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1 **Cryopreservation of Ghagus chicken semen: effect of cryoprotectants diluents and thawing**
2 **temperature**

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5 **Abstract**

6 The present study evaluated the effects of cryoprotectants, semen diluents and thawing
7 temperature during Ghagus chicken semen cryopreservation. Four different experiments were
8 conducted; Experiment 1- semen was cryopreserved using 6% dimethylacetamide (DMA) and
9 2% dimethylsulfoxide (DMSO) in Sasaki diluent (SD) and Lake and Ravie diluent (LRD),
10 Experiment 2 and 3- semen was cryopreserved using 8% Ethylene Glycol (EG) in SD, LRD and
11 Red Fowl Extender (RFE), Experiment 4- semen was cryopreserved using 6%
12 dimethylformamide (DMF) in SD, LRD and Beltsville Poultry Semen Extender (BPSE). Semen
13 was cryopreserved in 0.5 ml French straws. Thawing was done at 5°C for 100 sec in ice water in
14 Experiments 1, 2 and 4, whereas in Experiment 3 thawing was done at 37°C for 30 sec. The post-
15 thaw sperm motility, live sperm and percent acrosome intact sperm were significantly ($P<0.05$)
16 lower in cryopreserved samples in all the experiments. No fertile eggs were obtained from
17 cryopreserved samples in Experiments 1 and 2, except for 8% EG RFE treatment where the
18 fertility was 0.83%. In Experiments 3 and 4, highest fertility was obtained in LRD treatment
19 48.12 and 30.89% respectively. In conclusion, using cryoprotectant EG (8%) and thawing at
20 37°C for 30 sec, and DMF (6%) resulted in acceptable level of fertility in Ghagus chicken.
21 Though the diluents influenced post-thaw in vitro semen parameters the fertility was not
22 affected. In addition, results indicated that thawing temperature may be a critical stage in the
23 cryopreservation protocol.

24 *Keywords:* Chicken, Cryoprotectant, Diluent, Fertility, Semen cryopreservation

25 1 INTRODUCTION

26 Semen cryopreservation is an approach for long term *ex-situ* conservation of genetic resources
27 because of the cost effectiveness and simplicity of the technique (Silversides et al., 2012). The
28 fertilizing ability and viability of cryopreserved sperm is different between chicken breeds

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29 requiring standardization of breed or line specific semen cryopreservation protocol (Blesbois
30 2011; Long 2006). Ghagus is an important native chicken breed of India with native tract in
31 Karnataka state. These birds are of medium size with good mothering ability and broodiness
32 character (Haunshi et al., 2015). Efforts are being made for *ex-situ* conservation of this breed of
33 chicken.

34 The diluent used during semen cryopreservation should essentially help in maintaining
35 the structure and functional capacity of the sperm during and after cryopreservation. Few studies
36 have compared the effect of diluents/extenders during chicken semen cryopreservation (Nabi et
37 al., 2016; Rakha et al., 2016; Roushdy et al., 2014; Shanmugam and Mahapatra, 2019). In
38 addition to energy and buffering components in diluents used for semen cryopreservation
39 substances such as polyvinylpyrrolidone and glutamic acid are included that supports the sperm
40 during cryopreservation procedure. A diluent with minimal components that may support sperm
41 during liquid storage may not be of use during cryopreservation (Shanmugam and Mahapatra,
42 2019).

43 The cryoprotectant used for semen cryopreservation by itself profoundly influences the
44 post-thaw semen parameters and fertility (Long, 2006). Glycerol is the least toxic and effective
45 poultry semen cryoprotectant, however, concentration above 1% during insemination produces
46 contraceptive effect (Lake et al., 1980) necessitating it to be removed before insemination.
47 Various other cryoprotectants such as dimethylsulfoxide (DMSO), ethylene glycol and those
48 belonging to amide group such as dimethylacetamide, dimethylformamide and methylacetamide
49 were used for cryopreserving poultry semen and the semen can be used for insemination without
50 removal of the cryoprotectant.

51 Semen cryopreservation protocol for preserving Ghagus breed is not available. This
52 experiment aimed to develop a semen cryopreservation protocol for Ghagus chicken and study
53 the effect of different cryoprotectants, semen diluents and thawing temperature.

