World Rabbit Sci. 2015, 23: 111-120
doi:10.4995/wrs.2015.3464

SEMEN CHARACTERISTICS, SEXUAL HORMONES AND LIBIDO OF HY-PLUS RABBIT BUCKS INFLUENCED BY A DIETARY MULTI-ENZYME ADDITIVE

GADO H.*, MELLADO M.†, SALEM A.Z.M.‡, ZARAGOZA A.#, SELEEM T.S.T.§

*Department of Animal Production, Faculty of Agriculture, Ain Shams University, Cairo, Egypt
†Department of Animal Nutrition, Autonomous Agrarian University Antonio Narro, 25315 Saltillo, Mexico.
‡Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Toluca, Mexico.
#Instituto de Ciencias Agropecuarias, Área Académica de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de Hidalgo, Tulancingo, México.
§Animal Production Research Institute, Agricultural Research Centre, Dokki, Giza, Egypt.

Abstract: A total of 144 adult Hy-Plus rabbit bucks were randomly assigned into 4 treatments of 36 replicates each, in a completely randomised design. Animals were fed *ad libitum* on a basal diet supplemented with a multi-enzyme complex (EZ, including cellulases, xylanases, protease and α-amylase) at 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg/ton of feed. Total sperm count was higher ($P<0.05$) in EZ5 than in EZ0. Sperm motility increased with increasing levels of the EZ additive, being lowest for EZ0 and highest for EZ5 rabbits ($P<0.05$). Percentage of dead sperm was higher ($P<0.05$) in EZ0 than in EZ5 rabbits. Rabbit bucks that received the highest level of EZ in their diet had the shortest reaction time (22.31±3.17 s; $P<0.05$), whereas EZ5 bucks took 43.56±5.89 s to mount does for the first time after exposure. Blood testosterone, 17α-estradiol and progesterone levels were highest in EZ5 rabbits and lowest in EZ0 rabbits. Enzyme addition increased ($P<0.05$) sperm transit in estrus doe cervical mucus. Data suggested that the EZ additive in diets of adult Hy-Plus rabbit bucks was effective to improve both semen characteristics and sexual drive.

Key Words: enzyme, rabbit, semen.

INTRODUCTION

Rabbits have an important role in the supply of animal protein for humans. Rabbit occupies a vital midway position between ruminants and non-ruminant animals and can effectively utilise cellulose-rich feed in rations containing less than 20% grain (Saleh *et al.*, 2010). Rabbit digestive system is suitable for high cellulose diets (Abdel-Aziz *et al.*, 2014, 2015). Simple biological characteristics, short breeding cycle, high prolificacy and high feed conversion efficiency place rabbit just below poultry in term of feed efficiency (Hasanat *et al.*, 2006).

Enzymes are organic catalysts which are used as feed additive in non-ruminant animal diets. A variety of enzymes have been found to improve the absorption of nutrients in the intestines and the nutritive value of diets for non-ruminant animals (Bedford and Morgan, 1996; Abdel-Aziz *et al.*, 2014, 2015). The beneficial effects of adding enzymes have been attributed to a reduction in viscosity of digesta in the intestine, which results from arabinoxylans; the non starch polysaccharides present in the endosperm cell walls and which represent 70% of the total non starch polysaccharides in wheat (Zijstra *et al.*, 1999).

The addition of cellulolytic enzymes to rabbit diets has a pronounced beneficial effect on weight gain both in cage-reared and backyard-raised rabbits (Chandra *et al.*, 2014). Given that digestive capability of fibre and starch in young rabbits is limited (Marounek *et al.*, 1995; Abdel-Aziz *et al.*, 2014, 2015), enzyme addition improved the dietary digestion and

Correspondence: A.Z.M. Salem, asalem70@yahoo.com. Received *December 2014* - Accepted *April 2015.*
http://dx.doi.org/10.4995/wrs.2015.3464

performance of young rabbits on starter diets (Abdel-Aziz *et al.*, 2014, 2015; Gutierrez *et al.*, 2002). This response to enzyme addition has also been observed in 30-d old rabbits weaned at 25 d (Fernandez *et al.*, 1996).

The mode of action of enzymes on different segments of the rabbit gut has been addressed by several researchers. Sequeira *et al.* (2000) detected a lowering of gastric pH; however, an enzyme complex composed of amylase, xylanase, β-glucanase and pectinase did not affect the digestive parameters measured. Exogenous enzymes frequently fail to significantly affect enzyme activities in the gastric, intestinal and caecal contents (Sequeira *et al.*, 2000), even in the period following early weaning (Falcão-e-Cunha *et al.*, 2007).

Table 1: Feed ingredients and chemical composition of the pellet ration fed to rabbit bucks during the experimental period (manufactured by IBEX International Co. L.®).

Ingredients (%)	
Clover hay	40.50
Wheat bran	25.00
Yellow corn	14.00
Soybean meal (44% protein)	11.00
Molasses	3.00
Vines	3.00
Bone meal	1.75
Limestone	0.70
Sodium chloride	0.55
Vitamins and mineral premix	0.35
DL-Methionine	0.15
Chemical composition*	
Crude protein (%)	18.00
Ether extract (%)	3.00
Crude fibre (%)	14.00
Digestible energy (kcal/kg)	2720.00
Vitamins and minerals premix per kg	
Vit. A (I.U.)	10000
Vit. D3 (I.U.)	9000
Vit. E (I.U.)	10000
Vit. K (I.U.)	3
Vit. B1 (I.U.)	2
Vit. B2 (I.U.)	6
Vit. B6 (I.U.)	2
Biotin (mg)	0.2
Choline (mg)	1200
Niacin (mg)	40
Zn (mg)	60
Cu (mg)	0.1
Mn (mg)	85
Fe (mg)	75
Folic acid (mg)	5
Pantothenic acid (mg)	20

* Calculated according to NRC (1984) for rabbits.

