Fine-scale dynamics of competitive fertilisation in social groups of red junglefowl (*Gallus gallus*) shed new light on avian sperm competition

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Studies of birds have made a fundamental contribution to elucidating sperm competition processes, experimentally demonstrating the role of individual mechanisms in competitive fertilisation. However, the relative importance of these mechanisms and the way in which they interact under natural conditions remain largely unexplored. Here, we conduct a detailed behavioural study of freely-mating replicate groups of red junglefowl, *Gallus gallus*, to predict the probability that competing males fertilise individual eggs over the course of 10-day trials. Remating frequently with a female and mating last increased a male's probability of fertilisation, but only for eggs ovulated in the last days of a trial. Conversely, older males, and those mating with more polyandrous females, had consistently lower fertilisation success. Similarly, resistance to a male's mating attempts, particularly by younger females, reduced fertilisation probability. After considering these factors, male social status, partner relatedness and the estimated state of a male extragonadal sperm reserves did not predict sperm competition outcomes. These results shed new light on sperm competition dynamics in taxa such as birds, with prolonged female sperm storage and staggered fertilisations.

1. Introduction

Parker (1970) [1] revolutionized sexual selection theory by proposing that when females obtain sperm from multiple males (polyandry), competition among males continues after mating as their ejaculates compete to fertilise a set of ova, a process which became known as sperm competition [2-9]. While Parker's (1970) intuition was inspired by insects, the realization that polyandry is ubiquitous among sexually-reproducing organisms and often intense [10] has catalysed the study of sperm competition across different taxa [11-13]. An increasing number of studies have sought to experimentally isolate the causal effect of individual sperm competition mechanisms on male reproductive success by quantifying the overall share in paternity at a particular point in time, e.g. the end of a reproductive cycle. This research has been instrumental in identifying male- [4,6,7,13-18] and female-driven [19,20] mechanisms underpinning paternity share under controlled conditions.

In natural populations however, multiple variables act simultaneously and interact with each other to determine the outcome of sperm competition. Understanding these interactions is particularly challenging for many internally-fertilising taxa where prolonged female sperm storage increases the temporal window for sperm competition [21,22] and generates opportunity for complex patterns of sperm storage and precedence [23]. For example, in the fruit fly, *Drosophila melanogaster,* multiple mechanisms including passive sperm release from the female storage organs, active displacement of resident sperm by the second-male sperm, and female-controlled sperm ejection, lead to approximately 80% of eggs being fertilised by the second male to copulate with doubly-mated females [24,25]. An understanding of the temporal dynamics of competitive fertilisation in natural settings remains elusive however, largely due to the difficulty in disentangling multiple mechanisms occurring within the female reproductive tract. Addressing this outstanding challenge is fundamental to resolving the operation of sexual selection and

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related processes, such as alternative mating strategies, sexual conflict and the maintenance of genetic variation in natural populations [5,26,27].

Historically, birds have represented a key vertebrate model system for the empirical study of sperm competition [12,28-30], and a crucial counterpoint to studies of insects [1,13,24,25,31-34]. For example, the juxtaposition of avian and insect sperm competition dynamics has helped clarify the differential roles of sperm size and numbers in these clades [35]. The importance of birds is in part due to field studies of extra-pair copulation and extra-pair paternity in socially monogamous species [8,12,29,36-39] and the wealth of information on the reproductive biology of the domestic fowl, *Gallus domesticus*, and other domestic galliforms, which have informed the development of mechanistic models of sperm competition [30,40,41]. As in most other species of birds, female fowl typically ovulate one egg on each successive day of the laying cycle, with fertilisation occurring shortly after ovulation [42]. Females store viable sperm in epithelial invaginations at the utero-vaginal junction of the oviduct - the sperm storage tubules (SSTs) - for up to approximately two weeks [40-43]. During this time, sperm are thought to be passively lost from SSTs at a near constant rate, and sperm from competing ejaculates mix randomly within individual SSTs. Passive sperm loss combined with random sperm mixing within SSTs predicts that – all else being equal – the last male to copulate with a female should retain a fertilisation advantage for subsequent eggs, which is proportional to the time elapsed between competing inseminations [30,41]. The extent of this advantage is modulated by a range of factors. For example, shortly before and after oviposition, the fertilisation success of an ejaculate is reduced due to oviduct contractions associated with egg laying (reviewed in [30]). Moreover, studies of domestic populations, which experimentally controlled for insemination order through the simultaneous artificial insemination of two competing ejaculates, have demonstrated that relative differences in either sperm numbers [44] or fertilising efficiency [45,46] explain variation in paternity share between two competitors. Subsequent artificial insemination work in which both sperm number and fertilising efficiency (mobility) were experimentally manipulated has provided evidence of temporal dynamics in a population of domestic fowl: differences in sperm numbers predicted the paternity of eggs produced shortly following insemination, while differences in mobility predicted paternity later in the laying sequence [47]. Collectively, these experimental studies form the basis of our operational understanding of the mechanisms of avian sperm competition, and suggest that the probability that a given male fertilises a particular egg is determined by the number of other competitors inseminating the same female, the time elapsed between these inseminations and ovulation, and the size and fertilising efficiency of the inseminations of this male in relation to those of his competitors, broadly confirming patterns identified by previous work [48]. However, the extent to which this view adequately captures sperm competition dynamics in natural bird populations remains unknown. Some studies of natural populations have shown that the number of sperm reaching an egg and paternity share can change systematically over the course of a laying cycle in some species [49-56]. Other studies however, have failed to confirm these results [54,56], or found less conclusive [57] or more complex patterns [58,59]. A fundamental source of inconsistency is that most studies of free-ranging groups typically lack information about individual mating events, which makes it impossible to assess the level of sperm competition associated with the fertilisation of individual ova. In the current study we aim to address this knowledge gap by combining fine-grained information on socio-sexual interactions and parentage of individual zygotes in replicate flocks of red junglefowl, *G. gallus*, the main species that has given rise to the domestic fowl.

