

Behavioural avoidance of sperm ageing depends on genetic similarity of mates in a monogamous seabird

MAXIME PINEAUX^{1*}, PIERRICK BLANCHARD¹, ÉTIENNE DANCHIN¹, SCOTT A. HATCH², FABRICE HELFENSTEIN³, HERVÉ MULARD¹, JOËL WHITE¹, SARAH LECLAIRE^{1†} and RICHARD H. WAGNER^{4†}

¹Laboratoire Évolution & Diversité Biologique (EDB UMR 5174), Université de Toulouse Midi-Pyrénées, CNRS, IRD, UPS, 118 route de Narbonne, Bat 4R1, 31062 Toulouse cedex 9, France

²Institute for Seabird Research and Conservation, 12850 Mountain Place, Anchorage, AK 99516, USA

³Laboratory of Evolutionary Ecophysiology, Institute of Biology, Faculty of Sciences, University of Neuchâtel, Neuchâtel, Switzerland

⁴Konrad-Lorenz-Institute of Ethology, Department of Integrative Biology and Evolution, University of Veterinarian Medicine Vienna, Savoyenstr. 1a, A-1160 Vienna, Austria

[†]Co-last authors.

Received 8 April 2019; revised 6 May 2019; accepted for publication 6 May 2019

Inbreeding, i.e. the mating of genetically related individuals, can lead to reduced fitness and is considered to be a major selective force of mate choice. Although inbreeding avoidance has been found in numerous taxa, individuals may face constraints when pairing, leading to mating with suboptimal partners. In such circumstances, individuals that are able to avoid factors exacerbating detrimental effects of inbreeding should be favoured. Using the socially and genetically monogamous black-legged kittiwake (*Rissa tridactyla*), we explored whether the detrimental effects of inbreeding are exacerbated by sperm ageing (i.e. the post-meiotic senescence of sperm cells, mainly occurring within the female tracts after copulation), and whether they can be mitigated by behavioural tactics. First, by experimentally manipulating the age of the fertilizing sperm, we found that hatching failure due to sperm ageing increased with higher genetic similarity between mates. We then investigated whether more genetically similar pairs exhibited mating behaviours that prevent fertilization by old sperm. The more genetically similar mates were, the less likely they were to copulate early in the reproductive season and the more females performed post-copulatory sperm ejections. By flexibly adapting their behaviour in response to within-pair genetic similarity, kittiwakes may avoid exacerbation of inbreeding costs due to sperm ageing.

ADDITIONAL KEYWORDS: fitness – gamete – genetic relatedness – post-copulatory choice – reproductive behaviours – sperm ageing – sperm senescence.

INTRODUCTION

Studies on reproductive strategies based on the genotypes of partners have been steadily growing (Jennions & Petrie, 2000; Neff & Pitcher, 2005; Kempenaers, 2007; Kamiya *et al.*, 2014; Firman *et al.*, 2017). In particular, evidence has accumulated for avoidance of inbreeding, i.e. the mating of genetically related individuals (Pusey & Wolf, 1996; Hoffman *et al.*, 2007; Mulard *et al.*, 2009; Leclaire *et al.*, 2013), in line with studies reporting a negative effect of

inbreeding on fitness (Charlesworth & Charlesworth, 1987; DeRose & Roff, 1999; Keller & Waller, 2002). Inbreeding can reduce fitness because of the expression of detrimental recessive alleles or the loss of overdominance at loci with heterozygote advantage (Charlesworth & Charlesworth, 1987; Roff, 2002). The detrimental effects of inbreeding on fitness are exacerbated under stressful conditions (Armbruster & Reed, 2005; Fox & Reed, 2011; Ihle *et al.*, 2017), including adverse abiotic factors (e.g. temperature, drought), intraspecific competition (Armbruster & Reed, 2005; Fox & Reed, 2011) and high pathogen

*Corresponding author. E-mail: maxime.pineaux@univ-tlse3.fr

load (Coltman *et al.*, 1999; Ilmonen *et al.*, 2008; Bello-Bedoy & Nunez-Farfan, 2011).

Recently, sperm ageing, which is receiving growing attention in evolutionary biology (Reinhardt, 2007; Pizzari *et al.*, 2008; White *et al.*, 2008; Gasparini *et al.*, 2014, 2017, 2018; Firman *et al.*, 2015; Vega-Trejo *et al.*, 2019), has been shown to exacerbate inbreeding (Tan *et al.*, 2013). Sperm ageing refers to the post-meiotic senescence of haploid sperm cells and is independent from ageing of the diploid organism (Pizzari *et al.*, 2008). In *Drosophila melanogaster*, inbreeding decreases offspring viability when females are fertilized by old sperm but not by young sperm (Tan *et al.*, 2013). However, the mechanisms by which sperm age and inbreeding interplay to modulate embryo survival or growth remain unclear. As they age, sperm cells accumulate damage in DNA and changes in DNA methylation profiles (Twigg *et al.*, 1998; Aitken & Baker, 2006; Menezo *et al.*, 2016), mainly as a result of oxidative stress (Reinhardt, 2007; Pizzari *et al.*, 2008). These alterations of sperm DNA have deleterious effects on fertilization potential and the viability of zygotes and offspring (Tarin *et al.*, 2000; White *et al.*, 2008; Gasparini *et al.*, 2017), but can be repaired by post-fertilization mechanisms (Menezo *et al.*, 2016). However, defections in DNA repair mechanisms may be associated with the expression of detrimental recessive alleles at genes controlling such mechanisms (Okayasu *et al.*, 2000; Perez *et al.*, 2007). Thus, one could speculate that an interplay between inbreeding and sperm ageing may decrease embryo and offspring viability.

The increased deleterious effects of sperm ageing in inbred reproductive events may have created selective pressures inducing the evolution of counter strategies. In numerous species, individuals avoid inbreeding through pre-copulatory mate choice (Hoffman *et al.*, 2007; Mulard *et al.*, 2009; Leclaire *et al.*, 2013), post-copulatory strategies (Pizzari *et al.*, 2004; Bretman *et al.*, 2009; Welke & Schneider, 2009) or both (Gasparini & Pilastro, 2011; Daniel & Rodd, 2016). However, individuals do not necessarily have a choice of their sexual partner or can face permanent or temporary constraints in choosing within a limited pool of potential mates. These constraints include various ecological restrictions such as limited search areas (Pusey & Wolf, 1996; Frankham, 1998), asynchrony in reproductive phenology (Stutchbury & Morton, 1995; Lehmann & Perrin, 2003) or biased sex-ratio (Kvarnemo & Simmons, 1999; Tinghitella *et al.*, 2013). When breeding individuals have no option but to mate with genetically similar partners, they may limit fitness costs by avoiding factors exacerbating the deleterious effects of inbreeding. Several strategies preventing fertilization by old sperm have been proposed (Reinhardt, 2007), but only a few have

been empirically described. These include female preferential selection of spermatophores containing young sperm (Reinhardt & Siva-Jothy, 2005) and sperm ejection by females following copulations occurring long before the female fertile period (Wagner *et al.*, 2004; White *et al.*, 2008). However, whether these strategies are preferentially used by individuals paired with genetically similar mates (i.e. facing a higher probability of suffering from fertilization by old sperm) has yet to be examined.

