



Effect of azolla (*Azolla pinnata*) supplementation on puberty, semen characteristics and sexual behaviour in Alpine × Beetal crossbred bucks

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ABSTRACT

The present experiment was conducted at ICAR-National Dairy Research Institute, Karnal, Haryana. Eighteen male crossbred bucks (Alpine × Beetal) (average 103.94±2.87 days old) were divided into three groups with six animals in each group, viz. T₁, T₂ and T₃. Animals of T₁ group were fed different green fodder and concentrate mixture to meet their daily nutrient requirement. Animals of T₂ group were fed according to T₁ except 20% replacement of concentrate mixture by sun-dried azolla on DMB. Animals of T₃ group were fed according to T₁ except the animals were fed with concentrate supplemented with 20% sun-dried azolla on DMB. Effect of azolla on age at puberty, seminal parameters and sexual behaviour was assayed by standard methods. The animals of T₃ group attained puberty significantly earlier as compared to T₁ and T₂. Non-significant effect of azolla supplementation on semen volume, pH, mass activity, abnormality and acrosomal integrity was observed. The progressive motility and sperm concentration was significantly higher in T₃ group as compared to T₁. The HOST% and live spermatozoa were significantly higher in T₂ and T₃ as compared to T₁. Sexual behaviours were significantly better in T₂ and T₃ as compared to T₁. In conclusion, the supplementation of bucks with azolla reduces their age at puberty, improves seminal characteristics and sexual behaviour.

Keywords: Azolla, Bucks, Feeding, Puberty, Semen, Sexual behaviour

Azolla is an aquatic fern found in tropical and sub-tropical zones of the world. Azolla has been used as a fertilizer, manure, herbicide, water purifier, water saver, mosquito inhibitor and feed for different livestock species. Presently, mainly six species of azolla are more popular, viz. *A. micropylla*, *A. pinnata*, *A. filiculoides*, *A. caroliniana*, *A. maxicana* and *A. nilotica* (Lumpkin and Plucknet 1982).

Azolla is a rich source of essential amino acids, protein, vitamins like vitamin A, B₁₂ and beta carotene (Leterme *et al.* 2010), growth promoters, intermediaries and minerals. It also contains certain compounds such as carotenoids, poly-unsaturated fatty acids (PUFA), biopolymers, and probiotics (Parashuramul and Nagalakshmi 2012). Parthasarathy *et al.* (2002) reported that azolla is a rich source of protein (21.6%) with all essential amino acids like lysine, arginine and methionine. Alalade and Iyayi (2006) reported that azolla contains 1.59, 4.59, 1.59, 5.71, 4.07, 5.37, 4.35, 4.72, 3.18, 4.60, 4.21, 0.84, 1.82 and 5.51 g amino acid/100 g CP; methionine, lysine, phenylalanine,

cystine, tryptophan, isoleucine, leucine, tyrosine, glycine, serine, threonine, arginine and valine, respectively. Azolla is a good source of minerals like calcium, phosphorous, magnesium, potassium, iron, zinc, etc. Subudhi and Singh (1978) found that Ca and P content in azolla ranged from 0.4 to 1.0% and 0.5 to 0.9% respectively.

It is used as antioxidant agent, antibacterial and alternate medicine (Nayak *et al.* 2015). Zn and other micronutrients play an important role in male reproductive system including growth of reproductive system, spermatogenesis, maintenance of libido, etc. Supplementation of azolla has been found to improve the reproductive performance in buffalo heifers (Gupta *et al.* 2014). Few studies have revealed that feeding Barbari goats with azolla had positive effect on semen quality (Kumar *et al.* 2016). Ahmad *et al.* (2005) found that feeding is a major factor that affects the sexual behaviour of animals. Sexual behaviour is indicator of reproductive efficiency in animals. However, the effect of azolla on age at puberty has not been evaluated yet, very less literature is available related to effect of azolla on semen quality and sexual behaviour parameters. So the present study was designed to evaluate the effect of azolla (*Azolla pinnata*) supplementation on puberty, semen characteristics and sexual behaviour in Alpine × Beetal crossbred bucks.

