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1 **No evidence for pre-zygotic post-copulatory avoidance of kin despite high**
2 **inbreeding depression**

3

4 Running title: Absence of post-copulatory avoidance of kin

5

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19 **Abstract**

20 Offspring resulting from mating among close relatives can suffer from impaired fitness
21 through the expression of recessive alleles with deleterious effects. Post-copulatory sperm
22 selection (a pre-zygotic mechanism of cryptic female choice) has been suggested to be an
23 effective way to avoid inbreeding. To investigate whether post-copulatory female choice
24 allows avoiding fertilization by close kin, we performed an artificial insemination experiment
25 in a promiscuous bird, the houbara bustard (*Chlamydotis undulata undulata*). Females were
26 inseminated with a mix of sperm from triads of males, each constituted of a male genetically
27 unrelated to the female, a first cousin, and a half-sibling. Paternity was assigned based on
28 microsatellite genotyping. When counting the number of eggs sired by unrelated males,
29 cousins or half-siblings, we found a significant deviation from the expected random
30 distribution, with more eggs sired by unrelated males. However, offspring sired by cousins,
31 and especially by half-siblings suffered from high pre-hatching mortality, suggesting that the
32 observed paternity skew towards unrelated males might reflect differential offspring mortality
33 rather than sperm selection. In agreement with this hypothesis, we found that within-triad
34 siring success was similar for the three parental relatedness categories, but the relationship
35 between siring and hatching success differed across parental relatedness categories. In
36 clutches that had a high hatching success ($\geq 80\%$), males that were unrelated to the female
37 had the highest siring success; on the contrary, in clutches that had a high failure rate ($\leq 20\%$
38 hatching success), the highest siring success was for half-siblings. In addition, offspring sired
39 by males in the half-sibling relatedness category also suffered from reduced growth rate
40 during the first three months of life and higher post-hatching mortality. Hence, despite
41 substantial fitness costs associated with fertilization by close relatives, females do not seem to
42 preferentially select sperm of unrelated males.

43

44 **Keywords:** cryptic female choice, inbreeding avoidance, inbreeding cost, parental
45 relatedness, sperm selection, siring success

46 **Introduction**

47 Inbreeding, due to mating among close relatives, increases the homozygosity of
48 offspring, allowing recessive alleles with potentially deleterious effects to be fully expressed,
49 and reducing the expression of overdominance (Charlesworth & Charlesworth, 1999;
50 Charlesworth & Willis, 2009). As a consequence, inbreeding is generally associated with
51 impaired offspring fitness (i.e. inbreeding depression), directly through reduced survival and
52 reproduction or indirectly, via increased vulnerability to environmental hazards (Billing et al.,
53 2012; Crnokrak & Roff, 1999; Keller, Arcese, Smith, Hochachka, & Stearns, 1994; Keller &
54 Waller, 2002; Kruuk, Sheldon, & Merila, 2002). Therefore, inbreeding likely represents a
55 major selective force that should promote the evolution of mechanisms of inbreeding
56 avoidance.

57 The first mechanism of inbreeding avoidance consists in minimizing the probability to
58 encounter a close relative when searching for mates (Gandon, 1999). This is primarily
59 achieved through sex-dependent dispersal from the natal site (Lebigre, Alatalo, & Siitari,
60 2010; M. Szulkin & Sheldon, 2008). In spatially structured, viscous populations, the potential
61 for kin to encounter during the search for mates can be high (Blyton, Banks, & Peakall, 2015;
62 Geffen et al., 2011; Riehl & Stern, 2015). In this case, an additional mechanism of inbreeding
63 avoidance is kin recognition (Bateson, 1982; Hatchwell, Ross, Fowlie, & McGowan, 2001;
64 Johnston, 2008; Krause, Kruger, Kohlmeier, & Caspers, 2012; Penn & Frommen, 2010).
65 When females mate with multiple males, post-copulatory mechanisms (i.e. cryptic female
66 choice; Eberhard, 1996) of inbreeding avoidance might be particularly effective as females
67 are thought to pay stronger costs of inbreeding than males (Billing et al., 2012), and therefore
68 to be less tolerant to inbreeding (Lehmann & Perrin, 2003). Cryptic female choice
69 encompasses both pre-zygotic (i.e. selective storage (Bretman, Newcombe, & Tregenza,

70 2009; Welke & Schneider, 2009) or use (Firman & Simmons, 2015) of sperm from unrelated
71 mates) and post-zygotic (e.g. differential abortion or maternal investment; Firman, Gasparini,
72 Manier, & Pizzari, 2017) mechanisms.

73 The idea that in sexually reproducing species, individuals should avoid mating with
74 relatives essentially rests on the observation that inbred offspring have impaired fitness.
75 However, it has now become clear that pre-copulatory mechanisms promoting inbreeding
76 avoidance also incur costs (Bengtsson, 1978). Dispersal and/or search for mates can be
77 time/energy consuming and can expose to increased predation risk (Bonte et al., 2012).
78 Further, refusing to mate with a close relative (kin recognition and avoidance) might imply no
79 reproduction at all if unrelated mates are rare. In addition, coerced mating might leave little
80 room for kin avoidance (Parker, 2006). Finally, while inbreeding avoidance has some intrinsic
81 costs, mating with kin can also provide benefits in terms of kin selection (Kokko & Ots,
82 2006). Therefore, the net selection acting on inbreeding depends, not only on the costs
83 associated with mating with kin, but also on its benefits and on the costs of kin avoidance
84 (Szulkin, Stopher, Pemberton, & Reid, 2013).

