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Semen characteristics, cryopreservation, and successful artificial insemination in the Blue rock pigeon (*Columba livia*)

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Abstract

The present study was undertaken in the Blue rock pigeon (*Columba livia*) to evaluate the annual semen characteristics, to identify a suitable extender for semen short-term storage, to determine a protocol for cryopreservation of semen and finally to check whether intracloacal insemination would lead to the birth of a chick. Semen characteristics such as semen volume, sperm concentration, sperm motility, and percentage of normal spermatozoa were maximum during the monsoon season. TALP was observed to be the most suitable semen extender and the sperm survived best at 37 °C at a dilution of 1:100 in TALP. Further, cryopreservation studies on pigeon semen indicated that 8% DMSO with or without egg yolk (20%) proved to be a better cryoprotectant compared to glycerol and polyethylene glycol. In addition, the slow freezing protocol was better than the fast-freezing protocol and about 40% of the cryopreserved spermatozoa were motile following thawing. Computer-aided semen analysis indicated that pigeon spermatozoa were extremely active immediately after dilution in TALP and exhibited linear trajectories persisting up to 9 h. But, with time there was a time-dependent decrease in the velocity parameters (VAP, VSL, and VCL). Cryopreserved spermatozoa following thawing also exhibited linear trajectories but had reduced velocity as evident from the significant decrease in VAP, VSL, and VCL. Further, artificial inseminations using fresh semen resulted in 45% fertilization and birth of a live chick.

Keywords: Blue rock pigeon; Semen characteristics; CASA; Cryopreservation; Artificial insemination

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1. Introduction

Extinction of species is irreversible and is now occurring at an accelerated rate due to habitat destruction and poaching which facilitate inbreeding depression and thus affect the reproductive fitness, fecundity and the survival of young ones [1]. In such cases, captive breeding remains the only alternative to conserve endangered species, which have no immediate opportunity to survive in nature.

Assisted reproductive technologies such as artificial insemination (AI) and semen cryopreservation play an important role in preserving and transfusing valuable genes to the future generation and thus help to conserve and propagate endangered species which fail to breed in captivity. In birds, these techniques have been well established in chicken [2], turkey [3], and duck [4]. However, very little information is available on non-domesticated birds [5–7]. Successful application of these techniques requires prior knowledge of sperm physiology, identification of a suitable semen extender, a suitable cryoprotectant and the development of a freezing regime for semen cryopreservation. The family Columbidae includes 28 Indian species of pigeon, of which, species such as the Pale-backed pigeon (*Columba eversmanni*) and the Pale-capped pigeon (*Columba punicea*) are listed as vulnerable under IUCN 2002 category, while the Nicobar pigeon (*Caloenas nicobarica*) is listed in Schedule-I of the Wildlife Protection Act (1972) of India as an endangered species. This necessitates conservation and propagation of these species by assisted reproductive techniques. The present study was undertaken in the Blue rock pigeon (*Columba livia*) to evaluate seasonal changes in the semen characteristics, to identify a semen extender for short-term storage, to identify a suitable cryoprotectant for cryopreservation of semen and to check the suitability of intracloacal insemination. It is hoped that these studies will be of direct relevance in propagating the endangered species of pigeons.

2. Materials and methods

2.1. Experimental birds

Ten adult male (average weight 360 ± 15 g) and five female (average weight 310 ± 12 g) pigeons were captured randomly from a breeding population of approximately 100 birds of either sex from an aviary in the Nehru Zoological Park, Hyderabad, India and used for all the experiments. All the birds were housed individually in metal cages and were exposed to natural environmental conditions and a photoperiod of 11.2–12.3 h of light and 12.8–11.7 h of darkness depending on the season. The males and females were kept in adjacent cages so as to maintain visual contact. The animals were fed ad libitum with a diet consisting of 60% jowar (*Sorghum*), 40% green gram (*Vigna*), and a calcium supplement. Water was made available ad libitum.

2.2. Semen collection and evaluation

The same person collected semen from the above 10 randomly selected birds, twice a week, throughout the year (November 2000 to October 2001). The males were restrained

