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ORIGINAL ARTICLE

Effects of seasonal changes on the ovulation rate and embryo quality in superovulated Black Suffolk ewes

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Abstract

OBJECTIVE: The objective of this study was to investigate the effects of seasonal changes on the superovulation in Black Suffolk ewes, particularly the ovulation rate and embryo quality.

DESIGN: Black Suffolk ewes were superovulated either in May (n=22) or in September (n=21), 2013. After estrus synchronization with CIDR, the donor ewes were superovulated with PMSG and seven decreasing doses of FSH (twice daily at 07:00 and 19:00 for four consecutive days. Then, they were subjected to laparoscopic intrauterine artificial insemination. The viable morula and blastocysts were recovered and immediately transferred to recipients.

RESULTS: Ewes that were superovulated in May had a much higher ovulation rate than those were superovulated in September (16.8±3.23vs. 10.2±2.94, $p<0.01$); however, the viability rate of the embryo was lower than that of September (56.0±1.92% vs. 92.5±3.26%, $p<0.01$). There was no significant difference in the survival rate of the transferred viable embryos (33.9±1.00% vs. 36.7±1.64%, $p>0.05$) and the number of offspring per donor ewe (3.1±0.54 vs. 2.9±0.72, $p>0.05$) between May and September. In contrast, the offspring/ova ratio of the donor ewes superovulated in May was lower than that of September (18.5±1.64% vs. 32.8±2.14%, $p<0.01$).

CONCLUSIONS: The superovulation of Black Suffolk ewes may be affected by the seasonal changes. Generallly, The ewe's ovulation rate was higher in May, whereas the viability rate of embryo was higher in September.

INTRODUCTION

As typical seasonal breeding mammals, the reproductive cycle of ewes always begins in August or September and temporarily terminates during the spring and summer. Anestrus of ewes may last for several months. For example, the mean durations of anestrus for St. Croix and Suffolk ewes are 132.7 days and 140.3 days, respectively (Goff *et al.* 2013).

Regulation of the reproductive activity of sheep involves various factors, including the environment, hormones and genes, and may be driven by photoperiodism, which is the light/dark cycle. The increased expression of the central nervous system E_2 (estradiol) receptor and the availability of estradiol access to the brain during long days activate the dopaminergic system and inhibit GnRH (gonadotropin-releasing hormone) cells, decreasing the frequency of LH (pulsatile luteinizing hormone) secretion (Thiery & Malpaux 2003). Without stimulation from high frequency and low amplitude LH secretion, the regression but not ovulation of mature follicles occurs successfully in the ovaries of anestrus ewes. Moreover, homozygous ewes with the absence of the polymorphic *Mnl*I site on the Mel_{1a} exon II do not undergo ovulatory activity during the spring (Pelletier *et al.* 2000).

Compared to autumn and winter, the estrus and ovulatory activity of ewes is lower during the spring and summer, particularly in February–May (Lassoued *et al.* 2014). Few sheep display estrus behavior yearly round Only a few breeds, including Hu sheep, Small tailed Han sheep and Dorse exhibit yearly round estrus behavior. It is likely that the off-season reproduction of Hu sheep is caused by the higher expression of the *Ppap2b*, *Nid1*, *Serpine2* and *Foxola* genes in the ovaries compared to the other seasonal anestrus breeds (Chen *et al.* 2012). Furthermore, sheep have fewer offspring, with 1.28 to 1.60 for Dorper (Cloete *et al.* 2000; Schoeman, 2000; Snyman and Olivier, 2002) and 1.46 to 1.92 for Suffolk (Notter 2000; Schmidová *et al.* 2014) compared to multiparous mammals, as only one or two lambs are born for each ewe. Therefore, there is economic value for the constant and thorough superovulation of ewes.

