Lack of effect of short-term lupin grain feeding on ovulation rate in non-prolific Polish Mountain ewes during the breeding season: Ultrasonographic and endoscopic assessment of ovarian activity

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Abstract

Specific changes in antral follicle numbers and wave-like pattern of development in cyclic ewes receiving high-protein, high-energy lupin grain for 6 days during the luteal phase of the estrous cycle (short-term nutritional flushing) remain unknown. The main objective of this experiment was to employ transrectal ovarian ultrasonography and videoendoscopy to determine ovarian effects of the 6-day lupin grain feeding in non-prolific Polish Mountain ewes. Estrus and ovulations were synchronized in 24 ewes with progestin-releasing intravaginal sponges inserted for 12 days during the middle portion of the breeding season (September-October; 50.0458° N, 19.8406° E). Eight ewes received the
maintenance (hay-only) diet (Control), while the lupin-fed groups (each n=8) received 500 g of lupin grain once a day (Treatment 1) or 250 g of lupin grain twice a day (Treatment 2), from Days 9 to 14 of the synchronized cycle (Day 0=first ovulation of the interovulatory period studied). There were no differences (P>0.05) in the mean ovulation rate between the three groups of Polish Mountain ewes studied. Ovarian antral follicles emerging in the penultimate wave of the estrous cycle in Treatment 2 ewes had a longer (P<0.05) growth phase and attained a greater (P<0.05) diameter before ovulation in comparison to the other two groups of animals. A final wave of the interovulatory interval emerged ~1 day earlier in Treatment 2 than in Treatment 1 ewes (P<0.05). Nutritional supplementation with lupin grain increased (P<0.05) the number of 3-mm follicles in Treatment 2 ewes. The present results indicate that short-term nutritional flushing with lupin grain from mid- to late luteal phase does not consistently enhance ovulatory responses in non-prolific genotypes of ewes. Even though the administration of lupins altered the timing of wave emergence, ovulatory follicle diameter or duration of different stages of follicular lifespan, it failed to increase the number of ovulatory follicles emerging in the penultimate and final waves of the estrous cycle in non-prolific Polish Mountain sheep.

**Key words:** sheep; ovulation rate; lupin grain; flushing; ultrasonography

**Introduction**

Nutrition is one of the most important extrinsic factors influencing fertility in small ruminants (Scaramuzzi et al., 2006, 2011; Arellano-Rodriguez et al., 2007; Meza-Herrera
et al., 2012). More than one effect of nutrition on the ovulation rate in sheep have been recognized to date including: the static effect, the dynamic effect, and the intermediate effect (Viñoles, 2003). The static effect refers to the generally higher ovulation rate in heavy-weight ewes compared with lighter animals while the dynamic effect is a rise in the ovulation rate due to an increase in live weight and body condition score shortly before the estrus period and mating. In addition, short-term supplementation with high-energy, high-protein diet can result in a higher ovulation rate without any changes in live weight or body condition score; this phenomenon is referred to as the intermediate effect (Viñoles, 2003). Lupins are a leguminous grain used as a high-energy, high-protein feed supplement (e.g., *Lupinus angustifolius*: 13 MJ ME/kg DM and 320 g CP/kg DM; Kendall et al., 2003). Short-term lupin feeding (4 to 6 days) during the luteal phase of the estrous cycle increases mean ovulation rate in cycling ewes (Scaramuzzi et al., 2006, 2011).

Many researchers have reported significant increases in the mean ovulation rate of ewes fed lupins for varying periods of time before breeding (Hill, 1988; Scaramuzzi et al., 2011). However, not all studies have shown marked responses (Hill, 1988). Physiological processes that are responsible for the intermediate effects are not completely understood and the precise ovarian mechanism by which nutritional flushing with lupins increases the ovulation rate in sheep remains to be elucidated.

