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RELATIONSHIP OF PUBERTY AND SEMEN CHARACTERISTICS TO SEASONAL AND NUTRITIONAL VARIATIONS IN SAIDI RAM LAMBS

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SUMMARY

This study was designed to determine puberty and semen characteristics in Saidi ram lambs in relation to seasonal and nutritional variations. Sixteen Saidi ram lambs of 6-7 months age and average body weight 31.55 .kg were divided into four equal groups. The 1st group was served as a control (Maintenance), the 2nd group was fed a high protein/low energy ration (HL), the 3rd group was fed a low protein/high energy ration (LH) and the 4th group was fed a high protein/high energy ration (HH). Ram lambs were checked daily early in the morning for 15 minutes for observing sexual behavior by introducing a ram lamb to a teaser ewe chosen randomly from a flock of sheep. The ram which showed erection and extrusion of the penis out of the sheath was caught immediately and a smear of fluids secreted around the penile sheath was prepared for microscopic examination. The ram which produced sperm in the smear was immediately trained for semen collection by an artificial vagina. One ejaculate/week/ram was taken to determine semen characteristics (ejaculate volume, pH value, mass motility, sperm concentration, live & dead sperms and sperm abnormalities). Rams fed HL ration reached puberty earlier (338.3 days) than rams fed LH ration (349.0 days), rams fed HH ration (373.0 days) and rams fed LL ration (394.5 days). Significant effects ($p<0.05$) were found for the different levels of feeding on puberty. Quality of semen was much better in spring and autumn than in summer and winter. Furthermore, rams fed low protein and/or low energy had a poor semen quality compared to the other groups. From these data, low feeding levels are unacceptable in practice owing to increase of age at puberty and reduction of semen quality as well. So, the economic use of Saidi ram semen required collecting and utilizing semen in autumn and 10 % addition of protein and TDN over the maintenance requirements (14 % protein and 73 % TDN).

Keywords: Puberty, semen quality, season, nutrition and Saidi rams

INTRODUCTION

Puberty is defined as an age at which the first sperm appears in the seminal ejaculate (Pretorius and Marincowitz, 1968). Puberty in the male sheep is exactly as in the female depends on body development, birth date and photo-period length (Foster *et al*., 1988). Ram lambs have the ability to mount for the first time at 7-9 months of age. Haynes and Schanbacher (1983) reported that puberty in ram lambs

ranged from 84 to 456 days depending on breed. Puberty in a ram was dependent on body weight much better than age (Dyrmundsson and Lees, 1972; Dyrmundsson, 1978). Courot (1979) indicated that puberty in a ram may represent 40-45 % of the adult body weight, this is dependent on breed.

Nelson *et al*. (1987) reported that puberty can be determined through several criteria such as presence of live active sperm in the first ejaculate of animal life, age, live body weight and scrotal circumference measurements. Indian native ram lambs produced the first ejaculate of semen by 5-6 months of age, while Merino ram lambs do not ejaculate for the first time until 8-9 months of age (Symington, 1961). Ramakrishnan *et al*. (1993) indicated that Siamese long tail ram lambs produced semen at 5 months of age and desirable semen quality was observed in those rams by 12 months of age.

Literature on rams (AlKass *et al*., 1982) and bulls (Coulter and Kozub, 1984) indicated that males animals fed at or below their maintenance requirements for energy will have a reduced reproductive performance compared to animals fed well above maintenance. Kemp *et al*. (1989) found that energy intake has a large effect on the number of produced spermatozoa. Protein and energy intake seems to have an effect on semen quality, where low energy and /or low protein intake decreased sperm concentration and libido (Kemp and Verstegen, 1990). Generally an inadequate protein intake results in a lowered reproductive efficiency. Otherwise, rams fed a high protein intake was not necessary for high sperm production, and increasing the energy intake had a higher effect on sperm production than did increasing the protein intake (Braden *et al*., 1974). The goal of the present study was to investigate the relative importance of season, protein and energy in influencing puberty and semen characteristics in Saidi ram lambs.

