



No evidence for prezygotic postcopulatory avoidance of kin despite high inbreeding depression.

Pauline Vuarin, Alice Bouchard, Loïc Lesobre, Gwènaëlle Levêque, Toni Chalah, Michel Saint Jalme, Frédéric Lacroix, Yves Hingrat, Gabriele Sorci

► To cite this version:

Pauline Vuarin, Alice Bouchard, Loïc Lesobre, Gwènaëlle Levêque, Toni Chalah, et al.. No evidence for prezygotic postcopulatory avoidance of kin despite high inbreeding depression.. *Molecular Ecology*, 2018, 27 (24), pp.5252-5262. 10.1111/mec.14951 . hal-01973580

HAL Id: hal-01973580

<https://hal.science/hal-01973580>

Submitted on 21 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Running title: Absence of post-copulatory avoidance of kin

Pauline Vuarin^{1,2*†}, Alice Bouchard¹, Loïc Lesobre³, Gwènaëlle Levêque¹, Toni Chalah³,
Michel Saint Jalme⁴, Frédéric Lacroix³, Yves Hingrat^{3,§}, Gabriele Sorci^{2,§}

1. Emirates Center for Wildlife Propagation, Missour, Morocco

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

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

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

* Corresponding author: pauline.vuarin@gmail.com

§ Shared senior authorship

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

Abstract

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

43

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

Introduction

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

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

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

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

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

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

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

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

Materials and methods

Mating system and reproductive biology of the houbara bustard

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

Experimental birds and breeding program management

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

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

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

Artificial inseminations

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

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

Egg collection, incubation and fertility assessment

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

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

Offspring growth and survival under captive conditions

188 chicks hatched out of the 394 fertile eggs. Hatchlings were transferred into indoor facilities where they were kept in small plastic boxes (0.25 m², maximum 6 individuals per box) for 10 days. They were fed by hand with a mix of pellets (Evalis, 24% of protein content), fresh chopped alfalfa, mealworms and crickets. Then, they were kept in outdoor cages (16 m², maximum 6 individuals per cage) up to 40 days of age, and finally in large outdoor aviaries (270 m², maximum 12 individuals per aviary) where food was delivered once a day. Water was provided *ad libitum*. Chick body mass (± 1 g) and size, estimated through measurements of head-skull length using a manual caliper [from the tip of the beak to the back of the head with ± 0.5 mm precision), were measured at day 1, 9, 18, 26, 40, 60 and 90 post-hatching. Body mass and head-skull length were used to compute an index of body condition as the residuals of the linear regression of body mass against size (Schulte-

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

Paternity analyses

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

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

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

Ethical statement

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

Statistical analyses

All statistical analyses were performed with R 3.1.3.

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

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

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

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

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

Results

Siring success

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

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

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

Cost of inbreeding

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments

The Emirates Center for Wildlife Propagation (ECWP) provided the data and funding for this study, under the leadership of IFHC. We are grateful to H.H. Sheikh Mohammed bin Zayed Al Nahyan, Crown Prince of Abu Dhabi and Chairman of the International Fund for Houbara Conservation (IFHC) and H.E. Mohammed Al Bowardi Deputy Chairman of IFHC for their support. We are thankful to all ECWP employees who helped collecting the data, especially Charles Jabbour, Nourdine Abbou, Omar Elhassnaoui, Yann Le Galloudec, Emilie Arnoux and Jesse Gabbard who greatly contributed to the experiment. We are also grateful to Léo Bacon for his valuable advice regarding statistical analyses.

References

- Ala-Honkola, O., Manier, M. K., Lüpold, S., & Pitnick, S. (2011). No evidence for postcopulatory inbreeding avoidance in *Drosophila melanogaster*. *Evolution*, 65(9), 2699–2705. <https://doi.org/10.1111/j.1558-5646.2011.01317.x>
- Andersson, M., & Simmons, L. W. (2006). Sexual selection and mate choice. *Trends in Ecology & Evolution*, 21(6), 296–302. <https://doi.org/10.1016/j.tree.2006.03.015>
- Bateson, P. (1982). Preferences for cousins in Japanese quail. *Nature*, 295, 236–237. <https://doi.org/10.1038/295236a0>

