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Highly precocious activation of reproductive function in autumn-born goats (*Capra hircus*) by exposure to sexually active bucks.

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Key words: Puberty, Goats, Seasonal reproduction, Behavior, Estrous cycle, Ovulation

Abstract:

Goats are seasonal breeders with the main cue controlling the timing of breeding season being photoperiod. Hence, the season of birth impacts puberty onset: spring-born goats reach puberty in autumn, at 7 months of age, while autumn-born goats reach puberty at 1 year during the next reproductive season. The aim of this study was to determine whether exposure of autumn-born young females to sexually-active males could counteract the delay in puberty onset observed in autumn-born goats. Females exposed to sexually-active males (n=8) reached puberty earlier than isolated females (n=8); with exposed females ovulating at a mean age of 3.5 months. To our knowledge, such precocious puberty onset obtained through social stimulation has never been described in the literature. Moreover, those exposed females exhibited estrus behavior for most ovulations. Our results indicate that, in goats born out-of-season, exposure to sexually-active buck is really efficient to induce an early puberty, suggesting that social interactions could have a crucial impact in the regulation of pubertal transition.

Highlights:

- Exposure to sexually-active bucks is a highly efficient way to induce ovulations in young does
- Puberty can be induced by exposure to male as early as 3.5 months in young female goats
- Exposure to sexually-active bucks failed to prevent a return to anestrous in young does

1. Introduction

The pubertal transition is a key period which marks the beginning of the reproductive life. During this period, the hypothalamic-pituitary-gonadal (HPG) axis that remained quiescent since early post-natal life is reactivated and individuals acquire the physiological and behavioral ability for reproduction. For females, puberty is usually defined as the occurrence of the first ovulation [1]. The HPG axis is highly sensitive to internal and external factors [2], with one of the most important cues being photoperiod.

Small ruminants are seasonal breeders; alpine goats kept under temperate latitudes exhibit a succession of estrus and ovulatory cycles from early autumn to late winter [3]. Alpine bucks also display seasonal variation of their sexual activity with a breeding period starting late summer and finishing late winter [4]. For those seasonal breeders, photoperiod does not only affect adults' reproductive functions but it also influences the start of the reproductive life of each individual by regulating the onset of puberty. It has been shown that under both temperate [5] and subtropical [6] latitudes, puberty will occur only during the breeding season. So, for females, the age at first ovulation depends on their month of birth: spring-born goats reached puberty around 7 months of age during the normal season of sexual activity, while does born out-of-season (autumn) reach puberty only around 1 year of age, due to the inhibitory effect of photoperiod on reproduction in spring and summer [5,6]. Therefore, out-of-season birth induces a significant delay of puberty of 5 months.

While photoperiod is considered to be the main environmental cue regulating reproductive functions in seasonal breeders, other factors such as social interactions can also play an important role. In small ruminants, the possibility to regulate reproduction by socio-sexual signals is well known, especially the capacity of a male to induce ovulation when introduced into a group of anestrous females [7,8]. This phenomenon, called the « male effect », has been well characterized in goats, highlighting that the main limiting factor for an efficient male-effect is the level of sexual activity of bucks [9]. The use of photo-stimulated bucks increases significantly the success of the male-effect. The percentage of ovulatory females drops from 85-90% using photo-stimulated bucks to 5-10% using non-stimulated bucks [10–13]. Hence, in goats, the reproductive axis of females seems to be highly sensitive to signals provided by bucks.

Some studies reported that the presence of males could also affect the onset of puberty of young females [ewes: 14–16; goats: 17,18]. This highlights that social factors can affect the immature gonadotropic axis by inducing its precocious reactivation. In goats, the level of sexual activity of males is crucial to induce precocious puberty in young females, as exposure to castrated bucks fails to impact the age at puberty [18]. Nevertheless, most of the studies investigating pubertal induction in ruminants were conducted with spring-born young females, so little information is available about the possibility of modifying the onset of puberty in autumn-born females which are known to have a delayed puberty. Recently, one study conducted in sheep highlighted that exposure to stimulated rams was efficient to induce a precocious puberty in autumn-born ewes [19]. Females exposed to sexually-active rams were pubescent 1.4 months before the control group. In our study, we tested the hypothesis that exposure to sexually-active bucks will induce the onset of puberty of autumn-born females earlier than in females isolated from bucks. In a previous study carried out in spring-born females we highlighted that exposure to inactive bucks had no impact on the female pubertal transition [18]. Hence, in this experiment, we choose to not include a group of female exposed to inactive bucks.