MATERIALS AND METHODS

Sixteen Saidi ram lambs of 6-7 months of age and mean body weight of 31.55 kg were purchased from Al Alomenium factory farm station, Nagh Hammady, Quina governorate and transported to Animal Production Experimental Farm, Fac. of Agriculture, Assiut University. Animals were divided into four equal groups (four animals/group). The 1st group was served as a control (Maintenance, 14 % protein and 73 % TDN), the 2nd group was fed a high protein low energy ration (HL) (10 % over maintenance with constant TDN) , the 3rd group was fed low protein high energy ration (LH) (10 % over maintenance with constant level of protein) and the 4th group was fed high protein high energy ration (HH) (10 % over maintenance for both). According to NRC (1975) requirements, the percentage of protein and energy was altered corresponding to the change in the body weight during the experimental period (Table 1). Ram lambs were weighed and the weight was recorded biweekly throughout the experimental period.

Ram lambs were checked daily early in the morning for 15 minutes for sexual behavior by introducing a ram lamb to a teaser ewe chosen randomly from the flock. A teaser ewe was restrained in a wooden collection crate in a space of 4m (wide) x 5m (Length). The collection crate dimensions were 88.8 cm (length) x 87.5 cm (wide) x 37 cm (Height). All ram lambs were allowed to run freely to the collection crate for observing their sexual behavior toward the teaser ewe. A ram which showed erection and extrusion of the penis out of the sheath was caught

immediately and a smear of fluids secreted around the penile sheath was prepared for microscopic examination. The ram which produced sperm in the smear was immediately trained for semen using the artificial vagina. During semen collection, the ram was sexually stimulated either by running or by allowing to make one false mount on the teaser ewe. Semen was then collected at the second mount and the ejaculate was received in the graduated tube of the artificial vagina.

Table 1. Feeding requirements in feeding Saidi ram lambs according to NRC, 1975 (control group)

Body weight (kg)	Daily dry matter/animal (kg)	TDN %	Crude protein %
30-39	1.4	73	14.0
40-59	1.8	65	10.2
> 60	2.3	60	9.5

One ejaculate/week/ram was taken to determine semen characteristics including ejaculate volume, pH, mass motility, sperm concentration (using Haemocytometer), live & dead sperm percentage and sperm abnormalities including primary abnormalities in the sperm head, secondary abnormalities in the mid-piece of the sperm and tertiary abnormalities in the tail of sperm (Wilson *et al*., 1959).

Data were statistically analyzed by analysis of variance using the general linear models of SAS (1985). Body weight, age at puberty, sperm motility, sperm concentration, live sperm, sperm abnormalities were dependent variables, while the main effects in the model were rams, season, level of feeding and the interaction of season with level of feeding. All comparisons among means for all variables in the experiment were tested using Duncan's procedure.

RESULTS AND DISCUSSIONS

Puberty:

The overall mean of the pubertal age in Saidi ram lambs was 363.8±13.3 days and body weight of 50.3 ± 3.0 kg. Data in the Table 2 indicated that the ram lambs fed HL ration reached puberty at earlier age (338.3 ± 6.3 days with mean body weight 48.3 ± 1.0 kg) than the other groups fed LH (349.3 ± 3.3 days with mean body weight 49.8 ± 3.6 kg), HH (373.0 ± 13.7 days with mean body weight 54.3 ± 3.0 kg) and maintenance (394.5 ± 30.0 days with mean body weight 48.8 ± 4.3 kg) rations (Fig.1). No significant differences have been found among groups in the mean body weight, while the significant differences ($p < 0.05$) were noticed in the age at puberty. These results are consistent with those reported by Tegegne *et al*. (1992). They found that age at puberty was earlier in the rams fed 16 % crude protein ration. Abi-Saab *et al*. (1997) found that goat kids fed high level of protein reached puberty earlier than those fed low level of protein.