One possibility is that the effect of short-term nutritional flushing is mainly metabolic, and ovulation rate is increased in response to the energy and/or protein content of the lupin grain (Scaramuzzi et al., 2006). Alternatively, it has been suggested that lupin ingredients may directly modulate folliculogenesis in ewes by increasing ovarian sensitivity to gonadotropic hormones (i.e., hormonal effect; Kendall et al., 2003);
cultivated varieties of grain legumes have been identified as an excellent source of phytoestrogens that can enhance ovarian responsiveness to luteinizing hormone (LH; Petterson and Fairbrother, 1994; Jayasena et al., 2004). Ovarian antral follicles in sheep grow in the wave-like fashion throughout the 17-day interovulatory interval Bartlewski et al., 2011). In cyclic ewes, one to four small antral follicles (2-3 mm in diameter) emerge approximately every 4 days to attain an ostensibly ovulatory size of ≥5 mm in diameter (Bartlewski et al., 2011). In prolific breeds of sheep, ovarian follicles from the last two waves of the estrous cycle can ovulate to give a higher ovulation rate compared with less prolific genotypes in which only follicles form a final wave of the cycle ovulate and a proportion of potential ovulatory follicles regress before the estrous phase; in prolific ewes nearly 100% of such follicles ovulate (Bartlewski et al., 2011). Therefore, there exist at least three possible mechanisms whereby nutritional supplementation with lupin grain exerts its effects on ovulation rate in non-prolific strains of sheep: 1) addition of antral follicles from the penultimate wave to the ovulatory follicle cohort; 2) increase in the number/percentage of ovulatory follicles emerging in a final wave; or 3) both changes in antral follicle kinetics.

This experiment was undertaken to examine the effects of short-term nutritional flushing with lupin grain on ovarian activity and ovulation rates in non-prolific Polish Mountain sheep. Our objective was to use ultrasonographic imaging and videoendoscopy of ovaries to document antral follicle development, ovulation rate and corpus luteum formation in cyclic Polish Mountain ewes that received lupins from Days 9 to 14 after ovulation.
Materials and Methods

Animals and experimental procedures

All procedures followed the EC directives for animal experimentation and were conducted under the local animal care/bioethics committee authorization. The present experimental design was based largely on the study by Somchit et al. (2007). Twenty-four clinically healthy, non-prolific Polish Mountain ewes (aged 2-3 years (multiparous), mean body weight of 62.5±4.1 kg) were gathered from the field research station of the Agricultural University of Kraków, Poland, situated in Bielany (50.0458° N, 19.8406° E). The ewes were obtained in September, amid the regular breeding season, and were kept in separate groups in a barn with ambient light and temperature. Estrus and ovulations were synchronized with intravaginal progestin-releasing sponges (Chronogest®, 45 mg of flugestone acetate; Intervet International B.V., Boxmeer, Holland) inserted for 12 days. Estrus was detected twice daily (2 x 20 min), starting 2 days after sponge withdrawal, with two fertile rams fitted with an apron preventing penetration during mounting. Timing of the ovulation was confirmed by transrectal ovarian ultrasonography conducted twice a day from detection of behavioral estrus. Ovulations were defined as a disappearance of the largest visible antral follicles followed by a formation of corpora hemorrhagica approximately 24 h after follicle rupture (Duggavathi et al., 2003b).

