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1 **Original Research Paper**

2 **Effect of season and age on Indian red jungle fowl (*Gallus gallus murghi*) semen**
3 **characteristics: A 4-year retrospective study**

4
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15
16 **Running Title:** Effect of season and age on semen characteristics of Indian Red Jungle Fowl

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26 ABSTRACT

27 The reproductive potential of the adult males is expected to vary with age/season and largely
28 differ not only in closely related avian species but even in subspecies, breeds and/or strains of
29 the same species. Thus, it is pre-requisite to have knowledge of seminal parameters to
30 achieve maximum production potential of at-risk species for *ex situ in vitro* conservation
31 programs. A 4-year study was designed to evaluate the effect of age and season (spring,
32 summer, autumn and winter) on semen characteristics of Indian red jungle fowl (*Gallus*
33 *gallus murghi*) in retrospective manner. Semen ejaculates (n=1148) were regularly collected
34 from eight adult Indian red jungle fowls from 6 to 54 months of age. Quantitative and
35 qualitative semen parameters viz; volume (μL), concentration ($1 \times 10^9 \text{ mL}^{-1}$), total sperm
36 number per ejaculate ($1 \times 10^9 \text{ mL}^{-1}$), motility (%), viability (%), plasma membrane integrity
37 (%), acrosome integrity (%) and semen quality factor were recorded. A chronological
38 increasing trend with age of most sperm quantitative and qualitative traits (semen volume,
39 sperm concentration, total sperm number per ejaculate, plasma membrane integrity, viability,
40 acrosomal integrity, semen quality factor) was observed. The highest values were observed at
41 four years of age ($P < 0.05$) with the exception of sperm motility that was not affected by the
42 age. Spring was the best season for sperm parameters viz; volume, motility, plasma
43 membrane integrity, viability and acrosomal integrity ($P < 0.05$), however a remarkable
44 sperm production was noticed all over the year. It is concluded that Indian red jungle fowl
45 exhibit an evolution of sperm production that greatly differs in many points from other fowl
46 sub-species. It is suggested that semen ejaculates of highest quality achieved for semen
47 banking at the age of four year in the spring season.

48

49 **Keywords:** Semen characteristics; Age; Season; Quantitative semen quality; Qualitative
50 semen quality; Indian red jungle fowl

51 1. Introduction

52 The red jungle fowl, a member of family Phasianidae and genus *Gallus* is considered
53 one of the ancestors of the domestic fowl [1-3]. The red jungle fowl is enlisted as least
54 concern species in IUCN Red List of Threatened Species [4] but its population is
55 continuously declining in its native habitat [2,5]. Red jungle fowl is considered as endangered
56 in Singapore list of Threatened Plant and Animal Species, facing high risk of extinction in the
57 wild [6]. From five fowl subspecies, Indian red jungle fowl (*Gallus gallus murghi*) is native
58 to Northern India and Azad Jammu and Kashmir, Pakistan [7-10]. The Indian red jungle fowl
59 population is found in restricted areas in fragments, and is more likely to collapse of genetic
60 diversity and population decline due to sudden unexpected threats [2,11-13]. The factors
61 contributing in population reduction are habitat destruction, egg collection, predation and
62 genetic hybridization with domestic fowl [7,13].

63 The decline in Indian red jungle fowl needs special attention; several reports suggest
64 adopting appropriate *ex situ* conservation strategies [2,11,12]. For *ex situ in vitro*
65 conservation, semen banking is considered one of the most applicable options for bird species
66 that are facing continuous decline in their natural habitat and the threats cannot be easily
67 mitigated and/or ameliorated [14-20]. Cryo-banking of valuable male germplasm could be an
68 important assisted reproductive biotechnology to maintain genetic diversity and *in vitro ex*
69 *situ* conservation program [8,9]. It is well established that avian semen can be stored in
70 frozen state for infinite period of time without significant decline in fertilizing potential
71 [17,21,22]. The characteristics of the semen ejaculate viz; volume, concentration, motility,
72 viability, plasma membrane and acrosome integrity, semen quality factor determine the
73 reproductive potential of the cocks either used in natural or artificial breeding program
74 [8,9,23-25]. Nevertheless, semen quality varies with age and season of collection in birds that
75 may affect fertilizing capacities accordingly [26-28]. The success of semen banking depends

76 on successful cryopreservation and acceptable frozen thawed semen quality and fertility
77 outcomes that depend on the initial quality of the ejaculate to be used in processing for
78 cryopreservation [17,20,29]. The reproductive performance of the male fowl varies with age
79 and seasons; largely differ not only in closely related bird species but also even in subspecies,
80 breeds and/or strains of the same species [30]. Scarce available literature depicted immense
81 variations in semen characteristics of five subspecies of red jungle fowl [8,31]. The
82 knowledge about retrospective seminal parameters is therefore the prerequisite to achieve
83 maximum production potential of at-risk species for *ex situ in vitro* conservation.