- 484 Bengtsson, B. O. (1978). Avoiding inbreeding: at what cost? *Journal of Theoretical Biology*,
485 73(3), 439–444. [https://doi.org/10.1016/0022-5193\(78\)90151-0](https://doi.org/10.1016/0022-5193(78)90151-0)
- 486 Billing, A. M., Lee, A. M., Skjelseth, S., Borg, Å. A., Hale, M. C., Slate, J., ... Jensen, H.
487 (2012). Evidence of inbreeding depression but not inbreeding avoidance in a natural
488 house sparrow population. *Molecular Ecology*, 21(6), 1487–1499.
489 <https://doi.org/10.1111/j.1365-294X.2012.05490.x>
- 490 Birkhead, T. R., Hall, J., Schut, E., & Hemmings, N. (2008). Unhatched eggs: Methods for
491 discriminating between infertility and early embryo mortality. *Ibis*, 150(3), 508–517.
492 <https://doi.org/10.1111/j.1474-919X.2008.00813.x>
- 493 Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews*
494 *Genetics*, 3(4), 262–273. <https://doi.org/10.1038/nrg774>
- 495 Blyton, M. D. J., Banks, S. C., & Peakall, R. (2015). The effect of sex-biased dispersal on
496 opposite-sexed spatial genetic structure and inbreeding risk. *Molecular Ecology*, 24(8),
497 1681–1695. <https://doi.org/10.1111/mec.13149>
- 498 Bonte, D., Van Dyck, H., Bullock, J. M., Coulon, A., Delgado, M., Gibbs, M., ... Travis, J.
499 M. J. (2012). Costs of dispersal. *Biological Reviews*, 87, 290–312.
- 500 Bretman, A., Newcombe, D., & Tregenza, T. (2009). Promiscuous females avoid inbreeding
501 by controlling sperm storage. *Molecular Ecology*, 18(16), 3340–3345.
502 <https://doi.org/10.1111/j.1365-294X.2009.04301.x>
- 503 Bretman, A., Wedell, N., & Tregenza, T. (2004). Molecular evidence of post-copulatory
504 inbreeding avoidance in the field cricket *Gryllus bimaculatus*. *Proceedings of the Royal*
505 *Society B*, 271(1535), 159–164. <https://doi.org/10.1098/rspb.2003.2563>
- 506 Chargé, R., Jalme, M. Saint, Lacroix, F., Cadet, A., & Sorci, G. (2010). Male health status,
507 signalled by courtship display, reveals ejaculate quality and hatching success in a lekking
508 species. *Journal of Animal Ecology*, 79(4), 843–850. <https://doi.org/10.1111/j.1365-2656.2010.01696.x>
- 510 Charlesworth, B., & Charlesworth, D. (1999). The genetic basis of inbreeding depression.
511 *Genetical Research*, 74(3), 329–340.
- 512 Charlesworth, D., & Willis, J. H. (2009). The genetics of inbreeding depression. *Nature*
513 *Reviews Genetics*, 10, 783–796.
- 514 Cornec, C., Hingrat, Y., Robert, A., & Rybak, F. (2015). The meaning of boom calls in a
515 lekking bird: identity or quality information? *Animal Behaviour*, 109, 249–264.
516 <https://doi.org/10.1016/j.anbehav.2015.07.017>
- 517 Cornec, C., Hingrat, Y., & Rybak, F. (2014). Individual signature in a lekking species: Visual
518 and acoustic courtship parameters may help discriminating conspecifics in the houbara
519 bustard. *Ethology*, 120(7), 726–737. <https://doi.org/10.1111/eth.12244>
- 520 Crnokrak, P., & Roff, D. A. (1999). Inbreeding depression in the wild. *Heredity*, 83, 260–270.
521 <https://doi.org/10.1038/sj.hdy.6885530>
- 522 Denk, A. G., Holzmann, A., Peters, A., Vermeirssen, E. L. M., & Kempenaers, B. (2005).
523 Paternity in mallards: Effects of sperm quality and female sperm selection for inbreeding

avoidance. *Behavioral Ecology*, 16(5), 825–833. <https://doi.org/10.1093/beheco/ari065>

Eberhard, W. G., & Cordero, C. (1995). Sexual selection by cryptic female choice on male seminal products - a new bridge between sexual selection and reproductive physiology. *Trends in Ecology and Evolution*, 10(12), 493–496. [https://doi.org/10.1016/S0169-5347\(00\)89205-8](https://doi.org/10.1016/S0169-5347(00)89205-8)

Eberhard, W. G. (1996). *Female Control: Sexual Selection by Cryptic Female Choice*. Princetown University Press.