54 2 MATERIALS AND METHODS

55 2.1 Experimental birds and husbandry

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56 The experiment was conducted at the experimental poultry farm of ICAR-Directorate of Poultry
57 Research, Hyderabad, India. The birds were housed in individual cages in an open-sided house
58 and feed and water was available *ad libitum* throughout the experimental period. The study
59 consisted of four independent experiments and was carried out following the approval of the
60 Institutional Animal Ethics Committee.

61 **2.2 Experimental procedures**

62 Experiment 1

63 Semen from fifteen Ghagus rooster (32 weeks age) was collected by abdominal massage
64 (Burrows and Quinn, 1937), pooled and used for the experiment. An aliquot of pooled semen
65 was evaluated for sperm concentration, progressive motility, live and abnormal sperm. Semen
66 was cryopreserved using 6% DMA and 2% DMSO in two diluents, Sasaki diluent (D (+)-
67 glucose- 0.2 g, D (+)-trehalose dehydrate- 3.8 g, L-glutamic acid, monosodium salt- 1.2 g,
68 Potassium acetate- 0.3 g, Magnesium acetate tetrahydrate- 0.08 g, Potassium citrate
69 monohydrate- 0.05 g, BES- 0.4 g, Bis-Tris- 0.4 g in 100 ml distilled water, final pH 6.8; Sasaki
70 et al., 2010) and Lake and Ravie (LR) diluent (sodium glutamate 1.92 g, glucose 0.8 g,
71 magnesium acetate $4H_2O$ 0.08 g, potassium acetate 0.5 g, polyvinylpyrrolidone [relative
72 molecular mass (Mr) = 10 000] 0.3 g and double distilled water 100 ml, final pH 7.08, osmolality
73 343 mOsm/kg; Lake and Ravie, 1984). The semen mixed with the cryoprotectant with final
74 sperm concentration 2000 x 10^6/ml was immediately loaded into 0.5 ml French straws and sealed
75 with polyvinyl alcohol powder. The straws were placed 4.5 cm above liquid nitrogen (LN_2) on a
76 styrofoam raft floating on LN_2 in a thermocol box and exposed to nitrogen vapours for 30
77 minutes. The straws were then plunged into LN_2 and stored till further use. Semen straws were
78 stored for a minimum of seven days before evaluation. Post-thaw evaluation and insemination of
79 semen was done after thawing at 5°C for 100 sec in ice water (Sasaki et al., 2010). The samples
80 were evaluated on seven different occasions for progressive sperm motility, live sperm, abnormal
81 sperm, and intact sperm acrosome. Thawed semen was inseminated per vagina into 33 weeks old
82 Ghagus hens (13 hens/treatment) using a dose of 200 million sperm/0.1 ml volume. Insemination
83 was repeated three times at three days interval. Freshly collected and inseminated semen served
84 as control. Eggs were collected from the second day onwards after first insemination and stored
85 in cold chamber (15°C) till incubation. Eggs were candled on the 18th day of incubation for

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86 embryonic development. Infertile eggs were broke open for examination and confirmation of the
87 absence of embryonic growth. Hatchability results were obtained on 21st day of incubation.

88 Experiment 2

89 Ghagus (41 weeks age) semen was cryopreserved similar to Experiment 1 with few
90 modifications. Ethylene glycol at 8% final concentration was used as cryoprotectant and three
91 semen diluents namely Sasaki diluent, Lake and Ravie diluent and Red Fowl Extender (RFE;
92 Fructose-1.15 g, Sodium glutamate-2.1 g, Polyvinylpyrrolidone- 0.6 g, Glycine- 0.2 g, Potassium
93 acetate- 0.5 in 100 ml distilled water, final pH 7, osmolality 380 mOsm/kg; Rakha et al., 2016)
94 were evaluated. Post-thaw semen was evaluated on six occasions for progressive sperm motility,
95 live sperm, abnormal sperm, and intact sperm acrosome. Thawed semen was inseminated into 46
96 weeks old Ghagus hens (15 hens/treatment) using a dose of 200 million sperm in 0.1 ml volume.
97 Insemination was repeated three times at four days interval. Insemination using fresh semen
98 served as control. Post insemination eggs were incubated and data on fertility parameters
99 obtained as in Experiment 1.

