# Manuscript Details

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| **Manuscript number** | ANIREP_2018_560_R1 |
| **Title** | Effects of short-term intravaginal progestagens on the onset and features of estrus, preovulatory LH surge and ovulation in sheep |
| **Article type** | Research Paper |


**Abstract**

In the present study, there was a comparison among classical long-term progestagen (fluorogestone acetate) protocols for synchronization of estrus and ovulation (14 days; group FGA14, n = 9 ewes) and short-term protocols based on 7 days of progestagen treatment plus a dose of prostaglandin F2α at either insertion (PG-FGA7, n = 11) or removal (FGA7-PG, n = 12). There were no significant differences in the ovulation rate and progesterone secretion among treatments. The FGA7-PG group, however, had a similar percentage of ewes expressing estrous behavior than the group FGA14 (90.9 and 100%, respectively, with a trend for a lesser percentage in the PG-FGA7 group, 63.6%) and about 90% of the ewes in the FGA7-PG group had the preovulatory surge release of LH 8 h after the onset of estrous behavior. These features may be related to a greater number of preovulatory follicles during growing phases ($P < 0.05$) and a greater plasma estradiol concentration ($P < 0.05$) in this group than in the classical 14-day group, which suggest these are more functional preovulatory follicles. In conclusion, therefore, the use of the FGA7-PG treatment may favor efficiency of progestagen-based protocols for reproductive management.



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| **Keywords** | Estrus synchronization; Ovine |
| **Taxonomy** | Animal Pharmacology, Sheep Reproduction, Animal Science |



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Editorial Office
Animal Reproduction Science

Madrid, 29th August, 2018

Dear Dr. Kinder,

We are uploading a new version in which all the Editor in Chief's edits and comments were accepted. We would deeply like to thank the editing done on our manuscript, which significantly improves its readability, and overall the help received to publish our work.

Yours sincerely,

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All the Editor in Chief's edits and comments were accepted.

# Effects of short-term intravaginal progestagens on the onset and features of estrus, preovulatory LH surge and ovulation in sheep

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## ABSTRACT

In the present study, there was a comparison among classical long-term progestagen (fluorogestone acetate) protocols for synchronization of estrus and ovulation (14 days; group FGA14, $n = 9$ ewes) and short-term protocols based on 7 days of progestagen treatment plus a dose of prostaglandin $F_{2\alpha}$ at either insertion (PG-FGA7, $n = 11$) or removal (FGA7-PG, $n = 12$). There were no significant differences in the ovulation rate and progesterone secretion among treatments. The FGA7-PG group, however, had a similar percentage of ewes expressing estrous behavior than the group FGA14 (90.9 and 100%, respectively, with a trend for a lesser percentage in the PG-FGA7 group, 63.6%) and about 90% of the ewes in the FGA7-PG group had the preovulatory surge release of LH 8 h after the onset of estrous behavior. These features may be related to a greater number of preovulatory follicles during growing phases ($P < 0.05$) and a greater plasma estradiol concentration ($P < 0.05$) in this group than in the classical 14-day group, which suggest these are more functional preovulatory follicles. In conclusion, therefore, the use of the FGA7-PG treatment may favor efficiency of progestagen-based protocols for reproductive management.

**Keywords:** Estrous synchronization; Ovine

## 1. Introduction

Artificial insemination in sheep is a common practice to improve genetics of flocks, by using semen from selected sires, and to improve reproductive management, by reducing the number of required rams on farms. The implementation of artificial insemination makes necessary the induction of reproductive activity in animals during the anestrous season and the synchronization of time of ovulations for a greater fertility when there are timed artificial inseminations without previous estrous detection, which is the usual protocol for insemination in sheep. Induction and synchronization of estrus and ovulation, in field practice, is mostly based on the insertion of progestagen-impregnated intravaginal sponges for 12 to 14 days, followed by the intramuscular injection of equine chorionic gonadotrophin (eCG) at sponge removal. Insemination can be performed from 47 (intrauterine) to 55 (intracervical) hours after removal of the device (Abecia et al., 2012).

