partum stages.

For ultrasonographic measurements, repeated measures mixed effect linear regression models were used to determine whether outcomes changed over the course of the study. Fixed effect was the time-point of the study (i.e., L0, L1, etc.). Effect of experimental subjects (animals) was included as random effect in the model. Models were adjusted for repeated measures within animals. Initially, separate analyses were performed for each stage post-lambing (S_1 – S_4), which were followed by an analysis that took into account all the measurements (13 time-points) carried out.

The Mann-Whitney test was used to evaluate differences between groups in scores for average leucocyte numbers. Further, these scores were compared by the Friedman Test using each sampling day's score as the unit and with group as 'treatment' and day number as 'block'.

Results for reproductive performance were evaluated using comparison of proportions or the Mann-Whitney test, as appropriate per type of result.

In all cases, level of significance was set at $P = 0.05$.

RESULTS

Clinical findings

In two A1 ewes (0.286), systemic clinical signs (recumbence, selective appetite, increased [> 40.5 ^{o}C] rectal temperature) were recorded on L0. No systemic clinical findings were observed in A2 ewes (P = 0.32 for A1 *versus* A2). In all A1 ewes (1.000), genital clinical signs (presence of malodorous, thick, purulent, yellow- to brown- to black-coloured vaginal discharge [n = 7], vaginal hyperaemia and vulval oedema [n = 7], retention of foetal membranes [n = 1]) were evident; median duration of genital clinical signs was 27.5 days. Also in all A2 ewes (1.000), genital clinical signs (vaginal discharge as above [n = 5], vaginal hyperaemia and vulval oedema [n = 5], retention of foetal membranes [n = 1]) were evident (P = 1.00 for A1 *versus* A2); median duration of genital clinical signs was 22.5 days (P = 0.48 for A1 *versus* A2). Recurrence of local clinical signs occurred in one A1 and one A2 ewes (P = 0.68 for A1 *versus* A2).

Systemic clinical signs were not observed in any group C ewe (0.000) (P = 0.31 for A versus C). In 2 ewes (0.222), there was transient presence of malodorous vaginal discharge; the median duration of the genital clinical signs was 10 days. No recurrence of the genital clinical signs occurred in these ewes.

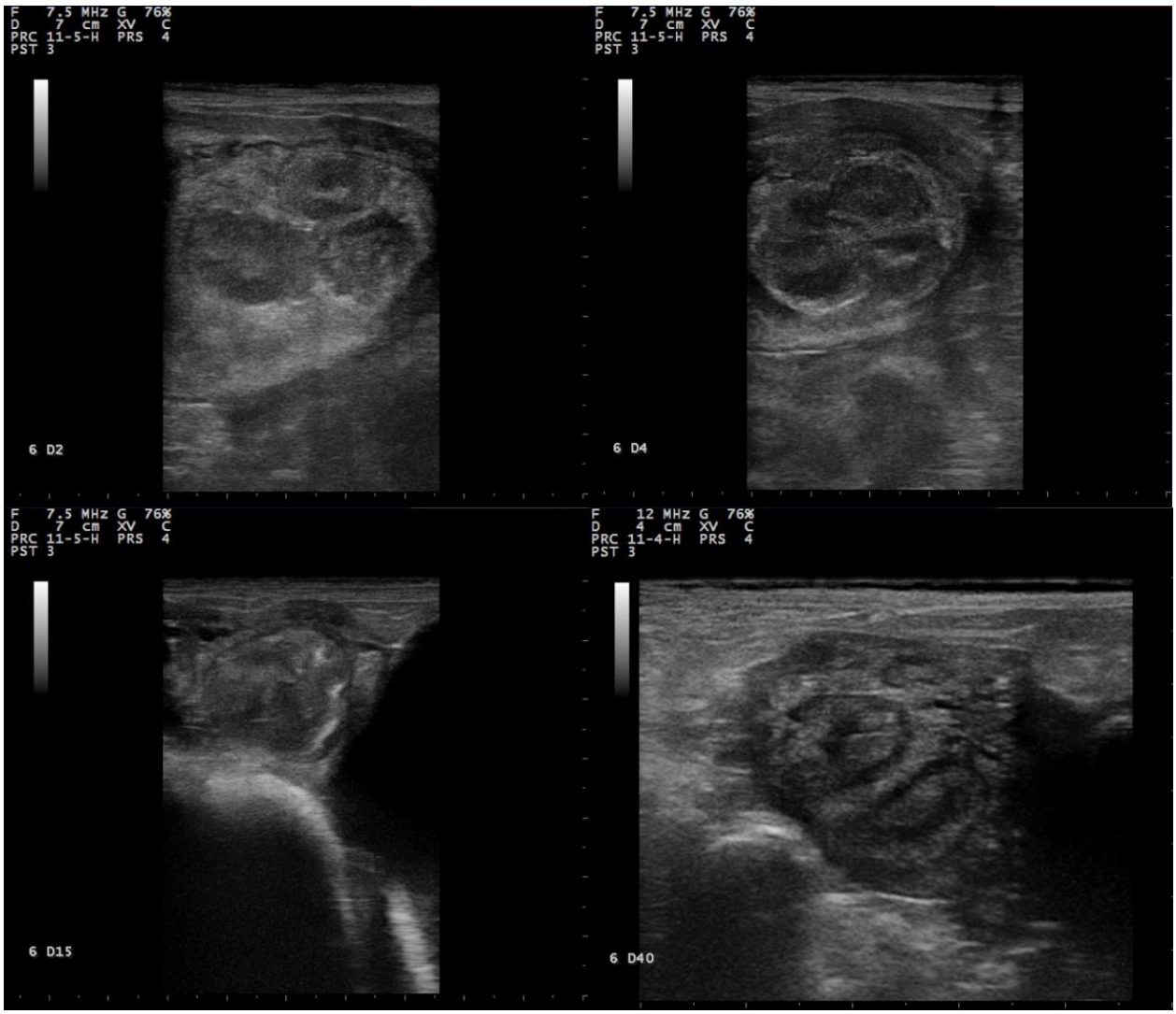
Ultrasonographic findings

B-mode examination

In A group ewes, the uterus could be easily imaged during the first week post-partum, with the endometrium appearing more hyperechoic than in healthy control ewes. The layers of the uterine wall appeared thickened and particularly echogenic until the 15th day post-partum. The content of the uterine lumen was imaged with hyperechoic foci (possibly due to the presence of fluid and cellular components in most ewes until the 7th to 15th day post-partum). Progressively, the uterine content was imaged with inconsistent and blurred structures of increased or mixed echogenicity; the uterine content could not be imaged beyond the 15th day post-partum. Immediately post-partum, the caruncles were less

echogenic than the endometrium, but differences decreased during the first week post-partum (Figure 13).

Figure 13. Sequential post-partum B-mode ultrasonographic presentation of the uterus of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had developed pregnancy toxaemia (subgroup A1) during the preceding gestation.



Top left: 2 days, top right: 4 days, bottom left: 15 days, bottom right: 40 days after lambing; caruncles and uterine wall layers are imaged (transverse sections obtained by transcutaneous examination at the inguinal area; image taken and processed on a MyLab® 30 ultrasonography system with linear transducer, imaging frequency: 7.5 – 12.0 MHz - scanning depth: 40 - 70 mm).

In group C, layers in the uterine wall were clearly imaged up to the 30th day post-partum: the perimetrium and the outer layer of the myometrium were highly echogenic, the middle layer of the myometrium was anechoic to hypoechoic, like a dark rim, the inner

layer of the myometrium and the endometrium appeared as echogenic structures. The uterine lumen was seen irregularly shaped. After the tenth day post-partum, the organ appeared circular or elliptical (longitudinal sections) or polygonal to elliptical, progressively changing to circular (transverse sections). Caruncles were seen on the endometrium, often appearing in mushroom-like shape. In general, they were echogenically similar to the endometrium and its folds (Table 11); differences in their echogenity were evident: the most superficial layer was hyperechoic, with the deeper tissue being less echoic; caruncles were last observed on the 20th day post-partum. There was echogenic content within the anechoic lumen. The uterine body bifurcation and the horns could be imaged up to 30th day post-partum. The myometrium and the endometrium were imaged as clearly separate layers until the 30th day post-partum. Subsequently, the organ was imaged as a round entity near the bladder, as its size decreased. The echopattern of the uterine wall was finely textured, homogeneous and hypoechoic to moderately echogenic (Figure 14).

The GS_{CT} / GS_{IT} ratio was significantly greater in group C than in group A ewes during S_1 ($P = 0.007$), but not in subsequent stages ($P > 0.15$) (Table 11).

Table 11. Post-partum echogenicity: ratio GS_{CT} / GS_{IT}[1] (mean ± standard error of the mean) in the uterus of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

		Stage post-partum[2,3]		
		S_1	S_2	S_3
Group	A1	$0.70 \pm 0.05^{b,m,n}$	0.90 ± 0.06^{m}	0.93 ± 0.04^{n}
	A2	$0.77 \pm 0.07^{b,m}$	0.92 ± 0.05^{m}	1.02 ± 0.17
	C	$0.85 \pm 0.03^{b,m,n}$	0.96 ± 0.03^{m}	1.00 ± 0.06^{n}

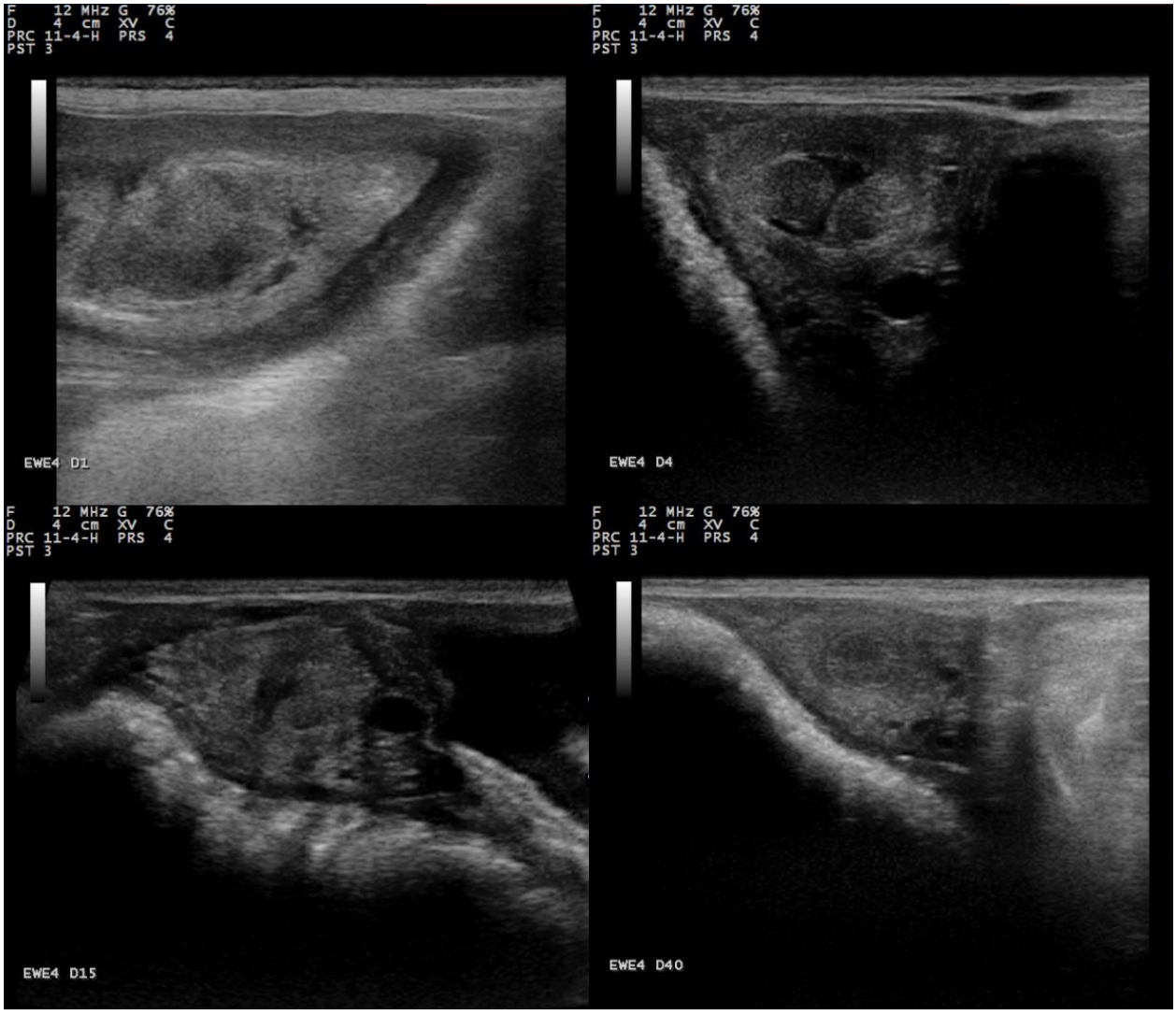
a - b: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.020 < P < 0.035$, b: $0.001 < P \leq 0.020$) – m - n: same superscripts within a row indicate significant difference between stages ($P < 0.035$).