85 Sexual promiscuity, and more specifically polyandry, is a widespread mating system
86 in the animal kingdom (Andersson & Simmons, 2006; Pizzari & Wedell, 2013). Multiple
87 mating during the same reproductive bout implies that sperm of different males compete for
88 egg fertilization, and that females might modulate male fertilization success through covert
89 processes operating within their genital tracts (Birkhead & Pizzari, 2002; Eberhard &
90 Cordero, 1995). Sperm selection by females has been reported in several species and involves
91 different mechanisms including gametic interactions, or interactions between sperm and
92 ovarian fluids (Firman & Simmons, 2015; Gasparini & Pilastro, 2011; Løvlie, Gillingham,
93 Worley, Pizzari, & Richardson, 2013; Wedekind, Chapuisat, Macas, & Rüllicke, 1996). While
94 cryptic female choice is generally thought as a mechanism allowing promiscuous females to

95 fertilize their eggs with the gametes of the most desirable males (supposedly improving
96 offspring fitness), this might include genetically compatible (i.e., unrelated) mates. Post-
97 copulatory mechanisms of inbreeding avoidance have been investigated in several species,
98 and these studies have reported both positive (i.e., avoidance of fertilization by kin; Bretman,
99 Wedell, & Tregenza, 2004; Firman & Simmons, 2008; Fitzpatrick & Evans, 2014; Tregenza
100 & Wedell, 2002) and negative (i.e., absence of avoidance of fertilization by kin; Denk,
101 Holzmann, Peters, Vermeirssen, & Kempenaers, 2005; Evans, Brooks, Zajitschek, & Griffith,
102 2008; Stockley, 1997) results. These heterogeneous findings might be due to the intrinsic
103 difficulty of properly testing for female post-copulatory choice based on kinship. For instance,
104 when females mate with related and unrelated males it might be impossible to tease apart pre-
105 and post-copulatory avoidance mechanisms. Similarly, if males can sense that a female is
106 closely related, they might strategically adjust their investment into the ejaculate (Pizzari, Lo,
107 & Cornwallis, 2004), which may affect siring success in the absence of any female cryptic
108 choice. Therefore, only artificial inseminations offer the possibility to remove the effects of
109 pre-copulatory choice and to isolate post-copulatory mechanisms (Fitzpatrick & Evans, 2014).
110 Finally, siring success is usually assessed based on the genotyping of offspring and putative
111 fathers. This might produce biased results if eggs fertilized by kin suffer from early
112 developmental arrest that might go unnoticed. For this reason, a proper assessment of post-
113 copulatory kin avoidance should also explicitly take into account differential embryo
114 mortality.

115 Here, we used a promiscuous bird, the North African houbara bustard (*Chlamydotis*
116 *undulata undulata*), to i) investigate whether females avoid fertilization with kin and ii) assess
117 the cost of inbreeding. This species is a relevant model to study post-copulatory sexual
118 selection since females mate with multiple males in the wild (Lesobre, Lacroix, Le Nuz, et al.,
119 2010). We artificially inseminated females with a mix of sperm from triads of males with

120 different levels of relatedness (unrelated, first cousin and half-sibling) and assessed male
121 siring success within triads. Artificial inseminations ensured that any fertilisation bias would
122 be the result of post-copulatory selection only. We assessed the cost of inbreeding by
123 measuring life history traits (pre- and post-hatching survival, body mass, size and body
124 condition at hatching, growth rate) of offspring produced by fertilizations with sperm from
125 related or unrelated males.

126

127 **Materials and methods**

128 *Mating system and reproductive biology of the houbara bustard*

129 The North African houbara bustard is a bird species living in arid habitats, its range
130 extends from Mauritania to Egypt, and the breeding season from January to June. Males
131 express a conspicuous sexual display on dedicated but separated sites (exploded-
132 leks) (Hingrat et al. 2004, 2007); females mate with several males, as shown by the
133 occurrence of multiple paternities within broods (Lesobre, Lacroix, Le Nuz, et al., 2010), and
134 can store sperm for several weeks (Saint Jalme, Gaucher, & Paillat, 1994). Females typically
135 lay a single clutch of one to three egg(s), but can produce a replacement clutch if the first one
136 is lost (Saint Jalme, Williams, Mickaelian, & Paillat, 1996). Only females provide parental
137 care, the contribution of males being restricted to the transfer of genetic material, and chicks
138 remain with their mother for 6 to 10 weeks (Hingrat & Saint Jalme, 2005).

139 *Experimental birds and breeding program management*

140 All birds used in this study were part of the Emirates Center for Wildlife Propagation
141 (ECWP), a conservation breeding program located in eastern Morocco, aiming at reinforcing
142 natural populations of the houbara bustard. This program relies entirely on artificial

143 inseminations (Saint Jalme et al., 1994), where pairing is designed to promote genetic
144 diversity while avoiding inbreeding within the captive flock (Lesobre, Lacroix, Caizergues, et
145 al., 2010). Ejaculates are routinely collected using a dummy female, and immediately
146 transferred into a lab adjacent to the sperm collection site. Sperm motility is scored from 0 to
147 5 under a light microscope, using the following scale: (0) total lack of movement, (1) few
148 motile sperm without forward movement, (2) less than 50% of sperm showing moderate
149 activity, (3) above 50% motile sperm showing forward movement, (4) above 80% motile
150 sperm showing fast forward movement, (5) almost all sperm showing fast forward movement
151 with waves and whirlwinds (Saint Jalme et al., 1994). Then, the volume ($\pm 1 \mu\text{L}$) of undiluted
152 ejaculates is measured using a precision microliter pipette. After dilution with Lake 7.1
153 diluent (Lake & Ravie, 1984) the number of sperm in the ejaculate is assessed using a
154 spectrophotometer and a specific calibration for the species (Chargé, Saint Jalme, Lacroix,
155 Cadet, & Sorci, 2010).

156 Females are regularly checked for reproductive status and start to be inseminated with
157 freshly collected semen when ready to lay. Laying sequences are carefully recorded, so that
158 inseminations are generally performed 3-6 days before clutch initiation, as to maximize
159 fertility (Saint Jalme et al., 1994). Freshly laid eggs are collected and incubated in large
160 incubators at $37.7 \text{ }^\circ\text{C}$ for 22 days (eggs that do not hatch after 24 days of incubation are
161 considered failed).

162 *Artificial inseminations*

163 The study was conducted during the 2016 breeding season. One hundred and one
164 females (mean age 4.3 ± 1.5 years, min-max = 3-8 years) were used for this study. Each
165 female was inseminated once with semen from a triad of males (mean age = 5.5 ± 1.6 years,
166 min-max = 3-8 years, male age being homogeneously distributed across relatedness

167 categories; Table S1). Sperm was slowly delivered inside the vagina using an insemination
168 tube mounted on a pipette (Saint Jalme et al., 1994). All birds in the captive flock have a
169 known pedigree, therefore it was possible to choose triads of males based on their level of
170 relatedness to the female. Triads were composed of an unrelated male (relatedness coefficient
171 $r = 0$), a first cousin ($r = 0.125$) and a half-sibling ($r = 0.25$). In total, 140 different males were
172 used to perform the inseminations, and the semen of each male was used 2.2 ± 1.9 times on
173 average (min-max = 1-13 times). In heterospermic inseminations, sperm number is positively
174 related to fertilization success (Martin et al. 1974) and embryo development in birds
175 (Hemmings & Birkhead, 2015). Hence, we balanced the contribution of each male in terms of
176 sperm number. In other words, in each triad, males equally contributed to the insemination by
177 providing an equal number of sperm (mean number of sperm per insemination = $30.0 \pm 8.0 \times$
178 10^6 sperm, each male contributing to the third of this overall sperm number).

