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# Evaluation of Factors Affecting Pregnancy Rate after Cervical Insemination of Dairy Ewes in Greece

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## Abstract

The objective of this study was to assess the factors affecting success of cervical Artificial Insemination (AI) with chilled semen in intensively reared dairy ewes in Greece. The study involved 1,785 adult ewes from 20 flocks of Lacaune and Chios sheep. A typical estrous synchronization protocol, including intravaginal placement of progestogen sponge for 14 days and injection of equine chorionic gonadotropin at sponge removal, was applied in ewes during mating period. All ewes were cervically inseminated 53-57 hours after sponge removal with chilled semen (15oC) collected from 10 adult purebred Lacaune rams. Pregnancy Diagnosis (PD) was performed by trans-dermal ultrasonography at 35-38 d after AI. The following data were available for each ewe: breed; parity; Body Condition Score (BCS) at sponge placement, at AI and at PD; presence of rams during synchronization and number of previous synchronizations. Recordings during the AI procedure included: onset of synchronization to AI interval; semen collection to AI interval; semen deposition depth; cervical mucus presence; order of ewe; average time required for each animal; ram semen used. The results showed that farm, parity, semen deposition depth, ram, ewe BCS and changes of BCS during the sponge placement-PD period, significantly affected AI success ($P<0.05$). Overall fertility was 44%. Ewes of 2nd (50.3%) and 3rd lactation (48.1%) had the highest conception rates, that declined as age increased. Ewes in moderate BCS (2.50-3.50) at onset of synchronization (48.4%) and AI (49.4%) had significantly higher fertility than ewes with low (<2.50) or high (>3.50) BCS. Positive energy balance following the onset of synchronization seems to benefit animals with low and moderate BCS, but decrease pregnancy rates of high BCS ewes. In conclusion, selection of appropriate ewes and rams, BCS evaluation prior to synchronization and adjustment of dietary management are key factors dictating pregnancy rates following AI in greek dairy sheep.

## Introduction

Assisted reproductive technologies in farm animals are used to cater the needs for higher productivity and better quality of products. In sheep production, the use of Artificial Insemination (AI) has enabled the rapid introduction of valuable genes that improved production traits and prevented disease transmission [1].

However, in comparison to other food producing animals, the implementation of AI in sheep globally, is relatively limited [2,3]. The only exception is France, where more than 410,000 inseminations are performed annually in both nucleus and commercial dairy flocks of the Lacaune breed [4]. An obstacle to the widespread use of AI in sheep is the structural complexity of the ewe cervix that prevents deep deposition of semen in uterus and leads to poor fertility rates when frozen-thawed semen is used for cervical AI [5,6]. The notion is that fertility rates can be enhanced by the application of laparoscopic insemination, but the procedure has increased costs, requires personnel with technical skills and raises welfare concerns. Using chilled semen for cervical AI enhances fertility, but increases semen production cost, has time limitations during transportation and often gives irregular results, since the success of the method is affected by many factors. Environmental conditions, management factors, health of males and females and physiological status of ewes are among the factors that need to be controlled before AI implementation [7-11].

Dairy sheep industry is of significant importance to national economy of most Mediterranean countries and Greece is one of them [12]. With around 6.6 million milking sheep, Greece is ranking 2nd on milking sheep population and 1st on ewe milk production (670,000 tons)inside EU28, comprising the 45% of national milk production [13]. Despite its importance, greek dairy sheep industry is characterized by relatively low productivity and is in a transitional phase of gradual intensification [14]. Usage of higher milk-production breeds, like the native Chios and the imported Lacaune breed, and application of better management techniques are becoming more common among farms. Nevertheless, application of AI for the genetic improvement of the animals is not used often and, despite the fact that some breeding programs are in operation, they are supported on the usage of home-bred or imported rams [15]. Although AI is commercially available in most areas, the high irregularity on fertility results makes difficult its widespread application.

Our objective was to carry out an artificial insemination programme to study the factors affecting the success of cervical Artificial Insemination (AI) with chilled semen in intensively reared dairy ewes in Greece. It is the first study that assesses the effect of female, ram, AI procedure conditions and farm nutrition program at the same time on fertility rates after AI on intensively reared dairy sheep in the area.

