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# Cloacal Gland Size Significantly Alters Semen Production, Sperm Activities and Fertility in Different Lines of Japanese Quail (*Coturnix coturnix japonica*)

N. Shit, R.P. Singh, K.V.H. Sastry, J. Mohan, N. Pandey and R.P. Moudgal
Division of Physiology and Reproduction, Central Avian Research Institute, Izatnagar 243122 (UP), India

**Abstract:** In sexually active male Japanese quail cloacal gland hypertrophy and foam production is androgen dependent and highly positively correlated with testis size and sexual activity. Nevertheless, the physiological functions of this gland in male reproduction are still a controversial issue. Therefore, the aim of this investigation was made to rule out the effect of cloacal gland size on semen production, sperm activities and fertility in both Heavy Body Weight (HBW) and White Feathered (WF) lines of Japanese quail. Sixty matured males and thirty females (8 weeks) from each lines of Japanese quail were randomly selected. Males were categorized into three groups (20/group) based on the increasing order of cloacal gland area. They were maintained in uniform husbandry condition with *ad libitum* feed and water at 14 h photo-schedule. Highest (11.04±0.20 and 9.6±0.41) semen production was recorded in birds having largest cloacal gland area and was positively correlated to the cloacal gland size in both the experimental quail lines. Metabolic activity by adopting Methylene Blue Reduction Time (MBRT) test and proteolytic activity by Acrosine Proteolytic Activity (APA) test of quail spermatozoa was found significantly different ($p \leq 0.05$) among groups and also revealed a positive correlation to the increasing area of the cloacal gland. In both the experimental lines, fertility was noted maximum ($p \leq 0.05$) in group III categorized by largest cloacal gland area. From this current study, it may be concluded that cloacal gland can be considered as external indicator or selection marker of testicular functions in male Japanese quail.

**Key words:** Cloacal gland, semen, methylene blue reduction time, acrosome proteolytic activity, fertility, quail

## INTRODUCTION

Japanese quail has recently been identified as a leading model for expensive biomedical, toxicological and basic research due to their faster juvenile growth, short generation interval and high rate of egg production (Wilson *et al*., 1961). In sexually matured male, cloacal gland (*Glandula proctodealis dorsalis*) the unique reproductive facet among avian species, is a greatly swollen mass on the outer dorsal lip of the cloaca (Fujihara, 1992). It is a series of tubular gland complex with numerous discrete glandular units which secrets white meringue like foam (McFarland *et al*., 1968). Evidently, this gland is androgen dependant and the area of cloacal gland is positively correlated with testicular weight, plasma level of testosterone

**Corresponding Author:** Nonigopal Shit, Division of Physiology and Reproduction, Central Avian Research Institute, Izatnagar 243122 (UP), India
Tel: +91-9756304702 Fax: 91-0581-2301321

and fertility in sexually active males (Sachs 1967; McFarland *et al*., 1968; Klemm *et al*., 1975; Siopes and Wilson, 1975; Massa *et al*., 1980; Balthazart *et al*., 1984; Mohan *et al*., 2002; Biswas *et al*., 2007). This suggested that measurement of cloacal gland may provide a valuable non-invasive method of predicting testicular activity and subsequently fertilizing ability of male quail (Mohan *et al*., 2002; Biswas *et al*., 2007).

Increased fertility in birds having large cloacal gland (Mohan *et al*., 2002; Biswas *et al*., 2007) suggested presence of more number of metabolically active spermatozoa at the time of fertilization. In the process of fertilization, unlike most mammals cock spermatozoa have only to penetrate the vitelline membrane of the hen's ovum a single layer of connective tissue-like protein (Bellairs *et al*., 1963). They have also been shown to contain a high proteases or Trypsin-Like Enzymatic (TLE) activity in the acrosomes (acrosomal extracts) for the best penetration of vitelline membrane (Ho and Meizel, 1970; Polakoski, 1972). Many reports have ensured the presence of acrosomal proteases or Trypsin-Like Enzyme (TLE) activity in Japanese quail by considering gelatine digestion around each head of normal and freeze-thawed quail spermatozoa (Maeda *et al*., 1990; Win *et al*., 2006). Nevertheless, there would not have any attempt so far to correlate the area of cloacal gland with metabolic and proteolytic activities of quail spermatozoa. Therefore, this was an attempt to appraise the effect of cloacal gland size on semen production, metabolic as well as proteolytic activity of spermatozoa and fertility in both Heavy Body Weight (HBW) and White Feathered (WF) lines of Japanese quail.