84 Therefore, present, 4-year retrospective study was designed to elucidate the effect of
85 age and season (autumn, spring, summer and winter) on semen characteristics (motility,
86 semen volume, sperm concentration, total sperm number per ejaculate, viability, semen
87 quality index, plasma membrane and acrosome integrity) of Indian red jungle fowl.

89 2 Materials and methods

90 2.1 Experimental birds

91 The chicks of Indian red jungle fowl were reared at Avian Research Center, Pir Mehr
92 Ali Shah Arid Agriculture University Rawalpindi. The Rawalpindi region (33° 36' 0" North,
93 73° 4' 0" East) is located in Southern slopes of the North-Western Himalayas including large
94 mountain tracks with rich valleys traversed by mountain rivers. This region falls in humid
95 and subtropical region having four seasons; autumn (13.9°C to 33.5°C), spring (10.1°C to
96 23.7°C), summer (21.5°C to 46.5°C) and winter (-2.8°C to 20.0°C). The average annual
97 rainfall of this region is about 1,249 millimeters. The average rainfall in each season was
98 73.13 mm/autumn, 39.2 mm/spring, 224.73 mm/summer and 37.3 mm/winter recorded
99 during the study period. The average temperature of the area ranges from -3.9°C to 48.3°C
100 (Pakistan Metrological Department, 2012-2015). The chicks were kept in individual pens

101 106.68 cm x 121.92 cm, exposed to natural day-light and standardized environmental
102 conditions without any significant changes during the entire 4-year study period. The birds
103 were offered commercially available poultry cock breeder feed (100g/day). Fresh water was
104 available to the birds all the daylong throughout the experimental period. The birds were
105 subjected to abdominal massage and were trained for semen collection until the birds were
106 able to donate neat and clean semen without fecal contamination. This training was continued
107 until we were able to get sufficient semen for further processing and experimental work.
108 Semen collection of eight cocks was performed on alternate days, depending on individual
109 cock quality and libido.

110 2.2. *Semen collection and quantitative evaluation*

111 The birds were subjected to semen collection training through abdominal massage as
112 described by Burrows and Quinn [8, 32,33]. The successful ejaculate was collected after 4
113 weeks of training. Semen was collected from individual birds in a graduated plastic tube. A
114 total of 1148 ejaculates were studied during the 4-year study period (2012-2015). Semen
115 volume was measured in microlitres using micropipette.

116 2.2.1 *Sperm progressive motility*

117 Sperm motility of each ejaculate was determined as described by Zemjanis [34] by
118 mixing 10 μ L of semen samples in 500 μ L of phosphate buffer saline (pH 7.2, 300
119 mOsm/kg). The percentage of motile sperm was subjectively evaluated on a scale ranging
120 from 0 to 100% by putting a drop of semen sample on a pre-warmed glass slide (37°C) under
121 phase contrast microscope (x400, Olympus BX20, Japan).

122 2.2.2 *Sperm concentration*

123 Sperm concentration was measured by taking 1 μ L of semen and 200 μ L of formal
124 citrate solution (1mL of 37% formaldehyde in 99 mL of 2.9% (w/v) sodium citrate) with

125 Neubauer haemocytometer (Marienfeld, Germany) under phase contrast microscope (x400,
126 Olympus BX20, Japan) throughout the 4-year study period [8].

127 2.2.3. *Total sperm number per ejaculate*

128 Total sperm number per ejaculate was obtained by multiplying the total volume with
129 the concentration of ejaculates obtained during the study period [8, 35].