In nature, red junglefowl live in polygynandrous groups, socially structured in sex-specific hierarchies [60]. Recent work indicates that variance in paternity share is the largest source of variance in male reproductive success, indicating the importance of sperm competition and post-copulatory sexual selection in these populations [61,62]. Sperm competition favours males that remate with the same female at a high frequency [61,62], and a similar advantage derived by frequent remating with the same female has been reported for some wild bird species [63] and in other taxa, e.g. some insects [64-66]. Mating in fowl is strongly modulated by the interaction of female and male behaviours. Studies of domestic fowl and red junglefowl indicate that while in small, strongly female-biased groups mating is largely driven by females, in larger groups and groups with a higher proportion of males, the majority of mating attempts is initiated by males [60]. However, differential female behaviour plays an important role in determining the outcome of these male mating attempts. For example, females can influence the success of an insemination by differentially resisting copulation attempts, manipulating male-male competition and copulation interference, and by ejecting semen after insemination [60]. These mechanisms can be modulated by characteristics of individual males and females, and their interactions. For example, male social status mediates competitive access to mates and mating opportunities, and is favoured by female responses before and after mating [60]. On the other hand, male status may be negatively related to sperm fertilising efficiency [46,67], as observed in species with more distinct alternative mating tactics [68,69], suggesting a possible trade-off between male investment in pre- *vs.* post-copulatory intrasexual competition. Similarly, male age has been linked to lower fertilisation success, as older males transfer fewer sperm than younger males [70,71]. Moreover, sperm of older males tend to swim more slowly, possibly due to lower levels of seminal antioxidants [72]. Female age may also affect the outcome of sperm competition. On the one hand, older females are less polyandrous and avoid males more often than younger females [73], and have a faster rate of sperm loss from the SSTs than younger females [74]. On the other, resistance to males and male coercion are costly [75] and older females might be less able to resist male attempts. Finally, genetic relatedness between a male and a female may influence fertilisation success. Experimental evidence suggests that red junglefowl females favour sperm from unrelated males after mating, possibly in order to reduce the risks of inbreeding depression [76,77], a pattern observed in some other taxa [20]. Despite this experimental work however, we currently lack an understanding of how these mechanisms collectively influence sperm competition dynamics in social groups of freely-interacting birds.

Here, we study mechanisms underpinning fowl sperm competition by using detailed mating behaviour and parentage data of freely-mating replicate groups of a captive population of red junglefowl. This dataset has been previously analysed to investigate the relationship between male phenotypes and overall male reproductive success [62], the role of female sociality on the structure of sociosexual networks [73], and changes in female and male phenotype associated with mating trials [75]. This earlier work identified a role for both male and female behaviours in determining variation in individual reproductive success [62,73,75]. The current study consolidates and builds on this earlier work, with a drastically different analytical approach which investigates day-to-day variation in the probability of fertilisation of individual eggs laid over successive days of the mating trial. By doing so, the present study moves beyond previous analyses of variation in overall reproductive success and develops a novel methodological platform to examine the relative importance of different factors dynamically through time. We predicted that males that remate more often and those that copulate last with a female will have a higher probability of fertilising an egg. Based on the passive sperm loss model [30], we predicted that the last male advantage should increase with the time elapsed between competing inseminations. We further predicted that probability of fertilisation declines with male age, the level of female resistance (which we predicted to be weaker in older females), the relatedness of the male, the number of males mating with the female, and the depletion of a male’s extragonadal sperm stores through previous copulations. Male status might have a positive effect, e.g. through preferential female sperm retention, or a negative effect, through a possible trade-off with fertilising efficiency.