The enzyme preparation product of ZADO® is a biotechnological product derived from anaerobic bacteria rich in cellulolytic enzymes (Salem *et al.*, 2012, 2013; Valdes *et al.*, 2015). The main actions of this product are on rumen kinetics and improvements in how effectively the rumen microflora can utilise feed ingredients, and should be reflected in the animal's performance in terms of either milk or meat production (Gado *et al.*, 2009; Salem *et al.*, 2013). This enzyme preparation improved ruminal fermentation, feed intake, nutrient digestibility, milk production and live weight gain (Gado *et al.*, 2009, 2011; Salem *et al.*, 2013). However, the aim of this study was to evaluate the effect of this product as dietary addition at different levels in adult rabbit buck diets on semen characteristics, libido and blood sexual hormones concentration.

MATERIAL AND METHODS

Rabbits and General Management

The present study was carried out in a commercial rabbitry (capacity 300 does), near El-Khatatbah city, Egypt, from October 2012 until March 2013. The laboratory work was conducted in the Animal Production Research Institute, Agriculture Research Centre, Giza, Egypt. The climate in the study area is a hot desert climate with summer rains with an annual average rainfall of 22 mm and a mean annual temperature between 14 and 28°C. Rabbits were raised in a semi-closed rabbitry with wire-netted windows on their sides for natural ventilation. The windows were 2 m above the floor.

Diet Characteristics

One hundred and forty-four sexually mature Hy-Plus rabbit bucks were randomly divided into 4 equal experimental groups of 36 each. The first group did not receive the feed enzyme additive (control- EZ0) and were offered a commercial breeder standard diet *ad libitum* to satisfy or exceed the nutritional recommendations of NRC (1984) -Table 1. Rabbits in groups 2, 3 and 4 were fed the same control diet but were offered an EZ preparation of ZADO® at 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg/ton. The diet

ingredients and chemical composition of the pelleted ration offered to rabbits are shown in Table 1. Diets were offered twice per day at 8:00 and 16:00 h. Feed and water were offered *ad libitum.*

The enzyme (EZ) product of ZADO® is an enzyme preparation obtained from *Ruminococcus flavefaciens*, recently developed by the laboratory of Rumen Ecology Centre, Animal Production Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The EZ product is a powdered multi-mix of cellulases, xylanases, proteases and α-amylase enzymes, in addition to the related anaerobic bacteria which produce these enzymes, coated with starch and glycol. The product EZ complex was spread directly onto the feed before offering it to the rabbits and was active immediately after feeding (Abdel-Aziz *et al.*, 2014, 2015).

Enzyme activities in the enzyme preparation were determined for endoglucanase (7.1 U/g; Robyt and Whelan, 1972), α-*amylase* (61.5 U/g; Bernfeld, 1955), protease (29.2 U/g; Lin *et al.* 1969), and xylanase (2.3 U/g; Robyt and Whelan, 1972) activities by catalysing hydrolysis of xylan from oat spelt: the reducing groups liberated were determined using alkaline copper reagent.

Reaction Time and Mounting Activity of Rabbit Bucks

Reaction time in seconds was recorded from the time of introducing the doe to the buck until mounting (Seleem, 2003). The number of ejaculations in 15 min was also recorded for rabbit bucks.

Semen Collection, Evaluation and Preservation

Total semen was collected from all rabbits artificially, twice a week for up to 5 wk, by means of an artificial vagina maintained at 45°C and a teaser doe, as described by Seleem (2003). Samples ejaculated from each rabbit buck were evaluated individually microscopically before semen ejaculate volume was determined (semen was transferred to graduate vial for volume determination to the nearest 0.1 mL, after removal of the gel mass).

Mass motility and forward moving sperm (%) were examined at 37°C under a microscope with phase-contrast optics, at 40×, and assessed from 0 to 100%. Percentage of dead sperm cells and sperm cell abnormalities were determined using an eosin-nigrosin blue-staining mixture. Sperm-cell concentration ($N\times10^6$/mL) and total-sperm output ($N\times10^6$/ejaculate) were determined counting the cells for the evaluation of sperm concentration according to Smith and Mayer (1955), using the improved Neubauer haemocytometer slide. Acrosomal integrity was determined by using a Giemsa stain procedure as described by Watson (1975).

Semen samples ejaculated within each experimental group were pooled and diluted with sodium citrate diluents (2.9 g di-sodium citrate+1.25 lactose+0.04 citric acid anhydrous+5 mL egg yolk+50000 I.U. sodium penicillin+50000 µg streptomycin sulphate/100 mL distilled water) at 1:5 dilution rates. Each semen sample was divided into 2 portions; the first was incubated at 37°C for up to 4 h and the second was kept at 4-6°C for 3 d. After each preservation period, the treated diluted semen samples were centrifuged at 6000 *g* for 20 min before removal of the supernatant which was used for the enzymatic assay. Activities of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) enzymes were determined according to Reitman and Frankel (1957). Acid phosphatase (ACP) and alkaline phosphatase (ALP) enzymes were determined calorimetrically according to Graham and Pace (1967).

Sperm Penetrability Assay

Sperm penetration into estrus doe cervical mucus of extended Hy-Plus rabbit buck semen was assessed during incubation condition at 37°C for 4 h, as described by Seleem (2003).

Blood Collection and Analysis of Hormones and Enzymes

Blood samples from all rabbit bucks were taken in less than 2 min from the marginal ear vein of 6 rabbit bucks within each experimental group twice monthly. Blood samples were collected using a stainless steel needle, into heparinised tubes which were centrifuged at 3000 *g* for 20 min and kept in deep freeze (–20°C) until further analysis of sexual hormone levels. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in rabbit bucks' semen were estimated according to Reitman and Frankel (1957).