100 Experiment 3

101 In this experiment Ghagus (54 weeks age) semen was cryopreserved and evaluated similar to
102 Experiment 2 except that the semen straws were thawed at 37°C for 30 sec. Thawed semen was
103 inseminated into 54 weeks old Ghagus hens (15 hens/treatment) using a dose of 200 million
104 sperm in 0.1 ml volume. Insemination was repeated three times at four days interval. Post
105 insemination eggs were incubated and data on fertility parameters obtained as in Experiment 1.

106 Experiment 4

107 Ghagus (54 weeks age) semen was cryopreserved similar to Experiment 1 with few
108 modifications. Dimethylformamide at 6% final concentration was used as cryoprotectant and
109 three semen diluents namely Sasaki diluent, Lake and Ravie diluent and Beltsville Poultry
110 Semen Extender (BPSE; Fructose-0.3 g, Potassium citrate-0.0384 g, Sodium glutamate-0.5202 g,
111 Magnesium chloride- 0.0204 g, di-Potassium hydrogen phosphate- 0.762 g, TES- 0.317 g,
112 Potassium di-hydrogen phosphate- 0.039 g, Sodium acetate- 0.258 g in 100 ml distilled water,
113 final pH 7.3, osmolality 330 mOsm/kg; Rakha et al., 2016) were evaluated. Post-thaw semen was

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114 evaluated on six occasions for progressive sperm motility, live sperm, abnormal sperm, and
115 intact sperm acrosome. Thawed semen was inseminated into 57 weeks old Ghagus hens (20
116 hens/treatment) using a dose of 200 million sperm in 0.1 ml volume. Insemination was repeated
117 three times at four days interval. Insemination using fresh semen served as control. Post
118 insemination eggs were incubated and data on fertility parameters obtained as in Experiment 1.

119 **2.3 Semen quality assays**

120 Sperm motility

121 Sperm motility was subjectively scored as percentage of progressively motile sperm after placing
122 a drop of diluted semen on a Makler chamber and examined under 20x magnification.

123 Live and abnormal sperm

124 The live and abnormal sperm percent was estimated by differential staining using Eosin-Nigrosin
125 stain (Campbell et al. 1953). Semen smear prepared by mixing one drop of semen with two drops
126 of Eosin-Nigrosin stain was air dried and observed under high power (1000x). All pink stained
127 and partially stained sperm were considered dead and unstained sperm as live. The percentage of
128 live sperm was determined by counting at least 200 sperm. The same slides were used for
129 estimating the percentage of abnormal sperm that was showing different morphological
130 abnormalities.

131 Intact sperm acrosome

132 The intact acrosome in sperm was assessed as described by Pope et al. (1991). Briefly, 10 µl of
133 diluted semen was mixed with 10 µl of stain solution (1% (wt/vol) rose Bengal, 1% (wt/vol) fast
134 green FCF and 40% ethanol in citric acid (0.1 M) disodium phosphate (0.2 M) buffer
135 (McIlvaine's, pH 7.2-7.3) and kept for 70 sec. A smear from the mixture was made on glass
136 slide, dried and examined under high magnification (1000x). The acrosomal caps were stained
137 blue in acrosome-intact sperm and no staining in the acrosome region of acrosome reacted
138 sperm. A minimum of 200 sperm were counted in each smear sample for calculating the percent
139 acrosome intact sperm.

140 **2.4 Statistical analysis**

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141 Data were analyzed using SAS 9.2 software and $P<0.05$ was considered significant. Statistical
142 analyses of semen parameters were performed by one-way ANOVA with Tukey's post hoc test.
143 Data with percent values were arcsine transformed before analysis.