## Materials and Methods

### Animals

The study was conducted during the usual breeding season in Greece, from May to November, for two consecutive years (2017-2018). A total of 1785 adult ewes (1247 Lacaune and 538 Chios) were used from 20 commercial flocks located in North and Central region of Greece. Selected ewes belong to the most common intensively reared breeds in Greece and were born and raised in the above regions. During the study, the animals were at the 5-7th month of their lactation with an average daily milk yield of 1.32±0.22 liters.

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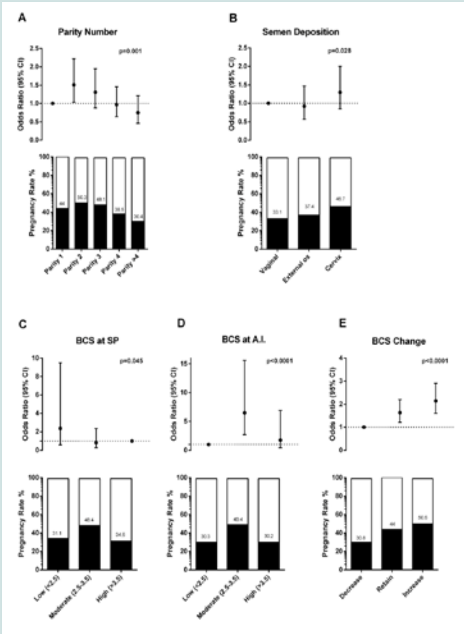


**Figure 1:** Pregnancy rates and adjusted odds ratio with 95% Confidence Interval of significant factors in final model: parity number (A), semen deposition site (B), BCS at SP (C), BCS at AI (D), BCS change (E).

Ewes received rations containing mainly alfalfa hay and alfalfa silage, supplemented with 0.800-1.400 kg. Of a concentrate mix of maize, soybean meal, barley and wheat middlings depending on their milk production. Ewes had free access to water and wheat straw that was offered ad-libitum in all cases.

### Estrous synchronization

Each ewe was treated with a sponge containing 20 mg Fluogestone Acetate (FGA) (CHRONOGEST CR®, MSD Animal Health), that was placed intravaginally for 14 days. At the day of sponge withdrawal, 500 IU (Lacaune) or 400 IU (Chios) of equine Chorionic Gonadotropin (eCG) (GONASER®, Hipra) were intramuscularly injected to the ewes.

### AI procedure

Semen was collected from 10 Lacaune rams that were located in the same semen collection center (OVIS PC, Greece), using an artificial vagina. Immediately after collection, motility and concentration of the undiluted semen were assessed. Only ejaculates with concentration greater than $3x10^9$ spermatozoa/ml and mass motility greater than 4, on the 0-5 scale described by Evans and Maxwell were used for the study [16]. After this evaluation, semen was diluted to concentration of 1,6x109 spermatozoa/ml using skimmed milk supplemented with antibiotics gradually cooled at 15 °C and loaded into 0,25 ml mini straws (400x106 spz/ dose) (IMV Technologies, France) [17]. The straws were transported on farm for use inside thermos flasks with acetic acid ampoules at 15 °C.

Cervical fixed-time AI was performed on each farm 53-57 hours after the sponge removal. Ewes were immobilized by two assistants, with hind legs lifted. In case of mucus presence inside vagina, the animal was put again in horizontal position and the mucus was removed using a speculum. AI was performed afterwards with the help of a speculum equipped with light source and an ovine AI gun (IMV Technologies, France). All artificial inseminations were carried out by the same technician within 8 hours after semen collection. During the procedure, ewes were kept on a restrained area and released to their boxes after insemination, or they were head-locked in feed alley whenever this was applicable Ultrasonography was performed 35-38 days after AI for Pregnancy Diagnosis (PD) using 5MHztransducerwith sector probe (ANIMAL PROFI, DRAMINSKI, Poland).

### Data collection

For all inseminated ewes, data concerning breed, parity and number of previous synchronizations were recorded. Body Condition Score (BCS) was assessed for each ewe at the time of sponge placement, AI and PD. BCS was assessed by palpation in the lumbar region by the same experienced evaluator. Scores assigned to the ewes were based on the existing scale of Russel et al. ranging from 0 to 5, according to which score (0) represents extremely emaciated animals, while the highest score (5) represents obese ones [18]; 0.25 and 0.5 unit increments were used. Changes of BCS between sponge placement and pregnancy diagnosis (51-54 days) were evaluated to determine whether the animals were in negative, zero or positive energy balance during that period.