Such protocols have been used without major changes since development in the early 1960s (Robinson, 1965). The only more recent change has been a reduction of the dose to 20 mg (half the amount of the concentration in previous devices) of progestagen in the case of fluorogestone acetate sponges . With the use of the changed dose of progestogen, there were similar physiological, behavioral, and endocrine effects as with the 40 mg dose that had been previously used (Letelier et al., 2009). Fertility after progestogens, however, has been reported to be less than after natural estrus (Killian et al., 1985; Scaramuzzi et al., 1988). Possible causes have been hypothesized to be related to the long-term duration of the treatment (Menchaca and Rubianes, 2004; Gonzalez-Bulnes et al., 2005). Sometimes the release of progestagen from the sponges is not enough to be effective in controlling the reproductive physiology at the end of the treatment period. This occurs because secretion of LH is not adequately suppressed which results in abnormal follicular development with large persistent follicles being present in the ovaries for longer than typical periods of time during the luteal phase of the estrous cycle (Johnson et al., 1996; Viñoles et al., 1999). There also are associated alterations in the patterns of LH release (Scaramuzzi et al., 1988) and ovulations are atypical as compared with the ovulations that occur during estrous cycles of untreated ewes (Killian et al., 1985; Gonzalez-Bulnes et al., 2005). Maintenance of intravaginal progestagens for such a long period as 14 days is also related to the development of vaginitis and problems with lack of sponge retention (Suarez et al., 2006; Martins et al., 2009), which are not consistent with what is desired from an animal welfare and health perspective.

A possible alternative to minimize the time of insertion of intravaginal devices is the use of protocols with a short-term progestogen treatment (Ungerfeld and Rubianes, 1999; Knights et al., 2001; Viñoles et al., 2001). In brief, the treatment consists of the insertion of progestagen-impregnated sponges for 6 or 7 days. Treatment periods of this length result in increased circulating progestogens for shorter periods than what occurs with endogenous progesterone from the corpus luteum during a typical estrous cycle. It, therefore, is necessary to induce lysis of the corpus luteum in estrous cycling animals when the regimen of shorter periods of progestogen treatments is used. Treatment with a single dose of prostaglandin $F_{2\alpha}$ or its analogues at either the time of insertion (Letelier et al., 2009) or the removal of the sponge (Menchaca and Rubianes, 2004; Cox et al., 2012) will allow for a synchronous decrease in circulating progestogen and synchrony in the time of estrous expression.

Short-term protocols are more and more frequently used for sheep artificial insemination under field conditions but even with the advantages with use of this protocol there is still less use of this progestogen treatment regimen than that of the classical long-term treatments. There are, to the best of our knowledge, no previous comparative studies of possible differences in follicular and endocrine events with use of long-term and short-term treatments with administration of prostaglandins at insertion and removal of the progestagen sponge. Such data may give substantial information for adapting timing of artificial insemination and enhancing fertility outcomes with such treatments. Hence, the objective of the current experiment was to compare possible differences among these three protocols (using fluorogestone acetate sponges) in preovulatory follicular dynamics and functionality, timing and characteristics of estrous behavior and the preovulatory LH surge, and number and progesterone secretion of the induced corpora lutea after progestagen treatment.

## 2. Material and methods

### *2.1. Animals and experimental design*

The experiment was conducted during the non-breeding season and involved a total of 32 ewes, 2 to 5 years-old with a mean body score of 3.5 ± 0.5 (scale 1 to 5). Sheep were maintained outdoors with access to indoor facilities at the experimental farm of the Universidad CEU Cardenal Herrera in Naquera (Valencia, Spain; latitude 39 ºN), which meets local, national and European requirements. The experiment was performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE about the protection of animals used for research, and was specifically assessed and approved by the CEU Cardenal Herrera Committee of Ethics in Animal Research (report CEEA17/019).

Ovarian cyclic functions and ovulation were synchronized in all the animals by the insertion of one intravaginal progestagen impregnated sponge (20 mg fluorogestone acetate, FGA, Chronogest®, MSD Animal Health, Madrid, Spain) plus the administration of one i.m. injection of 400 IU of eCG (Foligon®, MSD Animal Health, Madrid, Spain) at the time of sponge withdrawal. Sheep were divided in three groups according to the duration of the sponge insertion. The first group (FGA14; *n* = 9) received a classical protocol with 14 days of duration of progestogen treatment, whilst the progestagen sponge was maintained 7 days in the other two groups. These two short-term progestagen groups received an i.m. injection of 5 mg of prostaglandin $F_{2\alpha}$ (dinoprost tromethamine, Dinolytic®, Zoetis, Madrid, Spain) at either the insertion or the withdrawal of the sponge (groups PG-FGA7, *n* = 12, and FGA7-PG, *n* = 11, respectively).

