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Sperm competition accentuates selection on ejaculate attributes

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17 **Abstract**

18 Ejaculate attributes are important factors driving the probability of fertilizing eggs. When
19 females mate with several males, competition between sperm to fertilize eggs should
20 accentuate selection on ejaculate attributes. We tested this hypothesis in the North African
21 houbara bustard (*Chlamydotis undulata undulata*) by comparing the strength of selection
22 acting on two ejaculate attributes when sperm from single males or sperm from different
23 males were used for insemination. In agreement with the prediction, we found that selection
24 on ejaculate attributes was stronger when sperm of different males competed for egg
25 fertilization. These findings provide the first direct comparison of the strength of selection
26 acting on ejaculate attributes under competitive and non-competitive fertilizations, confirming
27 that sperm competition is a major selective force driving the evolution of ejaculate
28 characteristics.

29

30 **Keywords:** ejaculate, fertilization success, multiple mating, natural selection, sexual
31 selection, sperm competition

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35 **1. Introduction**

36 In order to fertilize eggs, sperm released in the female reproductive tract or in the
37 external environment must strive against adverse environmental conditions, imposing strong
38 selection on ejaculate characteristics (1,2). Ejaculate attributes and sperm phenotypes are,
39 therefore, thought to be under the action of natural selection. In addition to their intrinsic
40 capacity to fertilize eggs, ejaculates can also be under the action of post-copulatory sexual
41 selection (3). When females mate with multiple males during the same reproductive bout,
42 ejaculates of different males compete for the fertilization of eggs (4). Thus, sperm competition
43 can be an additional source of selection on ejaculate traits.

44 Parker (5) suggested that sperm number should be an important determinant of
45 fertilization success under competitive conditions, since ejaculates with more sperm should
46 have higher chances to win the raffle against competitor sperm. Support for this hypothesis
47 has been provided by several studies (6,7, but also see 8). However, ~~sperm number is not all~~
48 ~~(9), and~~ several other attributes of the ejaculate (e.g., sperm motility) have also been found to
49 play a role when sperm of different males compete for fertilization (9,10,11). Recent work has
50 provided an overview of the main features of the ejaculate that correlate with fertilization
51 success (12,13).

52 Studies on the association of ejaculate attributes and/or sperm phenotype with
53 fertilization success have been conducted either under competitive (sperm of different males
54 compete for egg fertilization) or non-competitive (only sperm of single males can fertilize the
55 eggs) conditions, but a direct comparison of the strength of selection acting on ejaculate
56 attributes in the presence and absence of sperm competition is still missing (12). Here, we
57 compared the strength of selection under competitive and non-competitive fertilizations on
58 two ejaculate attributes, the proportion of motile sperm and the proportion of sperm with
59 normal morphology. We used a large dataset that has been collected over 14 years in a captive

60 breeding population of the North African houbara bustard (*Chlamydotis undulata undulata*).
61 During the breeding season, semen is routinely collected and used to inseminate females,
62 either with semen of a single male or with semen of different males, in successive
63 inseminations. We predicted that sperm competition, arising when the semen of multiple
64 males is used to inseminate a single female, should accentuate selection acting on the two
65 ejaculate attributes.

66

67 **2. Material and methods**

68 All data used in this study were collected on North African houbara bustards that were
69 part of a long-term conservation breeding program in eastern Morocco (Emirates Center for
70 Wildlife Propagation). The program relies entirely on artificial insemination (14). Our dataset
71 included 1302 males born between 1986 and 2012; data on ejaculate attributes were collected
72 over a 14-year period (2000-2013), giving a total number of 3519 records.

73 We focused on two ejaculate attributes: the proportion of motile sperm and the
74 proportion of morphologically normal sperm (see Table S1 for descriptive statistics, and
75 Table S2 for repeatability of each trait). Although the two traits positively covaried, their
76 correlation coefficient was low (Pearson's $r = 0.18$, $n = 3519$).