physically and the semen was collected in to a glass funnel used for semen collection in birds [8] by massaging the dorsal aspect of the abdomen towards the cloaca followed by gentle squeezing at the base of the cloaca according to the method described in detail by Cheng et al. [9]. The neat ejaculate was analyzed immediately after collection with respect to its volume, pH, osmolarity, sperm concentration, percentage of motile spermatozoa and percentage of normal and abnormal spermatozoa. In addition progressive motility of the spermatozoa was graded on a 5-point scale (where 0 indicates no motility, 1 indicates non-progressive motility, 2 indicates slow progressive motility, 3 indicates side to side movement accompanied by slow progressive motility, 4 indicates faster progressive motility, and 5 indicates very fast progressive motility). The ejaculate volume was determined by aspirating the semen in to a calibrated positive displacement pipette, the pH by using pH indicator strips (Qualigens Fine Chemicals, Glaxo India Ltd., Mumbai) and the osmolarity by using a vapour pressure osmometer (Wescor 5500, Utah, USA). Sperm concentration was assessed following dilution of 10 μl of semen to 100 times in a semen dilution fluid containing 5 g $NaHCO_3$ and 1 ml formalin in 100 ml of distilled water. Subsequently, 10 μl of the diluted semen was transferred to a Makler chamber and the semen concentration was then determined using a Computer Aided Sperm Analyzer (CASA; HTM-IVOS, Version 10, Hamilton Thorne Research Inc., Danvers, MA). The percentage of motile spermatozoa was also determined using CASA in which the slide was mounted on a stage warmer set at 37 °C. For sperm morphology studies, approximately 300 spermatozoa per ejaculate were analyzed. For this purpose, 2 μl of semen was fixed in 100 μl of 0.5% gluteraldehyde, smeared on a glass slide, air-dried and observed under the microscope (400×).

2.3. Computer-aided sperm motility analysis

For motility analysis, sperm suspensions diluted 1:100 in TALP were transferred to a pre-warmed slide chamber (130 μm depth) [10,11] and analyzed using the CASA as previously reported [12,13]. Various sperm motility parameters such as average path velocity (VAP), progressive velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), straightness of track (STR), amplitude of lateral head displacement (ALH), and linearity of track (LIN) of the spermatozoa were determined as previously reported [11,14].

2.4. Seasonal variation in semen characteristics

Hyderabad, which is located at 78.3E and 17.2N, is a semi-arid region with a tropical climate having three distinct seasons namely summer (March–June; temperature range 24–37 °C), monsoon (July–October; temperature range 23–32 °C), and winter (November–February; temperature range 17–29 °C). Ejaculates from 10 adult male pigeons were collected twice a week during November 2000 to October 2001 and were evaluated for semen parameters such as volume, sperm concentration, percentage of motile spermatozoa and the percentage of normal and abnormal spermatozoa. Semen was collected only once in 15 days and therefore assessed for variation in sperm morphology only once in fifteen days.

2.5. Optimization of semen extender

In order to determine a suitable extender for short-term storage or transport of pigeon semen, semen was collected and pooled from four birds and then suspended in a semen extender such as turkey sperm extender (TSE) [7], chicken sperm extender (CSE) [7], mouse sperm extender (CZB) [15], Ham's F-10 [16], Tyrode's medium [17], and TALP (a modified Tyrode's medium containing Tyrode's medium supplemented with albumin, lactate, and pyruvate) [11] and evaluated for the sustenance of motility using CASA. The percentage of motile spermatozoa was determined after every hour of incubation up to 8 h and the data was statistically evaluated by Kruskal–Wallis test on an hourly basis. Each experiment was repeated six times for each extender.

2.6. Effect of dilution on sperm motility

The optimum dilution required for the maintenance of semen was determined by collecting fresh ejaculates from four birds, pooling them together and diluting the semen with TALP in the ratio of 1:5, 1:10, 1:20, 1:100, and 1:200 (semen:TALP), respectively. The diluted semen aliquots were maintained at 37 °C in a CO_2 incubator (equilibrated with 5% CO_2 in air) and the percentage of motile spermatozoa was determined at every hour for 6 h, using a microscope with a stage warmer set at 37 °C. The entire experiment was repeated six times.

2.7. Effect of temperature on sperm motility

Semen from four birds was pooled and diluted 1:100 in TALP and maintained at 4, 24, and 37 °C and the percentage of motile spermatozoa was recorded on an hourly basis up to 7 h. The experiment was repeated six times at the above temperatures and Kruskal–Wallis test was applied to calculate significant differences in the percentage of motility at the temperatures tested.

2.8. Artificial insemination

Pigeons breed throughout the year and lay only two eggs per clutch with an inter-clutch period of 20–25 days. Therefore, we considered the day of egg laying as day 0 and inseminated the birds from day 18 onwards on alternate days till day 25 or the day of egg laying whichever is earlier. Each female was restrained manually and inseminated intra-cloacally [4,18] with 100 μl of semen diluted in TALP such that the sperm number was approximately 250–300 million spermatozoa with 70–80% motility per insemination. In these experiments five females were inseminated and the semen was pooled from four males. A human insemination catheter (Gynetics Medical Products, N.V. Hamont-Achel, Belgium) attached to a Gilson-P100 pipette or a 1 ml syringe was used for transferring the semen in to the cloaca. Before insemination, the females were stimulated by cloacal massage to facilitate opening of the cloaca [19]. After the eggs were laid, they were allowed to be incubated by the respective female till hatching. After 12–15 days of incubation, eggs were candled to ascertain fertilization and embryo development [4].