2. Material and Methods

2.1 Animals

Experiments were carried out from September 2016 to April 2017 in Nouzilly, France (latitude 47° 32N and longitude 0° 46E) on alpine goats (*Capra hircus*). We used a total of 26 animals for this study, 16 juvenile females and 10 sexually experienced adult males. Young females were born between August 24th and September 4th. They were weaned at 2.5 month old and one week later they were divided into two groups balanced for body weight. Sisters were separated to avoid any genetic effect. Animals were fed daily with barley straw, lucerne hay and commercial concentrate, with free access to water and mineral blocks. All procedures were performed in accordance with the European directive 2010/63/EU on the protection of animals used for scientific purposes and approved by an ethical committee for animal experimentation (CEEA VdL, Tours, France, n°2016091910327211).

2.2 Photoperiodic treatment

To ensure that females were permanently exposed to sexually active bucks (SAB), the males were divided into three groups and two of them were successively submitted to a photoperiodic treatment of long days. This treatment consists of an exposure to artificial long days (16 hours of light and 8 hours of darkness per day) for 80 days in a light proof building. Light intensity in the lightproof building was at least 300 lx at the level of the eyes of the animals. Following this light treatment, bucks were moved into open barns and exposed to the natural photoperiodic conditions.

One group of bucks was exposed to artificial long days from September 15th to December 1st (SAB2, n=3) and another from November 4th to January 23rd (SAB3, n=3). Six weeks after, they were exposed to females: from January 15th to March 5th for SAB2 and from March 6th to April 24th for SAB3. SAB1 (n=4) bucks were used to stimulate females during the breeding season (from November 22nd to January 15th), hence they were naturally sexually actives and did not need to be stimulated with a photoperiodic treatment. This photoperiodic treatment is known to be efficient in inducing a sexually-active state in males during the non-breeding season after 45 to 60 days following the switch of exposure from artificial long days to natural photoperiod [4,10]. The level of sexual behavior of photo-stimulated bucks has been shown to be as intense as the one of naturally sexually active bucks [20].

To evaluate the efficiency of the photoperiodic treatment, testicular volumes were assessed at the end of the photoperiodic treatment and during the first week of exposure to females. Measures were taken by the same operator to avoid inter-individual variations [21,22].

2.3 Pre-pubertal exposure to bucks

One group of 8 females was exposed to bucks (STIM) just after weaning while the other group of 8 females remained isolated (ISOL) from any male. In the STIM group, exposure to bucks was continuous behind a fence from November 22nd until April 24th. Throughout this period, we allowed direct contact between bucks and does for 1 hour, 3 times a week; in this case, males were fitted with an apron to prevent intromissions and conceptions. During those close interactions, we observed the behavior of both males and females to detect estrus behavior and to ensure the sexually-active state of the bucks. Ano-genital sniffing, flehmen and lateral approaches, indicative of buck behavior, were measured. The number of contacts between male and females was quantified to ensure that the bucks' and does' motivation to interact were similar throughout the experiment. A doe was considered in estrus when it showed a state of excitement during which it vocalized more, wagged her tail and accepted the mount by the buck.

2.4 Evaluation of puberty onset and weight gain

Age at puberty was assessed by the determination of the onset of ovulatory activity. Ovulations were detected by measuring plasma progesterone (P4) concentrations twice a week. Blood samples were obtained by jugular venipuncture from all females from the age of 3.5 months till the age of 7.5 months in 5mL tubes containing heparin. Plasma was obtained after 30 minutes of centrifugation at 3500g and concentration of plasma progesterone was determined in samples using an immunoenzymatic assay as described previously [23]. Sensitivity of this assay was 0.25ng/mL. The mean intra-assay and inter-assay coefficients of variation were <10%. Females with progesterone concentrations ≥0.5ng/mL in 2 consecutive samples were considered to have ovulated and then to be pubescent [24,25]. Throughout the experiment, females were also weighed monthly.