77 Ejaculates were routinely collected using a dummy female and immediately analysed
78 in an adjacent laboratory. The proportion of motile sperm was assessed using a mass motility
79 index, scored from 0 (no motile sperm) to 5 (high proportion of motile sperm), under a light
80 microscope (14). The proportion of morphologically normal sperm was assessed using an
81 eosin-nigrosin method (15) (see supplemental material and methods for a full description).

82 Females were inseminated with either semen of a single male or successively
83 inseminated with semen of different males. Male reproductive success was assessed as the
84 number of hatchlings produced by each male per year. In the case of single male

85 inseminations, paternity was obviously assigned with certainty. In the case of multiple male
86 inseminations, paternity was assigned based on the genotyping of 9 microsatellite loci
87 designed for the houbara bustard (16) (see ESM for full description).

88 *Statistical analyses*

89 All phenotypic traits were standardized (zero mean, unit variance) within each year for
90 each fertilization context (competitive and non-competitive fertilizations), and male
91 reproductive success was converted into relative fitness by dividing each individual value by
92 the mean annual value. Selection coefficients were computed using a multiple regression
93 approach (17), where relative fitness was the dependent variable and proportion of motile
94 sperm and proportion of morphologically normal sperm were independent variables. This
95 model also included a variable that described the fertilization context (0 = non-competitive
96 fertilizations; 1 = competitive fertilizations), and the interactions between the two ejaculate
97 attributes and the fertilization context. In order to correct for possible confounding factors, the
98 model also included i) the number of times the semen of a given male was used to inseminate
99 females (correcting for both inter-individual differences in mating opportunities and number
100 of eggs laid, see supplemental methods); ii) the proportion of times his semen was used as last
101 in the insemination sequence for each breeding season (number of times the semen of a given
102 male was used as last in the insemination sequence over the total number of times it was
103 involved in multiple male inseminations) (correcting for a last male precedence effect); iii) the
104 number of inseminated sperm (correcting for the actual number of sperm used for
105 insemination); iv) male age (correcting for age-dependent variation in ejaculate attributes)
106 (18).

107 Statistical significance was inferred using a linear mixed effects model ('lmer'
108 function of the 'lme4' package for R) that in addition to the fixed effects mentioned above
109 also included male identity and year of birth as random effects.

110

111 **3. Results**

112 Selection gradients were positive for both ejaculate attributes, showing that males
113 producing ejaculates with higher proportions of motile and morphologically normal sperm
114 achieved better annual relative fitness (Table 1). This result holds when correcting for
115 potential confounding factors such as the number of times the semen of a given male was
116 used to inseminate females or the proportion of times the semen of a given male was used as
117 last in the insemination sequence. However, the model also showed statistically significant
118 interactions between the fertilization context and the two ejaculate attributes, with the
119 coefficients indicating higher slope between relative fitness and both ejaculate attributes under
120 competitive fertilization (Table 1). To better visualize the difference between fertilization
121 contexts, we ran two additional models, one for each competitive context. These models
122 showed that the selection gradients were 3-fold (for the proportion of morphologically normal
123 sperm) and 1.7-fold (for the proportion of motile sperm) higher under competitive
124 fertilizations (Table 2, Fig. 1).

125

126 **4. Discussion**

127 The aim of our study was to compare the strength of selection acting on ejaculate
128 attributes when sperm of different males compete for egg fertilization and when they do not.
129 We predicted that under competitive fertilizations, selection on ejaculate attributes should be
130 stronger. A direct test of this simple prediction was lacking, because previous work has either
131 focused on non-competitive (single male) or competitive (multiple males) fertilizations. We
132 found that the proportion of motile sperm and the proportion of morphologically normal
133 sperm were under positive selection and that selection coefficients were higher when
134 estimated under competitive than under non-competitive fertilizations.