1. GS_{CT}: Grey-scale of caruncular tissue, GS_{IT}: Grey-scale of inter-caruncular tissue

2. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum.

3. no data are presented for S_4, because no measurements could be taken.

Figure 14. Sequential post-partum B-mode ultrasonographic presentation of the uterus of normally involuting ewes (group C).



Top left: 1 day, top right: 4 days, bottom left: 15 days, bottom right: 40 days after lambing; caruncles and uterine wall layers are imaged (longitudinal sections obtained by transcutaneous examination at the inguinal; image taken and processed on a MyLab® 30 ultrasonography system with linear transducer, imaging frequency: 12.0 MHz - scanning depth: 40 mm).

Immediately after lambing (stage S_1), there were significant differences between A and C ewes in the caruncular diametre (1.48 and 1.35 cm, respectively; P = 0.015), the endometrium thickness (1.17 and 0.65 cm, respectively; P = 0.021) and the lumen diametre (0.83 and 0.67 cm, respectively; P = 0.034) (Table 12). Progressively, in all ewes, there was a reduction of dimensions of above structures as the post-partum period advanced. Particular reduction of dimensions occurred during S_2 (i.e., during the first week after lambing), present in all ewes in the study: mean daily reduction of dimensions of the uterine

structures varied from 1.1% to 6.6%; subsequently, during S_3 and S_4, this was 0.4% to 4.2% and 0.3% to 1.4%, respectively (Table 13). Further, mean daily reduction of the caruncular diametre (during S_3) and the myometrium thickness (during S_2) was smaller in A than in C ewes ($P < 0.02$) (Table 13). Throughout the study period, the overall reduction (from S_1 to S_4) in myometrium thickness was 42.8% and 52.9% for A and C ewes, respectively ($P = 0.018$). No significant differences were found for the overall reduction in other structures.

Table 12. Post-partum ultrasonographically measured dimensions (mean ± standard error of the mean) of the uterine structures of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Uterine dimensions	diametre of caruncles (cm)	A1	$1.50 \pm 0.06^{b,m}$	$1.37 \pm 0.06^{c,n}$	$1.16 \pm 0.07^{c,m,n}$	-[2]
		A2	$1.46 \pm 0.02^{b,m,n}$	$1.39 \pm 0.05^{c,n}$	$1.14 \pm 0.07^{c,m,n}$	-
		C	$1.35 \pm 0.05^{b,m,n}$	$1.16 \pm 0.04^{c,m,o}$	$0.83 \pm 0.04^{c,n,o}$	-
	myometrial thickness (cm)	A1	$0.50 \pm 0.03^{m,n}$	$0.49 \pm 0.02^{c,o,p}$	$0.38 \pm 0.02^{c,m,o,q}$	$0.27 \pm 0.02^{c,n,p,q}$
		A2	$0.52 \pm 0.03^{m,n}$	$0.49 \pm 0.01^{c,o,p}$	$0.38 \pm 0.03^{c,m,o,q}$	$0.33 \pm 0.03^{c,n,p,q}$
		C	$0.49 \pm 0.02^{m,n,o}$	$0.40 \pm 0.01^{c,m,p,q}$	$0.30 \pm 0.01^{c,n,p,r}$	$0.23 \pm 0.01^{c,o,q,r}$
	endometrium thickness (cm)	A1	1.01 ± 0.03^{a}	$0.78 \pm 0.06^{c,m,n}$	$0.45 \pm 0.04^{a,m,o}$	$0.25 \pm 0.03^{b,n,o}$
		A2	$1.34 \pm 0.01^{a,m,n,o}$	$0.90 \pm 0.03^{c,m.p.q}$	$0.45 \pm 0.04^{a,n,p}$	$0.39 \pm 0.06^{b,o,q}$
		C	$0.65 \pm 0.03^{a,m,n}$	$0.53 \pm 0.03^{c,o,p}$	$0.40 \pm 0.02^{a,m,o,q}$	$0.26 \pm 0.01^{b,n,p,q}$
	diametre of uterine lumen (cm)	A1	$0.93 \pm 0.01^{a,m,n}$	$0.87 \pm 0.08^{c,o,p}$	$0.58 \pm 0.05^{c,m,o,q}$	$0.38 \pm 0.02^{c,n,p,q}$
		A2	0.74 ± 0.03^{a}	$0.72 \pm 0.09^{c,m}$	$0.59 \pm 0.04^{c,n}$	$0.37 \pm 0.02^{c,m,n}$
		C	$0.67 \pm 0.08^{a,m,n}$	$0.52 \pm 0.03^{c,o,p}$	$0.36 \pm 0.02^{c,m,o,q}$	$0.25 \pm 0.01^{c,n,p,q}$

a - c: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.020 < P < 0.035$, b: $0.001 < P \leq 0.020$, c: $P \leq 0.001$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.035$).

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.

2. no measurements could be taken during S_4.

Table 13. Mean daily reduction of the dimensions of the uterine structures, as calculated based on ultrasonographic measurements, of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

			Stage post-partum[1]		
			S_2	S_3	S_4
Uterine dimensions	diametre of caruncles (cm)	A1	0.02	< 0.01[a]	-
		A2	0.02	0.01[a]	-
		C	0.05	0.02[a]	-[2]
	myometrial thickness (cm)	A1	0.02[a]	0.01	0.01
		A2	0.01[a]	0.02	0.01
		C	0.04[a]	0.02	0.01
	endometrium thickness (cm)	A1	0.05	0.03	0.01
		A2	0.05	0.04	< 0.01
		C	0.03	0.03	0.01
	diametre of uterine lumen (cm)	A1	0.04	0.03	0.01
		A2	0.07	0.01	0.01
		C	0.05	0.02	0.01

Group

a: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups ($P < 0.02$).

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.

2. no measurements could be taken during S_4.

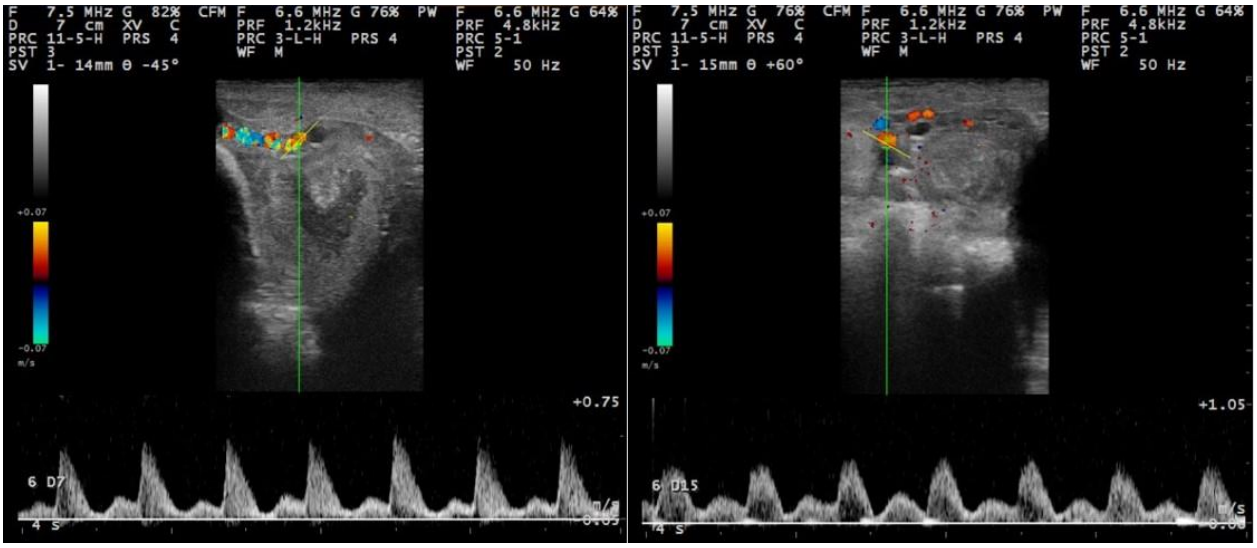
Doppler mode examination

The spectral display in Doppler mode was observed as a broad band structure (Figures 15 and 16). During the study period, in group A ewes, the changes in the haemodynamic parametres were significant for the vessel diametre, the pulsatility rate, the systolic acceleration and the blood flow volume ($P < 0.045$), but not for the other parametres ($P > 0.135$) (Figure 17, Table 14). Further, there were no differences in any parametre between subgroups A1 and A2 ($P > 0.055$). In group C ewes, the progressive changes in the haemodynamic parametres were significant ($P < 0.04$), bar for the systolic acceleration ($P > 0.12$).

There were differences for all parametres between groups C and A ($P < 0.017$) on S_2 and thereafter, bar for the mean velocity ($P = 0.061$). Post-partum, the blood flow volume

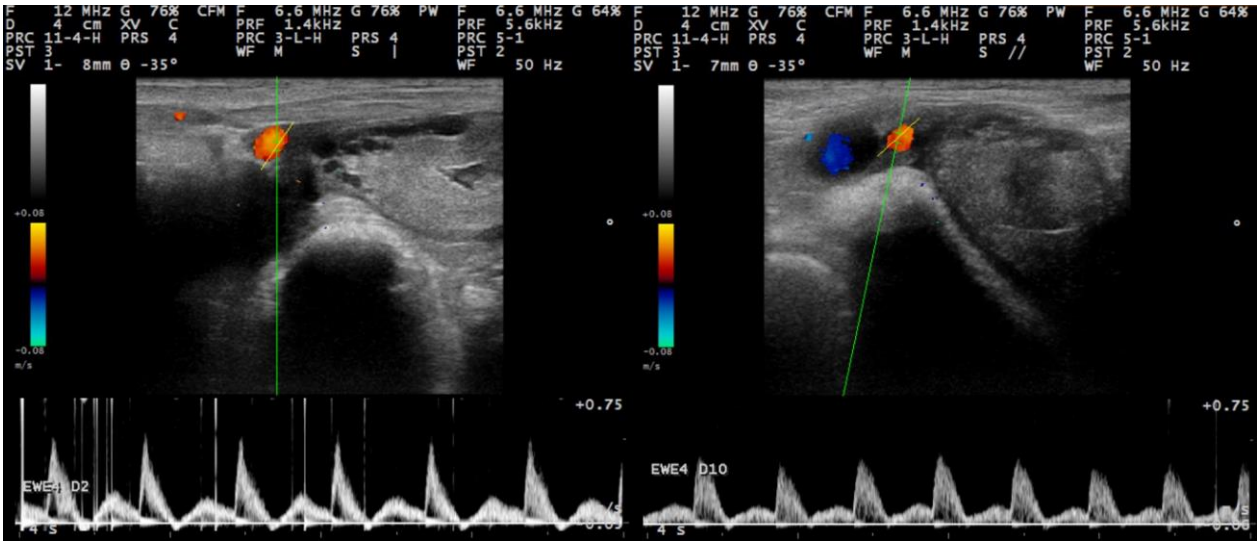
was significantly greater in ewes of group A (256.0 mL min.$^{-1}$ in S_1, 147.9 mL min.$^{-1}$ in S_2, 62.7 mL min.$^{-1}$ in S_3, 29.4 mL min.$^{-1}$ in S_4) than in ewes of group C (190.1 mL min.$^{-1}$, 65.5 mL min.$^{-1}$, 30.5 mL min.$^{-1}$, 13.8 mL min.$^{-1}$ in respective stages) ($P < 0.006$ in S_2 and thereafter) (Figures 15 – 17, Table 14).