The physiological mechanisms controlling the maturation and ovulation of follicles continue to function during the anestrus season (Webb *et al.* 1992). However, the total number of antral follicles and the percentage of small follicles in the ovaries, which are affected by the basic levels and pulses of reproductive hormones, change synchronously with the alternating seasons. This is due to the differences in the progesterone, LH and FSH (follicle stimulating hormone) concentrations during the breeding and nonbreeding seasons. Interestingly, whether the stimulation with the exogenous hormones counteracts the seasonal variations of the reproductive activities of the ewes or further disrupts the balance between the number of antral follicles and the percentage of small follicles were currently unknown. In addition, whether different superovulation procedures are adapted during breeding and anestrus seasons related to the higher ovulation and viability rates remain to be determined.

The objective of this study was to investigate the effects of seasonal changes on the quantity and quality of ova recovered from Black Suffolk ewes that were superovulated either during the May or during the September, 2013.

MATERIALS AND METHODS

Animals and experimental design

Black Suffolk ewes provided by Aoxin Animal Husbandry Company Ltd. (Beijing, 40°N) were superovulated during May (n=22) and September (n=21). All experimental protocols concerning the handling of animals were performed in accordance with the requirements of the Institutional Animal Care and Use Committee at the China Agricultural University.

The experiment was designed to determine the ovulation rate and embryo quality in superovulated during May and September of 2013. After morphological evaluation, the embryos collected from the superovulated multiparous ewes were transplanted into the adult multiparous ewes to assess their viability *in vivo*.

Superovulation and estrus synchronization

The estrus cycles of donor and recipient ewes were synchronized with the insertion of controlled release intravaginal devices containing 30 mg progesterone (CIDR, InterAg, New Zealand) left *in situ* for fourteen days. Superovulation was initiated by FSH (FSH, Sansheng Ltd., China) injection. Total seven gradually reduced doses (3.8 IU/kg, decline 5 IU each time) were injected twice daily (07:00 and 19:00) for four consecutive days. This procedure was completed three days before pessary withdrawal. The CIDRs were removed at the time of the 7th FSH injection, followed by the administration of PMSG (PMSG, Sansheng Ltd., China) (300–330 IU) in the afternoon. Twenty-four hours after the onset of estrus, laparoscopic intrauterine artificial insemination was performed with fresh diluted semen. LH (110–130 IU) was administered nine hours later.

Endoscopic-assisted insemination

Each ewe was restrained on an operation cradle in dorsal recumbence with the head down at an angle of 45°. An endoscope (30 forward oblique, Karl Storz Endoskope GmbH, Tuttlingen, Germany) was introduced into the abdominal cavity at the ventral midline approximately 5–10 cm cranial to the mammary gland as previous described (Kuehholzer et al.2007). The endoscope intrauterine horn method was used to inseminate donor ewes with 0.3 mL of fresh semen (motility >0.6, diluted 2-fold with saline) at 51–52 h after CIDR removal.

Embryo collect and evaluation

The embryos were collected by oviduct flushing with a cannula that attached to a syringe and inserted into the lumen near the uterotubal junction 5.5 days after LH injection. All females were deprived of food for 12 h before embryo recovery to facilitate surgery and reduce post-operative intestinal adhesions. A medial ventral incision was made to expose the reproductive tract, the number of functional *corpora lutea* was recorded and the uterine horns were flushed with modified PBS (DPBS®, XXX, New Zealand). The flush was guided with a stereomicroscope (SZ61; Olympus, Kawasaki, Japan) to search for embryos at magnifications of 10–40. Then, the ova were transfer into holding medium (Immuno-Chemical Products Ltd., Auckland, New Zealand) and qualitatively evaluated with an inverted microscope (LX71;Olympus, Kawasaki, Japan). The evaluation criteria were based on previous studies with goat embryos (Ishwar & Memon 1996; Baril & Vallet 1990). The number of *corpora lutea* (CL) and the total number of recovered embryos (RE) were recorded for each ewe. The ovaries and uterine horns were flushed with saline solution during the process of recovery and transplant surgery to prevent development of abdominal adhesions. The wound was treated with penicillin and streptomycin when the incision was sutured.

