Theses of Doctoral (PhD) Dissertation

IMPROVING THE EFFECTIVENESS OF LAPAROSCOPIC-ASSISTED REPRODUCTION TECHNIQUES AND INVESTIGATING THE IMPACT OF STRESS IN SHEEP

Dr. Melinda Bagi

Supervisor: Dr. Nóra Vass Dr. Pálfyné
assistant professor

UNIVERSITY OF DEBRECEN
Doctoral School of Animal Science

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1. INTRODUCTION AND AIM OF THE THESIS

Since world population shows a rising tendency, meat consumption, including the consumption of sheep and goat meat is going to increase too, especially in developing countries. The delicate flavor and low-fat content of sheep and goat meat in addition to the traditions of certain ethnic groups also influence the amount of meat that is consumed. This tendency requires new research advancement and innovation in the production of sheep and goat products (AMIRIDIS and CSEH, 2012; TEIXERIA et al., 2019).

In reproductive biology, the use of assisted reproductive techniques (ARTs) is a major advancement that can facilitate genetic progress, allow the spread of genotypes that benefit the market and prevent complications associated with livestock transportation. In Hungary however, the use of these techniques is not common compared to the countries where sheep breeding is prevalent (e.g.: Australia, United Kingdom, France) (VASS et al., 2017). The success of assisted reproductive techniques can be affected by several external factors (e.g.: weather), ARTs technological background (e.g.: farming and feeding technology, separation time of lambs), protocol (e.g.: synchronization of the estrus cycle, artificial insemination) (ABECIA et al., 2017; SPANNER et al., 2024). It is important to examine these factors to determine the ones that have the largest impact on the success of ARTs and improve the reproductive output of breeding animals. However besides successful implementation, the stress and pain inflicted on the animals needs to be minimized and the risk of infection reduced. This is not only important for researchers, but also a major concern for farmers and the general public as well (MUHAMMAD et al., 2022).

Research objectives:

1. In the first section of my research a stress examination was carried out on recipient ewes during embryo transfer program. The objectives of the experiment were:

- Determining cortisol levels from saliva samples
- Monitoring the lying and standing behavior during the preparation and after the procedure
- Examining the extent of stress caused by laparoscopic and semi-laparoscopic embryo transfer, determining the differences between methods and comparison with control group

Based on the results of these stress tests, we can make future recommendations to breeders and veterinarians to choose the procedures that are less painful and stressful for animals.

2. In the second part of the research work, we investigated the factors influencing the success of laparoscopic artificial insemination in the Ile de France sheep breed. The objectives of the study were:

- Examination of the impact of season, moon phase, maternal effect, livestock management and ram effect on the success of insemination
- Effect of season and moon phase on twin gestation and the sex of lambs to be born

By evaluating the results, we can assist breeders to make frozen semen-based artificial insemination as effective as possible in order for them to meet their own and the market needs efficiently.

2. MATERIAL AND METHODS

2.1. Monitoring stress level during embryo transfer

2.1.1. Presentation of the experimental sites and animals

The study was approved by the Department of Food Chain Safety and Animal Health at Hajdú-Bihar County Governmental Office (Permit Number: 33-M/2022/DEMÁB). The interventions in the experiment were carried out in compliance with the legislation and guidelines in force.
Our experiments were carried out at the Kismacs Animal Husbandry Experimental Site of the Agricultural Research Institutes and Training Facility at the University of Debrecen.
40 recipient and 15 donor ewes from the Kismacs Animal Husbandry Experimental Site were involved in the experiment. Only healthy animals not showing signs of any disease were involved. The age of ewes were 2 to 6 years, their nutritional status raged from medium to well-nourished (Body Condition Score, BCS: 2.5-3.5). They were given ad libitum hay and water, and concentrated feed twice a day. The animals were kept in semi-intensive housing which is typical for Hungarian domestic sheep farming, in a deep litter system, separated from the rest of the animals by a grid 2 months prior to the intervention.

2.1.2. Housing and preparation of animals

The experimental programs took place between October 2022 and April 2023. In order to monitor stress levels caused by embryo transfer, estrus synchronization was performed in a total of 40 recipient ewes. For estrus synchronization Chronogest® (MSD Animal Health, France) vaginal sponge was inserted into the recipient ewes and removed after 14 days. Simultaneously with the removal of the progestogen source ewes were given 300 NE eCG (equine chorionic gonadotropine, Folligon® inj. AU.V., MSD Animal Health, France) injection intramuscularly (i.m.). The embryo transfer took place on day 7 after the removal of the vaginal sponge.
Two days before operation the recipient ewes were placed in individual 1.5x2 m pens where they could interact with each other and were given ad libitum hay and water, together with twice daily feeding. However, 24 hours of fodder- and 12 hours of water deprivation were essential prior to the day of surgery. This is necessary to ensure that the operations can be carried out without complications, as the filled rumen and intestines can place a pressure on the abdominal organs and lungs during the surgical positioning, increasing the risk of damaging these organs and preventing easy access to the reproductive tract, which affects the duration and success of the operation. Individual housing was necessary in order to avoid disturbing the other animals

involved in the experiment when moving them, which is an important factor when monitoring the stress levels. The isolation 2 days preceding the experiment was necessary to allow the animals to adapt to the new environment before the stress test, causing this factor not to affect the outcome of the experiment. After the intervention recipient ewes were returned to their individual pens for 24 hours and were placed again into groups 1 day later, but still separated from the rest of the animals.

2.1.3. Multiple ovulation and embryo transfer program

Prior to the program superovulation treatment, we performed in the donors in addition to estrus synchronization. Similarly, to recipient ewes, estrus synchronization was carried out using Chronogest® (MSD Animal Health, France) vaginal sponge which was removed after 14 days. Animals received follicle stimulating hormone (FSH) treatment performed based on simplified superovulation protocol (3.75 ml Stimufol®, Reprobiol SPRL, Belgium) and were given it intramuscularly once 12 days after sponge insertation. Simultaneously with the FSH treatment they were given 300 NE eCG-t (Folligon® inj. A.U.V., MSD Animal Health, France) intramuscularly. 24 hours after the removal of the gestagen source donors received GnRH (Receptal®, MSD Animal Health, France) i.m. injection. Insemination using fresh or frozen semen was performed by laparoscopic technique 48-50 hours after the removal of the vaginal sponge. Following the procedure donors were placed in a harem. Surgical embryo extraction was performed on the 7th day after the removal of the progestagen source. Extracted embryos were evaluated under microscope then inserted into recipient ewes.