The variables evaluated during the induced follicular phase and the subsequent luteal phase were timing of onset of estrous behavior, preovulatory follicular dynamics and functionality (in terms of estradiol secretion), timing and extent of the preovulatory LH surge, and number and functionality (in terms of progesterone secretion) of the induced corpora lutea.

*2.2. Timing of estrous behavior*

Symptoms of estrous behavior were determined every 4 h from 12 to 60 h after sponge withdrawal by the use of trained rams in a proportion of one ram/one ewe. Interval from treatment to estrus onset was defined by the time elapsed between device removal and the first observed mating.

*2.3. Evaluation of preovulatory follicular dynamics and functionality*

In all the animals, number, size and position of all follicles ≥3.5 mm in size were recorded in a diagram of each one of both ovaries, daily from sponge withdrawal to 48 h later. Ovaries were examined by transrectal ultrasonography using a real-time, B-mode scanner (Aloka SSD 500, Aloka Co. Ltd., Tokyo, Japan) fitted to a 7.5 MHz linear-array probe, as previously described and validated in the laboratory of the researchers that conducted the present study (Gonzalez-Bulnes et al., 1994). Follicular dynamics during the follicular phase was characterized retrospectively for the animals responding to the treatment by determining changes in diameter of the largest and the second largest follicles (LF1 and LF2, respectively) and changes in both the number of total large and medium follicles (≥ 5.5 mm and 3.5–5.4 mm, respectively) and number of large and medium growing follicles (those that increased in size when compared to the previous day).

Follicular function was evaluated in terms of estradiol secretion. Thus, jugular blood samples (5 mL) were collected, twice daily from device withdrawal to onset of estrus, with heparinized vacuum blood evacuation tubes (Vacutainer® Systems Europe, Becton Dickinson, Meylan Cedex, France). Blood samples were centrifuged at 2000 *g* for 15 min. Thereafter, the plasma was stored at -20 ºC until assayed for estradiol-17β determination. Such determination was performed by using a highly sensitive commercial solid-phase radioimmunoassay kit for the direct quantitative determination of estradiol-17β (ESTR-US-CT Ultrasensible, IBA Molecular, Madrid, Spain), as described by Romeu et al. (1995) and adapted for use in sheep plasma (Gonzalez-Bulnes et al., 2003). Sensitivity of the assay was 0.5 pg/mL and inter- and intra-assay variation coefficients were 6.1% and 3.5%, respectively (concentrations used as controls for variation coefficients ranged from 1 to 50 pg/mL).

*2.4. Timing and interval of preovulatory LH surge*

The characteristics of the preovulatory LH surge were evaluated by collecting jugular blood samples at 4 h intervals from 32 to 80 h after sponge withdrawal. Plasma LH was measured using a commercial enzimoimmunoassay kit (LH Detect®, INRA, Tours, France). The sensitivity of the assay was 0.01 ng/mL and the inter- and intra-assay variation coefficients were 7.4% and 8.5%, respectively (concentrations used as controls for variation coefficients ranged from 0.05 to 40 ng/mL). In the characterization of the preovulatory LH surge, the onset of the surge was defined as the nadir point before LH concentration exceeded an increase of 10% as compared with the values for the basal concentration (Veiga-Lopez et al., 2006). Basal LH concentration for each ewe was calculated as the mean of the LH concentrations determined over sampling time, except concentrations included in the LH surge. Timing of maximum LH was established as the point of the greatest LH concentration.

*2.5. Ovulation rate and corpora lutea functionality*

In all the animals responding to the treatments, number of corpora lutea was determined by ultrasonography at Day 11 of the induced estrous cycle. The luteal functionality was evaluated in terms of progesterone secretion, by collecting blood samples coincidentally with ultrasonic assessments and processing these as previously described in this manuscript. Plasma progesterone concentrations were measured using a commercially available direct solid-phase RIA kit (PROG-CTRIA, IBA Molecular, Madrid, Spain). Sensitivity of the assay was 0.05 ng/mL and the inter- and intra-assay variation coefficients were 4.5% and 3.5%, respectively (concentrations used as controls for variation coefficients ranged from 1 to 18 ng/mL).