Figure 15. Sequential post-partum spectral waveforms of the uterine artery (Doppler ultrasonography) of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had developed pregnancy toxaemia (subgroup A1) during the preceding gestation.



Left: 7 days, right: 15 days after lambing (image taken and processed on a MyLab® 30 ultrasonography system with linear transducer, Doppler imaging frequency: 6.6 MHz - scanning depth: 70 mm)

Figure 16. Sequential post-partum spectral waveforms of the uterine artery (Doppler ultrasonography) of normally involuting ewes (group C).



Left: 2 days, right: 10 days after lambing (image taken and processed on a MyLab® 30 ultrasonography system with linear transducer, Doppler imaging frequency: 6.6 MHz - scanning depth: 40 mm)

Table 14. Post-partum Doppler ultrasonographic measurements (mean ± standard error of the mean) in the uterine artery of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

Doppler ultrasonographic parametre	Group	Stage post-partum[1]			
		S_1	S_2	S_3	S_4
uterine artery diametre (cm)	A1	$0.5 \pm 0.0^{m,n,o}$	$0.4 \pm 0.0^{b,m,p,q}$	$0.3 \pm 0.0^{b,n,p,r}$	$0.2 \pm 0.0^{o,q,r}$
	A2	$0.5 \pm 0.0^{m,n,o}$	$0.4 \pm 0.0^{b,m,p,q}$	$0.3 \pm 0.0^{b,n,p,r}$	$0.2 \pm 0.0^{o,q,r}$
	C	$0.4 \pm 0.0^{m,n,o}$	$0.3 \pm 0.0^{b,m,p,q}$	$0.2 \pm 0.0^{b,n,p,r}$	$0.2 \pm 0.0^{o,q,r}$
resistance index	A1	0.8 ± 0.1	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}	0.8 ± 0.0^{b}
	A2	0.8 ± 0.0^{m}	0.8 ± 0.0^{b}	$0.8 \pm 0.0^{b,m}$	0.8 ± 0.0^{b}
	C	$0.8 \pm 0.0^{m,n,o}$	$0.9 \pm 0.0^{b,m}$	$0.9 \pm 0.0^{b,n}$	$0.9 \pm 0.0^{b,o}$
pulsatility index	A1	2.00 ± 0.2	2.1 ± 0.2^{b}	2.2 ± 0.2^{c}	2.1 ± 0.4^{b}
	A2	$1.9 \pm 0.2^{m,n}$	$2.6 \pm 0.3^{b,m}$	$2.5 \pm 0.2^{c,n}$	2.2 ± 0.5^{b}
	C	$1.8 \pm 0.1^{m,n}$	$2.8 \pm 0.2^{b,m}$	$2.9 \pm 0.2^{c,n}$	3.2 ± 0.2^{b}
systolic : diastolic velocity ratio	A1	6.6 ± 1.6	5.5 ± 0.6	6.7 ± 1.0^{b}	5.2 ± 1.0^{b}
	A2	$4.5 \pm 0.4^{m,n}$	8.9 ± 2.2^{m}	$6.8 \pm 0.8^{b,n}$	8.3 ± 3.9^{b}
	C	$4.2 \pm 0.3^{m,n,o}$	$8.5 \pm 0.9^{m,p}$	$9.9 \pm 1.0^{b,n}$	$15.6 \pm 4.0^{b,o,p}$
general pressure (mm Hg)	A1	1.0 ± 0.4	1.0 ± 0.2^{b}	0.9 ± 0.1^{b}	0.6 ± 0.2
	A2	0.9 ± 0.3	1.2 ± 0.2^{b}	0.9 ± 0.3^{b}	0.8 ± 0.4
	C	$0.9 \pm 0.1^{m,n,o}$	$0.6 \pm 0.1^{b,m,p,q}$	$0.5 \pm 0.0^{b,n,p,r}$	$0.4 \pm 0.0^{o,q,r}$
mean pressure (mm Hg)	A1	0.3 ± 0.1	0.2 ± 0.1^{b}	0.2 ± 0.0^{c}	0.2 ± 0.1^{b}
	A2	0.2 ± 0.1	0.2 ± 0.1^{b}	0.2 ± 0.0^{c}	0.2 ± 0.1^{b}
	C	$0.2 \pm 0.0^{m,n,o}$	$0.1 \pm 0.0^{b,m,p}$	$0.1 \pm 0.0^{c,n}$	$0.1 \pm 0.0^{b,o,p}$
mean velocity ($m\ s^{-1}$)	A1	0.7 ± 0.2	0.8 ± 0.1	0.8 ± 0.1^{a}	0.7 ± 0.1
	A2	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1^{a}	0.7 ± 0.1
	C	$0.6 \pm 0.0^{m,n}$	0.7 ± 0.0	$0.7 \pm 0.0^{a,m}$	0.7 ± 0.0^{n}

Table 14 (continued).

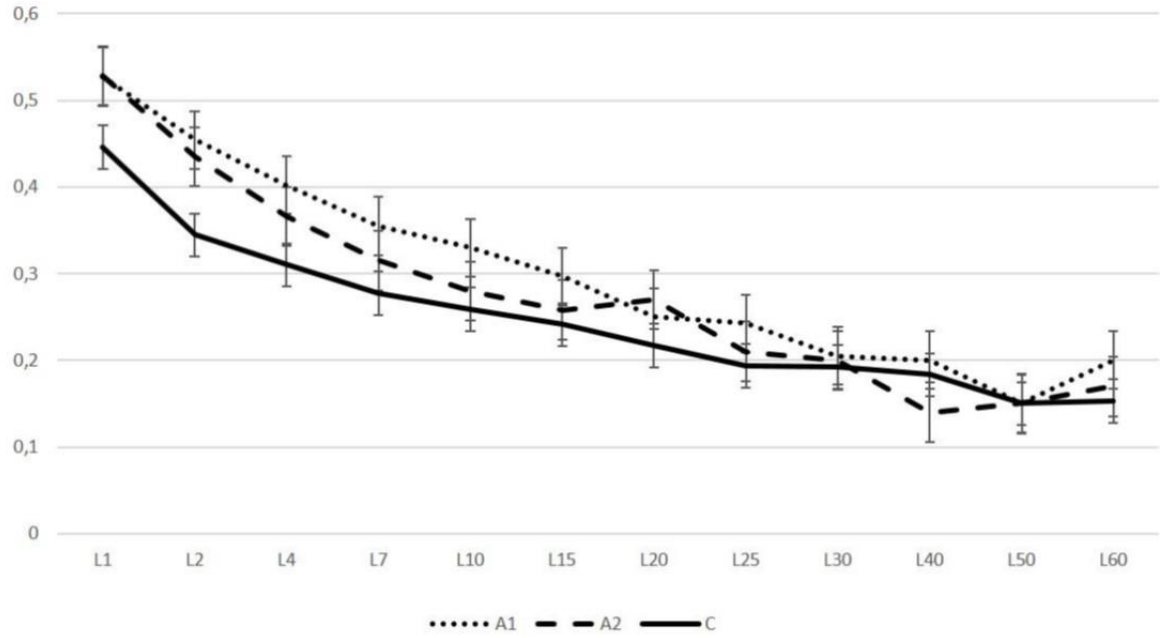
			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Doppler ultrasono-graphic parametre	systolic acceleration (m s^{-1})	Groups A1	$5.3 \pm 0.9^{m,n}$	6.8 ± 0.7	7.7 ± 1.0^{m}	$8.0 \pm 1.2^{a,n}$
		Groups A2	$4.5 \pm 0.6^{m,n}$	$8.1 \pm 0.8^{m,o}$	$6.9 \pm 0.6^{n,p}$	$5.1 \pm 0.4^{a,o,p}$
		Groups C	7.3 ± 0.9	7.2 ± 0.6	7.5 ± 0.5	8.5 ± 0.9^{a}
	blood flow volume (mL $min.^{-1}$)	Groups A1	$262.7 \pm 51.3^{m,n}$	$161.5 \pm 29.2^{c,o,p}$	$72.0 \pm 8.2^{c,m,o,q}$	$32.4 \pm 8.1^{b,n,p,q}$
		Groups A2	$248.2 \pm 62.1^{m,n}$	$130.4 \pm 28.7^{c,o,p}$	$48.6 \pm 6.6^{c,m,o,q}$	$25.3 \pm 8.2^{b,n,p,q}$
		Groups C	$190.1 \pm 32.0^{m,n,o}$	$65.5 \pm 9.3^{c,m,p,q}$	$30.5 \pm 3.2^{c,n,p,r}$	$13.8 \pm 1.6^{b,o,q,r}$

a - c: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $0.020 < P < 0.040$, b: $0.001 < P \leq 0.020$, c: $P \leq 0.001$) – m - r: same superscripts within a row indicate significant difference between stages ($P < 0.045$).

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.

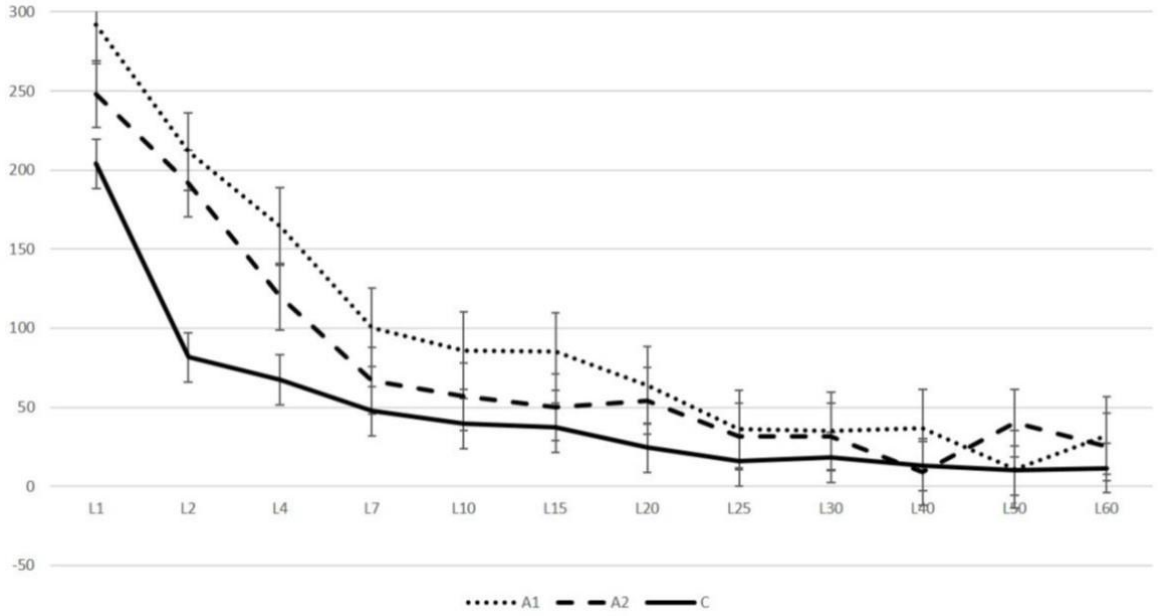
Figure 17. Time-series graphs (L0: day of lambing) of Doppler ultrasonography results of the uterine artery of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C) (a) diametre of the vessel (cm), (b) blood flow (mL min.$^{-1}$).

(a)



Vertical axis: diametre of the uterine artery (cm), horizontal axis: days post-lambing (L0 = day of lambing).