179 *Egg collection, incubation and fertility assessment*

180 We collected up to five eggs per female (after a single insemination, the probability
181 that eggs laid after the five first ones are fertilized being very low), giving a total number of
182 469 eggs (99 out of 101 inseminated females having laid at least one egg). Five eggs were
183 found broken in the cage and thus could not be incubated. Thirty-one eggs had poor-quality
184 shell, representing a sanitary risk for the other eggs in the incubator. These eggs were
185 incubated apart for 5-6 days before opening them, allowing embryonic development to initiate
186 and revealing whether they were fertile. A final number of 433 eggs were incubated following
187 a standardized protocol. Incubated eggs were candled (using a light beam applied against the
188 shell) at day 8 and 21 to check for embryo development. If candling showed either no sign of
189 development or arrested development, eggs were removed from the incubator and opened.
190 Eggs whose yolk had a non-fertile appearance were further analyzed to discriminate between
191 infertility and early embryo mortality, by revealing embryonic cells in division using a DNA-

192 specific dye (Birkhead, Hall, Schut, & Hemmings, 2008). Briefly, we sampled a squared piece
193 (1 x 1 cm) of perivitelline layer (PVL) containing the germinal disc (GD). After carefully
194 washing the PVL through 3 consecutive baths in 1 mL of Hanks' balanced salt solution
195 (Sigma-Aldrich), it was plunged for 15 min in 1 mL of a diluted dye solution (Hoechst 33342,
196 Thermofisher, diluted into Hanks at a concentration of $10 \mu\text{g.mL}^{-1}$; based on a solution of dye
197 previously diluted into Lake 7.1 at a concentration of $100 \mu\text{g.mL}^{-1}$) and kept under light
198 cover. The piece of PVL was then spread on a slide and examined under a fluorescence
199 microscope (Nikon) to search for fluorescent clusters corresponding to groups of embryonic
200 cells. Candling of the 433 incubated eggs revealed embryonic development in 394 cases. Out
201 of the remaining 39 eggs, fertility analyses revealed that only 6 eggs had not been fertilized,
202 while for 33 eggs it was not possible to unambiguously assign them as fertile or not. Even
203 including these uncertain eggs in the non-fertile category, the overall fertility rate was high
204 (394 out of 433, 91.0%).

205 *Offspring growth and survival under captive conditions*

206 188 chicks hatched out of the 394 fertile eggs. Hatchlings were transferred into indoor
207 facilities where they were kept in small plastic boxes (0.25 m^2 , maximum 6 individuals per
208 box) for 10 days. They were fed by hand with a mix of pellets (Evalis, 24% of protein
209 content), fresh chopped alfalfa, mealworms and crickets. Then, they were kept in outdoor
210 cages (16 m^2 , maximum 6 individuals per cage) up to 40 days of age, and finally in large
211 outdoor aviaries (270 m^2 , maximum 12 individuals per aviary) where food was delivered once
212 a day. Water was provided *ad libitum*. Chick body mass ($\pm 1 \text{ g}$) and size, estimated through
213 measurements of head-skull length using a manual caliper [from the tip of the beak to the
214 back of the head with $\pm 0.5 \text{ mm}$ precision), were measured at day 1, 9, 18, 26, 40, 60 and 90
215 post-hatching. Body mass and head-skull length were used to compute an index of body
216 condition as the residuals of the linear regression of body mass against size (Schulte-

217 Hostedde, Zinner, Millar, & Hickling, 2005). We also monitored daily survival of birds up to
218 September 5th, where all of them were at least 3 months old, i.e. when they reached adult size
219 (mean age of 141 ± 17 days, min-max = 92-184 days).

220 *Paternity analyses*

221 DNA samples for microsatellite genotyping were either obtained from dead embryos
222 by cutting off a small piece of chest muscle or from hatchlings by taking a small volume of
223 blood (~100 μ L) from the axillary vein, using a single-use sterilized needle and capillary
224 tubes. Blood samples (~200 μ L) from the brachial vein of the wing were also taken from all
225 females and putative fathers, using a single-use 25-gauge insulin syringe. Samples were
226 stored in 2 mL Eppendorf tubes containing 1.5 mL of absolute ethanol. DNA extraction and
227 genotyping were carried out by GENOSCREEN (Lille, France), the detailed procedure being
228 described elsewhere (Lesobre, Lacroix, Le Nuz, et al., 2010). We genotyped 13 microsatellite
229 loci designed for the houbara bustard. Paternities were assigned using CERVUS 3.0, based on
230 the following simulation parameters: 50000 cycles, 3 candidate fathers, 98.3% of fathers
231 genotyped, 98.1% of loci typed and 0.05% of genotyping error rate. Individuals were included
232 in parentage analyses whenever they were successfully genotyped at a minimum of 11 loci,
233 and only paternities assigned with a confidence level of 95% were considered.

234 Among the 188 hatchlings, 187 were sampled for the genetic analyses, 185 were
235 successfully genotyped and for 181 we could assign a father. For the 206 fertile eggs that did
236 not hatch, only a small fraction ($n = 63$) provided sufficient genetic material (due to arrested
237 embryo development at early stages), and we could successfully genotype 62 of them.
238 Paternity was then assigned for 60 chicks that did not hatch. We also had genotypes of 4
239 embryos from eggs that were incubated apart and we could assign paternity for all of them.
240 All offspring that were successfully genotyped at a minimum number of 11 microsatellite loci

241 (n = 251) had their paternity assigned. However, for 6 of them there was a mismatch with the
242 genotype of their mother, suggesting possible mistakes in the labeling of the tubes. To be
243 conservative these 6 offspring were removed from the analyses. Hence, paternity could be
244 assigned for a final number of 245 offspring: 181 hatchlings and 64 failed embryos (see Table
245 S2 and Fig. S1 in supporting information for a detailed description of sample sizes).