Blood serum testosterone, 17-α estradiol and progesterone concentration were determined using RIA Kits (Immunotech, A Coulter Co., France) according to the manufacturer procedures.

Statistical Analyses

Data were subjected to analysis of variance of completely randomised design according to Snedecor and Cochran (1982) using the General Linear Model procedure of SAS (2001). Data were analysed using the following linear model:

$$Y_{ij} = \mu + B_i + e_{ij}$$

Where: Y_{ij}=Observation on i^{th} animal, μ=overall mean; B_i=effect of i^{th} treated group (i=1 to 4) and e_{ij}=random error.

Percentage values were transformed to Arc-Sin values to approximate normal distribution before statistical analysis. Comparisons between individual treatments were tested using Duncan's new multiple range tests by the SAS program at a significance level of $P<0.05$.

RESULTS

Control rabbits (i.e., EZ0) had the longest reaction time ($P<0.05$), most dead spermatozoa, acrosomal damage and sperm abnormalities compared to the other rabbit groups. Rabbit bucks that received the highest dietary enzyme (i.e., EZ5) presented the fastest ($P<0.05$) mountings in 15 minutes, whereas the EZ0 rabbit bucks showed the shortest mounting activity. The volume of ejaculate, sperm mass motility and forward moving sperm increased ($P<0.05$) as the ENZ levels in rabbit diet were increased. Sperm cell concentration and total-sperm output were increased ($P<0.05$) compared to the control, following the EZ rabbits (Table 2).

Addition of EZ reduced the number of abnormal sperm and sperm with damaged acrosome ($P<0.05$). Rabbit bucks fed the EZ diets had the highest sperm cell concentration per mL and total spermatozoa output compared with the EZ0 rabbits ($P<0.01$); however, the difference between the EZ groups was not as large as that of the EZ0 rabbits.

Rabbit bucks fed diets with EZ presented lower ($P<0.05$) concentrations of semen GOT, GPT, acid phosphates and alkaline phosphates than EZ0 bucks (Table 3).

At any incubation period of diluted semen, the spermatozoa from EZ rabbit bucks had the greatest penetration through cervical mucus compared with the EZ0 rabbits. The bucks receiving the highest level of the enzyme (EZ5) presented the highest ($P<0.05$) penetrability compared with sperm from rabbit bucks receiving EZ at lower doses (Table 4).

Table 2: Reaction time, mating frequency and physical semen characteristics of Hy-Plus rabbit bucks as affected by enzyme additive to diets (means±standard error).

	Dietary enzyme levels[1]			
	EZ0	EZ1	EZ3	EZ5
No. of rabbits	36	36	36	36
Reaction time (s)	43.56±5.89^{c}	32.64±4.18^{b}	25.17±3.46^{ab}	22.31±3.17^{a}
Frequency of mating in 15 minutes	2.43± 0.067^{a}	2.98± 0.032^{b}	3.69± 0.083^{c}	3.80±0.014^{d}
Semen ejaculate volume (mL)	0.44±0.14^{a}	0.61±0.12^{ab}	0.76±0.11^{bc}	0.89±0.13^{c}
Mass motility, score	2.98±0.03^{a}	3.26±0.06^{b}	4.41±0.04^{c}	4.45±0.06^{c}
Forward moving sperm (%)	56.12±1.47^{a}	68.52±2.01^{b}	73.18±1.98^{c}	76.24±2.21^{c}
Dead spermatozoa (%)	23.24±2.16^{c}	18.42±1.19^{b}	15.05±1.07^{a}	14.34±1.11^{a}
Sperm abnormalities (%)	21.28±1.98^{a}	17.89±1.87^{b}	13.91±1.00^{c}	11.82±1.04^{d}
Acrosomal damages (%)	17.62±1.72^{c}	14.71±1.33^{b}	12.99±1.43^{b}	10.15±1.28^{a}
Sperm-cell concentration (N×10^{6}/mL)	415±31^{a}	482±28^{b}	562±38^{c}	597±37^{c}
Total-sperm output (N×10^{6}/ejaculate)	182.4±19.5^{a}	294.3±22.7^{b}	426.9±31.9^{c}	531.9±34.0^{d}

^{abc}Means bearing different letter superscripts within the same row are significantly ($P<0.05$) different.
[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ton of diet.

Table 3: Enzymatic semen activity of Hy-Plus rabbit bucks as affected by enzyme additive to diets (means±standard error).

	Dietary enzyme levels[1]			
	EZ0	EZ1	EZ3	EZ5
No. of rabbits	36	36	36	36
Glutamic-oxaloacetic transaminase (I.U./L)	30.91±2.08^{c}	28.16±1.97^{bc}	24.71±1.22^{ab}	22.98±0.65^{a}
Glutamic-pyruvic transaminase (I.U./L)	21.37±1.00^{c}	19.22±1.04^{b}	18.02±0.61^{ab}	16.99±0.92^{a}
Acid phosphates (I.U./L)	28.41±1.11^{c}	25.32±1.36^{b}	21.19±1.21^{a}	20.37±0.99^{a}
Alkaline phosphates (I.U./L)	37.81±1.67^{c}	33.14±1.47^{b}	32.11±1.14^{b}	28.91±1.22^{a}

^{abc}Means with different superscripts within row are significantly different (P<0.05).

[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ ton of diet.