2. Methods

We studied a population of red junglefowl at the John Krebs Field Station of the University of Oxford. We analysed the data collected in 20 mating trials conducted in 2011-2013. Detailed accounts of the protocol of the mating trials have been published elsewhere [62,73,75]. Briefly, in each mating trial one of us (GCM) observed a group (males=10, females=12) for 3h in the morning and 3h in the evening on each of 10 consecutive days (day1-10). We recorded all male and female mating related behaviours (males: successful and unsuccessful mating attempts, courtship waltzes; females: mating solicitations, level of resistance to mating attempts [78]), and male agonistic interactions (pecks, fights, aggressive waltzes and avoidances). Eggs (n=982) were collected daily from the second day of the trial (day2) to the day following the end of the trial (day11), artificially incubated for 5-9 days, and parentage of the embryos was assigned molecularly [62].

We analysed the effect of multiple predictor variables on the probability of fertilisation of individual eggs by competing males, which were defined as all males that mated successfully with the female up to, and including, the day before the laying of an egg. Thus, copulations on the day an egg was laid were not considered as competing for the fertilisation of that egg, because it is unlikely that sperm from these inseminations could have reached the egg in time for fertilisation [30,40,42]. We considered the following predictor variables (i.e. fixed effects, see electronic supplementary material for fuller discussion): i) number of male competitors, ii) remating rate (the frequency at which a male mates with the same female up until the day prior to egg-laying), iii) mating order, iv) male social status (calculated using Elo scores [79]), v) male age, vi) average female resistance to mating attempts of the focal male (scored following [78]), vii) female age, viii) male-female relatedness (calculated using Wang’s estimator [80,81]), ix) an index of the male extragonadal sperm reserves (MSR, Figure S1), and x) time (i.e. the day of the mating trial when the egg was laid).

(a) Model comparison

All analyses were conducted in R v3.6.2 [82], using the “lme4” v1.1-21 package [83]. We built a generalized linear mixed model (GLMM) with binomial distribution and logit function with the fertilisation outcome of each egg (unsuccessful males=0, successful male=1) as the response variable, predictors (i-ix) and their interaction with time (x) as fixed effects (mod13, Tables 1, S1). We used relative values of a predictor variable (i.e., the value of each male divided by the mean value of all males competing for that particular egg), to standardise a male’s value in relation to his direct competitors and to avoid conversion problems due to large scaling differences among predictors. Mating order (iii) and relatedness (viii) were not standardised because already expressed in relative terms. Collinearity across predictors was calculated using variance inflation factors (VIFs) implemented in the package “car” v3.0-3 [84]. All predictors showed

VIFs<3 and were therefore retained [85]. We compared this model (mod13) against simpler models with fewer predictors using the Akaike's information criterion (AIC [86]), such that a difference (ΔAIC) < 2 was interpreted as no difference of fit between models, $2 \leq \Delta$AIC < 4 as a difference of fit, $4 \leq \Delta$AIC < 10 as a considerable difference of fit, and ΔAIC > 10 as a strong preference for one model over the other [87]. Overall, we defined 44 models *a priori* (Table S1) using different combinations of predictors, and compared them simultaneously using the package "AICcmodavg"v2.2-2 [88]. All 44 models also included male identity, female identity, female group identity (i.e. the identity of the unique unit of 12 females used in each trial [42]), and mating group identity (i.e., the identity of the unique group of 10 males 12 females used in each trial [42]) as random effects to account for sources of non-independence [42]. We quantified the variance explained by each model as marginal R^2 (R^2GLMM(m)), i.e. the variance explained by fixed effects alone, and conditional R^2 (R^2GLMM(c)), i.e. the variance explained by the combination of the fixed and random effects [89,90]. Finally, while a male's remating rate (predictor (ii)) reflects his sperm investment in a female, it does not take into consideration that sperm are passively lost from the female SSTs over time. Therefore, we also constructed GLMMs replacing (ii) with an estimated value of a male's sperm numbers in the female SSTs at the time of fertilisation (female sperm reserves), which was calculated using the rate of sperm loss described for domestic fowl [91,92] (electronic supplementary material, Figure S2). We compared AIC scores between models using (ii) against models replacing (ii) with female sperm reserves (Table S2).

(b) The effect of individual predictors

We tested the statistical significance of individual predictors in the most complex model (mod13, Tables 1,S1), by performing likelihood ratio tests (LRTs) removing each variable of interest while holding constant the effect of all other predictors. Interaction terms that were not significant were dropped before conclusions were drawn on the significance of the main effects. To further confirm temporal patterns, we explored the effect of individual predictors (i-ix) on fertilisation probability, by running separate GLMMs for each individual day of the trial. Results were qualitatively similar to the original approach (see Table S3, Figure S4).