2.5 Statistical Analysis

Results are reported as mean ± SEM (standard error of the mean). A Shapiro-Wilk test has been performed to test the data distribution. If data were normally distributed ($p>0.05$) a parametric test was performed, otherwise we used non-parametric tests. Weight data were compared between groups using a two-way repeated-measures ANOVA. Intra-group variations of the testicular volume were performed using paired Student t test and inter-groups variations were analyzed by the Kruskal-Wallis test. The age at puberty was examined using a Kaplan-Meier survival analysis and differences between groups were compared by the log rank test. Behavior of bucks was analyzed using the Kruskal-Wallis test followed by multiple comparisons using the Tukey-Type test (« nparcomp » package). Number of cycles was analyzed using the Mann-Whitney test and percentages of cycling goats were compared using Fisher exact tests. Area under the curve (AUC) of progesterone secretion was calculated for each group following the trapezoidal rule, comparison between groups was performed by a Mann-Whitney test. All analyses were performed using R software [26]. Statistical significance was set at $p<0.05$.

3. Results and discussion

The aim of this study was to investigate the consequences of exposure to sexually active bucks on the onset of puberty of female goats born out-of-season. We observed that goats exposed to sexually active bucks had an earlier first ovulation than isolated goats ($p<0.01$, **Fig. 1A**). On December 19th, 5/8 of STIM females were considered pubescent while none of the ISOL females reached puberty; at the end of the experiment (late April), all STIM females were pubescent but only half of the ISOL females. In our study, young females exposed to sexually active bucks were pubescent at a mean age of 112 days (3.6 months).

Our results are consistent with the only other study in ruminants in which induction of precocious puberty in autumn-born females by sexually-active males occurred in sheep, with an advance of 1.4 months. In this case, teased females were pubescent at a mean age of 7.8 months while controls females reached puberty at 9.2 months [19]. This difference from our findings can be partly explained by the different experimental design; indeed, we chose to expose females to bucks at weaning (3 months). This was earlier than in this previous study in which rams were introduced when females were 5.5 month old. In our experiment, more than 85% of goats exposed to sexually active bucks reached puberty within a month after the first contact with males **(Fig. 1A)**. We might predict that if ewes had been exposed to rams earlier, they might have reached an even earlier puberty. Interestingly we observed a great synchronicity of the first ovulation in the group exposed to sexually-active males: all STIM females reached puberty in a range of 3 weeks, while in the control group only 50% reached puberty over the 5 months of the experiment **(Fig. 1A)**. We have already observed this three-week delay between the beginning of exposure to sexually-active males and the ovulatory response of young females in our previous study conducted with young does born in season [18].

The difference in the age at puberty that we observed here between STIM and ISOL groups is not due to other factors such as the metabolic state, which can impact the age at puberty. Indeed, regarding the body weight variation of females, a strong effect of time ($F_{(7,98)}$= 299.9, $p<0.001$) and a treatment-by-time interaction were detected ($F_{(7,98)}$= 3.4, $p<0.01$) but no group effect ($F_{(1,14)}$= 0.58, p=0.46) can be observed **(Supplemental Fig. 1)**. One single difference was observed between groups for the month of April, STIM females were heavier than ISOL females (39.88 ± 2.5Kg versus 34.75 ± 2.5Kg; $p<0.05$). Hence, no difference in body weight was detected between groups when they started ovulating, thus this factor is therefore not responsible for the precocious puberty observed in females exposed to sexually active male.