*2.6. Statistical analysis*

Statistical analysis was performed using SPSS® 22.0 (IBM Corporation, New York NY, USA). Heterogeneity for confounding factors (age and body condition) was assessed and such factors were included in the model. Differences in the numerical variables (hormonal and follicular data) were estimated by analysis of variance (ANOVA) using the Greenhouse significance level and Student-Newman-Keuls and Duncan *post hoc* tests to contrast the differences within the groups. Correlations between number of follicles and plasma estradiol concentrations were conducted by Pearson correlation analysis. Binomial dependent variables (response to treatment) were analyzed using chi square test. Additionally, the significance of the effect of the experimental treatments was ascertained with binary logistic regression procedures, by prospective steps based on in Wald statistics with criteria of $P > 0.1$, including first-degree interactions, including into the model the confounding factors (age and body condition). All results were expressed as mean ± standard deviation and with $P < 0.05$ there were considered to be differences and with $P < 0.1$ there was considered a be a trend toward differences in response to treatment.

## 3. Results

### *3.1. Timing of estrous behavior*

The ewes in the FGA14 and FGA7-PG groups had a trend for a greater percentage expressing estrous symptoms when compared to the PG-FGA7 group ($P = 0.065$; Table 1). The cumulative percentage of animals expressing estrous behavior over time after sponge withdrawal is depicted in Figure 1. There were no statistical differences in the mean timing for onset of estrous behavior after progestagen withdrawal among groups. All the ewes expressing estrous behavior had ovulations regardless of the treatment.

### *3.2. Preovulatory follicular dynamics and functionality*

The pattern of growth of the largest follicles during the follicular phase was similar between groups (Figure 2). Briefly, the number of follicles of ovulatory size (≥5.5 mm) and the mean diameter of the largest follicles (LF1 and LF2) increased prior to estrus ($P < 0.05$ for all the groups). The number of both total and growing medium and large follicles (3.5–5.4 mm and ≥5.5 mm, respectively), however, was different among treatments ($P < 0.05$). At 24 h after sponge withdrawal, the number of total and growing medium follicles was greater in the FGA7-PG group. At 48 h after sponge withdrawal, there was a marked increase in the number of total and growing large follicles in the ewes where the short-term protocols were imposed (from 0.7 to 1.7 in the group PG-FGA7, $P = 0.076$, and from 1.0 to 2.6 in the FGA7-PG group, $P < 0.005$) and therefore the number of preovulatory follicles at 48 h was greater in such groups than in the FGA14 group ($P < 0.05$).

Changes in size and number of large preovulatory follicles were correlated with changes in plasma concentration of estradiol ($P < 0.05$), with marked and linear increase from sponge withdrawal to 36 h later in the short-term groups but a lesser increase in the FGA14 group due to lesser values at 24 h following sponge removal ($P < 0.005$).

*3.3. Timing and interval of preovulatory LH surge*

The data for mean timing of the onset of the preovulatory surge LH release after onset of estrous behavior are included in Table 1. There was, similar to  the timing of onset of estrus, no differences among groups. The duration of time from onset of estrous behavior to the preovulatory LH surge release (Figure 3) was less in the FGA14 and FGA7-PG groups than the PG-FGA7 group ($P < 0.05$), with about 90% of the ewes in the FGA7-PG group having the LH peak 8 h after the onset of estrous behavior. The PG-FGA7 group, had a greater variation in the time of onset of preovulatory surge releases of LH and also had lesser maximum concentrations of LH during the surge release ($P < 0.05$).

*3.4. Ovulation rate and corpora lutea functionality*

There were no significant differences among treatment responses for the number of corpora lutea (neither for the mean progesterone concentrations (Table 1).

## 4. Discussion

The present study supports the thought that short-term (7-days) progestagen-based protocols (fluorogestone acetate, FGA) are equally effective for inducing estrus and ovulation and there are a similar number of corpora lutea with similar progesterone secretion as when classical (14-days) protocols are used for estrous synchronization. There, however, were differences in the efficiency and extent of synchronization among treatments which may influence the most desirable protocol option for estrous synchronization.