Table 2. Body weight at puberty (Mean±SEM) in Saidi ram lambs fed different levels of nutrition

Item	Number of rams/group	Age at puberty (day)	Body weight (kg)
Control (maintenance)	4	394.5 ± 30.0[a]	48.8 ± 4.3
Animals fed HH	4	373.0 ± 13.7[b]	54.3 ± 3.0
Animals fed HL	4	338.3 ± 6.3[d]	48.3 ± 1.0
Animals fed LH	4	349.3 ± 3.3[c]	49.8 ± 3.6
Overall mean (±SE)	16	363.8 ±13.3	50.3 ± 3.0

[a b c and d] are significantly differences at [**] p (<0.01)

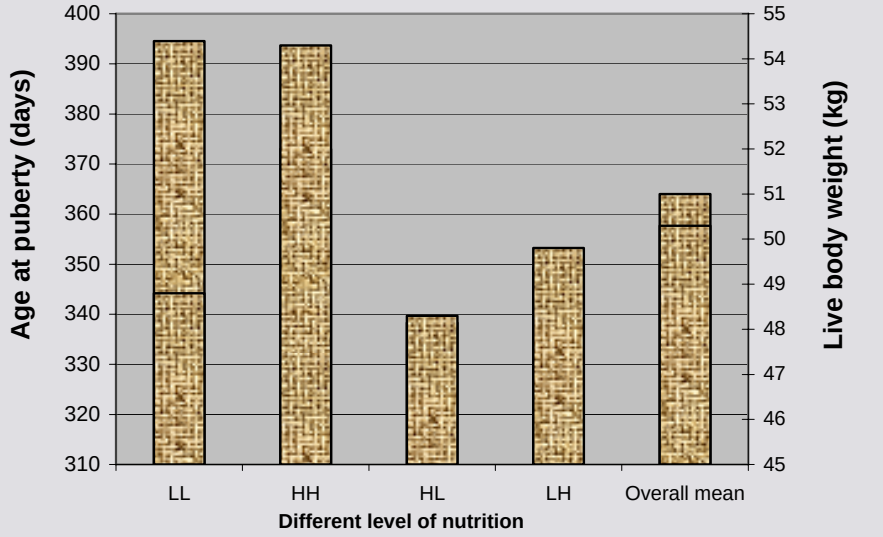

Fig. 1. Relationship of puberty (days) and live body weight (kg) to the level of nutrition in Saidi ram lambs.

Semen characteristics:

The overall mean of ejaculate volume was larger in autumn (0.95±0.04 ml) and spring (0.91±0.03 ml) than that in summer (0.83±0.02 ml) and winter (0.75±0.02 ml) (Table 3). Therefore, there were significant differences ($p<0.05$) among seasons in the semen ejaculate production. Data in the Table 3 indicated also that rams fed low protein/high energy (LH group) and high protein/high energy (1.03±0.02 ml) (HH group) resulted in much better semen volume with overall mean 1.08±0.03 ml and 1.03±0.02 ml, respectively than the rams fed high protein/low energy (HL group) and low protein /low energy (LL group) with overall mean 0.82±0.02 ml and 0.53±0.02 ml, respectively. Rams left without treatment (control group) did not show any remarkable improvement in semen volume, thus the overall mean of ejaculated semen volume was the lowest (0.53±0.02 ml). It is evident that level of energy is

more effective than level of protein in the improvement of semen volume. These results are in agreement with those obtained by Alkass *et al*. (1982) and Zhu *et al*. (1997) who reported that feeding on high energy meals is much better than low energy. The interaction between season and level of nutrition on the ejaculated semen volume was significant ($p<0.05$). The highest value of semen volume was obtained in autumn/LH group (1.21±0.10 ml) while the lowest was in summer/control group (0.48±0.03 ml).