Recipient treatment and embryo transfer

The estrus cycles of recipient were synchronized by the insertion of controlled release intravaginal devices containing 30 mg progesterone (CIDR, InterAg, New Zealand) and the CIDRs were removed 10 h before the door while at the same time the recipient was given 280 IU PMSG. Identification on oestrus by ram 3 times a day 12 h after CIDR removal. 36 h after CIDR removal, 10 μg LRH-A3 was given to the recipients.

The embryos were transplanted into recipients with a assistant of endoscopic apparatus. One or two embryos were transferred into the uterine horn ipsilateral to the ovary with a good CL(1–3CL).The uterine wall was punctured with a 17-ga trocar l cm cranial to the uterotubal junction. A transfer catheter (Agtech, Inc.,Manhattan, KS, USA) attached to a 1-mL syringe was advanced approximately 2 cm into the uterine lumen. Then, one or two embryos in 0.3 to 0.5 mL of holding medium were deposited into the uterine horn without insufflating air. Recipient ewes transplanted in May gave birth to lambs during mid-October of that year, whereas the ewes transplanted in September lambed during mid-February of the next year.

Statistical analysis

All data are expressed as the mean ± SEM, An independent sample T test or Chi-square test was used to determine the statistical significance.The software was SPSS (version 18.0). Statistical significance was set up by $p<0.05$.

RESULTS

Ovarian response and ova recovery

There were significantly more *corpora lutea*, ova and viable embryos in ewes that **superovulated** in May than those in September. Moreover, both the number of *corpora lutea* (16.8±3.23 vs. 10.2±2.94, $p<0.01$) and ova recovered (11.5±1.79 vs. 6.4±1.70, $p<0.05$) differed significantly between May and September, but the number of viable embryos did not differ significantly (6.2±1.57 vs. 5.9±1.16, $p>0.05$) (Figure 1).

The recovery rate was slightly higher during May than that in September but the differences did not reach to r statistical significance (65.4±2.91% vs. 62.6±2.59%, $p>0.05$) By contrast, the viability rate of the embryo was significantly lower in May than that in September (56.0±1.92% vs. 92.5±3.26%, $p<0.01$) (Figure 1).

Embryo transplantation and survival

There were 127 viable embryos which were transferred to 72 recipient ewes in May, whereas 32 ewes ultimately lambed, with 43 lambs in total. In September 120 viable embryos were transferred to 83 recipient ewes. A total of 44 lambs were born by 43 ewes. The data were listed in Table 1. The pregnancy rate and lambing rate in the Sep. was significantly higher ($p<0.05$) than those in May (54.1±3.91% vs. 47.2±1.20%; 51.7±3.51% vs. 44.4±1.24%). In addition to the pregnancy rate and lambing rate, the number of lambs born per embryo and per recipient were also investigated. There were no significant difference in the number of lambs born per embryo from the total embryos and recipients between May and Sep.($p>0.05$).

The survival rate of the embryos transferred singly per ewe was significantly low in May compared to September (16.7±5.92% vs. 47.8±4.12%, $p<0.05$). However, neither the viable embryos nor the embryos transferred as twins per ewe differed significantly (33.9±2.24% vs. 36.7±1.56% and 37.7±3.64% vs. 29.7±6.32%, $p>0.05$) between May and Sept. (Figure 2).

Tab. 1. Effects of seasonal changes on the viability of embryos recovered from donor in superovulated Black Suffolk ewes.