(b)



Vertical axis: blood flow volume (mL min.$^{-1}$), horizontal axis: days post-lambing (D0 = day of lambing

Bacteriological findings

Vaginal swab samples

In group A, bacteria were isolated from all ewes (1.000) at least once. In total, 56 / 91 (0.615) and 33 / 65 (0.507) samples from subgroup A1 and A2, respectively, yielded bacteria (P = 0.12 for A1 *versus* A2). From these 56 and 33 samples, 57 and 34 bacterial isolates were recovered respectively (subgroup A1, subgroup A2) (1.02 and 1.03 isolates per positive sample, respectively) (P = 0.37 for A1 *versus* A2) (Table 15). The median period from lambing to the first isolation of bacteria from the genital tract was 1.5 days both for A1 and A2 ewes (P = 0.065 for A1 *versus* A2). The median duration of bacterial isolation was 27.0 days for A1 and 8.0 days for A2 ewes (P = 0.044 for A1 *versus* A2).

In group C, bacteria were isolated from all ewes (1.000) at least once. In total, 75 / 117 samples yielded bacteria (0.641) (P = 0.15 for A *versus* C). From these 75 samples, 96 bacterial isolates were recovered (1.28 isolates per positive sample) (P < 0.001 for A *versus* C) (Table 15). The median period from lambing to the first isolation of bacteria from the genital tract was 0.5 days (P < 0.001 for A *versus* C). The median duration of bacterial isolation was 12.0 days (P < 0.001 for A *versus* C).

The most commonly recovered organism was *Escherichia coli*. It consisted of 0.725 of the isolates from A ewes and of 0.474 of the isolates from C ewes (P = 0.001) (Table 16).

Table 15. Frequency of isolation of bacteria from anterior vagina swab samples of ewes, which, subsequently to suboptimal feeding during last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

	Stage post-partum[1]			
Group	S_1	S_2	S_3	S_4
A1	4 / 14[2]	14 / 21	26 / 28	12 / 28
A2	2 / 10	9 / 15	11 / 20	11 / 20
C	6 / 18	22 / 27	27 / 36	20 / 36

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.
2. m / n = samples that yielded bacteria / total number of samples collected.

Uterine tissue samples

In group A, bacteria were isolated three times (from 2 A1 and 1 A2 ewes), from the uterine tissue sample collected on the first biopsy (L10 or L20) performed in each animal (0.125); *E. coli* and *T. pyogenes* were identified.

In group C, no bacteria were isolated from any uterine tissue sample (P = 0.41 for A *versus* C).

There was no association between results of uterine tissue samples and vaginal swab samples.

Table 16. Frequency of isolation of various bacterial species from anterior vagina swab samples of ewes, which, subsequently to suboptimal feeding during the last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

	Group											
	A1				A2				C			
	Stage post-partum[1]											
Microorganism	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4	S_1	S_2	S_3	S_4
E. coli	4	13	20	11	2	3	6	7	2	15	20	9
Staphylococcus spp.								1	6	10	8	10
Streptococcus spp.		1	5	1		4	4	4			3	1
Bacillus spp.									1	2	2	2
T. pyogenes			2			2				3		
Proteus spp.							1				1	
Klebsiella sp.									1			

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.

Cytological findings

In vaginal swab samples, neutrophils were the predominant leucocyte type observed in all ewes (Table 17). There were no differences in proportion of leucocyte types between subgroups A1 and A2 ($P > 0.055$). Neutrophil proportion in group A ewes was, in general, smaller than in group C ewes throughout the study period, with significant differences identified on S_2 and thereafter ($P < 0.04$ for C *versus* A).

Table 17. Proportions (%) of leucocyte types observed in vaginal swab samples of ewes, which, subsequently to suboptimal feeding during the last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

			Stage post-partum[1]			
			S_1	S_2	S_3	S_4
Leucocyte types	Neutrophils	A1	$99.04 \pm 0.37^{m,n,o}$	$92.14 \pm 2.24^{b,m}$	$91.77 \pm 1.75^{a,n}$	$83.46 \pm 4.79^{b,o}$
		A2	91.72 ± 4.20^{m}	$88.73 \pm 2.74^{b,n}$	$93.80 \pm 1.83^{a,o}$	$74.63 \pm 6.49^{b,m,n,o}$
		C	98.77 ± 0.86^{m}	98.13 ± 0.75b	96.30 ± 1.29a,m	93.18 ± 3.44b
	Lymphocytes	A1	$0.36 \pm 0.19^{m,n,o}$	$3.40 \pm 1.39^{m,p}$	4.75 ± 1.48^{n}	$11.86 \pm 4.17^{o,p}$
		A2	2.17 ± 1.93	8.10 ± 3.36	3.50 ± 1.60	7.13 ± 2.20
		C	0.80 ± 6.02	1.63 ± 0.64	2.64 ± 1.10	6.70 ± 0.02
	Macrophages	A1	$0.61 \pm 0.20^{m,n}$	$4.38 \pm 1.60^{b,m}$	3.48 ± 1.24^{n}	4.64 ± 2.50^{b}
		A2	6.06 ±4.10	$3.17 \pm 1.05^{b,m}$	1.53 ± 0.46^{n}	$18.25 \pm 6.45^{b,m,n}$
		C	0.43 ± 0.33	0.24 ± 0.16^{b}	1.06 ± 0.78	0.45 ± 0.45^{b}

a, b: with reference to the same parametre, same superscripts within a column indicate significant difference between A and C groups (a: $P = 0.035$, b: $P < 0.02$) – m - p: same superscripts within a row indicate significant difference between stages ($P < 0.05$).

1. S_1: included samples collected on lambing day and 1st day post-partum, S_2: included samples collected from 2nd to 7th day post-partum, S_3: included samples collected from 10th to 25th day post-partum, S_4: included samples from 30th to 60th day post-partum.

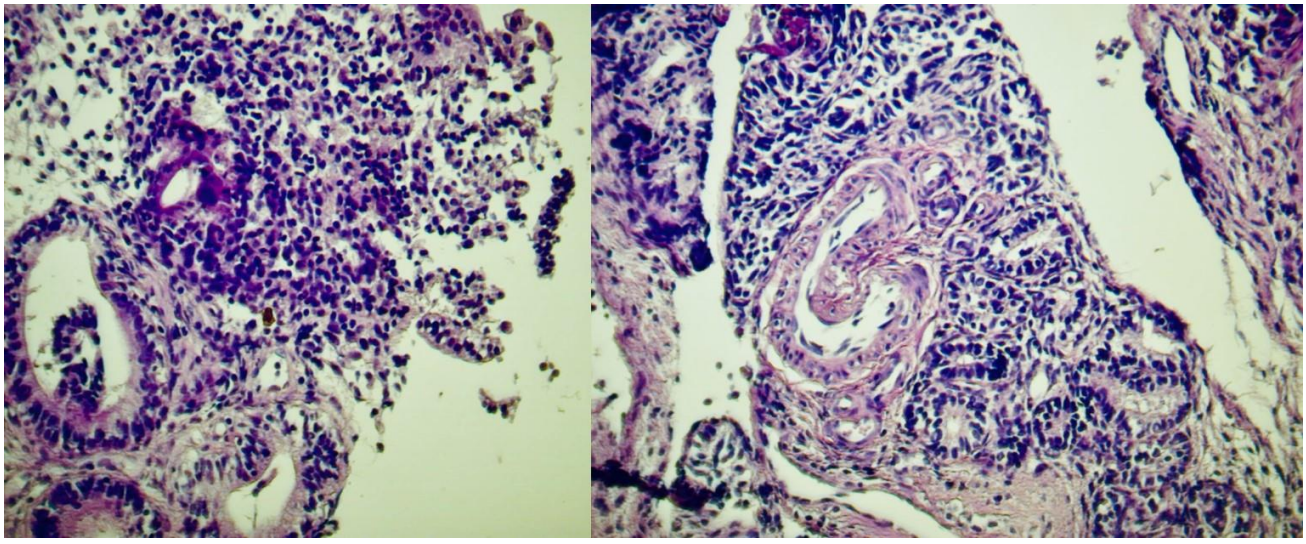
Histological findings

In group A ewes, in samples collected on the tenth day post-partum, the epithelium of the endometrium in the inter-caruncular areas was characterised by single-layered cells, although, occasionally, multi-layered ones were also seen; these were cylindrical with 'foamy' appearance (large nucleated cells with vacuolated cytoplasm). Occasionally, the epithelium in the inter-caruncular areas adjacent to the caruncles was completely destroyed. There was increased endometrial hyperaemia with mild cellular infiltration (mostly neutrophils). The uterine glands were distended, contained large amount of fluid and were surrounded by leucocytes. The myometrium was markedly thickened and there was presence of mild leucocyte infiltration. In one A1 ewe, no sample was collected, because the uterine wall was deemed to be particularly friable; the uterus appeared hyperaemic, with extensive orogonitis. On the 20th day post-partum, epithelium was single-layered with cuboidal cells; endometrial hyperaemia was still seen. Progressively, the proportion of lymphocytes increased, with most of them present in the endometrium. Increased apoptosis was seen. The diametre of uterine glands was smaller compared to that on the tenth day. On the 30th day post-partum, the uterine glands had an ovoid shape. Leucocytic infiltration in the endometrium and myometrium continued to be intense and apoptosis was still evident. On the 40th day post-partum, only a few uterine glands with flattened shape could be seen. The epithelium of the endometrium was characterised by single-layered cylindrical to cuboidal cells with small numbers of foamy cells. Infiltration was still evident and characterised by lymphocytes (Figure 18).

In group C ewes, in samples collected on the tenth day post-partum, the epithelium of the endometrium was characterised by single- or multi-layered cuboidal- to cylindrically-shaped cells with 'foamy' appearance with gaps in-between them. There was endometrial hyperaemia with little cellular infiltration (mostly neutrophils and lymphocytes). The uterine glands were readily visible, with cuboidal- to cylindrically-shaped cells. The myometrium was markedly thickened, with presence, in some cases, of 'foamy' cells, containing vacuoles, as well as activated lymphocytes or degenerative macrophages. On the 20th day post-partum, the epithelium of the endometrium was mainly single-layered and only occasionally multi-layered, with the height of epithelial cells being small. Size of

uterine glands was reduced compared to earlier samples. There was hyperaemia in the lamina propria and little cellular infiltration (mostly lymphocytes) in the endometrium and around the uterine glands. Thickness of the myometrium was reduced. On the 30th day post-partum, the epithelium of the endometrium was characterised by single-layered cells; no foamy material was seen on epithelial cells. The uterine glands had regressed and collapsed. Infiltration was characterised by lymphocytes, primarily around remnants of uterine glands and in the myometrium. On the 40th day post-partum, the epithelium of the endometrium was characterised by single-layered cuboidal cells. Infiltration was characterised by lymphocytes.

Figure 18. Post-partum histological pictures of the uterus of ewes which, subsequently to suboptimal feeding during last stage of pregnancy, had developed pregnancy toxaemia (subgroup A1) during the preceding gestation,.



Left: stage S_3 (section of the endometrium, with evidence of degradation of the single- or multi-layered epithelium, presence of fully developed uterine glands with cylindrical single-layered epithelium and content in their lumen, presence of intense subepithelial infiltration primarily by lymphocytes with a few neutrophils), right: stage S_4 (section of the endometrium, presence of collapsed uterine glands with cuboidal single-layered epithelium and reduced diametre with no content therein, presence of mild infiltration by lymphocytes), respectively (H & E, 200× and 400×, respectively)

No differences were evident in median scores for number of leucocytes in samples from A1 (4, 2 – 4) and A2 (4, 2 – 4) ewes ($P = 0.47$). In contrast, median score in samples from A (4, 2 – 4) was higher than in C (2, 1 – 4) ewes ($P = 0.015$); differences between A and C

ewes in median scores were also significant for number of neutrophils and lymphocytes, but not for number of macrophages ($P = 0.030$, $P = 0.025$, $P = 0.32$, respectively). There was also some significance in differences in scores for number of leucocytes across the sampling points ($P = 0.0498$). Neutrophils predominated in uterine tissue samples from all ewes. Neutrophil proportion in group A ewes was, in general, smaller than that in group C ewes throughout the study period, with specific significant differences identified on L20 and L30 ($P < 0.035$ for A *versus* C on those occasions). There were no differences in proportion of leucocyte types between subgroups A1 and A2 ($P > 0.095$).