246 *Ethical statement*

247 The birds used in the present study were bred in captivity in agreement with Moroccan
248 authorities: Ministère de l'Agriculture, Développement Rural et des Pêches Maritimes,
249 Direction Provinciale de l'Agriculture de Boulemane, Service Vétérinaire (Nu
250 DPA/48/285/SV) under permit no. 01-16/VV; OAC/2007/E; Ac/Ou/Rn. All releases were
251 recorded by the Haut Commissariat aux Eaux et Forêts et à la Lutte Contre la Désertification.
252 Bird handling and sampling were performed by trained bird keepers or experimenters of the
253 ECWP.

254 *Statistical analyses*

255 All statistical analyses were performed with R 3.1.3.

256 Paternity share was analysed using two metrics. First, based on the number of eggs
257 with assigned paternity, we compared the probability to be sired by a half-sibling, a cousin or
258 an unrelated male, using a test of equal proportions (the null hypothesis being that the
259 proportion of offspring sired by each relatedness category should be 1/3). Second, we
260 computed the within-triad siring success as the proportion of eggs sired by each male divided
261 by the number of eggs with assigned paternity. Siring success was then analysed using a
262 linear mixed effects model ('lmer' function of the 'lme4' package) with parental relatedness
263 (unrelated, cousin, half-sibling) as a fixed effect, and male identity as a random variable to
264 take into account the fact that the semen of some males was used more than once. Given that

265 sperm motility has been shown to be a good predictor of fertilization success in several
266 species (Tommaso Pizzari & Parker, 2009), including the houbara bustard, we also included it
267 as a fixed term.

268 Hatching success was analyzed using a generalized linear mixed effects model with a
269 binomial distribution of errors ('glmer' function of the 'lme4' package) that included parental
270 relatedness, sperm storage duration and laying date as fixed effects. Maternal and paternal
271 identities were included as random effects.

272 Offspring body mass, size and body condition at hatching were analyzed using linear
273 mixed effects models. In all models, parental relatedness was included as a fixed effect, and
274 laying date and sperm storage duration were also included as covariates. Maternal and
275 paternal identities were included as random effects. When analyzing offspring growth during
276 the first three months of life, we also included offspring age and its interaction with parental
277 relatedness as fixed effects. We tested for linear, logarithmic and curvilinear effects of
278 offspring on growth by comparing three models including age, log(age), or age and squared
279 age, respectively, based on their AICc. A linear effect of age received the best statistical
280 support for body mass, a quadratic effect of age for body size, and a logarithmic effect of age
281 for body condition. Offspring identity nested into maternal identity was included as a random
282 effect to account for the repeated measurements on the same chicks over time and the non-
283 independence of chicks produced by the same female. Paternal identity was also included as a
284 random effect. An information theoretic model averaging approach was used to test for the
285 significance of fixed effects as predictors of the response variables using the function 'dredge'
286 of the 'MuMIn' package. Significance of each effect was based on the *p*-value of the
287 corresponding conditional model-averaged coefficient.

288 Differences in post-hatching offspring survival among parental relatedness groups
289 were analyzed using a mixed effects Cox-regression model (function ‘coxme’ of the ‘coxme’
290 package) that included maternal and paternal identities as random effects.

291

292 **Results**

293 *Siring success*

294 When using the number of eggs with assigned paternity, we found that paternity
295 significantly deviated from the equal proportion of 1/3. Among the 245 offspring with
296 assigned paternity, 103 were sired by males that were unrelated to the female (42.0%), 71 by
297 cousins (29.0%) and 71 by half-siblings (29.0%) ($\chi^2_2 = 12.539$, $df = 2$, $p = 0.002$). This result
298 would therefore suggest a cryptic female choice against related mates. However, when using
299 the within-triad siring success, we did not find any evidence suggesting that females avoided
300 the sperm of related males ($\beta = -0.096 \pm 0.223$, $t = -0.429$, $p = 0.541$; Figure 1a). Sperm
301 motility tended to be positively correlated with siring success, but the effect did not reach the
302 significance threshold ($\beta = 0.083 \pm 0.045$, $t = 1.837$, $p = 0.059$).

303 Two hundred and six chicks died before hatching, and we could assign paternity for
304 only 60 of them (plus 4 embryos from eggs that were incubated apart). This raises the
305 question of a possible skewed paternity of dead embryos towards kin, which would
306 potentially change the actual paternity share across the three relatedness categories, and
307 explain the discrepancy between the results obtained when using the overall number of eggs
308 and the within-triad siring success. To address this issue, we performed two additional
309 analyses. First, we computed the proportions of embryos that were sired by unrelated, cousin
310 and half-sibling males among those that failed to hatch and used these proportions to allocate

311 the 146 dead embryos with unknown paternity to males of the three relatedness categories
312 (see supporting information for computational details). These new proportions no longer
313 deviated from the 1/3 expectation ($\chi^2 = 2.530$, $df = 2$, $p = 0.282$; Fig. S2), suggesting that the
314 skewed paternity share towards unrelated males, found when considering the number of eggs
315 with assigned paternity, might reflect differential embryo mortality rather than female sperm
316 selection. Second, we ran a linear mixed effects model with within-triad siring success as the
317 dependent variable and parental relatedness and sperm motility as fixed effects. This model
318 also included hatching success and the interaction between parental relatedness and hatching
319 success as fixed effects and male identity as a random effect. In agreement with the view that
320 parental relatedness affected siring success depending on embryo mortality, we found a highly
321 significant interaction between parental relatedness and hatching success (Table 1; Fig. 1b).
322 When clutches had a high hatching success ($\geq 80\%$), within-triad siring success was
323 significantly higher for unrelated males (mean \pm se for unrelated males = 0.49 ± 0.09 ; cousins
324 = 0.34 ± 0.08 ; half-siblings = 0.17 ± 0.06 ; $t = -3.040$, $p = 0.003$). On the contrary, when most
325 of the eggs failed to hatch ($\leq 20\%$ hatching success), within-triad siring success was
326 significantly higher for half-sibs (mean \pm se for unrelated males = 0.19 ± 0.07 ; cousins = 0.27
327 ± 0.09 ; half-siblings = 0.53 ± 0.10 ; $t = 2.793$, $p = 0.006$).

328 *Cost of inbreeding*

329 A total of 188 chicks hatched out of 394 fertile eggs (47.7%). Among the 102
330 offspring that were sired by unrelated males, 88 hatched (86.3%), while hatching success of
331 chicks sired by related males was substantially lower [cousins: 50 out of 69 (72.5%), half-
332 siblings: 43 out of 70 (61.4%)]. A generalized linear mixed effects model with a binomial
333 distribution of errors and maternal and paternal identity declared as random effects, showed
334 that the probability to hatch was strongly affected by parental relatedness, while neither laying
335 date nor sperm storage duration explained hatching success (Table 2).