144 3 RESULTS

145 The post-thaw sperm motility and live sperm percent were significantly ($P<0.05$) lower in
146 cryopreserved samples in all the experiments (Tables 1-4). Cryopreservation did not affect the
147 percent abnormal sperm in first three experiments and in Experiment 4 samples cryopreserved in
148 LR and BPSE diluents had significantly ($P<0.05$) higher abnormal sperm. Intact sperm acrosome
149 was significantly ($P<0.05$) lower in cryopreserved sperm except for the 8% EG SD treatment in
150 Experiment 3. No fertile eggs were obtained from cryopreserved samples in Experiment 1 and 2,
151 except for 8% EG RFE treatment where the fertility was 0.83% (Table 2). The percent fertility
152 after insemination with cryopreserved semen was significantly ($P<0.05$) lower in all the
153 treatments of Experiments 3 and 4 except for 8% EG LR treatment where it was similar to that of
154 control (Tables 3 and 4). No difference in percent hatchability was observed between the
155 treatments in Experiments 3 and 4.
156

157 4 DISCUSSION

158 In the present study a series of experiments were conducted to evaluate the effect of different
159 cryoprotectants and diluents during Ghagus chicken semen cryopreservation. In this process
160 semen cryopreservation protocols producing acceptable fertility were developed for this breed of
161 chicken.

162 The DMSO and amide cryoprotectant DMA has been successfully used in
163 cryopreservation of chicken semen protocols producing good fertility (Woelders et al., 2006;
164 Santiago-Moreno et al., 2012; Herrera et al., 2005; Zhandi et al., 2017). Earlier, high fertility
165 using 4% DMSO was obtained in another line maintained in the Institute farm (Pranay Kumar et
166 al., 2019). In the present study the DMSO level was reduced to 2% based on preliminary in vitro
167 studies, however, no fertility was obtained. In the present study DMA did not produce any fertile
168 egg and is similar to an earlier report where DMA was used at 9% in Nicobari chicken
169 (Shanmugam et al., 2018).
170

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171 Ethylene glycol has been used for cryopreserving chicken semen and thawed at 5°C or
172 37°C (Mphaphathi et al., 2016; Miranda et al., 2018; Olexikova et al., 2019). The studies have
173 reported only in vitro results and fertility trails were not conducted. Woelders et al. (2006) have
174 reported use of EG as cryoprotectant during chicken semen cryopreservation where it was found
175 to yield lower post-thaw motility and viability, however, the thawing conditions are not known.
176 Ethylene glycol has also been used for storing chicken semen as pellets or in glass ampoules and
177 there was no clear advantage over other cryoprotectants in terms of fertilizing ability (Wishart,
178 2001). The present study reports both in vitro and fertility results of experiments using EG as
179 cryoprotectant. The interesting finding of this study was the influence of the thawing temperature
180 on fertility outcome. Ethylene glycol cryopreserved semen when thawed at 5°C did not result in
181 any fertility whereas semen thawed at 37°C resulted in 18-48% fertility. The fertility results were
182 not affected by the semen diluent. Miranda et al. (2017) have hypothesized based on the in vitro
183 results that due to the low molecular weight, greater membrane permeability and affinity towards
184 sperm plasma membrane EG act as a better cryoprotectant when thawed at lower temperature.
185 However, in the present study it was observed that semen cryopreserved with EG when thawed
186 at 37°C rather than 5°C and inseminated produced higher fertility. It is not possible to compare
187 results between these studies since fertility trails were not reported by Miranda et al. (2017). The
188 results of the Experiments 2 and 3 indicate that thawing of cryopreserved semen is a critical
189 point in the semen cryopreservation protocol. The in vitro results in the two experiments were
190 almost similar or the values are lower in Experiment 3 where thawing was done at 37°C,
191 however, better fertility was obtained. The thawing temperature has induced changes at cellular
192 or molecular levels that are worth studying in further experiments.
193
194 Chicken semen cryopreserved in straws with DMF gave better post thaw motility
195 parameters and fertility (Ehling et al., 2002). Higher post-thaw motility parameters were reported
196 for semen cryopreserved with DMF and thawed at 5°C. Chicken semen cryopreserved only with
197 DMF in plastic vials or straws produced higher fertility (Chalah et al. 1999; Chuaychu-noo et al.,
198 2017; Thananurak et al., 2017, 2020). A higher live sperm, mitochondrial activity and lower
199 acrosome damage was obtained in Korean native chicken semen cryopreserved with DMF (Choi
200 et al., 2013). However, semen vitrified using DMF as a cryoprotectant resulted in negligible
201 fertility (Shanmugam and Mahapatra, 2019). In the present report using DMF as cryoprotectant

