**Electronic Supplementary Material**

**Sperm competition accentuates selection on ejaculate attributes**

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**Supplemental material and methods**

*Model species*

The North African houbara bustard (*Chlamydotis undulata undulata*) lives in arid lands across North Africa. The species is sexually dimorphic both in size and plumage (males are bigger and harbour longer ornamental feathers). During the breeding season, males aggregate in “exploded” leks where they perform extravagant courtship displays to attract females (Hingrat et al. 2008; Gaucher et al. 1996). Females mate with one or several males as shown by the occurrence of broods with multiple fathers in the wild (Lesobre et al. 2010). Sperm of different males, therefore, regularly compete for the fertilization of eggs.

*Ejaculate collections and artificial inseminations*

Ejaculates were routinely collected in captivity using a dummy female. Briefly, the dummy female was presented to males and the ejaculate collected in a Petri dish hold under the male cloaca. This should ensure that the quantity of semen collected closely approaches the amount ejaculated during a natural copulation (at least compared to other semen collecting techniques, such as massage). Ejaculates were transferred into an Eppendorf tube and brought immediately to an adjacent laboratory. The proportion of motile sperm was assessed using a mass motility index, scored from 0 to 5, under a light microscope (x100), 5 indicating higher proportion of motile sperm in the ejaculate (Saint Jalme et al. 1994). The proportion of morphologically normal sperm was assessed using an eosin-nigrosin method (Lindsay et al. 1999). Semen was stained with eosin-nigrosin and a minimum of 100 sperm were screened under a light microscope (x1000). Normal live sperm do not absorb the stain and appear white, while sperm with corrupted membrane integrity absorb the stain and take a pinkish coloration. Abnormal sperm morphology mainly included double flagella, double heads, swollen membrane or extended nuclei.

Females are regularly checked by breeders throughout the breeding season and when deemed ready to lay, they are inseminated twice within 48h, either with the sperm of the same male or with the sperm of a different male for each insemination (depending on the availability of ejaculates of the first male 2 days later). This sequence (2 inseminations within 48h) is repeated after completion of the clutch (usually 2 to 3 eggs). Again, these repeated inseminations are performed with the sperm of the first male or of different males, depending on whether the ejaculate of the first male is successfully collected when needed. If no eggs are laid, another insemination sequence is performed after 10 days. The whole process making that the sperm of a male was used in single or multiple male inseminations was therefore completely random. We used, a posteriori, all the records that have been included in the ECWP database to reconstruct whether the sperm of a given male used to inseminate a given female a given year had to compete with the sperm of other males, or not. This allowed to assess the association between ejaculate attributes and fertilization success under non-competitive and competitive conditions. Given that there was not an a priori assignment of males to one fertilization context, the majority of individuals was actually used under both fertilization contexts (749 out of 1302).

For the purposes of the breeding program, collected ejaculates are usually split in several aliquots that are used to inseminate different females. Therefore, the number of inseminated sperm does not correspond to the actual sperm production (i.e. the actual number of sperm in the ejaculate). Corroborating this point, both the mean number of inseminated sperm (the insemination dose established by the breeders) and its inter-individual variation are much smaller than the mean number of sperm in the ejaculate and its variation [mean number of inseminated sperm (± SD), 17.23 x 106 ± 4.94 x 106; mean number of sperm in the ejaculate (± SD), 31.45 x 106 ± 14.47 x 106; n = 3519]. For this reason, we did not consider the number of inseminated sperm as a trait upon which it was meaningful to assess the effect of selection, because it simply reflects the standard protocol used to inseminate females. Nevertheless, given that the number of inseminated sperm was not identical across inseminations, we included it as a covariate in the statistical models (see also below).

Male reproductive success was assessed as the number of hatchlings produced by each male per year [mean number of hatchlings sired per year and per male, 4.22 ± 5.56 (SD), n = 3519]. In competitive fertilizations, males competed on average with the semen of 2.49 [± 1.35 (SD), range 1-9, n = 1419] competitors.

In the case of single male inseminations, paternity was obviously assigned with certainty. In the case of multiple male inseminations, paternity was assigned based on the genotyping of 9 microsatellite loci designed for the houbara bustard (Lesobre et al. 2010). A small volume of blood (~100 µL) was taken from all birds (parents and offspring) and stored in 2 mL Eppendorf tubes containing 1.5 mL of absolute ethanol. DNA extraction and genotyping were carried out by GENOSCREEN (Lille, France), the detailed procedure being described elsewhere (Lesobre et al. 2010). Paternities were assigned using CERVUS 3.0.