	May	September
No. of recipient ewes	72	83
No. of Pregnant Ewes (pregnancy rates, %)	34(47.2±0.54)[a]	45(54.1±1.75)[b]
No. of lambing ewes (lambing rate,%)	32(44.4±0.55)[a]	43(51.7±1.57)[b]
No. of lambs born (lambs/recipient ewes,%)	43(59.8±1.51)[a]	44(53.0±0.86)[a]
Total Embryos Transplanted (lambs/total embryos,%)	127(33.9±1.00)[a]	120(36.7±0.70)[a]

Values with different letters in the same row (a, b, c) are significantly different; $p<0.05$.

Offspring of donor ewes

In order to determine the effect of seasonal changes on the offspring of donor ewes, the result of MOET was also investigated. There were no significant difference in the percentage of ewes with offspring and the mean number of offspring per donor ewe between May and September (53.2±12.22% vs. 66.7±4.47%; 3.1±1.21 vs. 2.9±1.62, $p>0.05$). However, the offspring/ova ratio of the donor ewes superovulated in May was significantly low compared to September (18.5±3.67% vs. 32.8±4.78%, $p<0.01$) (Figure 3).

DISCUSSION

Superovulation during the anestrus and breeding seasons are very similar, and 0.05 more follicles were recovered from Kivircik ewes ovulated in March–May (trial 1) than those during the September–November (trial 2) (Üstüner *et al.* 2014). Similarly, 0.4 more follicles were recovered from Mule ewes ovulated in April than October (Mitchella *et al.* 2002), and 7.6 more follicles were recovered from Merino ewes ovulated in February-April than those during September–November (Buffonia *et al.* 2014). However, there was no significant difference between seasons in each experiment. The mean ovulation rate of superovulated Merino × Polled Dorset ewes was significantly low during May–June compared to November–December (Fukui *et al.* 1994). Light is known to stimulate reproductive function (Danilenko *et al.* 2015) and the light cycle changes with the alternation of seasons. The reproductive activity of sheep is governed by seasonal variations, where photoperiodic information is conveyed to the reproductive neuroendocrine system by the circadian secretion of