The recipient ewes were randomly divided into 3 groups. The first group of recipients (n=15) underwent laparoscopic embryo transfer, for the second group (n=10) a semi-laparoscopic method was applied. The third group consisted of the control ewes (n=15). For sedation 40-50 µg/kg body weight dose of detomidin injection (Domosedan®, Orion Corporation, Finland) was used intramuscularly in case of the laparoscopic and control groups. Surgery preparation was identical for the laparoscopic (L) and semi-laparoscopic groups (SL). The wool was trimmed from the area the surgery was performed on, from around and beside the udder, dirt was cleaned from the skin with disinfectant soap and then antiseptized with alcohol (Bradoderm Soft, Florin Ltd., Hungary). The animals were placed in dorsal recumbency in a special cradle used for laparoscopic surgery. Ewes were placed in a 45° head tilted position (Trendelenburg position) during surgery. This positioning is important since it helps create a smaller risk of injury, and leaving the ovaries and uterus more easily accessible, because the abdominal organs slide towards the diaphragm. In group L the procedure was started at least 15 minutes (19.6 minutes

in average) after the injection of detomidine, when detomidine had reached its full effect. During the laparoscopic embryo transfer, a 1 cm skin incision was made on each side in a distance of 5 cm from the udder and also 5 cm from the midline. A trocar was inserted into the abdominal cavity through each of the incisions, allowing access for the laparoscopic optics and hand instruments to the abdominal cavity. Aided by laparoscopic optics with light source and atraumatic forceps (laparoscopic instruments: Storz, Germany and EMD, Hungary) we detected the ovaries and checked for the presence of the corpus luteum, since embryo implantation into the recipients is only possible after luteinisation. Meanwhile, the embryo to be implanted was transferred into 0.25 ml artificial straws, leaving an air bubble in front of and behind the embryo that allowed to check the success of insertation. The artificial straw was placed in a fertilization catheter (Aspic®, IMV, France). The insemination catheter was inserted through the trocar and the embryo was injected into the third part of the uterine horn anterior to the ovary with the corpus luteum. At the end of the procedure, the ewes were given a 15 mg/kg body weight dose of the antibiotic amoxicillin (Betamox LA®, 150 mg/ml A.U.V. inj., Norbrook, Ireland) and a 1 mg/kg body weight dose of the non-steroidal anti-inflammatory substance meloxicam (Melovem® 20 mg/ml A.U.V. inj., Dopharma Research B.V., Netherlands). The average duration of the laparoscopic procedure was 6.6 minutes.

The semi-laparoscopic group received a combination of 20 µg/kg body weight Domosedan® (Orion Corporation, Finland) and 2 mg/kg body weight of ketamine (Ketamidor® 100 mg/ml inj. A.U.V., Richter Pharma AG, Austria) intramuscularly. The procedure was started after a lag time of at least 10 minutes. During the semi-laparoscopic embryo transfer, two skin incisions were made as described for the laparoscopic technique, through which the trocars and the optic and atraumatic forceps were inserted. The abdominal incision on the same side as the corpus luteum was extended to 2-2.5 cm and the atraumatic forceps were used to gently lift the uterine horn from the abdominal cavity on the same side. A hole was made in the oviductal third of the uterine horn with a blunt-tipped probe, through which a Tomcat embryo transfer catheter (Minitube, Germany) was inserted into the uterine lumen. Then it was inserted into the uterine cavity with a syringe the embryo had been previously absorbed into. After the embryo transfer, the uterus was released back into the abdominal cavity and the wound was closed with 2 knot stitches. The recipients received antibiotics and non-steroidal anti-inflammatory medication at the end of the operation identical to group L. The average duration of SL embryo transfer was 8.3 minutes.

Control group ewes were also placed in the laparoscopic cradle 10 minutes after sedation, where they spent 6 minutes in Trendelenburg position, and then returned to their individual pens

without any other intervention. For the control group, our aim was to imitate the restraint and sedation required for surgery and the stress caused by this procedure.

2.1.4. Saliva cortisol sampling and testing

Saliva samples were collected from the animals with synthetic swabs (Sailvette Cortisol, Sarstedt GmbH, Nümbrecht-Rommelsdorf, Germany) four times during the experimental period. The first samples were collected from each ewe on the morning of the day of the operation. The second and third samples were taken 1 and 2 hours after the animals returned to their pens (after the surgery). The fourth sample was collected 24 hours after the surgery. The sampling swabs were held with Collin tongue forceps and then put into the sheep's mouths between the molars and bucca when the sheep were not ruminating to avoid sample contamination. The sheep were allowed to chew on the rolls for approximately 20 seconds for proper saliva collection. The Salivettes were immediately cooled to 4°C and transported to the laboratory. The swabs were centrifugated on 3000 g for 10 minutes, and the saliva (at least 1.5 mL per sample) was stored at –80°C in Eppendorf tubes until the assay. A direct homemade RIA method was used for cortisol analysis, which was developed for cortisol determination in the plasma of food-producing animals using 1,2,6,7–3H-cortisol (TRK 407; Radiochemical Centre, Amersham, UK) and a highly specific polyclonal antibody raised against cortisol-21-HS-BSA in rabbits. Cross-reactivity of the assay: cortisol: 100%; corticosterone: 19%; prednisolone: 9.5%; deoxycortisol: 6.4%; 17α-OH progesterone: 5.7%; progesterone: 2.6%; any other 22 steroids: 0.54 to 0.0001%). The assay standards (cortisol FW 362.5; Sigma Chemical Company, St. Louis, MO) were prepared in cortisol-free plasma. The antibody-bound and the free fractions were separated by cold dextran-coated charcoal suspension after an 18 to 24 h incubation period. Radioactivity was measured by a TriCarb liquid scintillation counter (Perkin Elmer Inc., Downers Grove, IL, USA). The sensitivity of this assay system was 11.37 fmol/tube. Within the concentration range of about 2.0 and 100.0 nmol/mL, the intra- and interassay coefficients of variation varied between 3 and 8% and 5 and 10%, respectively. Samples with cortisol concentrations higher than 100.0 nmol/L were re-assayed after dilution.