Incubation of semen at 37°C for up to 6 h affected semen quality. The highest effects (P<0.05) in semen variables were observed with advancing incubation periods. Addition of EZ in rabbit diets increased (P<0.05) both percentage of forward moving sperm and percentage of storage ability (Table 5). When semen of rabbit bucks was chilled, semen of the EZ0 rabbits had the highest (P<0.05) percentage values of dead spermatozoa, sperm abnormalities and acrosomal damage. These effects were higher (P<0.05) as chilled storage advanced (Table 6).

The concentration of different measured sexual hormones including testosterone, estradiol 17-α, and progesterone were increased (P<0.05) in EZ rabbit bucks compared with EZ0 (Table 7).

DISCUSSION

Good sex drive of rabbit bucks and high quality semen are required year-round to achieve maximum productivity and libido, either through artificial insemination (Rodríguez-De Lara *et al.*, 2008) or natural mating (Saleh *et al.*, 2010). In the present study, the EZ0 rabbit bucks presented a lower sexual drive than the EZ-supplemented rabbit bucks, coincident with a linear increase in blood testosterone levels with ascending levels of the diet EZ additive. The reaction time of rabbit bucks was much longer than the 4.2 s observed by Ogbuewu *et al.* (2009) with New Zealand white×Chinchilla rabbit bucks and 14-21 s reported by Safaa *et al.* (2008) in Black Baladi and White New Zealand rabbit bucks. This may be due to the testosterone and estradiol, which act synergistically to stimulate male sexual behaviour (Cross and Roselli, 1999). However, estradiol may be stimulated by chemo investigation, frequency of mountings and reduced mount latency, while testosterone acts in part through *in situ* conversion to estradiol by aromatase in the preoptic area stimulating mounting, ultimately improving the copulatory behaviour. The slightly higher blood levels of testosterone in EZ-supplemented rabbit bucks apparently enhanced reaction time, and confirmed that decreased testosterone concentration was linked to low sexual drive (Zeidan *et al.*, 1997).

The blood testosterone concentration in EZ -supplemented groups was higher than in the EZ0 rabbit bucks. The beneficial effect of the exogenous EZ additive might be due to a stimulatory effect of nutrients made available to the

Table 4: Spermatozoa penetration into estrus doe estrous cervical mucus (mm/h) as affected by the addition of enzyme additive to diets of Hy-Plus rabbit bucks (means±standard error).

	Dietary enzyme levels[1]			
	EZ0	EZ1	EZ3	EZ5
No. of rabbits	36	36	36	36
Incubation period of diluted semen (h)				
1.5	19.62±1.07^{a}	20.71±1.21^{a}	24.84±1.15^{b}	25.13±1.42^{b}
3.0	29.14±2.76^{a}	33.98±1.92^{b}	39.81±1.68^{c}	45.08±1.87^{d}
4.5	35.21±2.11^{a}	40.34±2.44^{b}	49.19±3.57^{c}	57.18±2.76^{d}
6.0	39.93±2.46^{a}	45.74±2.17^{b}	56.27±3.01^{c}	64.87±3.44^{d}

^{abc}Means bearing different letter superscripts within the same row are s'gnificantly (P<0.05) different.

[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ton of diet.

Table 5: Semen quality of Hy-Plus rabbit bucks, during incubation condition at 37°C for up to 6 h as affected by levels of a dietary enzyme supplement (means±SE).

Item	Incubation period (h)	Dietary enzyme levels[1]			
		EZ0	EZ1	EZ3	EZ5
No. of rabbits	36	36	36	36	36
Forward moving sperm (%)	0.0	55.82±2.54^{a}	66.29±3.94^{b}	70.75±1.98^{bc}	72.78±2.18^{c}
	2.0	50.23±2.86^{a}	63.86±3.72^{b}	68.22±3.91^{bc}	71.62±2.44^{c}
	4.0	42.12±2.34^{a}	57.26±2.86^{b}	64.48±3.86^{bc}	70.16±2.98^{c}
	6.0	32.77±1.99^{a}	48.96±2.93^{b}	58.06±4.12^{c}	65.41±2.89^{d}
Means±SE		45.24±2.34^{a}	59.09±2.19^{b}	65.38±2.99^{bc}	69.99±2.81^{c}
Storage ability (%)		58.71±3.34^{a}	73.86±3.73^{b}	82.06±2.22^{c}	89.87±3.87^{d}
Dead spermatozoa (%)	0.0	25.41±2.87^{c}	19.78±1.26^{b}	16.84±1.54^{a}	15.73±1.98^{a}
	2.0	27.34±2.56^{c}	21.12±1.41^{b}	17.87±1.38^{a}	16.63±2.17^{a}
	4.0	30.76±2.31^{c}	23.29±1.52^{b}	19.15±2.11^{a}	18.11±2.01^{a}
	6.0	36.12±2.60^{c}	27.27±2.11^{b}	22.11±1.96^{a}	20.35±2.31^{a}
Means±SE		29.91±2.43^{c}	22.87±1.98^{b}	18.99±2.01^{bc}	17.71±1.87^{c}
Sperm abnormalities (%)	0.0	23.36±1.62^{c}	19.14±1.54^{b}	15.13±1.44^{a}	12.98±1.62^{a}
	2.0	23.99±1.54^{c}	20.37±1.73^{b}	15.32±1.57^{a}	13.23±1.50^{a}
	4.0	24.41±1.81^{c}	21.11±1.68^{c}	16.24±1.50^{b}	13.73±1.34^{a}
	6.0	26.01±1.82^{c}	22.71±1.74^{c}	17.12±1.65^{a}	14.23±1.64^{a}
Means±SE		24.44±1.65^{c}	20.83±1.61^{b}	15.95±1.52^{a}	13.54±1.63^{a}
Acrosomal damages (%)	0.0	18.46±1.08^{c}	15.90±1.65^{bc}	14.02±1.87^{ab}	11.43±1.45^{a}
	2.0	19.99±1.17^{c}	17.01±1.81^{bc}	14.95±1.76^{ab}	11.61±1.87^{a}
	4.0	20.64±1.49^{c}	18.31±1.77^{bc}	14.96±1.79^{ab}	12.01±1.84^{a}
	6.0	22.12±1.23^{b}	20.12±2.04^{b}	16.03±1.88^{a}	12.77±1.97^{a}
Means±SE		20.30±1.79^{c}	17.83±1.98^{bc}	14.99±1.86^{ab}	11.96±1.93^{a}