*Confounding variables in the statistical analyses*

Male reproductive fitness obviously depends on the number of mating opportunities and on the number of eggs laid by the different females a male has mated with. Under the specific requirements of the breeding program, the sperm of some males was used more often to inseminate females than the sperm of other males. We therefore included the number of inseminations in the model to correct for inter-individual differences in mating opportunities. By doing so, we also corrected for differences in the number of eggs laid, since number of inseminations and number of eggs laid were strongly correlated (Pearson’s r = 0.90, n = 3519), and including the two variables in the same models would raise collinearity issues.

In competitive fertilizations, an additional confounding variable was the position in the insemination sequence. Usually last males have a disproportionally higher fertilization success, a phenomenon called “last male precedence” (Birkhead and Biggins 1998). Paternity studies in ECWP’s captive population (N = 5300 chicks analysed) highlighted the occurrence of last male precedence with 75,5 % of the chicks sired by the last male in the insemination sequence (Lesobre, personal observation). To correct for such confounding effect, we computed for each male the proportion of times his sperm was used as last in the insemination sequence for each breeding season (number of times the semen of a given male was used as last in the insemination sequence over the total number of times it was involved in multiple male inseminations). A value of 1 indicates that a given year, the sperm of a given male has always been used as last in the insemination order; a value of 0 indicates that the sperm of a given male has never been used as last in the insemination order. In non-competitive fertilizations, this proportion always takes the value of 1. Finally, we also included the number of inseminated sperm, and male age (as to control for age-dependent variation in ejaculate attributes).

While the regression model gives the values of the selection coefficients, it provides inflated *p* values due to the non-independence of repeated observations for a given individual over time. Therefore, statistical significance was inferred using a linear mixed effects model (‘lmer’ function of the ‘lme4’ package for R) that in addition to the fixed effects described above also included male identity and year of birth as random effects.

*References*

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**Table S1. Descriptive statistics for the two ejaculate attributes for the 1302 North African houbara bustard used in the study.** The proportion of motile sperm in the ejaculate was scored using a mass motility index (0 to 5). sd: standard deviation, CV: coefficient of variation (%).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Mean | sd | CV (%) | N |
| Proportion of motile sperm | 3.80 | 0.43 | 11.37 | 3519 |
| Proportion of morphologically normal sperm | 0.80 | 0.11 | 13.52 | 3519 |

**Table S2. Yearly repeatability for the two ejaculate attributes for the 1302 North African houbara bustard used in the study.** Repeatability was computed as the intraclass correlation coefficient (R).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Proportion of motile sperm | | | |  | Proportion of morphologically normal sperm | | | |
| Year | R | F | df | *P* |  | R | F | df | *p* |
| 2000 | 0.19 | 5.33 | 55.109 | <0.001 |  | 0.57 | 29.61 | 50.932 | <0.001 |
| 2001 | 0.53 | 9.77 | 111.137 | <0.001 |  | 0.20 | 1.81 | 51.118 | 0.004 |
| 2002 | 0.33 | 9.76 | 184.392 | <0.001 |  | 0.48 | 3.84 | 53.118 | <0.001 |
| 2003 | 0.61 | 25.08 | 261.531 | <0.001 |  | 0.58 | 5.43 | 152.332 | <0.001 |
| 2004 | 0.58 | 25.37 | 354.821 | <0.001 |  | 0.60 | 5.29 | 247.480 | <0.001 |
| 2005 | 0.59 | 16.51 | 395.607 | <0.001 |  | 0.40 | 3.47 | 54.171 | <0.001 |
| 2006 | 0.61 | 25.70 | 489.889 | <0.001 |  | - | - | - | - |
| 2007 | 0.71 | 62.27 | 756.206 | <0.001 |  | - | - | - | - |
| 2008 | 0.70 | 54.83 | 1072.270 | <0.001 |  | 0.60 | 5.37 | 461.889 | <0.001 |
| 2009 | 0.65 | 47.81 | 1472.410 | <0.001 |  | 0.67 | 6.01 | 335.495 | <0.001 |
| 2010 | 0.62 | 40.71 | 1759.467 | <0.001 |  | 0.59 | 6.12 | 83.161 | <0.001 |
| 2011 | 0.60 | 43.49 | 2004.613 | <0.001 |  | 0.75 | 7.45 | 233.261 | <0.001 |
| 2012 | 0.54 | 29.78 | 2201.636 | <0.001 |  | 0.50 | 4.60 | 114.292 | <0.001 |
| 2013 | 0.52 | 31.70 | 2384.717 | <0.001 |  | 0.87 | 16.59 | 449.544 | <0.001 |