3. Results

Models including female sperm reserves had lower AIC scores than equivalent models with remating in 11 cases (ΔAIC≥6.46, Table S2). In the remaining cases (n=15), mostly when time was included in the model as an interaction term, both models had similar AIC scores (ΔAIC≤2.18, Table S2). This suggests that female sperm reserves captures a temporal dimension of sperm competition, and becomes redundant when time is included in the model explicitly. Therefore, we only report models with remating rate below, as this approach does not rely on additional assumptions of patterns of sperm loss from the SSTs.

(a) Model comparison

Using an AIC approach, the best model predicting the fertilisation probability of individual eggs (mod1, Table 1,S1) included a male's remating rate and its interaction with time (i.e. day of the trial), male mating order and its interaction with time, number of competing males, male age, and the interaction between overall female resistance and female age. Male-female relatedness, his sperm reserves (MSR) and male social status were not included in the best model. The best model was only slightly better than the model without the interaction between female resistance and female age (mod2, ΔAIC=2.86, Table S1), but performed considerably better than all other models (ΔAIC>4, Table S1).

The best model explained 14.98% of the variance by fixed effects alone (i.e., R^2GLMM(m)), and 19.59% when random effects were included (i.e. R^2GLMM(c)). When considering R^2GLMM(m) for models including only a single predictor, the model with number of competing males explained the most variance (5.5%, mod24, Tables 1,S1) in the probability of fertilisation. The model with female resistance (mod30, Tables 1,S1) explained 2.05%, male age (mod38, Tables 1, S1) explained 1.89%, remating rate (mod33, Tables 1,S1) explained 1.12%, male social status (mod39, Tables 1,S1) explained 0.14%, and mating order (mod41, Tables 1,S1) explained 0.04% of the variance in fertilisation success, respectively. The remaining two predictors, relatedness and MSR, explained each 0.02% (mod43, mod44, Tables 1, S1) of the variance.

(b) The effect of individual predictors

Results of the model including all main effects and the statistically significant interactions are summarised in Table 2.

We found a positive and significant interaction between a male's remating rate and time, such that males that copulated more often with the female had a higher probability of fertilisation later in the trial (remating rate*time: χ^2_1=5.72, p=0.016, Figure 1a). Similarly, males that tended to mate last with a female had a higher probability of fertilising eggs laid later in the trial (mating order*time: χ^2_1=13.17, p<0.001, Figure 1b), as predicted by the passive sperm loss model. Such last male advantage later in a trial may be caused by increasing variation in the time elapsed between the last insemination by competing males and fertilisation. We explored this hypothesis by testing post-hoc whether the time interval (gap) between the last insemination by the first and last competitor male in the mating order tended to increase over successive days in the trial. Consistent with expectations the time gap spanning the mating order increased over the course of the trial (Time gap~mating order*time: χ^2_1=98.85, p<0.001, Figure S5), suggesting that some males that mate with a female early in a trial are disadvantaged in sperm competition later in the trial as they fail to remate with the female.

Males that experienced more intense female resistance across copulation attempts were significantly less likely to fertilise eggs overall (resistance: χ^2_1=101.82, p<0.001), and this effect was constant over time (resistance*time: χ^2_1=1.99, p=0.159), but was dependent on female age. Males were less likely to fertilise when resisted by younger females (resistance*female age: χ^2_1=4.86, p=0.027, Figure 1c). Competing against more males decreased a male's probability of fertilisation (competitors: χ^2_1=193.18, p<0.001, Figure 1d), but there was no evidence that this effect varied over the course of a trial (competitors*time: χ^2_1=0.24, p=0.628). Similarly, older males had a lower probability of fertilisation overall (male age: χ^2_1=51.43, p<0.001, Figure 1e), which was consistent throughout the course of a trial (male age*time: χ^2_1=0.46, p=0.496).

The state of a focal male's sperm reserves (MSR) did not increase his overall probability of fertilisation (MSR: χ^2_1=0.42, p=0.515, Figure 1f), and this relationship did not change over time (MSR*time: χ^2_1=0.26, p=0.614). Similarly, a focal male's relatedness with the female did not predict fertilisation probability (relatedness: χ^2_1=0.64, p=0.423, Figure 1g), and this pattern did not change over time (relatedness*time: χ^2_1=0.16, p=0.694). Finally, social status was not associated with a focal male's probability of fertilisation (Elo score: χ^2_1=0.01, p=0.936, Figure 1h), and this pattern did not change over time (Elo score*time: χ^2_1=0.31, p=0.580). Male identity explained almost all the variance of random effects (Figure S3).

