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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

6 Pauline Vuarin<sup>1,2\*†</sup>, Alice Bouchard<sup>1</sup>, Loïc Lesobre<sup>3</sup>, Gwènaëlle Levêque<sup>1</sup>, Toni Chalah<sup>3</sup>,  
7 Michel Saint Jalme<sup>4</sup>, Frédéric Lacroix<sup>3</sup>, Yves Hingrat<sup>3,§</sup>, Gabriele Sorci<sup>2,§</sup>

8

9 1. Emirates Center for Wildlife Propagation, Missour, Morocco

10 2. Biogéosciences, UMR 6282 CNRS, Université de Bourgogne Franche-Comté, Dijon,  
11 France

12 3. Reneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates

13 4. Centre d'Ecologie et des Sciences de la Conservation, UMR 7204 MNHN CNRS-UPMC,  
14 Museum National d'Histoire Naturelle, Paris, France

15

16 \* Corresponding author: pauline.vuarin@gmail.com

17 § Shared senior authorship

18

† Present address: School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa

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  $37.7^\circ\text{C}$  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  $4.3 \pm 1.5$  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 =  $5.5 \pm 1.6$  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  $2.2 \pm 1.9$  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 ( $0.25 \text{ m}^2$ , 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 ( $16 \text{ m}^2$ , maximum 6 individuals per cage) up to 40 days of age, and finally in large  
211 outdoor aviaries ( $270 \text{ m}^2$ , 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 5<sup>th</sup>, where all of them were at least 3 months old, i.e. when they reached adult size  
219 (mean age of  $141 \pm 17$  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  $\mu$ L) from the axillary vein, using a single-use sterilized needle and capillary  
224 tubes. Blood samples (~200  $\mu$ 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 =  $0.49 \pm 0.09$ ; cousins  
324 =  $0.34 \pm 0.08$ ; half-siblings =  $0.17 \pm 0.06$ ;  $t = -3.040$ ,  $p = 0.003$ ). 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 =  $0.19 \pm 0.07$ ; cousins =  $0.27$   
327  $\pm 0.09$ ; half-siblings =  $0.53 \pm 0.10$ ;  $t = 2.793$ ,  $p = 0.006$ ).

### 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	<b>0.013</b>
Hatching success	0.357	0.125	2.853	<b>0.007</b>
Sperm motility	0.087	0.043	1.995	<b>0.048</b>
Relatedness:Hatching success	-2.955	0.770	-3.840	<b>&lt;0.001</b>
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).

<b><i>Hatching success</i></b>				
Fixed effects	Estimate	SE	z value	<i>P</i>
Intercept	1.408	0.801	1.758	<b>0.002</b>
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	<b>&lt;0.001</b>
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	<b>&lt;0.001</b>
Sperm storage duration	0.007	0.036	0.187	0.204
Laying date	-0.046	0.029	-1.577	<b>0.025</b>
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	<b>&lt;0.001</b>
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	<b>0.018</b>
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).