2.9. Cryopreservation of semen

Ejaculates from four birds were pooled, diluted 1:100 in TALP at 37 °C, and aliquots of 250 μl were transferred to 1.8 ml screw capped cryo vials (Nalge International, Denmark) containing equal volume of TALP supplemented with the cryoprotectants namely glycerol (4 or 8%), dimethyl sulfoxide (DMSO; 4 or 8%) or polyethylene glycol (PEG; 5 or 10%). In addition, TALP supplemented either with 8% glycerol or 8% DMSO and containing 20% egg yolk was also used as a cryoprotectant medium and then subjected to cryopreservation. TALP without egg yolk and any of the above two cryoprotectants served as the control. The cryo vials containing the semen suspended in the cryoprotectant medium were then subjected either to a fast freezing or a slow freezing regime in a programmable cryogenic unit (Consarctic, Gottingen, Germany). During fast freezing, the samples were cooled from 24 to 4 °C at 3 °C/min and later plunged directly into liquid nitrogen. In contrast, during slow freezing, the samples were cooled from 24 to 4 °C at 1 °C/min, subsequently to −80 °C at 8 °C/min and then plunged into liquid nitrogen. Twenty-four to 48 h later, the samples were thawed in a water bath at 37 °C for 1 to 2 min and assessed for percentage of motile spermatozoa by using a CASA system. All experiments were repeated six times for each cryoprotectant and concentration.

2.10. Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Kruskal–Wallis one-way analysis of variance was used to test the differences in semen characteristics in different seasons and also applied in the semen extender experiment. Spearman's rank correlation was used to test correlation among the semen characteristics and the Student's *t*-test was applied to test the difference in time-dependent motility at every hour and to compare the motility parameters between fresh and frozen-thawed spermatozoa.

3. Results

3.1. Collection and characteristics of semen

Ejaculates were obtained within 30 s from 90% of the birds following the massaging procedure as described by Cheng et al. [9]. A total of 1000 ejaculates were obtained from 10 pigeons following about 1100 massages over a period of 12 months. The color of the semen varied from pale cream to white and the semen volume ranged from 5 to 20 μl. The concentration of spermatozoa in each ejaculate was in the range of 0.5 to 14×10^9 spermatozoa per milliliter and the pH of semen varied from 6.0 to 7.3. CASA analysis indicated that the percentage of motile spermatozoa varied from 25 to 95% and progressive motility graded subjectively on a scale of 0 to 5 varied from 3 to 4.5. The osmolarity of neat semen was in the range of 338 to 352 mOsm. The mean percentage of morphologically normal spermatozoa was about 75.2% and that of pleiomorphic spermatozoa was about 24.8%. The pleiomorphic spermatozoa were either macrocephalic (3.2%) or possessed an amorphous head (8.9%). Some of the spermatozoa had either a bent mid piece (4.3%), a

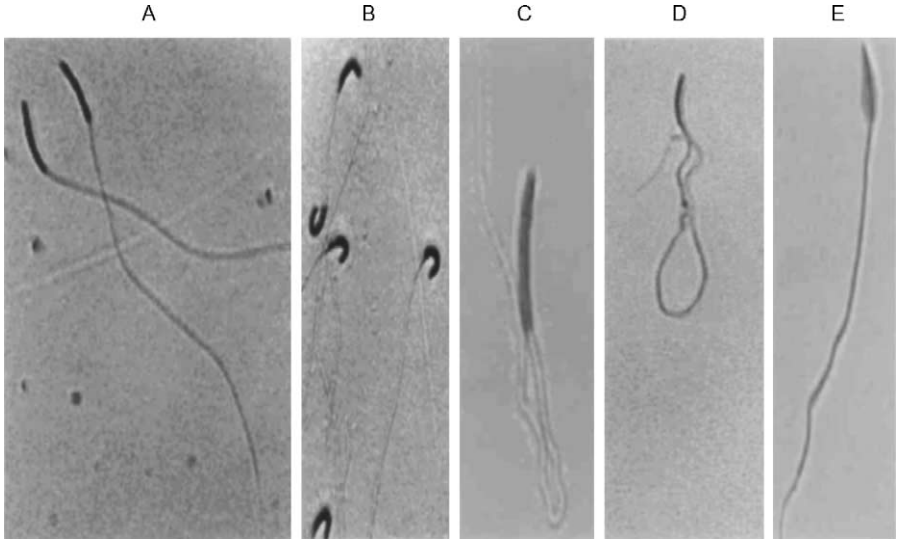

Fig. 1. Phase contrast micrographs showing morphology of Blue rock pigeon spermatozoa. (A) Normal; (B) bent head; (C) coiled tail; (D) coiled mid piece and tail; (E) amorphous head (400×).

coiled tail (4.8%), or a cytoplasmic droplet (3.5%) (Fig. 1; Table 1). A significant positive correlation was observed between semen volume and sperm motility ($r_s = 0.87$, $P < 0.001$); sperm motility and concentration ($r_s = 0.72$, $P < 0.01$); semen volume and normal spermatozoa ($r_s = 0.45$, $P < 0.01$); and sperm concentration and normal spermatozoa ($r_s = 0.53$, $P < 0.01$) using the Spearman correlation test.