^{abc}Means bearing different letter superscripts within the same row are significantly ($P<0.05$) different.
[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ton of diet. SE: standard error.

animal on testicular steroidogenesis, as improved nutrition enhances testicular functions, stimulating testosterone synthesis (Attia and Kamel, 2012).

All the semen quality variables followed an upward trend as the inclusion rate of the rabbit's EZ increased. Adding the EZ to rabbit buck diets markedly increased sperm concentrations and total sperm counts. This indicates that diets containing the enzymatic complex had a marked effect on the spermatogenesis process. However, exogenous fibrolytic enzymes included in the feed have been shown to improve fibre digestion by cellulolytic ruminal bacteria (Wang *et al.*, 2012), microbial growth and production of microbial protein (Gado *et al.*, 2009). These actions could have improved the nutrition status of rabbit bucks receiving the enzyme preparation. Moreover, the enzyme preparation in diets could have improved intestinal mucosal development, which enhances nutrient digestibility, which in turn would promote nourishment of the sertoli cells and seminal fluid that nurse the germ cells. Other researchers have documented remarkably high improvement in semen volume and total sperm cells in the ejaculate of rabbit bucks, like that observed in the present study, with subtle changes in the diet, such as the inclusion of prebiotics (Ewuola, 2013) or antioxidants (Castellini *et al.*, 2003).

There is a strong correlation between animal nutrition and spermatogenesis, sperm maturation and male reproductive system development (Cheah and Yang, 2011). However, apparently the EZ addition to rabbit diets promoted the availability of macronutrients and micronutrients caused by the action of proteases, amylases and cellulases required for the synthesis of diverse components of the spermatozoa. This may be because the improved nutrient availability with the exogenous EZ supplied enhanced the endocrine activity of rabbit buck gonads, which created a supportive

Table 6: Semen characteristics of Hy-Plus rabbit bucks, during chilled storage at 4-6°C for up to 3 d, as affected by the addition of different levels of an enzyme additive in diets (means±SE).

	Chilled storage	Dietary enzyme levels[1]			
	(d)	EZ0	EZ1	EZ3	EZ5
No. of rabbits		36	36	36	36
Forward moving sperm (%)	0.0	54.31±2.75^{a}	65.12±3.24^{b}	69.85±2.12^{bc}	71.93±2.44^{c}
	1.0	49.17±2.54^{a}	62.99±3.44^{b}	67.98±3.99^{bc}	70.45±2.78^{c}
	2.0	40.87±2.58^{a}	55.43±3.18^{b}	62.45±3.48^{c}	68.23±3.41^{d}
	3.0	30.45±1.99^{a}	46.71±3.14^{b}	55.28±3.99^{c}	62.17±3.37^{d}
Means±SE		43.70±2.18^{a}	57.56±2.22^{b}	63.89±3.41^{c}	68.20±2.99^{c}
Storage ability (%)		56.07±3.92^{a}	71.73±3.22^{b}	79.14±3.61^{c}	86.43±3.22^{d}
Dead spermatozoa (%)	0.0	25.97±2.07^{c}	20.21±1.12^{b}	17.14±1.72^{a}	16.93±1.33^{a}
	1.0	28.04±2.84^{c}	21.63±1.91^{b}	18.73±1.19^{ab}	17.21±1.98^{a}
	2.0	32.14±2.11^{c}	23.88±1.34^{b}	19.84±2.76^{a}	18.59±1.88^{a}
	3.0	37.19±2.12^{c}	28.11±2.24^{b}	22.87±1.44^{a}	20.67±2.56^{a}
Means±SE		30.84±2.57^{c}	23.46±1.46^{b}	19.65±1.94^{a}	18.35±1.92^{a}
Sperm abnormalities (%)	0.0	23.41±1.39^{c}	19.41±1.33^{b}	15.38±1.71^{a}	13.43±1.82^{a}
	1.0	24.56±1.68^{c}	20.91±1.90^{b}	15.87±1.82^{a}	13.82±1.49^{a}
	2.0	24.99±1.72^{b}	21.68±1.71^{b}	17.26±1.49^{a}	14.68±1.69^{a}
	3.0	25.13±1.84^{c}	24.15±1.99^{c}	17.71±1.93^{b}	14.59±1.84^{a}
Means±SE		24.52±1.78^{b}	21.54±1.52^{b}	16.56±1.96^{a}	14.13±1.84^{a}
Acrosomal damages (%)	0.0	18.82±1.01^{c}	16.37±1.81^{bc}	14.43±1.73^{ab}	11.48±1.36^{a}
	1.0	20.34±1.29^{c}	17.54±1.42^{bc}	14.91±1.71^{ab}	11.70±1.84^{a}
	2.0	21.48±1.53^{c}	18.48±1.40^{b}	14.99±1.71^{a}	12.19±1.67^{a}
	3.0	22.63±1.54^{c}	20.81±1.95^{c}	16.38±1.19^{b}	12.91±1.48^{a}
Means±SE		20.82±1.81^{c}	18.30±1.72^{bc}	15.18±1.73^{ab}	12.07±1.96^{a}

^{abc}Means bearing different letter superscripts within the same row are significantly ($P<0.05$) different.