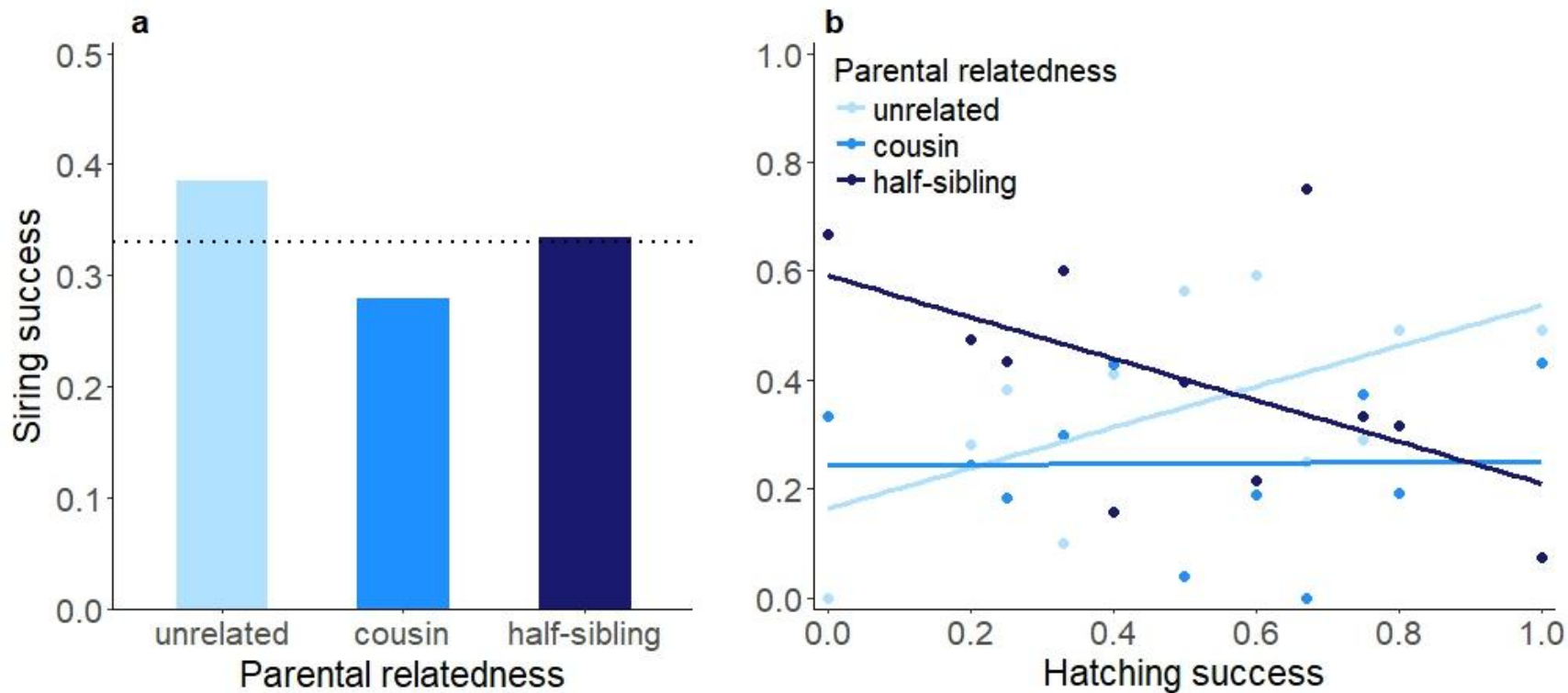
<b>Body mass</b>				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	39.004	34.480	1.13	0.685
Sperm storage duration	1.428	0.569	2.51	<b>0.035</b>
Laying date	-0.497	0.343	-1.45	0.201
Offspring age	12.034	0.116	103.75	<b>&lt;0.001</b>
Parental relatedness	59.360	57.143	1.04	0.310
Parental relatedness x offspring age	-8.667	0.861	-10.07	<b>&lt;0.001</b>
Random effects	Variance		SD	
Offspring ID:Maternal ID	1608.20		40.10	
Maternal ID	442.10		21.03	
Paternal ID	783.00		27.98	
<b>Body size</b>				
Fixed effects	Estimate	SE	t value	<i>P</i>
Intercept	38.451	1.272	30.22	<b>&lt;0.001</b>
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	<b>&lt;0.001</b>
Offspring age <sup>2</sup>	-0.007	0.0001	-55.51	<b>&lt;0.001</b>
Parental relatedness	0.737	2.073	0.36	0.777
Parental relatedness x offspring age	-0.249	0.085	-2.92	<b>&lt;0.001</b>
Parental relatedness x offspring age <sup>2</sup>	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	
<b>Body condition</b>				
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	<b>0.001</b>
Parental relatedness	66.075	42.228	1.565	0.128

Parental relatedness x log(offspring age)	-53.706	10.788	-4.978	<b>&lt;0.001</b>
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.