Here, we present evidence that sperm ageing exacerbates the detrimental effects of inbreeding in the black-legged kittiwake (*Rissa tridactyla*), and report behavioural tactics that might reduce such effects. Kittiwakes are strictly monogamous during a given breeding season (Helfenstein *et al.*, 2004b) and frequently retain the same mate over several years, although divorce can occur after breeding failure (Naves *et al.*, 2007). Breeding failure in kittiwakes is associated with sperm ageing (Wagner *et al.*, 2004; White *et al.*, 2008), and might be limited by females preferentially ejecting sperm following precocious copulations (i.e. sperm that would have been old by the time of fertilization) (Wagner *et al.*, 2004). Kittiwakes also suffer reproductive costs from inbreeding, and they preferentially mate with genetically dissimilar mates (Mulard *et al.*, 2009), possibly via an odour-based mechanism (Leclaire *et al.*, 2012). However, not all individuals pair with a genetically dissimilar mate, perhaps because of constraints on mate choice (Mulard *et al.*, 2009). Being strictly monogamous, kittiwakes cannot avoid inbreeding through post-pairing strategies (e.g. extra-pair mating, cryptic female choice), and are thus expected to have evolved strategies that limit the factors exacerbating the deleterious effects of inbreeding. If sperm ageing exacerbates the detrimental effects of inbreeding in kittiwakes, we therefore predict that the more genetically similar mates are, the more they will use the behavioural strategies preventing fertilization by aged sperm (i.e. avoidance of precocious copulations, and sperm ejection after precocious copulations).

Our long-term monitoring of kittiwake populations has created the first opportunity of which we are aware to examine the potential behavioural adaptations to interactions between sperm ageing and inbreeding. First, using a well-established protocol (White *et al.*, 2008), we manipulated the age of the fertilizing sperm to investigate whether sperm ageing exacerbates the detrimental effects of inbreeding on three proxies of fitness: eggs viability, and hatchling body condition and size (Helfenstein, 2002; Helfenstein *et al.*, 2004a). Hence, this experimental design makes it possible to highlight fitness costs not otherwise detectable in nature if counter-strategies have evolved. Then, to determine whether behavioural strategies might

mitigate these costs, we used behavioural observations conducted on unmanipulated breeding pairs in another kittiwake population and tested whether the timing of copulations and sperm ejections varied with genetic similarity between mates.

MATERIAL AND METHODS

EXPERIMENTAL MANIPULATION OF SPERM AGE

The manipulative part of this study was conducted in the 2006, 2009 and 2010 breeding seasons (May–August) on a colony of black-legged kittiwakes nesting on an abandoned U.S. Air Force radar tower on Middleton Island (59°26'N, 146°20'W), Gulf of Alaska. Nest sites created on the upper walls of the tower can be observed from inside through sliding one-way mirrors (Gill & Hatch, 2002). All nest sites were checked twice daily (09:00 and 18:00 h) to record laying and hatching events.

We used a protocol developed on kittiwakes during previous breeding seasons (White *et al.*, 2008). Briefly, after pairs ($N = 27$) had commenced copulating, males were fitted with an anti-insemination ring (i.e. a rubber ring placed around the cloaca and maintained with a harness) that prevents cloacal contact and insemination, and hence females from receiving fresh sperm (White *et al.*, 2008). Males were recaptured after completion of the clutch to remove their ring. Thus, the minimum age of sperm available for fertilization corresponded to the number of days the ring was worn before laying. Rings were fitted randomly over a period of 19 days preceding egg laying. As in most wild species, the exact duration between fertilization and egg laying is unknown in kittiwakes. We assumed that fertilization occurred 1–2 days before egg laying (Bakst *et al.*, 1994). Because fertilization might have occurred before the ring was fitted if the male wore a ring for less than 2 days before laying, we excluded the corresponding egg from analyses ($N = 2$). Rings were inconspicuous, as they were covered by surrounding feathers, and they allowed normal behaviour (White *et al.*, 2008).

Because inbreeding and sperm ageing are known to have strong effects on early-life stages (Spottiswoode & Møller, 2004; White *et al.*, 2008; Hemmings *et al.*, 2012), we used hatching success, and chick body condition (body mass adjusted for tarsus length) and size (i.e. tarsus length) at hatching as proxies of fitness. Tarsus length is a good estimator of overall body size in adults (Rising & Somers, 1989), which is a good indicator of breeding success in kittiwakes (Helfenstein, 2002; Helfenstein *et al.*, 2004a). On the day of laying, A- and B-eggs (i.e. the first and the second laid egg, respectively) were labelled individually with a non-toxic marker. To facilitate monitoring of egg

development, eggs were removed from their nest and placed in artificial incubators, which does not affect hatching success in kittiwakes (White *et al.*, 2008). We checked for embryonic development using egg candling, which consists in using a bright light to see through the shell. Eggs that did not exhibit early signs of embryonic development (i.e. 'yolk spreading'; $N = 4$ eggs) were conservatively excluded from the analyses to ensure that sperm age, and not sperm presence, was the only factor manipulated as in White *et al.* (2008). This led to a final sample size of 36 eggs in 24 nests. Eggs were placed back in their nest after 25 days of incubation (the incubation period of kittiwakes lasts 27 days; Coulson & White, 1958) or as soon as external pipping occurred. Within 12 h of hatching, all chicks were weighed to the nearest gram using an electronic scale, and tarsus length was measured to the nearest millimetre using a caliper ($N = 24$ chicks).

To control for any potential effects caused by the ring itself on reproductive performance, other males were fitted with a thinner control ring that did not prevent insemination (White *et al.*, 2008). In this control group ($N = 43$ eggs and 32 chicks), we found no significant association between reproductive performance and ring wear duration (see Supporting Information for details). This indicates that the potential effects on reproductive performance in the experimental group are due to sperm ageing and not to the wearing of the ring per se, as previously found by White *et al.* (2008).

OBSERVATION OF MATING BEHAVIOUR IN UNMANIPULATED PAIRS

Behavioural observations were conducted in the 1999–2001 breeding seasons (May–August) on the kittiwake population nesting at Cap Sizun in Brittany, France (48°5'N, 4°36'W), where birds can be individually identified using colour bands (Danchin *et al.*, 1998).