Table 1
Semen characteristics of the Blue rock pigeon[a]

Parameter	Mean ± S.D.	Range
Ejaculate volume (μl)	10.5 ± 2.6	5–20
Semen pH	6.8 ± 0.2	6–7.3
Semen osmolarity (mOsm)	340 ± 1.2	338–352
Sperm concentration ($\times 10^9$ ml^{-1})	3.2 ± 0.46	0.5–14
Motile spermatozoa (%)	71.9 ± 3.1	25–95
Progressive motility (0–5 scale)	3.9 ± 0.6	3–4.5
Morphologically normal spermatozoa (%)[b]	75.2 ± 0.2	65–82
Morphologically abnormal spermatozoa (%)[b]		
Macrocephalic	3.2 ± 0.7	3–5
Amorphous head	8.9 ± 0.3	7–10
Bent tail	4.8 ± 0.2	3–5
Bent mid piece	4.3 ± 0.2	3–5
Proximal cytoplasmic droplet	3.5 ± 0.3	3–4

[a] The values represent mean ± S.D. of data from 1000 ejaculates of Blue rock pigeons ($n = 10$) collected twice a week during November 2000 to October 2001.

[b] Analysis was carried out on a total of 240 ejaculates from 10 birds collected fortnightly over a period of 1 year.

Table 2
Seasonal variation in the semen characteristics of the Blue rock pigeon

Season	Semen volume (μl)	Sperm motility (%)	Sperm concentration ($\times 10^9$ ml^{-1})	Normal spermatozoa (%)
Summer (March–June)	9.3 ± 5.1[a]	2.2 ± 18.6[a]	2.2 ± 1.8[a]	69.3 ± 5.2[a]
Monsoon (July–October)	13.1 ± 1.9[b]	84.4 ± 3.4[b]	6.1 ± 3.4[b]	81.4 ± 3.8[b]
Winter (November–February)	8.4 ± 4.8[a]	77.3 ± 16.4[a,b]	2.7 ± 1.8[c]	77.0 ± 3.2[c]

Values represent mean ± S.D. Different letters (a, b, and c) in superscript indicate significant difference between the seasons (Student's t-test: $P < 0.05$).

3.2. Seasonal variation in semen characteristics

To determine the influence of season, if any, on the semen characteristics of the pigeon, data collected from ten pigeons over a period of 1 year were analyzed following grouping of the 1000 ejaculates in to three seasons namely summer (March–June), monsoon (July–October), and winter (November–February) depending on their date of collection. Semen volume, percentage of motile spermatozoa, sperm concentration and percentage of normal spermatozoa varied depending on the season (Kruskal–Wallis test: $P < 0.05$) and were significantly increased during the monsoon season compared to the summer and winter seasons (Table 2; Students t-test: $P < 0.05$).

3.3. Optimization of conditions for semen handling

Attempts were made in the present study to determine suitable conditions for culturing of pigeon spermatozoa with respect to the buffer (extender) to be used, the degree of dilution to be made and the incubation temperature. In contrast to turkey sperm extender, chicken sperm extender, mouse sperm extender, Ham's F-10 and Tyrode's medium, the percentage of motile spermatozoa was best maintained in TALP (Table 3). Therefore, TALP was chosen as the buffer for all studies with pigeon semen. Further, in semen, diluted to 1:100 with TALP, the percentage of motile spermatozoa was significantly higher compared to semen diluted 1:5, 1:10, 1:50, and 1:200 (Table 4; Student's t-test: $P < 0.001$). It was also observed that semen diluted 1:100 with TALP when incubated at 24 and 37 °C exhibited no significant difference in the percentage of motile spermatozoa

Table 3
Effect of various semen extenders on the motility of the Blue rock pigeon spermatozoa

Extenders	Percentage of motile spermatozoa								
	0.25 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
TALP	95 ± 8	65 ± 10	55 ± 8.9	50 ± 11.7	40 ± 11.6	35 ± 12.0	15 ± 13.6	10 ± 9.2	5 ± 5.6
Tyrode	95 ± 9.3	55 ± 4.9	5 ± 2.5	2 ± 5.8	0	0	0	0	0
CZB	95 ± 16.3	25 ± 2.8	20 ± 3.8	15 ± 5.0	10 ± 2.3	5 ± 8.2	0	0	0
Chicken	95 ± 20	5 ± 19.5	2 ± 9.7	0	0	0	0	0	0
Ham's F-10	95 ± 7.5	35 ± 6.1	10 ± 5.2	0	0	0	0	0	0
Turkey	95 ± 10.5	10 ± 12.5	0	0	0	0	0	0	0

Table 4
Effect of semen dilution[a] on motility of the Blue rock pigeon spermatozoa

