1 **Influence of insemination time on the fertility of sex sorted frozen-thawed Y-sperm**
2 **in red deer**
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24 Short Title: Insemination time with sexed sperm in red deer.
25

26 **Abstract**

27 The aim of this study was to assess the effect of insemination timing on pregnancy rates
28 in red deer (*Cervus elaphus*) when using sex-sorted sperm samples. Semen was collected by
29 electroejaculation from 8 mature stags and processed to obtain: Conventional samples,
30 following standard freezing procedures for commercial purposes; Control sorted samples,
31 diluted and handled as per sorted samples but without being submitted to the sorter passage; and
32 Y Sex Sorted (YSS) samples. Hinds were synchronized via intravaginal CIDR (Controlled
33 Internal Drug Release) placement and given eCG (Folligon® PMSG Serum Gonadotrophin) on
34 day 12, upon CIDR removal. They were then inseminated with one of each sperm treatment, at
35 the following post-eCG intervals: I_1, 55:01 – 55:30 h; I_2, 55:31 – 56:00 h; I_3, 56:01 – 56:30
36 h; or, I_4, 56:31 – 57:00 h. Pregnancy rates were assessed at parturition. Average pregnancy
37 rates were highest (P<0.05) for Conventional samples (77.6%), but similar between YSS
38 (49.8%) and Control sorted (51.3%) samples. However, when insemination interval was taken
39 into account, pregnancy rates within the YSS group, pregnancy rates were 80 and 83.1% for I_1
40 and I_2, respectively were obtaioned. Notably, these rates were similar (P>0.05) to the average
41 pregnancy rates obtained with Conventional samples (77.6%). As expected, YSS sperm yielded
42 94% male offspring contrasting with the 57% males obtained with Conventional and Control
43 sorted samples. Our findings support the importance of developing specific insemination timing
44 protocols to improve pregnancy rates when using frozen-thawed sex-sorted sperm. These
45 findings provide the foundation for further investigations in order to determine why the YSS
46 sperm are able to fertilize the oocyte in a shorter period of time than the conventional samples.

47

48 **Key words:** Red deer; Artificial insemination; Sex-sorted sperm; Insemination time; Fertility.

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50

51 **1. Introduction**

52 The captive deer breeding industry has experienced an important period of growth
53 worldwide within the past 40 years [1,2]. In recent years, there has been an increased trend
54 toward deer farming in Spain, becoming a viable alternative to raising more conventional
55 livestock species. In Spain, these are deer gaming farms where the main financial profits rely on
56 antler trophies. Given that males have the highest economic value, the possibility of sex
57 selection at breeding would represent significant management cost-savings and avoid the
58 massive slaughter of females [3].

59 Presently, the most practical and effective methodology to predetermine the sex of
60 progeny in livestock species is to perform sperm sex sorting by flow cytometry, which is based
61 on the differences in DNA content between X and Y chromosome-bearing sperm [4]. This
62 technique has been successfully applied in Sika deer (*Cervus nipon*) and red deer (*Cervus*
63 *elaphus*), to produce offspring of the desired sex [5,6]. In the latter study, Anel-Lopez et al. [6]
64 reported 50% fertility rates when using Y-bearing frozen-thawed sex-sorted sperm for artificial
65 insemination (AI) in red deer. While this fertility rate is acceptable it is still far from the 75%
66 reported when using conventional samples of frozen-thawed sperm. Surprisingly, in that study,
67 post-thaw sperm evaluation parameters were not different between sorted and unsorted samples
68 and hence could not account for the differences in fertility observed.