Prior to the present experiment, the ewes’ diet consisted of the concentrate formulated to provide 100% of nutritional requirements (Norms, 1993). In addition, all animals had free access to good quality alfalfa hay (crude protein: 8.2% of dry matter, total extractable lipids: 2.9% of dry matter, and moisture: 15.0%), water and mineralized salt licks containing anti-parasitic herbal extracts (Star Bloc Phyto Vers, Guyokrma Ltd.,
Prague, the Czech Republic; Nosal et al., 2016). The ewes were divided by weight into three groups: a control group (n=8) and two lupin-fed groups (each n=8; Treatment 1 and Treatment 2). The ewes in the lupin-fed groups received the maintenance diet from the day of sponge insertion until Day 9 (Day 0=first ovulation of the interovulatory interval studied), at which time the ewes in these groups were switched to a lupin grain diet (*Lupinus angustifolius*; 500 g/ewe/day; 500 g at 8:00 a.m.-Treatment 1 or 250 g at 8:00 a.m. and 250 g at 6:00 p.m.-Treatment 2) until Day 14 (a total of 6 days); animals in the control group received the hay-only diet throughout the study period. The reason for testing two treatment regimens (or lupin grain feeding intervals) was the fact that the rate of *in situ* nitrogen (N) disappearance for raw lupins after 12-h incubation in the rumen is ∼83% (Kung et al., 1991). Because of rapid ruminal absorption and utilization of lupin grain, the two groups were designed to evaluate and compare the “intermittent” (Treatment 1) and more “stable” (Treatment 2) influence of its constituents. All animals allocated to Treatment 1 or 2 were trained to eat the lupin grain (crude protein: 48.3% of dry matter, total extractable lipids: 5.0% of dry matter, and moisture: 11.8%) over a 1-2-day period a week before the beginning of the experiment.

**Monitoring of ovarian function**

Transrectal ovarian ultrasonography was performed every morning from Day 8 of the estrous cycle studied until ovulation using the Aloka ProSound 2 scanner (Hitachi Aloka Medical, Tokyo, Japan) equipped with a stiffened (plastic extension) 7.5-MHz linear-array transducer. Digital ultrasonograms of both ovaries (DICOM) at a resolution of 640 x 480 pixels were recorded directly on a computer connected to an ultrasound diagnostic system. Laparoscopy was performed approximately 7 days after the second ovulation of
the estrous cycle studied to enumerate corpora lutea (CL). Each ewe received a single i.m injection of 0.4 ml of Nefrasin vet. (containing 20 mg/ml of xylazine hydrochloride; aniMedica Polska, Gdynia, Poland) and local s.c. injections of 1.5-2 ml of polocainum hydrochloricum (2%) with adrenaline (0.005%; Biowet, Drwalew, Poland). Laparoscopy utilized the Tele Pack Vet X Led video endoscope (Karl Sorz GmbH & Co. KG, Tuttlingen, Germany) and all recordings were saved onto a USB drive.

Follicle classification, assessment of follicular wave kinetics and statistical analyses

Follicles were grouped into four size classes: 2-mm, 3-mm, 4-mm and ≥ 5-mm follicles (Duggavathi et al., 2003a). A follicular wave was defined as a cohort of follicles that grew synchronously from 2-3 mm to ≥ 5 mm in diameter before regression or ovulation; follicles emerging within 24 h were included in a wave (Baby and Bartlewski, 2011a,b). The day of wave emergence, the number of all follicles as well as the number and proportion of ovulatory follicles in the penultimate and last wave of the estrous cycle studied wave were noted. All antral follicles of the wave were analyzed for their maximum diameter, duration of the growth phase, static phase and regressing phase as well as the mean growth rate (Bartlewski et al., 1999). The growth phase of follicular lifespan was defined as the time taken to grow from 2-3 mm to the maximum diameter, and the regressing phase was the number of days taken to regress from its maximum diameter to 2-3 mm. The time between the end of growth and the onset of regression or ovulation was the static phase of follicular lifespan. The number and diameter of all ovulating follicles of waves were also recorded.

Statistical analyses were done using the SigmaPlot® statistical software package (version 11.0 for Windows®, 2010 Systat Software, Inc. Richmond, CA, USA). The results are presented as means±SEMs unless otherwise indicated and the differences between
mean values were regarded significant at P<0.05. All data sets were initially tested for outliers using the Dixon Q-test. All single-time responses and follicular wave characteristics were analyzed using one-way analysis of variance (ANOVA) and changes in follicle populations (daily follicle counts) were carried out using two-way repeated measures ANOVA, with time (Day) as the within subjects (Sheep) repeated measure and Group (Control, Treatment 1 or Treatment 2) as the between-subjects factor. Whenever the data had non-homogenous variances, they were transformed by log, and if still not normalized, they were analyzed using an equivalent non-parametric test (e.g., the Mann-Whitney rank sum test).