2.1.5. Monitoring the lying and standing behaviour

Pedometers that can be inserted on legs (HOBO Pendant G logger, Onset Computer Corp., Bourne, USA) were fitted onto the right hind leg of the animals one day before the procedure. The devices were attached to the outside of the leg with an elastic strap (Copoly), during which cotton wool was used for padding. The device continuously measured and recorded acceleration

and pitch on three axes (x, y, z) which enabled to separate the standing and lying positions. The measurements were taken every 30 seconds by the device (BONK et al., 2013). The devices were on the animals for a total of three days and removed 24 hours after the procedure. The data was then downloaded and organized in an Excel sheet. The measurements of the x-axis were used to determine the time spent lying. Lying position was detected when it was maintained at least up to 3 measurements (1.5 minutes) in order to avoid faulty measurements. The data were evaluated by comparing the following variables: total time spent lying, number of lying periods, average time spent lying per lying period, minimum and maximum lying time per lying period. The variables were measured 24 hours before and 24 hours after the procedure. The first measurement period was started when the devices were fitted on all animals and lasted until the first operated started (24 hours pre-procedure). The post-operative 24-hour measurement period began when the animal was returned to the individual pen after the procedure.

2.2. Study of factors influencing artificial insemination in Ile de France sheep breed

2.2.1. Sites of artificial insemination and the presentation of animals

The laparoscopic frozen-semen artificial inseminations were performed between 2017 and 2023 on the sheep farms of three Ile de France sheep breeders. The inseminations were carried out on Dr. Sándor Harangi's sheep farm in Hajdúszoboszló, Balázs Angyal's farm in Kunhegyes and Zsolt Győri's farm in Polgár. The inseminations were carried out within the breeding season in autumn (October, November) and off-season in spring (March, April). All ewes were of the ile de france breed, aged between 10 months and 10 years with BCS 2.5-3.5. The animals were kept in semi-intensive, deep litter housing in paddock, with small pasture grazing opportunity from spring to autumn. Hay was available ad libitum and fodder was given them daily once. The inseminated ewes were selected by the breeders, so were the rams, the frozen semen of which were used in the inseminations. The frozen semen was imported hereditary material from France. A total of 250 Ile de France ewes were inseminated laparoscopically.

2.2.2. Estrus synchronization and laparoscopic artificial insemination

Estrus synchronization was done identically to the process described above. The Chronogest® (MSD Animal Health, France) vaginal sponge was removed 14 days after the procedure, at the same time as the ewes received 300 NE eCG (Folligon® inj. A.U.V., MSD Animal Health, France) i.m. injections. Insemination occurred 54-56 hours after the removal of the progestagen

source. Prior to the intervention 24 hours of starvation and 12 hours of water deprivation from the ewes were required. In order to enable this the animals were housed in a pen without access to feed one day before the artificial insemination. In order to accelerate the insemination process wool was removed from the surgical area 2-5 days before operation. Sedation was carried out using detomidine i.m. injection (Domosedan®, Orion Corporation, Finland) at a dose of 60-70 µg/kg body weight. As our experience has shown that the response of sheep to anesthetics may vary, we used a lower dose for the first time and repeated the injection if it was necessary. The cleaning and disinfection of the surgical area on animals followed the same process that was described for embryo transfer. The animals were placed in the laparoscopic cradle and then lifted in a 45° head tilted position. A 1 cm incision was made on each side in a distance of 5 cm from the midline in front of the udder, using a sharp bladed tool, and 1 trocar was inserted through the incisions into the abdominal cavity, paying attention not to damage the abdominal organs. A laparoscopic optic with a light source (Storz, Germany; EMD, Hungary) was inserted through one of the trocars into the abdominal cavity and, if it was necessary, the uterine horns were detected using atraumatic forceps. The semen frozen in a 0.25 ml artificial straw was thawed in a water bath at 36-37 °C for 1 minutes and loaded into the inseminating catheter (Aspic®, IMV, France). The inseminating catheter was inserted into the abdominal cavity with the pointed end facing it and the semen was distributed and injected into both uterine horns. At the end of the operation, the animals received an injection containing 15 mg/kg body weight of amoxicillin (Betamox LA®, 150 mg/ml A.U.V. inj., Norbrook, Ireland) and 1 mg/kg body weight of meloxicam (Melovem® 20 mg/ml A.U.V. inj., Dopharma Research B.V., Netherlands). The wound was treated with antiseptic spray (Alamycin® aerosol A.U.V., Norbrook Ireland). Animals were allowed to return to their group after complete awakening. Pregnancy ultrasound was performed 45 days after insemination however only lambs that were given birth to were involved into our statistical analysis.

2.2.3. Season, meteorological parameters and moon phase

The procedures were carried out in October, November, March and April, and the impact of season on fertilization effectiveness was investigated. Of the 250 ewes, 55 ewes were inseminated in autumn and 195 ewes in the spring. Meteorological parameters were analyzed before and after insemination. Our first observation period (4 days) lasted from the 3rd day before insemination until the day of insemination, which is important for the development of the dominant follicle(s) and ovulation. In the second observation period (40 days), we collected data from the first day following fertilization to day 40 after it, which is the period of early

embryo development. Meteorological parameters were obtained from the database of the Centre for Precision Farming R&D Services, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen. Data from the meteorological station situated nearest to the sheep farm were always used for statistical analyses (Hajdúszoboszló - Nádudvar, Polgár - Folyás, Kunhegyes - Kunmadaras).

Investigated parameters:
- minimum daytime temperature
- maximum temperature
- daily average temperature
- daily temperature fluctuation
- precipitation
- relative humidity

There was little variation in the weather parameters of consecutive days during the studied period. Therefore, standard deviation square (variance) of the minimum, maximum and average temperatures, as well as the precipitation and relative humidity, were calculated for the periods preceding and succeeding fertilizations. The average temperature fluctuation in both periods was analysed.

In studying the phase of the moon, we have collected which phase the day of fertilization falls into:
- new moon
- waining moon (first and second moon phase)
- full moon
- waxing moon (third and fourth moon phase)

The number of days were also recorded between fertilization and the emergence of full moon.

2.2.4. Site management, maternal effect, ram effect

The date birth of ewes was used to calculate their current age (in days) on the day of insemination. Frozen semen from a total of 16 different rams was used in the inseminations and the ram effect was also investigated. Regarding maternal effects mothers born as the result of artificial insemination were recorded among those that were included in the program. In addition, we examined the effect of the previous pregnancies of mothers, the effect of the number of days passed after lamb separation until inseminated, number of artificial inseminations the mother has had before the procedure, and the type of the previous pregnancies

(natural breeding or artificial insemination). Data were collected from the farm management systems of the sheep farms and organized in Excel tables.