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MATERIALS AND METHODS

The present experiment was conducted at ICAR-National Dairy Research Institute, Karnal, Haryana. For production of *Azolla pinnata*, a pit of 3 × 1 m size was prepared. A deep plastic bed with 2.5 kg cow dung, 15-20 g SSP in 10 L of water was used for azolla production. Harvesting of azolla was done at 5-7 days interval. Just after harvesting, azolla was washed with clean water and then sun-dried for removal of maximum moisture. Powdered dried azolla was made up by crushing azolla with the help of hands. After that, dried azolla was packed in moisture free plastic bags for further feeding. This procedure was continued till the end of experiment.

Animals and experimental diets: Eighteen male crossbred bucks (Alpine × Beetal) (average 103.94±2.87 days old) were taken from Livestock Research Centre (LRC), NDRI, Karnal. The animals were divided into three groups with six animals in each group, viz. T₁, T₂ and T₃. The experimental diet was offered just after starting of experiment. Animals of T₁ group were fed green fodder like maize, berseem, cow pea, sorghum and concentrate mixture, etc. to meet their daily nutrient requirement as per ICAR (2013) (Table 1). Animals of T₂ group were fed according to T₁ except 20% replacement of concentrate mixture by sun-dried azolla on DMB. Animals of T₃ group were fed according to T₁ except animals were fed with concentrate supplemented with 20% sun-dried azolla on DMB.

Composition of azolla and different feed and fodder: Dry matter, total ash, organic matter, crude protein, ether extract, TDN of *Azolla pinnata* and different feed and fodders were assayed according to standard procedure and methods (AOAC 2005).

Assessment of age at puberty: The age at puberty was assessed based on semen parameters. Pubertal age is the time when the ejaculate contained >50×10⁶ spermatozoa/ml with 10% progressively motile spermatozoa (Foster 1994, Ahmad *et al.* 2010).

Semen collection and its analysis: The bucks were trained to ejaculate in artificial vagina 15 days before start of experiment. The ejaculates were collected by artificial vagina as per standard procedure. Six samples from each bucks were collected at weekly interval for final semen parameters study. Immediately after collection, the volume of each ejaculate was measured directly from the reading of the graduated collection vial. The pH of each ejaculate was recorded by digital pH meter. For evaluation of mass motility (0-5 scale), a drop of freshly collected semen was put over a clean grease-free slide maintained at 37°C and examined under low power of the microscope (10×). For evaluation of progressive motility, a drop (10 µl) of diluted semen was placed on a clean grease-free slide maintained at 37°C covered with a coverslip and examined under a phase contrast microscope (Nikon Eclipse, E600) at 40× magnification. Sperm motility was assessed in four different fields for each sample.

Sperm concentration was estimated as described by Chemineau *et al.* (1991). Viability and abnormality was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.* 1953). For hypoosmotic swelling (HOS) response, semen sample (50 µl) was added to 1 ml of HOST solution (150 mOsm), mixed and incubated at 37°C for 1 h. After incubation, one drop was put on a warm glass slide and examined under 40×. The spermatozoa expressing different tail coiling patterns were counted as HOS responsive. Acrosomal integrity was assessed by Giemsa stain (Watson 1975).

Sexual behaviours: Sexual behaviour of each animal was observed after attainment of puberty. Different sexual behaviours were studied according to standard methods (Suyadi 2012, Moghaddam *et al.* 2012).

Statistical analysis: The data were analysed using One-way analysis of variance (ANOVA) using statistical software package (SPSS, Version 20) and results were expressed as Mean±SE. The P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Composition of sun dried azolla and concentrate feed: The proximate composition of sun-dried *Azolla pinnata* and concentrate mixture fed for various dietary treatments is presented in Tables 1 and 2. The DM, crude protein, organic matter, ether extract, and total ash were similar to the values reported by Arvindraj (2012), Sujatha *et al.* (2013), Cherryl *et al.* (2014) and Srinivas *et al.* (2015).