At the time of AI, the following data were collected for each ewe: ram semen identification, semen collection-AI interval, sponge removal-AI interval, time required per AI, order number of the AI, presence of mucus in vagina, availability of headlocks for the procedure, presence of rams near the females during synchronization period and semen deposition site. The latter was distinguished in 3 classes depending on the deposition depth of the catheter and retrograde flow of semen: vaginal deposition, external cervical os deposition with partial semen backflow or deeper cervical placement

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**Table 1:** Description of risk factors assessed in the analysis.

| Risk Factors | Class | N | Mean | Included in full model | |
|---|---|---|---|---|---|
| **Farm** | 20 classes | 1.845 | | YES | *** |
| **Year** | 1 | 869 | | NO | n.s. |
| | 2 | 916 | | | |
| **Month** | May | 414 | | YES | ** |
| | June | 370 | | | |
| | July | 89 | | | |
| | August | 88 | | | |
| | September | 503 | | | |
| | October | 209 | | | |
| | November | 112 | | | |
| **Breed** | Lacaune | 1247 | | YES | *** |
| | Chios | 538 | | | |
| **Parity** | 1 | 175 | 2.99±1.17 | YES | *** |
| | 2 | 505 | | | |
| | 3 | 480 | | | |
| | 4 | 418 | | | |
| | >4 | 207 | | | |
| **BCS at SP** | Low (<2.5) | 295 | 2.99±0.6 | YES | *** |
| | Moderate (2.5-3.5) | 1278 | | | |
| | High (>3.5) | 212 | | | |
| **BCS at A.I.** | Low (<2.5) | 284 | 3.01±0.59 | YES | *** |
| | Moderate (2.5-3.5) | 1286 | | | |
| | High (>3.5) | 215 | | | |
| **BCS change** | BCS decrease | 393 | | YES | *** |
| | BCS retain | 589 | | | |
| | BCS increase | 803 | | | |
| **A.I. order** | 1 (1-25) | 500 | | NO | N.S |
| | 2 (26-50) | 484 | | | |
| | 3 (51-75) | 429 | | | |
| | 4 (76-100) | 297 | | | |
| | 5 (101-125) | 75 | | | |
| **Semen deposition depth** | Vagina | 118 | | YES | *** |
| | External os | 343 | | | |
| | Cervix | 1325 | | | |
| **Mucus presence** | No | 1340 | | NO | N.S |
| | Yes | 445 | | | |
| **Previous eCG administration** | No | 1523 | | YES | * |
| | Yes | 262 | | | |
| **Sponge removal to A.I. interval** | <55 hours | 447 | 54.98±0.7 | | *** |
| | 55 hours | 918 | | | |
| | ≥56 hours | 420 | | | |
| **Semen collection to A.I. interval** | <4 hours | 435 | 4.58±1.18 | YES | *** |
| | 4-6 hours | 1222 | | | |
| | >6 hours | 128 | | | |
| **A.I. speed sec/ animal** | | | 67.00±12.84 | YES | * |
| **Headlocks** | No | 1167 | | NO | N.S |
| | Yes | 502 | | | |
| **Presence of rams** | No | 1379 | | YES | *** |
| | Yes | 406 | | | |
| **Ram used** | 10 classes | | | YES | *** |

N.S: Not significant P≥ 0.25, *: P< 0.25, **: P< 0.05, ***: P<0.01

without semen backflow.

**Statistical analysis**

Binary logistic regression analysis (SPSS ver. 25.0, IBM) was used to determine the effect of risk factors to fertility of AI. Pregnancy diagnosis outcome at ultrasonography was used as the dependant variable. Logistic regression was conducted according to the method of Hosmer and Lemeshow with the following five steps: 1) preliminary screening of all variables for univariate associations, 2) construction of a full model using all the variables

**Table 2:** Semen deposition site and pregnancy rate in Lacaune and Chios ewes.

| Breed | Semen deposition site % (No. ewes) | | | Pregnancy rate (%) |
|---|---|---|---|---|
| | Vagina | External os | Cervix | |
| **Lacaune** | 5.1% (63)[a] | 16.4% (205)[a] | 78.5% (979)[a] | 48.3 |
| **Chios** | 10.2% (55)[b] | 25.5% (137)[b] | 64.3% (346)[b] | 34.2 |

Different superscripts within same column indicate significant differences (P< 0.05)