69 In this regard, the insemination timing is essential for pregnancy success, and depends
70 on parameters such as the sperm and oocyte quality and lifespan, the time it takes for viable
71 sperm to reach the place of fertilization and/or the timing of ovulation relative to insemination
72 [7]. In our context, improving pregnancy rates by ascertaining the best time to perform the AI
73 procedure would offer an economic incentive to overcome the perceived drawbacks associated
74 with AI, including the additional expense associated with sperm sex-sorting and more intensive
75 management. Because many of the factors affecting reproductive management protocols are
76 species-specific, such protocols must be tailored to the species of interest [8]. Moreover, the
77 sperm sex-sorting procedure accelerates several of the physiological sperm processes required

78 for fertilization such as capacitation or acrosomal exocytosis, which in turn may decrease sperm
79 longevity [9]. This emphasizes the need to improve sperm handling procedures prior to
80 insemination.

81 Therefore, the aim of this study was to assess whether optimizing insemination timing
82 protocols we could improve the fertility rates with frozen-thawed sex-sorted red deer sperm. For
83 this, we compared the fertility results with 3 different types of sperm samples (Control sorted,
84 Y-Sexed sperm and Conventional samples), when inseminated at different times following
85 estrus synchronization.

86

87 **2 Materials & Methods**

88 **2.1. Reagents and media**

89 All the reagents were purchase from Sigma-Aldrich (Madrid, Spain) unless otherwise indicated.
90 The collecting medium used during sorting was a Tris-Citrate-Glucose (TCG) (pH: 7.3 and
91 pOsm: 380 mOsm/kg) containing: glucose (250 mM), sodium citrate (12 mM), EDTA (1.6
92 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk (EY) at 5% (V/V) penicillin (0.7 mM),
93 and streptomycin (1.14 mM). The ejaculate washing medium was the transport extender with
94 the addition of 2.5% (V/V) egg yolk. The transport medium was a Tris-Citrate-Fructose (TCF)
95 (pH: 7.3 and pOsm: 330 mOsm/kg) containing: Tris (213 mM), citric acid monohydrate (71.83
96 mM), fructose (55.51 mM), egg yolk at 20% (V/V), penicillin (0.7 mM), and streptomycin (1.14
97 mM).

98 **2.2. Ejaculate collection and sperm sample preparation**

99 Samples were obtained from 8 mature stags during the breeding season (mid-
100 September). Animals were housed in a semi-free ranging regimen at Las Lomas Farm
101 (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in
102 accordance with Spanish law in regards to the care and use of research animals (RD 53/2013)
103 conforming to European Union regulation 2010/63. Semen collection by electroejaculation was
104 carried out as described Anel-Lopez et al [10]. Briefly, males were anesthetized with 0.75
105 mg/Kg of Xylazine (Rompun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared

106 of feces and the prepucial area was shaved and washed with physiological saline solution. A
107 three-electrode probe (P.T. Electronics, Boring, OR, USA) connected to a power source that
108 allowed voltage and amperage control was used (P.T. Electronics). Probe diameter, probe length
109 and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The electroejaculation regimen
110 consisted of a consecutive series of 5 pulses of similar voltage applied for 5 s with 5 s of rest in
111 between each pulse. Initially, 1V was applied that was then progressively increased at the next
112 series until reaching a maximum of 5V. Semen was collected by fractions in graduated glass
113 tubes. Then, fractions with urine contamination, that is, positive to Urea Test Strips (Diagnostic
114 Systems GmbH, Holzheim, Germany) were discarded. Fractions with total sperm motility under
115 80% were also discarded.

116 Semen was diluted 1:3 in TCG containing 2.5% egg yolk and then centrifuged at 600xg
117 for 5 min. The supernatant was removed and sperm concentration of the pellet was assessed
118 using a hemocytometer (Bürker chamber; Brand Gmbh, Wertheim, Germany), after diluting an
119 aliquot of the sample in a glutaraldehyde solution (5 µL of sample in 500 µL of 2%
120 glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate
121 and 2 g/L sodium bicarbonate). Then, sperm aliquots were diluted to a concentration of 800 x
122 10^6 sperm/mL in TCF medium supplemented with 20% (v/v) of egg yolk and transported to the
123 sorting facility (about 8 h at 17ºC). Upon arrival to the laboratory each of the sperm samples
124 was split into 3 aliquots, to be processed to obtain: (1) conventional samples, following standard
125 freezing procedure for commercial purposes; (2) Control sorted samples, diluted and handled as
126 per sorted samples but without being submitted to the sorter passage; and, (3) Y Sex Sorted
127 (YSS) samples. Sperm samples for sorting (YSS) were further diluted to 100 x 10^6 sperm/mL
128 with TCG (0% egg yolk) medium and stained with 2.6 µL of Hoechst-33342 (H-42) (Stock
129 solution: 25 mg/mL) during 50 min at 34ºC as previously described by Parrilla et al. [11].