Table 1. Composition of concentrate mixture (%) for different groups during feeding trials

Ingredient	Group T ₁	Group T ₂	Group T ₃
Maize grain	33.00	26.00	33.00
Barley grain	12.00	9.60	12.00
Pearl millet grain	5.00	4.00	5.00
Ground nut cake	9.00	7.20	9.00
DMOC	10.00	8.00	10.00
SBM	6.80	5.40	6.80
DORB	4.00	3.20	4.00
Gram chuni	5.00	4.00	5.00
Wheat bran	12.20	9.60	12.20
Sun dried azolla	0.00	20.00	20.00
Minerals	2.00	2.00	2.00
Salt	1.00	1.00	1.00
Total	100.00	100.00	120.00
CP (%)	19.98	20.30	24.39
TDN (%)	74.40	70.24	85.57

Table 2. Proximate composition of sun dried *Azolla pinnata* on DMB (Gupta *et al.* 2018)

Nutrient	(Dry matter %)
Dry matter	90.00±0.77
Crude protein	22.05±0.72
Organic matter	81.05±0.44
Ether extract	3.25±0.76
Total ash	18.94±0.31

Effect on age at puberty: The important reproductive parameters of bucks during age at puberty is presented in Table 3. The animals of T_3 group attained puberty significantly ($P<0.05$) earlier as compared to T_1 and T_2 . The animals of T_3 group attained puberty at 25.75 ± 0.19 kg body weight, whereas at this time average body weights of T_1 and T_2 group were 22.11 ± 0.27 kg and 22.68 ± 0.43 kg, respectively. The animals of all groups reached puberty within the normal age period. Previously, many authors reported that bucks of different breeds attained puberty at up to 8 month of age (Bezerra *et al.* 2009, Elsheikh and Elhammali 2015). There was non-significant ($P<0.05$) difference between groups related to sperm concentration and scrotum circumference at puberty.

Table 3. Reproductive parameters of bucks during age at puberty

Attribute	Group T_1	Group T_2	Group T_3
Age at puberty (d)	241.67 ^b $\pm$ 2.48	237.17 ^b $\pm$ 2.65	207.83 ^a $\pm$ 1.53
Body weight (kg)	25.31 $\pm$ 0.31	24.30 $\pm$ 0.43	25.75 $\pm$ 0.19
Sperm concentration (10 ⁶ /ml)	275.00 $\pm$ 25.00	308.33 $\pm$ 27.13	333.33 $\pm$ 27.88
Scrotum circumference (cm)	22.45 $\pm$ 0.55	20.15 $\pm$ 0.65	22.00 $\pm$ 0.61

Values with different superscripts in a row differ significantly ($P<0.05$).

There was significant ($P<0.05$) effect of nutrition on age at puberty. The bucks in T_3 group received significantly ($P<0.05$) higher DM, CP and TDN (g/animal/day) in diet and reached puberty earlier (Table 1). Many factors like mineral and vitamins supplementation through azolla may be contributed for early age at puberty in T_3 group. azolla is a very good source of minerals, vitamins and other health

promoting nutrient which are responsible for growth, better digestibility, cellular metabolism, improvement of antioxidant status and maintenance of physiology. The animals of T_3 group reached puberty significantly earlier, which may be due to higher body weight gain (g/d) ($T_3=93.65\pm3.39$) than other groups ($T_2=84.44\pm2.79$ and $T_1=82.74\pm2.68$) till age at puberty. This result showed that attainment of proper body weight is more important than age for early attainment of puberty. Hafez and Hafez (2004) found that puberty of animals depends on body weight rather than the age. The importance of body weight for early age at puberty was reported by many authors (Lemond 1970, Maquivar *et al.* 2006, Bhatti *et al.* 2007).

Seminal parameters and sexual behaviour traits

Semen volume and pH: Semen volume (ml) and pH values were similar ($P<0.05$) between groups (Table 4). The values obtained in the present study are in agreement with the report of Puranik *et al.* (1993) and Kale (1995) in Pashmina goat and Alpine $\times$ Beetal crossbred bucks. Non-significant ($P<0.05$) effect of azolla supplementation on semen volume was observed in Barbari bucks (Kumar *et al.* 2016) and Marwari stallion (Songara *et al.* 2020). Contrary to this finding, significantly increased volume was observed in azolla fed Barbari bucks (Gangwar *et al.* 2019).