Firstly, there was a trend for a lesser number of ewes responding with estrus and ovulation after 7-days treatments with prostaglandin F2α injection at sponge insertion (group PG-FGA7) than after 7-day treatments with prostaglandin $F_{2\alpha}$ injection at the time of sponge withdrawal (group FGA7-PG) and after 14-day protocols (FGA14). This factor cannot be ignored even though there was a lack of statistical significance because from a productivity and economic perspective it is important to recognize that about 26% of ewes did not respond to the PG-FGA7 treatment in the first 80 h after sponge removal. Hence, although further studies with a larger number of animals are obviously necessary, such data may indicate that it is not efficacious from an estrous synchrony perspective to use 7-day progestogen treatments with prostaglandin $F_{2\alpha}$ injections at sponge insertion. A possible explanation for the markedly lesser percentage of ewes responding to the treatment in the PG-FGA7 group may be related to some ewes being in the very early-luteal phase of the estrous cycle at the time of sponge insertion and prostaglandin injection (i.e., had ovulations 1 or 2 days before the time of PG treatment). Administration of prostaglandin $F_{2\alpha}$ is known to be effective for inducing luteolysis if there has been a 3 days from the time when ovulation occurred (Rubianes et al., 2003; Contreras-Solis et al., 2009). Thus, animals with early-stage corpora lutea development would not respond to prostaglandins and would continue to have a normal-length estrous cycle after sponge removal. Conversely, ewes with early-stage corpora lutea at sponge insertion in the FGA7-PG group would be in mid-luteal phase 7 days later and would respond to prostaglandin treatment at the time of sponge removal by having a normal follicular phase and subsequently a synchronized time of estrus and ovulation.

After the onset of estrous behavior, the assessment of the preovulatory surge release of LH resulted in new findings regarding the response in the PG-FGA7 group. Ewes in this group had a lesser maximum concentration of LH during the preovulatory surge release of LH and more variation in the range when the surge occurred after sponge removal to the onset of the preovulatory LH surge than the other two treatment groups (FGA14 and FGA7-PG). The assessment of the preovulatory LH surge indicated that the most synchronous grouping occurred as a result of the FGA7-PG treatment rather than the FGA14 treatment, with about 90% of the ewes in the FGA7-PG group having the LH discharge 8 h after the onset of estrous behavior. These features may be related to the differences in the preovulatory follicle dynamics among groups.

In all the groups, the largest follicles increased in diameter during the follicular phase. The assessment of the follicular dynamics indicated that the FGA7-PG group had a larger number of growing medium follicles than the other two groups at 24 h after sponge removal and a larger number of growing large follicles than the FGA14 group at 48 h after sponge removal. This means that, inconsistent with what occurred with ewes of the FGA14 group, most of the ovulatory follicles in both short-term treatment groups (especially in the group FGA7-PG) emerged from newly recruited follicles and were undergoing active growth phase during the follicular phase. This finding is supported by the increase in plasma estradiol concentrations that was observed during the follicular phase, which was greater in both short-term treatment than the classical treated groups. Concomitantly, the FGA14 group had a lesser estradiol concentration at 24 h after sponge withdrawal, when most of the ewes in this group were in estrus. In ewes, circulating estradiol is considered as a reliable marker of follicular quality (Campbell et al., 1995; Gonzalez-Bulnes et al., 2004) and a lesser estradiol secretion during the preovulatory phase has been related to aberrant preovulatory follicular development and development of persistent follicles (Gonzalez-Bulnes et al., 2005). In cows, ovulation of defective persistent follicles has been related to alterations in the developmental competence of the oocytes (Revah and Butler, 1996; Mihm et al., 1999) and, hence, alterations in fertility (Bridges and Fortune, 2003). Such effects have also been described in ewes (Johnson et al., 1996; Ungerfeld and Rubianes, 1999; Viñoles et al., 1999).