The protocol used for behavioural observations is described in Helfenstein *et al.* (2003, 2004b). In summary, we used daily continuous observations (Altmann, 1974) to record copulations and sperm ejections in pairs nesting on a cliff (daily number of hours of observation, mean \pm SD: 3.76 ± 2.06 ; range: 1–10). We observed 13 pairs in 1999, 19 pairs in 2000 and 21 pairs in 2001 (35 unique pairs in total as 14 pairs were observed during more than one year), in which both mates were banded, genotyped and observable from a single observation point ~30 m away. Sperm ejection is defined as females forcefully ejecting a white fluid within 90 s of the male dismounting. These distinctive cloacal expulsions occur non-randomly after copulation and are different from defaecations, which occur without noticeable muscular contraction (Helfenstein *et al.*, 2003). We used copulations occurring within 20 days before the laying of the first

egg to allow comparison with the experimental study, and because kittiwakes rarely mate before this period (Helfenstein *et al.*, 2004b). Cap Sizun birds were caught on the nest using a hook system to collect blood (Helfenstein *et al.*, 2004b).

GENETIC ANALYSES

For all birds, blood was taken from the alar vein using a 1-mL syringe and a 25-gauge needle, and kept in a preservative solution. DNA was extracted from each blood sample using either a 'salting out' protocol (Mulard *et al.*, 2009) or the DNeasy Blood and Tissue Kit (Qiagen Group) following the supplier's guidelines. Birds were genotyped at ten microsatellite loci (Mulard *et al.*, 2009; Leclaire *et al.*, 2012) by using the protocol described by Mulard *et al.* (2009) ($N = 114$ birds). Additional DNA samples ($N = 48$ birds) were analysed subsequently by using a more recent protocol described by Leclaire *et al.* (2012). The correspondence between the two methods was tested by genotyping 30 individuals using the two protocols. Correspondence did not match for loci K32 and RBG20 (Leclaire *et al.*, 2012). Genetic relatedness between mates that were not genotyped with the same protocol ($N = 6$ out of 81 pairs) was therefore calculated without these two loci.

We used GENEPOP v.4.6 (Rousset, 2008) to test linkage disequilibria and deviation from Hardy–Weinberg equilibrium (Markov chain parameters: 10 000 dememorization steps, 100 batches and 5000 iterations per batch). After correcting for multiple tests, the K16 locus appeared to be out of Hardy–Weinberg equilibrium in both populations ($P < 0.001$) and no locus was genetically linked to another locus ($P > 0.05$). Therefore, we excluded K16 from the genetic relatedness analyses.

Genetic similarity between mates was calculated using the identity index (R_{ID}) (Mathieu *et al.*, 1990) in the IDENTIX software (Belkhir *et al.*, 2002). This index has been validated as a good estimator of the consanguinity of offspring in cases where identical alleles are likely to be identical by descent, something especially relevant when focusing on the fitness consequences of inbred mating (Belkhir *et al.*, 2002). In addition, this index has been used in previous studies that revealed patterns and effects of biological meaning in kittiwakes (Mulard *et al.*, 2009; Leclaire *et al.*, 2012). R_{ID} was transformed to an estimate of genetic distance (D_{ID}) using the formula, $D_{ID} = 1 - R_{ID}$, which can theoretically range from 0 (corresponding to mates sharing the same microsatellite alleles) to 1 (corresponding to mates sharing not a single microsatellite allele). In our study, D_{ID} ranged from 0.39 to 0.83 (mean \pm SD: 0.64 ± 0.10 ; $N = 46$ pairs) for pairs from the Middleton population, and from 0.23 to

0.61 (mean \pm SD: 0.43 ± 0.09 ; $N = 35$ pairs) for pairs from the Cap Sizun population.

STATISTICAL ANALYSES

Experimental manipulation of sperm age

We tested the effect of genetic similarity and sperm age on each of the three proxies of fitness (i.e. hatching success, body condition and tarsus length at hatching) using mixed models. Explanatory variables were the genetic distance between pair members, the duration of ring wear (i.e. minimum sperm age), their two-way interaction and egg rank. Tarsus length at hatching was included in the model built for body mass at hatching, which can thus be interpreted as size-adjusted body mass, or body condition (Garcia-Berthou, 2001). Year and pair identity were included as random effects. For analyses of hatching success, we used a generalized linear mixed model (GLMM) and a binomial distribution, while for analyses of body mass and tarsus length, we used linear mixed models (LMMs). We checked for outliers by calculating Cook's distance with the *influence.ME* package in R (Nieuwenhuis *et al.*, 2012). We considered as too influential those data points with a Cook's distance that exceeded the cut-off value $4/N$, with N being the sample size (Nieuwenhuis *et al.*, 2012). Analyses were redone when excluding the influential data, and results were similar (see Results). For LMMs, we checked for normality and homoscedasticity of residuals.

Observation of mating behaviour in unmanipulated pairs

As found in previous studies (Helfenstein *et al.*, 2004b, White, 2008), kittiwakes were rarely observed copulating more than once a day (18 cases over 926 observations). Therefore, to test for a relationship between genetic similarity and copulation behaviour in unmanipulated pairs, we calculated the daily probability of observing at least one copulation for each pair ($N = 53$ pairs). We built a GLMM using a binomial distribution with this binary variable as the response variable. Over the 146 copulations recorded during this period, 38 (26%) were followed by sperm ejection, and no sperm ejection was observed between 20 and 15 days before laying. Analyses of sperm ejection were thus restricted to the 15 days before laying. We tested whether sperm ejection probability was related to genetic similarity using a GLMM with a binomial distribution. For each pair ($N = 45$ pairs), the proportion of copulations followed by sperm ejection on a given day was used as the response variable and was weighted by the number of copulations [using the *weights* parameter in the *glmer* function from the

lme4 package (Bates *et al.*, 2015) in R (R Core Team, 2017)]. The number of days before laying, genetic distance between pair members and their two-way interaction were included as explanatory variables in models explaining copulation and sperm ejection. Pair identity nested in year and date were included as random effects.

In all analyses, we standardized variables before analysis, and models were fitted with a maximum-likelihood estimator, and normality of the random effects was checked. The significance of a term in the model was assessed by the change in deviance after removal of that term (likelihood-ratio test, LRT) using a chi-square test. The interaction was removed when not significant. All statistical analyses were performed with R 3.4.3 (R Core Team, 2017) and the *lme4* R package (Bates *et al.*, 2015).