130 **2.3. Flow cytometric sperm sex sorting**

131 Just prior to sorting, stained sperm samples were filtered through a 30-µm nylon mesh
132 filter. Then, 1 µL of food dye (0.002% w/v; FD&C #40, Warner Jenkinson Company Inc., St.
133 Louis, MO, USA) was added to each sample to quench the fluorescence of H-42 in sperm with

134 compromised cell membranes, allowing them to be gated out during the sorting process [20].
135 Sperm were sorted for YSS according to the Beltsville Sperm Sorting Technology method [21]
136 using a high-speed cell sorter (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA)
137 modified for sperm sorting. The cell sorter was operated at 40 psi and was equipped with a UV-
138 laser set at an output of 175 mW (Spectra Physics 1330, Mountain View, CA, USA). The
139 samples were sorted in the presence of a HEPES-buffer based sheath [22] supplemented with
140 0.1% EDTA (w/v), and were collected in 50-mL tubes prefilled with 2.5 mL of collection
141 medium (TCG medium containing 5% (v/v) of EY). A total of 20 x 10^6 sorted sperm was
142 collected per tube in an approximate volume of 25 mL with a purity of ≥90%.
143

144 **2.4 Sperm cryopreservation**

145 Conventional samples were frozen at a concentration of 100 x 10^6 sperm/mL using
146 Triladyl® (Minitüb, Tiefenbach, Germany), supplemented with 20% (v/v) EY, following the
147 manufacturer's instructions. Sorted sperm were centrifuged at 3000xg for 4 min at 21ºC. The
148 supernatant was discarded and the pellets were re-extended to 40 x 10^6 sperm/mL using
149 Triladyl® supplemented with 20% (v/v) of EY. Then sperm samples were immersed in a
150 programmable temperature-controlled water bath (Programmable Model 9612, PolyScience,
151 Niles, IL, USA) and slowly cooled from 21ºC to 5 ºC for over 90 min, and left for an
152 equilibration time of 2 h. After this time-period, processed samples were packaged in 0.25 mL
153 straws (Minitüb, Tiefenbach, Germany), placed on a rack 4 cm above liquid nitrogen for 10
154 min, and then plunged into liquid nitrogen and stored accordingly.

155 The control sorted group consisted of sperm handled and diluted as per the YSS group
156 but without being submitted to the sorter passage. For this purpose, control sorted sperm were
157 centrifuged to remove the supernatant and then gradually diluted to 20 x 10^6 sperm/mL using
158 HEPES-buffer based sheath fluid in the presence of collection medium. Then control sperm
159 samples were stored at room temperature (21–22°C) for approximately 4 h mimicking the
160 conditions to which sorted sperm were exposed. Freezing of control sorted samples was
161 performed concomitantly and following the exact same protocol as per sorted samples (YSS).