[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ton of diet. SE: standard error.

environment for spermatogenesis. Given that duration of spermatogenesis process in rabbit bucks takes about 47 d (Boiti *et al*., 2005), the beneficial effect of the EZ complex dietary addition on semen characteristics was expressed in the long term. These results reveal higher values of ejaculate volume in EZ rabbits *vs.* EZ0 rabbit bucks. The ejaculate volume observed in the rabbit bucks that received the highest EZ level (EZ5) was much higher than values observed by other researchers (Paál *et al*., 2014). No published reports were available in the literature on the effect of exogenous EZ addition in diets on semen volume in rabbit bucks. The commercial EZ used in the current study probably stimulated development of accessory sex glands, enhancing semen volume.

Table 7: Blood sexual hormone concentrations of Hy-Plus rabbit bucks as affected by levels of an enzyme complex added to diets (means±standard error).

	Dietary enzyme levels[1]			
	EZ0	EZ1	EZ3	EZ5
No. of rabbits	36	36	36	36
Testosterone (ng/mL)	5.74±0.01^{a}	5.86±0.01^{b}	5.99±0.00^{c}	6.15±0.00^{d}
Estradiol 17α (pg/mL)	28.61±1.87^{a}	32.54±1.11^{b}	36.72±1.52^{c}	38.65±2.23^{c}
Progesterone (pg/mL)	0.67±0.01^{a}	0.74±0.03^{b}	0.79±0.03^{ab}	0.82±0.03^{a}

^{abc}Means bearing different letter superscripts within the same row are significantly ($P<0.05$) different.

[1]Diet supplemented with 0 (EZ0), 1 (EZ1), 3 (EZ3) and 5 (EZ5) kg enzyme complex/ ton of diet.

Addition of EZ improved sperm motility and reduced abnormal sperm and dead spermatozoa, which suggests that dietary manipulation using an EZ additive improved the proliferation of high quality sperm cells. This response could be due to nutritional influences on reproduction, including the cellular processes within seminiferous tubules in the testis and the neuroendocrine pathways through which nutritional inputs affect the brain centres that control reproduction (Martin *et al.*, 2004).

In vitro oestrus cervical mucus penetration test provides a good assessment for fertility of rabbit bucks. The maximum cervical mucus sperm penetration distance value was observed by sperm of animals that received the highest EZ levels (i.e., EZ5) in their diets, while the lowest penetration distance was observed in EZ0 rabbit bucks. As no report is available in the literature on the effect of dietary addition of EZ complexes, the present results could not be compared.

In farm animals, diverse studies reveal a good correlation between several sperm quality parameters (positive: progressive motility and velocity according to the straight path; negative: damaged acrosomes and apoptotic cells and scores of penetration tests (Richardson *et al.*, 2011; Martínez-Rodríguez *et al.*, 2012). The increased level of the EZ complex in the present study seems to enhance sperm migration through cervical mucus.

Semen preservation is still a limiting factor for extensive commercial application programs in rabbits (López-Gatius *et al.*, 2005). Sperm cells form EZ rabbit bucks presented a greater semen quality after 6 h of incubation at 37°C, or after 3 d of storage at 4-6°C. Rabbit sperm survival drastically decreased after 36 h and its fertilising capacity tends to diminish after about 16 h of storage (Carluccio *et al.*, 2004). In the present study, the semen from rabbit bucks receiving the highest level of the EZ preparation presented higher motility than semen from other EZ groups, after 3 d of refrigeration, which is not in line with other studies (Carluccio *et al.*, 2004). This higher sperm motility promoted by the EZ preparation could be due to a greater nutrient availability for rabbit bucks, which was reflected in greater vitality of sperm cells. Other researchers have documented the positive impact of improved nutrition of sperm motility in other mammals (Contri *et al.*, 2011).

These data are of potentially great public interest, because they indicate that the use of the EZ complex in rabbit buck diets would facilitate the preservation of liquid semen at 5°C for several days, or several hours at ambient temperature. This is an important step for the breeding management of rabbit bucks.

The mean GOT and GPT activity in seminal plasma of different groups of rabbit bucks revealed lower values in EZ-supplemented groups *vs.* EZ0 rabbits. This is mainly attributed to a membrane stabilising action of nutrients made available by the EZ complex, which apparently caused lesser release of these enzymes in seminal plasma. Leakage of these enzymes is used as a good indicator of semen quality because they measure plasma membrane stability of spermatozoa (Juyena and Stelletta, 2012). The possible source of these enzymes is thought to be the testes or epididymides because they show a positive correlation with sperm concentration and a negative correlation with semen volume (Kareskoski and Katila, 2008).

CONCLUSIONS

Dietary manipulation using the enzyme additive provides an easily applicable measure for improving sexual activity and spermatozoa production and quality of Hy-Plus rabbit bucks. Addition of enzyme could facilitate the preservation of liquid semen and improve rabbit buck breeding management in large-scale rabbit production farms.