4. Discussion

Parker's intuition of sperm competition [1] kickstarted a new field of research, which has exploded over recent decades [8-10]. This effort has elucidated the independent role of numerous mechanisms including physiological, genetic, behavioural and morphological traits in determining competitive fertilisation. Despite this, we still have a poor understanding of how these mechanisms interact with each other to drive dynamics of sperm competition in natural populations. This is particularly the case for the many taxa, such as birds, with internal fertilisation, prolonged female sperm storage and iterated insemination and fertilisation events, mostly due to the difficulty in linking mating to fertilisation and following temporal patterns of ejaculate utilisation. In this study we combined detailed information on mating behaviour with molecular parentage data from groups of red junglefowl to explain variation in the probability of individual males fertilising individual eggs over successive days. We show that around 20% of the variance is explained by information gleaned from mating behaviour data. The degree of female polyandry has an intuitive negative impact on a male's probability of fertilisation, however the outcome of sperm competition among males is modulated by four other factors.

First, a male is more likely to fertilise a female's eggs - especially those ovulated in the last days of a trial - if he mates more frequently with her. Previous work had shown that post-copulatory sexual selection favours males that remate more frequently with their partners in this population [61,62], but there has been no attempt at quantifying temporal effects. Repeated matings with between partners are often observed in birds and the significance of this behaviour has long been debated [93]. Because females can continue to ovulate over successive days following an insemination, and because sperm are lost at a constant rate from the female sperm storage tubules (SSTs), male birds may be selected to top up their sperm representation in a female's SSTs by remating rather than inseminating a single large ejaculate. In addition, we now show that this effect is progressively accentuated over successive days; multiple days of remating are required for males to accumulate sufficiently more sperm than rivals within the female SSTs in order to have an advantage in sperm competition. Positive post-copulatory sexual selection on remating rates has also been demonstrated in some insects [64,66], although in these studies the advantage to remating may be related to mating order, as males that mate more frequently with a female may be more likely to mate last and displace the sperm previously inseminated by rivals. Thus, in polyandrous populations characterised by a degree of sperm competition intensity [61,62], selection may favour males that mate with fewer females, but are able to remate with these females more frequently [94].

Second, controlling for remating rates, we found an interaction between mating order and time; such that mating last increases the fertilisation probability of males late in a trial. Establishing the effect of mating order is critical to evaluate different models of sperm competition. A last male advantage is commonly observed in a number of taxa [13]. In birds, different sperm competition models have been explored [29,30,41]. First, sperm from different inseminations may stratify within the female's SSTs, with sperm from later copulations "sitting" on top of sperm from earlier copulations. Second, a new insemination may displace sperm of previous inseminations from the female SSTs. Finally, sperm from earlier inseminations may be lost over time due to passive sperm loss from the SSTs [92,95]. All three models predict some degree of last male advantage in sperm competition, but only the passive sperm loss model predicts that this advantage increases with the time elapsed between competing inseminations [92,95]. Despite early suggestions of sperm stratification in individual SSTs [96], analysis of artificial insemination experiments in domestic fowl indicated that avian sperm competition may conform to passive sperm loss [29,30,41,92,95]. A subsequent careful differential sperm staining experiment in domestic fowl and domestic turkeys, *Meleagris gallopavo*, found no evidence of sperm stratification [97], with sperm of rival inseminations largely segregated in different SSTs in both species, although some of visual the patterns presented raised the possibility of sperm stratification when rival inseminations mixed within individual SSTs (e.g. figure 2c). The sperm stratification model predicts that the first insemination may experience a renewed increase in fertilisation probability later in the laying sequence, as the sperm from subsequent inseminations are depleted allowing the sperm from the first insemination to leave the SSTs. Our post-hoc analysis showed that the time lag between the last copulations of competing males tended to increase over successive days of the trial, which is consistent with passive sperm loss but less so with sperm stratification. This suggests that most males mate early with a female but only some manage to remate with her later on in the trial. Mating later in the trial provides these males with a fertilisation advantage as the sperm of previous competitors will have been depleted from a female's SSTs through passive loss. Mating last in the first days of the trial does not convey the same advantage, instead our results suggest that, early in the trial, mating first might be advantageous. This conclusion is also supported by our attempts to model sperm loss directly, which yielded considerably better fits than simply modelling remating rates in almost half of the models. Importantly, segregation of rival ejaculates across different SSTs may be a common pattern observed in galliform [97] and passerine birds [98,99]. Random occupation of different individual SSTs by rival ejaculates would likely result in an overall pattern of sperm loss similar to that predicted under conditions of random sperm mixing within individual SSTs [6]. Non-random segregation patterns however, might buffer or accentuate patterns of paternity share predicted by the passive sperm loss model. For example, last male sperm precedence might be favoured if sperm from the last insemination were preferentially stored in the SSTs closer to the influndibulum, which mature later, resulting in a possible fertilising advantage [98]. The lack of remating by many males may occur for two non-mutually exclusive reasons. i) It may take some time for the social hierarchies to influence differential access to mating opportunities, and mating later in the trial may become increasingly difficult for low-ranking males, as dominant males progressively monopolise females [60]. Consistent with this, dominant males were more likely to fertilise eggs on the 8th day of the trial (see Analysing each day of the trial separately in the electronic supplementary material). ii) Mating can be costly for males in these groups [75], and only some males (e.g. younger and in better condition) may be able to maintain high mating rates throughout the trial. These findings highlight the importance of accounting for the temporal dynamics underpinning sperm competition in species with prolonged sperm storage.