The differences of sperm mass motility among seasons were significant ($p<0.05$). It was higher in autumn (4.41±0.06) than in other seasons with averages of 4.27±0.09, 4.14±0.07 and 3.94±0.09 in spring, summer and winter, respectively (Table 3). Hafez *et al*. (1954) reported that sperm motility of Rahmani sheep was high in autumn and relatively lower in winter. They found a positive correlation (r=+ 0.72) between motility and the live sperm percent. Moreover, rams fed high protein/low energy produced relatively high mass motility (4.58±0.06) in comparison to the other treated groups of (4.52±0.07 for HH group, and 4.32±0.07 for LH group), while rams fed maintenance meal (control group) had the lowest mass motility, 3.37±0.09 (Table 3). Salamon (1964) reported that gross sperm motility of rams fed high protein/ high energy was greater than that of rams fed low protein/low energy. Zhu *et al*. (1997) indicated that the value of gross sperm motility was highly significant between rams fed high protein/high energy, high protein/low energy and low protein/low energy. Furthermore, the interaction effect between season and nutrition on the mass motility was highly significant ($p<0.01$). The maximum value of mass motility was found in the spring /HH group (4.83±0.08), while the minimum value was found in the winter/control group (2.98±0.17) (Table 3).

Data in the Table 3 indicated that the mean sperm cell concentration reached the maximum value in autumn (3.93±0.08 x 10^9 sperm/ml) and the minimum value in summer (3.19±0.08 x 10^9 sperm /ml). Seasonal variations in the sperm concentration were significant ($p < 0.05$). These results are in a harmony with those obtained by Elwishy *et al*. (1971) and Roca *et al*. (1992) who found that the highest value of sperm concentration was recorded in spring and autumn. On the other hand, Abdalla (1996) reported that the highest quality of semen were obtained during summer and autumn in Awassi rams. Moreover, there was a positive correlation between sperm concentration and semen volume. Karagiannidis *et al*. (2000) reported that the correlation between sperm count and ejaculate volume was positive (r=+0.86). Furthermore, there were significant differences ($p < 0.05$) between treated groups. Rams fed high protein/low energy had higher sperm concentration (4.35±0.10 x 10^9 sperm/ml) in comparison to the other treated groups. Rams fed maintenance meal exhibited the lowest value of sperm concentration (2.57±0.12 x 10^9 sperm/ml). The increase of sperm concentration could be attributed to the effect of protein on testis size. The results obtained by Ortavant (1959) and Amann (1970) indicated that there was a considerable correlation between testicular weight and sperm yield. Hotzel *et al*. (1998) found that sperm concentration increased with increasing level of nutrition and decreased as the level of nutrition decreased. Salamon (1964) found also that sperm concentration in Merino rams increased with the feeding on high protein/high energy and decreased with feeding on low protein/low energy. Furthermore, There was significant difference ($p < 0.05$) in the sperm concentration between rams fed

high energy and those received low energy (Alkass *et al*., 1982; Ketut Sutama and Endey, 1985). On the other hand Zhu *et al*. (1997) found that rams fed high protein/high energy and high protein low energy meals were much better in sperm production than those fed high energy/low protein meal. The interaction between season and nutrition in the present study had a significant ($p < 0.05$) effect on sperm concentration. The maximum value of sperm concentration has occurred in spring/HL group ($4.66\pm0.12 \times 10^9$ sperm/ml), while the minimum value was in spring/control group ($2.20\pm0.26 \times 10^9$ sperm/ml). There was reciprocal correlation between sperm concentration and semen volume. These results are coincident with those obtained by Wells *et al*. (1971), Tiwari and Sahni (1982), Langford *et al*. (1989) and karagiannidis *et al*. (2000), who reported that there was positive correlation between sperm count and semen volume.

