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Short-term dietary protein supplementation improves reproductive performance of estrous-synchronized ewes when there are long intervals of prostaglandin or progesterone-based treatments for timed AI

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ABSTRACT

To evaluate the reproductive effects of a short-term dietary protein supplementation (Days -10 to -3) before timed AI (TAI = Day 0), 471 Merino ewes grazing native pastures were estrous-synchronized when there were either long intervals between prostaglandin administrations (two prostaglandin injections 15 or 16 d apart; PG15 and PG16, respectively) or with a progesterone-eCG (P4-eCG) protocol, resulting in a 3 × 2 experimental design. Ovulation rate on Day 8 (OR), non-estrous-return to Day 21 (NRR21), and fertility, prolificacy and fecundity on Day 70 were evaluated. The interaction between estrous synchronization protocol and supplementation was not significant for any of these variables ($P > 0.05$). Supplementation increased OR, prolificacy and fecundity (+0.14, +0.15 and +0.14, respectively, $P < 0.01$), but did not affect NRR21 or fertility of ewes (+6.2% and +6.7% respectively, $P > 0.05$). Ewes treated using the PG15 and PG16 protocols had a lesser OR (-0.27), prolificacy (−0.22) and fecundity (−0.20) than ewes treated using P4-eCG protocol ($P < 0.01$ for each), and similar NRR21 and fertility (−5.4% and −7.9% respectively, $P > 0.05$ for both variables), without significant differences between the PG15 and PG16 groups. In conclusion, a short-term dietary protein supplementation before TAI improved OR, prolificacy and fecundity of ewes which were estrous-synchronized by imposing long interval PG (15 or 16 d apart) or P4-eCG-based protocols. There was a greater OR,

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prolificacy and fecundity when there was use of the P4-eCG compared to long interval PG-based protocols. Estrous-non-return rate after AI and fertility as a result TAI were not affected by either the supplementation or the estrous synchronization protocols used.

1. Introduction

Timed Artificial Insemination (TAI) is a practical technique for genetic improvement allowing for synchronized timing of AI without estrous detection. There is, therefore, a more efficient use of genetically superior males, nutritional resources and labor. Use of TAI includes hormonal treatments to ensure synchronized timing of ovulation (Menchaca and Rubianes, 2004). Conventional TAI protocols involve use of intravaginal sponges impregnated with progestagens (P4) maintained during 12–14 d, in conjunction with equine chorionic gonadotropin at the time of device withdrawal (P4-eCG). With use of these treatment regimens, there is acceptable pregnancy rates for breeding during the breeding or non-breeding seasons (Gordon, 1999; Abecia et al., 2012). Use of the P4-eCG based protocols, however, have been associated with alterations in oocyte quality, which leads to lesser fertilization rates due to impaired embryo development (González-Bulnes et al., 2005; Berlinguer et al., 2007). In addition to practical operational aspects (placement and removal of progestogen devices, losses and adhesions of sponges), the use of P4-eCG based protocols can potentially result in environmental and tissue contaminant issues due to residues of sponges, eCG, or antibiotics used to prevent vaginitis (González-Bulnes et al., 2005; Contreras-Solís et al., 2009; Martemucci and D'Alessandro, 2011; Viñoles et al., 2011), thus, there are objections about use of these products by some individuals. The application of TAI in commercial farming conditions requires easy implementation procedures, acceptable reproductive outcomes and minimal environmental effects (Martin and Ferasyi, 2016). With these considerations, and due to its rapid rate of metabolization (Piper et al., 1970; Davis et al., 1980), easiness of application and relatively lesser cost, use of prostaglandin is an option for the reproductive management in sheep (Abecia et al., 2012; Fierro et al., 2013).