Third, our results suggest that female resistance may impair the fertilisation probability of successful mating attempts by compromising sperm transfer. Male fowl are on average larger than females, and frequently coerce females into copulating [60], which can undermine female pre-copulatory choice. Female responses to male harassment in fowl range from acquiescence to costly resistance [60]. Previous work in feral domestic fowl has shown that female resistance reduces the probability that a copulation attempt leads to successful sperm transfer [100]. Another possibility is that females are more likely to eject sperm following resisted copulations. Previous studies have demonstrated that female fowl eject sperm differentially [101,102], and the risk of sperm ejection increases over successive matings [101]. Female resistance is common in birds and can be intense in some species [28,103], however its influence on the reproductive outcome has seldom been quantified. In mallards, *Anas platyrhynchos*, female resistance reduces the probability that a mating attempt leads to successful copulation [104], while experiments in Japanese quails, *Coturnix japonica*, indicate that males that are more aggressive to females have a lower fertilisation probability [105]. Early models of sperm competition ignored female behaviour. Our results indicate that female behaviour is a key factor in determining the outcome of sperm competition and paternity share, even in species characterised by widespread male sexual coercion. Importantly, we show that the effect of female resistance decreased with female age, suggesting that older females are less effective at dislodging harassing males, or are less able to bias the outcome of sperm competition against their sperm (e.g., through differential sperm ejection [101,102]). Our results suggest that on the one hand, it may be easier for males to coerce older partners, which may be less efficient at resisting, while on the other, mating with older females may result in lower reproductive returns given their reduced fecundity and faster sperm loss rates [74].

Fourth, older males have reduced fertilisation probability for individual eggs, confirming the overall effect of male age on male reproductive success previously reported for these trials [62]. In addition, we now show that this negative effect occurs constantly over the course of a trial, suggesting a consistent detrimental effect of ageing on male ability to compete in sperm competition. These findings are consistent with previous studies of domestic fowl showing that older males have lower sperm quality [72], and fail to transfer sperm more often, or transfer smaller ejaculates, than younger males [70,71]. Interestingly, several studies of extra-pair paternity in natural passerine populations have detected the opposite effect, i.e. older males tend to cuckold younger males and sire more extra-pair offspring [106-109]. This incongruence may reflect differences between fowl and passerine birds, between the polygynandrous and socially monogamous mating systems, or between captive and natural populations. While the effect of phylogenetic and mating system differences are harder to assess, it is likely that high extrinsic mortality rates in natural populations mean that males seldom age beyond their prime, and male age in these conditions may positively covary with experience and genetic quality. Low extrinsic mortality rates in captive populations on the other hand, allow individuals to age beyond their prime, revealing a stronger impact of reproductive ageing. A study of captive house sparrow, Passer domesticus, however, shows that old males (>6 years) can deliver more sperm than young males [110], suggesting that captivity effects may not be sufficient to explain differences in the role of male age in sperm competition between socially monogamous passerines and polygynandrous galliforms. While male reproductive ageing has long been recognised in several populations [111-113], its role in sperm competition dynamics has only recently begun to emerge [114]. Our findings indicate that male age is a key determinant of paternity share in sperm competition in age-structured bird populations and suggest that females could minimise fitness costs associated with mating with older males by promoting sperm competition [112].