Evans, J. P., Brooks, R. C., Zajitschek, S. R. K., & Griffith, S. C. (2008). Does genetic relatedness of mates influence competitive fertilization success in guppies? *Evolution*, 62(11), 2929–2935. <https://doi.org/10.1111/j.1558-5646.2008.00496.x>

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution*, 32(5), 368–382. <https://doi.org/10.1016/j.tree.2017.02.010>

Firman, R. C., & Simmons, L. W. (2008). Polyandry facilitates postcopulatory inbreeding avoidance in house mice. *Evolution*, 62(3), 603–611. <https://doi.org/10.1111/j.1558-5646.2007.00307.x>

Firman, R. C., & Simmons, L. W. (2015). Gametic interactions promote inbreeding avoidance in house mice. *Ecology Letters*, 18(9), 937–943. <https://doi.org/10.1111/ele.12471>

Fitzpatrick, J. L., & Evans, J. P. (2014). Postcopulatory inbreeding avoidance in guppies. *Journal of Evolutionary Biology*, 27, 2585–2594. <https://doi.org/10.1111/jeb.12545>

Gandon, S. (1999). Kin competition, the cost of inbreeding and the evolution of dispersal. *Journal of Theoretical Biology*, 200(4), 345–364. <https://doi.org/10.1006/jtbi.1999.0994>

Gasparini, C., & Pilastro, A. (2011). Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. *Proceedings of the Royal Society B*, 278(1717), 2495–2501. <https://doi.org/10.1098/rspb.2010.2369>

Geffen, E., Kam, M., Hefner, R., Hersteinsson, P., Angerbjörn, A., Dalèn, L., ... Wayne, R. K. (2011). Kin encounter rate and inbreeding avoidance in canids. *Molecular Ecology*, 20(24), 5348–5358. <https://doi.org/10.1111/j.1365-294X.2011.05358.x>

Hardouin, L. a., Nevoux, M., Robert, A., Gimenez, O., Lacroix, F., & Hingrat, Y. (2012). Determinants and costs of natal dispersal in a lekking species. *Oikos*, 121(6), 804–812. <https://doi.org/10.1111/j.1600-0706.2012.20313.x>

Hatchwell, B. J., Ross, D. J., Fowlie, M. K., & McGowan, A. (2001). Kin discrimination in cooperatively breeding long-tailed tits. *Proceedings of the Royal Society B*, 268(1470), 885–890. <https://doi.org/10.1098/rspb.2001.1598>

Hemmings, N., & Birkhead, T. R. (2015). Polyspermy in birds: sperm numbers and embryo survival. *Proceedings of the Royal Society B*, 282, 20151682. <https://doi.org/10.1098/rspb.2015.1682>

Hingrat, Y., & Saint Jalme, M. (2005). Mating system of the houbara bustard *Chlamydotis undulata undulata* in eastern Morocco. *Ardeola*, 52(1), 91–102.