Prostaglandin F2α and its synthetic analogues (PG) are potent luteolytic agents in ruminants and use of this compound has previously been proposed for TAI (revised by Fierro et al., 2013). Relatively lesser reproductive outcomes when conventional PG based protocols were imposed (9–12 d apart between injections) as compared with progestogen-based protocols have discouraged the utilization of the PG treatment regimens (Evans and Maxwell, 1987; Menchaca and Rubianes, 2004). Extending the interval between PG injections prolonged the time that pre-ovulatory follicle development was controlled by luteal-stage progesterone concentrations, thus two administrations of PG injections 14–16 d apart ("long interval") resulted in an enhanced reproductive outcome after TAI (Fierro et al., 2016, 2017), to pregnancy rates comparable to that with use of P4-eCG based protocols (Fierro and Olivera-Muzante, 2017). Nevertheless, ovulation rate (OR), prolificacy and fecundity when there was use of the long interval PG protocol for TAI may be less than that with use of P4-eCG based protocols or when ewes are bred based on expression of a spontaneous estrus (Fierro et al., 2011; Fierro and Olivera-Muzante, 2017), a factor to be considered when there is an emphasis on genetic improvement in commercial farming sectors.

The use of alternative approaches such as short-term nutrition treatments ("focus feeding") may be a desirable approach due to lack of use of compounds that could result in contamination of the environment and an effective option to improve the OR and prolificacy associated to long interval PG based protocols for TAI in sheep. With these considerations, Errandonea et al. (2018) reported that a short-term protein supplementation before TAI via the cervical lumen with fresh semen after imposing a long interval PG based protocol (two PG injections administered 15 d apart; PG15), resulted in similar OR, prolificacy and fecundity to that when there was breeding based on detection of a spontaneous pre-synchronized estrus. The effects on reproduction are unknown when there is use of these nutritional alternatives combined with administrations of PG at relatively longer (16 d apart for example) intervals than occurs with typical estrous synchrony protocols, or when there is use of the P4-eCG based estrous synchronization protocol.

The aim of the present experiment, therefore, was to evaluate the reproductive effects with use of a short-term dietary protein supplementation before the time when AI occurred when this treatment was combined with use of two long interval PG (15 and 16 d apart) or P4-eCG based protocols for TAI in sheep. We hypothesize that this supplementation regimen would result in improvements in OR, prolificacy or fecundity of the ewes when there is use of these TAI protocols.

2. Materials and methods

The experiment was conducted during the breeding season (April to June) at a commercial farm ("El Recuerdo"; Artigas-Uruguay, 30 °S–57 °W). The experimental procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary-Universidad de la República (CUEA-FVet Protocol N° 475/17).

2.1. Animals

Multiparous Merino ewes (2.5–4.5 years old, and at least one parturition; $n = 471$), accustomed to consuming supplements, with a moderate body condition score (2.9 ± 0.2, mean ± SD; scale 0–5, Russel et al., 1969) and weighing 41.2 ± 4.9 kg (fasted overnight; Q&F AD-4406 weighing scale, Japan) at the beginning of the experiment were used (Day -18; Day 0 = TAI).