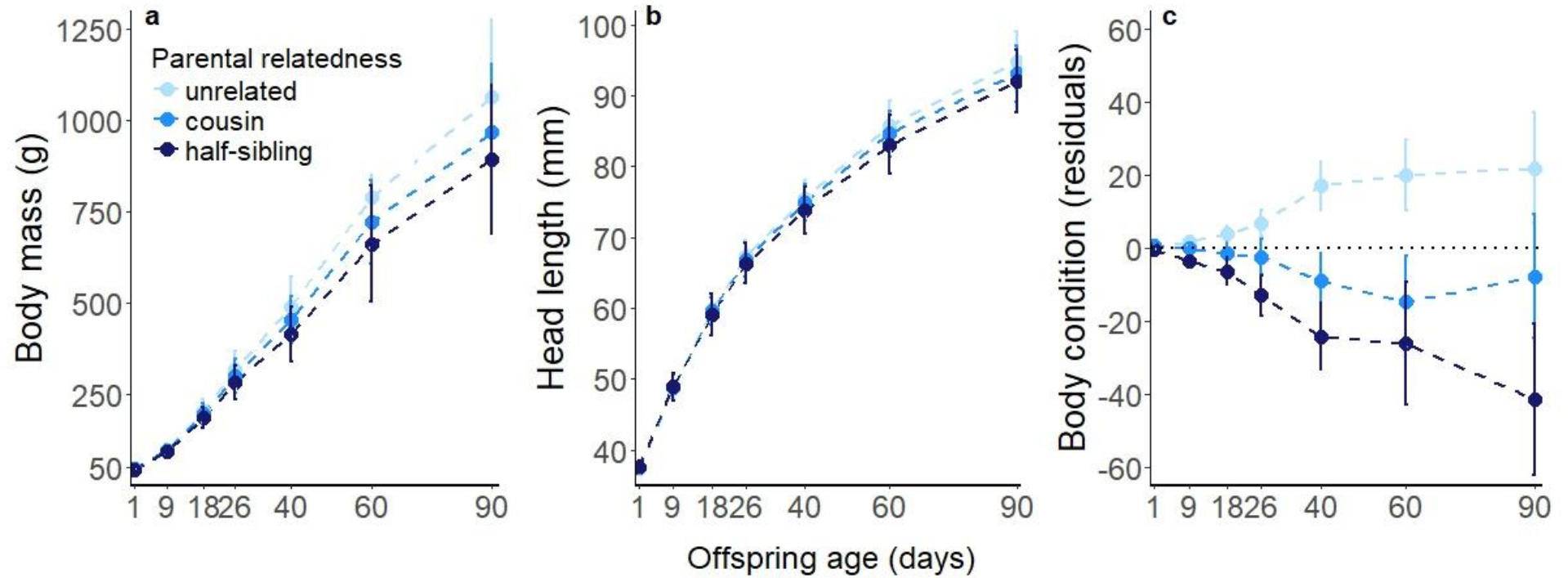


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699 Figure 2. Offspring body mass (a), size (b) and condition (c) according to parental relatedness (unrelated, cousin or half-sibling) during the first  
700 three months of age. Values are means  $\pm$  s.d. of raw data for panels a and b, and means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

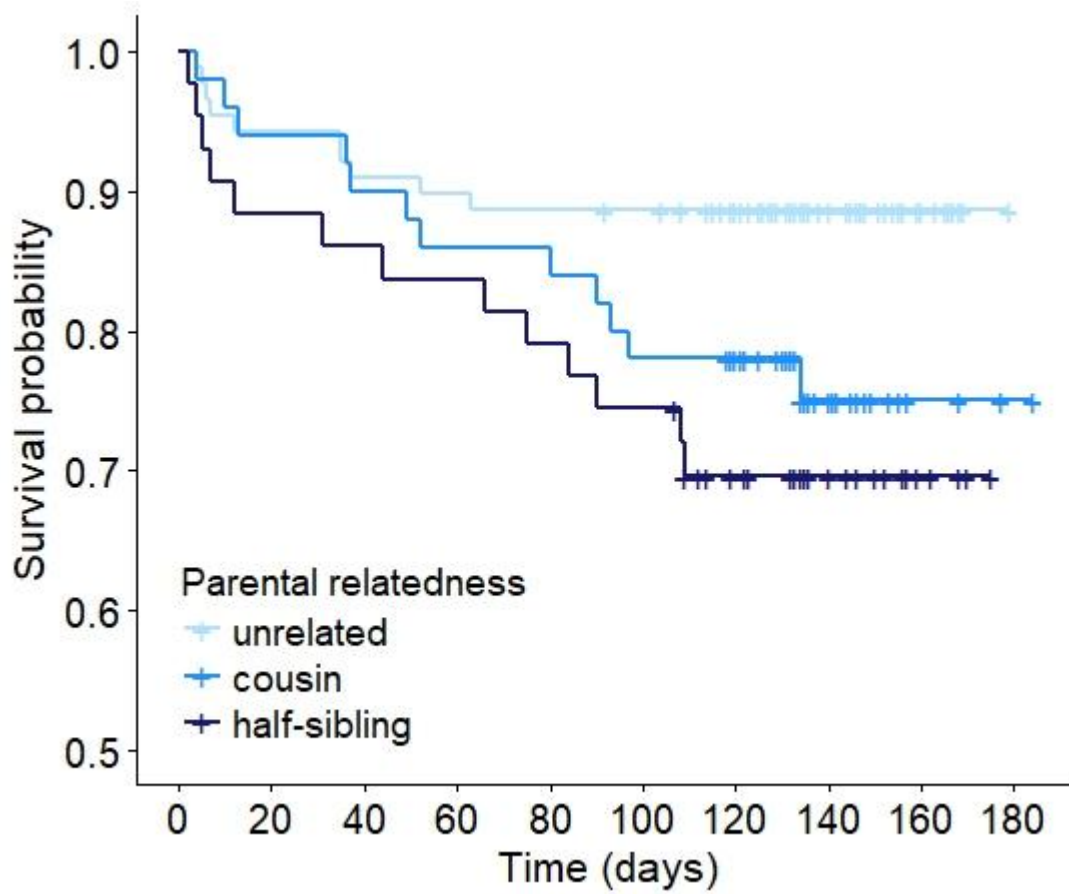


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705 Figure 3. Offspring survival according to parental relatedness. The lines represent survival  
706 curves for offspring sired by males of each of the parental relatedness categories (unrelated,  
707 cousin or half-sibling), and the crosses represent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



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