

Journal of the Hellenic Veterinary Medical Society

Vol 65, No 1 (2014)



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doi: [10.12681/jhvms.15509](https://doi.org/10.12681/jhvms.15509)

To cite this article:

TSILIGIANNI (Θ. ΤΣΙΛΙΓΙΑΝΝΗ) T. (2017). Induction of oestrus in ewes of the rare Greek breeds Skopelos, Zakynthos, Kymi - Electrical resistance of cervical mucous. *Journal of the Hellenic Veterinary Medical Society*, 65(1), 23–30. <https://doi.org/10.12681/jhvms.15509>

■ Induction of oestrus in ewes of the rare Greek breeds Skopelos, Zakynthos, Kymi - Electrical resistance of cervical mucous

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■ Πρόκληση οίστρου σε προβατίνες των σπάνιων φυλών Σκοπέλου, Ζακύνθου και Κύμης - Ηλεκτρική αντίσταση της τραχηλικής βλέννας

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ABSTRACT. Objective of the study was the investigation of the response of Skopelos, Zakynthos and Kymi ewes to oestrus induction treatment. Furthermore, feasibility of measuring electrical resistance of cervical mucus for oestrus detection was examined. Oestrus was induced by intravaginal insertion of sponges impregnated with 60 mg medroxyprogesterone acetate for 12 days [groups: Skopelos (n=17), Zakynthos (n=18), Kymi (n=22)], followed by intramuscular injection of 400 iu equine chorionic gonadotropins at the time of withdrawal. Blood samples were collected 10 days before sponge insertion, at insertion and withdrawal and at oestrus for measurement of oestradiol 17-β and progesterone concentration. Electrical resistance of cervical mucus was measured after oestrus detection, then ewes were mated. Proportion of Zakynthos ewes (100%) in oestrus was greater than that of Kymi (64%) or Skopelos (78%) ewes. Pregnancy rate ranged from 50% (Kymi ewes) to 67% (Skopelos ewes). Electrical resistance of cervical mucus of ewes that conceived was significantly smaller than of those that did not. Based on results of progesterone blood concentration measurement 10 days before and at sponge insertion, only Kymi ewes were at full anoestrus, whilst Zakynthos and Skopelos ewes were in the breeding season. Progesterone blood concentration at sponge withdrawal and oestradiol 17-β blood concentration at oestrus were significantly greater in Zakynthos ewes that conceived compared to those in ewes that did not conceive. Furthermore, progesterone concentration at sponge insertion was significantly greater in all ewes that conceived compared to that in ewes that did not. There were significant differences in oestradiol-17β and progesterone blood concentrations among breeds and at the various time-points of blood collection. It is concluded that response of the above sheep breeds to oestrus induction was satisfactory. Hormonal status of ewes during sponge insertion is of importance for conception. Measurement of electrical resistance of cervical mucus could be useful for selection of ewes for mating, even if rams used to detect oestrous.

Keywords: electrical resistance of cervical mucus, ewe, oestradiol 17β, oestrus induction, progesterone, rare breed

ΠΕΡΙΛΗΨΗ. Σκοπός της μελέτης ήταν η αξιολόγηση της ανταπόκρισης στην αγωγή για πρόκληση οίστρου σε προβατίνες των φυλών Σκοπέλου, Ζακύνθου και Κύμης. Επίσης, διερευνήθηκε η δυνατότητα προσδιορισμού της ηλεκτρικής αντίστασης της τραχηλικής βλέννας για προσδιορισμό προβατινών σε οίστρο. Η πρόκληση οίστρου έγινε με ενδοκολπική τοποθέτηση σπόγγων εμποτισμένων με 60 mg οξικής μεδροξυπρογεστερόνης επί 12 ημέρες [προβατίνες Σκοπέλου (n=17), Ζακύνθου (n=18), Κύμης (n=22)], ακολουθούμενη από ενδομυϊκή ένεση 400 IU ίππειας χοριακής γοναδοτροπίνης. Δείγματα αίματος, για προσδιορισμό

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Date of initial submission: 9 August 2013
Date of revised submission: 10 September 2013
Date of acceptance: 10 September 2013

Ημερομηνία αρχικής υποβολής: 9 Αυγούστου 2013
Ημερομηνία αναθεωρημένης υποβολής: 10 Σεπτεμβρίου 2013
Ημερομηνία αποδοχής: 10 Σεπτεμβρίου 2013