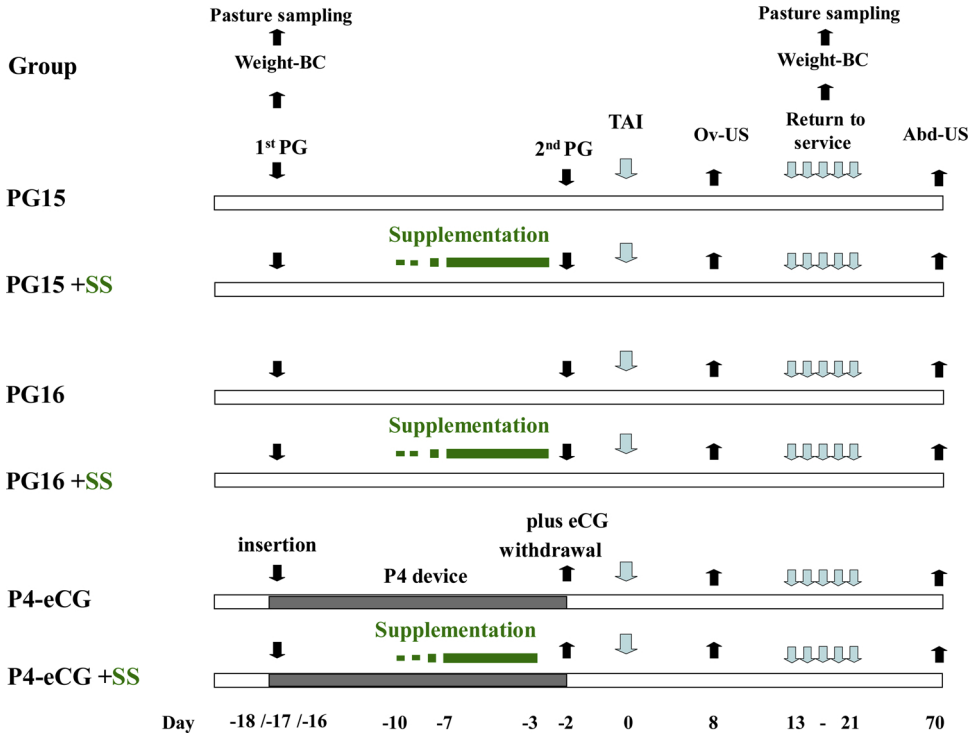

Fig. 1. Experimental design: Group PG15 or PG16: ewes in which timing of estrus was synchronized with two PG injections 15 or 16 d apart; P4-eCG: ewes in which timing of estrus was synchronized with an intravaginal sponge impregnated with progestagens plus an im injection of equine chorionic gonadotropin at time of sponge withdrawal; TAI: timed insemination via the cervical lumen with fresh pooled semen (Day 0); Group PG15 + SS, PG16 + SS or P4-eCG + SS: ewes in which timing of estrus was synchronized using PG15, PG16 or P4-eCG treatments plus a short-term dietary supplementation ("Supplementation"; +SS) during 8 d (Days -10 to -3 relative to TAI); Weight: evaluated using an electronic scale on Day -18 and 21 with ewes fasted overnight; BC: body condition score; Pasture sampling: to evaluate forage mass; Ov-US: ovarian trans-rectal ultrasonography to evaluate ovulation rate; Return to estrus: assessed using breast-painted rams; Abd-US: trans-abdominal ultrasonography on Day 70 to evaluate fertility and prolificacy.

2.2. Experimental design

Ewes were randomly assigned on Day -18 to one of the six groups in a 3 × 2 factorial design: type of estrus (three levels) -induced using two different intervals of PG injections (15 or 16 d apart; PG15 and PG16) or P4-eCG- and a short-term protein supplementation (two amounts) -yes (+SS) or not- before insemination, resulting in the following groups (body condition score, live weight and age, means ± SEM): PG15 (2.9 ± 0.02, 41.1 ± 0.6 kg and 3.8 ± 0.1 years old; $n = 78$;), PG15 + SS (2.9 ± 0.02, 41.0 ± 0.6 kg and 4.0 ± 0.1 years old; $n = 80$), PG16 (2.9 ± 0.02, 41.5 ± 0.6 kg and 4.1 ± 0.1 years old; $n = 78$), PG16 + SS (2.9 ± 0.02, 41.7 ± 0.5 kg and 3.9 ± 0.1 years old; $n = 78$), P4-eCG (2.9 ± 0.02, 41.0 ± 0.5 kg and 3.8 ± 0.1 years old; $n = 78$) or P4-eCG + SS (2.9 ± 0.02, 41.1 ± 0.5 kg and 4.0 ± 0.1 year old; $n = 79$), without significant differences between values for these variables ($P > 0.05$). A schematic representation of the experimental design is depicted in Fig. 1.

2.3. Estrous synchronization protocols

Ewes were estrous synchronized using two PG injections administered 15 or 16 d apart on Day -17 or -18 and -2, respectively (D-L Cloprostenol im, 125 µg per injection, Estrumate®, Schering-Plough, Germany), in PG groups, or using the Medroxi-progesterone acetate impregnated intravaginal sponges (MAP 60 mg; Syntex Lab., Uruguay), dusted with antibiotics (Oxitetraciclina, Tetrabion Oxi 200®; Fatro, Uruguay), which remained inserted for 14 d (Days -16 to -2) and with an im injection of eCG at the time of sponge withdrawal (300 UI; Novormon 5000®; Syntex Lab., Uruguay) in P4-eCG based groups. To inseminate all ewes at the same time intravaginal sponge was removed 6 h after the second PG injection in the PG-treatment groups.