RESULTS

EFFECTS OF SPERM AGE AND GENETIC DISTANCE ON FITNESS PROXIES

Hatching success was significantly related to the interaction between genetic distance and minimum sperm age ($N = 36$, $\chi^2_1 = 6.20$, $P = 0.01$; Fig. 1). The more genetically similar mates were, the more sperm age impaired hatching success. There was no significant effect of egg rank on hatching success ($\chi^2_1 = 0.73$, $P = 0.39$). Similar results were obtained when influential data points were excluded ($N = 33$; genetic distance \times minimum sperm age: $\chi^2_1 = 17.92$, $P < 0.001$; egg rank: $\chi^2_1 = 0.42$, $P = 0.52$). Body condition at hatching was not significantly related to any of the parameters we considered ($N = 24$, all $P > 0.36$) except egg rank ($N = 24$, $\chi^2_1 = 6.36$, $P = 0.01$), A-chicks being in better condition than B-chicks. Redoing this analysis without influential data points did not change the results ($N = 21$, all $P > 0.08$, except for egg rank: $\chi^2_1 = 7.47$, $P = 0.01$). Tarsus length at hatching was not significantly associated with the two-way interaction between genetic distance and minimum sperm age ($N = 24$, $\chi^2_1 = 2.89$, $P = 0.09$), nor with any other parameters (all $P > 0.74$). Redoing this analysis without influential data points did not change the results ($N = 20$, all $P > 0.25$).

EFFECTS OF GENETIC DISTANCE AND TIME ON REPRODUCTIVE BEHAVIOURS

The probability of copulation varied significantly with the interaction between genetic distance and the number of days before laying ($\chi^2_1 = 7.82$, $P = 0.005$; Fig. 2). The probability of copulation decreased markedly with increasing pairwise genetic similarity

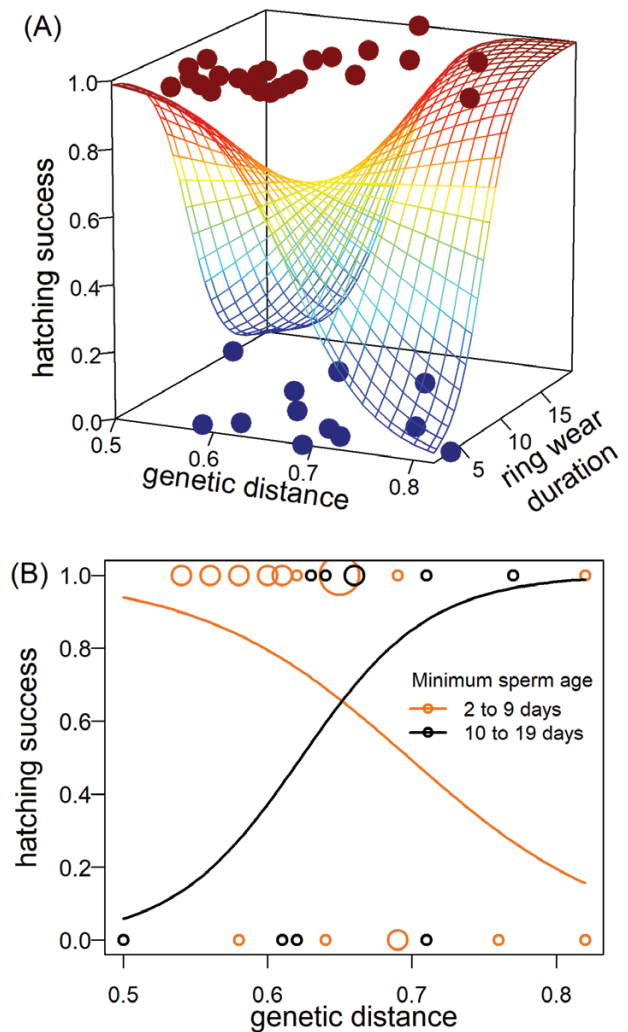


Figure 1. Effects of genetic distance and minimum sperm age (the duration of anti-insemination ring wear) on hatching success in (A) a 3D view and (B) a 2D view. Predicted values of hatching success were derived from the model including genetic distance between mates, the duration of ring wear and their interaction as fixed effects. For clarity, random effects (year and pair identity) were not considered in the model used for graphical representations. Removing influential data points did not change the results (see Results). In A, each point represents whether an egg hatched (red) or not (blue) and colours of the grid represent estimated hatching success, with blue corresponding to low values, red to high values and yellow/green to intermediate values. In B, we divided ring wear duration (2–19 days) in half, producing a short ring wear duration (2–9 days, orange points, $N = 26$ eggs) and a long ring wear duration (10–19 days, black points, $N = 10$ eggs). The size of the points represents the number of eggs. Predicted values were derived from the model described above by fixing the duration of ring wear to 5 days (i.e. short ring wear duration, orange curve) or 15 days (i.e. long ring wear duration, black curve).

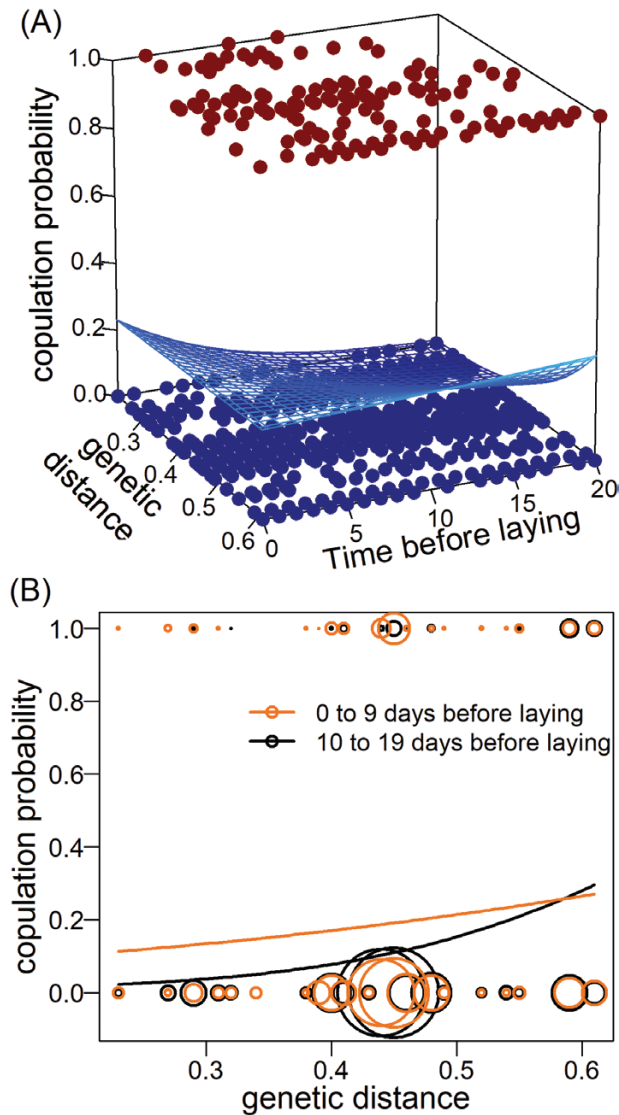


Figure 2. The probability of copulation over time and according to genetic distance (A) in a 3D view and (B) in a 2D view. Predicted values of copulation probability were derived from the model including genetic distance between mates, number of days before laying of the first egg and their interaction. For clarity, random effects (pair identity nested in the year and Julian day) were not considered in these models for graphical representations. In A, each point represents whether a pair copulated (red) or not (blue) during a given day of observation. In B, we divided the period before laying (20–0 days) in half, producing a late period (0–9 days before laying, in orange, $N = 456$ observations) and an early period (10–20 days before laying, in black, $N = 470$ observations). The size of the points represents the number of observations. Predicted values were calculated by fixing the time before laying to 5 days (i.e. early period, orange line) or 15 days (i.e. late period, black line).

early in the reproductive season but not as laying date approached (Fig. 2). The probability of sperm ejection decreased with pairwise genetic distance ($\chi^2_1 = 4.19$, $P = 0.04$; Fig. 3A) and decreased as laying date approached ($\chi^2_1 = 6.52$, $P = 0.01$; Fig. 3B), but it did not vary with the interaction between these two variables ($\chi^2_1 = 0.17$, $P = 0.68$).