Mass activity: The per cent values of mass activity (Mean $\pm$ SE) were similar ($P<0.05$) among groups, however numerically higher values were observed in T_2 and T_3 groups due to availability of Zn in the azolla (Table 4). Zn maintains sperm motility by controlling utilization of energy through ATP system (El-Masry *et al.* 1994).

Progressive motility: The progressive motility was significantly ($P<0.05$) higher in T_3 group as compared to T_1 whereas T_2 group showed intermediate value (Table 4). This happened due to higher levels of trace minerals in azolla. Kumar (2016) found that progressive motility increased

Table 4. Seminal characteristics and sexual behaviour parameters of bucks in different groups (Mean $\pm$ SE)

Attribute	Group T_1	Group T_2	Group T_3
Volume (ml)	0.60 $\pm$ 0.01	0.63 $\pm$ 0.02	0.66 $\pm$ 0.01
pH	6.57 $\pm$ 0.02	6.55 $\pm$ 0.02	6.52 $\pm$ 0.02
Mass activity (1-5)	3.02 $\pm$ 0.11	3.19 $\pm$ 0.11	3.30 $\pm$ 0.10
Progressive motility (%)	69.30 ^a $\pm$ 0.99	72.08 ^{ab} $\pm$ 1.06	74.02 ^b $\pm$ 1.05
Concentration (10 ⁶ /ml)	1941.73 ^a $\pm$ 58.80	2015.33 ^{ab} $\pm$ 69.20	2176.43 ^b $\pm$ 73.55
Abnormality (%)	12.58 $\pm$ 0.36	11.56 $\pm$ 0.40	11.94 $\pm$ 0.33
Live (%)	74.69 ^a $\pm$ 0.92	77.58 ^b $\pm$ 0.84	78.86 ^b $\pm$ 0.73
HOST (%)	56.08 ^a $\pm$ 0.62	59.55 ^b $\pm$ 0.53	61.05 ^b $\pm$ 0.42
Acrosomal integrity (%)	81.77 $\pm$ 0.44	82.11 $\pm$ 0.54	82.75 $\pm$ 0.47
Day of penile protrusion (d)	214.33 ^b $\pm$ 3.62	207.50 ^b $\pm$ 2.76	181.00 ^a $\pm$ 3.35
First attempt to mount (d)	226.33 ^b $\pm$ 3.37	217.83 ^b $\pm$ 4.43	192.00 ^a $\pm$ 4.06
Reaction time (sec)	29.91 ^b $\pm$ 0.68	22.50 ^a $\pm$ 0.42	21.97 ^a $\pm$ 0.47
Ejaculation time (sec)	42.72 ^b $\pm$ 0.89	36.50 ^a $\pm$ 0.84	35.86 ^a $\pm$ 0.81
Refractory period (sec)	98.91 ^b $\pm$ 2.10	96.13 ^{ab} $\pm$ 2.00	90.47 ^a $\pm$ 2.16
Fixing strength (1-3 scale)	2.16 $\pm$ 0.08	2.33 $\pm$ 0.09	2.41 $\pm$ 0.08
Number of mountings	3.50 $\pm$ 0.14	3.19 $\pm$ 0.13	3.38 $\pm$ 0.14

Values with different superscripts in a row differ significantly ($P<0.05$).

with Zn supplementation in Barbari bucks. Similar result was observed in bucks (Saleh *et al.* 1992) and crossbred cattle bulls (Kumar 2006) due to Zn supplementation. Zn feeding results in increment of sorbitol dehydrogenase and lactate dehydrogenase activity which is important for sperm motility (Nagamine *et al.* 1990).

Sperm concentration: The sperm concentration was significantly ($P<0.05$) higher in T_3 group as compared to T_1 , whereas T_2 group showed intermediate value (Table 4). Azolla feeding in treatment groups might have provided good quality amino acids and different minerals. Higher protein intake may have resulted in increased seminiferous tubules cell proliferation (Nistane and Honmode 1982). The significant effect of Zn on sperm concentration (Saleh *et al.* 1992) and spermatogenesis process (Underwood and Somers 1969, Wong 2002, Kumar 2006) were reported earlier. Zn influences cell division and synthesis of DNA and RNA by enhancing activity of DNA polymerase and RNA polymerase. Zn is also responsible for enhancing testosterone concentration and stimulating Leydig cells of testis (Fang and Furuhashi 1978).