2.3. Statistical analysis

Our statistical analyses were performed using R statistics (R version 4.4.1 -- "Race for Your Life"). We used a paired t-test for the change in lying behavior. We used ANOVA to evaluate the results of cortisol measurements (STAFFORD et al., 2006). We used probit analysis to analyze meteorological parameters and evaluate maternal and ram effects. Khi-square test was used to examine the season, and ANOVA model (ALBERGHINA et al., 2021) to evaluate the impact of moon phases.

3. RESULTS

3.1. Monitoring stress level during embryo transfer

3.1.1. Saliva cortisol concentration measurement

In the statistical analysis cortisol results of those animals are only featured, from which at least 1.5 ml of saliva was sampled. A total of 15 animals from group C, 15 animals from group L and 7 animals from group SL were used. In Table 1. the mean cortisol concentrations are summarized and presented by group at each sampling time (Table 1).

Table 1.: Mean of salivary cortisol concentrations (ng/ml)

Time of sampling	Total group mean	Group C	Group L	Group SL
1	3.61±1.73[a]	3.82±1.46	3.74±2.11	2.83±1.38
2	4.86±2.43[b]	4.78±2.88	5.28±2.06*	4.13±2.19
3	5.65±2.22[b]	5.53±1.99*	5.90±2.20*	5.35±3.01
4	3.60±2.07[a]	3.39±1.64	3.99±2.83	3.26±0.60

Remarks:
Sampling time:
1. in the morning on the day of the procedure
2. 1 hour after the procedure
3. 2 hours after the procedure
4. 24 hours after the procedure
a, b: different letters mean that the difference is significant ($p<0.05$)
* difference is significant compared to the first sample in the given column

No significant differences were found between the groups at each measurement time points. The increase and decrease in mean of salivary cortisol concentrations showed a similar curve in all three groups (Figure 1). Cortisol concentrations were the highest at 1 and 2 hours after the procedure in each group, and then almost returned to baseline levels at the first measurement time point 24 hours after the procedure. Significant differences were found between measurement time points. Regarding the mean of groups cortisol levels were significantly higher ($p<0.05$) at the second and third measurement time points 1 and 2 hours after the procedure than at the first and fourth sampling times. Comparing each group result to the first measurement time point results, it was found that cortisol concentrations were significantly

higher in group C at 2 hours after the procedure and in group L at 1 and 2 hours after the procedure. In group SL a trend was observed in cortisol concentrations showing an increase at sampling times 2 and 3, but there was no significant difference compared to sampling time 1.

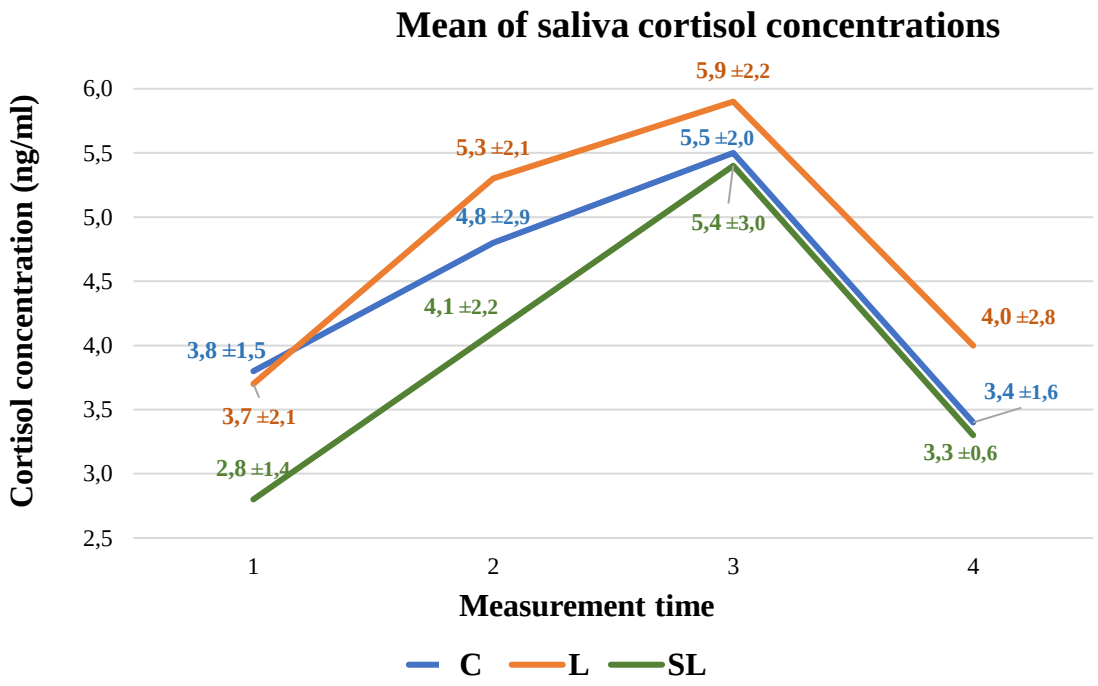

Figure 1.: Change in mean salivary cortisol concentration (±SD) per group

Remark: C: control group, L: laparoscopic group, SL: semi-laparoscopic group

In sheep, even routine handling procedures (such as restraint, shearing, and bathing) are associated with stress. However, the physiological response to stress (e.g. an increase in cortisol levels) may vary between the animals (KILGOUR and LANGEN, 1970; HARGREAVES and HUTSON, 1990). The literature has already reported that laparoscopic minimally invasive procedures result in elevated cortisol levels. Nevertheless, laparoscopic artificial insemination did not induce greater stress in animals than restraint and placement in the laparoscopic cradle without surgery (MARTIN et al., 1981; KHALID et al., 1998). Based on the results of our own salivary cortisol measurements, elevated cortisol levels were also observed during laparoscopic and semi-laparoscopic procedures. However, according to the results of our experiment, this increase was not significantly higher than the values measured in individuals belonging to the control group.