REFERENCES

Abdel-Aziz N., El-Adawy M., Mariezcurrena-Berasain M.A., Salem A.Z.M., Olivares-Pérez J., Kholif A.E., Borhami B.E. 2015. Effects of exogenous enzymes, *Lactobacillus acidophilus* or their combination on feed performance response and carcass characteristics of rabbits fed sugarcane bagasse. *J. Integr. Agric., 14: 544-549. doi:10.1016/S2095-3119(14)60827-3*

Abdel-Aziz N., El-Adawy M., Salem A.Z.M., Cerrillo-Soto M.A., Camacho L.M., Borhami B.E. 2014. Effects of exogenous enzymes, *Lactobacillus acidophilus* or their combination on feed intake, digestibility and performance of rabbits fed sugarcane bagasse. *Anim. Nutr. Feed Technol., 14: 137-145.*

Attia Y.A., Kamel K.I. 2012. Semen quality, testosterone, seminal plasma biochemical and antioxidant profiles of rabbit bucks fed diets supplemented with different concentrations of soybean lecithin. *Animal, 6: 824-833. doi:10.1017/S1751731111002229*

Bedford M.R., Morgan A.J. 1996. The use of enzymes in poultry diets. *World's Poult. Sci. J., 52: 61-68. doi:10.1079/WPS19960007*

Bernfeld P. 1955. Amylases, alpha and beta. *Method. Enzymol., 1: 149-158.*

Boiti C., Castellini C., Besenfelder U., Theau-Clément M., Liguori L., Renieri T., Pizzi F. 2005. Guidelines for the handling of rabbit bucks and semen. *World Rabbit Sci., 13: 71-91. doi:10.4995/wrs.2005.527*

Carluccio A., Robbe D., De Amicis I., Contri A., Tosi U., Russo F., Paoletti M. 2004. Artificial insemination in rabbits: laboratory and field trial with three different semen extenders. *World Rabbit Sci., 12: 65-79. doi:10.4995/wrs.2004.580*

Castellini C., Lattaioli P., Bosco A.D., Minelli A., Mugnai C. 2003. Oxidative status and semen characteristics of rabbit buck as affected by dietary vitamin E, C and n-3 fatty acids. *Reprod. Nutr. Dev., 43: 91-103. doi:10.1051/rnd:2003008*

Chandra S., Mahender M., Prakash M.G., Raghunandan T., Reddy K.K. 2014. Productive performance of broiler rabbits fed diets supplemented with probiotic and enzymes under two systems of housing. *Indian J. Anim. Res., 48: 355-361. doi:10.5958/0976-0555.2014.00455.5*

Cheah Y., Yang W. 2011. Functions of essential nutrition for high quality spermatogenesis. *Adv. Biosci. Biotechnol., 2: 182-197. doi:10.4236/abb.2011.24029*

Contri A., De Amicis I., Molinari A., Faustini M., Gramenzi A., Robbe D., Carluccio A. 2011. Effect of dietary antioxidant supplementation on fresh semen quality in stallion. *Theriogenology, 75: 1319-1326. doi:10.1016/j.theriogenology.2010.12.003*

Cross E., Roselli C.E. 1999. 17 beta-estradiol rapidly facilitates chemo investigation and mounting in castrated male rats. *Amer. J. Physiol., 226: 497-509.*

Ewuola E.O. 2013. Daily sperm production, gonadal and extra-gonadal sperm reserves of rabbits fed prebiotic and probiotic supplemented diets. *Int. J. Appl. Agric. Apic. Res., 9: 48-53.*

Falcão-e-Cunha L., Castro-Solla L., Maertens L., Marounek M., Pinheiro V., Freire J., Mourao J.L. 2007. Alternatives to antibiotics growth promoters in rabbit feeding: a review. *World Rabbit Sci., 15: 127-140. doi:10.4995/wrs.2007.597*

Fernández C., Merino J.M., Carabaño R. 1996. Effect of enzyme complex supplementation on diet digestibility and growth performance in growing rabbits. *In: Proceedings of the Sixth World Rabbit Congress, 9-12 July, 1996, Toulouse, France, Vol. 1: 163-166.*

Gado H.M., Salem A.Z.M., Robinson P.H., Hassan, M. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Anim. Feed Sci. Technol., 154: 36-46. doi:10.1016/j.anifeedsci.2009.07.006*

Gado H.M., Salem A.Z.M., Odongo N.E., Borhami B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Anim. Feed Sci. Technol., 165: 131-136. doi:10.1016/j.anifeedsci.2011.02.016*

Graham E.F., Pace M.M. 1967. Some biochemical changes in spermatozoa due to freezing. *Cryobiology, 4: 75-84. doi:10.1016/S0011-2240(67)80214-1*

Gutiérrez I., Espinosa A., García J., Carabaño R., De Blas C. 2002. Effect of levels of starch, fiber and lactose on digestion and growth performance of early-weaned rabbits. *J. Anim. Sci., 80: 1029-1037.*

Hasanat M.S., Hossain M.E., Mostari M.P., Hossain M.A. 2006. Effect of concentrate supplementation on growth and reproductive performance of rabbit under rural condition. *Bangl. J. Vet. Med., 4: 129 132.*

Juyena N.S., Stelletta C. 2012. Seminal plasma: an essential attribute to spermatozoa. *J. Androl. 33: 536-551. doi:10.2164/jandrol.110.012583*

Kareskoski M., Katila T. 2008. Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. *In: Proc.: 5th International Symposium on Stallion Reproduction, 249-256.*

Lin Y., Gary E., Robert E. 1969. Action of proteolytic enzymes on N,N-dimethyl proteins. Basis for a micro-assay for proteolytic enzymes. *J. Biolog. Chem. 244: 789-793.*

López-Gatius F., Sances G., Sancho M., Yaniz J., Santolaria P., Gutiérrez R., Núñez M., Núñez J., Soler C. 2005. Effect of solid storage at 15°C on the subsequent motility and fertility of rabbit semen. *Theriogenology, 64: 252-260. doi:10.1016/j.theriogenology.2004.11.015*

Marounek M., Vovk S.J., Skřivanová V. 1995. Distribution of activity of hydrolytic enzymes in the digestive tract of rabbits. *Brit. J. Nutr. 73: 463-469. doi:10.1079/BJN19950048*

Martin G.B., Rodger J., Blache D. 2004. Nutritional and environmental effects on reproduction in small ruminants. *Reprod. Fertil. Dev., 16: 491-501. doi:10.1071/RD04035*

Martínez-Rodríguez C., Alvarez M., Ordás L., Chamorro C.A., Martinez-Pastor F., Anel L., de Paz P. 2012. Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test. *Theriogenology, 77: 1575-1586. doi:10.1016/j.theriogenology.2011.11.026*

NRC. 1984. Nutrient Requirements of Rabbits. 2nd Ed. National Academy of Science, Washington, DC. USA.