Dilution	Percentage of motile spermatozoa						
	0.25 h	1 h	2 h	3 h	4 h	5 h	6 h
1:5	84.2 ± 4.1	25.0 ± 15.4	1.6 ± 1.2	0	0	0	0
1:10	85.8 ± 8.5	30.9 ± 14.0	7.5 ± 6.2	1.0 ± 1.9	0	0	0
1:20	84.2 ± 4.7	35.8 ± 12.5	19.2 ± 4.7	5.8 ± 2.8	0	0	0
1:100	84.2 ± 4.7	55.0 ± 2.4	40.0 ± 4.1	30.8 ± 2.4	22.5 ± 4.1	14.1 ± 6.2	12.5 ± 7.2
1:200	84.5 ± 12.3	47.3 ± 4.0	35.0 ± 7.1	19.0 ± 6.2	14.0 ± 2.4	9.0 ± 6.2	7.5 ± 6.1

[a] Freshly collected semen was diluted with TALP and incubated for 6 h at 37 °C.

(Table 5; Student's *t*-test: $P > 0.05$). But, when incubated at 4 °C, all the spermatozoa were immotile and a significant difference in the percentage of motile spermatozoa was observed when compared to diluted semen incubated either at 24 or 37 °C (Student's *t*-test: $P < 0.001$).

3.4. Sperm motility kinetics by CASA

Using the conditions described in Table 6, the CASA system was used successfully to monitor the motility and motility parameters of pigeon spermatozoa. In freshly diluted pigeon semen, spermatozoa were extremely active, progressively motile and exhibited linear trajectories. By 9 h, the spermatozoa become sluggish and only about 10% of the spermatozoa were motile. Table 7 shows the time-dependent changes in the motility parameters of pigeon spermatozoa. VAP and VSL increased at 2 h whereas VCL remained unchanged. Subsequently all the velocity parameters including BCF decreased by 4 h and remained so till 9 h. Further, linearity (LIN) decreased after the second hour but ALH remained unchanged all through the incubation period of 9 h.

3.5. Artificial insemination

Following 23 intracloacal inseminations in five birds, the inseminated birds laid a total of 11 eggs. Five of these eggs were fertilized (5/11 or 45%) and a live chick was born following 18 days of incubation. In the remaining four fertilized eggs, the embryos were almost fully developed but did not hatch.

Table 5
Effect of semen[a] holding temperature on motility of the Blue rock pigeon spermatozoa

Temperature (°C)	Percentage of motile spermatozoa							
	0.25 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h
4	82.6 ± 2.5	30.0 ± 5.6	20.0 ± 4.9	10.0 ± 7.8	0	0	0	0
24	81.7 ± 5.9	51.0 ± 6.8	45.0 ± 2.1	35.0 ± 10.0	20.0 ± 10.0	10.0 ± 5.6	5.0 ± 8.9	0
37	81.7 ± 3.6	58.0 ± 12.0	51.0 ± 6.0	39.0 ± 1.2	30.0 ± 5.6	22.0 ± 9.0	15.0 ± 6.3	8.0 ± 5.6

[a] Freshly collected semen was diluted 1:100 with TALP and incubated at 4, 24, and 37 °C for 7 h.

Table 6
Conditions and parameter setup of HTM-IVOS motility analyzer used for the analysis of the Blue rock pigeon spermatozoa

Conditions/parameter setup	
Temperature (°C)	37
Apply sort	None
Frames acquired	30
Frame rate (Hz)	60
Minimum contrast	25
Minimum cell size (pixels)	4
Minimum static contrast	50
Threshold straightness (%)	80
Low VAP cut off (μm/s)	5
Medium VAP cut off (μm/s)	20
Low VSL cut off (μm/s)	20
Non-motile head size (pixels)	4
Non-motile head intensity	50
Static head size	0.1–2.92
Static head intensity	0.48–3.0
Static elongation (limits)	50–99
Slow cells motile	Yes
Magnification	2.04
Video source	Camera
Video frequency	60
Bright field	No
Image type	Phase contrast
Brightness for LED	2681
Chamber depth (μm)	100
Field selection mode	Auto
Minimum track points	15

3.6. Cryopreservation of semen

Semen samples showing more than 80% of motile spermatozoa prior to cryopreservation were subjected to cryopreservation. Following cryopreservation in TALP containing 8% DMSO with or without egg yolk, the post-thaw percentage of motile spermatozoa was about 40% with 70–80% exhibiting progressive motility. In contrast, when glycerol was used as a cryoprotectant, the post-thaw sperm motility was very poor (7–13%; Table 8). It was also observed that the slow-freezing protocol was better than the fast-freezing protocol and none of the semen samples frozen by the fast-freezing protocol showed any post-thaw sperm motility (Table 8).

The cryopreserved spermatozoa following thawing also exhibited linear progressive type of motility like the spermatozoa from neat semen but appeared to have a reduced velocity. This is further confirmed by the observation that all the velocity parameters such as VAP, VSL, and VCL were significantly decreased in spermatozoa following cryopreservation and thawing compared to spermatozoa from normal semen (Table 9). BCF and LIN were also reduced in the cryopreserved spermatozoa (Table 9).