3.1.2. Monitoring the lying and standing behaviour

The total time spent lying, the minimum and maximum time spent lying, the number of lying periods, the mean of the time spent lying per lying period was measured in the 24 hours before and after the procedure. There was no significant difference in the total lying time between pre- and post-operation periods in either group (C: p=0.69; L: p=0.51; SL: p=0.48). Total lying time increased almost equally after surgery (C: 10%; L: 12%; SL: 12%). The minimum length of lying in group L increased significantly after surgery (p=0.0006241). No strong correlation was found in the control group, but a tendency to increase was found (p=0. 09618). There was no significant difference in group SL (p=0.567). A maximum length of lying time was found to longer 24 hours after surgery in the group L (p=0.01571) and group SL (p=0. 0001477), but no significant difference was found in group C (p=0.3832). The maximum length of the lying periods increased most significantly in group SL compared to the other two groups (C: 30%; L: 45%; SL: 115%). Evaluating the data from the three groups, we found that the mean length of of lying bouts increased significantly in all cases in the post-procedure measurement period (C: p=0.043; L: p=0.00059; SL: p=0.0029). This increase was more pronounced in the experimental groups (C: 54%; L: 110%; SL: 110%). Looking at the recipients in the experimental group, we found that 33 out of 41 individuals showed an increase in the mean length of lying bouts compared to the pre-operation mean (Figure 2). At the same time, there was a change in the number of lying bouts (number of lying and standing up), which decreased significantly in all groups (C: p=0.032; L: p=0.022; SL: p=0.03). There was no significant difference in the extent of the decrease in the number of lying bouts between groups (C: 31%; L: 39%; SL: 40%).

Our statistical analysis covered the extent to which anesthetics affect changes in lying behavior. Thus, the 24-hour post-operation period did not begin at the end of the procedure, data from 1 hour after procedure were not included in the analysis. There were no significant changes in the mean length of the lying bouts and the number of lying bouts. The mean length of the lying bouts increased significantly in all groups, while the number of lying bouts decreased ($p<0.05$). The increase in the mean length of the lying bouts was significant in the experimental groups compared to the control group (C: 57%; L: 88%; SL: 103%). The decrease in the number of lying bouts was more significant in the experimental groups, including the semi-laparoscopic technique (C: 34%; L: 43%; SL: 55%). The change in total lying period was not significant when comparing the pre- and post-operative periods. However, a tendency was observed which showed that if the effect of anesthetics is ignored the total lying time increased (3%) in group K, but decreased (L: 2%; SL: 19%) in groups L and SL in the post-operation periods. The maximum length of the lying period increased in all three groups (not significantly) within 24

hours' post-operation. This increase was pronounced when using the semi-laparoscopic method (C: 25%; L: 28%; SL: 91%).

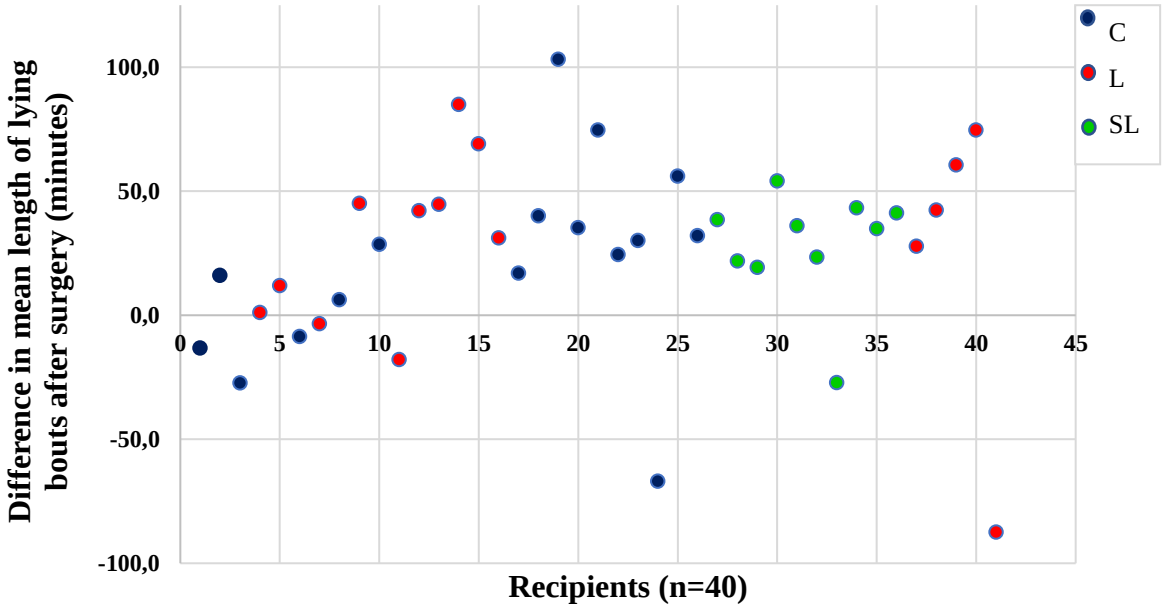

Figure 2.: Change in the average length of lying time compared to the pre-operation mean

During painful procedures (e.g. dehorning), an increase in mean lying time was observed during the 24 hours following the intervention. In addition, the number of lying bouts decreased, while no differences were found between treatments in the duration of lying bouts (HEMPSTEAD et al., 2018). In goats, following castration without the use of transdermal analgesia, animals spent more time lying after the procedure, and the duration of lying bouts increased, whereas the number of lying bouts decreased (LEE et al., 2020).

Our own results showed similar changes in lying behaviour to those reported in the literature following dehorning or castration. As a response to stress, during the 24 hours after the interventions, animals in all three groups spent more time lying, the duration of lying bouts increased, and in parallel, the frequency of transitions between lying and standing positions decreased.

3.2. Investigating factors affecting the effectiveness of artificial insemination

3.2.1. The season and meteorological parameters

The results of laparoscopic artificial insemination of a total of 250 Ile de France ewes were processed. In autumn (October, November) 55 inseminations were performed. Out of the 55 inseminated ewes, a total of 11 ewes became pregnant, resulting in a pregnancy rate of 20.0%. In the off-season in spring months (March, April) a laparoscopic insemination of 195 ewes resulted 100 ewes became pregnant, with a pregnancy percentage of 51.3%. The spring inseminations as a result had a significantly higher pregnancy percentage ($p<0.01$).

Due to the poor pregnancy rates in autumn inseminations, the influence of meteorological parameters on pregnancy probability was investigated by taking into account the results of inseminations in spring months. A correlation matrix was prepared with the variables of the meteorological parameters, which indicates how closely the combined variation of weather factors are related.

In the pre-fertilization period co-variation is in **a medium range (0.51-0.75):**
-of minimum temperature (Tmin1) and average temperature (Ta1) (0.69),
-of relative humidity (Rh1) and average temperature (0.69),
-of precipitation volume (Prec1) and average temperature (0.51),
- of precipitation volume and relative humidity (0.52).