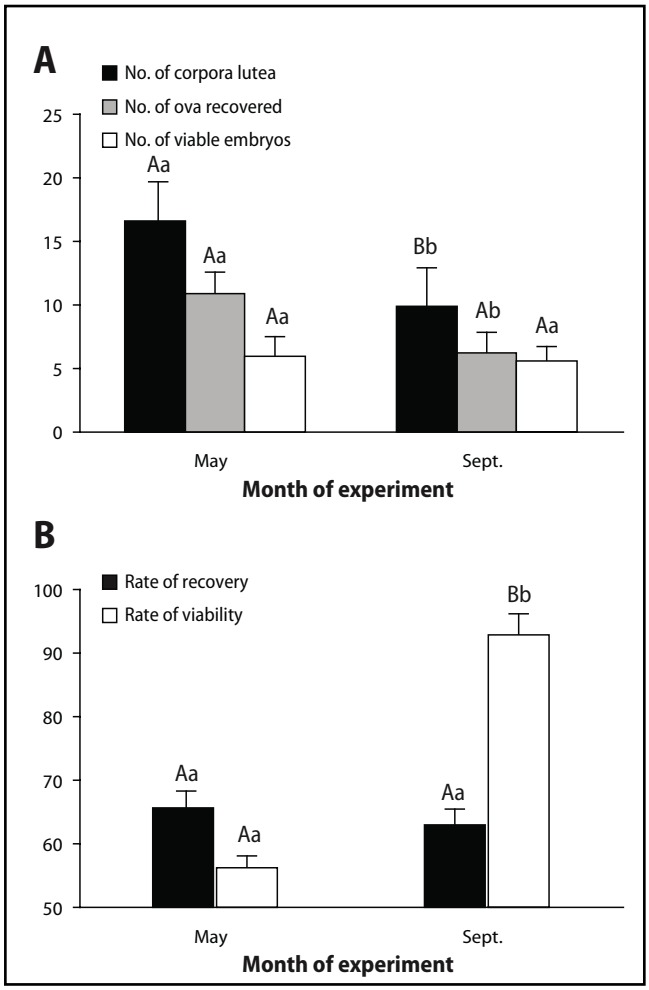

Fig. 1. Effects of seasonal changes on the number of sheep *corpora lutea* and recovered embryos. **Panel A:** Number of the *corpora lutea*, the rceovered embryos and the viable embryos during May and Sep. (n=21). **Panel B:** Embryo recovery rate (the number of recovered embryos/*corpus luteum*) and viability rate(the number of viability embryos/recovered embryos). Data were expressed as mean± SEM. Different letters indicate differences within groups where a and b represent $p<0.05$, and A and B represent $p<0.01$.

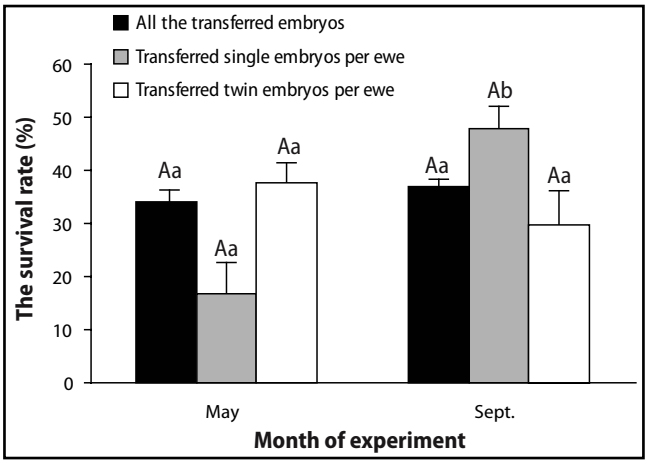

Fig. 2. Embryo survival rate after transplant in May and September. Data were expressed as mean± SEM. Different letters indicate differences between groups where a and b represent $p<0.05$, and A and B represent $p<0.01$.

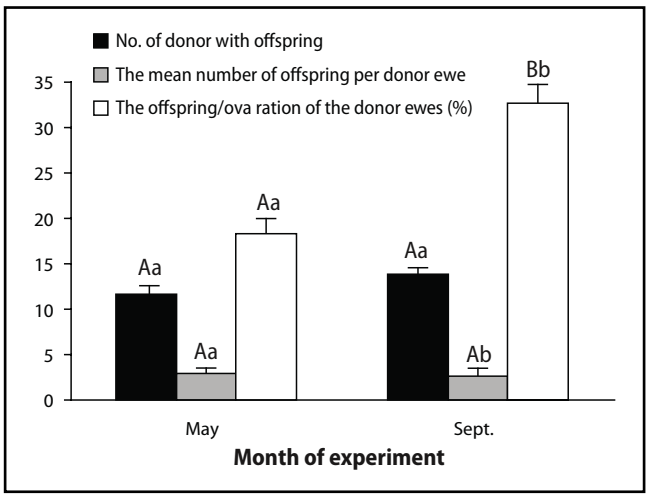

Fig. 3. Effect of seasonal changes on the offspring of donor ewes. Data were expressed as mean± SEM. No. of donor ewes flushed was 21. Different letters indicate differences between groups where a and b represent $p<0.05$, and A and B represent $p<0.01$.

melatonin from the pineal gland melatonin in increasing superovulation and transgenic embryo transplantation efficiency in sheep (Bittman *et al.* 1983). Melatonin implantation improved the donor's response to superovulation and provided more high quality embryos in sheep(Zhang Lu *et al.* 2013).