- 563 Hingrat, Y., Saint Jalme, M., Ysnel, F., Lacroix, F., Seabury, J., & Rautureau, P. (2004).
 564 Relationships between home-range size, sex and season with preference to the mating
 565 system of the houbara bustard *Chlamydotis undulata undulata*. *Ibis*, 146, 314–322.
- 566 Johnston, R. E. (2008). Individual odors and social communication: individual recognition,
 567 kin recognition, and scent over- marking. *Advances in the Study of Behavior*, 38, 439–
 568 505. [https://doi.org/10.1016/S0065-3454\(08\)00009-0](https://doi.org/10.1016/S0065-3454(08)00009-0)
- 569 Keller, L. F., Arcese, P., Smith, J. N. M., Hochachka, W. M., Stearns, S. C. (1994). Selection
 570 against inbred song sparrows during a natural population bottleneck. *Nature*, 372, 356–
 571 357. <https://doi.org/10.1038/372356a0>
- 572 Keller, L. F., & Waller, D. M. (2002). Inbreeding effects in wild populations. *Trends in*
 573 *Ecology & Evolution*, 17(5), 230–241. [https://doi.org/10.1016/S0169-5347\(02\)02489-8](https://doi.org/10.1016/S0169-5347(02)02489-8)
- 574 Kempenaers, B. (2007). Mate choice and genetic quality: a review of the heterozygosity
 575 theory. *Avances in the Study of Behavior*, 37, 189–278. [https://doi.org/10.1016/S0065-](https://doi.org/10.1016/S0065-3454(07)37005-8)
 576 [3454\(07\)37005-8](https://doi.org/10.1016/S0065-3454(07)37005-8)
- 577 Kempenaers, B., Adriaensen, F., Van Noordwijk, A. J., & Dhondt, A. A. (1996). Genetic
 578 similarity, inbreeding, and hatching failure in blue tits: are unhatched eggs infertile?
 579 *Proceeding of the Royal Society B*, 263, 179–185.
- 580 Kokko, H., & Ots, I. (2006). When not to avoid inbreeding. *Evolution*, 60(3), 467.
 581 <https://doi.org/10.1554/05-613.1>
- 582 Krause, E. T., Kruger, O., Kohlmeier, P., & Caspers, B. A. (2012). Olfactory kin recognition
 583 in a songbird. *Biology Letters*, 8(3), 327–329. <https://doi.org/10.1098/rsbl.2011.1093>
- 584 Lake, P. E., & Ravie, O. (1984). An exploration of cryoprotective compounds for fowl
 585 spermatozoa. *British Poultry Science*, 25(1), 145–150. Retrieved from
 586 [http://www.tandfonline.com/gate1.inist.fr/doi/abs/10.1080/13632758408454852#.Ve7L1](http://www.tandfonline.com/gate1.inist.fr/doi/abs/10.1080/13632758408454852#.Ve7L1pem1j4)
 587 [pem1j4](http://www.tandfonline.com/gate1.inist.fr/doi/abs/10.1080/13632758408454852#.Ve7L1pem1j4)
- 588 Lebigre, C., Alatalo, R. V., & Siitari, H. (2010). Female-biased dispersal alone can reduce the
 589 occurrence of inbreeding in black grouse (*Tetrao tetrix*). *Molecular Ecology*, 19, 1929–
 590 1939. <https://doi.org/10.1111/j.1365-294X.2010.04614.x>
- 591 Lehmann, L., & Perrin, N. (2003). Inbreeding avoidance through kin recognition: choosy
 592 females boost male dispersal. *The American Naturalist*, 162(5), 638–652.
 593 <https://doi.org/10.1086/378823>
- 594 Lesobre, L., Lacroix, F., Caizergues, A., Hingrat, Y., Chalah, T., & Jalme, M. Saint. (2010).
 595 Conservation genetics of houbara bustard (*Chlamydotis undulata undulata*): Population
 596 structure and its implications for the reinforcement of wild populations. *Conservation*
 597 *Genetics*, 11(4), 1489–1497. <https://doi.org/10.1007/s10592-009-9979-9>
- 598 Lesobre, L., Lacroix, F., Le Nuz, E., Hingrat, Y., Chalah, T., & Jaime, M. Saint. (2010).
 599 Absence of male reproductive skew, along with high frequency of polyandry and
 600 conspecific brood parasitism in the lekking houbara bustard *Chlamydotis undulata*
 601 *undulata*. *Journal of Avian Biology*, 41(2), 117–127. [https://doi.org/10.1111/j.1600-](https://doi.org/10.1111/j.1600-048X.2009.04794.x)
 602 [048X.2009.04794.x](https://doi.org/10.1111/j.1600-048X.2009.04794.x)
- 603 Løvlie, H., Gillingham, M. a F., Worley, K., Pizzari, T., & Richardson, D. S. (2013). Cryptic