## MATERIALS AND METHODS

### Experimental Birds

Sixty healthy adult male and thirty female Japanese quail (8 weeks old) from each line, i.e., HBW and WF were randomly selected (2006) from the institute experimental quail farm. They were managed in 4 tier individual battery cages (44×32×24 $cm^3$) under uniform husbandry conditions and were offered *ad-libitum* water and quail breeder ration (CP = 19.92%; ME = 2723.50 Kcal) with 16 h photo-schedule. Based on the area of clocal gland, males were equally divided into three groups, i.e., Gr-I, <225 $mm^2$, Gr-II, 225-425 $mm^2$, Gr-III,> 425 $mm^2$ (20/group). The total area of the cloacal gland was resolved by measuring both width (lateral) and height (dorsoventral) of the gland using vernier calipers with 0.01 mm accuracy (Siopes and Wilson, 1975). Each group was again replicated into two (10/replicate) where 10 birds were used for semen quality assessment and remaining was allowed to mate females (1:1) for fertility determination in both the lines. The experiment was conducted according to the guidelines of Institutional Animal Ethics Committee (IAEC, CARI, Izatnagar).

### Semen Collection

Semen was collected twice in a week by the technique of Burrows and Quinn (1937) with minor modification. Each male quail birds were gently restrained on the palm of the left hand and foam was squeezed out before semen collection. The lumber region towards tail was massaged 3-4 times smoothly and applied gentle pressure on either side of the vent by using thumb and fore finger. Chalky or yellowish white semen from the spade like papillae was collected into a graduated glass micropipette. Utmost care was taken to curtail the contamination from feaces, urate crystals and watery fluid.

### Semen Production and Sperm Activities

The volume of ejaculated fresh semen from individual male birds was measured in a graduated glass pipette (0.1 mL) with 0.001 mL accuracy. Metabolic activity of quail

spermatozoa was confirmed by conducting modified Methylene Blue Reduction Time (MBRT) test (Beck and Salisbury, 1943) with suitability to Japanese quail. Immediately after collection, neat semen (5 μL) was diluted with Tyrode's diluents at 1:5 ratios. Exactly 2.5 μL methylene blue solution (50 mg% w/v) was added to the diluted semen and gently mixed by inverting the tubes. The upper layer was immediately covered with 0.2 mL mineral oil and incubated at 37°C till the methylene blue (blue) turned in to leuko-methylene blue (white). Acrosomal Proteolytic Activity (APA) test was performed in both the lines to predict the proteolytic activity of acrosomal enzymes (TLE, proteases, acrosin etc) from acrosome of quail spermatozoa. Apart from semen dilution with Tyrode diluents (1:2 ratios), the whole APA test was done by following the protocol described by Maeda *et al*. (1990). The horizontal diameters of individual halos formed upon gelatin digestion around each sperm head in both the lines were measured by using the available software (Image Pro Express, version-5.1.0.12, Media Cybernetics Corporation Inc. UK).

**Determination of Fertility in Male Birds**

Male birds were allowed to mate naturally with equal number of females (1:1). After a week adaptation, egg collection was started twice daily till the end of experiment. Eggs were stored for 7 day in the egg holding room at institute hatchery at low temperature (15-18°C) before setting. After fumigation and equilibration, eggs were set and fertility was assured by broken open eggs at 7th day of incubation following 99.5°F temperature and 55% relative humidity. The percent fertility was determined by the ratios of number of fertile eggs to the number of total egg set in the incubator.

**Statistical Analysis**

All the data generated during the course of experiment was analyzed by statistical software package developed at computer centre of the institute by following standard procedures for ANOVA (Snedecor and Cochran, 1994) and Duncan's multiple range tests (Duncan, 1955) for comparing the mean values of cloacal gland size with other parameters.

## RESULTS

The mean volume of semen per ejaculation in both HBW and WF lines of Japanese quail is presented in Fig. 1. Significantly higher ($p \leq 0.05$) volume of semen per ejaculation was

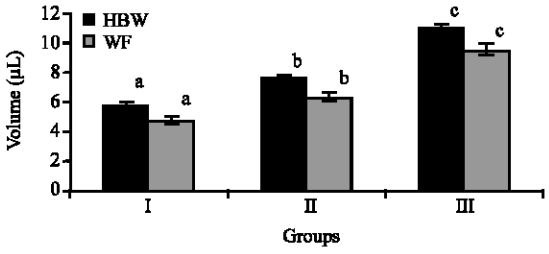


Fig. 1: Volume of Semen production (μL) in both Heavy Body Weight (HBW) and White Feathered (WF) lines of Japanese quail. Gr I, <225 $mm^2$; Gr II, 225-425 $mm^2$; Gr III, >425 $mm^2$. Means bearing different superscript in each line differ significantly ($p \leq 0.001$)