Finally, our study suggests that factors previously identified as important determinants of sperm competition outcomes may play a more negligible role in freely-mating groups. A male’s relatedness to a female, his social status, and the state of his sperm reserves (measured as MSR) were not significant predictors of his fertilisation probability. Individuals are expected to avoid reproducing with close relatives to prevent inbreeding depression [115-117]. Theory predicts that asymmetries in reproductive costs mean that, for intermediate levels of inbreeding depression, females should avoid fertilisation by close relatives more than unrelated males [117]. In line with this, previous experimental studies in fowl have shown that males are as likely to mate with full-sib sisters as with unrelated females [76], whereas females may counteract this by favouring the sperm of unrelated males [76,77], a pattern that has been also found in other taxa [20,118-124, but see 125-127]. While previous studies on post-copulatory inbreeding avoidance have typically used highly controlled experiments with two rival males that differ markedly in their relatedness to the female (e.g., unrelated *versus* full sibling), studies of natural populations are scarcer (e.g., [123]). It is possible that in the freely interacting groups of the present study, inbreeding risk may be reduced largely at a pre-copulatory stage by mating with less related partners. Additionally, post-copulatory inbreeding avoidance may be captured by other mechanisms, such as female resistance, included in the models and/or may have a negligible independent effect on fertilisation probability compared to other factors. Similarly, the lack of an effect of social status on male fertilisation success in the present results also contrasts with previous studies suggesting that dominant male fowl are favoured in post-copulatory sexual selection [61] and that females tend to retain more sperm from dominant males [101,102]. One possibility is that the reproductive advantage associated with dominance in fowl and other species [128,129] could be driven mostly by traits, such as younger male age, lower female resistance and higher remating rates [61,62,66], which were directly included in our models, or pre-copulatory strategies such as mate-guarding [130,131]. Alternatively, the benefits of social dominance may be cancelled out if subordinate males produce ejaculates of higher fertilising efficiency [45,46]. Future studies should disentangle potential positive and negative consequences of status for paternity share variation in polyandrous populations. While our groups were not assembled systematically with respect to male age, social competitive ability, and male-female relatedness, future studies could manipulate group composition experimentally to resolve the causal role these factors in sperm competition, while maintaining a more realistic setting. Exploring additional factors such as the role of sperm fertilising efficiency (e.g. measures of sperm motility [45,46]) will also be important in improving our understanding of sperm competition in bird populations.

Birds have provided a model system of vertebrate sperm competition. In studying sperm competition in socially monogamous bird species, Birkhead [48] identified four predictors of the probability of extrapair paternity: (1) the relative timing and success of insemination by competing males, (2) the relative remating rates by competing males, (3) the duration of female sperm storage, and (4) patterns of sperm precedence. Our study has provided evidence that all these factors are important in sperm competition dynamics in polyandrous social flocks. We have provided corroborating evidence of passive sperm loss under more natural conditions, and identified a role for both male and female processes in determining the probability that competing males fertilise individual eggs. Crucially, our results show that the temporal dependency of these effects should be taken into consideration when studying avian sperm competition. Nevertheless, approximately 80% of the variance in probability of fertilisation remained unexplained, demonstrating that much work is still needed in order to understand complex patterns of sperm competition in nature. We hope this paper provides a methodological platform for future studies. While the need for behavioural information to study sperm competition in wild birds has long been recognised [36], this has been notoriously difficult to obtain. Recent advances in tracking technologies however, are beginning to yield behavioural data of unprecedented high-resolution for wild animals (e.g., [132]), and we foresee exciting opportunities to understand dynamics of sperm competition and their eco-evolutionary significance in natural populations.

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Tables

Table 1. Comparison of selected binomial generalized linear mixed models (GLMMs), predicting the probability male red junglefowl, *Gallus gallus*, fertilise individual eggs, ranked according to AIC. For all models see Table S1.

Model ID	Fixed effects	K	AIC	ΔAIC	R^2_m	R^2_c
mod1	RR*T + MO*T + C + A + FR*FA	15	3621.33	0	0.1498	0.1959
mod13	RR*T + MO*T + C*T + A*T + FR*FA + SS*T + MSR*T + R*T	23	3633.24	11.92	0.1460	0.1917
mod24	C	6	3851.02	229.69	0.0550	0.1318
mod30	FR	6	3968.72	347.4	0.0205	0.1355
mod33	RR	6	3997.33	376.03	0.0112	0.1168
mod38	A	6	4016.99	395.66	0.0189	0.0907
mod39	SS	6	4040.27	418.95	0.0014	0.1097
mod41	MO	6	4041.31	419.98	0.0004	0.1089
mod43	R	6	4042.1	420.78	0.0002	0.1107
mod44	MSR	6	4042.24	420.91	0.0002	0.1076

A=Male age; C=Number of competitors; FA=Female age; FR=Female resistance; MO=Mating order; MSR=Male sperm reserves; R=Relatedness; RR=Remating rate, SS=Social status; T=Time; K=Number of parameters; AIC=Akaike information criterion; ΔAIC=with respect to mod1; R^2_m=Marginal R^2; R^2_c=Conditional R^2.

Table 2. Summary statistics and likelihood ratio tests results of the generalized linear mixed-effects model (GLMM) predicting egg fertilisation success in multiple replicate groups of red junglefowl, *Gallus gallus*. Model contains all main effects and statistically significant interactions (mod5, Table S1).