2.4. Feed intake and composition

Forage mass of experimental paddocks were sampled at Day -18 and 21 as described by Haydock and Shaw (1975). Pasture and supplements were analyzed for percentage of dry matter (DM), crude protein (CP), acid detergent fiber (ADF) and neutral detergent fiber (NDF) at the Laboratory of Nutrition of INIA "La Estanzuela". Percentage of DM and CP (N*6.25) were measured using the

Official Methods 934.01 and 955.04 of the AOAC System (1990), respectively. The ADF and NDF were measured using the protocols described by Van Soest et al. (1991). Content of metabolic energy (ME) was estimated using the NRC recommendations (2007).

Ewes were maintained in field conditions where they grazed native pastures in a paddock of 16 ha with a forage allowance of 10 kg of DM/100 kg of body weight/d (initial forage mass of 2300 kg DM/ha; CP: 5.8%, ADF: 44.7%, NDF 69.7%, ME: 1.98 Mcal/kg DM), and fresh water was available *ad libitum*. Ewes with dietary supplementation (PG15 + SS, PG16 + SS and P4-eCG + SS) were transferred to a similar parcel of the same paddock during the period of differential feeding. Short-term supplementation was conducted using a mixture of pelleted soybean meal and whole sorghum grain (ratio 80/20; DM: 89.2%; CP: 45.4%, ADF: 13.9%, NDF: 22.7%, ME: 2.83 Mcal/kg DM). The experimental dietary regimen was gradually imposed from Day -10 to -8 (0.5, 0.8 and 1.0% of body weight/ewe/d of supplement on a DM basis) with the complete diet being fed from Day -7 to -3 relative to TAI (1.3% of body weight/ewe/d of supplement in DM basis) as has been previously described by Banchero et al. (2012). The supplement was provided collectively at 8:00 am, in a linear feeding trough placed in the paddock, with a feeding access space of 0.35 m/ewe. To evaluate supplement consumption, ewes were identified with a painted number on their flank. All ewes ate supplement and they consumed the entire amount of feed provided. To calculate CP intake, it was assumed that the ewes would consume 3.1% to 3.2% of DM matter as a percentage of their body weight (NRC, 2007; Banchero et al., 2012), then, the theorical (pasture) or real intake (supplement) was multiplied by the CP concentration of each treatment diet. The estimated average intakes of CP between Days -7 to -3 (irrespective of changes in pasture selection between supplementation treatments) were 75 and 288 g/ewe/d for non-supplemented and supplemented groups, respectively. The estimated CP and ME consumed by the ewes of all groups during the supplementation period were similar to the feeding regimen described for maintenance gain by the NRC (2007).

2.5. Semen collection, evaluation and dilution

Semen was collected from 11 Merino Dohne adult rams (checked for normal breeding soundness) using an artificial vagina and assessed as described by Evans and Maxwell (1987). Two consecutive ejaculates from each ram were collected, evaluated for use (> 80% subjective progressive motility) and pooled according to the individual sperm concentration, so that each ram contributed a similar number of spermatozoa to the pool. Soon after pooling, semen was extended with UHT skim milk with antibiotic (enrofloxacin 250 mg/L, Baytril® 10%, Bayer, Uruguay) to the final sperm concentration (mean of 1066×10^6 spermatozoa/ml). Extended semen was maintained at room temperature and protected from sunlight until AI. The same semen pool was used for ewes of all groups.