162

163 **2.5. Artificial insemination**

164 The trial was conducted at the Medianilla farm in Cádiz (Spain) during September of
165 2013, 2014 and 2015. Six hundred thirty-four Iberian deer (4-6 years old; 75-101 kg body
166 weight) housed in outdoor enclosures that provide exposure to natural fluctuations in light and
167 temperature were inseminated. Estrous synchronization of hinds was performed as previously
168 described by Garde et al. [1]. Briefly, single controlled internal drug release (CIDR) devices
169 (type G, 330 mg progesterone per device, InterAg Effective Agricultural System, Hamilton,
170 NZ) were inserted vaginally. Then CIDRs were replaced in each animal on day 9 to ensure high
171 progesterone concentrations and at this time we administered 0.75 mL of $PGf_2\alpha$ (IM;
172 Prosolvin®; VIRBAC S.A, Cataluña, SPAIN) per hind. Upon CIDR removal on day 12 (3 days
173 following PgF2α administration), hinds were given 250 IU of eCG (Folligon®, Intervet,
174 Salamanca, Spain) intramuscularly. One straw per hind was used to carry out the AI. Females
175 were then randomly inseminated transcervically with Control sorted sperm (158 hinds) and Y-
176 sexed sperm (239 hinds) at a concentration of 40 $x10^6$ sperm/mL or Conventional frozen-
177 thawed sperm (237 hinds) at a concentration of 100 x 10^6 sperm/mL. At the time of
178 insemination, hinds were individually identified and the exact time after eCG administration
179 was recorded. Hence timing of insemination was divided into 4 intervals, corresponding to
180 hours post-eCG administration: I_1, 55:01 – 55:30 h; I_2, 55:31 – 56:00 h; I_3, 56:01 – 56:30 h;
181 and, I_4, 56:31 – 57:00 h. Inseminated hinds were kept in groups until approximately 1 week
182 before the due date, at which time they were moved to individual pens for better monitoring.
183 Fertility rates were assessed upon delivery rates. The sex of fawns was recorded at birth.

184

185 **2.6. Statistical analysis**

186 Data were analysed using the SAS™ v.9.1. Package (SAS Institute Inc., Cary, NC,
187 USA). Sperm fertility data were compared using a LOGISTIC procedure considering a binary
188 response model. The statistical model included sperm sample (Control sorted; YSS;
189 Conventional) and time of insemination (I_1, I_2, I_3 or I_4) as factor, and fertility rates as a

190 response variable. Between-group differences in the frequency were tested using Wald Chi-
191 Square. Results are presented as percentages. Significance level was set at $P<0.05$.
192
193 **3. Results**
194 Overall fertility rates were similar ($P>0.05$) between YSS samples (49.8%) and Control
195 sorted samples (51.3%) (Figure 1). Conversely, Conventional sperm samples yielded higher
196 ($p<0.05$) fertility rates (77.6%) when compared to both Sexed and Control sorted samples
197 (Figure 1).
198 There were no differences in pregnancy rates in Control sexed samples among the
199 different insemination intervals (Table 1a). Conversely, for YSS samples there were remarkable
200 differences ($P<0.05$) among insemination intervals (Table 1b). For instance, both I_1 and I_2
201 yielded the highest pregnancy rates (80 and 83.1%, respectively) within the sperm type group.
202 However, I_3 and I_4 yielded the lowest pregnancy rates (33.3 and 3.3%, respectively)
203 ($P<0.05$) (Table 1b). In fact, pregnancy rates for YSS sperm used in intervals I_1 and I_2 were
204 similar ($P>0.05$) to the average pregnancy rates obtained with Conventional samples (77.6%)
205 (Table 1c). Within Conventional samples, there were also differences in pregnancy rates
206 according to the insemination interval. In fact, I_3 yielded the highest pregnancy rates (91.7%;
207 $P<0.05$) within the group, while I_1 yielded the lowest pregnancy rates (66.7%; $P<0.05$).
208 Results were intermediate for I_2 and I_4 (Table 1c).
209 As expected, the males were accounted for 56.8% and 57% of the offspring for both
210 Control sexed samples and Conventional samples, respectively. Conversely, 94% of the
211 offspring were males when using YSS samples ($P<0.05$) (Figure 2).
212
213 **4. Discussion**
214 Sperm sex-sorting by flow cytometry is currently the best technology available to
215 modify the offspring sex-ratio in livestock species, thus allowing for faster genetic progress and
216 increased production while reducing wastage. However, fertility rates obtained after AI with
217 sexed samples are lower than those obtained with unsorted samples under the same management