In the post-fertilization period, there is also a moderately close correlation between minimum (Tmin2) and maximum temperature (Tmax2) (0.74).

There is very **close co-variance (0.76-0.99)** in the pre-fertilization period:
-of maximum temperature (Tmax1) and Ta1 (0.88),
-of Rh1 and Tmin1 (0.87).

There is also a very close correlation in the post-fertilization period:
-of Tmax2 and the average temperature (Ta2) (0.96),
-of Tmin2 and Ta2 (0.87).

In the period of four days before insemination it was found that an extreme change in Tmin1 affects negatively the probability of conception (p=0.0692). Significant correlations were found within the 40 days' period after insemination. For both Tmax2 and Tmin2, an increase in temperature variance decreases the probability of conception (Tmax: p=0.0472; Tmin: p=0.033). A statistically verifiable correlation was also found for Ta2, but in this case an increase in the mean temperature variance increases the probability of conception (p=0.0191). Precipitation, relative humidity and daily temperature fluctuations did not influence the success of conception either before or after fertilization.

De et al. (2016) reported that during artificial insemination performed both in- and out of season, no differences were observed in the estrus synchronization response of Malpura and Kheri ewes under semi-arid tropical conditions. However, lambing rates were higher during the breeding season (66.67%) than outside the season (57.57%) (DE et al., 2016). In the Spanish Rasa Aragonesa breed, insemination with fresh semen during the spring months (March to May) resulted in a pregnancy rate of 45%, whereas based on our own results, this value was 51.3% in the Ile de France breed when frozen semen was used (ABECIA et al., 2016a). When investigating factors influencing insemination success in the Rasa Aragonesa and Churra breeds, lower pregnancy rates were reported during from spring to summer (out-of-season) months (ANEL et al., 2005; PALACÍN et al., 2012). In contrast, Hill et al. (1998) found no differences in pregnancy rates of Australian Merino ewes between spring and winter inseminations (HILL et al., 1998). In the Ile de France breed, under Hungarian temperate humid continental climatic conditions, spring laparoscopic inseminations proved to be more successful when frozen semen was used.

Based on the literature, among the parameters of temperature, precipitation, humidity, and the temperature–humidity index, temperature appears to have the greatest influence on the success of artificial insemination. However, the effects of changes in these individual parameters may differ across seasons (ABECIA et al., 2016a; PALACIOS and ABECIA, 2014; SANTOLARIA et al., 2014). According to our research findings, during spring inseminations, extreme changes in temperature affected the success of artificial insemination. In the 40 days following insemination, an increase in the variance of Tmin and Tmax values resulted in a reduced probability of fertilization.

3.2.2. Moon phase

The effect of moon phase on the probability of pregnancy, twin pregnancy and the sex of lambs born was investigated. The waning moon (1st and 2nd quarters), waxing moon (3rd and 4th

quarters) and full moon had no significant impact on fertility. Besides the number of days passed form the full moon did not affect the efficiency of artificial insemination. The sex ratio of lambs born from the 250 inseminations that were carried out did not result in the presumed 50-50% rate, but resulted in a lower number of rams among the lambs that were born (46.97% in autumn and 43.33% in spring), but no significant impact caused by the moon phase was detected among the factors included in the study.

Season had the strongest impact on the rate of twin pregnancies, out of the parameters described above. Spring inseminations produced a significantly higher number of twin pregnancies (29.23%) compared to autumn inseminations (12.73%) ($p=0.00613$). Among the moon phases, the number of twin pregnancies was lower (21.90%) in the waning moon period compared to the waxing moon period (28.47%) ($p=0.0957$) with a significance level 10%.

Contrary to reports in the literature (SUBRAMANIAM et al., 1991; PALACIOS and ABECIA, 2014), our results indicated that lunar phase had no effect on the success of insemination. However, when insemination was performed during the waxing moon, a higher number of twin lambs were born compared with inseminations carried out during the waning moon. Neither season nor lunar phase influenced the sex of the lambs. In the literature, an effect of lunar phase on offspring sex has been demonstrated only in dogs, whereas no such association was found in sheep or horses (AGUILAr et al., 2015; ABECIA et al., 2016b; ALBERGHINA et al., 2021). Season affected not only insemination success but also the number of twin pregnancies. Following artificial insemination performed in the spring months, more twin lambs were born compared with autumn inseminations. According to the results reported by Zapasnikienė (2002), higher litter sizes can be expected during the winter lambing period (following late-summer inseminations) than during spring lambing (ZAPASNIKIENĖ, 2002). In contrast, Dimsoski et al. (1999) reported greater litter sizes in spring lambings compared with winter and summer lambing periods (DIMSOSKI et al., 1999). Based on our own results, the proportion of male lambs among the offspring was lower in both seasons. Contrary to the findings reported in the literature (ABECIA et al., 2016b), no significant differences in sex ratio were observed between seasons.

3.2.3. Ewe age, ram effect

Mothers inseminated in spring were aged between 328 days to 3760 days, with an average age of 1162 days (about 3.2 years). Most ewes were aged between 300 and 600 days. Considering the age of 195 mothers, a significant correlation was found in terms of fertilization success.

There is a negative correlation between the age of the mother and fertilization success, which means that the probability of pregnancy decreases with a progress in age (p=0.0227) (Figure 3.).