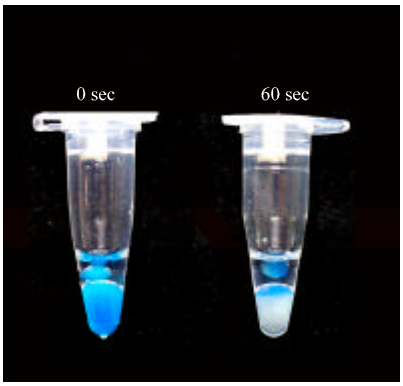


Fig. 2: Methylene Blue Reduction Time (MBRT) test showing reduction of methylene blue to leukomethylene blue in Japanese quail semen

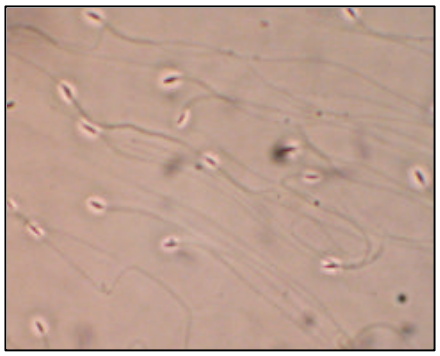

Fig. 3: Acrosin Proteolytic Activity (APA) test showing gelatin digestion (halo formation) by acrosin released from sperm acrosome in Japanese quail

Table 1: Methylene Blue Reduction Time (MBRT) test in HWB and WF experimental lines of Japanese quails

| | MBRT (sec) | |
|---|---|---|
| Groups | HBW | WF |
| I | 60.33±1.01$^{c}$ | 61.86±1.97$^{c}$ |
| II | 45.55±1.88$^{b}$ | 47.33±2.80$^{b}$ |
| III | 34.71±0.98$^{a}$ | 37.24±1.37$^{a}$ |
| p-value | * | ** |

Values in the same column with uncommon roman superscripts are significantly different. HBW: Heavy body weight; WF: White feathered. Group-I <225 mm$^2$; Group-II 225-425 mm$^2$; Group-3 >425 mm$^2$ *p = 0.000; **p = 0.001

recorded in birds having the larger cloacal gland area (group III) compared to others in both the lines. The line difference in regards to semen volume per ejaculation was also higher in former line than its counterpart. The metabolic activity of quail spermatozoa determined by conducting Methylene Blue Reduction Time (MBRT) test is shown in Table 1, Fig. 2. The birds with larger cloacal gland area from both the lines reduced the methylene blue into leukomethylene blue in significantly ($p \leq 0.05$) lesser time than the remaining groups. The mean diameters (μm) of halos formed around each head of quail spermatozoa during assessment of proteolytic activity are shown in Table 2 and Fig. 3. Average horizontal

Table 2: Horyzontal halos diameter (μm) determined by Acrosin Proteolytic Activity (APA) test in HBW and WF experimental lines of Japanese quails

| | Halo diameter (μm) | |
|---|---|---|
| Groups | HBW | WF |
| I | 10.56±0.40$^{a}$ | 11.08±0.30$^{a}$ |
| II | 14.82±0.27$^{b}$ | 12.64±0.27$^{b}$ |
| III | 17.82±0.34$^{c}$ | 15.74±0.76$^{c}$ |
| p-value | * | * |

Values in the same column with uncommon roman superscripts are significantly different. HBW: Heavy body weight; WF: White feathered. Group-I, <225 mm$^2$; Group-II, 225-425 mm$^2$; Group-3, >425 mm$^2$. *p = 0.05

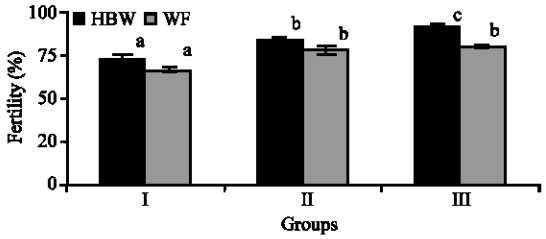


Fig. 4: Percentage of fertility based on cloacal gland size in heavy body weight (HBW) and white feathered (WF) lines of Japanese quail. Gr I, <225 mm$^2$; Gr II, 225-425 mm$^2$; Gr III, >425 mm$^2$. Means bearing different superscript in each experimental line differ significantly ($p \leq 0.05$)

diameter of halos found on the gelatin coated slides was evident greater ($p \leq 0.05$) in birds with lager cloacal gland (group III) in both the experimental quail lines. The fertility percentage was also perceived significantly ($p \leq 0.05$) higher in groups classified with utmost cloacal gland area in both the lines of Japanese quail (Fig. 4). Though, arbitrarily fertility percent was higher in group III, but statistically there was no significant difference between group II and group III in white feathered line of Japanese quail.