Possible causes for these differences in follicular dynamics between long- and short-term treatment groups may be related to the kinetics of progestagen release from the sponge. The release of progestagen after sponge insertion results in maximum plasma concentrations about 48 h later (Robinson, 1965; Greyling and Van der Nest, 2000). Progestagen release and, therefore, plasma progestogen concentrations decrease throughout the period of sponge placement and, at the end of long-term treatment periods there may be concentrations that are too low to simulate the functions of the corpus luteum (Robinson et al., 1968). Consequently, the long-term progestagen protocols may not adequately suppress LH secretion, as has been shown to occur in cows (Kojima et al., 1992), leading to inadequate follicular development with persistent large follicles in the static or early atretic phase (Johnson et al., 1996; Leyva et al., 1998; Viñoles et al., 1999; Flynn et al., 2000). Conversely, short-term protocols would be more adequate to maintain sustained progestagen concentrations, suppress LH secretion and induce an adequate follicular development (Ungerfeld and Rubianes, 1999; Viñoles et al., 2001; Letelier et al., 2009). In fact, results of previous studies in cattle and sheep indicate the preovulatory LH surge and ovulation are advanced in animals with large follicles in growing phase at the beginning of the follicular phase (Stevenson et al., 1998; Viñoles and Rubianes, 1998; Veiga-Lopez et al., 2008), because these growing follicles require a shorter time for final preovulatory growth and ovulation (Scaramuzzi et al., 1980; Kastelic and Ginther, 1991; Stevenson et al., 1998), which may have occurred in the ewes in the FGA7-PG group of the present study.

In view of these considerations, the current results are consistent with previous reports of the suitability of a short-term progestagen treatment associated with prostaglandin $F_{2\alpha}$ injection at device removal (Menchaca and Rubianes, 2004; dos Santos-Neto et al., 2015). In conclusion, the results of the present study support the use of such protocols, rather than of short-term progestagen protocols with prostaglandin $F_{2\alpha}$ injection at sponge withdrawal, as an alternative to classical long-term protocols.

**Acknowledgments**

The authors thank the CEU-Cardenal Herrera farm staff for their assistance with animal care and handling.

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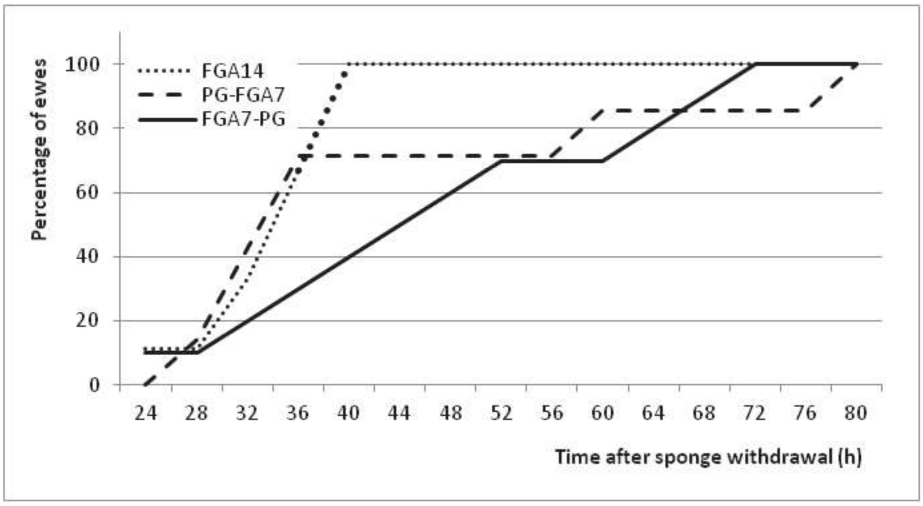
**Figure captions**

Fig. 1. Cumulative percentage of ewes expressing estrous behavior after sponge withdrawal (FGA14 group: 14 days of progestagen sponge + eCG at sponge withdrawal; PG-FGA7 group: 7 days of progestagen sponge + $PGF_{2\alpha}$ at sponge insertion + eCG at sponge withdrawal; FGA7-PG group: 7 days of progestagen sponge + $PGF_{2\alpha}$ and eCG at sponge withdrawal).