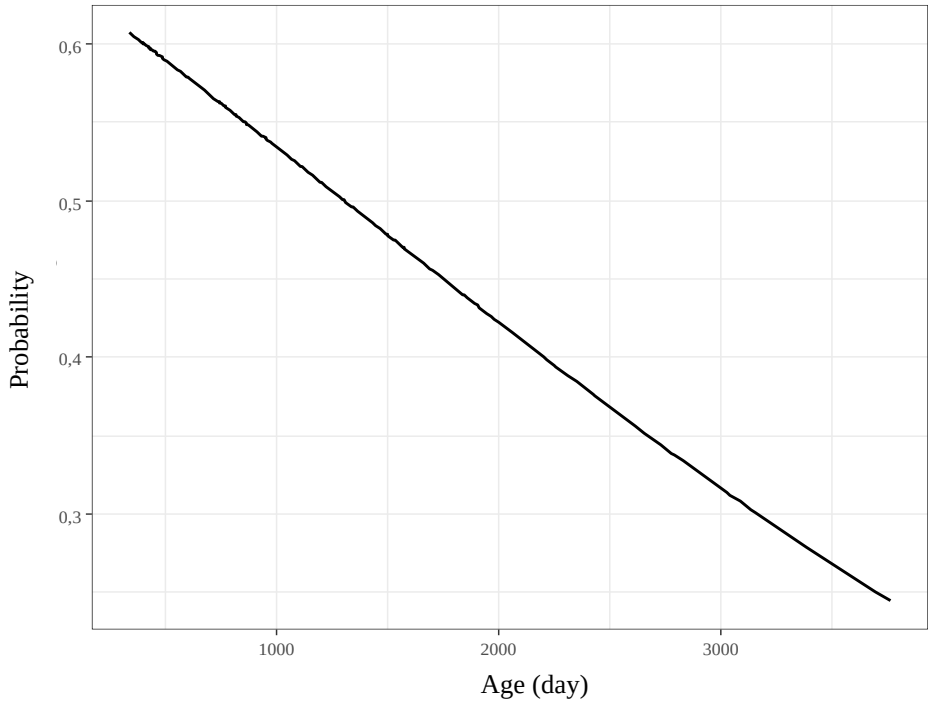

Figure 3.: The relationship between maternal age and fertility potential

During artificial insemination, frozen semen from 16 rams were used. Rams were numbered 1-16 in our analysis. In case of semen from three rams pregnancy percentages were significantly higher (ram 6: p=0.01948; ram 9: p=0.00795; ram 12: p=0.00313). However, in case of one ram no ewes became pregnant out of the seven inseminations (ram 13). We have also plotted the relationship between maternal age and the probability of pregnancy by individual rams on a diagram (Figure 4). For 15 rams (with ram 13 having zero successful inseminations) the probability of pregnancy decreased steadily with increasing maternal age. For example, for rams number 9 and 10, the age of the inseminated ewes ranged within a wide interval which indicated that ewes below 1000 days of age had a significantly higher probability of successful insemination (above 60% probability). While for mothers between 2000 and 3000 days of age the probability of insemination decreased steadily (above 3000 days, below 30%).

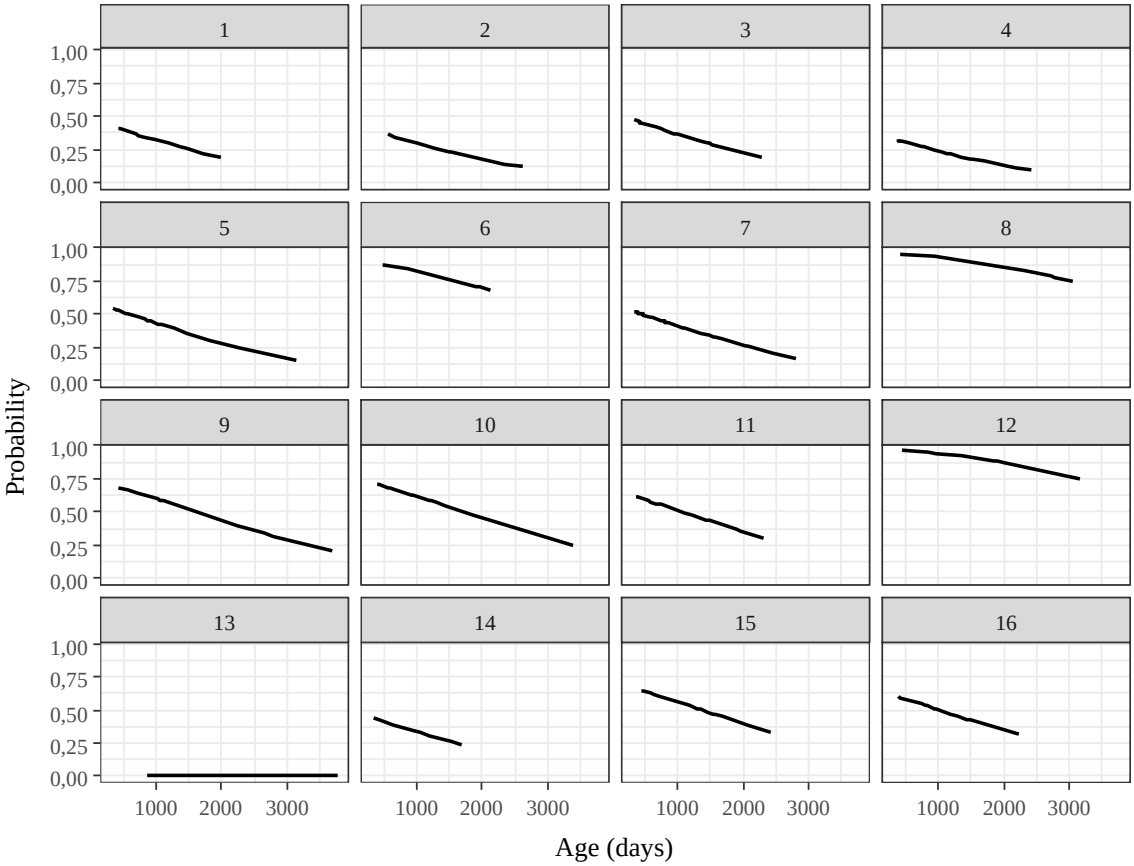

Figure 4.: Probability of fertility per ram in relation to age

Our results are consistent with those reported by Anel et al. (2005), who published similar conclusions in the Churra breed during both laparoscopic and vaginal insemination (Appendix 14) (ANEL et al., 2005). Using laparoscopic intrauterine insemination performed out of season, Fukui et al. (2010) also obtained comparable results, reporting significantly higher pregnancy and lambing rates in ewes younger than three years compared with ewes older than three years (FUKUI et al., 2010). In addition, litter size is greatest between 3 and 5 years of age, while the probability of embryo survival is highest at three years of age (SHORTEN et al., 2013).

3.2.4. Farm management of the farming sites involved in the study

In order to facilitate statistical analysis ewes that had previously been pregnant were separated from the rest during farm management. This group included 140 ewes with a pregnancy rate of 48.57%. Among the variables studied none of the variables below had a significant impact on the success of insemination:

-if the mother ewe was born as a result of artificial insemination

-the date of lamb weaning prior to artificial insemination

-number of previous artificial inseminations performed

-number of lambs born out of artificial inseminations.

The group of jerke lambs included 55 ones (pregnancy rate: 58.18%) where the only investigated variables were whether the mother was born from artificial insemination and the number of artificial insemination of the ewer has already had. None of the examined variables had an effect on pregnancy.