2.6. AI procedures

Insemination via the cervical lumen was randomly performed using a speculum equipped with a light source and an insemination instrument (Walmur® Veterinary Instruments, Montevideo, Uruguay) by two teams of technicians, as described by Evans and Maxwell (1987). The insemination dose was 0.15 cc of extended semen, containing a mean of 160×10^6 spermatozoa that was slowly released in as cranial a position as possible into the cervix. Ewes were inseminated at the same time at 56 ± 2 h after administration of the second PG injection (Fierro et al., 2016) or 50 ± 2 h after sponge withdrawal (Evans and Maxwell, 1987) in PG and P4-eCG based groups, respectively.

2.7. Ovarian response, estrous-non-return rate and fertility measurements

Ewes having ovulations (ewes with ovulations/total ewes in experimental group x 100), and OR (number of CL/number of ewes with ovulations) were evaluated on Day 8 using transrectal ultrasonography (7.5 MHz linear array. ALOKA SSD-500, Overseas Monitor Corp. Ltd., Tokyo, Japan) as described by Viñoles et al. (2010a). Non-return rate to service on Day 21 (number of ewes not expressing behavioral estrus/total number of ewes in experimental group x 100; NRR21) was assessed from Day 14 to 21 using breast painted Merino Dohne rams (three rams/100 ewes). Fertility (number of pregnant ewes/total number of ewes in experimental group x 100), prolificacy (number of fetuses/number of pregnant ewes), and fecundity (number of fetuses/ewe in service in experimental group) were evaluated on Day 70 using transabdominal ultrasonography with a 3.5 MHz convex array transducer and the same ultrasonic scanning device.

2.8. Statistical analyses

Ovulation rate, NRR21, fertility, prolificacy and fecundity were analyzed using an analysis of variance for categorical variables, utilizing the CATMOD (categorical modelling) procedure of SAS (SAS 8.3 V; SAS Institute Inc., 2000). The statistical model included the following terms: estrous synchronization protocol (three types), supplementation (two amounts) and the interactions in values for these variables. For variables in which the estrous synchronization protocol was significant, differences between groups were compared using pairwise contrasts and the probabilities were corrected using the Bonferroni-Holm method for multiple tests. The effect of dietary supplementation on body weight and body condition score was analyzed using the repeated-measured analysis of variance using MIXED procedure of SAS (SAS 8.3 V; SAS Institute Inc., 2000). Data for body weight, body condition, OR and prolificacy are presented as means ± SD, while data ewes with ovulations, NRR21 and fertility data are presented as percentages. Differences were considered significant if $P < 0.05$.

Table 1
Reproductive performance in multiparous Merino ewes estrous synchronized with administration of 15 or 16 d apart (PG15, PG16) or with a progestagen treatment plus eCG (P4-eCG) when there was or was not short-term dietary supplementation (+SS) during 8 d (Days -10 to -3 relative to TAI), and inseminated with fresh pooled semen.

Group (*n*)	OR	NRR21 (%)	Fertility (%)	Prolificacy	Fecundity
PG15 (78)	1.10 ± 0.31	42.3	38.5	1.00 ± 0.01	0.38
PG15 + SS (80)	1.27 ± 0.48	48.8	46.3	1.19 ± 0.40	0.55
PG16 (78)	1.07 ± 0.27	50.0	46.2	1.00 ± 0.01	0.46
PG16 + SS (78)	1.19 ± 0.40	51.3	43.6	1.15 ± 0.36	0.50
P4-eCG (75)	1.35 ± 0.48	48.0	44.0	1.24 ± 0.44	0.55
P4-eCG + SS (78)	1.51 ± 0.50	59.0	59.0	1.37 ± 0.49	0.81
Significance ($P <$)					
Treatment (T)	0.0001	0.36	0.24	0.0003	0.0096
**PG15 compared to PG16*	0.24	-	-	0.63	0.83
**PG15 compared with P4-eCG*	0.0003	-	-	0.001	0.014
**PG16 compared with P4-eCG*	0.0003	-	-	0.0003	0.016
Supplementation (S)	0.0002	0.18	0.14	0.0006	0.0074
Interaction (T × S)	0.83	0.69	0.30	0.84	0.30