336 At hatching, chicks sired by unrelated males, cousins or half-siblings had similar body
337 mass, size and condition (Table 3). However, growth rate (change in body mass, size and
338 condition) during the first three months of age was significantly lower in chicks sired by
339 cousins and even more so in chicks sired by half-siblings, as indicated by the significant age
340 by parental relatedness interaction (Table 4, Fig. 2).

341 Survival was overall high (79%) under captive conditions. Nevertheless, there was a
342 gradual decrease of survival across the three parental relatedness groups, with chicks sired by
343 males unrelated to the female having the highest survival and chicks sired by half-siblings the
344 lowest survival (mixed effects Cox-regression, parental relatedness: $\beta = 4.492 \pm 1.739$, $z =$
345 2.58 , $p = 0.010$) (Fig. 3).

346

347 **Discussion**

348 We found that females inseminated with a mix of sperm from unrelated, first cousin
349 and half-sibling males had an apparently biased fertilization of their eggs towards unrelated
350 males. Nonetheless, this paternity bias likely resulted from differential embryo mortality
351 between offspring sired by related and unrelated males, rather than from differential siring
352 success driven by female sperm selection. Offspring sired by males genetically related to the
353 female suffered from higher pre- and post-hatching mortality, and reduced growth rate,
354 compared to those sired by unrelated fathers. These results show that fertilization by close
355 relatives incurs severe fitness costs, and that the magnitude of the costs progressively
356 increased with increasing degree of parental relatedness.

357 It is now well established that, although inbreeding can undeniably incur costs, it can
358 also confer benefits by improving inclusive fitness (Kokko & Ots, 2006; Szulkin et al., 2013).

359 Avoiding mating with close kin can also generate costs, and fertilization with genetically
360 distant mates can disrupt the association of co-adapted genes (outbreeding cost) (Rosengaus
361 & Traniello, 1993). Therefore, whether individuals should avoid, tolerate or favor inbreeding
362 will depend on the net balance between the associated costs and benefits. We found that
363 offspring resulting from the fertilization by sperm of close kin had severely impaired pre- and
364 post-hatching survival, and reduced growth rate. These negative effects were expressed under
365 the benign environmental conditions experienced in the captive breeding, and it can be easily
366 expected that their magnitude would be higher in the wild (Meagher, Penn, & Potts, 2000)
367 where individuals face the full range of environmental threats. These high costs of inbreeding
368 strongly suggest that selection should promote mechanisms of inbreeding avoidance because
369 it seems unlikely that benefits of mating with kin and/or costs of inbreeding avoidance might
370 outweigh the severe fitness costs reported here.

371 Costs of inbreeding are generally thought to be stronger for females than for males,
372 and particularly so in species with no paternal care (Lehmann & Perrin, 2003). Females and
373 males differ in their relative investment into a given reproductive bout. Females produce
374 large, costly gametes, and provide extensive parental care, which tremendously limits the
375 number of reproductive bouts females can achieve per breeding season. Therefore, the cost of
376 losing a brood due to inbred mating can be substantial for females. When male contribution to
377 reproduction is limited to the transfer of gametes, the cost of inbreeding is expected to be
378 lower because males can potentially mate multiply during each breeding season. This male-
379 female asymmetry can generate a sexual conflict over inbreeding avoidance, with females
380 selected to avoid inbreeding and males to tolerate it (Szulkin et al., 2013). The houbara
381 bustard has a mating system that is compatible with the idea that males and females should
382 have conflicting interests in terms of inbreeding avoidance. Lekking males only contribute
383 through their gametes, can mate with several females and do not provide any parental care.

384 On the contrary, females raise a single brood per year, and mate with multiple males
385 (Lesobre, Lacroix, Le Nuz, et al., 2010) which potentially give them the opportunity to select
386 sperm of desirable mates.

387 The ability to discriminate sperm based on relatedness would, therefore, represent an
388 effective mechanism for polyandrous females to avoid the cost of inbreeding. One of the most
389 prevalent hypothesis to explain the evolution of polyandry is that females seek genetic
390 benefits from multiple mating (Firman et al., 2017; Kempenaers, 2007). Females would either
391 select sperm carrying genes increasing offspring viability or attractiveness (i.e. “good genes”),
392 or sperm carrying genetically compatible genes to their own (i.e. “compatible genes”)
393 (Kempenaers 2007; Mays et al. 2008). Both types of post-copulatory selection would require
394 molecular and cellular mechanisms to assess sperm genetic quality or compatibility,
395 respectively (Tregenza & Wedell, 2000; Zeh & Zeh, 1997). Despite the potential benefits of
396 sperm selection by promiscuous females, experimental tests of post-copulatory kin avoidance
397 have produced mixed results, with many studies failing to provide support to the hypothesis,
398 including the present one (Ala-Honkola, Manier, Lüpold, & Pitnick, 2011; Denk et al., 2005).
399 There are several explanations to this pattern. First, it is possible that females simply lack the
400 possibility to discriminate between sperm. However, in an elegant experiment, Firman and
401 Simmons (2015) realized competitive *in vitro* fertilizations of mouse oocytes with sperm of
402 kin and non-kin and found a significant deviation from the ½ siring success expected in the
403 absence of sperm selection. This shows that gametic interactions (that might include MHC
404 receptors) can possibly provide a mechanism for sperm selection. In another study, we
405 conducted an experiment where female houbara bustards were inseminated with a mix of
406 sperm of young and old males (i.e. more than 12 years old; Vuarin et al. submitted). We found
407 that young males sired a significantly higher fraction of offspring, even when early embryo
408 mortality was taken into account. This strongly suggests that female houbara bustards do have

409 the molecular and/or cellular mechanisms allowing them to bias fertilization towards sperm of
410 desirable males.