Subsequent reproductive performance

During the subsequent reproductive (breeding) season, all ewes in group A and group C were mated and diagnosed to be pregnant by using ultrasonographic examination. No increased concentrations of β-hydroxybutyrate in blood samples from any ewe were detected. Finally, all ewes lambed normally and produced healthy and viable lambs. There were no significant differences between A1 and A2 ($P > 0.10$) or between A and C ($P > 0.25$) in any reproductive performance parametre (Table 18).

Table 18. Reproductive performance, during the subsequent reproductive season, of ewes, which, subsequently to suboptimal feeding during the last stage of pregnancy, had (A1) or had not (A2) developed pregnancy toxaemia, or of healthy controls (C).

	Group		
Measure of reproductive performance	A1	A2	C
Mating rate	100%	100%	100%
Pregnancy rate	100%	100%	100%
Abortion rate	0%	0%	0%
Lambing rate	100%	100%	100%
Total lambs per ewe	1.71	2.20	1.89
Stillbirth rate	0%	0%	0%

CHAPTER IV

GENERAL DISCUSSION

During the puerperium, the genital system is returning to its non-pregnant state. Nevertheless, it does not completely return to the original pre-gravid state, as some of the changes taking place during gestation are not completely reversible (e.g., size of the uterus). In this study, an established model for inducing uterine infection was employed; the potential effects of the disorder in uterine involution were studied. Development of the disease was confirmed by means of clinical, bacteriological and cytological findings.

Ultrasonographic, histological and cytological findings

Ultrasonographic findings

The results of B-mode ultrasonographic evaluation revealed a delay in the process of the involution of the uterus in inoculated ewes. In these animals, the principal regression took place during S_3, i.e., in contrast to control ewes, in which regression occurred mostly during S_2. In previous studies, in which ultrasonographic examination was employed to study the genital tract of ewes, uterine involution was found to have been completed within 35 days post-lambing (e.g., Ababneh and Degefa 2005, Derar et al. 2012, Badawi et al. 2014, Fasulkov 2014, Medan and El-Daek 2015).

The results of Doppler evaluation further corroborated the delay in uterine involution. In inoculated ewes, the greater diametre of the uterine artery and the increased blood volume were obviously the result of the inflammation (Hurley 1983).

Histological findings

The ultrasonographic findings can be associated with the results of histological evaluation of uterine tissue samples. Based on these findings, regression was considered to be complete between D34 and D44 in control ewes (according to the day of biopsy-sampling). Gray et al. (2003) have defined as a major criterion for regression the eversion of caruncles and the 're-epitheliazation' of the uterus. Although that study did not extend beyond the 28th day post-partum, those authors reported that, on that day, the process was nearing its completion, a conclusion that is in line with the present findings. In contrast, in

the inoculated ewes, regression was not fully completed at the above days, which indicates a delay in the involution of the organ.

Moreover, the increased blood flow recorded during the Doppler evaluation was associated with the frequent findings of hyperaemia and extravasation in tissue samples from inoculated ewes.

Nevertheless, at the end of the 60-day monitoring period, the uterus in the inoculated ewes had also regressed, as confirmed by the ultrasonographic and histological findings. This was corroborated by the lack of differences between the two groups in the reduction of dimensions for any uterine structure for the entire 60-day monitoring period.

Cytological findings

Post-challenge, lymphocytes were more abundant in the inoculated ewes (compared to control animals), as seen in vaginal and uterine samples. Bacterial phagocytosis by migrating leucocytes is the principal mechanism involved in the elimination of intrauterine bacteria (Noakes 2009). Moreover, Cai et al. (1994) have reported that the phagocytic ability and intracellular bacterial killing capacity of neutrophils in cows with post-partum infections were impaired. Therefore, lymphocytes become of particular value to clear uterine infections. In cows, it has been found that subepithelially located lymphocytes (this also being a finding of the present study) included CD4+ cells and B lymphocytes (Leung et al. 2000), playing a paramount role in the clearance of intrauterine bacteria. Brodzki et al. (2014) reported that, in cows with metritis, CD4+ cells were reduced and CD8+ prevailed. Endometrial epithelial cells may also participate in the fight against pathogens by expressing receptors, which would recognise components of bacterial cells (e.g., lipopolysaccharides in the case of *E. coli*) (Singh et al. 2008). Hence, the damage in the uterine epithelium, as seen in samples from inoculated ewes, might have contributed in the delayed clearance of the pathogen recorded in the study.

The importance of lymphocytes in controlling the infection is also underlined by the increased number of these cells in the blood. In a previous study, this increase has been found to take place specifically in *vena cava* blood samples (Ramadan et al. 1997). This may reflect a movement of lymphocytes from secondary lymphatic tissue into the blood circulation, whence it could move into the uterus, as identified in this study in the relevant

samples. The importance of lymphocytes is reflected in the progressive and significant increase of their proportion in genital tract samples, which is compatible with the progressive increase of lymphocyte counts in blood.

Bacteriological findings

The present results indicated that *E. coli* constituted the major proportion of the bacterial populations of the vagina in inoculated ewes, which may reflect its presence in uterine exudate leaking into the vagina. In these animals, other bacteria were also recovered, but they were significantly less frequent than in uninoculated control ewes. In cases of infections with established pathogens, bacterial flora in affected sites decreases. Antagonism of invading pathogens *versus* commensal bacteria present in the affected body site has been reported in cases of intestinal infections; for example, *Vibrio cholerae* 'attacks' members of the gut microbiota, thus facilitating its colonisation of the intestine (Zhao et al. 2018), and *Salmonella typhimurium*, in a T6SS-mediated manner, kills commensal organisms, in order to establish itself in the intestine of affected hosts (Sana et al. 2016). As the T6SS nanomachine has also been identified in *E. coli* (Navarro-Garcia et al. 2019), one cannot rule out that this pathogen in a similar manner possibly limited the populations of genital commensal bacteria in the inoculated ewes, in order to secure its dominance in the infected genital tracts.

Haematological findings

In infected ewes, the smaller eosinophil counts in blood, present only post-challenge, can be allied to increased presence of these cells in the uterine tissue, which in women has been suggested as a preliminary diagnostic means for long-standing endometritis (Adegboyega et al. 2010). In the endometrium, eosinophils possess high affinity receptors for IgE (Bondurant 1999), with binding of antibodies to these receptors resulting in the degranulation of these cells, leading to the release of inflammatory mediators, superoxides, lytic enzymes and kallikreins (Bondurant 1999).

Significance of the period when lambing occurred

Ewes often lamb during the natural anoestrus period (i.e., outside the breeding period), at which time no hormones are involved in the genital tract post-partum. In contrast, during the breeding period, when ovulations occur with a subsequent increase of progesterone concentrations, a local immunosuppressive effect on the endometrium can occur, which may increase the risk of bacterial complications during uterine involution (Faigl et al. 2011). Hence, in ewes that lamb during the anoestrous period, the cellular mechanisms would be highly effective against intrauterine bacteria.

In contrast, in ewes that would be subjected to reproductive control for accelerated lambing, one may express concerns regarding a possible impairment of these defences. This can be of importance in management systems, in which ewes are subjected to reproductive control soon after lambing, with the aim to be mated for accelerating production and achieving two lambings within a year.

Uterine involution in ewes with pregnancy toxaemia during the preceding gestation

Pregnancy toxaemia is a common metabolic disorder of ewes occurring as the result of energy-deficient feeding of pregnant animals (Schlumbohm and Harmeyer 2004, Brozos et al. 2011). It is a frequent metabolic disorder of sheep and can be fatal. Its consequences may be beyond pregnancy: in a previous study, it was found that pregnancy toxaemia could predispose ewes to mastitis during the subsequent lactation period (Barbagianni et al. 2015b). That study focussed in the immediately post-partum period, up to seven to ten days after lambing. In a more recent study, Karagiannis et al. (2018) have indicated the presence of associations between development of pregnancy toxaemia and suboptimal milk production in affected ewes during the subsequent lactation period.

Ultrasonographic, histological and cytological findings

Ultrasonographic findings

Immediately post-partum, uterine structures in ewes that had developed pregnancy toxaemia during the preceding gestation, were found ultrasonographically to be greater than in healthy animals. Possibly, this might indicate a compensatory mechanism during the last stage of gestation, aiming to support near-term foetuses, as a lack of nutrients could have led them to death. Similar findings have been reported in cows with ketosis by Paiano et al. (2019).

The significantly larger blood flow in the uterus of ewes that previously had pregnancy toxaemia, was possibly a continuation and consequence of the proposed compensatory mechanism. Consequently, this higher volume of blood in ewes that previously had pregnancy toxaemia than in control ewes, was eventually reflected histologically in the higher amount of hyperaemia and presence of increased amount of fluid in the uterine glands of the ewes of that group than in control animals. This larger blood flow could also be reflected in the echogenicity difference between the two groups: increased blood flow in the uterine tissue would have resulted in higher greyscale in intercaruncular areas, ultimately resulting in smaller GS_{CT} / GS_{IT} ratio in ewes that had pregnancy toxaemia. In general, there were few differences between the groups of ewes in the rate of reduction of the various structures of the uterus; possibly, the increased blood flow in ewes of group A has contributed to involution of the genital tract of the ewes that had pregnancy toxaemia, to a rate similar to that of healthy controls. No differences in haemodynalic parametres were evident in S_1; differences after that period were likely the result of the necessity for increased blood flow to finally have the same involution in both groups. In all ewes however, blood flow into the uterus progressively decreased as the involution process advanced, which is in accord with the previous findings of Elmetwally and Bollwein (2017).

Histological findings

The histological findings have indicated that uterine involution in ewes that previously had pregnancy toxaemia, did not differ to that in healthy animals. Higher blood

flow seen during Doppler mode ultrasonographic examination in ewes that had pregnancy toxaemia (compared to healthy controls) has might have led to increased number of leucocytes (as evidenced by scores for their numbers), which would have counteracted the infection and thus contributed in involution of the genital tract. It is noteworthy that relevant reports in cows have suggested that uterine involution in ketotic animals was more rapid than in non-ketotic ones (Emery and Williams 1964).

The release of oxytocin as the result of frequent sucking by lambs (Bareham 1976) would also promote the involution process and contribute to the rapid reduction of dimensions. In fact, no differences in reduction rate of uterine structures were evident between groups.

Cytological findings

An interesting finding was the smaller proportion of neutrophils in ewes that had previously developed pregnancy toxaemia. In cows with ketosis, it has been found that during the period of negative energy balance, the animals had reduced blood neutrophil numbers (Galvao et al. 2010). Moreover, reduced glucose concentrations in blood may directly affect polymorphonuclear cell chemotaxis (Kim et al. 2006). All these may be reflected in the smaller numbers of neutrophils in samples from the genital tract of ewes that previously had pregnancy toxaemia.

Bacteriological findings

The results of microbiological examinations of vaginal samples provided conflicting evidence for differences between groups of ewes: whilst the presence of bacteria was seen later in ewes with pregnancy toxaemia, microorganisms were recovered from ewes that had pregnancy toxaemia for a longer duration. Possibly, these were consequences of metabolic derangements that occurred prior to lambing on uterine involution post-partum. Also, no differences were seen in results of microbiological examinations of uterine tissue samples. Previously, in a field study, it was reported that ewes with pregnancy toxaemia were at higher risk to subsequently develop mastitis, but not metritis (Mavrogianni et al. 2014). The former finding was subsequently corroborated in experimental work (Barbagianni et al.

2015b), the latter one is allied to the present results. It is noteworthy that in cattle, bacteria can be isolated and reisolated from the uterus for a varying period post-partum without the cows developing uterine disease (Griffin et al. 1974, Boitor et al. 1976; Sheldon et al. 2009). One may thus postulate that also in the present study bacterial isolations were related to carrier-state rather than true infections of the genital tract.