- female choice favours sperm from major histocompatibility complex-dissimilar males. *Proceedings of the Royal Society B*, 280(1769), 20131296. <https://doi.org/10.1098/rspb.2013.1296>
- Meagher, S., Penn, D. J., & Potts, W. K. (2000). Male-male competition magnifies inbreeding depression in wild house mice. *Proceedings of the National Academy of Sciences*, 97(7), 3324–3329.
- Parker, G. A. (2006). Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society B*, 361(1466), 235–259. <https://doi.org/10.1098/rstb.2005.1785>
- Penn, D. J., & Frommen, J. G. (2010). Kin recognition: an overview of conceptional issues, mechanisms and evolutionary theory. In *Animal Behaviour: Evolution and Mechanisms* (pp. 55–85). Springer, Heidelberg. <https://doi.org/10.1007/978-3-642-02624-9>
- Pizzari, T., Lo, H., & Cornwallis, C. K. (2004). Sex-specific, counteracting responses to inbreeding in a bird. *Proceedings of the Royal Society B: Biological Sciences*, 271(1553), 2115–2121. <https://doi.org/10.1098/rspb.2004.2843>
- Pizzari, T., & Parker, G. A. (2009). Sperm competition and sperm phenotype. In T. R. Birkhead, D. J. Hosken, & S. Pitnick (Eds.), *Sperm Biology* (pp. 207–245). London: Academic Press. <https://doi.org/10.1016/B978-0-12-372568-4.00006-9>
- Pizzari, T., & Wedell, N. (2013). The polyandry revolution. *Philosophical Transactions of the Royal Society B*, 368, 20120041.
- Riehl, C., & Stern, C. A. (2015). How cooperatively breeding birds identify relatives and avoid incest: New insights into dispersal and kin recognition. *BioEssays*, 37(12), 1303–1308. <https://doi.org/10.1002/bies.201500120>
- Rosengaus, R. B., & Traniello, J. F. (1993). Disease risk as a cost of outbreeding in the termite *Zootermopsis angusticollis*. *Proceedings of the National Academy of Sciences*, 90(14), 6641–6645. <https://doi.org/10.1073/pnas.90.14.6641>
- Saint Jalme, M., Gaucher, P., & Paillat, P. (1994). Artificial insemination in Houbara bustards (*Chlamydotis undulata*): influence of the number of spermatozoa and insemination frequency on fertility and ability to hatch. *Journal of Reproduction and Fertility*, 100(1), 93–103. <https://doi.org/10.1530/jrf.0.1000093>
- Saint Jalme, M., Williams, J., Mickaelian, I., & Paillat, P. (1996). Seasonal variation of LH, sex steroids, body mass, molt, display, and laying in two subspecies of Houbara bustard, *Chlamydotis undulata macqueenii* and *Chlamydotis undulata undulata*, housed in outdoor cages under natural conditions. *General and Comparative Endocrinology*, 102(1), 102–112. <https://doi.org/10.1006/gcen.1996.0051>
- Schulte-Hostedde, A. I., Zinner, B., Millar, J. S., & Hickling, G. J. (2005). Restitution of mass-size residual: validating body condition indices. *Ecology*, 86(1), 155–163.
- Stockley, P. (1997). No evidence of sperm selection by female common shrews. *Proceedings of the Royal Society B*, 264, 1497–1500. <https://doi.org/10.1098/rspb.1997.0207>
- Szulkin, M., & Sheldon, B. C. (2008). Dispersal as a means of inbreeding avoidance in a wild bird population. *Proceedings of the Royal Society B*, 275(1635), 703–711.

<https://doi.org/10.1098/rspb.2007.0989>

Szulkin, M., Stopher, K. V., Pemberton, J. M., & Reid, J. M. (2013). Inbreeding avoidance, tolerance, or preference in animals? *Trends in Ecology and Evolution*, 28(4), 205–211. <https://doi.org/10.1016/j.tree.2012.10.016>

Tregenza, T., & Wedell, N. (2000). Genetic compatibility, mate choice and patterns of parentage: Invited Review. *Molecular Ecology*, 9, 1013–1027.

Tregenza, T., & Wedell, N. (2002). Polyandrous females avoid costs of inbreeding. *Nature*, 415, 71–73.