130 2.2.4 *Plasma membrane integrity*

131 Plasma membrane integrity of spermatozoa was assessed by using hypo-osmotic
132 swelling test (HOS) as described by Santiago-Moreno et al. [36]. The HOS solution was
133 prepared by adding 1 g of sodium citrate to 100 mL of distilled water. Semen (25 μ L) was
134 mixed with 500 μ L of HOS solution (100 mOsm/kg) and incubated at 37°C for 30 minutes. A
135 drop of incubated solution was placed on a pre warmed (37°C) slide and fixed in buffered 2%
136 glutaraldehyde. The spermatozoa showing swollen heads, swollen and coiled tails were
137 classified as normal spermatozoa having intact plasma membrane. A total of 200
138 spermatozoa were counted at four separate fields under a phase-contrast microscope (1000x
139 with oil immersion).

140 2.2.5 *Sperm viability*

141 Viability (% Live / total sperm) of Indian Red Jungle Fowl spermatozoa was
142 examined by adding eosin-nigrosin to the lake glutamate solution [37]. Twelve drops of stain
143 were mixed with 1 drop of semen. A smear was made on a glass slide, fixed and air dried. A
144 total of 200 spermatozoa were studied per slide under a phase-contrast microscope (1000x
145 with oil immersion). The mixture provides a clear background in the smear to enhance the
146 contrast of white and pink. Unstained and straight sperm were considered as “live and
147 morphologically normal” while the pinkish stained as “dead ” sperm.

148 2.2.6 *Acrosomal integrity*

149 Acrosomal integrity of Indian Red Jungle Fowl spermatozoa was assessed through
150 Giemsa stain [38]. The stain was prepared by adding Giemsa (3g) and phosphate buffer saline
151 at pH 7.0 (2 mL) into 35 mL water. Smear was prepared by taking a drop of semen sample on
152 a clean glass slide, dried and fixed in neutral formal-saline (5% formaldehyde) for 30 min.
153 Fixed slides were kept in Giemsa stain for 1.5 hrs. Sperm with normal acrosome appeared to
154 be evenly stained; abnormal spermatozoa were unevenly stained while spermatozoa having
155 ruptured acrosome remained unstained. A total of 200 spermatozoa were observed at least in
156 four separate fields under a phase-contrast microscope (x400; Olympus BX20, Japan) at
157 magnification of 1000x with oil immersion.

158

159 2.2.7 Sperm quality factor

160 Sperm quality factor (SQF) of each semen ejaculate was calculated to check the
161 number of spermatozoa potentially capable of fertilization in ejaculate. Sperm quality factor
162 was calculated by the following formula [24].

$$\text{SQF} = \frac{\text{Sperm concentration} \left(\frac{\text{billion}}{\text{mL}} \right) \times \text{ejaculate volume (mL)} \times \text{normal livespermatozoa}}{100}$$

163 2.3 Statistical analysis

164 The data were analyzed with statistical software Megastat[®] version 7.25 Mc-Graw-Hill
165 New Media, New York, for excel. The descriptive statistical parameters (mean, standard error
166 of mean) were calculated for different age groups and seasons. To compare differences
167 between seasons and age groups, data were analyzed using analysis of variance in completely
168 randomized design with unequal replications. Post hoc comparisons between the means were
169 done though least significant difference test to determine differences among individual
170 groups at different ages and seasons. Differences were considered statistically different when
171 $P < 0.05$, and presented as Mean \pm SEM.

172

173 3. Results

174 The data on ejaculate volume, sperm concentration and total sperm number per
175 ejaculate of red jungle fowl from 6 to 54 months of age are given in Table 1. Semen volume,
176 sperm concentration and total sperm number per ejaculate was recorded significantly
177 ($P<0.05$) higher at 43-54 months of age followed by 31-42, 19-30 and 6-18 months of age.
178 The highest ($P<0.05$) semen ejaculate volume, sperm concentration and total sperm number
179 were recorded in spring season followed by winter, autumn and summer from 6 to 54 months
180 of age.

181 The data on qualitative semen quality parameters viz; motility, plasma membrane
182 integrity, viability, acrosome integrity and semen quality factor were given in Table 2. Sperm
183 motility did not differ ($P>0.05$) with increase in age from 6 to 54 months of age.
184 Nevertheless, an increase was observed in plasma membrane integrity, viability, acrosome
185 integrity and semen quality factor with the increasing age. The highest ($P<0.05$) values for
186 aforementioned parameters were recorded during 43-54 months of age compared to 31-42,
187 19-30 and 6-18 months of age. A highly positive ($P<0.05$) effect of spring season was
188 recorded on most of the quality parameters with the exception of motility that was
189 equivalently efficient in autumn, spring and summer and only altered in winter.