With regard to the identity of bacteria isolated from vaginal samples, one may postulate that increased *E. coli* numbers were present in the vagina as the result of immunocompromised occurring immediately post-partum subsequently to pregnancy toxaemia (Barbagianni et al. 2015b). In cases of infections with established pathogens, other bacterial species in affected sites decrease, as the result of potential antagonism of invading pathogens *versus* commensal organisms present in the affected body site. Hence, it should be taken into account that *E. coli*, through various mechanisms, possibly limited the populations of commensal bacteria in the affected ewes, in order to secure its dominance in the infected genital tract as described in greater detail hereabove.

Lack of agreement between results of bacteriological examination of uterine samples and vaginal samples can be attributed to isolation of vaginal bacterial flora in the latter samples. It is noteworthy that Madoz et al. (2014) have not recommended the bacteriological examination of uterine samples for diagnosis of uterine infections, as they did not isolate bacteria from relevant samples of cows with confirmed endometritis.

In ewes that had pregnancy toxaemia, reduced milk production was recorded (Karagiannis et al. 2018), which, in turn, does not provide to lambs all the milk quantity they would require and thus would make them suck their dams more often. Hence, increased oxytocin release may be seen in such ewes. This oxytocin acts on the uterus and supports uterine contractions. That way, removal of uterine content is promoted and hence expulsion of invading bacteria. Isolation of fewer bacteria per sample from group A than group C ewes lends some support to this hypothesis; possibly, frequent oxytoxin release contributes in bacterial expulsion and hence presence of smaller bacterial populations in the genital tract. The long duration of genital infection would also predispose to the development of clinical signs in the genital tract, as seen by the increased frequency of bacterial isolations in ewes of group A.

Ultrasonographic examination of the uterus in the post-partum period

Findings in healthy ewes

B-mode ultrasonographic examination

In general, the ultrasonographic examination was found to be a useful means of monitoring of the genital tract of ewes post-partum. In healthy animals, the findings were allied to those previously reported by other authors (Ali et al. 2001, Hauser and Bostedt 2002, Ababneh and Degefa 2005, Hayder and Ali 2008, Fernandes et al. 2013, Badawi et al. 2014).

Further to the above, it is noted that during B-mode examination with a convex transducer by using the transabdominal technique, in longitudinal sections, especially after the tenth day post-partum, the uterus often could appear circular or elliptical in shape and it might be difficult to differentiate the horns. Moreover, in transverse sections, images of the organ often may appear polygonal to elliptical, progressively becoming compressed circular to circular. Moreover, separate layers of the uterine wall could be clearly imaged with a linear transducer, which would allow for a close monitoring and detailed description of the uterine wall; in most cases, this is imaged as finely textured, homogeneous and hypoechoic to moderately echogenic.

Doppler mode ultrasonographic examination

Doppler ultrasonographic examination can be applied in measuring uterine blood flow and determining changes in blood perfusion of the organ during various reproductive stages. That way, physiological changes can be accurately identified and studied. The findings have indicated that the blood flow and the diametre of the uterine artery were the most useful parametres to employ for the evaluation of the health status of ewes during the post-partum period.

It is noteworthy that reference ranges for Doppler indices applicable when examining the uterine artery may depend on the technique used, hence, appropriate reference ranges should be taken into account according to the technique employed (Bhide et al. 2013).

Moreover, when evaluating results of Doppler examination of the uterine artery blood flow, animal temperament must also be taken into account (Elmetwally 2012).

Findings in ewes with uterine infection

The most important pathological condition in ewes post-partum is uterine infection. The ultrasonographic examination was found to be useful to assess severity of the disease. The principal uterine characteristics that could be assessed were uterine distention, asymmetry of the organ, distention of uterine lumen, presence, quantity and texture of the uterine content, thickness of the uterine wall, localisation of inflammatory foci on the uterine wall (endometrium, myometrium or both), texture of uterine wall (differences in its echogenity), alterations in uterine wall vascularisation and confirmation of uterine involution completion.

In cases of mild infection, findings are, in general, similar to those of the involuting uterus in healthy animals (Hauser and Bostedt 2002, Ababneh and Degefa 2005, Badawi et al. 2014). In such cases, the uterine lumen may not be imaged and the uterine content would be seen anechoic (Kähn 1992, 2004); alternatively, only presence of intrauterine fluid (with no relevant clinical signs) may be evident (Kasimanickam et al. 2004) during the ultrasonographic examination. In more severe cases, one or both horns of the uterus can be seen distended, even along their entire length (Kähn 1992, 2004), with accumulation of fluid evident therein; the uterine wall may be seen thicker than in healthy animals and more echogenic (Kähn 1992, 2004). Further, increased vascularisation can be observed by using Doppler mode examination.

Post-partum use of contrast-enhanced ultrasonographic examination

Due to the small number of animals involved and measurements performed, the CEUS work should be considered as preliminary and indicative only. The increased cost of the technique was a limiting factor for furthering the work at this stage.

Nevertheless, the work has indicated that the dose of the contrast-agent administered, allowed clear visualisation of the uterine structures. Further, decreased enhancement in the

inoculated uteri indicated reduced perfusion of contrast agent into the organ, which occurred despite the increased blood flow therein as the consequence of early stage of inflammation. This may possibly indicate that tissue damage to the organ has occurred already within 24 hours of inoculation.

Post-partum utrasonographic examination of the uterus in sheep health management

Ultrasonographic examination of the ovine uterus post-partum may be applied in a flock to investigate potential genital disorders of the animals. The examination may also be performed in flocks, where, as a consequence of disorders during pregnancy or parturition, post-partum subclinical uterine infection may develop. This would be of importance in intensively managed flocks, where ewes are scheduled to become pregnant two months after lambing (Samartzi and Fthenakis 2003).

The ultrasonographic examination of the uterus can be useful, in order to assess response to treatment in cases of genital infection. Successful treatment is characterised by a reduction in uterine size and decrease in the amount of uterine content, coupled with clinical improvement of the animal.

Ultrasonographic examination has advantages over other techniques that may be employed for monitoring the genital system, e.g., laparascopy, the applicability of which in clinical conditions is difficult and potentially risky (Ababneh and Degefa 2005). In contrast, ultrasonographic examination is an easily applied and accurate technique, which can be easily performed in the field. It is noteworthy that in cows, the technique is used with significant success as a tool for diagnosis of subclinical post-partum metritis (Barlund et al. 2008, Meira et al. 2012).

Effects of uterine infection or pregnancy toxaemia in the subsequent reproductive performance of ewes

Effects of uterine infection

The present results did not show that uterine infection had any adverse consequences in the subsequent reproductive performance of ewes. In fact, all the inoculated ewes were mated and lambed as planned.

Outside the breeding period, there is a prolonged period of sexual rest in ewes; although there is a minimal follicular development during that period, the reproductive system of ewes is quiescent. One may suggest that the genital tract, despite the infection, had recovered during that period, before the new exposure to progesterone (Fthenakis 2004). One may postulate that, if the 'resting' period of the genital tract would be shorter (e.g., as in accelerated production systems in meat-producing flocks), some adverse effects could possibly be noted in the subsequent reproductive performance of the animals.

Under field conditions, in cases of uterine infections, veterinarians would often prescribe to affected animals a broad spectrum antimicrobial agent, often coupled with a non-steroid anti-inflammatory agent. These actions are in accord with good clinical veterinary practice. In the present study, no therapeutic intervention was performed, as there was interest in following the course of the disease as it developed. The results have indicated that ewes recovered spontaneously and subsequently did not show adverse reproductive effects. This can be taken into account, in order to reduce the use of antimicrobial agents, which would contribute in limiting development of resistance to antimicrobial agents. Nevertheless, each case should be considered individually and appropriate interventions should be made if necessary, having always in mind the welfare of the animals under consideration.

Effects of pregnancy toxaemia

The present results did not show that pregnancy toxaemia had any adverse consequences in the subsequent reproductive performance of ewes. No further cases of the

disease were recorded in the subsequent gestation among previously affected ewes. In fact, all those ewes lambed as planned. On this occasion, all recommended health management procedures for ewes during gestation (Fthenakis et al. 2012) had been applied and appropriate care for the animals had been taken.

In cows, ketosis during pregnancy (a disease of similar aetiopathogenesis to pregnancy toxaemia) can have an adverse impact in the subsequent reproductive activity; for example, Walsh et al. (2007) have found reduced conception rates in cows with ketosis during the preceding pregnancy and Roche et al. (2018) have reported the presence of long post-partum anoestrus periods in such animals. Various hypotheses have been presented regarding reasons for this suboptimal performance and were associated with various pathways. Improper follicle development has been associated with negative energy balance (Beam and Butler 1999), whilst effects in the insulin-like growth factor system might adversely affect uterine receptivity to the conceptus (Fenwick et al. 2008). Uterine infections, which are particularly common in cows with pregnancy ketosis (Hammon et al. 2006, Duffield et al. 2009) may also lead in difficulties in conceptus survival and implantation in such animals. Delayed recovery of the function of reproductive organs post-partum may be an effect of reduced energy availability and can lead to delayed resumption of reproductive activity (Butler and Smith 1989, Wathes et al. 2007), whilst post-partum hormonal imbalance may also be present in these circumstances (Butler 2003).

In cows with hyperketonaemia, a delay in uterine involution was found, which was attributed to a delay in the return to cyclicity of these animals (Paiano et al. 2019). Ewes that had lambed outside the breeding season, as in the present study, were not cycling anyway at that time. When cyclicity started after the 'resting' of the genital system and the start of the breeding season, these ewes cycled normally and their reproductive performance was not hindered. Moreover, no delay in the completion of uterine involution in ewes that previously had pregnancy toxaemia, was evident; this could have contributed further in the lack of adverse effects in the subsequent reproductive performance.

Also, in cows, ketosis occurs more frequently during lactation, whilst in ewes pregnancy toxaemia occurs during gestation. In the present work, the interval from the disorder to the subsequent reproductive activity was long and, hence, any adverse effects

would have possibly subsided; this contributed further to the restoration of normal reproductive activity.

The study was performed in dairy ewes, which usually lamb once annually, as the milking period may take up to 6 to 8 months. In meat production systems, where accelerated breeding programs are often applied and matings may be planned within a short period post-lambing (Keisler and Buckrell 1997), the 'resting' period of the genital tract would be much shorter; therefore, one may postulate that, in such cases, some adverse effects may possibly be noted in the subsequent reproductive performance of the animals.

Conclusions

The study has investigated the long-term effects of uterine infection and pregnancy toxaemia in uterine involution and in the subsequent reproductive performance of affected ewes.

In ewes with uterine infection, the uterus was able to counteract the bacterial invasion; nevertheless, the process of involution took longer in affected ewes than in healthy animals. In ewes that had pregnancy toxaemia during the preceding gestation, no overall differences were evident in the involution process compared to healthy animals.

The ultrasonographic examination (B-mode, Doppler mode, contrast-enhanced examination) was found to be a useful means for assessment of the genital tract of ewes post-partum.

After undertaking correct health management in all affected ewes during the following gestation, no adverse effects were noted in the subsequent reproductive performance of ewes previously with uterine infection or pregnancy toxaemia.

REFERENCES

Ababneh, M.M., Degefa, T., 2005. Ultrasonic assessment of puerperal uterine involution in Balaby goats. *Journal of Veterinary Medicine* 52, 244-248.

Adegboyega, P.A., Pei, Y., McLarty, J. 2010. Relationship between eosinophils and chronic endometritis. *Human Pathology* 41, 33-37.

Ali, A., Salem, A.A., El-Din Zain, A., 2001. Ultrasonographic assessment of postpartum uterine involution and onset of ovarian activity in the Ossimi ewe. *Proceedings of the Egyptian Society for Animal Reproduction and Theriogenology* http://esarf2.tripod.com/ali1.htm.

Amiridis, G.S., Fthenakis, G.C., Dafopoulos, J., Papanikolaou, T., Mavrogianni, V.S., 2003. Use of cefquinome for prevention and treatment of bovine endometritis. *Journal of Veterinary Pharmacology and Therapeutics* 26, 387-390.

Badawi, M.E., Makawi, S.E.A., Abdelghafar, R.M., Ibrahim, M.T., 2014. Assessment of postpartum uterine involution and progesterone profile in Nubian goats (*Capra hircus*). *Journal of Advances in Veterinary and Animal Research* 1, 36-41.