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202 resulted in fertility ranging from 19 to 30%. Based on the fertility reports from these studies it
203 may be concluded that DMF helps in cryopreserving sperm when packed in straws.
204
205 The composition of the diluent has been reported to influence the post-thaw in vitro
206 semen parameters (Nabi et al., 2016; Rakha et al., 2016; Roushdy et al., 2014). The effectiveness
207 of a particular diluent in these reports was based on in vitro parameters and no information is
208 available about the fertility. Laboratory assays are only suggestive and cannot determine the
209 fertilizing capacity of a semen sample, therefore, to know the fertility outcome from
210 cryopreserved semen adequate number of inseminations is required (Graham and Mocé, 2005).
211 The fertility after insemination with DMF cryopreserved semen was lower in a PVP based
212 diluent compared to other diluents (Thananurak et al., 2017). In our study no difference in
213 fertility was observed between PVP present or absent diluents in semen cryopreserved using
214 DMF. Even in other experiments in our study it was observed that differences existed in post-
215 thaw in vitro parameters between the semen diluents but not in fertility.
216 Acrosome reaction is an important parameter to be considered during semen
217 cryopreservation. A low acrosome intact post-thaw sample will not produce fertile eggs,
218 however, having higher post-thaw acrosome intact sperm will not guarantee fertility either. This
219 could be observed in the first two experiments of this study. Induction of acrosome reaction in
220 cryopreserved semen has been shown to be affected by type of birds/flock (Lemoine et al.,
221 2011). Nevertheless, evaluation of acrosome integrity in post-thaw sperm may be used as a
222 screening test along with other simple in vitro parameters such as motility to eliminate the
223 cryoprotectant/protocol before proceeding further in the process of semen cryopreservation for
224 any particular breed/line.
225
226 In conclusion, results from this study indicate that cryoprotectants and not diluents affect
227 the fertility obtained from cryopreserved semen and the thawing temperature has been found to
228 influence the success of cryopreservation protocol.
229

230 **CONFLICT OF INTEREST**

231 There is no conflict of interest to be declared by the authors.
232

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233 **AUTHOR CONTRIBUTIONS**

234 SM designed the experiment, collected the samples, analysed the data and wrote the manuscript.
235 RM designed the experiment and participated in the writing of the manuscript.

236

237 **DATA AVAILABILITY**

238 Data for this study can be obtained from the corresponding author upon request.

239

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329 **TABLE 1** Effect of cryoprotectants and semen diluents on post thaw semen and fertility
330 parameters in cryopreserved Ghagus semen.

Parameters	Control	6% DMA SD	2% DMSO SD	6% DMA LR	2% DMSO LR
Progressive sperm motility (%)	64.29 ± 1.7 [a]	21.43 ± 1.43 [b]	12.14 ± 1.01 [c]	17.86 ± 1.01 [b]	9.28 ± 0.71 [c]
Live sperm (%)	71.83 ± 2.92 [a]	34.93 ± 1.69 [b]	22.66 ± 0.95 [c]	34.46 ± 2.13 [b]	21.23 ± 1.51 [c]
Abnormal sperm (%)	2.07 ± 0.63	2.13 ± 0.62	1.51 ± 0.22	2.51 ± 0.35	1.04 ± 0.18
Acrosome intact sperm (%)	99.57 ± 0.2 [a]	4.57 ± 1.21 [c]	94.85 ± 0.55 [b]	4.14 ± 0.74 [c]	91.61 ± 0.76 [b]
Fertility (%)	85.76 ± 5.47 [a]	0 [b]	0 [b]	0 [b]	0 [b]
Hatchability on FES (%)	98.18 ± 1.81 [a]	0 [b]	0 [b]	0 [b]	0 [b]
No. of eggs incubated	43	34	35	29	52

331 Values given are Mean±SE.
332 Figures bearing different superscripts in a row differ significantly (*P*<0.05).
333 FES- Fertile egg set
334
335

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336 **TABLE 2** Effect of ethylene glycol and semen diluents on post-thaw semen and fertility
337 parameters in Ghagus chicken thawed at 5°C.