Results
The mean duration of the interovulatory period studied, ovulation rate and number of detected luteal structures did not vary (P>0.05) between lupin-fed and control Polish Mountain ewes (Table 1). Ovarian antral follicles in the penultimate wave of the estrous cycle had longer (P<0.05) growing phase in Treatment 2 ewes compared with both the Control and Treatment 1 animals, by approximately 2 days. The duration of regressing phase of follicle lifespan was greater (P<0.05) in Control compared with Treatment 1 ewes. The diameter attained by ovulating follicles in this wave was greater (P<0.05) in Treatment 2 than in Control and Treatment 1 Polish Mountain ewes. The emergence of the final wave of the interovulatory interval studied occurred ~1 day earlier (P<0.05) in Treatment 2 compared with Treatment 1 ewes. There were no other differences among the three groups of animals for any characteristic of the last two follicular waves of the estrous cycle.

During the period from 1 day before to 3 days after nutritional flushing with lupins,
there was a significant main effect of Day for the number of 2-, 4- and ≥5-mm follicles, and a significant effect of Group for the number of 3-mm follicles detected ultrasonographically in Polish Mountain ewes studied (Fig. 1). In Treatment 1 ewes, mean numbers of 2-mm follicles were greater (P<0.05) on Day 17 compared with Days 9 and 10 whereas in Treatment 2 animals, the number of 2-mm follicles was greater (P<0.05) on Day 17 than on Days 8, 9, 10 and 13-16; there were no significant differences over time in Control animals (Fig. 1A). In addition, Treatment 1 ewes exceeded Control sheep in the mean number of 2-mm follicles on Day 15 (Fig. 1A). Mean daily numbers of 3-mm follicles were overall greater (P<0.05) in Treatment 2 ewes (3.4±0.2 follicles/day) compared with Treatment 1 and Control animals (2.4±0.2 and 2.6±0.2 follicles/day, respectively); the difference between Treatment 2 and two other groups was most prominent on Day 10 (Fig. 1B). Even though the main effect of Day was significant for daily numbers of 4-mm follicles, subsequent statistical tests did not reveal any differences among the three subsets of animals on any day of the observation period (Fig. 1C). Lastly, mean daily numbers of large antral follicles (≥5 mm in diameter) were lower (P<0.05) on Day 17 compared with Days 12, 13, 15 and 16, and were lower (P<0.05) on Days 8 and 9 than on Days 15 and 16 in Treatment 1 ewes, whereas in the remaining two groups, the only significant difference was observed between Days 17 and 13 (Control ewes) or between Days 17 and 14 (Treatment 2 ewes; Fig. 1D).

Discussion

Generally, nutritional flushing with lupins boosts fertility in non-prolific genotypes of sheep by increasing the incidence of twinning (Hill, 1988; Scaramuzzi et al., 2011). Significant
increases in ovulation rates after the flushing are mainly observed in ewes receiving poor quality feed (e.g., animals grazing wheat straw or dry subterranean clover; Hill, 1988). Older animals usually respond better to nutritional flushing than younger ewes, although in some studies the age of ewes did not appear to be a determining factor (Hill, 1988). Our study shows that despite some changes in antral follicle numbers and wave dynamics, lupin grain feeding from 9 to 14 days after ovulation does not consistently stimulate more ovulations in sexually mature, non-prolific ewes.

Failure to achieve a flushing response could occur because the Polish Mountain ewes have already reached their full genetic potential for ovulation rate with their previous nutritional regimen (the static effect of nutrition; Coop, 1966). Alternatively, inability to maintain the threshold level for digestible energy intake might explain lower than expected ovulatory responses (Coop, 1966). In the past, the amount of lupin used to improve sheep fertility varied considerably (from 170 to 750 g/day) but there has been no systematic study of the effects of daily lupin grain doses in different breeds of sheep.