Barbagianni, M.S., Giannenas, E., Papadopoulos, E., Petridis, I.G., Spanos, S.A., Gouletsou, P.G., Valasi, I., Fthenakis, G.C., 2015a. Pregnancy toxaemia in ewes: development of an experimental model and potential interactions with gastrointestinal nematode infections. *Small Ruminant Research* 133, 102–107.

Barbagianni, M.S., Ioannidi, K.I., Vasileiou, N.G.C., Mavrogianni, V.S., Orfanou, D.C., Fthenakis, G.C., Valasi, I., 2017. Ultrasonographic examination of pregnant ewes: from early diagnosis to prediction of dystocia. *Small Ruminant Research* 152, 41-55.

Barbagianni, M.S., Mavrogianni, V.S., Katsafadou, A.I., Spanos, S.A., Tsioli, V., Galatos, A.D., Nakou, M., Valasi, I., Gouletsou, P.G., Fthenakis, G.C., 2015b. Pregnancy toxaemia as predisposing factor for development of mastitis in sheep during the immediately post-partum period. *Small Ruminant Research* 130, 246–251.

Barbagianni, M.S., Spanos, S.A., Ioannidi, K.S., Vasileiou, N.G.C., Katsafadou, A.I., Valasi, I., Gouletsou, P.G., Fthenakis, G.C., 2015c. Increased incidence of peri-parturient problems in ewes with pregnancy toxaemia. *Small Ruminant Research* 132, 111-114.

Bareham, J.R., 1976. The behaviour of lambs on the first day after lambing. *British Veterinary Journal* 132, 152-162.

Barlund, C.S., Carruthers, T.D., Waldner, C.L., Palmer, C.W., 2008. A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. *Theriogenology* 69, 714-723.

Barrow, G.I., Feltham, R.K.A., 1993. *Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge University Press, Cambridge, United Kingdom.

Beam, S.W., Butler, W.R., 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *Journal of Reproduction and Fertility* Suppl. 54, 411-424.

Bhide, A., Acharya, G., Bilardo, C.M., Brezinka, C., Cacifi, D., Hernandez-Andrade, E., Kalache, K., Kingdom, J., Kiserud, T., Lee, W., Lees, C., Leung, K.Y., Malinger, G., Mari, G., Prefumo, F., Sepulveda, W., Trudinger, B., 2013. ISUOG practice guidelines: use of Doppler ultrasonography in obstetrics. *Ultrasound in Obstetrics and Gynecology* 41, 233-239.

Boitor, I., Draghici, C.,Gaboreanu, M., Cristea, E., Boitor, M.,1976. Die dynamic der leukozyten und der microflora des uterusinhaltes im normalen und pathologischen puerperium und bei chronischen endometritiden des rindes. *Deutsche Tierärztliche Wochenschrift* 83, 24-25.

Bondurant, R.H., 1999. Inflammation in the bovine female reproductive tract. *Journal of Animal Scienc*e 77, 101-110.

Brodzki, P., Kostro, K., Brodzki, A., Lisiecka, U., Kurek, Ł., Marczuk, J., 2014. Phenotyping of leukocytes and granulocyte and monocyte phagocytic activity in the peripheral blood and uterus of cows with endometris. *Theriogenology* 82, 403-410.

Brozos, C., Mavrogianni, V.S., Fthenakis, G.C., 2011. Treatment and control of peri-parturient metabolic diseases: pregnancy toxemia, hypocalcemia, hypomagnesemia. *Veterinary Clinics of North America Food Animal Practice* 27, 105-113.

Buckrell, B.C., 1988. Applications of ultrasonography in reproduction in sheep and goats. *Theriogenology* 29, 71-84.

Butler, W.R., 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livestock Production Science* 83, 211–218.

Butler, W.R., Smith, R.D., 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *Journal of Dairy Science* 72, 767-783.

Cai, T.Q., Weston, P.G., Lund, L.A., Brodie, B., McKenna, D.J., Wagner, W.C., 1994. Association between neutrophil functions and periparturient disorders in cows. *American Journal of Veterinary Research* 55, 934-943.

Dawson, L.J., 2007. Clinical examination of the female reproductive tract. In: R.S. Youngquist (ed.) *Current Therapy in Large Animal Theriogenology*, 2nd edn. Saunders, Saint Louis, United States of America; pp. 537–540.

Degefa, T., 2003. *Postpartum Uterine Involution in Goat in Jordan*. Master of Science Dissertation, Jordan University of Science and Technology, Irbid, Jordan.

Degefa, T., Ababneh, M.M., Moustafa, M.F., 2006. Uterine involution in the post-partum Balaby goat. *Veterinarski Archiv* 76, 119-133.

Derar, D.R., Hayder, M., Ali, A., Hamdoun, H., 2012. Postpartum uterine involution and luteal activity in Farafra ewes lambing in autumn. *Assiut Veterinary Medical Journal* 58, 1-10.

Duffield, T.F., Lissemore, K.D., McBride B.W., Leslie, K.E., 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. *Journal of Dairy Science* 92, 571-580.

Elmetwally, M.A.A., 2012. *Clinical Applicability of Non-invasive Doppler Ultrasonography in Small Ruminants throughout Pregnancy*. Doctor of Veterinary Medicine Thesis, University of Veterinary Medicine Hannover, Hannover, Germany.

Elmetwally, M., Bollwein, H., 2017. Uterine blood flow in sheep and goats during the peri-parturient period assessed by transrectal Doppler sonography. *Animal Reproduction Science* 176, 32-39.

Emery, R.S., Williams J.A., 1964. Incidence of ketosis, other disease, and some postpartum reproductive ailments in normal and triiodothyronine-treated cows. *Journal of Dairy Science* 47, 879-881.

Euzeby, J.P., 1997. List of bacterial names with standing in nomenclature. *International Journal of Systematic Bacteriology* 47, 590-592.

Faigl V., Keresztes, M., Márton, A., Fébel, H., Kulcsár, M., Nagy, S., Cseh, S., Solti, L., Hyszenicza, G., 2011. Effect season and photoperiod on the time of first postpartum ovulation in Awassi ewes. *Acta Veterinaria Hungarica* 59, 497-510.

Fasulkov, I., 2014. Ultrasonography of uterine involution in goats. *Journal of the Faculty of Veterinary Medicine of Istanbul University* 40, 63-69.

Fenwick, M.A., Fitzpatrick, R., Kenny, D.A., Diskin, M.G., Patton, J., Murphy, J.J., Wathes, D.C., 2008. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domestic Animal Endocrinology* 34, 31-44.

Fernandes, C.E., Cigerza, C.F., Pinto, G.D.S., Miazi, C., Barbosa-Fereira, M., Martins, C.F., 2013. Parturition characteristics and uterine involution in native sheep from Brazilian Pantanal. *Ciência Animal Brasileira* 14, 245-252.

Ferretti, L.M., Newell, S.M., Graham J.P., Roberts G.D., 2000. Radiographic and ultrasonographic evaluation of the normal feline postpartum uterus. *Veterinary Radiology and Ultrasound* 41, 287-291.

Fthenakis, G.C., 2004. Effects of retention of fetal membranes on subsequent reproductive performance of dairy ewes. *Theriogenology* 61, 129–135.

Fthenakis G.C., Arsenos, G., Brozos, C., Fragkou, I.A., Giadinis, N.D., Giannenas, I., Mavrogianni V.S., Papadopoulos, E., Valasi, I., 2012. Health management of ewes during pregnancy. *Animal Reproduction Science* 130, 198-212.

Fthenakis, G.C., Leontides, L.S., Amiridis, G.S., Saratsis, P., 2000. Incidence risk and clinical features of retention of foetal membranes in ewes in 28 flocks in southern Greece. *Preventive Veterinary Medicine* 43, 85-90.

Galatos, A.D., 2011. Anesthesia and analgesia in sheep and goats. *Veterinary Clinics of North America Food Animal Practice* 27, 47-59.

Galvao, K.N., Flaminio, M.J.B.F., Brittin, S.B., Sper, R., Fraga, M., Caixeta, L., Ricci, A., Guard, C.L., Butler, W.R., Gilbert, R.O., 2010. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. *Journal of Dairy Science* 93, 2926-2937.

Gatti, M., Zunino, P., Ungerfeld, R., 2011. Changes in the aerobic vaginal bacterial mucous load after treatment with intravaginal sponges in anoestrous ewes: effect of

medroxiprogesterone acetate and antibiotic treatment use. *Reproduction in Domestic Animals* 46, 205-208.

Ginther, O.J., 2007. *Ultrasonic Imaging and Animal Reproduction: Color-Doppler Ultrasonography*. Equiservices Publishing, Wisconsin, United States of America.

Goddard, P.J., 1995. *Veterinary Ultrasonography*. CABI, Wallingford, United Kingdom.

Gray C.A., Stewart, M.D., Johnson, G.A., Spencer, T.E., 2003. Postpartum uterine involution in sheep: histoarchitecture and changes in endometrial gene expression. *Reproduction* 125, 185-198.

Griffin, P.G., Ginther, O.J., 1991. Uterine morphology and function in postpartum mares. *Equine Veterinary Science* 11, 330-339.

Griffin, J.F.T., Hartigan, P.J., Nunn, W.R., 1974. Non-specific uterine infection and bovine fertility I. Infection patterns and endometritis during the first seven-weeks post-partum. *Theriogenology* 1, 91-106.

Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., Walters, J.L., 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. *Veterinary Immunology Immunopathology* 113, 21-29.

Hauser, B., Bostedt, H., 2002. Ultrasonographic observations of the uterine regression in the ewe under different obstetrical conditions. *Journal of Veterinary Medicine A* 49, 511-516.

Hayder, M., Ali, A., 2008. Factors affecting the postpartum uterine involution and luteal function of sheep in the subtropics. *Small Ruminant Research* 79, 174-178.

Hurley, J.V., 1983. *Acute Inflammation*, 2nd edn. Chruchill Livingstone, Edinburgh, United Kingdom.

Hussain, S.O., Al-Zubaili, S.F., Asofi, M., 2013.Different endometritis treatments in ewe: Comparative study. Journal of Agricultural and Veterinary Sciences 3, 91–94.

Irie, M., 1987. Observations on the uterus of postpartum sows using a linear electronic scanner. *Japanese Journal of Zootechnical Science* 58, 407-412

Ishwar, A.K., 1995. Pregnancy diagnosis in sheep and goats: a review. *Small Ruminant Research* 17, 37-44.

Kähn, W., 1992. Ultrasonography as a diagnostic tool in female animal reproduction. *Animal Reproduction Science* 28, 1-10.

Kähn, W., 2004. *Veterinary Reproductive Ultrasonography*. Schlutersche, Hannover, Germany.

Karagiannis, I., Brozos, C., Kiossis, E., Boscos, C., Fthenakis, G., Panousis, N., 2018. Associations of periparturient β-hydroxybutyric acid and non-esterified fatty acids blood serum concentrations with milk yield, milk composition and milk somatic cells count of intensively managed Chios dairy ewes. *Journal of the Hellenic Veterinary Medical Society* 69, 1195-1204.

Kasimanickam R., Duffield T.F., Foster R.A., Gartley C.J., Leslie K.E., Walton J.S., Johnson W.H., 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology* 62, 9-23.

Keisler, D.H., Buckrell, B.C., 1997. Breeding strategies. In: R.S. Youngquist (ed.) *Current Therapy in Large Animal Theriogenology*. Saunders, Philadelphia, United States of America; pp. 602–611.

Kim, S.Y., Nguyen, A.D., Gao, J.L., Murphy, P.M., Mansfield, B.C., Chou, J.Y., 2006. Bone marrow-derived cells require a functional glucose 6-phosphate transporter for normal myeloid functions. *Journal of Biological Chemistry* 281, 28794-28801.

Leung, S.T., Derecka, K., Mann, G.E., Flint, A.P.F., Wathes, D.C., 2000. Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. *Journal of Reproduction and Fertility* 119, 25-33.