Fig. 2. Mean diameter of the largest and the second largest follicle (LF1 and LF2, respectively; left column), mean number of large and medium follicles (≥5.5mm and 3.5-5.4 mm, respectively; middle column) and mean number of growing medium and large follicles (right column) during the follicular phase (FGA14 group: 14 days of progestagen sponge + eCG at sponge withdrawal; PG-FGA7 group: 7 days of progestagen sponge + $PGF_{2\alpha}$ at sponge insertion + eCG at sponge withdrawal; FGA7-PG group: 7 days of progestagen sponge + $PGF_{2\alpha}$ and eCG at sponge withdrawal). There were only significant differences among treatments in the number of both total and growing medium and large follicles; the number of total and growing medium follicles was greater in the FGA7-PG group at 24 h after sponge withdrawal while, at 48 h, the number of large growing follicles was higher in both short-term treatments than in the FGA14 group ($P<0.05$ for all).

Fig. 3. Cumulative percentage of ewes having a preovulatory LH surge release after the onset of estrous behavior (FGA14 group: 14 days of progestagen sponge + eCG at sponge withdrawal; PG-FGA7 group: 7 days of progestagen sponge + $PGF_{2\alpha}$ at sponge insertion + eCG at sponge withdrawal; FGA7-PG group: 7 days of progestagen sponge + $PGF_{2\alpha}$ and eCG at sponge withdrawal).



Percentage of ewes
100
80
60
40
20
0
FGA14
PG-FGA7
FGA7-PG
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
Time after sponge withdrawal (h)


Figure 1

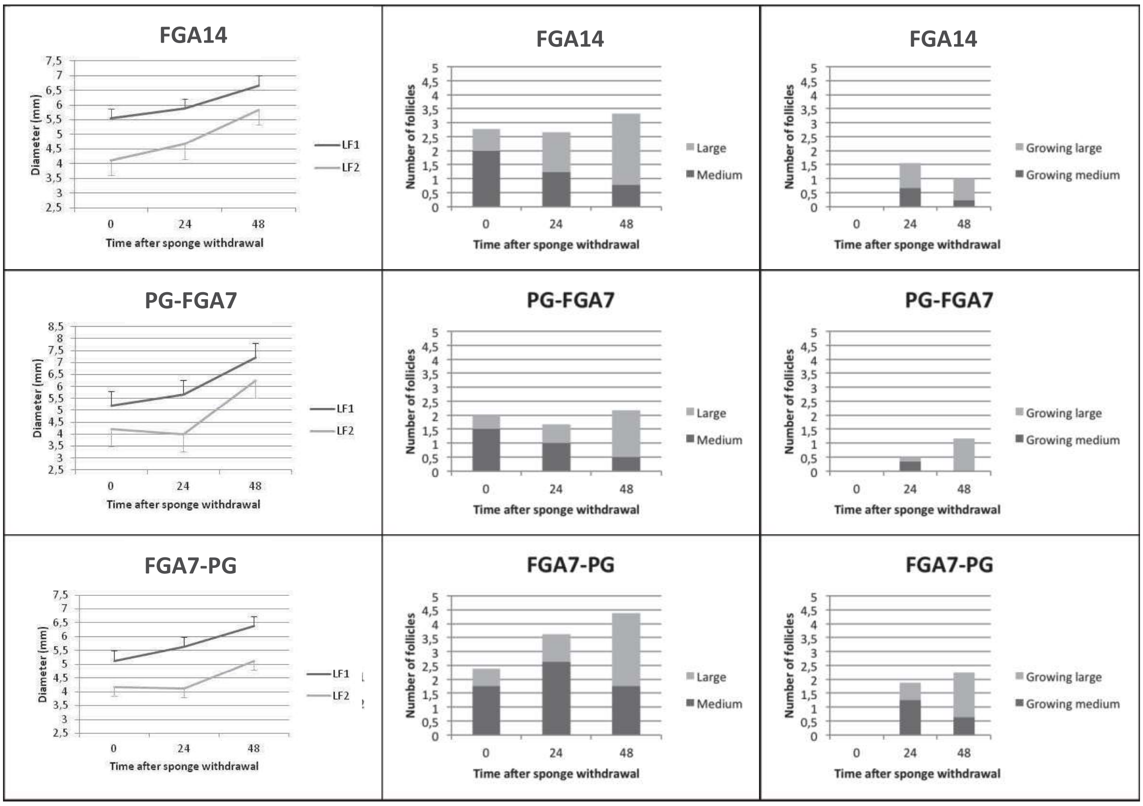

FGA14
Diameter (mm)
LF1
LF2
Time after sponge withdrawal
Number of follicles
Large
Medium
Growing large
Growing medium
PG-FGA7
FGA7-PG