Seasonal variation in the live sperm percent was significant ($p < 0.05$). The lowest percent of live sperm was recorded in winter (68.11 %) , while the highest percent was in autumn (75.73 %) followed by spring (75.71 %). Daader *et al*. (1985) found that Ossimi rams produced higher live sperms percent in spring and autumn than in summer and winter. The effect of nutrition on the live spermatozoa was statistically significant, where rams fed high protein/low energy resulted in high live sperms in comparison to the other treated groups (Table 3). The lowest value of live spermatozoa was noted for rams fed low protein/low energy (control group). Zhu *et al*. (1997), Prajapati *et al*. (1998) and Al-Haboby *et al*. (1999) reported that rams fed high protein diet had higher content of live spermatozoa than those fed low level of protein. Furthermore, the effect of interaction between level of nutrition and season on the live spermatozoa was highly significant ($p < 0.01$). The highest value of live spermatozoa (83.90%) was obtained from rams fed high protein/low energy x spring. On the other hand, the lowest value of live spermatozoa, 53.48% was achieved in winter x (control group).

Data in Table 3 indicated, significant differences ($p < 0.05$) among seasons in the percent of sperm abnormality. The percent of abnormal sperms was relatively higher in winter (14.09 ± 0.60) followed by summer (13.07 ± 0.85) while the lowest percent of abnormal spermatozoa was recorded in autumn (8.53 ± 0.44). These results were similar to those obtained by Hafez *et al*. (1954), Galal *et al*. (1978) and Daader *et al*. (1985), who found that the total abnormal spermatozoa of Rahmani, Ossimi and Rahmani x Finn rams was very low in autumn and spring , while it was very high in summer and winter. Rams fed high protein/high energy had very low frequency of abnormal spermatozoa (8.17 ± 0.43) in comparison to other tested groups (Table 3). The highest percent of the total abnormal spermatozoa was noted for rams fed low level of protein/low energy (control group), 18.88 ± 0.75. Al-Haboby *et al*. (1999) indicated that the total abnormal spermatozoa was lower in rams fed protein supplementation than in grazing rams. Data in Table 3 indicated also that there has been a significant influence ($p < 0.01$) of season x level of nutrition on the abnormal spermatozoa. Karagiannidis *et al*. (2000) and Rege *et al*. (2000) found a negative correlations between abnormal sperms and mass motility, live sperm and sperm concentration. The present results indicated that the correlations among abnormal sperms and sperm concentration ($r= -0.32$) or live sperm ($r= -0.55$) were also significant.

There was little fluctuation among seasons in pH value of semen. So the pH value of Saidi ram semen tended to be acidic. Data in Table 3 indicated that there were significant differences ($p<0.05$) among nutritional levels and pH value. The highest value of pH (6.96±0.01) resulted from rams fed the low protein/energy meal, whereas the lowest value of pH (6.93±0.01) was obtained from rams fed the high protein low energy. The correlation was a little positive between the pH value and abnormality (r=0.14) and negative with mass motility (r=-0.23), ejaculate volume (r=-0.13) and sperm count (r=-0.30). These results are in agreement with those reported by Wells *et al*. (1971).

From these data it could be concluded that low levels of feeding (protein and energy required) are likely to be unacceptable in practice owing to increase in age at puberty and reduction semen quality as well. So, the economic use of Saidi ram semen required collecting and utilizing semen in autumn and adding 10 % of protein and TDN over maintenance (14 % protein and 73 % TDN).

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علاقة البلوغ الجنسى وخصائص السائل المنوى بالتباين الموسمى والغذائى فى ذكور حملان الأغنام الصعيدية

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قسم الإنتاج الحيوانى – كلية الزراعة – جامعة أسيوط