Table 7
Time-dependent motility parameters of the Blue rock pigeon spermatozoa incubated in TALP at 37 °C[1] ($n = 100$)

Time (h)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	STR (%)	LIN (%)	BCF (Hz)
0.25	114.1 ± 23.7[a] (70–199)	109.3 ± 24.8[a] (64.5–196)	153.5 ± 26.0[a] (89.8–226.2)	5.3 ± 1.6[a] (2.5–11.1)	95.6 ± 5.6[a] (59–100)	71.4 ± 10.9[a] (40–89)	38.6 ± 2.2[a] (34.3–43.2)
2	121.3 ± 23.8[b] (74.9–171)	116.4 ± 25.3[b] (64.2–169.6)	158.6 ± 25.6[a] (97.3–201.3)	5.3 ± 1.4[a] (2.7–10.8)	95.6 ± 4.1[a] (74–100)	73.0 ± 8.2[a] (44–92)	37.3 ± 3.5[a] (32.1–40.1)
4	103.6 ± 20.2[c] (61.8–174.6)	97.6 ± 22.8[c] (49.6–173.2)	144.6 ± 22.6[b] (81.1–224.7)	5.5 ± 1.4[a] (3.2–10.5)	93.6 ± 6.5[b] (66–99)	67.3 ± 10.3[b] (40–90)	32.5 ± 3.0[b] (27.7–37.9)
6	101.3 ± 18.4[c] (58.4–155.3)	94.3 ± 20.2[c] (43.2–152.3)	141.8 ± 22.3[b] (80.5–190.2)	5.4 ± 1.6[a] (2.5–11.1)	92.8 ± 7.3[b] (52–100)	66.7 ± 10.8[b] (28–88)	31.5 ± 3.3[b] (28.8–37.4)
8	98.2 ± 21.5[c] (49.1–138.5)	90.8 ± 24.0[c] (29.2–135.5)	137.2 ± 23.7[b] (68.3–184.9)	5.5 ± 1.8[a] (2.9–16.5)	91.9 ± 9.9[b] (45–99)	66.2 ± 12.9[b] (23–87)	30.4 ± 3.9[b] (23.5–33.5)
9	85.3 ± 20.2[d] (41–146.4)	78.0 ± 20.7[d] (32.1–127.7)	129.1 ± 25.2[c] (62.9–201.1)	5.5 ± 1.6[a] (2.2–14.9)	91.0 ± 8.4[b] (48–99)	60.3 ± 10.6[c] (28–82)	30.4 ± 3.6[b] (26.5–34.5)

Different letters (a, b, c, and d) in superscript indicate that the values are significantly different (Student's t-test: $P < 0.05$).
[1] Values represent mean ± S.D. and values in parentheses indicate the range.

4. Discussion

In the present study, the basic spermatology of the Indian Blue rock pigeon was studied. Semen was collected without any fecal contamination from 10 Blue rock pigeons using the simple manual massage technique. Ninety percent of the attempts yielded an ejaculate in contrast to Cheng et al. [9] who reported only 40% success in semen collection. The massage technique has also been successfully used earlier in several other birds like the eagle, crane, and stork [20], Northern pintail duck [4], pigeon [9,21], and pheasant [22].

The ejaculate volume in pigeon ranged from 5 to 20 μl similar to that reported earlier for pigeon [9,21]. This is in contrast to the high volumes of ejaculates obtained in Northern

Table 8
Evaluation of glycerol, dimethyl sulfoxide (DMSO), and polyethylene glycol (PEG) as cryoprotectants for cryopreservation of the Blue rock pigeon semen[a] ($n = 6$)

Cryoprotectant	Concentration of cryoprotectant	Percentage of motile spermatozoa	
		Slow freezing	Fast freezing
Glycerol	4%	7.6 ± 1.5	0.00
	8%	9.0 ± 1.5	0.00
	8% + egg yolk (20%)	13.2 ± 1.5	0.00
DMSO	4%	8.6 ± 1.5	0.00
	8%	39.2 ± 4.3	0.00
	8% + egg yolk (20%)	37.8 ± 3.0	0.00
PEG	5%	0	0.00
	10%	0	0.00

[a] Semen suspensions were made in TALP to which the cryoprotectants were added. The samples were cooled from 24 to 4 °C at 1 °C/min, subsequently to −80 °C at 8 °C/min and then plunged in to liquid nitrogen. The frozen samples were thawed in a water bath at 37 °C for 1–2 min and evaluated for percentage of sperm motility. Values represent mean ± S.D.