411 The other possible explanation for the heterogeneity in the results of post-copulatory
412 selection against kin resides in the intrinsic difficulty to properly test this hypothesis. First,
413 when females naturally mate with related and unrelated males it is difficult to tease apart pre-
414 and post-copulatory avoidance mechanisms and it might be impossible to control for
415 differential transfer of sperm number. In the present study, we performed artificial
416 inseminations where each male contributed an equal number of sperm, and we statistically
417 controlled for variation in sperm motility, precluding any possible pre-copulatory mechanism
418 (females and males were never in contact) and any possible paternity bias due to sperm
419 competition. Second, testing the sperm selection hypothesis implies assessing whether related
420 and unrelated sperm have different fertilization success. Using paternity assignment of
421 successful offspring as a metric of fertilization success might be a correct approach if there is
422 no differential embryo mortality between related and unrelated males. Differential early
423 embryo mortality is among the recognized cost of inbreeding (Kempnaers, Adriaensen, Van
424 Noordwijk, & Dhondt, 1996) and therefore basing the assessment of skewed fertilization
425 success on paternity assignment might provide erroneous results when testing for female
426 sperm selection. Actually, when using the number of eggs with assigned paternity (mostly
427 involving successful hatchlings), we did find that unrelated males sired a significantly higher
428 proportion of offspring, and we might have concluded that females adaptively avoid
429 fertilization with sperm from kin. However, when considering the large number of fertilized
430 eggs that failed to hatch (and for which we could not assign paternity), we found similar
431 fertilization success across all parental relatedness categories. We used the observed
432 proportions of offspring sired by unrelated males, cousins, and half-siblings that failed to
433 hatch to allocate dead embryos with unknown paternity to the three relatedness categories.

434 Although theoretical, this approach remains nevertheless meaningful when attempting to take
435 into account differential embryo mortality. As a complement to the previous analyses, we also
436 showed that within-triad siring success was positively or negatively correlated with hatching
437 success, depending on the parental relatedness category. When most of the eggs in the clutch
438 hatched, males in the unrelated category had the best siring success; on the contrary when
439 most of the eggs failed to hatch, males in the half-sibling category had the highest siring
440 success. This result therefore corroborates the view that differential mortality of embryo sired
441 by related and unrelated males might account for apparently biased paternity towards
442 unrelated fathers.

443 The pending question is why females do not select against sperm of related males
444 despite very strong costs. It might be that close relatives never encounter in the wild, making
445 the evolution of post-copulatory avoidance mechanisms useless. In the houbara bustard, natal
446 dispersal is higher in males than females (Hardouin et al., 2012) and this might be enough to
447 reduce the risk of inbred mating. In addition, recent studies on communication behavior in
448 houbara bustards showed that male vocalizations (booming) carry information on individual
449 quality, identity and kinship, potentially used in male-male interactions within leks (Cornec,
450 Hingrat, Robert, & Rybak, 2015; Cornec, Hingrat, & Rybak, 2014). While the implication of
451 this communication channel in male-female interactions has not been established yet, it
452 represents a potential source of information for females in their mate choice (pre-copulatory
453 selection) and their sperm storage strategy (post-copulatory selection). An alternative
454 explanation for the absence of sperm selection observed here would be that cryptic female
455 choice operates after fertilization, i.e. through post-zygotic mechanisms (Firman et al., 2017).
456 Females could invest differently on embryos depending on their relatedness to the sire, and
457 inbred embryos would suffer from higher mortality as a result of lower maternal investment.

458 In conclusion, artificial inseminations revealed that females of a promiscuous bird
459 species were not able to avoid fertilization with related sperm through post-copulatory
460 mechanisms. Paternity share may be misinterpreted as the result of female cryptic choice
461 when differential embryo mortality is not accounted for. Altogether, the lower hatching
462 success and performance of offspring sired by related males, compared to those sired by
463 unrelated males, support that inbred fertilization can lead to substantial fitness costs in this
464 species.

465

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475

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662 **Data accessibility**

663 The datasets supporting this article will be made available in the Dryad Digital Repository
664 upon acceptance of the manuscript.

665

666 **Author contributions**

667 All authors designed the study. PV and AB conducted the experiments; PV performed
668 statistical analyses and drafted out the manuscript, which was revised by LL, YH and GS. All
669 authors gave final approval for publication.

670

671 **Tables and figures**

672

673 Table 1. Effects of parental relatedness, hatching success, sperm motility and the interaction
 674 between parental relatedness and hatching success on within-triad siring success (i.e.
 675 proportion of eggs sired by each of the three males within a given mix of sperm; n = 282).
 676 Significant *p* values are in bold.

<i>Within-triad siring success</i>				
Fixed effects	Estimate	SE	t value	<i>p</i>
Intercept	-0.140	0.179	-0.782	0.429
Parental relatedness	1.267	0.420	3.018	0.013
Hatching success	0.357	0.125	2.853	0.007
Sperm motility	0.087	0.043	1.995	0.048
Relatedness:Hatching success	-2.955	0.770	-3.840	<0.001
Random effect	Variance		SD	
Paternal ID	0.010		0.101	

677

678 Table 2. Effects of sperm storage duration (delay between insemination and laying), laying
 679 date and parental relatedness (unrelated, cousin or half-sibling) on hatching success.
 680 Significant *p* values are in bold (n = 245).

<i>Hatching success</i>				
Fixed effects	Estimate	SE	z value	<i>P</i>
Intercept	1.408	0.801	1.758	0.002
Sperm storage duration	-0.025	0.020	-1.293	0.274
Laying date	0.009	0.010	0.824	0.630
Parental relatedness	-5.478	1.551	-3.532	<0.001
Random effects	Variance		SD	
Maternal ID	0.000000001		0.00003	
Paternal ID	0.209		0.457	

681

682 Table 3. Effects of sperm storage duration (delay between insemination and laying), laying
683 date and parental relatedness (unrelated, cousin or half-sibling) on hatchling body mass, body
684 size (head length) and body condition (residuals of body mass on body size). Significant *p*
685 values are in bold (n = 181).