Figure 2

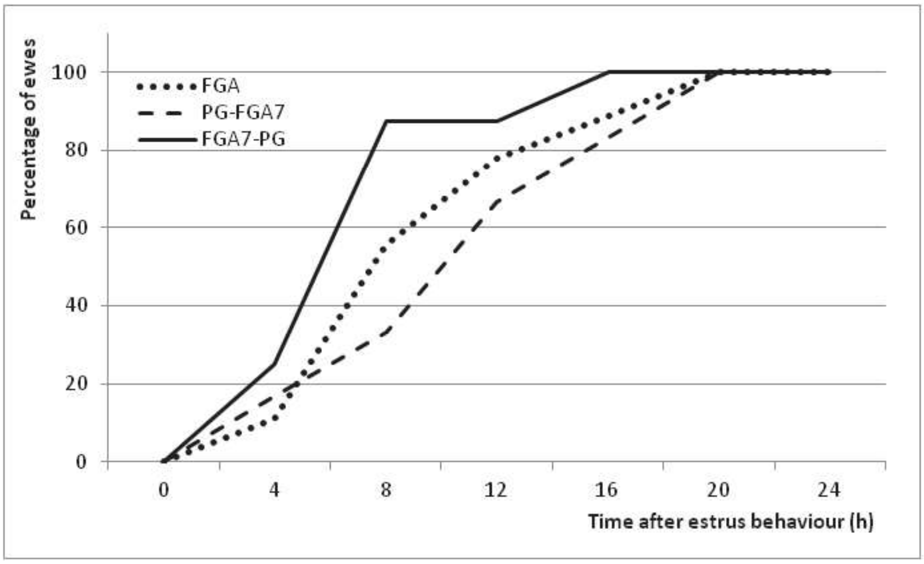

Percentage of ewes
100
80
60
40
20
0
FGA
PG-FGA7
FGA7-PG
0
4
8
12
16
20
24
Time after estrus behaviour (h)

Figure 3

Table I. Percentages and mean timing (± standard deviation) of estrus behavior and preovulatory LH surge and mean number of corpora lutea and plasma progesterone concentration after different treatments for estrus and ovulation synchronization (group FGA14: 14 days of progestagen sponge + eCG at sponge withdrawal; group PG-FGA7: 7 days of progestagen sponge + $PGF_{2\alpha}$ at sponge insertion + eCG at sponge withdrawal; group FGA7-PG: 7 days of progestagen sponge + $PGF_{2\alpha}$ and eCG at sponge withdrawal). Different superscripts indicate statistically significant differences ($P<0.05$).

| | **FGA14 (n=9)** | **PG-FGA7 (n=12)** | **FGA7-PG (n=11)** |
|---|---|---|---|
| **Estrus behavior (%)** | 100 | 63.6 | 90.9 |
| **Timing of onset of estrus behavior after sponge removal in hours (range)** | 35.1 ± 5.2 (24-40) | 43.4 ± 19.2 (28-80) | 48.0 ± 16.0 (24-72) |
| **Timing of LH surge after estrus onset in hours (range)** | 10.7 ± 4.9 (4-20) | 12.0 ± 5.7 (4-20) | 8.0 ± 3.7 (4-16) |
| **Maximum concentration of LH surge in ng/mL (range)** | 38.9 ± 27.7 [a] (13.1-77.9) | 12.4 ± 4.4 [b] (6.2-17.8) | 47.1 ± 30.5 [a] (13.6-80.0) |
| **Number of corpora lutea** | 2.6 ± 0.9 (1-4) | 2.4 ± 0.5 (1-3) | 2.6 ± 0.5 (1-3) |
| **Plasma progesterone concentration in ng/mL (range)** | 4.0 ± 3.7 (2.6-13.4) | 2.1 ± 1.5 (1.1-7.7) | 3.1 ± 1.8 (1.3-16.3) |

## Conflict of interests

The authors confirm that there are no conflicts of interests.