190 4. Discussion

191 In conservation programs, the success of AI and semen cryopreservation depends on
192 the initial semen quality of ejaculate [17]. Semen evaluation, based on *in vitro* measurement
193 of sperm motility, plasma membrane integrity, viability, acrosomal integrity and semen
194 quality factor (SQF) provides an indirect clue about the fertility potential of the males
195 [25,8,23,24]. The production of high quality semen ejaculates depends on the reproductive
196 cycle of the species; furthermore, species or subspecies specific variations depend on the
197 birds, their age, their environment including climate changes such as seasons [30, 36,39-46].

198 In contrast to domestic fowl, we showed for the first time in the present study that the highest
199 production and quality of semen of Indian red jungle fowl is not reached until the fourth year
200 of age and spring stays the best season.

201 Our results greatly differ from the observations made in most domestic fowl as Long
202 et al. [47] reported a decrease in sperm concentration from 1 to 1.5 years of age, a well-
203 known fact in the breeding practice. Nevertheless, this situation would be modulated in local
204 breeds of tropical area as Chotesangasa [48] and Sonseeda et al. [45] found an increase in
205 Thai indigenous chicken sperm concentration between 1 and 2 years of age. However, our
206 results with Indian red jungle fowl appeared to be “extreme” as 4 years of age is the best in
207 terms of sperm concentration and volume. Semen volume has already been reported to differ
208 with the age in Leghorns [30]. Contrary to our findings, broiler males showed maximum
209 semen volume in the first year of age and then decreased with age, a difference with here, one
210 of the presumed ancestors, the red jungle fowl. As a way of consequence, the total number of
211 sperm per ejaculate increased with the age, and was observed highest when the Indian red
212 jungle fowls were 4 years old. This leads to suggest an increase in the efficiency of sperm
213 production with the age in this wild fowl. Many hypotheses may arise from this observation
214 including suggestion of increasing adaptation of the species to its environment, nutrition, and
215 management conditions. However, due to low mean volume of semen, total sperm number
216 per ejaculate (billion) were still lower in Indian red jungle fowl compared to Malaysian red
217 jungle fowl [31] and domestic fowls [47,49]. Would there be a sort of “compensation” of this
218 low daily sperm production through and increasing of the longevity of sperm production?

219 In addition to the increase of sperm production with the age, it was evident that the
220 functionality of the sperm produced by Indian red jungle fowl increased with the age to reach
221 a maximum at 4 years as most of the quality parameter measured here (Hypo-osmotic test,
222 livability, acrosome integrity) were at their optimum at 43-54 months of age.

223 More precisely, Indian red jungle fowl spermatozoa showed greater resistance to the
224 hypo-osmotic solution at 37°C at 4 years of age. These changes would indicate that sperm
225 plasma membrane increases its “plasticity” with the age and thus its functionality. Indeed, the
226 response of spermatozoa towards hypo-osmotic solution in terms of swollen head and coiled
227 tail is due to the movement of physical and biochemical compounds which play essential
228 roles in maintaining the viability and fertilizing ability of sperm [50-52]. Shanunagum et al.
229 [44] reported that response of spermatozoa to the hypo-osmotic environment at one year of
230 age was maximum compared to 6 months of age; and described that age and genetic factors
231 contribute to the quality of semen in naked neck and dwarf chicken. But none of these studies
232 examined 3 or 4 years old fowl, thus limiting the potential comparison with our study.