The literature has examined several external factors related to reproductive management and maternal effects that influence fertility. One such factor is the timing of lamb weaning prior to artificial insemination. According to the findings of Anel et al. (2005), a weaning interval of less than 10 weeks and a higher number of previous lambings reduce fertility; therefore, the highest probability of conception is expected in nulliparous ewe lambs (ANEL et al., 2005). Palacín et al. (2012) reported that a lambing–artificial insemination interval longer than 240 days, as well as more than five previous pregnancies, negatively affects fertility (PALACÍN et al., 2012). In goats, a minimum weaning interval of 150 days prior to artificial insemination has been recommended (ARRÉBOLA et al., 2016). In contrast to the literature, our statistical analysis did not reveal any association between the examined factors and fertility.

4. NEW SCIENTIFIC RESULTS

1. The stress test, which was carried out during the laparoscopic and semi-laparoscopic embryo transfer within anesthetic protocols showed that salivary cortisol levels increased in all three groups as the result of the procedures. (pre-operation sampling: control=3.82 ng/ml, laparoscopic=3.74 ng/ml, semi-laparoscopic=2.83 ng/ml; sampling 2 hours after the procedure: control=5.53 ng/ml, laparoscopic=5.90 ng/ml, semi-laparoscopic=5.35 ng/ml). However, there was no significant difference ($p<0.05$) in measurement results between groups at each time point. Consequently, the two procedures did not lead to higher stress levels for ewes than the surgical preparation and anesthesia without these procedures caused to ewes of the control group.

2. As the result of a behavioral study of laparoscopic and semi-laparoscopic embryo transfer it was found that the **number of lying periods** decreased significantly in all three groups after the procedure (control: 31%, laparoscopic: 39%, semi-laparoscopic: 40% decrease). Simultaneously, an increase was found in the mean **length of the lying bouts** (control: 54%, laparoscopic: 110%, semi-laparoscopic: 110% increase). Our analyses showed that neither anesthesia nor the applied surgical technique influenced the change in the number of lying periods and the average duration of the lying periods.

3. When analyzing lying study results that were recorded during laparoscopic and semi-laparoscopic embryo transfer (minus the effect of anesthetic agents) significant changes were detected in terms of the semi-laparoscopic technique. The total post-operation lying time decreased (laparoscopic: 2%, semi-laparoscopic: 19%); the maximum length of the lying periods increased (laparoscopic: 28%, semi-laparoscopic: 91%); the mean length of the lying bouts increased (laparoscopic: 88%, semi-laparoscopic: 103%); and the number of lying bouts decreased (laparoscopic: 43%, semi-laparoscopic: 55%). The semi-laparoscopic technique changed the behavioral response of the animals towards the stress caused by surgery to a larger extent than laparoscopic procedure did.

4. In the humid-temperate continental climate of Hungary, estrus synchronization and frozen semen laparoscopic insemination are more successful in the off-season, in spring (pregnancy rate: 51.3%) than in autumn (pregnancy rate: 20.0%) for the Ile de France sheep breed.

5. The results of frozen-semen laparoscopic artificial insemination in Ile de France sheep breed showed that the percentage of twin pregnancies from spring inseminations was 29.23%, compared to 12.73% of autumn inseminations ($p=0.00613$). Besides the season effect at

p=0.0957 level of significance, it was detected that the number of twin pregnancies was lower (21.90%) in the waning moon period compared to the waxing moon period (28.47%).

6. Laparoscopic artificial insemination performed in spring in the humid-temperate continental climate of Hungary was found to be **affected negatively by extreme changes** in the daily minimum temperature within 4 days before (p=0.0692) and 40 days after insemination (p=0.033) and in daily maximum temperature within 40 days after insemination (p=0.0472).

5. PRACTICAL RESULTS

1. Besides the economic aspects of animal breeding, our aim in reproductive biology is to consider the issue of animal welfare when applying developing technologies. When using new techniques, it is important to assess their impact on the daily lives and welfare of animal, since we need to priorities interventions that cause as little pain, stress and distress as possible for the animals (CLARK and HARDING, 2006). In the European Union there is an increasing emphasis on interventions that are non-invasive or minimally invasive, require less medication and have the least negative impact on animal welfare. Our results showed that there was no difference between laparoscopic and semi-laparoscopic embryo transfer techniques in the extent of stress caused by the two procedures. However, considering the trends, semi-laparoscopic method has a more pronounced impact on behavioral changes. Laparoscopic embryo transfer can be performed quickly, it causes a small surgical wound, requires little manipulation of the abdominal cavity and therefore the risk of adhesions is reduced, which makes this technique minimally invasive and recommended for veterinarians. Considering animal welfare this method is suitable for breeders and veterinarians in MOET programs and experimental research for performing embryo transfer. However, adequate anaesthesia and analgesia are important during the procedure to reduce the pain and stress caused for the animals.

2. Ile de France is a popular sheep breed for meat in our country, therefore artificial insemination with frozen-semen laparoscopy was performed in this breed. Based on the results factors that influence the success of insemination the most were investigated as well as twin pregnancy and the sex of lambs. Our studies are primarily aimed at helping Ile de France breeders to identify which factors can maximize the success of artificial insemination. Based on our results it was found that frozen-semen laparoscopic artificial insemination of this breed should be scheduled for the spring months.

3. When selecting ewes, it is recommended to select primarily younger animals, under 3 years of age, because the probability of fertility decreases proportionally with increasing age.

4. Spring insemination should be chosen to achieve a higher number of born lambs. This season gives the opportunity to prepare for a higher number of twin pregnancies and to adjust the rearing and foddering technology accordingly.

5. Insemination during periods of extreme temperature fluctuations should be avoided in order not to reduce the pregnancy rate.

Through our research and experience, we would like to increase the use of laparoscopic artificial insemination among breeders of other sheep breeds, to increase its efficiency rate and to accelerate genetic progress.

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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (1)

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8. LIST OF ABBREVIATIONS

ART: assisted reproductive techniques

BCS: body condition score

eCG: equine chorionic gonadotropine

FSH: follicle stimulating hormone

GnRH: gonadotropine releasing hormone

i.m.: intramusculary

K: control group

L: laparoscopic group

MOET: multiple ovulation and embryo transfer

Prec: precipitation volume

RH: relative humidity

SL: semi-laparoscopic group

Ta: average temperature

Tmax: maximum temperature

Tmin: minimum temperature