<i>Hatchling body mass</i>				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	47.949	2.80	17.118	<0.001
Sperm storage duration	0.007	0.036	0.187	0.204
Laying date	-0.046	0.029	-1.577	0.025
Parental relatedness	0.179	2.462	0.069	0.847
Random effects	Variance		SD	
Maternal ID	13.787		3.713	
Paternal ID	0.629		0.793	
<i>Hatchling body size</i>				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	40.807	1.689	24.154	<0.001
Sperm storage duration	0.016	0.011	1.444	1.000
Laying date	-0.018	0.009	-2.102	0.149
Parental relatedness	-0.621	1.310	-0.474	0.613
Random effects	Variance		SD	
Maternal ID	0.277		0.527	
Paternal ID	169.330		13.013	
<i>Hatchling body condition</i>				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	4.850	2.786	1.741	0.637
Sperm storage duration	0.002	0.036	0.069	0.154
Laying date	-0.045	0.029	-1.560	0.018
Parental relatedness	0.787	2.460	0.320	0.657
Random effects	Variance		SD	
Maternal ID	13.587		3.686	
Paternal ID	0.682		0.826	

686

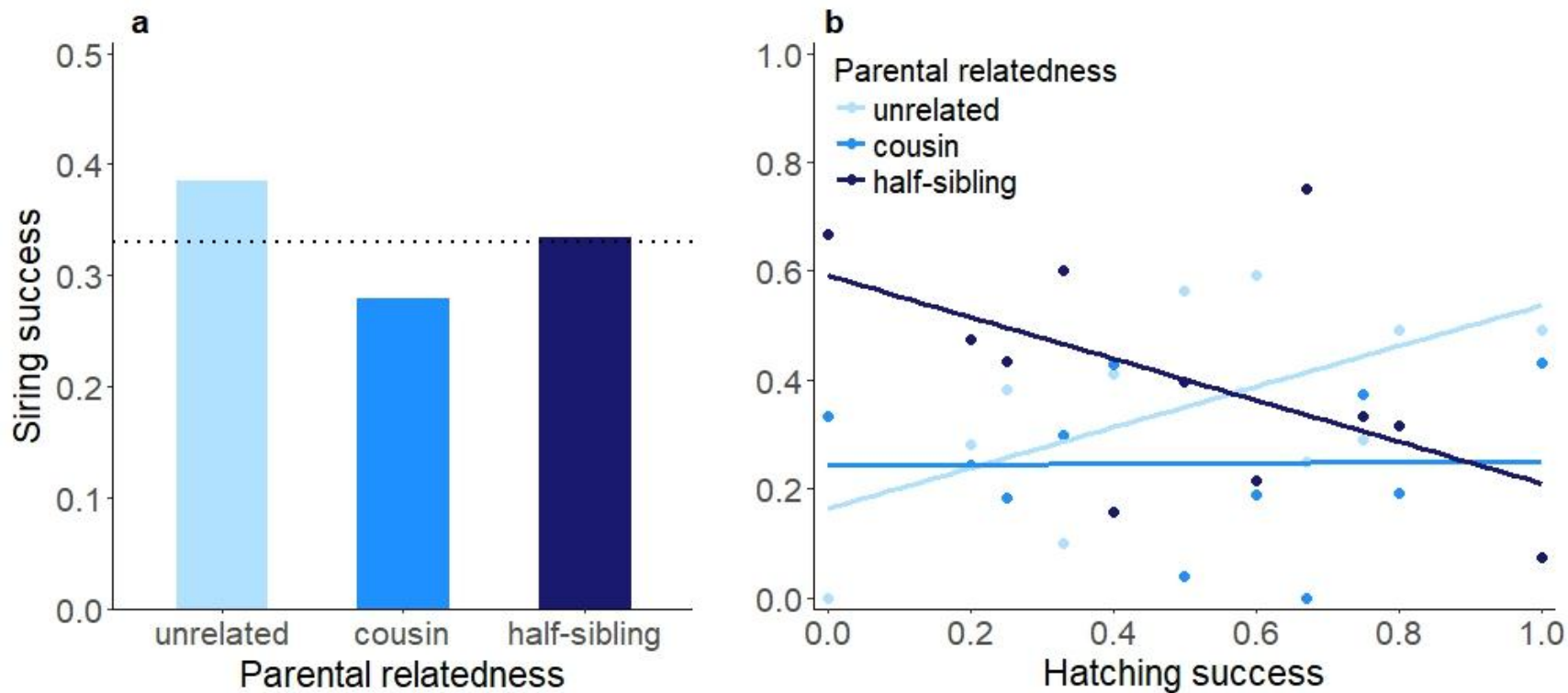
687 Table 4. Effects of sperm storage duration (delay between insemination and laying), laying
688 date and parental relatedness (unrelated, cousin or half-sibling) on offspring body mass, body
689 size (head length) and body condition (residuals of body mass on body size) during the first
690 three months of life. Significant *p* values are in bold (n = 181).

Body mass				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	39.004	34.480	1.13	0.685
Sperm storage duration	1.428	0.569	2.51	0.035
Laying date	-0.497	0.343	-1.45	0.201
Offspring age	12.034	0.116	103.75	<0.001
Parental relatedness	59.360	57.143	1.04	0.310
Parental relatedness x offspring age	-8.667	0.861	-10.07	<0.001
Random effects	Variance		SD	
Offspring ID:Maternal ID	1608.20		40.10	
Maternal ID	442.10		21.03	
Paternal ID	783.00		27.98	
Body size				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	38.451	1.272	30.22	<0.001
Sperm storage duration	0.035	0.020	1.73	0.124
Laying date	-0.010	0.013	-0.78	0.941
Offspring age	1.242	0.012	106.83	<0.001
Offspring age ²	-0.007	0.0001	-55.51	<0.001
Parental relatedness	0.737	2.073	0.36	0.777
Parental relatedness x offspring age	-0.249	0.085	-2.92	<0.001
Parental relatedness x offspring age ²	0.001	0.001	1.22	0.589
Random effects	Variance		SD	
Offspring ID:Maternal ID	2.362		1.537	
Maternal ID	1.210		1.100	
Paternal ID	0.359		0.599	
Body condition				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	28.645	21.416	1.403	0.809
Sperm storage duration	0.668	0.332	2.014	0.149
Laying date	-0.419	0.200	-2.095	0.103
Log(offspring age)	4.710	1.474	3.195	0.001
Parental relatedness	66.075	42.228	1.565	0.128

Parental relatedness x log(offspring age)	-53.706	10.788	-4.978	<0.001
Random effects	Variance		SD	
Offspring ID:Maternal ID	475.10		21.80	
Maternal ID	113.50		10.65	
Paternal ID	372.00		19.29	

691

692 Figure 1. Within-triad siring success for the three parental relatedness categories (unrelated, cousin or half-sibling), the dashed line representing
693 the equal 1/3 siring success (a) and within-triad siring success as a function of hatching success (the proportion of hatched eggs per clutch) for
694 the three relatedness categories (b). Siring success was calculated as the proportion of eggs sired by each of the three males within a given mix of
695 sperm.