Our observations also indicate that the frequency of lupin feeding may affect ovarian responses in cyclic ewes. It is feasible that a series of twice daily or even more frequent supplementations with lupins may be necessary to attain the required metabolic threshold and to elicit ovarian stimulation. Administration of 500 g of lupin grain once a day (Treatment 1) did not increase daily numbers of antral follicles and only shortened the regression phase of follicle lifespan (penultimate wave of the estrous cycle) by ~1.5 days, without apparent effects on other aspects of follicle wave development (i.e., inter-wave interval, maximum follicle diameter, etc.). Alternatively, feeding two meals of 250 g of lupin grain per day (Treatment 2), in the morning and evening, was associated with
increased small (3-mm) follicle numbers, prolonged growth phase of all emerging follicles and greater size of ovulatory follicles emerging in the penultimate wave before ovulation, and earlier emergence of the last wave of the estrous cycle studied. Even though there was a 3.4-fold increase in the percentage of ovulatory antral follicles emerging in the penultimate wave of the cycle in Treatment 2 ewes (Table 1), this difference was only numerical; such follicles were recorded in one control and two Treatment 2 animals. Our results contrast with previous studies in which short-term lupin feeding increased the number of large and medium-sized follicles (the latter was approximately doubled in response to lupin grain supplementation; Muñoz-Gutierrez et al., 2002, 2005) and they do not support an earlier suggestion by Scaramuzzi et al. (2011) that the stimulating effects of supplemental nutrition late in the luteal phase on the final follicular wave are likely to be more pronounced than those exerted on the preceding waves of the interovulatory period in ewes. In the present study, short-term flushing with lupin grain started mid-way through the interovulatory interval appeared to have limited effect on the development of ovulatory follicles emerging just before the onset of behavioral estrus. In fact, the proportion of ovulating antral follicles that emerged in the last wave of the cycle decreased numerically (by 21.7% and 27.9% in Treatment 1 and Treatment 2 ewes, respectively; Table 1); this can be explained by the fact that in the Control group, only 2/8 ewes ovulated ≤ 50% of all ovulatory-sized follicles in the final wave whereas 4/8 and 6/8 animals had ≤ 50% ovulatory follicles in the final wave of the cycle in the Treatment 1 and 2 groups, respectively.

In prolific strains of sheep, a proportion (50-75%) of large antral follicles in the penultimate wave of the estrous cycle are maintained and ovulate along with follicles that
emerge in the final follicular wave before estrus whereas ovulations of follicles from the second last wave in non-prolific ewes are sporadic (≤10%; Bartlewski et al., 1999). Ovulatory follicles from the penultimate wave emerge ∼48 h later than anovulatory follicles in the same wave and hence their regression may be prevented by the next increase in serum concentrations of FSH and/or gradual increase in LH pulsatility at luteolysis. In addition, approximately 25% of all ovulatory-sized follicles emerging in the last follicular wave in non-prolific ewes become atretic and do not ovulate, as opposed to near 100% ovulation of such follicles in this wave in prolific ewes (Bartlewski et al., 1999). Excepting the number of follicles ovulating in the last follicular wave, all of these aspects of antral follicular kinetics are due to inter-breed differences in circulating concentrations of luteal progesterone (P₄) governing the timing of FSH peaks and hence initial stages of wave development; the number and percentage of ovulating follicles emerging in the last wave of the cycle appear to be independent of P₄ and FSH secretion (Baby and Bartlewski, 2011a,b; Bartlewski et al., 2017). By comparing those earlier observations with our present results, it is doubtful that lower than normal luteal phase levels of P₄ seen in small ruminants receiving high-energy, high-protein diets (Hashem, 2012) were responsible for the alterations in wave dynamics in the ewes of the present study. These effects seem to be related mainly to high concentrations of serum glucose (Leury et al., 1990; Scaramuzzi et al., 2011) and insulin (Scaramuzzi et al., 2011) during the pre-ovulatory period, especially in animals receiving lupin grain twice a day. Elevated insulin levels associated with lupin grain flushing in the ewes increase ovarian glucose entry rates and stimulate FSH secretion and the numbers of large antral follicles (Scaramuzzi et al., 2011). The effect of nutrition on FSH concentrations are, however, very rapid and transient
(Scaramuzzi et al., 2011). FSH is responsible for the initial stages of antral follicle development up to the attainment of their maximum diameter (Bartlewski et al, 2011). It is not known why lupin-induced changes in FSH secretion and/or bioavailability potentially resulting in prolonged follicular growth in the ewes of the present study would be restricted to animals fed lupins twice a day.