Lewis, G.S., Wulster-Radcliffe, M.C., 2013. Prostaglandin F-2 alpha upregulates uterine immune defenses in the presence of the immunosuppressive steroid progesterone. *American Journal of Reproductive Immunology* 56,-102-111.

Madoz, L.V., Giuliodori, M.J., Giuliodori, M.J., Migliorisi, A.L., Jaureguiberry, M., de la Sota, R.L., 2014. Endometrial cytology, biopsy, and bacteriology for the diagnosis of subclinical endometritis in grazing dairy cows. *Journal of Dairy Science* 97, 195-201.

Manes J., Fiorentino M.A., Kaiser G., Hozbor F., Alberio R., Sanchez E., Paolicchi F., 2010. Changes in the aerobic vaginal flora after treatment with different intravaginal devices in ewes. *Small Ruminant Research* 94, 201-204.

Manes J., Fiorentino M.A., Martino S.S., Ungerfeldd R., 2018. Changes in the vaginal microbiota in ewes after insertion of intravaginal sponges at different stages of the oestrous cycle. *Livestock Science* 208, 55-59.

Mantziaras, G., Vasileiou, N.G.C., Ioannidi, K.S., Mavrogianni, V.S., Gougoulis, D.A., Fthenakis, G.C., Petridis, I.G., Barbagianni, M.S., 2018. Use of contrast-enhanced ultrasonographic examination to evaluate health status of mammary glands of ewes at the end of a lactation period. *Journal of Dairy Research* 85, 39-43.

Maulik, D., 2005. Spectral Doppler sonography: waveform analysis and hemodynamic information. In: D. Maulik (ed.) *Doppler Ultrasound in Obstetrics and Gynecology*, 2nd edn. Springer, Berlin, Germany; pp. 35-56.

Mavrogianni, V.S., Amiridis, G.S., Gougoulis, D.A., Fragkou, I.A., Fthenakis, G.C., 2007a. Efficacy of difloxacin for the control of postpartum uterine infections of ewes. *Journal of Veterinary Pharmacology and Therapeutics* 30, 583-585.

Mavrogianni, V.S., Brozos, C., 2008. Reflections on the causes and the diagnosis of peri-parturient losses of ewes. *Small Ruminant Research 76*, 77–82.

Mavrogianni, V.S., Cripps, P.J., Fthenakis, G.C., 2007b. Bacterial flora and risk of infection of the ovine teat duct and mammary gland throughout lactation. *Preventive Veterinary Medicine* 79, 163–173.

Mavrogianni, V.S, Papadopoulos E., Spanos S.A., Mitsoura A., Ptochos S., Gougoulis D.A., Barbagianni M.S., Kyriazakis I., Fthenakis G.C., 2014. Trematode infections in pregnant ewes can predispose to mastitis during the subsequent lactation period. *Research in Veterinary Science* 96, 171–179.

Medan, M.S., El-Daek, T., 2015. Uterine involution and progesterone level during the postpartum period in Barbady ewes in north Libya. *Open Veterinary Journal* 5, 18-22.

Meira, E.B.S., Henriques, L.C.S., Sá, L.R.M., Gregory, L., 2012. Comparison of ultrasonography and histopathology for the diagnosis of endometritis in Holstein-Friesian cows. *Journal of Dairy Science* 95, 6969-6973.

Miles, A.A., Misra, J.S., 1938. The estimation of the bactericidal power of the blood. *Journal of Hygiene, Cambridge* 38, 732-749.

National Institutes of Health, 2013. *Image J: Image Processing and Analysis in Java*. http://rsbweb.nih.gov/ij/.

Navarro-Garcia, F., Ruiz-Perez, F., Cataldi, Á., Larzábal, M., 2019. Type VI secretion system in pathogenic *Escherichia coli*: structure, role in virulence, and acquisition. *Frontiers in Microbiology* 10, 1965.

Noakes, D.E., 2009. The puerperium. In: D.E. Noakes, T.J. Parkinson, G.C.W. England (eds) *Veterinary Reproduction and Obstetrics*, 9th edn. Saunders, Edinburgh, United Kingdom; pp. 194-205.

Ojala, T., Pietikaeinen, M., Maeenpaea, T., 2002. Multiresolution gray-scale and rotation invariant texture classification with local binary patterns. *IEEE Transactions on Pattern Analysis and Machine Intelligence* 24, 971-987.

Okano, A., Tomizuka, T., 1987. Ultrasonic observation of postpartum uterine involution in the cow. *Theriogenology* 27, 369-376.

Orfanou, D.C., 2012. *Investigations into the Puerperium of Beagle-Breed Female Dogs*. PhD thesis, University of Thessaly, Volos, Greece.

Orihuela, A., Fierros-Garcia, A., Hallal-Calleros, C., Robles-Castro, S.R., Ungerfeld, R., 2019. Vaginal biota number is smaller in tailed than docked hair ewes (*Ovis aries*), but is not affected by copulation. *Tropical Animal Health and Production* 51, 993-995.

Paiano, R.B., Birgel, D.B., Birgel, E.H., 2019. Uterine involution and reproductive performance in dairy cows with metabolic diseases. *Animals* 9, 93.

Palmieri, C., Schiavi, E., Salda, L.D., 2011. Congenital and acquired pathology of ovary and tubular genital organs in ewes: a review. *Theriogenology 75*, 393–410.

Panousis, N., Brozos, C., Karagiannis, I., Giadinis, N.D., Lafi, S., Kritsepi-Konstantinou, M., 2012. Evaluation of Precision Xceed® meter for on-site monitoring of blood beta-hydroxybutyric acid and glucose concentrations in dairy sheep. *Research in Veterinary Science* 93, 435-439.

Perumamthadathil, C.S., Johnson, W.H., LeBlanc, S.J., Foster, R.A., Chenier, T.S., 2014. Persistence of oxytocin receptors in the bovine uterus during the first 7 d after calving: an immunohistochemical study. *Canadian Journal of Veterinary Research* 78, 72-77.

Petridis, I.G., Barbagianni, M.S., Ioannidi, K.S., Samaras, E., Fthenakis, G.C., Vloumidi, E.I., 2017. Doppler ultrasonographic examination in sheep. *Small Ruminant Research* 152, 22-32.

Pharr, J.W., Post, K., 1992. Ultrasonography and radiography of the canine post-partum uterus. *Veterinary Radiology and Ultrasound* 33, 35-40.

Prevost, M., Zelkowitz, P., Tulandi, T., Hayton, B., Feeley, N., Carter, S., Joseph, L., Pournajafi-Nazarloo, H., Ping, E.Y., Abenhaim, H., Gold, I., 2014. Oxytocin in pregnancy and the postpartum: relations to labor and its management. *Frontiers in Public Health* 2, 1.

Ramadan, A.A., Johnson, G.L., Lewis, G.S., 1997. Regulation of uterine immune function during the estrous cycle and in response to infectious bacteria in sheep. *Journal of Animal Science* 75, 1621-1632.

Roche, J.R., Burk, C.R., Crookende, M.A., Heiser, A., Loor, J.L., Meier, S., Mitchell, M.D., Phynand, C.V.C., Turner, S.A., 2018. Fertility and the transition dairy cow. *Reproduction, Fertility and Development* 30, 85-100.

Rubianes, E., Ungerfeld, R., 1993. Uterine involution and ovarian changes during early post-partum in autumn-lambing corridale ewes. *Theriogenology* 40, 365-372.

Said, S., Johansson, E.D., Gemzell, C., 1974. Return of ovulation during the post-partum period. *Acta Obstetricia et Gynecologica Scandinavica* 53, 63-67.

Samartzi, F., Fthenakis, G.C., 2003. Control of oestrous cycle in small ruminants. *Journal of the Hellenic Veterinary Medical Society* 54, 351-361.

Sana, T.G., Flaugnatti, N., Lugo, K.A., Lam, L.H., Jacobson, A., Baylot, V., Durand, E., Journet, L., Cascales, E., Monck, D.M., 2016. *Salmonella Typhimurium* utilizes a T6SS-mediated antibacterial weapon to establish in the host gut. *Proceedings of the National Academy of Sciences of the United States of America* 23, E5044-E5051.

Sargison, N., 2008. *Sheep Flock Health A Planned Approach*. Blackwell, Oxford, United Kingdom.

Schlumbohm, C., Harmeyer, J., 2004. Hyperketonemia impairs glucose metabolism in pregnant and nonpregnant ewes. *Journal of Dairy Science* 87, 350-358.

Schneider, M., 1999. Characteristics of SonoVue®. *Echocardiography* 16, 743-746.

Shao, C., Wang, H., Wang, X., Jang, S., Sun, J., Song, H., Li, J., 2017. Characterization of inflammatory responses by cervical cytology, cytokine expression and ultrastructure changes in a goat subclinical endometritis model. *Journal of Veterinary Medical Science* 79, 197-205.

Sheldon, I.M., Cronin J., Goetze, L., Donofrio, G., Schuberth, J., 2009. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biology of Reproduction* 81, 1025-1032.

Singh, J., Murray, R.D., Mshelia, G., Woldehiwet, Z., 2008. The immune status of the bovine uterus during the peripartum period. *Veterinary Journal* 175, 301-309.

Smith, K., 2009. Infertility and subfertility in the ewe and doe (female goat). In: D.E. Noakes, T.J. Parkinson, G.C.W. England (eds) *Veterinary Reproduction and Obstetrics*, 9th edn. Saunders, Edinburgh, United Kingdom; pp. 559-581.

Tian, W., Noakes, D.E., 1991. A radiographic method for measuring the effect of exogenous hormone therapy on uterine involution in ewes. *The Veterinary Record* 129, 463-466.

Tsousis, G., Brozos, C., Fthenakis, G.C., 2011. Post-partum metritis, prolapse of the uterus, retention of foetal membranes. In: G.C. Fthenakis (ed.) *Small Ruminant Reproduction*. Tziola Publications, Thessaloniki, Greece; pp. 311-314.

Tzora, A., Leontides, L.S., Amiridis, G.S., Manos, G., Fthenakis, G.C., 2002. Bacteriological and epidemiological findings during examination of the uterine content of ewes with retention of fetal membranes. *Theriogenology* 57, 1809-1817.

Valasi, I., Barbagianni, M.S., Ioannidi, K.S., Vasileiou, N.G.C., Fthenakis, G.C., Pourlis, A., 2017. Developmental anatomy of sheep embryos, as assessed by means of ultrasonographic evaluation. *Small Ruminant Research* 152, 56-73.

Valasi, I., Menegatos, I., Gougoulis, D.A., 2011. Physiology of the puerperium. In: G.C. Fthenakis (ed.) *Small Ruminant Reproduction*. Tziola Publications, Thessaloniki, Greece; pp. 127-132.

Walsh, R.B., Walton, J.S., Kelton, D.F., LeBlanc, S.J., Leslie, K.E., Duffield, T.F., 2007. The effect of subclinical ketosis inearly lactation on reproductive performance of postpartum dairy cows. *Journal of Dairy Science* 90, 2788-2796.

Wathes, D.C., Fenwick, M., Cheng, Z., Bourne, N., Llewellyn, S., Morris, D.G., Kenny D., Murphy J., Fitzpatrick R., 2007. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology* Suppl. 68, 232-241.

Wood, M., Romine, L.E., Lee, Y.K., Richman, K.M., O'Boyle, N.M.K., Paz, D.A., Chu, P.K., Pretorius, D.H., 2010. Spectral Doppler signature waveforms in ultrasonography: a review of normal and abnormal waveforms. *Ultrasound Quarterly* 26, 83-99.

Wright, P.J., Geytenbeek, P.E., Clarke, I.J., Findlay, J.K., 1981. Evidence for a change in oestradiol negative feedback and LH pulse frequency in postpartum ewes. *Journal of Reproduction and Fertility* 61, 97-102.

Yeager, A.E., Concannon, P.W., 1990. Serial ultrasonographic appearance of postpartum uterine involution in beagle dogs. *Theriogenology* 34, 523-535.

Zhao, W., Caro, F., Robins, W., Mekalanos, J.J., 2018. Antagonism toward the intestinal microbiota and its effect on *Vibrio cholera* virulence. *Science* 359, 210-213.