135 How sperm competition drives the evolution of ejaculate attributes has been
136 extensively studied both theoretically and empirically (4,5). Comparative and experimental
137 evidence shows that allocation to ejaculate attributes and sperm phenotypes usually increases
138 in response to sperm competition risk (19,20). When controlling for the number of sperm
139 competing, sperm motility and the proportion of morphologically normal sperm have been
140 shown to be important determinants of fertilization success in different species (12,13). For
141 instance, in the Iberian red deer (*Cervus elaphus hispanicus*), males with higher proportions
142 of morphologically normal sperm in the ejaculate have better reproductive success, during
143 non-competitive fertilizations (21). In addition to simply scrutinizing the association between
144 the proportion of motile sperm in the ejaculate, the proportion of morphologically normal
145 sperm and male reproductive success, we were able to quantitatively compare the strength of
146 such association and found, as predicted, stronger selection under competitive fertilizations.
147 The underlying mechanisms accounting for the selective advantage of higher proportion of
148 motile sperm might involve a privileged access of motile sperm to female sperm-storage
149 tubules (22) and/or improved capacity of motile sperm to escape the hostile environment they
150 experience in the female cloaca (1) (although this seems unlikely here because artificially
151 inseminated sperm bypass the transit through the cloaca); whereas morphologically normal
152 sperm may have an advantage because they swim straighter and with higher beat frequencies
153 (23).

154 To the best of our knowledge, only a single study has attempted to compare the gain in
155 paternity share of males with high motile sperm during competitive fertilizations with the
156 expected fertilization success in the absence of sperm competition (22). In agreement with the
157 prediction, this study reported that domestic fowls with high motile sperm sired 73% of the
158 offspring during competitive fertilizations, while the expected fertilization success based on
159 their performance in non-competitive fertilizations was 53%. However, it should be noted that

160 this comparison was based on findings reported in two different experiments conducted on
161 different individuals (22,24).

162 To conclude, we provide the first direct comparison of the strength of selection acting
163 on ejaculate attributes under both non-competitive and competitive fertilizations. Our results
164 support the view that sperm competition represents an additional selective force shaping the
165 evolution of ejaculate attributes and sperm phenotype.

166

167 **Ethics**

168 All birds used in the present study were bred in captivity in agreement with Moroccan
169 authorities: Ministère de l'Agriculture, Développement Rural et des Pêches Maritimes,
170 Direction Provinciale de l'Agriculture de Boulemane, and Service Vétérinaire (Nu
171 DPA/48/285/SV) under permit number 01-16/VV; OAC/2007/E; Ac/Ou/Rn.

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173 **Data accessibility**

174 The dataset supporting this article is available in the Dryad Digital Repository
175 (<https://datadryad.org/review?doi=doi:10.5061/dryad.bm1j1c7>) (25).

176

177 **Authors' contributions**

178 PV, YH, LL, MSJ, FL and GS conceived and designed the study; data were collected under
179 the supervision of FL, LL and YH; LL carried out paternity analyses; PV carried out
180 statistical analyses and wrote the first draft of the manuscript; PV, YH, LL, MSJ, FL and GS
181 edited and revised the manuscript. All authors gave final approval for publication and agree to
182 be held accountable for the work performed therein.