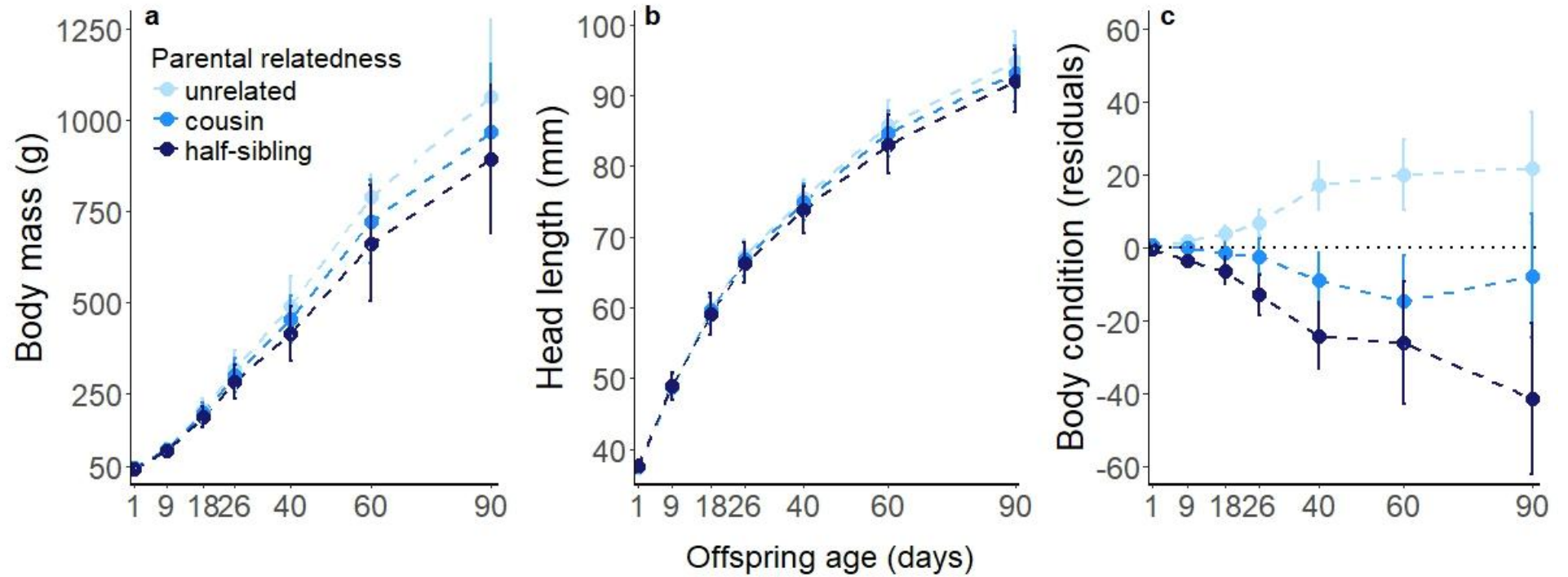


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698

699 Figure 2. Offspring body mass (a), size (b) and condition (c) according to parental relatedness (unrelated, cousin or half-sibling) during the first
700 three months of age. Values are means \pm s.d. of raw data for panels a and b, and means \pm s.e. of residuals of the regression of body mass on body
701 size for panel c.

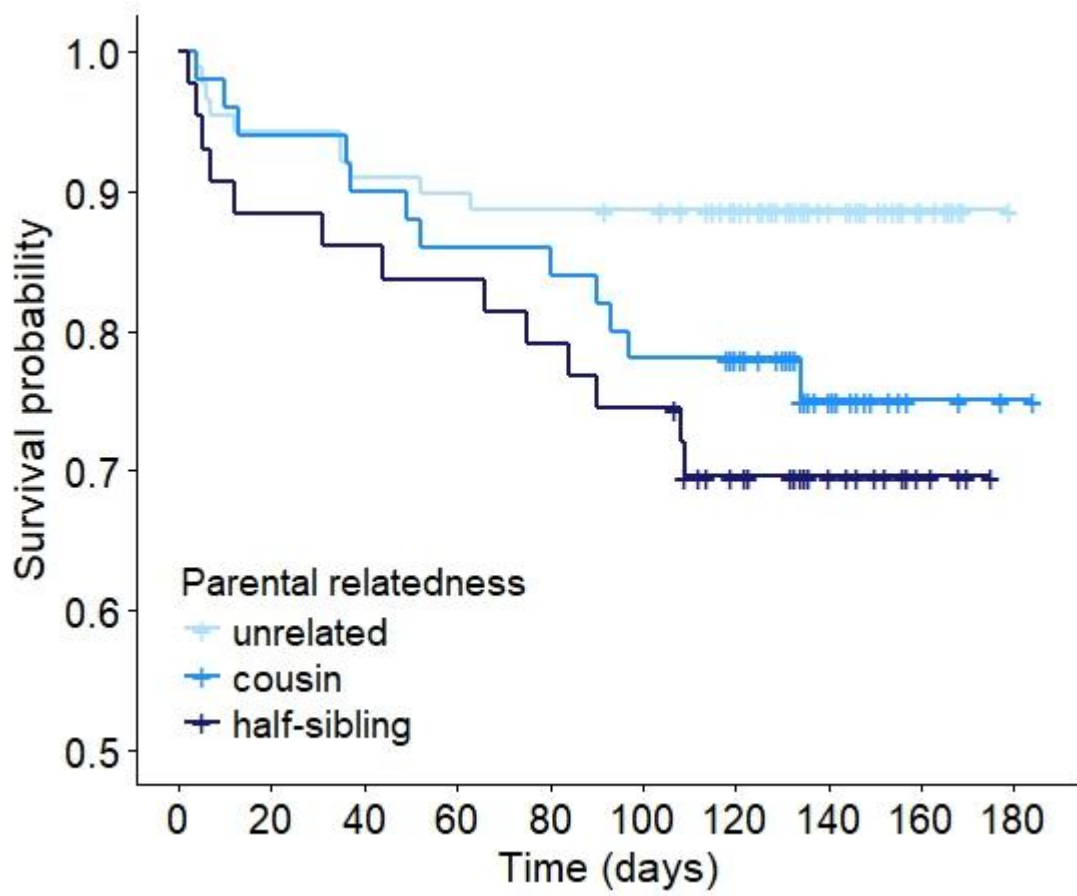


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704

705 Figure 3. Offspring survival according to parental relatedness. The lines represent survival
706 curves for offspring sired by males of each of the parental relatedness categories (unrelated,
707 cousin or half-sibling), and the crosses represent censored observations (i.e., offspring that
708 were still alive at the end of the monitoring period) (n = 181).



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710