In this study, we not only assessed the age at first ovulation, but we also characterized the subsequent estrous cycle expression of females. All young goats exposed to sexually-active bucks remained cyclic after the first ovulation, with at least two other cycles similar to those observed in mature goats [27]. In the ISOL group, only two females exhibited 2 cycles or more. Furthermore, for STIM females, 28 ovulations were detected by progesterone levels, in most cases (20/28), females exhibited estrus behavior few days before **(Fig. 1B)**. In young goats, we observed, as was expected, that most first ovulations are silent; i.e. that there is no estrus behavior associated with ovulation [28]. Observation of estrus behavior is still a commonly used approach to determine if a female is pubescent. According to our results, this method would fail to detect pubescent females, females that only had one cycle and that did not show any sign of estrus behavior which is the case of 3 out of 8 for our isolated females.

Because STIM females had more ovulations than ISOL females, the area under the curve (AUC) calculated for progesterone concentrations along the experiment **(Fig. 1C)** was significantly higher in the STIM group than in the ISOL group ($p<0.01$). This difference is not only due to the advancement of first ovulation, but also because exposure to males also induces the maintenance of the ovulatory cyclicity. Indeed, females continue cycling 3 weeks later when exposed to sexually-active bucks than when isolated from bucks **(Fig. 1D)**. The period within which all females from a group were cycling was 8.5 weeks in the STIM group and was only 1 week in the ISOL group. The percentage of cycling goats was significantly higher in the STIM group for late-December and mid-February (**Fig. 1D**, $p<0.05$). However, this enduring cyclicity ended late March. Our first hypothesis was that this was due to the bucks no longer being sufficiently sexually active to stimulate females. However, the analysis of the testicular volume of bucks used at this time of the experiment (SAB3) does not show any difference when compared to the testicular volume of bucks from the two other groups while exposed to females (**Fig. 2**; $p>0.05$). The photoperiodic treatment induced an increase of 18% of the testicular volumes in both treated groups (SAB2 and SAB3). Comparing the behavior of bucks among the three groups, no significant differences in the number of physical contacts between males and females were detected **(Fig. 3)**. An increase of the occurrence of appetitive behaviors is observed in SAB3 bucks, hence those bucks exhibit more ano-genital sniffing than the 2 other groups (SAB1-SAB3: $p<0.05$;

SAB2-SAB3: $p<0.01$) and more flehmen than SAB1 ($p<0.05$). No significant difference was detected in the occurrence of lateral approaches among bucks' groups **(Fig. 3)**. Therefore, we conclude that, for STIM females, the termination of estrous cycles was due to an inhibitory effect of photoperiod rather than a lack of stimulation. Indeed, in goats, the anestrous season starts in March when day length increases [29], providing an inhibitory signal to the reproductive axis.

Those results are however not consistent with a recent study [30] that raised the possibility of continued estrous cycle expression due to continuous exposure to sexually active bucks. However, in our experiment, while exposure to active bucks caused expression of estrous cycles, it failed to totally override the anestrous period. The difference between the two studies can be explained by several factors. First, magnitude of photoperiodic variations was different. The study by Delgadillo et al [30] was conducted in Northern Mexico, where day length varies from 10 h at the winter solstice to 14 h at the summer solstice. Under temperate latitudes variations are more important with, at the same dates, a photoperiod varying from 8 h to 16 h. The greater photoperiodic variation observed under temperate latitudes may result in a deeper anestrous and a stronger inhibitory effect of photoperiodic stimuli. Social factors could then be overridden by photoperiodic input. Another difference between the two studies was the level of sexual experience of females. The study of Delgadillo et al [30] used only sexually experienced multiparous adult goats. In our study, females were younger and had a very brief experience with the male (i.e. continuous exposure behind a fence for 4 months with only 3 hours per week of direct contact and no copulation). It is known that sexual experience can impact the quality of the female's response to the signal of the male. For example, multiparous ewes have a better response to a male-effect than nulliparous ewes [31]. In goats, results obtained during a male-effect experiment show in some cases a better response with multiparous females: 92% of those females exhibit estrus behavior while only 16% of inexperienced females do so [32].