183

184 **Competing interests**

185 We have no competing interests.

186

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189

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198

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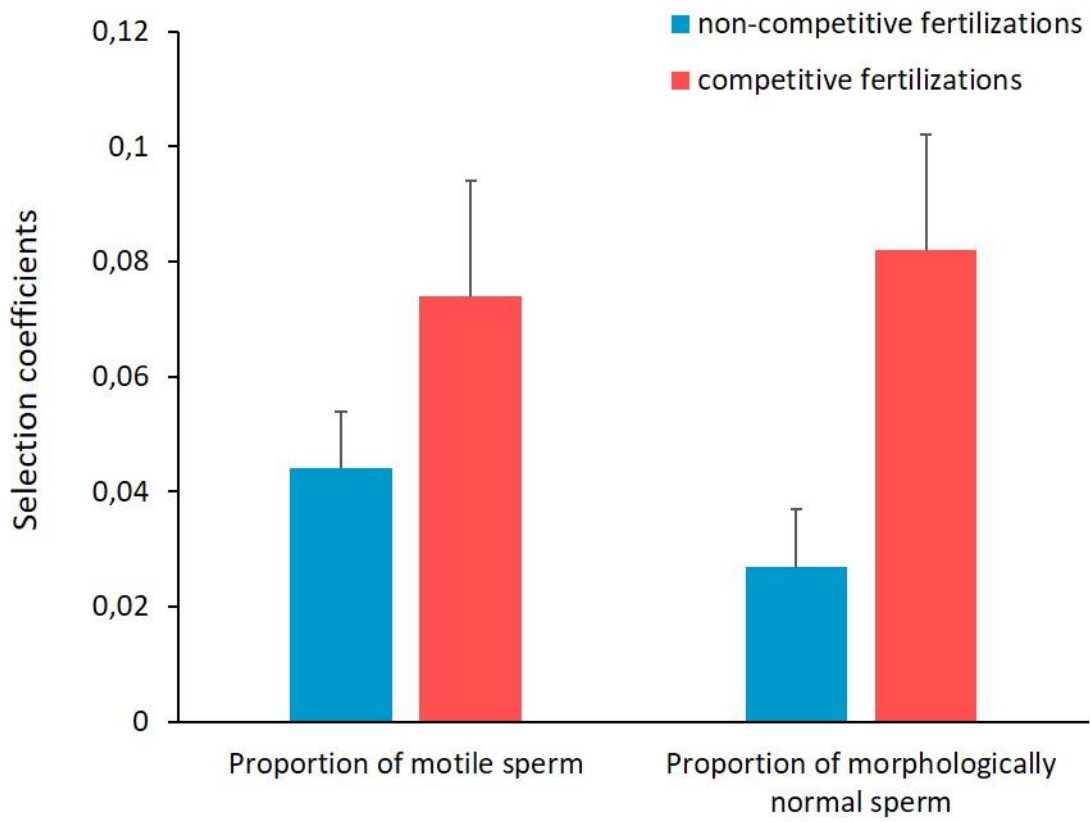
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- 265

266 Table 1. Estimates (\pm se) of a multiple regression model with relative fitness as dependent
 267 variable and two ejaculate attributes (proportion of motile sperm and proportion of
 268 morphologically normal sperm) as independent variables. The model also included the
 269 fertilization context, the number of times the semen of a given male was used to inseminate a
 270 female, the proportion of times his sperm was used as last in the insemination sequence, the
 271 number of inseminated sperm, and male age. F and p values were obtained from a linear
 272 mixed effects model that included male identity and year of birth as random factors.

Fixed effects	Estimate \pm se	df	F	<i>p</i>
Proportion of motile sperm	0.029 \pm 0.018	1, 2208	28.18	<0.0001
Proportion of morphologically normal sperm	0.033 \pm 0.017	1, 2208	26.75	<0.0001
Fertilization context	1.315 \pm 0.043	1, 2208	1006.08	<0.0001
Number of inseminations	0.819 \pm 0.014	1, 2208	3280.38	<0.0001
Proportion of last positions in the insemination sequence	0.491 \pm 0.021	1, 2208	627.69	<0.0001
Number of inseminated sperm	-0.024 \pm 0.013	1, 2208	3.17	0.0749
Age	-0.038 \pm 0.013	1, 2208	0.36	0.5511
Proportion of motile sperm x Fertilization context	0.098 \pm 0.027	1, 2208	13.01	0.0003
Proportion of morphologically normal sperm x Fertilization context	0.087 \pm 0.027	1, 2208	11.09	0.0009
Random effects	Estimate \pm se		Z	<i>p</i>
Male identity	0.031 \pm 0.009		3.58	0.0002
Year of birth	0.029 \pm 0.014		2.10	0.0177
Residual	0.555 \pm 0.015		36.72	<0.0001

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277 Figure 1. Selection gradients (\pm se) for the proportion of motile sperm and the proportion of
278 morphologically normal sperm under non-competitive and competitive fertilizations.



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