Table 9
Comparison of the motility parameters of Blue rock pigeon spermatozoa from fresh semen diluted in TALP and from semen cryopreserved in TALP containing 8% DMSO following thawing

Motility parameter	Unit	Spermatozoa from fresh-diluted semen ($n = 100$)	Spermatozoa from cryopreserved semen following thawing ($n = 100$)
Path velocity (VAP)	μm/s	114.1 ± 23.7 (70–199)	86.3 ± 4.0* (40.6–139.1)
Progressive velocity (VSL)	μm/s	109.4 ± 24.8 (64.5–196)	81.2 ± 4.0** (38–136.1)
Curvilinear velocity (VCL)	μm/s	153.6 ± 26.0 (89.8–226.2)	130.0 ± 5.3** (67.8–209)
Beat cross-frequency (BCF)	Hz	38.6 ± 2.2 (34.3–43.2)	32.4 ± 1.4** (13.6–55.4)
Amplitude of lateral head displacement (ALH)	μm	5.3 ± 1.6 (2.5–11.1)	5.3 ± 0.2 (2.3–8.1)
Straightness (STR)	%	95.6 ± 5.6 (59–100)	92.6 ± 1.2* (55–99)
Linearity (LIN)	%	71.4 ± 10.9 (40–89)	63.2 ± 2.0** (36–88)

Values represent mean ± S.D.
* $P < 0.01$, significantly different as determined by Student's t-test.
** $P < 0.001$, significantly different as determined by Student's t-test.

pintail duck (66 μl; [4]), Mallard duck (105 μl; [18]) and in pheasant (*Pheasianus colchicus mongolicus*) (390 μl; [23]). The average sperm concentration of spermatozoa in the ejaculate (3.2×10^9 ml^{-1}) was in agreement with the report of Cheng et al. [9], however, it was higher than that reported by Owen [21]. The mean semen pH was 6.8, which is similar to that observed in domestic fowl, Pekin duck, Mallard duck, and in pheasant [6,18,22]. About 25% of the spermatozoa exhibited abnormal morphology similar to that reported in pigeon by Ducci et al. [24] and the predominant sperm abnormalities were the presence of an amorphous head, macrocephaly, a bent mid piece and a bent tail, etc. All these sperm abnormalities have been described in other avian species [4,6]. Significant positive correlations did exist between semen volume and sperm concentration and motility, semen volume and normal spermatozoa, and sperm concentration and normal spermatozoa. Similar findings were recorded in pheasant species but the correlation between motility and semen volume was not significant [23].

CASA has been used effectively to study the motility patterns and kinetics of spermatozoa in various mammalian species such as the hamster [14], the lion [25], the tiger [25], the leopard [26], and gazelle [27]. However, to our knowledge, there is no detailed study on the motility parameters of avian spermatozoa using CASA except for a documentation of Froman and Feltmann [28] who monitored only VSL of rooster spermatozoa. CASA data indicated an increase in the velocity parameters—VAP and VSL 2 h following dilution in TALP. This indicates that neat semen following dilution is probably adjusting to the dilution effect and the extender and recovers by 2 h. Subsequently a concomitant decrease in VAP, VSL, VCL, LIN, and BCF was observed thus confirming the subjective observation that spermatozoa with time become sluggish and exhibit a curved trajectory. In this study, VSL of pigeon spermatozoa was recorded to be 85.23 μm/s which was much higher than that reported for rooster spermatozoa (39 μm/s; [28]).

Various avian as well as rodent sperm extenders were evaluated for their ability to sustain spermatozoal motility. Surprisingly, pigeon spermatozoa showed a total decline in percentage of motile spermatozoa by 2 h in avian sperm extenders (Turkey- and

chicken-sperm extender; [7]) indicating that an extender optimized for one species may not be suitable for another species. On the contrary, TALP (a modified Tyrode's medium containing albumin, lactate, and pyruvate) supported the motility of pigeon spermatozoa up to 6 to 8 h whereas in Tyrode's medium the motility declined by 2 h, thus implying that probably albumin, lactate and pyruvate are required for the sustenance of motility of pigeon spermatozoa.

Semen dilution studies indicated that 1:100 dilution of pigeon semen in TALP was the optimum and at dilutions of 1:5, 1:10, and 1:50 the motility declined drastically. Semen of drake when diluted 1:5 in Lake's diluent showed marked decrease in sperm motility [29]. This unique feature of pigeon semen wherein it could be diluted beyond 1:5 and still maintain motility could be a great advantage in semen banking and could be used for inseminating a larger number of birds. Further, the optimum storage temperature (in vitro) for pigeon spermatozoa was observed to be 37 °C when diluted 1:100 in TALP. These results are not in accordance with the observations of Cheng et al. [9] who observed vigorous motility of pigeon spermatozoa when diluted 1:20 with Beltsville Poultry Semen Extender and incubated at 30 °C. At this juncture, it may be worth while to mention that although the normal body temperature of birds is around 41–42 °C, ejaculated spermatozoa of various avian species show optimum motility at 30 °C where as they exhibit decrease in motility at 40 °C [30,31].