To our knowledge, we report for the first time that puberty of autumn-born young goats can be significantly advanced by exposure to sexually-active bucks, without any hormonal treatment. The presence of active bucks since weaning can induce an acceleration of the reactivation of the female reproductive axis enabling young goats to ovulate, maintain a normal cyclicity (i.e. regular cycles of approximately 21 days) and express an adequate estrus behavior as early as 3.5 months of age. This result is highly original as the commonly accepted age at puberty for goats born in spring is 7 months and one year for goats born in autumn. It highlights the fact that the gonadotropic axis is highly sensitive to male sensory cues even at a young age. The presence of bucks also helped maintain the cyclicity in young females but failed to prevent the anestrous season, as has been observed in mature females.

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Competing interests:

No competing interests declared.

Authors contribution:

M. Chasles and M. Keller designed the experiments, performed the results analysis and wrote the manuscript. P. Chemineau, J-A. Abecia and J-A. Delgadillo corrected the manuscript. C. Moussu and D. Chesneau helped during animal experimentations and corrected the manuscript.

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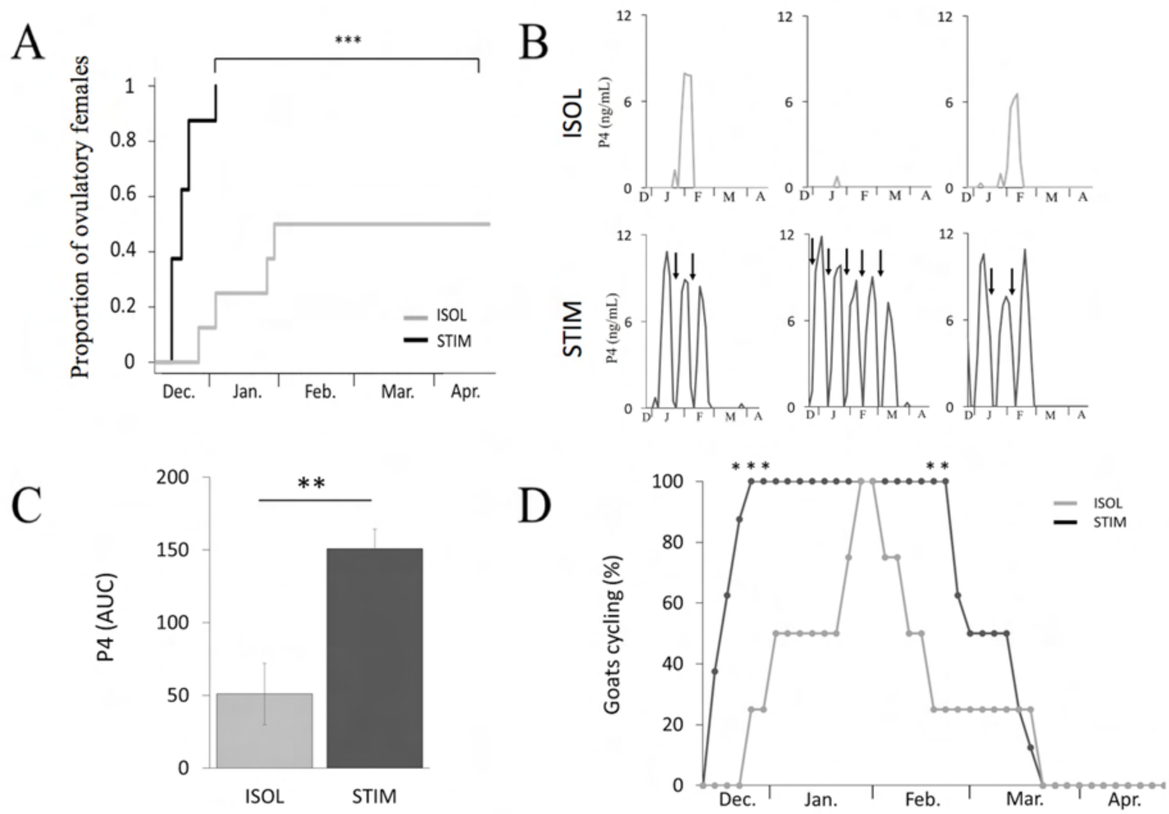
Figure legends