Group PG15 or PG16: ewes in which timing of estrus was synchronized with two PG injections 15 or 16 d apart followed by TAI via the cervical lumen; P4-eCG: ewes in which timing of estrus was synchronized with an intravaginal sponge impregnated with progestagens plus an im injection of equine chorionic gonadotrophin at the time of sponge withdrawal; TAI: Time Artificial Insemination with fresh pooled semen (Day = 0); Group PG15 + SS, PG16 + SS or P4-eCG + SS: ewes in which timing of estrus was synchronized using the PG15, PG16 or P4-eCG treatments plus a short-term dietary supplementation (+SS) for 8 d (Days -10 to -3 relative to TAI); OR: ovulation rate measurement using trans-rectal ultrasonography on Day 8 (number of CL/number of ewes with ovulations); NRR21: non-estrous-return rate after TAI between Day 14 and 21 as assessed suing breast-painted rams (number of ewes with no estrus return/total number of ewes in experimental group x 100); Fertility (number of pregnant ewes/total number of ewes in experimental group x 100), prolificacy (number of fetuses/number of pregnant ewes with TAI), and fecundity (number of fetuses/ewe in service in experimental group) evaluated on Day 70 using trans-abdominal ultrasonography; Data for OR and prolificacy are presented as means ± SD; NRR21 and fertility are presented as percentages; *Pairwise contrasts corrected for multiple tests using the Bonferroni-Holm method.

3. Results

Four ewes (2.5%) from P4-eCG groups had vaginal adherence of sponges and were excluded from the study. Nine ewes did not have ovulations (1.3, 1.2, 2.6, 1.3, 1.3 and 3.8% of the PG15, PG15 + SS, PG16, PG16 + SS, P4-eCG, P4-eCG + SS groups respectively; $P > 0.05$). Body weight and condition score of ewes did not change throughout the experiment (41.2 ± 4.9 kg compared with 42.3 ± 5.2 kg; 2.9 ± 0.2 compared with3.0 ± 0.2, Days -18 and 21 respectively), being similar on the various days when there were assessments during the treatment period and between times when there was imposing of estrous synchronization protocols or dietary supplementations ($P > 0.05$).

There was no interaction between estrous synchronization protocol and dietary supplementation for any of the evaluated variables ($P > 0.05$; Table 1). Dietary supplementation resulted in a greater OR, prolificacy and fecundity (+0.14, +0.15 and +0.14, respectively; $P < 0.01$), but did not affect NRR21 or fertility of ewes (+6.2% and +6.7%, respectively; $P > 0.05$). There was an effect of synchronization protocol on OR, prolificacy and fecundity ($P < 0.01$), but this did not affect NRR21 or fertility of ewes ($P > 0.05$). Ewes on which the PG15 and PG16 protocols were imposed had a lesser OR (−0.27), prolificacy (−0.22) and fecundity (−0.20) than ewes of P4-eCG group ($P < 0.01$ for each variable), and similar NRR21 and fertility (−5.4% and −7.9%, respectively; $P > 0.05$), and there were no differences between PG15 and PG16 groups ($P > 0.05$).

4. Discussion

The hypothesis for the present study was accepted that a short-term protein supplementation would improve reproductive performance of ewes when there was administration of PG (15 or 16 d apart) or P4-eCG for longer than typical intervals between administrations for TAI. Short-term dietary protein supplementation before the TAI resulted in a greater OR, prolificacy and fecundity when there was imposing of all estrous synchronization protocols, however, there was no effect on fertility.