της συγκέντρωσης οιστραδιόλης 17β και προγεστερόνης, συλλέχθηκαν 10 ημέρες πριν από την εισαγωγή των σπόγγων, κατά την εισαγωγή και την αφαίρεση αυτών και τον οίστρο. Η ηλεκτρική αντίσταση τραχηλικής βλέννας προσδιορίστηκε αμέσως μόλις οι προβατίνες ανιχνεύθηκαν σε οίστρο και, τελικά, πραγματοποιήθηκαν ελεύθερες οχείες. Η αναλογία προβατινών που εκδήλωσαν οίστρο στα ζώα φυλής Ζακύνθου (100%), ήταν μεγαλύτερη από αυτήν στα ζώα φυλής Κύμης (64%) ή Σκοπέλου (78%). Το ποσοστό εγκυμοσύνης κυμάνθηκε από 50% (προβατίνες Κύμης) έως 67% (προβατίνες Σκοπέλου). Σε προβατίνες που κυοφόρησαν, η ηλεκτρική αντίσταση τραχηλικής βλέννας ήταν σημαντικά μικρότερη από αυτήν σε προβατίνες που δεν ήταν έγκυες τελικά. Λαμβάνονταν υπόψη τη συγκέντρωση της προγεστερόνης 10 ημέρες πριν την εισαγωγή των σπόγγων και κατά την εισαγωγή αυτών, οι προβατίνες της φυλής Κύμης βρίσκονταν σε άνοιτρο, ενώ αυτές των φυλών Σκοπέλου και Ζακύνθου βρίσκονταν σε οιστρική περίοδο. Η συγκέντρωση της προγεστερόνης κατά την αφαίρεση των σπόγγων και της οιστραδιόλης-17β κατά τον οίστρο, στις προβατίνες της φυλής Ζακύνθου που κυοφόρησαν, ήταν σημαντικά μεγαλύτερη από τις αντίστοιχες σε αυτές που δεν κυοφόρησαν. Επίσης, η συγκέντρωση της προγεστερόνης κατά την εισαγωγή των σπόγγων ήταν σημαντικά μεγαλύτερη σε όλες τις προβατίνες που κυοφόρησαν σε σύγκριση με αυτές που δεν κυοφόρησαν. Σημαντικές διαφορές παρατηρήθηκαν στις συγκεντρώσεις της προγεστερόνης και της οιστραδιόλης 17β μεταξύ των τριών φυλών, σε διάφορες φάσεις του πειραματισμού. Συμπεραίνεται ότι, οι προβατίνες των σπάνιων φυλών ανταποκρίθηκαν ικανοποιητικά στην αγωγή πρόκλησης οίστρου. Το ορμονικό πρότυπο των προβατινών κατά την εισαγωγή των σπόγγων είναι σημαντικό για την κυοφορία. Η μέτρηση της ηλεκτρικής αντίστασης της τραχηλικής βλέννας θα μπορούσε να χρησιμοποιηθεί για την εντόπιση των προβατινών που θα οχευθούν, ακόμα και στην περίπτωση που χρησιμοποιούνται κριοί για την ανίχνευση των οίστρων.

Λέξεις ευρετηρίασης: ηλεκτρική αντίσταση τραχηλικής βλέννας, οιστραδιόλη 17β, πρόβατο, προγεστερόνη, πρόκληση οίστρου, σπάνια φυλή.

INTRODUCTION

There are many native breeds in each country and they are closely related to the socio-economical development and especially to milk products of each country and each region. It is important to study these breeds in order to maintain them and help the local economy of each region. The purpose of the present study was to investigate the response of three native Greek breeds of ewes (Skopelos, Zakynthos and Kymi) that considered rare to oestrus induction, in order to help producers to maintain these breeds. The originality of the present study is not the treatment used for oestrus induction (the most common treatment was used), but the response of three rare breeds of ewes on oestrus induction treatment. Furthermore, the effectiveness of an oestrus detector for cows (a device for the measurement of cervical mucus electrical resistance) was checked (Adams et al., 1981).

Certain parameters related to these breeds are presented in Table 1, in order to indicate the significance of maintaining them (Rogdakis, 2002; Georgoudis et al., 2011). It is important to notice that ewes of Skopelos breed are adapted perfectly to machine milking; Zakynthos breed is under threat for disappearance,

in spite of the excellent productivity and its resistance to diseases, because the 1st generation after crossbreed with Frisarta breed has increase productivity and ewe breeders use to follow this procedure. Kymi breed is not popular and also under threat for disappearance, because it is sensitive to unfavourable conditions.