Wedekind, C., Chapuisat, M., Macas, E., & Rülcke, T. (1996). Non-random fertilization in mice correlates with the MHC and something else. *Heredity*, 77(4), 400–409. <https://doi.org/10.1038/hdy.1996.160>

Welke, K., & Schneider, J. M. (2009). Inbreeding avoidance through cryptic female choice in the cannibalistic orb-web spider *Argiope lobata*. *Behavioral Ecology*, 20(5), 1056–1062. <https://doi.org/10.1093/beheco/arp097>

Zeh, J., & Zeh, D. (1997). The evolution of polyandry II: post-copulatory defences against genetic incompatibility. *Proceeding of the Royal Society B*, 264, 69–75.

Data accessibility

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

Author contributions

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

Tables and figures

Table 1. Effects of parental relatedness, hatching success, sperm motility and the interaction between parental relatedness and hatching success on within-triad siring success (i.e. proportion of eggs sired by each of the three males within a given mix of sperm; n = 282). 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	

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

Table 3. Effects of sperm storage duration (delay between insemination and laying), laying date and parental relatedness (unrelated, cousin or half-sibling) on hatchling body mass, body size (head length) and body condition (residuals of body mass on body size). Significant *p* 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	

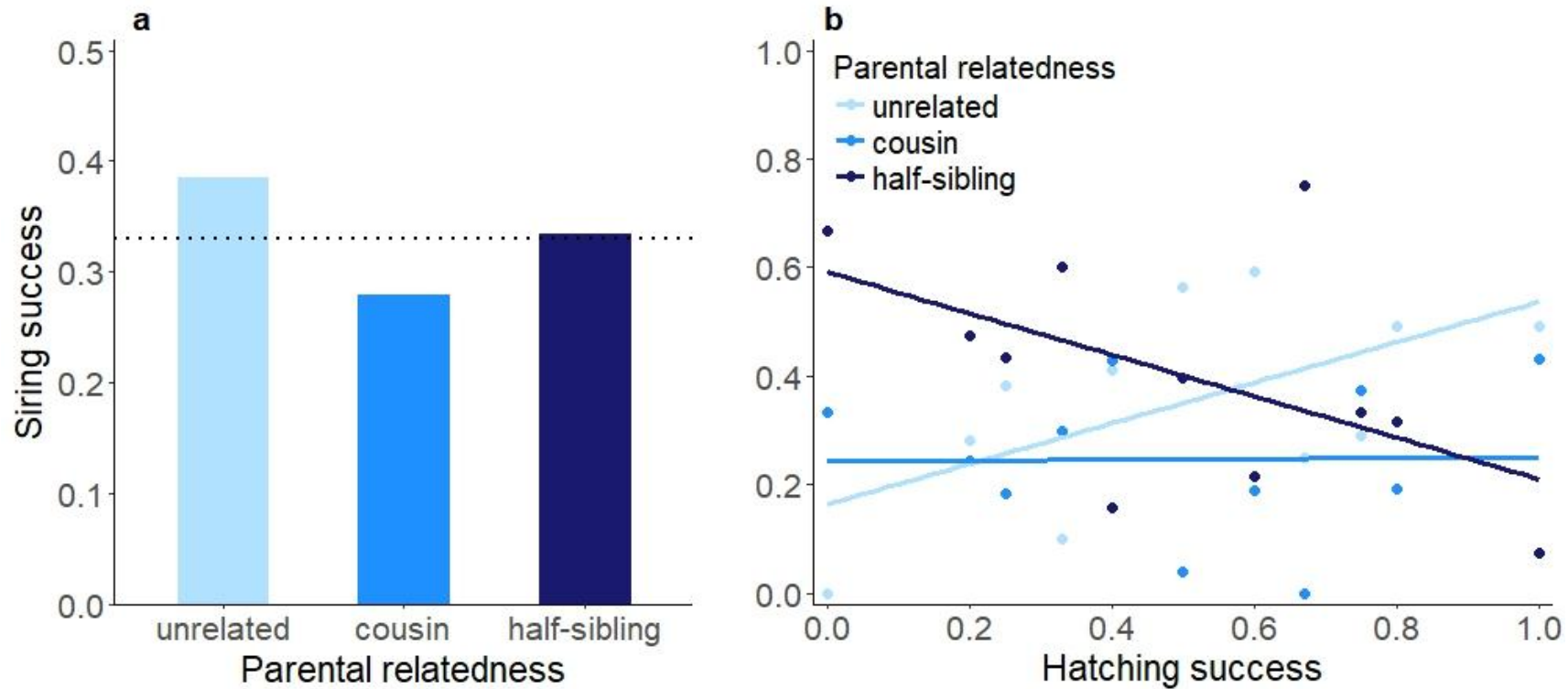
687 Table 4. Effects of sperm storage duration (delay between insemination and laying), laying
688 date and parental relatedness (unrelated, cousin or half-sibling) on offspring body mass, body
689 size (head length) and body condition (residuals of body mass on body size) during the first
690 three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