218 conditions in all species studied [6,12,13]. This is no different for red deer, a species in which
219 the main economic incentive relies on producing trophy males for hunting events [1,14]. The
220 reasons behind the lower fertility observed with sex-sorted sperm are not entirely clear. Both
221 factors related to sperm handling during the sorting process and to female breeding management
222 may account for the reduced fertility of sex-sorted sperm [15,16]. While this is sometimes
223 attributed to low sperm yields and the use of lower insemination dosages, increasing the number
224 of sperm to reach a concentration comparable to those used with non-sexed sperm did not
225 improve pregnancy rates in heifers [17]. This may be due partially to the fact that the sorting
226 process is not innocuous, given that sperm are exposed to many stressors such as fluorescent
227 dyes, high dilution rates, mechanical injuries, laser illumination and a subsequent passage
228 through an electric field [18]. Moreover, the sex-sorting process may induce capacitation-like
229 changes in sperm from several species, including ram and boar [19]. The development of
230 species-specific protocols in regards to sperm handling and insemination timing may help
231 overcome to some degree the decreased pregnancy rates observed with sexed-sorted sperm.

232 In agreement with previous studies, herein we showed that sperm sex-sorting via flow
233 cytometry is an excellent technology for Y-sperm separation when applied to a red deer sperm
234 production system [6,10]. Hence, in this study the male:female offspring sex ratio was 94:6 for
235 hinds inseminated with Y sex-sorted sperm, similar to a previous study [6]. However, overall
236 fertility rates were lower for sorted than conventional samples. In our previous study [6], that
237 fertility reduction could not be attributed to sperm quality parameters which were comparable
238 among the different treatments. Based upon these results, we decided to evaluate the effect of
239 insemination timing following induction of ovulation on pregnancy rates. Interestingly, within
240 Y-sorted sperm samples, pregnancy rates were significantly higher when hinds were
241 inseminated in the interval spanning 55:01 to 56:00 h following eCG administration. However,
242 pregnancy rates plummeted when sorted sperm was inseminated at later intervals. With the
243 exception of a peak in pregnancy rates for Conventional samples inseminated in the interval
244 spanning 56:01-56:30 h following eCG administration, differences among the other intervals
245 were not as marked for these samples. There were no differences among the different intervals

246 for control sorted samples. Overall these results underscore the importance of devising species-
247 specific protocols in regards to breeding management when using sex-sorted sperm samples. In
248 addition these results are in concordance with our previous work [6] where the in vitro sperm
249 quality of sex-sorted samples was even higher than that for the control samples. Interestingly,
250 insemination of sex-sorted sperm during intervals presumably closer to ovulation yielded
251 pregnancy rates that were not different to the average rates obtained with Conventional samples.
252 While this further supports the importance of insemination timing, the fact that the sex-sorting
253 process also selects viable sperm may also partially account for the remarkable pregnancy rates
254 obtained when inseminating closer to ovulation induction [10] and to the differences observed
255 with the control-sorted sperm, where such selection is not performed.

256 Notably, it has been suggested that the lower number of sperm used when inseminating
257 with sex-sorted sperm may be a major factor negatively impacting pregnancy rates in several
258 species, including cows [20], horse [21], sheep [22] or red deer [6]. However, we believe that
259 our work argues against this hypothesis. When inseminated at the optimal time, the pregnancy
260 rates obtained with 10 million Y sex-sorted sperm were higher than the average pregnancy rates
261 obtained with 25 million of Conventional sperm. Thus, adjusting the insemination timing is
262 especially important with sex-sorted semen given a lower insemination dose [23] and a shorter
263 life-span or at least a shorter period of fertilizing capacity of sperm within the female
264 reproductive tract [24]. Garner et al. [18] hypothesized that the increased precision of the time
265 of ovulation and of insemination relative to ovulation may play a critical role in obtaining
266 satisfactory results in fertility because of this fact. In this way, our results confirm that
267 hypothesis. For instance, Lu et al. [25] in bull or Maxwell et al. [24] in ram spermreported that
268 the sorting process induces sperm precapacitation changes. In the same way, Hollinshead et al.
269 [26] concluded that the sorting and freezing-thawing process accelerate the maturation of
270 sorted-frozen-thawed ram sperm, decreasing their fertilizing lifespan. This would reduce sorted
271 sperm longevity and account for the reduced pregnancy rates observed not only in deer [6] but
272 also in other species, like cattle [27]. Because of this previews and higher maturation, for use in
273 AI, insemination close to the site of fertilization and time of ovulation is critical for a successful