The results from the present study confirm those recently reported where there was a greater OR and prolificacy associated with a short-term dietary protein supplementation before TAI and when there was administration of PG with a 15-d interval between administrations followed by TAI (Errandonea et al., 2018). Furthermore, in the present study when there was administration of PG with a 16-d interval between administrations there was a greater OR and prolificacy when there was dietary supplementation of protein before TAI. When there was a short-term dietary protein supplementation with two administrations of PG 7 d apart, there was no improvement in reproductive outcome in sheep as a result of the supplementation before TAI (Fierro et al., 2014), confirming that the length of the interval between PG injections (at least with a 14 d interval between administrations), and consequently the progesterone concentrations in the reproductive tissues is important for improving pregnancy outcomes following breeding (Fierro et al., 2013). Results from previous studies where there was 16 d (PG16) between administrations indicated there was a lesser cumulative estrous response than with other treatments, but there was less variation in the timing of behavioral estrus and greater fertility as a result of TAI in comparison with when PG administrations were 7, 10, 12 or 14 d apart (Menchaca et al., 2004; Fierro

et al., 2011, 2016, 2017; Fierro and Olivera-Muzante, 2017). When these previous findings are considered, there is an explanation for the desirable reproductive outcomes when there is a short-term dietary supplementation. There, therefore, are more options for improving pregnancy outcomes in sheep using PG based protocols.

Similarly, with a short-term dietary protein supplementation before TAI, there was a greater OR, prolificacy and fecundity of ewes where there was estrous synchrony using the P4-eCG protocol. It, therefore, is speculated that there is an additive effect with use of the P4-eCG protocol and dietary protein supplementation which may result in minimization of the negative effects of P4 or eCG protocols on the quality of oocytes or embryos as previously reported (Kelly, 1997; Ungerfeld and Rubianes, 1999; Viñoles et al., 2001; Barret et al., 2002; González-Bulnes et al., 2005; Berlinguer et al., 2007). Thus, dietary supplementation of ewes would be advisable also when there is use of P4-eCG protocol. Dietary supplementation, however, did not result in an increased NRR21 or fertility on Day 70 in when any of the estrous synchronization protocols were used in the present study. This result from the present study indicates that short-term dietary supplementation before breeding would improve growth and development of the gonadotropin-sensitive follicles only in ewes with the genetic or metabolic capacity to respond (Viñoles et al., 2010b, 2012; Juengel and McNatty, 2013) but there would not be improvements in fertilization (Monget and Martin, 1997; Viñoles et al., 2012; Errandonea et al., 2018).

The OR, prolificacy and fecundity of ewes in which there was time of estrus synchronized using the PG15 and PG16 protocols was less than when there was use of the P4-eCG protocol. This was an expected result, in part due to the administration of eCG (300 UI) at the end of the imposing of the P4-based protocol because this compound has FSH as well as LH activity (Gordon, 1999; Abecia et al., 2012). The use of this treatment regimen could have promoted the growth of follicles and subsequent ovulation from follicles developing during more than one wave of ovarian follicular development. Consequently, and as expected, there was a greater OR and prolificacy outcome with use of the P4 protocol combined with use of the dose of eCG that was administered (Boland et al., 1979). There, however, were no observed differences in NRR21 or fertility with use of the estrous synchronization protocols imposed in the present study. This result confirms what was reported as a result of findings in previous studies (Fierro and Olivera-Muzante, 2017). In this regard, when the objective is to have more pregnant ewes during the breeding season, with use of any of these TAI protocols used in the present study, there would be desirable results. The use of the protocols where there are longer intervals between PG administrations would be an alternative, therefore, where there would be lesser environmental contamination concerns, less fiscal expenditures needed with use of the treatment regimen, and that is highly practical to implement (González-Bulnes et al., 2005; Contreras-Solís et al., 2009; Viñoles et al., 2011; Olivera-Muzante, 2018).

5. Conclusion

In conclusion, use of a short-term dietary protein supplementation from Days -10 to -3 relative to the timing of a cervical TAI resulted in a greater OR, prolificacy and fecundity of estrous-synchronized ewes using a protocol where there was 15 or 16 d between PG administrations or by using a P4-eCG treatment regimen. There was a greater OR, prolificacy and fecundity when there was use of the P4-eCG treatment regimen compared with the PG15 and PG16 treatments. Non-estrous-return rate and fertility after TAI in the present study were not affected by either the dietary supplementation or estrous synchronization protocols that were used.

Conflicts of interest

The authors have no conflict of interest to declare.
All authors have no financial or personal relationship with organizations or people that could influence or bias the study.

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