Parameters	Control	8% EG SD	8% EG LR	8% EG RFE
Progressive sperm motility (%)	62.5 ± 1.12^{a}	20.0 ± 1.3^{b}	18.33 ± 1.05^{bc}	15.0 ± 1.29^{c}
Live sperm (%)	74.22 ± 1.64^{a}	23.72 ± 1.12^{b}	26.52 ± 1.54^{b}	22.72 ± 1.43^{b}
Abnormal sperm (%)	2.7 ± 0.23	2.15 ± 0.19	3.02 ± 0.40	2.58 ± 0.42
Acrosome intact sperm (%)	99 ± 0.37^{a}	94.0 ± 0.86^{b}	92.33 ± 0.92^{b}	92.83 ± 1.08^{b}
Fertility (%)	71.63 ± 4.81^{a}	0^{b}	0^{b}	0.83 ± 0.83^{b}
Hatchability on FES (%)	98.81 ± 1.19^{a}	0^{b}	0^{b}	0^{b}
No. of eggs incubated	82	85	108	86

338 Values given are Mean±SE.
339 Figures bearing different superscripts in a row differ significantly ($P<0.05$).
340 FES- Fertile egg set

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341 **TABLE 3** Effect of ethylene glycol and semen diluents on post-thaw semen and fertility
342 parameters in Ghagus chicken thawed at 37°C.
343

Parameters	Control	8% EG SD	8% EG LR	8% EG RFE
Progressive sperm motility (%)	64.16 ± 1.54[a]	17.5 ± 1.12[b]	17.5 ± 1.12[b]	11.67 ± 1.05[c]
Live sperm (%)	80.17 ± 3.30[a]	26.0 ± 2.03[b]	24.67 ± 2.0[b]	20.33 ± 0.71[b]
Abnormal sperm (%)	1.48 ± 0.27	1.87 ± 0.24	1.93 ± 0.18	1.8 ± 0.17
Acrosome intact sperm (%)	97.17 ± 0.31[a]	84.58 ± 3.84[ab]	69.98 ± 8.11[b]	62.73 ± 9.60[b]
Fertility (%)	81.23 ± 5.39[a]	18.39 ± 8.89[b]	48.12 ± 11.1[ab]	38.29 ± 10.27[b]
Hatchability on FES (%)	72.53 ± 4.18	69.12 ± 14.98	68.98 ± 6.4	58.1 ± 9.85
No. of eggs incubated	82	101	84	111

344 Values given are Mean±SE.
345 Figures bearing different superscripts in a row differ significantly ($P<0.05$).
346 FES- Fertile egg set
347
348

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349 **TABLE 4** Effect of dimethylformamide and semen diluents on post-thaw semen and fertility
350 parameters in Ghagus chicken.

Parameters	Control	6% DMF SD	6% DMF LR	6% DMF BPSE
Progressive sperm motility (%)	66.43 ± 1.43[a]	18.57 ± 1.8[b]	20.0 ± 2.44[b]	15.0 ± 1.54[b]
Live sperm (%)	79.47 ± 4.48[a]	26.0 ± 1.73[b]	23.81 ± 2.79[b]	23.53 ± 1.06[b]
Abnormal sperm (%)	1.32 ± 0.15[b]	2.32 ± 0.34[ab]	3.44 ± 0.54[a]	3.34 ± 0.5[a]
Acrosome intact sperm (%)	97.77 ± 0.14[a]	55.61 ± 8.51[b]	22.03 ± 6.23[c]	12.8 ± 2.21[c]
Fertility (%)	78.85 ± 4.82[a]	24.76 ± 5.10[b]	30.89 ± 10.67[b]	19.32 ± 8.53[b]
Hatchability on FES (%)	75.28 ± 5.17	60.00 ± 12.10	81.25 ± 13.15	90.00 ± 10.00
No. of eggs incubated	105	108	83	124

351 Values given are Mean±SE.
352 Figures bearing different superscripts in a row differ significantly (*P*<0.05).
353 FES- Fertile egg set