صممت هذه الدراسة لتقدير البلوغ الجنسى فى حملان ذكور أغنام الصعيدى المصرية فى 16 ذكر حمل عند عمر 6-7 شهور ومتوسط وزن الجسم الحى 31.55 كجم تحت تأثير التباين الموسمى والتغذية. أجريت التجربة على 16 حمل ذكر قسمت إلى 4 مجاميع منفصلة (4 حيوانات/مجموعة) طبقاً لمستوى التغذية. حيث أعطيت المجموعة الأولى عليقة غذائية حافظة واعتبرت كنترول للمجموعات الأخرى، بينما غذيت المجموعة الثانية على عليقة تحتوى على بروتين عالى وطاقة منخفضة ، والمجموعة الثالثة غذيت على عليقة غذائية تحتوى على بروتين منخفض وطاقة عالية أما المجوعة الرابعة فقد غذيت على عليقة احتوت على بروتين عالى وطاقة عالية. وكانت التأثيرات الرئيسية فى هذه التجربة هى الموسم ومستوى التغذية من الطاقة والبروتين. وقد تم مراقبة الحملان الذكرية يومياً لمدة 15 دقيقة كل مجموعة على حدا لمشاهدة وتسجيل السلوك الجنسى من خلال ربط عنق أنثى شائعة فى صندوق شبه مفتوح يطلق عليه صندوق جمع السائل المنوى.وكان يمسك الحمل الذى يظهر عليه أحد خصائص الجنس الذكرية الثانوية نحو الأنثى المأسورة ويؤخذ من عضوه الذكرى مسحة تحتوى على الإفرازات السائلة ثم توضع على شريحة ميكروسكوبية بدون صبغ وأخيرا تفحص تحت الميكروسكوب .وكان الحمل الذى يظهر فى عينته حيوانات منوية يدرب لجمع قذفات منوية باستخدم المهبل الصناعى. ولقد تم أخذ قذفة منوية واحدة /حيوان/أسبوع لتقدير خصائص الحيوانات المنوية (حجم السائل المنوى بالملى- درجة الحموضة (pH) - حركة الحيوانات المنوية - تركيز الحيوانات المنوية – نسبة الحيوانات المنوية الحية والميتة- شواذ الحيوانات المنوية الكلية.

ولقد وجد أن الكباش التى غذيت على عليقة احتوت على بروتين عالى وطاقة منخفضة قد وصلت إلى البلوغ الجنسى فى عمر مبكر (338.3 يوم) عن الكباش التى غذيت على عليقة احتوت على بروتين منخفض وطاقة عالية (349.0 يوم) وكذلك الكباش التى غذيت على عليقة احتوت على بروتين عالى وطاقة عالية (373.0 يوم) ثم التى غذيت على عليقة احتوت على بروتين منخفض وطاقة منخفضة (394.5 يوم). ولقد وجدت فروق معنوية ($P<0.05$) بين مستويات التغذية فى العمر عند البلوغ الجنسىوخصائص السائل المنوى. ولقد كانت جودة خضائص السائل المنوى أفضل فى موسمى الربيع والخريف بالمقارنة بموسمى الصيف والشتاء. ولقد وجد أن الكباش المغذاة على مستوى بروتين منخفض أو مستوى طاقة منخفض قد أدى إلى انتاج سائل منوى فقير فى القيمة الفسيولوجية بالمقارنة بالمعاملات الغذائية الأخرى. من التحليلات الإحصائية تبين أن الموسم له تأثير معنوى ($P<0.05$) على خصائص السائل المنوى. مما سبق يتضح أن تحقيق أفضل النتائج مع مراعاة البعد الإقتصادى يتوقف على جمع السائل المنوى من ذكور أغنام الصعيدى خلال فصل الخريف مع تقديم عليقة تحتوى على بروتين و 10TDN % زيادة عن احتياجات العليقة الحافظة التى احتوت على 14 % بروتين و 73 % TDN .