Acute oestrus detection is the key point for increasing pregnancy rate after oestrus induction. Unfortunately, oestrus detection is a huge problem for ewe producers because it is time consuming, demands personnel and extra cost. This problem leads many ewe producers to avoid oestrous detection and leave the rams into the ewe flock, in order to increase pregnancy rates. However, another major problem come up; the inability of keeping data about breeding. During the past four decades, the majority of studies have been focused on testing various devices for predicting and confirming the fertile period during oestrous (Rezac, 2008). Although a variety of devices is commercially available for cattle (Rorie et al., 2002), this is not the case for the sheep. In the latter species only apron-fitted or vasectomized rams can be used for oestrus detection. On the other hand, Kohno et al. (2005) suggested that oestrus detection in ewes by teaser rams is not a reliable

Table 1. Certain parametres related to Skopelos, Zakynthos and Kymi breed ewes.

	Skopelos	Zakynthos	Kymi
Number of ewes (n)	2815	830 (in 9 flocks)	680 (in 15 flocks)
Body weight of male animals (kg)	60-65	76-82	60-75
Body weight of female animals (kg)	40-50	65-75	55-65
Breeding season		end May to July	
Lambing season	October-April	November-December	October-March
Litter size	1.6-1.84	1.8-1.9	1.6-2.3
Newborn body weight (kg)	4-4.6	3-4	
Weaning body weight (kg) / age (days)	14-17 / 50	16-25 / 60	15-18 / 60

method in the field, because the condition of the rams, the ewes to ram ratio, as well as, the rams' scramble behavior for the ewes being in oestrus could lead in late detection of oestrus. Thus, the need for alternative and accurate methods for oestrus detection after oestrous synchronization in ewes could be of great importance for the sheep industry. Electrical resistance / impedance measurement near the cervix uteri is a useful tool for oestrus detection and ovulation and for the appropriate time for artificial insemination in cow and sow (Gupta and Purohit, 2001; Rorie et al., 2002; Yamaouchi et al., 2009). However, there are very few data available for ewe (Bartlewski et al. 1999). Ovarian follicular growth and luteal tissue development are associated with histological (Adams and Tang, 1979) and histochemical (Murdoch and O'Shea, 1978) changes in the mucosal layer of the genital tract, which is followed by alterations in electrical properties of the mucosal tissues (Adams et al., 1981). Vaginal impedance is probably influenced by alterations in circulating levels of progesterone and/or oestradiol during the oestrous cycle and may provide useful information about spontaneous and hormonally induced luteolysis (Bartlewski et al., 1999; Gupta and Purohit, 2001; Rezac, 2008). In some cases, only a change in total impedance contains enough information to be correlated with the biological event (Valentimuzzi et al., 1996).

Rare breeds of Skopelos, Zakynthos and Kymi

have good productive indexes and they are important for the regions they are reared; however, there are no data about the response of these breeds in oestrus induction treatment. Furthermore, there are not enough evident on alternative methods that could be used for oestrus detection in ewes. In the present research, electrical resistance of cervical mucus was investigated just before mating, in order to provide or not an index for improving pregnancy rates after oestrus induction treatment. According to the available data as concern breeding period, oestrus induction treatment was performed on April, which is out of the usual breeding period for all breeds studied.

MATERIALS AND METHODS

Animals and oestrus induction treatment

The study was conducted at the Agricultural Research Station of Halkidiki in Northern Greece, at the beginning of April. Seventeen ewes of Skopelos, 18 ewes of Zakynthos and 22 ewes of Kymi breed aging from 30 to 42 months were used. Mean body weight of Skopelos ewes was 42.2 ± 1.5 Kg, of Zakynthos ewes 71.5 ± 2.3 Kg and of Kymi ewes 43.00 ± 1.30 Kg. All ewes were kept in a sheltered barn and had access to a natural pasture. The animals were in their second or third lactation and had their last lambing 2-3 months before the treatment. All ewes were at the anoestrous

period, according to the available data.

The oestrous cycles of all ewes were induced using medroxyprogesterone acetate (60 mg) impregnated intravaginal sponges (Veramix, Upjohn, USA) for 12 days, combined with a single dose of 400 iu equine chorionic gonadotropin (Intergonan, Intervet, Netherlands) at the time of sponge withdrawal.

Oestrus detection and mating

Estrus detection was carried out twice daily by a ram of each breed from 24h to four days after sponge withdrawal. Any ewe standing to be mounted by the ram was considered as being in oestrus. All ewes detected in oestrus in that case were mated by rams of known proven fertility. Three rams of Skopelos, three rams of Zakynthos and four rams of Kymi breed were used.