In summary, feeding a total amount of 500 g of lupin grain per day, from Days 9 to 14 after ovulation in estrus-synchronized ewes, had no effect on the ensuing ovulation rate. Lupin grain supplements offered once a day had little influence on antral follicular development monitored with transrectal ultrasonography, whereas two meals of 250 g of lupin twice a day altered the duration of follicular growth phase (penultimate wave) and advanced the emergence of the last wave of the estrous cycle but failed to significantly increase the number of ovulatory follicles. The present observations on antral follicle kinetics suggest that the effects lupin grain exerts on antral follicle growth are rapid and transient, and they do not seem to be governed by progesterone-mediated alterations in circulating FSH concentrations documented in earlier experimental studies using ultrasonographically monitored ewes. More studies are needed to corroborate a hormonal basis for ovarian effects of nutritional flushing in small ruminants.

**Funding**

This study was supported from the statutory funds of the Department of Animal Biotechnology, University of Agriculture in Kraków, and the Department of Biomedical Sciences, University of Guelph (PMB).

**Acknowledgements**
The authors wish to thank Mr. Marek Lipski for his technical assistance and care of experimental animals. The results of this experiment were presented, in a preliminary form, at the 5th Winter Workshop of the Society for Biology of Reproduction (“Central and Local Regulations of Reproductive Processes”) in Zakopane, Poland (13-15 February 2019).

Author contributions
Study concept and design: Murawski, Schwarz, Kridli and Bartlewski; Acquisition of data: Murawski, Schwarz, Erak, Sohal and Bartlewski; Analysis and interpretation of data: Erak, Ahmadi, Kridli and Bartlewski; Statistical analysis: Ahmadi and Bartlewski; Drafting of the manuscript: Erak and Bartlewski; Critical revision of the manuscript for important intellectual content: Murawski, Schwarz, Kridli and Bartlewski; Administrative, technical, and material support: Murawski, Schwarz and Bartlewski; Study supervision: Murawski, Schwarz and Bartlewski.