Sperm abnormality and live spermatozoa (%): The per cent values of sperm abnormality (Mean $\pm$ SE) was similar ($P<0.05$) among groups, however lower sperm abnormality was found in T_2 and T_3 groups (Table 4). The live spermatozoa count was significantly ($P<0.05$) higher in T_2 and T_3 as compared to T_1 . Similar result was reported in Barbari bucks due to azolla feeding (Gangwar *et al.* 2019). The increase in live spermatozoa values might be due to availability of good quality amino acids and trace minerals in the azolla. The presence of good quality amino acids in azolla was reported earlier (Ali and Lesson 1995, Alalade and Iyayi 2006). Zn is important for sperm viability (El-Mashry *et al.* 1994, Omu *et al.* 1998, Kumar 2006, Sabhapati *et al.* 2016), membrane stabilizing action. Manohar *et al.* (2018) reported that Zn and Cu supplementation have antioxidant protective effect.

Hypo-osmotic swelling test (HOST%): HOST was significantly ($P<0.05$) higher in T_2 and T_3 as compared to T_1 (Table 4). Significant effect of azolla on HOST was observed in Barbari Bucks (Gangwar *et al.* 2019). Azolla is a good source of Zn which resulted in significant improvement on HOST%. Contrary to present findings, Kumar *et al.* (2016) reported non-significant ($P<0.05$) effect of azolla feeding on HOST. It may be due to breed, age and feeding difference.

Acrosomal integrity: There was non-significant ($P<0.05$) difference in acrosomal integrity among the all groups, however numerically higher values were observed in T_2 and T_3 groups (Table 4). Similar result was reported in Barbari bucks due to azolla feeding (Gangwar *et al.* 2019). The higher acrosomal integrity in azolla supplemented groups may be due to presence of minerals in the azolla. The adequate amount of Zn maintains integrity of sperm membrane during epididymal storage (Mann 1964) and

ejaculation process (Lindholmer 1974).

Sexual behaviour traits: The day of penile protrusion and first attempt to mount was significantly ($P<0.05$) earlier in T_3 group as compared to T_1 and T_2 (Table 4). Louis *et al.* (1994) studied the effect of protein in libido of boar and found that boar with low protein diet required more time to start ejaculation process. The reproductive organs of animals in T_3 group developed faster due to higher intake of nutrients because azolla stimulated growth of penis and its protrusion. Animals with azolla feeding (Group T_2 and T_3) significantly ($P<0.05$) required less reaction time and ejaculation time as compared to T_1 . Gangwar *et al.* (2019) also reported about improved libido in Barbari bucks due to azolla feeding. It indicated that bucks were very active and alert for semen donation. Azolla supplied good quality protein, minerals and vitamins which resulted in reduction of reaction time and maintenance of libido. Azolla is a rich source of all essential amino acids (Mandal *et al.* 2012, Indira and Ravi 2014), minerals (Zn, Fe, Cu, Se, Ca and P), B complex and vitamin A.

The refractory period was significantly ($P<0.05$) lower in T_3 than T_1 . The animals of T_2 group showed intermediate value. The animals in T_3 group required significantly ($P<0.05$) less refractory period due to positive effect of protein, minerals, vitamins and bioactive molecules intake through azolla which were responsible for more alertness of animals in T_3 group resulting in reduction of refractory period. Non-significant ($P<0.05$) effect was observed between T_1 and treatment groups (T_2 and T_3) for fixing strength and number of mountings to complete ejaculation. However animals of T_3 group performed better followed by T_2 and T_1 . The animals in treatment groups were more active with high sexual stimulation and with potent libido.

On the basis of this study, it is concluded that feeding bucks with azolla reduces age at puberty, improves semen quality and sexual behaviour.

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