274 fertilization and ongoing pregnancy. In the opposite, in cattle pregnancy rates are higher when
275 insemination is carried out around 60h rather than 36 h following CIDR removal [28].
276 Interestingly, in our study, highest pregnancy rates with sorted sperm were obtained at around
277 55-56 h following eCG administration. It is possible that the fertility window in red deer is
278 smaller than that reported in other species, in addition to other factors possibly affecting fertility
279 in a wild species such as handling stress. Other studies in red deer with unsorted frozen-thawed
280 sperm reported the best intervals to be between 48 and 55 h after CIDR removal [29] or between
281 50 and 62h [30], which would be around the most fertile period identified herein.

282 In summary, from our results we infer the following: 1) sex-sorted red deer sperm are
283 viable for a short time span once inseminated, which may explain that the best results are
284 obtained at a time likely closer to ovulation; and, 2) stressors related to female handling for
285 insemination may further alter the ability of sorted sperm to fertilize the oocyte because of their
286 precapacitated status. Additional studies in regards to sperm and oocyte physiology in red deer
287 may further clarify our findings.

288

289 **Founding**

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292

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388 **Figure Legends**

389 **Figure 1:**

390 Fertility rates in red deer hinds inseminated (Control sexed n=158; YSS n=259; Conventional
391 Samples n=237) with sex-sorted frozen-thawed stag semen. One straw per hind was used to
392 carry out the artificial insemination. Sperm treatments included: Control sexed sperm (at a
393 concentration of 40 $x10^6$ sperm/mL), Y-sorted sperm (YSS; at a concentration of 40 $x10^6$
394 sperm/mL) and Conventional samples (at a concentration of 100 $x10^6$ sperm/mL). Pregnancy
395 rates were calculated based upon birth rates. Different textures show differences ($P<0.05$)
396 among treatments.

397

398 **Figure 2:**

399 Sex ratio resulting from insemination of Y-sexed or control sperm from red deer. Sperm
400 treatments included: Control sexed sperm (at a concentration of 40 $x10^6$ sperm/mL), Y-sorted
401 sperm (YSS; at a concentration of 40 $x10^6$ sperm/mL) and Conventional samples (at a
402 concentration of 100 $x10^6$ sperm/mL). Different textures show differences ($P<0.05$) among
403 treatments.

404

405

406 **Table 1**: Pregnancy rates for each insemination interval following CIDR removal for hinds
407 inseminated with Control sexed, Y sorted or Conventional frozen-thawed stag sperm from red
408 deer. Insemination intervals following eCG administration were set as follows: I_1 55:01 –
409 55:30 h, I_2 55:31 – 56:00 h, I_3 56:01 – 56:30 h, I_4 56:31 – 57:00 h. Sperm treatments
410 included: Control sexed sperm (at a concentration of 40 $x10^6$ sperm/mL), Y-sorted sperm (YSS;
411 at a concentration of 40 $x10^6$ sperm/mL) and Conventional samples (at a concentration of 100
412 $x10^6$ sperm/mL). Pregnancy rates were calculated based on birth rates. Different capital letters
413 within sperm treatment denote significant differences ($P<0.05$) among insemination intervals.