DISCUSSION

Our experimental manipulation of sperm age in kittiwakes, combined with behavioural observations of copulations and sperm ejections in unmanipulated pairs, has created a unique opportunity to investigate the deleterious interactions of sperm age and inbreeding, along with the behavioural tactics that can minimize them. We found that (1) sperm ageing and genetic similarity interacted to reduce fitness in the form of decreased hatching success, and (2) the frequency of two behavioural strategies used to avoid fertilization by old sperm, namely avoidance of early copulations and post-copulatory sperm ejection, increased with genetic similarity between mates.

Previous studies in kittiwakes showed that hatching success was independently reduced by inbreeding (Mulard *et al.*, 2009) and sperm ageing (Wagner *et al.*, 2004; White *et al.*, 2008). Here, by experimentally inducing the fertilization of eggs with old sperm, we found that the decrease in hatching success associated with sperm ageing increased with genetic similarity between mates, suggesting that fertilization by old sperm can exacerbate the detrimental effects of inbreeding. Our results add to those in *D. melanogaster* (Tan *et al.*, 2013), and suggest that the deleterious interaction between sperm age and inbreeding may be found across several taxa, which may provide new insights into inbreeding–stress interactions in vertebrates (Marr *et al.*, 2006; Pemberton *et al.*, 2017; Ihle *et al.*, 2017). However, we did not detect any effects of the interplay between inbreeding and sperm ageing on hatchling body condition and size. These results are in line with several studies that have reported that the effects of inbreeding and sperm ageing are especially high during embryo development (Tarin *et al.*, 2000; Hemmings *et al.*, 2012; Spottiswoode & Moller, 2004; Reinhardt, 2007).

We observed that more genetically similar pairs copulated more frequently as egg-laying approached compared to more genetically distant pairs. In addition, females that were more genetically similar to their mate ejected sperm more frequently. The increase in copulation rate over the course of the pre-laying period in kittiwakes (Helfenstein *et al.*, 2004b)

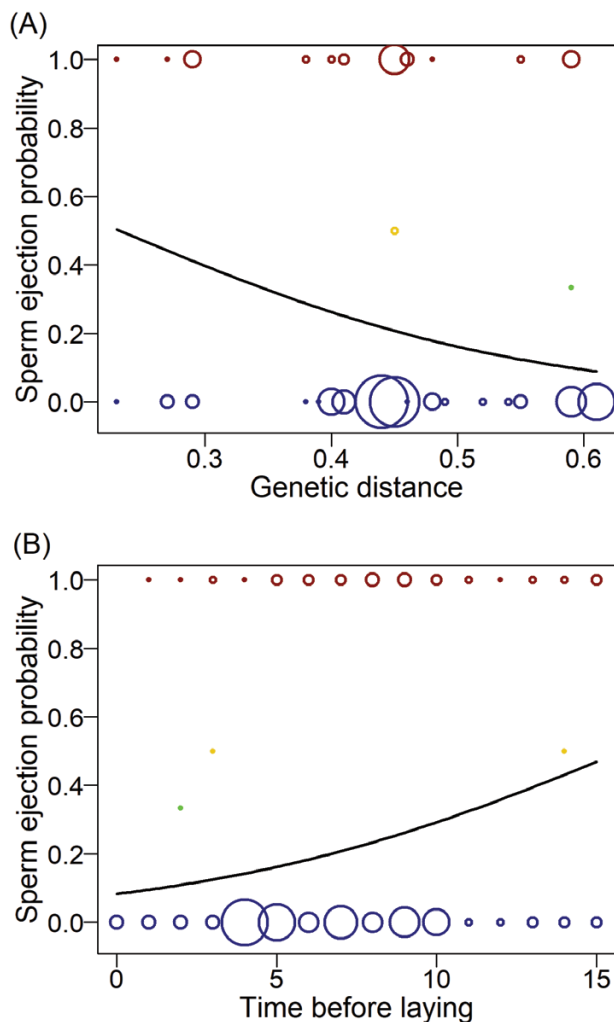


Figure 3. Post-copulatory sperm ejection probability (A) according to genetic distance and (B) over time. Each point represents the proportion of copulations followed by sperm ejection for a given pair on a given day and the size of the points represents the number of pairs. Kittiwakes rarely copulate more than once a day, meaning that sperm ejection probabilities were mostly equal to 1 (in red) or 0 (in blue). The regression lines represent the predicted values derived from the models including genetic distance between mates, number of days before laying of the first egg and their interaction. For clarity, random effects (pair identity nested in the year and Julian day) were not considered in these models for graphical representations. Predicted values were calculated in A by fixing the time before laying to the median number of days (i.e. 7 days) and in B by fixing genetic distance to the median value (i.e. 0.43).

and other birds (Birkhead & Moller, 1992), as well as post-copulatory sperm ejections, have been suggested to be two behavioural strategies that can prevent fertilization by aged sperm (Wagner *et al.*, 2004; White *et al.*, 2008). Here, we showed that these two

strategies are preferentially used by more genetically similar pairs, probably as an adaptation to limit the increasing reproductive costs of old sperm with inbreeding. Similar parental modulation of the effects of inbreeding has been suggested in the Japanese quail (*Coturnix japonica*) and the burying beetle (*Nicrophorus vespilloides*), where inbreeding effects are reduced when females allocate more resources to their offspring (Pilakouta *et al.*, 2015; Pilakouta & Smiseth, 2016; Ihle *et al.*, 2017). This plasticity in behaviours according to genetic distance implies that individuals can assess their genetic similarity to their mate. In a large range of species, genetic similarity is assessed using odour cues (Radwan *et al.*, 2008; Charpentier *et al.*, 2010; Leclaire *et al.*, 2017; Parrott *et al.*, 2007). However, although kittiwake odours do vary with genetic relatedness (Leclaire *et al.*, 2012), the ability of kittiwakes to use this cue has not yet been explored.