Ogbuewu I.P., Okoli I.C., Iloeje M.U. 2009. Semen quality characteristics, reaction time, testis weight and seminiferous tubule diameter of buck rabbits fed neem (*Azadirachta indica* A. Juss) leaf meal based diets. *Iranian J. Reprod. Med., 7: 23-28.*

Paál D., Kročková J., Ondruška Ľ., Slanina T., Strejček F., Massányi P. 2014. Effect of semen collection frequency on the progress in the motility of rabbit spermatozoa. *Slovak J. Anim. Sci., 47: 61-67.*

Reitman S., Frankel M. 1957. An colorimetric method for determination of serum oxaloactic and glutamic pyruvic transaminase. *Anim. Clin. Path. J., 16: 28-56.*

Richardson L., Hanrahan J.P., O'Hara L., Donovan A., Fair S., O'Sullivan M., Carrington S.D., Lonergan P., Evans A.C. 2011. Ewe breeds differences in fertility after cervical AI with frozen-thawed semen and associated differences in sperm penetration and physicochemical properties of cervical mucus. *Anim. Reprod. Sci., 129: 37-43. doi:10.1016/j.anireprosci.2011.10.012*

Robyt J.F., Whelan W.J. 1972. Reducing value methods for maltodextrins: 1. Chain-length dependence of alkaline 3,5-dinitrosalicylate and chain length independence of alkaline copper. *Anal. Biochem., 45: 510-516. doi:10.1016/0003-2697(72)90213-8*

Rodríguez-De Lara R., Fallas-López M., Rangel-Santos R., Mariscal-Aguayo V., Martínez-Hernández P.A., García Muñiz J.G. 2008. Influence of doe exposure and season on reaction time and semen quality of male rabbits. *In Proc.: 9th World Rabbit Congress, June 10-13, 2008, Verona, Italy.*

Safaa H.M., Emarah M.E., Saleh N.F.A. 2008. Seasonal effects on semen quality in Black Baladi and white New Zealand rabbit bucks. *World Rabbit Sci., 16: 13-20. doi:10.4995/wrs.2008.640*

Saleh S.Y., Attia K.A., Fouad M., Nassar M.M. 2010. Effects of Multi-enzyme Feed Additive "Kemzyme" or/and Sodium Bentonite "as a Feed Binder"on Sexual Activity and Some Fertility Parameters of Rabbit Bucks. *J. Agr. Sci., 2: 89-99. doi:10.5539/jas.v2n4p89*

Salem A.Z.M., Gado H.M., Colombatto D., Elghandour M.M.Y. 2013. Effects of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. *Livest. Sci., 154: 69-73. doi:10.1016/j.livsci.2013.02.014*

Salem A.Z.M., Hassan A.A., Gado H.M., Khalil M.S., Simbaya J. 2012. Effect of sun-drying and exogenous enzymes on nutrients intake and digestibility as well as nitrogen utilization in sheep fed *Atriplex halimus* foliages. *Anim. Feed Sci. Technol., 171: 128-135. doi:10.1016/j.anifeedsci.2011.10.011*

SAS. 2001. Statistical Analysis System, User's Guide Version 8.2 Cary NC. USA.

Seleem T.S.T. 2003. Studies on productive and physiological characteristics of rabbits under different managerial conditions. *Ph.D. Thesis, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.*

Sequeira J., Nicodemus N., Carabaño R., Villamide M.J. 2000. Effect of type of wheat and addition of enzymes on some digestive parameters at different sampling time. *In Proc.: 7th World Rabbit Congress. 4-7 July, 2000, Valencia, Spain, 437-444.*

Snedecor G.W., Cochran W.G. 1982. Statistical Methods. 2nd Ed. Iowa University, Press Ames, Iowa,USA.

Valdes K.I., Salem A.Z.M., Lopez S., Alonso M.U., Rivero N., Elghandour M.M.Y., Domínguez I.A., Ronquillo M.G., Kholif A.E. 2015. Influence of exogenous enzymes in presence of *Salix babylonica* extract on digestibility, microbial protein synthesis and performance of lambs fed maize silage. *J. Agr. Sci., 153: 732-742. doi:10.1017/S0021859614000975*

Watson P.F. 1975. Use of Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Vet. Res., 97: 12-15.*

Zeidan A.E.B., Marai I.F.M, Abd El-Kariem Z.A. 1997. Effects of intratesticular injection of gonadotropin-releasing hormone on reproductive performance of low fertile male rabbits under Egyptian summer conditions. *In Proc.: 1st International Conference on Animal Production and Health, 2-4 September, 1997, Dokki, Egypt, 557-566.*

Zijstra R.T., de Lange C.F.M., Patience J.F. 1999. Nutritional value of wheat for growing pigs: chemical composition and digestible energy content. *Can. J. Anim. Sci., 79: 187-194. doi:10.4141/A98-103*

Wang Y., Ramirez-Bribiesca J.E., Yanke L.J., Tsang A., McAllister T.A. 2012. Effect of exogenous fibrolytic enzyme application on the microbial attachment and digestion of barley straw In vitro. *Asian-Aust. J. Anim. Sci., 25: 66-74.*