Figure 1. Physiological response of young goats exposed (STIM) or not (ISOL) to sexually active bucks. Blood samples for progesterone were collected twice a week from mid-December to late-April. **(A)** Cumulative proportions of young goats reaching puberty from ISOL (n=8) and STIM (n=8) groups. Females were considered pubescent when progesterone levels ≥0.5ng/mL in two consecutive samples. Data were analyzed using log-rank test (***p < 0.001). **(B)** Representative patterns of the cyclicity of females. STIM females were submitted to direct contact with bucks 3 hours a week, estrus behavior (indicated by arrows) was determined during those close interactions. **(C)** Comparison of the cumulative serum progesterone secretion expressed by area under the curve (AUC, arbitrary unit). Stars indicate significant differences between groups using a Fisher exact test (**p < 0.01). **(D)** Percentage of pubescent females cycling in the STIM (n=8) and ISOL (n=4) groups**.** Stars indicate significant differences between groups using a Fisher exact test (*p < 0.05).

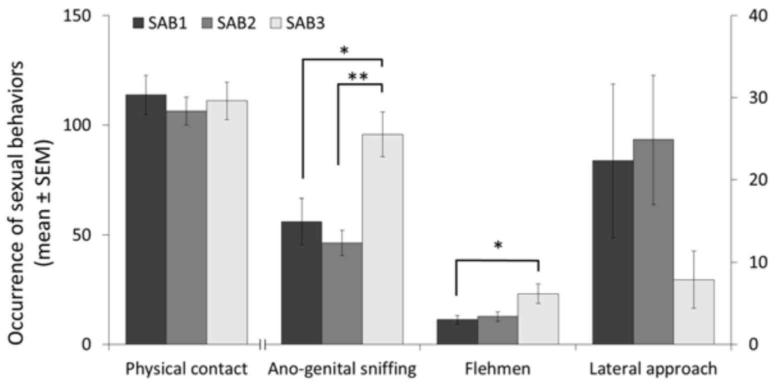
Figure 2. Variation of the testicular volume. Measure of the testicular volume was assessed by orchidometry the first day of exposure to females for each group of bucks: SAB1 (n=4), SAB2 (n=3) and SAB3 (n=3). For SAB2 and SAB3 bucks, a measure was also performed the last day of the long day treatment. Results are presented as mean ± SEM. Stars indicate significant differences within a group using the paired Student t test (*p < 0.05). Different letters denote a significant difference between groups using a Kruskal-Wallis.