Body mass				
Fixed effects	Estimate	SE	t value	P
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	P
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	P
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

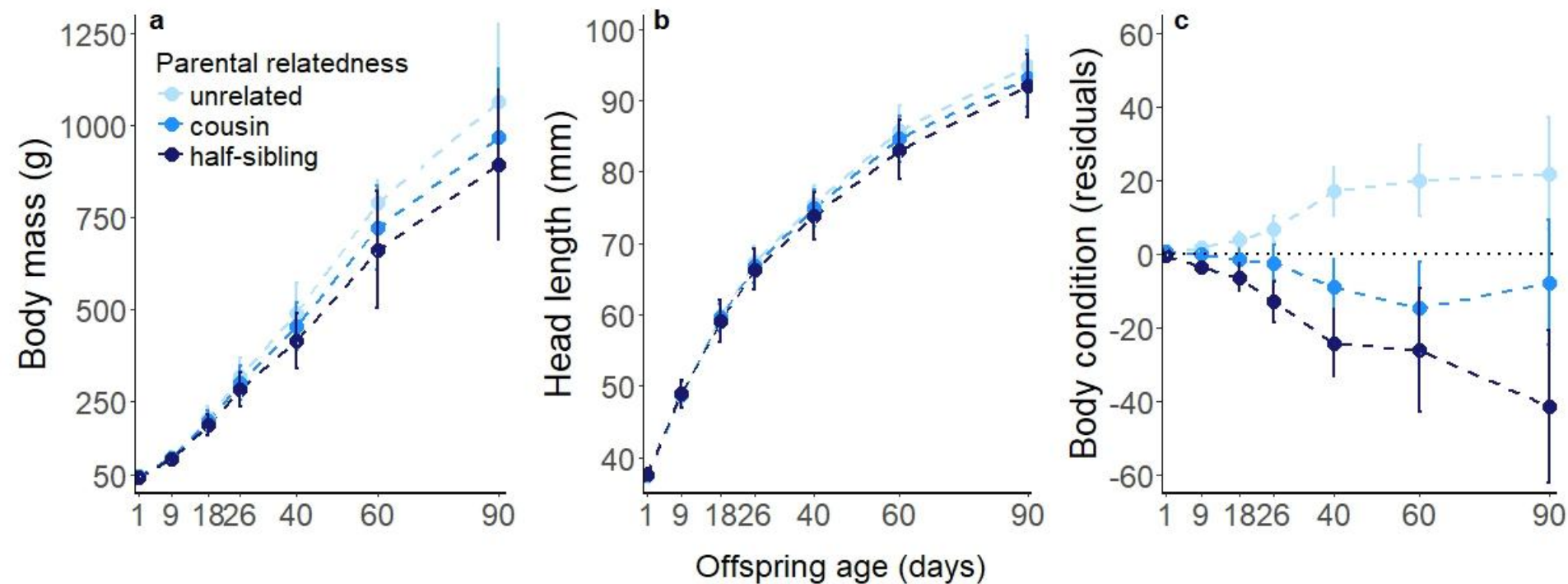
Parental relatedness x log(offspring age)	-53.706	10.788	-4.978	<0.001
Random effects	Variance		SD	
Offspring ID:Maternal ID	475.10		21.80	
Maternal ID	113.50		10.65	
Paternal ID	372.00		19.29	

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.



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.



702
703
704

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