414 **1a**

Control Sexed				
Interval	**Pregnant hinds**	**Total hinds**	**Fertility**	**Differences**
I_1	21	39	53,8%	A
I_2	20	40	50,0%	A
I_3	22	38	57,9%	A
I_4	18	41	43,9%	A
Total	81	158	51,3%	

415

416 **1b**

YSS				
Interval	**Pregnant hinds**	**Total hinds**	**Fertility**	**Differences**
I_1	48	60	80,0%	A
I_2	49	59	83,1%	A
I_3	20	60	33,3%	B
I_4	2	60	3,3%	C
Total	119	239	49,8%	

417

418 **1c**

Conventional Samples				
Interval	**Pregnant hinds**	**Total hinds**	**Fertility**	**Differences**
I_1	40	60	66,7%	A
I_2	45	59	76,3%	B
I_3	55	60	91,7%	C
I_4	44	58	75,9%	B
Total	184	237	77,6%	

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420

421 **Figure 1.**

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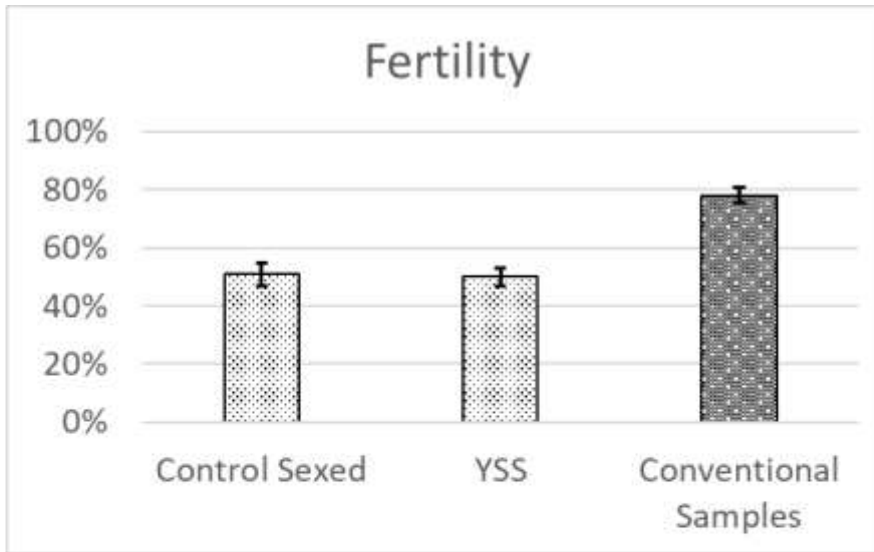

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427 **Figure 1b.**

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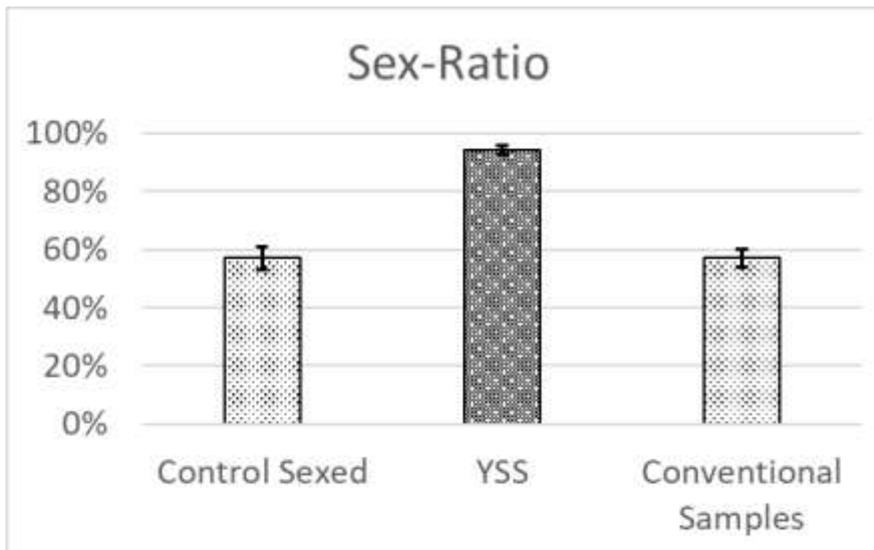

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