In addition to mating behaviours, other strategies based on parental phenotypic traits may modulate the reproductive consequences of the interplay between inbreeding and sperm ageing. For example, parental age, i.e. the pre-meiotic senescence of the diploid organism, has been suggested to heighten inbreeding effects (Fox & Reed, 2010) and sperm susceptibility to sperm ageing (Zubkova & Robaire, 2006; Paul & Robaire, 2013; Risopatron *et al.*, 2018), and might thus modulate the costs associated with their interaction. In *D. melanogaster*, the reproductive costs of the interaction between inbreeding and sperm ageing were modulated by parental age, with young parents suffering higher costs than older ones (Tan *et al.*, 2013). This may be explained by differential resource allocation into eggs between old and young parents (Bogdanova *et al.*, 2006; Ihle *et al.*, 2017; Beamonte-Barrientos *et al.*, 2010). If parental age modulates the reproductive costs of the interaction between inbreeding and sperm ageing in kittiwakes, we predict that breeding individuals would plastically adapt their behaviour in response to both age and within-pair genetic similarity. Female kittiwakes may be more likely to use the behavioural strategies preventing fertilization by aged sperm (i.e. avoidance of precocious copulations, and sperm ejection after precocious copulations) when paired with an old, genetically similar, male.

Our results raise also questions about the role of the interaction between sperm ageing and inbreeding in the strategies displayed by polyandrous species. For instance, when inbreeding interacts with sperm age to decrease fitness, we expect female cryptic preference for genetically dissimilar males to vary with sperm age. Females may not necessarily avoid inbreeding when inseminated with fresh sperm or they may bias fertilization towards the freshest sperm independently

of males' genetic characteristics (Gasparini *et al.*, 2018). The existence of such an interaction might partly explain why cryptic inbreeding avoidance is not ubiquitously found in nature (Mongue *et al.*, 2015) and emphasizes the importance of controlling for sperm age (or sperm quality) when testing for cryptic female sperm choice (Denk *et al.*, 2005).

An unresolved question is which sex is responsible for the behavioural patterns observed in our study. In kittiwakes, males and females may have a common interest in avoiding fertilization by old sperm because they share the same reproductive success (Kvarnemo, 2018). However, sexual conflict over mating behaviour can also arise in genetically monogamous species because the costs and benefits associated with these behaviours may differ between sexes (Helfenstein *et al.*, 2003; White, 2008). For instance, repeated copulations increase the likelihood of sexually transmitted pathogen transmission (Sheldon, 1993), especially in females (White, 2008; White *et al.*, 2010; van Dongen *et al.*, 2019). Therefore, although, in kittiwakes, both males and females have some control over copulations (White, 2008), the observed absence of seasonally early copulations in more genetically similar pairs (Fig. 2) is probably due to an absence of solicitations of copulation by females, which might suffer from repeated copulations more than males. Additionally, males also have some control over sperm ejection, which, despite being a female behaviour, can be prevented when males remain on the female's back after insemination (Helfenstein *et al.*, 2003).

Our results suggest that sperm ageing can exacerbate the deleterious effects of inbreeding in a vertebrate and that individuals can plastically adapt their behaviour in response to these selective pressures. Such best-of-a-bad-job strategy might allow monogamous species to avoid the fitness costs associated with delayed reproduction in a given breeding season (Cam *et al.*, 2003). Overall, our study highlights the selective pressures sperm ageing and inbreeding may exert on the evolution of reproductive behaviour.

ACKNOWLEDGMENTS

We thank M. Kriloff, M. du Toit, C. Gouraud, T. Merklings, F. Bailly, N. Vetter, V. Frochot and C. DeFranchesci for their help in the field on Middleton Island; A. Degeorges, J. Guillaumin, F. Martayan, J.-Y. Monnat and V. Nepoux for their assistance in the field at Cap Sizun; A. Ramey, S. Talbot, K. Sage, E. Lhuillier and C. Veyssière for their help with genetic analyses; and Bretagne Vivante-SEPNEB for providing access to the Réserve Naturelle du Cap Sizun. We thank the anonymous reviewers for their helpful comments. Experiments at Middleton Island were carried out

in accordance with US laws and under permits from the U.S. Fish and Wildlife Service and State of Alaska. Work at Cap Sizun complied with French laws. The authors declare no conflicts of interest.

FUNDING

This work was supported by grants from the French Polar Institute Paul-Emile Victor ('Program Arctique 429' and 'Program SexCoMonArc 1162' to E.D.), by a grant from the Agence Nationale de la Recherche Française (ANR-13-PDOC-0002 to S.L.), and by the French Laboratory of Excellence project 'TULIP' (ANR-10-LABX-41) and Idex UNITY (ANR-11-IDEX-0002-02).

REFERENCES

- Aitken RJ, Baker MA. 2006. Oxidative stress, sperm survival and fertility control. *Molecular and Cellular Endocrinology* **250**: 66–69.
- Altmann J. 1974. Observational study of behavior – sampling methods. *Behaviour* **49**: 227–267.
- Armbruster P, Reed DH. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.
- Bakst MR, Wishart G, Brillard J-P. 1994. Oviducal sperm selection, transport, and storage in poultry. *Poultry Science Reviews* **5**: 117–143.
- Bates D, Machler M, Bolker BM, Walker SC. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1–48.
- Beamonte-Barrientos R, Velando A, Drummond H, Torres R. 2010. Senescence of maternal effects: aging influences egg quality and rearing capacities of a long-lived bird. *American Naturalist* **175**: 469–480.
- Belkhir K, Castric V, Bonhomme F. 2002. IDENTIX, a software to test for relatedness in a population using permutation methods. *Molecular Ecology Notes* **2**: 611–614.
- Bello-Bedoy R, Nunez-Farfan J. 2011. The effect of inbreeding on defence against multiple enemies in *Datura stramonium*. *Journal of Evolutionary Biology* **24**: 518–530.
- Birkhead T, Moller A. 1992. *Sperm competition in birds. Evolutionary causes and consequences*. San Diego: Academic Press.
- Bogdanova MI, Nager RG, Monaghan P. 2006. Does parental age affect offspring performance through differences in egg quality? *Functional Ecology* **20**: 132–141.
- Bretman A, Newcombe D, Tregenza T. 2009. Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology* **18**: 3340–3345.
- Cam E, Monnat JY, Hines JE. 2003. Long-term fitness consequences of early conditions in the kittiwake. *Journal of Animal Ecology* **72**: 411–424.
- Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* **18**: 237–268.