**Table 3:** Effect of BCS change on pregnancy rates of animals with different BCS.

| | *BCS at sponge placement* | | | *BCS at A.I.* | | |
|---|---|---|---|---|---|---|
| | <2.5 | 2.5-3.5 | >3.5 | <2.5 | 2.5-3.5 | >3.5 |
| **BCS decrease** | 0%[a] | 31.1%[b] | 43.5%[b] | 0%[a] | 32.8%[b] | 42.2%[b] |
| **BCS retain** | 13.5%[a] | 54.2%[b] | 26.2%[a] | 13.3%[a] | 54.2%[b] | 26.4%[a] |
| **BCS increase** | 48.4%[a] | 53.0%[a] | 22.2%[b] | 45.5%[a] | 54.1%[a] | 22.2%[b] |

Different superscripts between different columns indicate significant differences (P< 0.05)

found to be significant in the univariate analysis at P <0.25 level, 3) stepwise removal of nonsignificant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding, 4) evaluation of interactions among variables and 5) assessment of model fit using Hosmer-Lemeshow statistics until all the main effects or interactions were significant at P <0.05. Chi square test was used to compare classes of the variables for better interpretation of the results if required [19].

## Results

The description of examined factors included in univariate analysis as well as in the multivariate model is presented in (Table 1). Among these factors, ewe parity number, semen deposition site, BCS at sponge placement and AI, BCS change from sponge placement to pregnancy diagnosis, farm and ram used had a significant effect on conception rates.

The overall pregnancy rate was 44%. Ewes that were on their second lactation period showed to have higher fertility rates (50.3%) compared to the rest, while odds were decreasing as the age increased (Figure 1A). Pregnancy rates increased as the deposition depth increased from vagina (33.1%) to external cervical os (37.4%) and to inside cervix (46.7%) (Figure 1B). The depth of semen deposition was found to differ significant between the 2 breeds (Table 2). Although breed was found to have a significant effect on fertility in the univariate analysis, it was not included in the final regression model (P> 0.05).

Higher fertility rates were observed at animals with moderate BCS (2.5-3.5) at sponge placement and at time of A.I (Figure 1C and 1D). Conception rate odds ratio of ewes increasing or retaining their BCS were 2.14 and 1.63 times to ewes in negative energy balance respectively (Figure 1E). However, at first step, interactions were found between BCS at sponge placement and BCS change as well as between BCS at A.I. and BCS change on their effect on conception rates. Fertility was increased only in animals with low (45.5%) or moderate (54.1%) BCS when they restored their body reserves. On contrast, fertility rates were low when high BCS animals gained more weight (22.2%) (Table 3). The effect of these interactions was significant on the fertility of the animals in the univariate model (p<0.25), but the interactions were (Hosmer and Lemeshow test= 10.62; df= 8; P= 0.224; Classification accuracy= 64.9%) finally excluded from the final model as non-significant. No other significant interactions between variables were found.

Regarding the rest factors in the final model, pregnancy rates were found to differ significant between farms (p<0.001) and used rams (p=0.007).

## Discussion

The present study addressed the main factors affecting fertility after AI using chilled semen in the most common intensively reared dairy breeds in Greece, Chios and Lacaune. It is the first study to examine the application of AI in Greece, including females of a native and foreign breed, born in the area and well adapted in local conditions and management systems.

Ewe parity and age have been widely assessed as factors influencing fertility. Shackell et al. reported a 3% decrease of fertility per year as average ewe age increased. Similar were the findings of Arranz et al. [20,21], Fukui et al [22], and Palacin et al. [23], who regarded the reduced fertility of older ewes as consequence of the decreased quality of ovulated oocytes, despite the improved cervical penetration that comes with increasing parity [24,25]. In our study, pregnancy rates of primiparous ewes were lower compared to ewes at second lactation period and declined rapidly beyond 4$^{th}$ parity. According to Anel et al. [7], young ewes show reduced fertility due to their inclusion with older ewes that usually lamb earlier. Additionally, nutritional deficiencies are often observed in young ewes during the mating period as a result of their larger requirements for growth. Our findings are in contrast with other studies that found no effect of ewe age on their fertility [26,27].