## DISCUSSION

Apart from the paracloacal vascular bodies, dorsal proctodeal gland and lymphatic folds, there are no secondary sexual organs in domestic birds (Fujihara, 1992). Therefore, the seminal fluid is probably derived entirely from the testosterone dependent epithelial cells of testis and the ex-current ducts, though lymphatic exudates from the phallic folds often contribute to the ejaculate (Etches, 1996). In contrast to the dorsal proctodeal gland of chicken and turkey, the androgen dependent cloacal glands of Japanese quail contributes a foamy secretion to semen (Whittow, 2000). The semen volume per ejaculation of each bird in both the lines of Japanese quail (Fig. 1) showed a significant difference ($p \leq 0.05$) among the groups within lines. Birds with larger cloacal gland area (>425 mm$^2$) ejaculated highest volume of semen which confirms the direct relationship between cloacal gland size and testicular activity. A high degree of correlation was also established between the area of cloacal gland with testicular size (Coil and Witherbee, 1959; Sachs, 1967; Siopes and Wilson, 1975; Follett and Maung, 1978) and testosterone levels (Mohan *et al*., 2002) in quails. Hence, the semen volume can be correlated in accordance to cloacal gland size via testosterone stimulation.

Methylene Blue Reduction Time (MBRT) test reflects the metabolic activity of spermatozoa (Beck and Salisburry, 1943; Bogdonoff and Shaffner, 1954). This test is based

on the fact that the color imparted to semen by the addition of dye (methylene blue) will disappear more or less quickly. Thus, the time of reduction is taken as a measure of the total metabolic reactions proceeding at the cell surface of the spermatozoa although it is also being influenced by the sperm concentration. Besides, MBRT is an oxidation-reduction reaction where seminal dehydrogenases (succinic acid dehydrogenase) releases hydrogen ions which proportionally reduce methylene blue (blue) into leucomethylene (white) as the former is a stable acceptor. The time taken by the quail spermatozoa for the above biochemical reaction (methylene blue to leukomethylene blue) ranged between 37-61 sec (Table 1, Fig. 3) which was less than chicken (Mohan *et al*., 2009) and indicated a very high metabolic activity of the quail spermatozoa. Larger cloacal gland confirms higher metabolic activity and sperm concentration which may be due to the higher level of testosterone which is positively correlated to the cloacal gland size.

In mammals, sperm penetration into zona pellucida surrounding ova is aided by hydrolytic enzymes such as Trypsin-Like Enzyme (TLE) or protease within the sperm acrosome (Stambaugh and Buckley, 1969; Stambaugh *et al*., 1969). Similarly, chicken spermatozoa have been shown to contain high trypsin-like enzymatic activity (Ho and Meizel, 1970). Furthermore, the necessity of this enzyme for digestion and penetration of the vitelline membrane of the hen's ovum has been demonstrated both *in vitro* (Howarth and Digby, 1973) and *in vivo* (Palmer and Howarth, 1973). Langford and Howarthm (1974) exhibited a uniform trend and functions of trypsin-like enzyme in the acrosome of quail spermatozoa. The presence of TLE or its proteolytic activity in quail sperm was ascertained by gelatin-substrate slide technique. The halo diameter on gelatin coated slide around each spermatozoon (Table 2, Fig. 2) was highest in birds having larger cloacal gland area and was significantly different ($p \leq 0.05$) from other groups. Besides, the proteolytic activity is directly influenced by testosterone level which is positively correlated to the cloacal gland index (Siopes and Wilson 1975; Mohan *et al*., 2002). Therefore, this result may co-relate that birds having larger cloacal gland area (>425 $mm^2$) might have greater concentration of TLE or protease in their spermatozoa and revealed highest ($p \leq 0.05$) halo diameter in both the experimental lines. It may also be evident that some areas of the gelatin substrate slides do not have any halos around the sperm heads that could be because of either incomplete acrosin-gelatin contact or inactive enzyme-inhibitor complex formation or oozing out of proteolytic enzymes (Maeda *et al*., 1990).

Cloacal gland has also been found to directly influence the fertility percent in both the lines of Japanese quail when they were allowed to mate naturally. Highest fertility was recorded in groups having birds with larger cloacal gland than other groups. This observation is in agreement to the previous findings by Biswas *et al*. (2007). Addition to this, testicular size, functions and testosterone level that facilitate higher levels of fertility are positively co-related to the cloacal gland size (Siopes and Wilson, 1975; Mohan *et al*., 2002). Therefore, it is clear that the area of cloacal gland is indirectly proportional to the fertility percent in Japanese quail. The ongoing discussion revealed that the androgen dependent cloacal gland accelerates significantly the male reproductive efficiency according to its size in terms of semen production, sperm activities and fertility. The findings of the present study may be concluded that the area of cloacal gland can be used as a selection marker for sexually active male during breeding programme.

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