Table 3. Semen characteristics in Saidi ram lambs fed different levels of nutrition and their relationship to the effect of season

Item	Ejaculate volume (ml)	Mass motility	Sperm concentration /ml (x 10^9)	Live sperm %	Total abnormal sperms %	pH value
Summer	0.83±0.02^{b}	4.14±0.07^{b}	3.19±0.08^{b}	72.46±0.86^{b}	13.07±0.85^{a}	7.00±0.00^{a}
Autumn	0.95±0.04^{a}	4.41±0.06^{a}	3.93±0.08^{a}	75.73±0.70^{a}	8.53±0.44^{c}	7.00±0.00^{a}
Winter	0.75±0.02^{c}	3.94±0.09^{c}	3.45±0.13^{b}	68.11±1.02^{c}	14.09±0.60^{a}	6.97±0.00^{b}
Spring	0.91±0.03^{a}	4.27±0.09^{ab}	3.90±0.15^{a}	75.71±1.04^{a}	10.93±0.52^{b}	6.83±0.01^{c}
Control	0.53±0.02^{c}	3.37±0.09^{c}	2.57±0.12^{d}	60.89±1.15^{c}	18.88±0.75^{a}	6.96±0.01^{b}
HH	1.03±0.02^{a}	4.52±0.07^{a}	3.98±0.09^{b}	78.36±0.60^{a}	8.17±0.43^{c}	6.95±0.01^{c}
HL	0.82±0.02^{b}	4.58±0.06^{a}	4.35±0.10^{a}	78.75±0.54^{a}	9.76±0.44^{b}	6.93±0.01^{bc}
LH	1.08±0.03^{c}	4.32±0.07^{b}	3.69±0.13^{c}	74.43±0.67^{b}	9.18±0.37^{cb}	6.94±0.01^{a}
Summer x Control**	0.48±0.03	3.38±0.14	2.48±0.16	60.48±1.92	13.55±1.05	7.00±0.00
Sum.xHH**	1.03±0.03	4.44±0.12	3.48±0.13	77.72±1.05	5.56±0.93	7.00±0.00
Sum.xHL**	0.82±0.04	4.47±0.12	3.46±0.16	77.39±1.04	7.39±0.96	7.00±0.00
Sum.xLH**	0.92±0.04	4.11±0.14	3.19±0.18	71.92±1.28	5.92±0.48	7.00±0.00
Autumn x Control**	0.85±0.04	3.96±0.12	3.25±0.17	66.83±1.41	12.33±1.10	7.00±0.00

Table 3. Cont.

Item	Ejaculate volume (ml)	Mass motility	Sperm concentration /ml (x 10^9)	Live sperm %	Total abnormal sperms %	pH value
Aut.xHH**	1.13±0.05	4.7±0.08	4.20±0.11	81.54±1.08	6.60±0.71	7.00±0.00
Aut.xHL**	0.89±0.04	4.64±0.11	4.66±0.12	79.33±0.96	8.33±0.80	7.00±0.00
Aut.xLH**	1.21±0.10	4.31±0.13	3.62±0.18	75.21±1.14	6.83±0.61	7.00±0.00
Winter x Control**	0.45±0.03	2.98±0.17	2.28±0.25	53.48±2.60	22.14±1.13	6.97±0.01
WintxHH**	0.81±0.03	4.10±0.19	3.62±0.18	73.24±1.21	11.05±1.05	6.98±0.01
WintxHL**	0.69±0.02	4.36±0.16	4.51±0.19	73.61±0.82	12.95±0.85	6.97±0.01
WintxLH**	1.05±0.05	4.34±0.14	3.38±0.26	72.43±1.02	10.09±0.74	6.97±0.01
Spring x Control**	0.58±0.05	3.14±0.19	2.20±0.26	62.00±2.41	17.10±1.08	6.89±0.01
Spr.xHH**	1.15±0.05	4.83±0.08	4.63±0.21	80.72±0.93	7.03±0.75	6.80±0.00
Spr.x HL**	0.87±0.02	4.79±0.07	4.55±0.24	83.90±0.88	7.69±0.68	6.80±0.01
Spr.x LH**	1.08±0.04	4.46±0.15	4.40±0.30	77.46±1.61	10.94±0.80	6.82±0.01

HH: high protein/high energy group, HL: High protein/low energy group and LH: low protein /high energy group.
[a b c and d] are significantly differences at ** p (<0.01)