The deposition site of semen into female's reproductive canal is considered very important factor by many authors. In our study, conception rates were improved when semen was deposited inside cervix without semen backflow. The deeper deposition allows more semen to reach the fertilization site and increases pregnancy chances [28,29]. However, Paulenz et al. and Masoudi et al. found no difference in pregnancy rates between vaginal and cervical AI when fresh semen was used, stating that fresh semen contains enough motile spermatozoa able to reach fertilization site. Conclusions of Richardson et al. were the same using frozen-thawed semen, but with lower pregnancy rates [30-32]. Attempts to deposit semen deeper in the cervix with the use of specific catheters increase conception rates of fresh semen especially when the catheter was introduced beyond 4 cm [33]. However, Kaabi indicated that pregnancy rates increased with deposition of semen in cervix up to 3 cm, but deeper deposition decreases fertility, maybe due to catheter and cervical manipulations that could activate pathways that interrupt pregnancy [34,35].

Reproductive performance is affected by the nutritional status of animals. Our findings are in agreement with other studies according to which animals in good condition respond well in onset of breeding season and have higher ovulation and lambing rates [22,36]. At the same time, ewes with low BCS activate their ovulation potential less effectively [26,37]. However, there seems to be a plateau on the effect of BCS on fertility as there is no benefit of increased BCS beyond a point, and conception rates decline in animals with BCS> 3.5. Very fat animals fail to show estrous at predicted time and exhibit lower lambing rate potential [38-40]. Our study agrees with many authors ending up that ewes should have a BCS of 2.5-3.5 at mating period [22,41,42].

A positive effect of BCS increase on fertility of animals with low and medium BCS was indicated in our study. Flushing during pre-mating period has positive effect on conception rates and can modify the number and quality of embryos especially on thin ewes [43,44]. At the same time, low feed intake and BCS reduction results to lower ovulation rate, decreased embryonic growth and increased fetal losses [45-47]. Negative energy balance seems to have no significant effect on animals with high BCS, indicating that the endocrine response to undernutrition depends on body energy reserves [48]. However, ewes with BCS> 3.5 demonstrated a decrease in pregnancy rate when they were on positive energy balance. We suggest that weight gain should be discouraged in fat sheep as can cause high ovulation rates and increase embryonic losses [49].

Pregnancy rates of AI tended to be different among the farms in our study. This could be attributed to variations on reproductive planning and animal handling between farms [7], human-animal interactions [23], or other stressful management conditions that suppress fertility [50]. According to Santolaria et al. [10], fertility rates can be affected positively by the improvement of handling conditions in the farms.

Ram affected conception rates significantly, despite the strictly selection of the ejaculates. Ram effect on AI success, evaluated independently from the quality of the ejaculate, has been described by many authors [23,51]. It remains unclear why same quality semen produced from different rams and processed under the same conditions, show significant variations in fertility rates. Differences on seminal characteristics of the ejaculate that remain uncertain or even genetic factors that influence ram's fertility could have been responsible for these variations.

Ewe breed has been described as a significant factor affecting AI success by many authors [6,8,26]. Most studies are focused on the differences among breeds on ovulation time, cervix morphology or physicochemical properties of cervical mucus that can impair semen transport [28,52,53]. In our study, breed did not affected pregnancy rates significantly. However, we reported significant differences on the deposition site of the semen between Chios and Lacaune breed. This observation can be explained by the differences on cervix morphology between the two breeds and could be responsible for pregnancy rates differences observed.

The month of the insemination performance had no effect on pregnancy rates, mainly due to the fact that this study was conducted from May to November, when both breeds exhibit strong cyclic activity in this region. The lack of obvious heat stress effect during July and August that could affect reproductive efficiency indicates the need of evaluating more precisely the micro-environmental conditions of each farm [54].

The presence of rams in the area near the synchronized ewes is a factor that could affect pregnancy rates. Lucidi et al. found increased pregnancy rate in Italian breed ewes that were exposed in ram after sponge removal [55]. Exposure of females to rams before sponge removal could reduce ecG administration-onset of estrous interval and reduce the success of classic fixed time insemination protocols [42,56,57]. That could be overcome by inseminating the ewes earlier. In our study, rams presence near the ewes during synchronization didn't have any effect to fertility rates. This could be due to the fact that rams didn't join ewes inside their lot, but were kept in a nearby area, limiting thus the male effect of a direct contact.

## Conclusion

In conclusion, farm, ewe parity, semen deposition site, ram and BCS of ewes, as well as changes of it during the period around AI, were the main factors affecting fertility after AI on intensively reared dairy sheep of Greece. Targeted selection of ewes, evaluation of farm's management practices and nutrition program, as well as timely identification of the most fertile rams could lead to better and more consistent results, contributing to the spread of AI application in greek intensive flocks in larger scale.

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