Blood sample and hormone measurement

One blood sample was collected from each ewe by jugular venipuncture, at 10 days before sponge insertion, at sponge insertion and withdrawal and at oestrus (after oestrus detection by teaser rams and just before mating). Blood was allowed to coagulate spontaneously at room temperature and serum was obtained by centrifugation (2500 rpm; 20 min; 4 °C). Serum samples were stored at -20 °C until assayed. Serum oestradiol-17 β and progesterone concentrations were determined using radioimmunoassay (Martin et al., 1987), with minor modifications (Rekkas et al., 1991), as described previously (Theodosiadou et al., 2004). The radiolabelled solutions of oestradiol [(2,4,6,7-³H) oestradiol] and progesterone [(1,2,6,7-³H) progesterone] were developed by Amersham Biotech, England. The lower limit of sensitivity for oestradiol-17 β was 3.90 pg/mL, the intra-assay variability was 1.8-2.2% (n=6) and the recovery rate was 96.20% \pm 2.0% (mean \pm sd, n=6). The lower limit of sensitivity for progesterone was 0.019 ng/ml, the intra-assay variability was 1.6-1.8% (n=6) and the recovery rate was 97.20% \pm 1.4% (mean \pm sd, n=6).

Electrical resistance of cervical mucus

The electrical resistance of cervical mucus was measured using a digital oestrus detector for cows (Cyclus, A.S. Lima, Sandnes, Norway). The detector is a 9 volt operated ohmmetre (measurement range 0-1990 ohm) consisting of a flexible probe permanently attached to the gauge. A standardized procedure was used for all ewes; the vulva of the ewe and the probe were cleaned, disinfected, rinsed with distilled water and dried. Thereafter, the lips of the vulva were spread apart and the probe was inserted into the vaginal vestibule of each ewe as far in as possible (until resistance was felt) in close proximity and posterior to the uterine cervix. It was turned clockwise for two and a half revolutions to scoop up mucus and then the measurement value was read. Three consecutive readings of electrical resistance were taken before the probe was removed from the vagina, and the mean value was recorded. Electrical resistance measurements were carried out once just after oestrus detection by teaser rams and before mating.

Pregnancy diagnosis

Pregnancy diagnosis was carried out by trans-abdominal ultrasonography (4.5-6 MHz convex transducer; SonoVet 2000; Medison CO, Seoul, Korea) on day 35-40 after mating.

Statistical analysis

Pearson's chi-square test was used to compare the oestrus detection rate (%) and the pregnancy rate (%). Student t-test for independent samples was used to compare the oestradiol-17 β and progesterone concentrations and the electrical resistance of cervical mucus between ewes conceived and those that did not. Analysis of variance was used to compare mean values of all parameters measured in the present study among breeds. Comparisons between means were performed by Duncan's new multiple range test. The results are expressed as mean \pm sem. Statistical differences were

Table 2. Oestrus detection rate, pregnancy rate and electrical resistance of cervical mucus of Skopelos, Zakynthos and Kymi breed ewes at oestrus.

	Skopelos	Zakynthos	Kymi
Oestrus detection rate (%)	77.80 ^{a,b}	100 ^a	63.60 ^b
Pregnancy rate (%)	66.70 ^a	55.56 ^a	50.00 ^a
Electrical resistance of cervical mucus (all ewes) (ohm)	313.89±45.73 ^a	400.00±54.84 ^a	476.70±68.28 ^a
Electrical resistance of cervical mucus (pregnant ewes) (ohm)	253.33±20.32	346.00±33.07	360.31±38.49
Electrical resistance of cervical mucus (non-pregnant ewes) (ohm)	535.00±22.91*	487.50±36.13*	710.83±188.54*

^{a, b} Significant difference ($P < 0.05$) among breeds, * Significance different between pregnant and non-pregnant ($P < 0.001$).

considered significant when $P < 0.05$. Statistical analysis was performed using SPSS ® 15.0 for Windows (SPSS Inc., Athens, A.E., Greece).

RESULTS

Oestrus detection rate (%), pregnancy rate (%) and the electrical resistance of cervical mucus value (ohm) of Skopelos, Zakynthos and Kymi breed at oestrus are presented in Table 2. It is important that oestrus detection rate ranged from 64% in Kymi ewes to 100% in Zakynthos ewes, while pregnancy rate ranged from 50% in Kymi ewes to 67% in Skopelos ewes. The electrical resistance of cervical mucus value was significantly lower in ewes of all breeds conceived compared to those did not.