233 Sperm viability and the integrity of the sub-cellular fraction of sperm such as the
234 acrosome are of absolute significance for the success of fertilization. In the present study,
235 viability and acrosome integrity were highest at the age of 43-54 months, a fact that is
236 opposite to the results described in previous reports. Indeed, decreases in viability and sperm
237 morphological integrity have previously been reported to coincide with the end of a
238 reproductive period between 1 and 1.5 years of age in free-range poultry [44], turkeys [53]
239 and roosters [54,55]. Such decrease has been related to changes in lipid composition and
240 decrease in fertility [55]. However, for these parameters also, the comparison between the
241 literature results and our results is limited since older ages were not explored in earlier
242 studies. One hypothesis to explain results could be due to late optimal sexual maturity in
243 Indian red jungle fowls. The SQF is dependent on the quantitative parameters of semen and
244 on one qualitative parameter, the number of morphologically normal and live spermatozoa. In
245 the present study, the SQF was significantly higher at 43-54 months of age compared to other
246 ages. As discussed earlier, the Indian red jungle fowl is a resident of Temperate and Tropical
247 forest and developed a high tolerance to environmental variations that could be “cost-

248 intensive” for the animal metabolism and consequently the sexual maturation. We suggest
249 that the Indian red jungle fowl would get fully mature at later ages compared to other fowls
250 [31].

251 Sperm motility was the only quality factor that was not affected by the age in the
252 present study, partially confirming the results obtained by Sonseeda et al. [45] on Thai
253 indigenous chicken of over 30 months. Sperm motility is a predictive parameter of viability
254 and quality of semen. However, in the present study, this parameter did not follow the
255 evolution of the viability measurements. This could indicate a specific reproductive
256 maturation process where the different functions of the sperm do not reach the maturity at the
257 same time; the signaling pathways and flagellar structures related to motility being fully
258 mature much earlier than plasmatic membranes and acrosome attributes.

259 The sperm production, volume and concentration of sperm were higher in spring and
260 low in the other seasons with a remarkably low semen volume in summer. The sperm
261 production is highly species-specific and even varies among different strains or breeds [46],
262 and may vary considerably throughout the year and seasons among avian species [30,40].
263 Environmental factors such as photoperiod and temperature affect semen production. In mild
264 climates with large range of photoperiods/temperatures, domestic fowl sperm production is
265 totally stopped for some months during autumn/winter and reaches maximum during spring.
266 In subtropical desert and in Saharan climates with a smaller range of annual photoperiods,
267 sperm production may be severely affected by the extremely hot temperatures and lack of
268 precipitation of the summer [30,56]. These environmental cues have also been expressed in a
269 breed specific manner. Some breeds show consistent semen production throughout the year
270 as in Thai chicken held in tropical humid climates [45,57,58].

271 Our study shows that the seasonal sperm production of Indian red jungle fowl is not
272 so far from the Thai indigenous chicken “model”, but also shows characteristics close to the

273 “Saharan model” with summer variations potentially related to the high temperatures of the
274 Pakistan summer. Indeed, deleterious effects of high temperatures on germ cells have already
275 been described [59]. The results of the present study are in agreement with previous studies
276 on Northern pintail ducks and Nigerian local domestic chicken in which high summer
277 temperatures caused temporary reduction in sperm production [40,43,59]. The low volume of
278 semen in hot summer could tentatively be related to the absence of cutaneous heat
279 evaporation that cannot mitigate the stressful events of heat disruption in summer season
280 [60].

281 In our study, spring was identified as the best season for the SQF that mixes
282 quantitative and viability sperm traits and for many other sperm quality traits (motility,
283 viability, acrosome integrity). In mild climates, the lengthening of photoperiod in spring
284 season acts as an environmental cue, stimulates the secretion of FSH, LH and testosterone,
285 which play important role in spermatogenesis and semen production [61]. Photoperiod
286 increase has previously been reported to increase the number of morphologically normal and
287 live sperm as well as motility and SQF in domestic fowls such as free range poultry,
288 Leghorns and others [30,62,63]. At the opposite, poor livability and viability of sperm in
289 winter or autumn have directly been associated with the shortening of the photoperiod in free-
290 range poultry [64] and turkey [53]. Poor motilities were also reported in correlation with loss
291 of energy and mitochondrial integrity in shortened photoperiod compared to spring collected
292 semen [65]. Seasonal differences in mitochondrial permeability [66] and its membrane
293 phospholipids composition [67] could also contribute to explain the greater sensitivity of
294 sperm in time of short photoperiod [64].

295 In the “Saharan climate,” the semen quality of Nigerian shika brown in terms of live
296 and normal sperm was reported maximum in winter season, which is in contrast to the present
297 study [68]. The reason of these differences could possibly be attributed to heterogeneity of

298 the semen quality among breeds, strains and species-specificity [47], but also to the specific
299 Saharan climate. The terrible dry and hot summer would have a significant role in the
300 adaptation of the reproductive season, the best season of reproduction being consequently
301 moved to winter. Regarding this parameter, the “Pakistan” environment of the Indian red
302 jungle fowl presents a more classical but “mixed” seasonal effect since sperm production is
303 present all over the year.