- Charpentier MJE, Crawford JC, Boulet M, Drea CM. 2010. Message 'scent': lemurs detect the genetic relatedness and quality of conspecifics via olfactory cues. *Animal Behaviour* **80**: 101–108.
- Coltman DW, Pilkington JG, Smith JA, Pemberton JM. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* **53**: 1259–1267.
- Coulson JC, White E. 1958. Observations on the breeding of the kittiwake. *Bird Study* **5**: 74–83.
- Danchin E, Boulinier T, Massot M. 1998. Conspecific reproductive success and breeding habitat selection: implications for the study of coloniality. *Ecology* **79**: 2415–2428.
- Daniel MJ, Rodd FH. 2016. Female guppies can recognize kin but only avoid incest when previously mated. *Behavioral Ecology* **27**: 55–61.
- Denk AG, Holzmann A, Peters A, Vermeirssen ELM, Kempenaers B. 2005. Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. *Behavioral Ecology* **16**: 825–833.
- DeRose MA, Roff DA. 1999. A comparison of inbreeding depression in life-history and morphological traits in animals. *Evolution* **53**: 1288–1292.
- van Dongen W, White J, Brandl H, Leclaire S, Hatch S, Danchin E, Wagner R. 2019. Experimental evidence of a sexually transmitted infection in a wild vertebrate, the black-legged kittiwake (*Rissa tridactyla*). *Biological Journal of the Linnean Society* **127**: 292–298.
- Firman RC, Gasparini C, Manier MK, Pizzari T. 2017. Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution* **32**: 368–382.
- Firman RC, Young FJ, Rowe DC, Duong HT, Gasparini C. 2015. Sexual rest and post-meiotic sperm ageing in house mice. *Journal of Evolutionary Biology* **28**: 1373–1382.
- Fox CW, Reed DH. 2010. Inbreeding depression increases with maternal age in a seed-feeding beetle. *Evolutionary Ecology Research* **12**: 961–972.
- Fox CW, Reed DH. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.
- Frankham R. 1998. Inbreeding and extinction: island populations. *Conservation Biology* **12**: 665–675.
- Garcia-Berthou E. 2001. On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. *Journal of Animal Ecology* **70**: 708–711.
- Gasparini C, Daymond E, Evans JP. 2018. Extreme fertilization bias towards freshly inseminated sperm in a species exhibiting prolonged female sperm storage. *Royal Society Open Science* **5**: 7.
- Gasparini C, Dosselli R, Evans JP. 2017. Sperm storage by males causes changes in sperm phenotype and influences the reproductive fitness of males and their sons. *Evolution Letters* **1**: 16–25.
- Gasparini C, Kelley JL, Evans JP. 2014. Male sperm storage compromises sperm motility in guppies. *Biology Letters* **10**: 4.
- 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: Biological Sciences* **278**: 2495–2501.
- Gill VA, Hatch SA. 2002. Components of productivity in black-legged kittiwakes *Rissa tridactyla*: response to supplemental feeding. *Journal of Avian Biology* **33**: 113–126.
- Helfenstein F. 2002. *Stratégies de reproduction et conflits sexuels: le cas d'une espèce coloniale, la mouette tridactyle Rissa tridactyla*. Paris: Université Pierre et Marie Curie.
- Helfenstein F, Danchin E, Wagner RH. 2004a. Assortative mating and sexual size dimorphism in Black-legged Kittiwakes. *Waterbirds* **27**: 350–354.
- Helfenstein F, Tirard C, Danchin E, Wagner RH. 2004b. Low frequency of extra-pair paternity and high frequency of adoption in Black-legged Kittiwakes. *Condor* **106**: 149–155.
- Helfenstein F, Wagner RH, Danchin E. 2003. Sexual conflict over sperm ejection in monogamous pairs of kittiwakes *Rissa tridactyla*. *Behavioral Ecology and Sociobiology* **54**: 370–376.
- Hemmings NL, Slate J, Birkhead TR. 2012. Inbreeding causes early death in a passerine bird. *Nature Communications* **3**: 4.
- Hoffman JI, Forcada J, Trathan PN, Amos W. 2007. Female fur seals show active choice for males that are heterozygous and unrelated. *Nature* **445**: 912–914.
- Ihle KE, Hutter P, Tschirren B. 2017. Increased prenatal maternal investment reduces inbreeding depression in offspring. *Proceedings of the Royal Society B: Biological Sciences* **284**: 5.
- Ilmonen P, Penn DJ, Damjanovich K, Clarke J, Lamborn D, Morrison L, Ghotbi L, Potts WK. 2008. Experimental infection magnifies inbreeding depression in house mice. *Journal of Evolutionary Biology* **21**: 834–841.
- Jennions MD, Petrie M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews* **75**: 21–64.
- Kamiya T, O'Dwyer K, Westerdahl H, Senior A, Nakagawa S. 2014. A quantitative review of MHC-based mating preference: the role of diversity and dissimilarity. *Molecular Ecology* **23**: 5151–5163.
- Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. *Trends in Ecology & Evolution* **17**: 230–241.
- Kempenaers B. 2007. Mate choice and genetic quality: a review of the heterozygosity theory. *Advances in the Study of Behavior* **37**: 189–278.
- Kvarnemo C. 2018. Why do some animals mate with one partner rather than many? A review of causes and consequences of monogamy. *Biological Reviews* **93**: 1795–1812.
- Kvarnemo C, Simmons LW. 1999. Variance in female quality, operational sex ratio and male mate choice in a bushcricket. *Behavioral Ecology and Sociobiology* **45**: 245–252.
- Leclaire S, Merklings T, Raynaud C, Mulard H, Bessiere JM, Lhuillier E, Hatch SA, Danchin E. 2012. Semiochemical compounds of preen secretion reflect genetic make-up in a seabird species. *Proceedings of the Royal Society B: Biological Sciences* **279**: 1185–1193.
- Leclaire S, Nielsen JF, Sharp SP, Clutton-Brock TH. 2013. Mating strategies in dominant meerkats: evidence for extra-pair paternity in relation to genetic relatedness between pair mates. *Journal of Evolutionary Biology* **26**: 1499–1507.