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Table 1. Comparisons of ovulation rates/numbers of detected luteal structures, estrous cycle duration and characteristics of follicular waves (penultimate and final wave of the estrous cycle studied; means±SEMs) among Control (hay-only diet) and lupin-fed Polish Mountain ewes.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (n=8)</th>
<th>Treatment 1 (1 x 500 g; n=8)</th>
<th>Treatment 2 (2 x 250 g; n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulation rate</td>
<td>1.6±0.2</td>
<td>1.7±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>No. of luteal structures at mid-cycle</td>
<td>1.5±0.2</td>
<td>1.7±0.2</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Interovulatory interval (days)</td>
<td>16.9±0.2</td>
<td>16.7±0.4</td>
<td>16.8±0.3</td>
</tr>
<tr>
<td><strong>Penultimate wave</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day of emergence*</td>
<td>9.2±0.2</td>
<td>9.4±0.4</td>
<td>9.4±0.7</td>
</tr>
<tr>
<td>No. of follicles</td>
<td>2.1±0.4</td>
<td>2.7±0.4</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>No. of ovulatory follicles</td>
<td>0.12±0.12</td>
<td>0.25±0.25</td>
<td>0.29±0.18</td>
</tr>
<tr>
<td>% of ovulating follicles</td>
<td>6.2±6.2</td>
<td>8.3±8.3</td>
<td>21.4±14.9</td>
</tr>
<tr>
<td>Growth phase (days)</td>
<td>2.0±0.5a</td>
<td>1.8±0.4a</td>
<td>3.8±0.5b</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.6±0.2</td>
<td>1.7±0.1</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>2.3±0.6</td>
<td>2.2±0.4</td>
<td>2.2±0.7</td>
</tr>
<tr>
<td>Regressing phase (days)</td>
<td>2.8±0.4a</td>
<td>1.4±0.1b</td>
<td>1.9±0.4ab</td>
</tr>
<tr>
<td>Regression rate (mm/day)</td>
<td>1.0±0.1</td>
<td>1.7±0.2</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Maximum follicle diameter (mm)</td>
<td>6.2±0.6</td>
<td>5.9±0.9</td>
<td>6.5±0.3</td>
</tr>
<tr>
<td>Ovulatory follicle diameter (mm)</td>
<td>5.5±0.2a</td>
<td>5.2±0.2a</td>
<td>6.2±0.3b</td>
</tr>
<tr>
<td><strong>Final wave</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day of emergence*</td>
<td>12.4±0.5ab</td>
<td>12.8±0.4a</td>
<td>11.7±0.4b</td>
</tr>
<tr>
<td>No. of follicles</td>
<td>2.2±0.5</td>
<td>2.9±0.5</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>No. of ovulatory follicles</td>
<td>1.5±0.2</td>
<td>1.5±0.3</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>% of ovulating follicles</td>
<td>80.0±9.8</td>
<td>58.3±14.1</td>
<td>52.1±9.7</td>
</tr>
<tr>
<td>Growth phase (days)</td>
<td>2.1±0.4</td>
<td>1.8±0.3</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>1.3±0.1</td>
<td>1.6±0.1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Static phase (days)</td>
<td>2.7±0.5</td>
<td>2.3±0.4</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Maximum follicle diameter (mm)</td>
<td>5.9±0.3</td>
<td>5.5±0.2</td>
<td>6.1±0.1</td>
</tr>
<tr>
<td>Ovulatory follicle diameter (mm)</td>
<td>6.1±0.3</td>
<td>4.9±0.7</td>
<td>5.9±0.9</td>
</tr>
<tr>
<td><strong>Inter-wave interval</strong></td>
<td>4.2±0.4</td>
<td>4.4±0.2</td>
<td>3.4±0.4</td>
</tr>
</tbody>
</table>

Treatment 1: 500 g of lupin grain at 8:00 a.m. and Treatment 2: 250 g of lupin grain at 8:00 a.m. and 250 g at 6:00 p.m. from Days 9 to 14.
*Day 0=first ovulation of the interovulatory interval studied.
Within rows, different letters denote significant differences (P<0.05) among groups.
Fig. 1. Daily numbers (means±SEMs) of ovarian antral follicles in different size classes recorded in ultrasonographically monitored cyclic Polish Mountain ewes that received lupin grain from Days 9 to 14 after ovulation. Treatment 1: 1 x 500 g of lupin grain/day and Treatment 2: 2 x 250 g of lupin grain/day. See text for additional statistical details.

- No. of 2-mm follicles
  - Treatment 1 (1 x 500 g; n=8)
  - Treatment 2 (2 x 250 g; n=8)
  - Control (n=8)

- No. of 3-mm follicles

- No. of 4-mm follicles

- No. of follicles 5 mm

- Days after ovulation

Group: P=0.71
Day: P<0.001
Group x Day: P=0.18

Group: P<0.001
Day: P=0.99
Group x Day: P=0.18

Group: P=0.81
Day: P<0.05
Group x Day: P=0.31

Group: P=0.71
Day: P<0.001
Group x Day: P=0.18