304 It is concluded that Indian red jungle fowls exhibit better semen quality in spring
305 season and chronological increasing trend with maximal quality at the age of 43-54 months in
306 terms of volume, sperm concentration, total sperm number per ejaculate, motility, viability,
307 semen quality factor, plasma membrane and acrosome integrity. It is suggested that semen
308 ejaculates of maximal quality can be achieved at the age of four years in the spring season.
309 These elements are very important in order to build an AI program for the conservation of
310 genetic diversity of the Indian red jungle fowl.

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1 Table 1: Effect of season and age (Mean \pm SE) on quantitative efficiency of spermatogenesis in Indian red
 2 jungle fowl: A 4-year retrospective study (n=8cocks/1148ejaculates)

	Season	Age in months			
		6-18	19-30	31-42	43-54
Semen volume (μ L)	Autumn	41.9 \pm 5.1 ^{c,z}	56.5 \pm 4.4 ^{b,y}	68.0 \pm 7.5 ^{b,y}	73.4 \pm 2.9 ^{c,x}
	Spring	88.7 \pm 9.5 ^{a,z}	78.5 \pm 7.6 ^{a,y}	106.6 \pm 6.8 ^{a,x}	160.1 \pm 8.2 ^{a,w}
	Summer	25.3 \pm 4.3 ^{d,z}	26.4 \pm 2.8 ^{d,y}	25.6 \pm 1.9 ^{c,x}	39.9 \pm 3.6 ^{d,w}
	Winter	41.1 \pm 4.1 ^{b,y}	44.2 \pm 2.9 ^{c,y}	63.3 \pm 5.4 ^{b,x}	93.8 \pm 4.2 ^{b,w}
Sperm concentration (1×10^9 mL ⁻¹)	Autumn	0.45 \pm 0.1 ^{b,z}	0.95 \pm 0.2 ^{b,y}	2.09 \pm 0.3 ^{a,x}	3.00 \pm 0.2 ^{b,w}
	Spring	1.11 \pm 0.2 ^{a,z}	1.83 \pm 0.3 ^{a,y}	2.50 \pm 0.2 ^{a,x}	3.41 \pm 0.3 ^{a,w}
	Summer	0.86 \pm 0.2 ^{a,z}	0.91 \pm 0.1 ^{b,y}	2.00 \pm 0.3 ^{a,x}	2.54 \pm 0.1 ^{b,w}
	Winter	0.53 \pm 0.1 ^{b,z}	1.14 \pm 0.1 ^{b,y}	2.37 \pm 0.2 ^{a,x}	3.01 \pm 0.2 ^{b,w}
Total sperm per ejaculate (1×10^9)	Autumn	0.03b,y	0.06b,y	0.14b,x	0.22b,w
	Spring	0.12a,z	0.15a,y	0.21a,x	0.37a,w
	Summer	0.02b,x	0.04b,x	0.07b,x	0.13c,w
	Winter	0.02b,y	0.04b,y	0.14b,x	0.25b,w

3 The values (mean \pm SE) for a given semen parameter in a column (season = a, b, c, and d) and
 4 row (age = w, x, y and z) with different superscript differ significantly (P<0.05).

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13 Table 2: Effect of season and age (Mean \pm SE) on qualitative semen parameters in Indian red jungle fowl:
 14 A 4-year retrospective study (n=8 cocks/ 1148 ejaculates)