Earlier studies had indicated that pigeons breed throughout the year [9,32]. Evaluation of their semen profiles all through the year indicated that the semen characteristics vary depending on the season and exhibit two annual peaks with motility and viability reaching peak values in March and November when the ambient temperature ranged between 19 and 24 °C [9]. In accordance with Cheng et al. [9], in the present study also it was observed that the semen characteristics of the Blue rock pigeon varied significantly depending on the season. But unlike the observations of Cheng et al. [9], semen characteristics such as semen volume, sperm motility, sperm concentration and percentage of normal spermatozoa were observed to be significantly increased only once during the year and it matched with the monsoon season (Table 2). During the monsoon season, that is in the months of August and September there is plenty of rain, high humidity, and moderate temperature (23–32 °C). The difference in the seasonality between the Indian Blue rock pigeon and that of the pigeons from Taiwan [9] may be due to the different environmental conditions in India and Taiwan. For instance, the average temperature in Taiwan ranges from 17 to 29 °C and the maximum barely exceeds 35 °C [9]. This temperature range is in contrast to the study site in Hyderabad, India where the temperature ranges from 22 to 42 °C with temperatures hovering above 35 °C and reaching a maximum of 40–45 °C in the months of March to May coinciding with summer. It is interesting to note that the lower limit of the temperature in India coinciding with the monsoon season is 23 °C similar to 24 °C observed in Taiwan when the Blue rock pigeon shows peak semen quality. The present study also confirms earlier observations that semen characteristics such as sperm viability, motility, concentration, and percentage of normal spermatozoa were lowest during summer [9,33]. In addition, heat-induced subfertility has also been reported in roosters at 32 °C [34].

Following 23 intracloacal inseminations in pigeon, a total of 11 eggs were laid. Five of these eggs were fertilized (45%) and a live chick was born from only one of the fertilized eggs (20%). In the remaining four fertilized eggs, the embryos were almost fully developed

but failed to hatch. The reason for this is not known but could be attributed to the female abandoning the eggs. Our approach of inseminating birds intra-cloacally is in agreement with earlier reports indicating that cloacal inseminations are effective [19] and was successful in Northern pintail duck and Mallard duck [4,18]. Similar fertilization success rates were reported in Northern pintail duck (51%; [4]) and pheasant species (30–40%; [22]) using intra-cloacal insemination with 100 μl diluted semen. A review of the earlier literature indicated that in avian species normally diluted semen is used for insemination and the extent of dilution ranges from a minimum of 1:2 as in Northern pintail duck [4] and a maximum of 1:20 in chicken [35]. But, irrespective of the extent of dilution the sperm number used for insemination ranged from 20 million [36] to 7400 million [4] as in Houbara bustard and Northern pintail duck, respectively. Therefore, in this study semen used for insemination was diluted so as to obtain a sperm count of 250 to 300 million spermatozoa. This number of spermatozoa is likely to represent three to four ejaculates of the Blue rock pigeon in the monsoon season when ejaculate volume is about 13 μl and sperm concentration is 6 million/μl. In future studies, it would be relevant to determine the optimum dilution of semen and sperm count required for fertilization and hatchability.

The critical steps in successful cryopreservation of avian spermatozoa are the choice of the cryoprotectant, cooling rate, and freezing and thawing conditions. In the present study, 8% DMSO with or without egg yolk (20%) was observed to be the preferred cryoprotectant for pigeon spermatozoa compared to the cryoprotectants PEG and glycerol. DMSO is a standard cryoprotectant for poultry semen and has an advantage over glycerol [37], which has been reported to possess a contraceptive effect [38,39]. Further the slow-cooling protocol of cryopreservation yielded a greater percentage of motile spermatozoa after thawing the frozen semen in accordance with earlier studies from avian species such as domestic turkey, golden eagle, Bonelli's eagle, peregrine falcon, and Northern pintail duck [4–7]. CASA analysis of the motility parameters of spermatozoa prior to cryopreservation and after cryopreservation and thawing indicated a decrease in the velocity parameters (VAP, VCL, and VSL) and STR and LIN implying that the spermatozoa were moving slowly and their trajectories were less planar. Similar observations were also made with respect to cryopreserved and thawed spermatozoa of lions [25], tigers [25], and leopards [26].

5. Conclusions

Seasonal variation was observed in the semen characteristics of the Blue rock pigeon. Semen volume, percentage of motile spermatozoa, sperm concentration, and the percentage of normal spermatozoa were observed to be the best during the monsoon season. TALP proved to be a suitable extender and the optimum dilution and temperature for short-term storage was 1:100 and 37 °C, respectively. CASA has been standardized for pigeon spermatozoa and to the best of our knowledge this is the first detailed report on analysis of sperm motility by CASA in any avian species. Further, 8% DMSO with or without egg yolk (20%) proved to be the best of the three cryoprotectants tried. Further, artificial insemination in pigeons using diluted semen resulted in the birth of a live chick.

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