It appears that the ovulation rate of superovulated ewes is not affected by the seasonal changes, as suggested by the four experiments described above. However, the ovulation rate of the crossbred ewes was observed to exhibit significant difference between seasons. This observation is inconsistent with the results of the current study. This inconsistence may be related to strain differences. The ovulation rate of superovulated ewes depends on the number of follicular waves and the follicles of each wave. The occurrence of follicular waves was indicated by FSH fluctuations because there is an increase in FSH concentration before the initiation of follicular waves, and there is also an approximate relationship between the number of follicular waves and FSH fluctuations per interval or the durations of the interwave and interpeak intervals (Ginther *et al.* 1995). Moreover, additional follicular waves are stimulated by the increasing frequency of FSH peaks after the administration of exogenous FSH, without altering the original rhythm of the follicular waves (Duggavathi *et al.* 2005).

Compared to December, the number of follicles on the day of sponge removal, the number of follicular waves during a 10 day interval and the number of follicles in the first wave after sponge removal were significantly higher than June when Western White Face ewes were superovulated with MAP (medroxyprogesterone acetate) and PMSG (Barrett *et al.* 2004). The occurrence of follicular waves and FSH peaks during the anestrus season is more frequent when ewes are treated with PMSG in the breeding season (Evans *et al.* 2000; 2001). Therefore, the total number of pre-ovulatory follicles for ewes superovulated with FSH or PMSG is significantly higher during the anestrus season.

However, follicles develop faster and more completely during the breeding season, even without stimulation from exogenous FSH and PMSG, because the growth of GTH (gonadotrophic hormone) dependent follicles is regulated by the combined secretion of FSH and LH. Furthermore, the basal level of FSH and LH is higher in cyclic ewes than seasonal anestrus ewes, which is confirmed by the observation that the peripheral concentrations of FSH in both intact and ovariectomized ewes is maintained at high levels during the breeding season (Findlay & Cumming 1976; Montgomery *et al.* 1987). It is also proposed that the secretion of LH in ewes is increased by frequent and amounts of GnRH release during the autumn and winter, the seasons with the highest melatonin levels (Thompson & Kaiser 2014). The hypothesis is verified by the results of the previous experiment, which showed that the maximum size and growth phase of the ovulatory follicles in cyclic ewes treated with PMSG are significantly higher and longer compared to superovulated seasonal anestrus ewes (Barrett *et al.* 2004). Moreover, the percentage of medium and large follicles (>3 mm) in ewes without FSH treatment was significantly higher during September–December than March–April (59.8% vs. 19.8%, $p<0.01$) (Stenbak *et al.* 2001).

Therefore, the ovulation rate of superovulated ewes differed between seasons because of the higher total number of follicles during the anestrus season. However, only the follicles with diameters greater than 3 or 4 mm respond to exogenous LH or hCG and ovulate. The differences observed during experiments may not be significant because of the higher percentage of ovulating follicles during the breeding season and the influence of the superovulation procedure, including hormones, length and dosages of the treatments. The significantly higher percentage of medium and large follicles (>3 mm) in ewes injected with four gradually decreasing dosages of FSH (88% vs. 61%, $p<0.01$) and the reversed results were observed in ewes administered six injections during September–December compared to March–April (63% vs. 83%, $p<0.01$) (Stenbak *et al.* 2001) which indicated that the longer the treatments duration, the more follicles that ovulate during the anestrus season.

Despite the higher ovulation rate in May, the number of viable embryos in superovulated Black Suffolk ewes did not differ significantly between seasons in this study. The percentage of surviving embryos after transfer also did not differ significantly. However, the viability rate and the offspring/ova ratio of donor ewes were significantly lower in May than that in September. This was similar to the results of the superovulation of crossbred ewes (Fukui *et al.* 1994), Mule ewes (Mitchella *et al.* 2002), Merino ewes (Buffonia *et al.* 2014) and Kivircik ewes (Üstüner *et al.* 2014), The percentages of unfertilized and degenerated ova in Black Suffolk ewes were significantly higher in the anestrus season than in the breeding season.