Oestradiol-17 β concentrations at oestrus in Zakynthos ewes and progesterone concentrations at sponge insertion in ewes of all breeds were significantly higher in ewes that conceived compared to those that did not. There were significant differences in oestradiol-17 β and progesterone concentration among breeds and at different time points.

DISCUSSION

The response of three rare breeds on oestrus induction by a common used treatment (medroxyprogesterone acetate impregnated intravaginal sponges

for 12 days) was investigated in the present study. These breeds are important for the regions they are reared; however, there are no available data about their response on oestrus induction. Furthermore, efficient and acute oestrus detection is the most important factor for successful mating in ewes. Therefore, simple and more effective techniques are required to detect oestrus and the optimal time for mating (Yamauchi et al., 2009). Changes in electrical resistance of vaginal and cervical mucus have been used to predict the best time for artificial insemination in all farm animals with varying degrees of success (Bartlewski et al., 1998; 1999; Gupta and Purohit, 2001; Rorie et al., 2002; Rezac, 2008). However, the available data for ewes are scarce (Bartlewski et al., 1998). The electrical resistance after oestrus detection by teaser ram was studied in order to estimate the efficiency and accuracy of detecting oestrus in ewes. This method could have practical implications in appointing the most appropriate time for mating.

According to the available data, breeding season for Zakynthos breed is from May to July and lambing season for Skopelos breed is from October to April and for Kymi breed is from October to March (Rogdakis, 2002). These lead to the logical assumption that breeding season for Skopelos and Kymi breed start on May also. In the present study oestrus induction treatment was performed at the beginning of April that is con-

sidered out of the normal breeding season. Menegatos et al. (1995) reported that progesterone concentration $>1 \text{ ng mL}^{-1}$ observed at the beginning of the breeding season. Taking into account progesterone concentration ($<1 \text{ ng mL}^{-1}$) at 10 days before sponge insertion and at sponge insertion only Kymi ewes might were at full anoestrus period. Zakynthos ewes could be at the beginning of breeding season and Skopelos ewes were at breeding season. All ewes were born and reared in Halkidiki, that is far away from the area of their origin and they were adapted to climate conditions of this area. It seems that breeding season is affected by the area where these breeds are reared; it started earlier than expected in Skopelos and Zakynthos ewes. Furthermore, there were significant differences in oestradiol- 17β and progesterone concentrations among breeds and at different time points. These differences could be related to the phase of the oestrous cycle or the breeding or nonbreeding season.

Oestrus detection rates were above 60% and pregnancy rates were above 50%. The response of all rare breeds studied on oestrus induction was satisfactory as concern oestrus detection rate and pregnancy rate. Oestrus induction treatment provides encouraging results for all three breeds, taking into account previous studies (Kohno et al., 2005). However, taking into account the breeding or nonbreeding season, Zakynthos ewes that were at the beginning of the breeding season presented the best results as concern oestrus detection rate; while Skopelos ewes that were at full breeding season and Kymi ewes that were at full anoestrus presented satisfactory results as concern oestrus detection rate. In my opinion, pregnancy rate was satisfactory only for Skopelos and Kymi ewes but not for Zakynthos ewes (all ewes detected in oestrus and only half conceived). Zakynthos ewes are heavier than the other two breeds; however the same dose of eCG was used for all ewes. Follicle development is often stimulated by using eCG to enhance the recruitment of small follicles, increase ovulation rates and increase the syn-

chrony of oestrus after oestrous synchronisation treatments (Evans 2004). In Zakynthos ewes, the eCG dose could affect follicle development and ovulation, and as a result, pregnancy rate. Further research is needed in order to improve pregnancy rate in these breeds. Other treatments and different doses could be checked in each breed. However, it was not in the aims of the present study to investigate different treatments.