- Leclaire S, Strandh M, Mardon J, Westerdahl H, Bonadonna F. 2017.** Odour-based discrimination of similarity at the major histocompatibility complex in birds. *Proceedings of the Royal Society B: Biological Sciences* **284**: 5.
- Lehmann L, Perrin N. 2003.** Inbreeding avoidance through kin recognition: choosy females boost male dispersal. *American Naturalist* **162**: 638–652.
- Marr AB, Arcese P, Hochachka WM, Reid JM, Keller LF. 2006.** Interactive effects of environmental stress and inbreeding on reproductive traits in a wild bird population. *Journal of Animal Ecology* **75**: 1406–1415.
- Mathieu E, Autem M, Roux M, Bonhomme F. 1990.** Epreuves de validation dans l'analyse de structures génétiques multivariées: comment tester l'équilibre panmictique? *Revue de Statistique Appliquée* **38**: 27.
- Menezo YJR, Silvestris E, Dale B, Elder K. 2016.** Oxidative stress and alterations in DNA methylation: two sides of the same coin in reproduction. *Reproductive Biomedicine Online* **33**: 668–683.
- Mongue AJ, Ahmed MZ, Tsai MV, de Roode JC. 2015.** Testing for cryptic female choice in monarch butterflies. *Behavioral Ecology* **26**: 386–395.
- Mulard H, Danchin E, Talbot SL, Ramey AM, Hatch SA, White JF, Helfenstein F, Wagner RH. 2009.** Evidence that pairing with genetically similar mates is maladaptive in a monogamous bird. *BMC Evolutionary Biology* **9**: 12.
- Naves LC, Cam E, Monnat JY. 2007.** Pair duration, breeding success and divorce in a long-lived seabird: benefits of mate familiarity? *Animal Behaviour* **73**: 433–444.
- Neff BD, Pitcher TE. 2005.** Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. *Molecular Ecology* **14**: 19–38.
- Nieuwenhuis R, te Grotenhuis M, Pelzer B. 2012.** influence.ME: tools for detecting influential data in mixed effects models. *R Journal* **4**: 38–47.
- Okayasu R, Suetomi K, Yu YJ, Silver A, Bedford JS, Cox R, Ullrich RL. 2000.** A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. *Cancer Research* **60**: 4342–4345.
- Parrott ML, Ward SJ, Temple-Smith PD. 2007.** Olfactory cues, genetic relatedness and female mate choice in the agile antechinus (*Antechinus agilis*). *Behavioral Ecology and Sociobiology* **61**: 1075–1079.
- Paul C, Robaire B. 2013.** Ageing of the male germ line. *Nature Reviews Urology* **10**: 227–234.
- Pemberton JM, Ellis PE, Pilkington JG, Berenos C. 2017.** Inbreeding depression by environment interactions in a free-living mammal population. *Heredity* **118**: 64–77.
- Perez GI, Acton BM, Jurisicova A, Perkins GA, White A, Brown J, Trbovich AM, Kim MR, Fissore R, Xu J, Ahmady A, D'Estaing SD, Li H, Kagawa W, Kurumizaka H, Yokoyama S, Okada H, Mak TW, Ellisman MH, Casper RF, Tilly JL. 2007.** Genetic variance modifies apoptosis susceptibility in mature oocytes via alterations in DNA repair capacity and mitochondrial ultrastructure. *Cell Death and Differentiation* **14**: 524–533.
- Pilakouta N, Jamieson S, Moorad JA, Smiseth PT. 2015.** Parental care buffers against inbreeding depression in burying beetles. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 8031–8035.
- Pilakouta N, Smiseth PT. 2016.** Maternal effects alter the severity of inbreeding depression in the offspring. *Proceedings of the Royal Society B: Biological Sciences* **283**: 7.
- Pizzari T, Dean R, Pacey A, Moore H, Bonsall MB. 2008.** The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends in Ecology & Evolution* **23**: 131–140.
- Pizzari T, Løvlie H, Cornwallis CK. 2004.** Sex-specific, counteracting responses to inbreeding in a bird. *Proceedings of the Royal Society B: Biological Sciences* **271**: 2115–2121.
- Pusey A, Wolf M. 1996.** Inbreeding avoidance in animals. *Trends in Ecology & Evolution* **11**: 201–206.
- R Core Team. 2017.** *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.
- Radwan J, Tkacz A, Kloch A. 2008.** MHC and preferences for male odour in the bank vole. *Ethology* **114**: 827–833.
- Reinhardt K. 2007.** Evolutionary consequences of sperm cell aging. *Quarterly Review of Biology* **82**: 375–393.
- Reinhardt K, Siva-Jothy MT. 2005.** An advantage for young sperm in the house cricket *Acheta domesticus*. *American Naturalist* **165**: 718–723.
- Rising JD, Somers KM. 1989.** The measurement of overall body size in birds. *Auk* **106**: 666–674.
- Risopatron J, Merino O, Cheuqueman C, Figueroa E, Sanchez R, Farias JG, Valdebenito I. 2018.** Effect of the age of broodstock males on sperm function during cold storage in the trout (*Oncorhynchus mykiss*). *Andrologia* **50**: 7.
- Roff DA. 2002.** Inbreeding depression: tests of the overdominance and partial dominance hypotheses. *Evolution* **56**: 768–775.
- Rousset F. 2008.** GENEPOP '007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* **8**: 103–106.
- Sheldon BC. 1993.** Sexually transmitted disease in birds – occurrence and evolutionary significance. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* **339**: 491–497.
- Spottiswoode C, Moller AP. 2004.** Genetic similarity and hatching success in birds. *Proceedings of the Royal Society B: Biological Sciences* **271**: 267–272.
- Stutchbury BJ, Morton ES. 1995.** The effect of breeding synchrony on extra-pair mating systems in songbirds. *Behaviour* **132**: 675–690.
- Tan CKW, Pizzari T, Wigby S. 2013.** Parental age, gametic age, and inbreeding interact to modulate offspring viability in *Drosophila melanogaster*. *Evolution* **67**: 3043–3051.
- Tarin JJ, Perez-Albala S, Cano A. 2000.** Consequences on offspring of abnormal function in ageing gametes. *Human Reproduction Update* **6**: 532–549.
- Tinghitella RM, Weigel EG, Head M, Boughman JW. 2013.** Flexible mate choice when mates are rare and time is short. *Ecology and Evolution* **3**: 2820–2831.
- Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. 1998.** Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reproduction* **13**: 1429–1436.

- Vega-Trejo R, Fox R, Iglesias-Carrasco M, Head M, Jennions M. 2019.** The effects of male age, sperm age and mating history on ejaculate senescence. *Functional Ecology*. doi:10.1111/1365-2435.13305.
- Wagner RH, Helfenstein F, Danchin E. 2004.** Female choice of young sperm in a genetically monogamous bird. *Proceedings of the Royal Society B: Biological Sciences* **271**: S134–S137.
- Welke K, Schneider JM. 2009.** Inbreeding avoidance through cryptic female choice in the cannibalistic orb-web spider *Argiope lobata*. *Behavioral Ecology* **20**: 1056–1062.
- White J. 2008.** *Stratégies d'accouplement et conflits sexuels dans un contexte monogame: Le cas de la Mouette tridactyle Rissa tridactyla*. Paris: Université Pierre et Marie Curie.
- White J, Mirleau P, Danchin E, Mulard H, Hatch SA, Heeb P, Wagner RH. 2010.** Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecology Letters* **13**: 1515–1524.
- White J, Wagner RH, Helfenstein F, Hatch SA, Mulard H, Naves LC, Danchin E. 2008.** Multiple deleterious effects of experimentally aged sperm in a monogamous bird. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 13947–13952.
- Zubkova EV, Robaire B. 2006.** Effects of ageing on spermatozoal chromatin and its sensitivity to in vivo and in vitro oxidative challenge in the Brown Norway rat. *Human Reproduction* **21**: 2901–2910.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Material 1. Analyses of the relationship between genetic similarity and control ring wear duration on fitness.