	Season	Age in months			
		6-18	19-30	31-42	43-54
Sperm motility (%)	Autumn	74.8 \pm 1.7 ^{b,x}	75.7 \pm 1.7 ^{b,x}	76.9 \pm 2.9 ^{b,x}	82.7 \pm 3.4 ^{a,x}
	Spring	77.8 \pm 2.4 ^{a,y}	82.1 \pm 2.7 ^{a,y}	83.9 \pm 5.0 ^{a,y}	88.9 \pm 1.8 ^{a,x}
	Summer	61.9 \pm 2.2 ^{c,x}	66.8 \pm 2.2 ^{c,x}	71.5 \pm 4.4 ^{b,x}	78.0 \pm 4.8 ^{b,x}
	Winter	36.0 \pm 1.1 ^{d,z}	42.1 \pm 5.5 ^{d,y}	46.9 \pm 3.4 ^{c,y}	58.8 \pm 3.1 ^{c,x}
Sperm plasma membrane integrity (%)	Autumn	16.7 \pm 1.9 ^{b,z}	27.3 \pm 3.0 ^{c,y}	48.3 \pm 5.1 ^{b,x}	84.7 \pm 1.3 ^{c,w}
	Spring	44.0 \pm 3.4 ^{a,y}	45.8 \pm 3.8 ^{a,y}	56.9 \pm 2.8 ^{a,x}	95.3 \pm 1.5 ^{a,w}
	Summer	21.3 \pm 1.8 ^{b,z}	29.5 \pm 4.1 ^{c,y}	44.9 \pm 3.5 ^{b,x}	90.9 \pm 0.8 ^{b,w}
	winter	17.8 \pm 2.4 ^{b,z}	38.1 \pm 3.9 ^{b,y}	47.7 \pm 6.7 ^{b,x}	67.8 \pm 4.4 ^{d,w}
Sperm livability (%)	Autumn	19.9 \pm 3.1 ^{b,z}	39.6 \pm 7.0 ^{b,y}	51.4 \pm 2.9 ^{b,x}	66.1 \pm 5.6 ^{b,w}
	Spring	61.9 \pm 6.0 ^{a,y}	69.5 \pm 4.3 ^{a,y}	84.7 \pm 1.5 ^{a,x}	88.6 \pm 1.7 ^{a,w}
	Summer	26.8 \pm 4.4 ^{b,y}	28.0 \pm 4.7 ^{b,y}	43.6 \pm 5.0 ^{b,x}	90.3 \pm 0.9 ^{a,w}
	winter	23.9 \pm 3.0 ^{b,z}	31.3 \pm 4.6 ^{b,y}	44.2 \pm 5.9 ^{b,x}	66.0 \pm 4.7 ^{b,w}
Sperm acrosomal integrity (%)	Autumn	48.7 \pm 7.2 ^{a,x}	54.9 \pm 7.1 ^x	51.7 \pm 6.5 ^x	74.9 \pm 2.3 ^w
	Spring	52.8 \pm 4.6 ^{a,y}	64.4 \pm 4.2 ^x	51.7 \pm 4.4 ^y	79.5 \pm 1.4 ^w
	Summer	17.8 \pm 2.7 ^{c,z}	57.1 \pm 6.3 ^x	51.0 \pm 4.5 ^y	73.9 \pm 2.8 ^w
	winter	41.9 \pm 3.7 ^{b,x}	47.3 \pm 5.6 ^x	47.5 \pm 7.0 ^x	75.1 \pm 2.9 ^w
Semen quality factor	Autumn	25.4 \pm 11.3 ^{b,z}	22.0 \pm 7.1 ^{b,y}	71.6 \pm 10.3 ^{b,x}	143.5 \pm 11.7 ^{c,w}
	Spring	81.8 \pm 18.0 ^{a,z}	109.0 \pm 26.8 ^{a,y}	179.6 \pm 36.0 ^{a,x}	326.4 \pm 26.9 ^{a,w}
	Summer	6.2 \pm 1.6 ^{c,z}	7.0 \pm 1.3 ^{d,y}	17.5 \pm 2.4 ^{c,x}	122.2 \pm 16.5 ^{d,w}
	winter	6.3 \pm 1.2 ^{c,z}	14.1 \pm 2.6 ^{c,y}	64.2 \pm 10.1 ^{b,x}	168.0 \pm 14.2 ^{b,w}

15 The values (mean \pm SE) for a given semen parameter in a column (season = a, b, c, and d) and
 16 row (age = w, x, y and z) with different superscript differ significantly (P<0.05).

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HIGHLIGHTS

- Retrospective seminal parameters with respect to age/season of Indian red jungle fowl (IJRF) were studied over the 4-year period.
- Semen quantitative and qualitative traits increased with the increasing age.
- Spring was the best season for the production of high quality ejaculate at 4-years of age.
- The reproductive potential of IJRF is highly variable in many points from its descendants.

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