All ewes detected in oestrus by teaser rams, while only those with electrical resistance of cervical mucus $<400 \text{ ohm}$ finally conceived. To be more specific, mating could be performed when electrical resistance of cervical mucus is: a) $<280 \text{ ohm}$ in Skopelos ewes; they were at breeding season, b) $<380 \text{ ohm}$ in Zakynthos ewes; they were at the beginning of breeding season and c) $<400 \text{ ohm}$ in Kymi ewes; they were at full anoestrus. It seems that electrical resistance of cervical mucus present differences as concern breeding or non-breeding season, or breed could affect electrical resistance of cervical mucus value. Electrical resistance could be used for oestrus detection in ewes; however, at the moment, electrical resistance of cervical mucus measurement every 12 hours for three consecutive days starting 24 h after sponge withdrawal are needed to find the best time for mating. Cervical mucus characteristics change during oestrous cycle and they are affected by oestrous synchronization, especially when exogenous progestagens are used (Stefanakis, 1988). In ewe cervical mucus volume, crystallization and spinnbarkeit increased after oestrus synchronization by medroxyprogesterone (Stefanakis, 1988), and these differences could be reflected to electrical resistance of cervical mucus. Furthermore, these changes could be involved in the disorders of sperm transportation in the genital tract and in particular into the cervix and might related to low pregnancy rates. A close association between the lowest value of vaginal impedance during the follicular phase of the oestrous cycle and manifestations of oestrous behavior was observed in many species (Rezac, 2008). Furthermore, the follicular development

is accompanied by an elevation in plasma oestradiol level (Menegatos et al., 2003; Ginther et al., 2005) that induces female receptivity and a decrease in vaginal impedance. Thus, electrical resistance of cervical mucus could provide useful information about the development of preovulatory antral follicles in ewes. Furthermore, low electrical resistance of cervical mucus in ewes conceived could be related to better sperm transformation and/or survival of spermatozoa into the cervix or related to the development of preovulatory antral follicles.

Significantly lower oestradiol-17 β concentration was observed 10 days before sponge insertion compared to that on sponge insertion in Zakynthos and Kymi breed. Serum oestradiol-17 β concentration at oestrus was lower compared to sponge insertion. These findings are not rear, because there are periods of increased oestradiol secretion throughout the oestrous cycle in ewes (Campbell et al., 1995) and oestrogen patterns during oestrus varies significantly depending on the breed of the sheep (Kouskoura et al., 1995). In the present study oestradiol-17 β concentration was higher compared to that reported previously (Iida et al., 2004).

It is important to notice that only ewes of Zakynthos breed that conceived presented higher oestradiol-17 β at oestrus compared to those did not. Oestradiol-17 β is related to the number of follicles developed and the oestrus signs. Ewes detected in oestrus and ovulated in non-breeding season had higher oestradiol-17 β levels than did ewes that had silent ovulation (Ungerfeld et al. 2005). In the present study all ewes detected in oestrus by a ram. However, it is not known how many of them have ovulation and how many follicles developed. Therefore, the increased oestradiol-17 β at oestrus in Zakynthos ewes could be related to the number of follicles developed in ewes that conceived.

An important finding of the present study was that progesterone concentration at sponge insertion was significantly higher in ewes of all breeds that finally conceived compared to those did not. Low progesterone concentration at sponge insertion could be related to

the absence of a corpus luteum; thus, it could be suggested that the ovarian function at the time of sponge insertion plays a significant role on oestrus induction and on pregnancy rate. Johnson et al. (1996) reported that creation of lower than normal serum progesterone concentration in ewes resulted in larger follicles than normal. The majority of ewes that conceived presented preovulatory follicles with faster terminal growth and larger final ovulatory size than ewes that failed to conceive (Ben Salem et al., 2010); in the latter ewes the number of medium-sized follicles was increased, which could be a sign of deficiencies in dominance effect. It is suggested that progesterone concentration at sponge insertion could be related to follicles development and subsequent ovulation. Thus, progesterone concentration level during sponge insertion may be related to pregnancy rate.

CONCLUDING REMARKS

In conclusion, oestrus induction by medroxyprogesterone provided encouraging results as concern oestrus detection rate and pregnancy rate in all three rare breeds studied in the present study. According to progesterone concentration only Kymi ewes were at full anoestrus, Zakynthos ewes were at the beginning of breeding season and Skopelos ewes were at breeding season. Progesterone at sponge insertion is important as concern pregnancy rate in all breeds studied in the present study. Electrical resistance of cervical mucus after oestrous induction was significantly lower in ewes conceived compared to those did not, no matter if all ewes detected in oestrus by teaser rams. Low electrical resistance of cervical mucus values seems to be identical for mating. The electrical resistance of cervical mucus could be used as a reliable index for oestrus detection before mating. Further research is needed to find the best time for measuring electrical resistance of cervical mucus and to increase pregnancy rates after oestrous induction in ewes of the three rare breeds studied.

CONFLICT OF INTEREST STATEMENT

The author reports no conflict of interests.

ACKNOWLEDGEMENTS

The author would like to thank Dr E.Theodosiadou for the hormone measurement.

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