The largest follicles always exist in the ovaries of ewes, regardless of the breeding season or anestrus season. Follicles with more FSH receptors survive via the phosphorylation of Erk1/2 and Akt (Evans & Martin 2000) and are then selected by low peripheral FSH concentration and high frequency LH pulses during the later luteal and follicular phases after gonadotrophic dependence transfer (Campbell *et al.* 1999). However, the significantly higher percentage of small follicles (≤3 mm) in non-superovulated ewes during March–April (80% vs. 40%, $p<0.01$) (Stenbak *et al.* 2001), the season with more total follicles, suggests the lack of dominance of large follicles during the anestrus season. This is because both follicle development and the initiation of follicular waves are suppressed by dominant follicles.

Co-dominance, which occurs in ewes ovulating multiple follicles (Evans *et al.* 2000), decreases the embryo

viability during the breeding season because of the premature ovulation induced by an earlier LH surge in superovulated ewes with a large size difference between the first and second largest follicles (Veiga-Lopez *et al.* 2006). Compared to the ewes without large follicles (≥6 mm) at the 1st FSH injection, the number of ova recovered and the viable embryos are also significantly lower in ewes with large follicles during the breeding season. However, this does not occur during the anestrus season, whereas the viability rate in ewes is higher for the presence of a *corpora lutea* prior to FSH treatment (Gonzalez-Bulnes *et al.* 2003). It has been proposed that the viability of embryos is positively associated with the concentration of progesterone (McEvoy *et al.* 1995); therefore, a higher viability rate is expected in cyclic ewes for higher basal levels of progesterone during the breeding season (Mitchell *et al.* 1999; Goff *et al.* 2013). However, the differences in embryo viability cannot be attributed to the different progesterone concentrations between seasons because the concentration of progesterone may be elevated by a high level of exogenous progesterone released from CIDR or FAG during both the breeding and anestrus seasons (Mitchell *et al.* 2002).

A positive relationship was demonstrated for the follicle sizes and the oocyte diameters (Karami-Shabankareh & Mirshamsi 2012), and the oocyte quality may also be associated with the follicle sizes. It has been reported that 84% of the follicles with a diameter larger than 150 μm reach the MII stage in sheep, whereas the oocytes recovered from follicles with 2–6 mm diameters are fully grown, and the nuclear maturation *in vitro* has been shown previously (Shirazi & Sadeghi 2007). However, not all ovulated oocytes are competent for fertilization, and a 3 mm diameter is the minimum size of follicles to produce viable oocytes (Veiga-Lopez *et al.* 2005). Possibly, the high degeneration rate of ova recovered from superovulated ewes during the anestrus season is caused by the high percentage of small follicles, and the immature oocytes do not survive *in vivo* after ovulation. Moreover, inconsistent ovulation may also account for the low viability rate during the anestrus season because it reduces the fertility of estrus synchronized ewes (Barrett *et al.* 2008). The differences in fertility of Black Suffolk ewes between the breeding and anestrus seasons is not caused by the mating system, which was performed using intrauterine artificial insemination in the present study. Additionally, according to the statistical results of a 7-year study, the pregnancy rate of ewes inseminated with thawed semen by laparoscopic AI did not differ significantly among the spring, summer and autumn (Palacios & Abecia 2014).

Because the physiological states of ovaries are different between individual ewes, it is likely that there is a stable and high viability rate in superovulated ewes during the breeding season. The positive or negative effects of large follicles or *corpora lutea* prior to the superovulation treatment may be offset in experiments with randomly selected ewes. However, the viability is significantly lower in seasonal anestrus ewes because of the adverse impacts of the anestrus season on oocyte maturation and fertilization.

In conclusion, the superovulation of Black Suffolk ewes is affected by the seasonal changes, and the ovulation rate was significantly higher in May, whereas the viability rate was significantly higher in September. The balance between the quantity of follicles and the quality of embryos is likely disrupted by the modified superovulation procedures, and the viability of ova recovered from seasonal anestrus ewes is improved after increasing the strength of hormonal stimulation of the follicles and the length of the oocyte growth phase during the anestrus season.

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