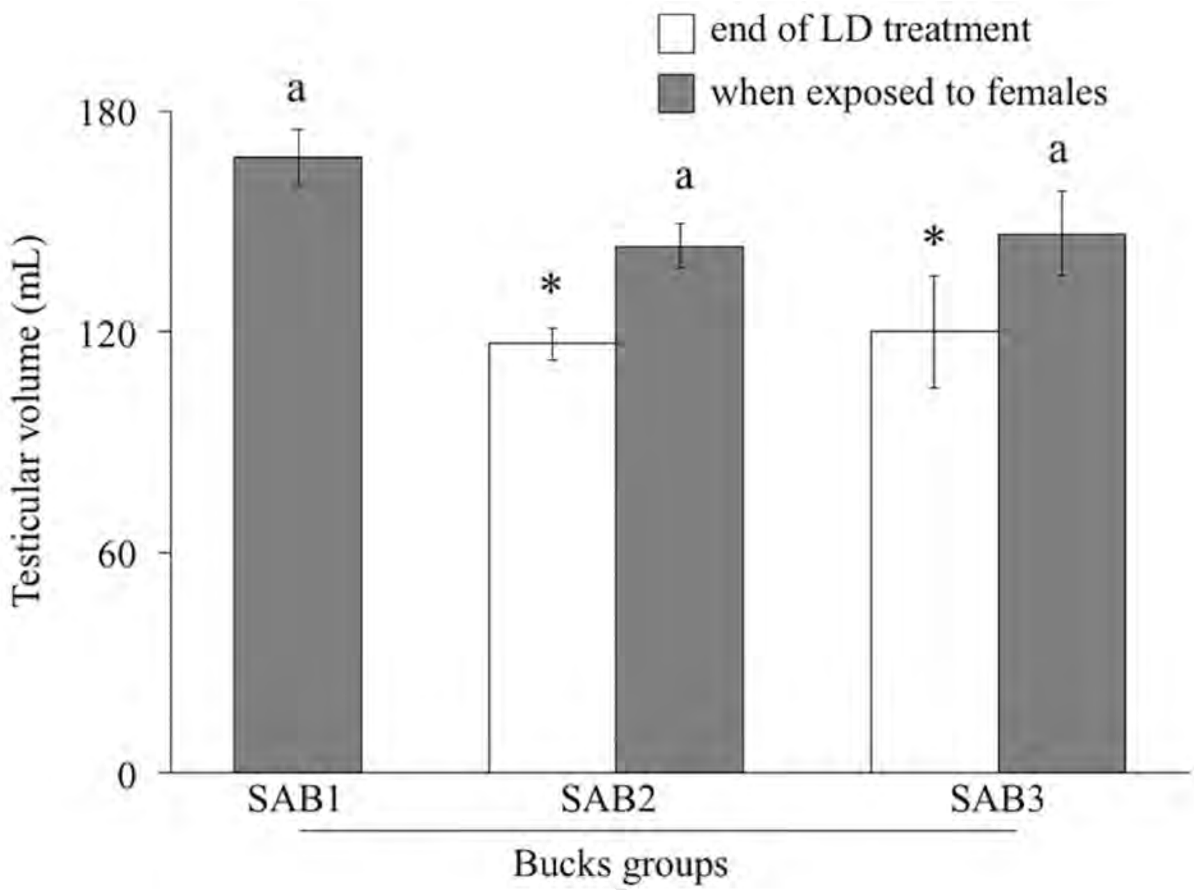
Figure 3. Occurrence of socio-sexual behaviors of the three groups of bucks. Behavior was observed for 1h three times a week when a single buck was introduced into the females' pen. SAB1 (n=4) were used from November 22nd to mid-January, SAB2 (n=3) from mid-January to mid-March and SAB3 from mid-March to late-April. Results are presented as the mean ± SEM occurrence of each behavior per hour. Stars indicate significant differences between groups using a Kruskal-Wallis test (*p < 0.05, **p < 0.01).

Supplemental Figure 1. Changes in body weight in female kids from birth to 7 months of age. Females from the 2 groups, ISOL (grey line; n=8) and STIM (black line; n=8) were weighed monthly. Data are presented as mean ± SEM. Stars indicate significant differences between groups using a two-way repeated-measures ANOVA followed by pairwise t-test (*p < 0.05).



A
***
Proportion of ovulatory females
1
0.8
0.6
0.4
0.2
0
ISOL
STIM
Dec.
Jan.
Feb.
Mar.
Apr.
B
ISOL
STIM
P4 (ng/mL)
12
6
0
D J F M A
C
200
150
100
50
0
**
P4 (AUC)
ISOL
STIM
D
***
**
100
80
60
40
20
0
Goats cycling (%)
ISOL
STIM
Dec.
Jan.
Feb.
Mar.
Apr.


SAB1
SAB2
SAB3
Occurrence of sexual behaviors
(mean ± SEM)
150
100
50
0
40
30
20
10
0
*
**
*
Physical contact
Ano-genital sniffing
Flehmen
Lateral approach



end of LD treatment
when exposed to females
Testicular volume (mL)
180
120
60
0
a
a
a
*
*
SAB1
SAB2
SAB3
Bucks groups

Highlights:

- Exposure to sexually-active bucks is a highly efficient way to induce ovulations in young does
- Puberty can be induced by exposure to male as early as 3.5 months in young female goats
- Exposure to sexually-active bucks failed to prevent a return to anestrous in young does

Credit author statement :

CM, CD, MC, DAJ, CP and KM designed the study. CM, CD, MC, and KM performed the experiment. CM, AJA, DJA, CP and KM analysed the data and wrote the manuscript. All authors read and validated the manuscript. PC and MK secured the funding of the study.