	Egg fertilisation success			
Predictors	*Estimate (odds ratio)*	*Confidence interval*	*LRT*	*p*
Intercept	30.09	12.19–74.25	-	-
Remating Rate (RR)	.93	.61–1.42	-	-
Time (T)	.82	.75–.89	-	-
Mating Order (MO)	.92	.80–1.05	-	-
Female Resistance (FR)	.12	.07–.21	-	-
Female Age (FA)	.85	.70–1.02	-	-
Number of Competitors (C)	.65	.61–.69	193.18	**<.001**
Male Age (A)	.44	.35–.55	51.43	**<.001**
Relatedness (R)	.88	.64–1.20	.64	.423
Social Status (SS)	.99	.73–1.33	.01	.936
Sperm reserves (MSR)	1.08	.86–1.36	.42	.515
MO:T	1.08	1.01–1.16	13.17	**<.001**
RR:T	1.04	1.02–1.06	5.72	**.018**
FR:FA	1.23	1.02–1.49	4.88	**.027**
Random Effects				
σ^2	3.29			
$\tau_{00\ \mathrm{MaleID}}$	0.35			
$\tau_{00\ \mathrm{FemaleID}}$	0			
$\tau_{00\ \mathrm{femalegroupID}}$	0			
$\tau_{00\ \mathrm{groupID}}$	0			
Observations	4134			

Notes: LRT=Likelihood ratio test; σ^2=residual variance; τ_{00}=random intercept variance.

Figure and table captions

Figure 1. Relationships between different predictors and the probability that competing males fertilise individual eggs in semi-natural groups of red jungle fowl, *Gallus gallus*. Panels represent the marginal effects of a predictor based on the generalised linear mixed model (GLMM) in Table 2. a) Relative remating rate and its interaction with time in days, b) mating order and its interaction with time in days, c) relative female resistance and its interaction with female age in years, d) number of competing males, e) relative male age, f) relative male sperm reserves (MSR), g) relatedness between a male-female pair, and h) relative male Elo score (i.e., social status). Shaded areas represent the 95% confidence intervals. Colour coding in panels a-c represents three arbitrary values of the interaction term ((a) and (b) time: day2, day5, day9; (c) female age: 1, 5, 7years old). Asterisks represent statistical significance (Table 2), such that * = $p<0.05$, and *** = $p<0.001$.

Figures

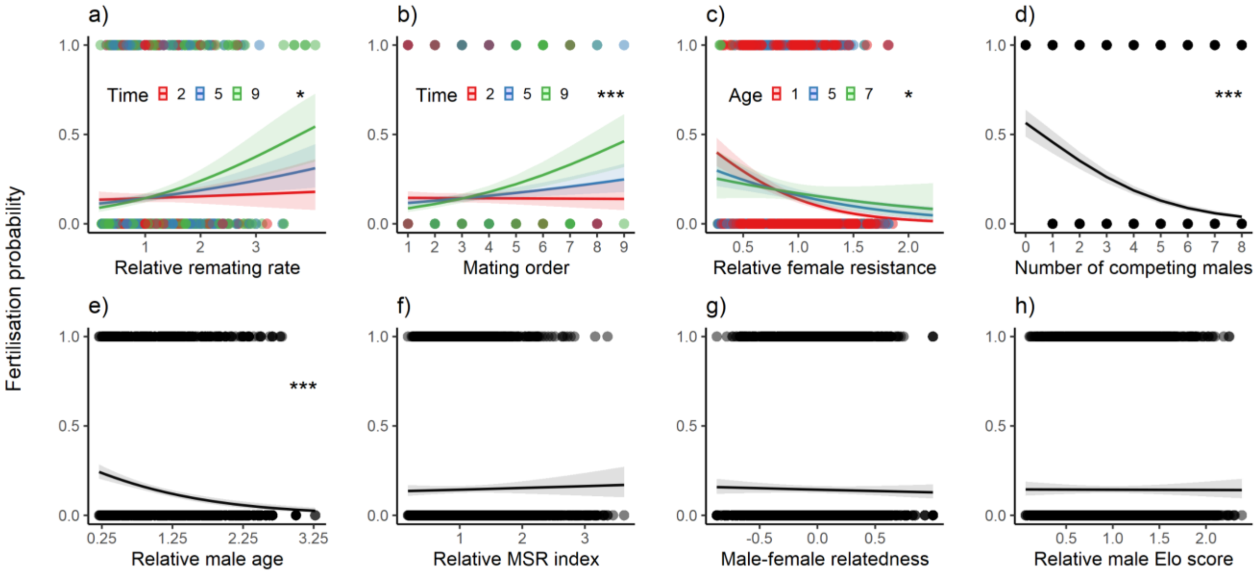

Figure 1

Additional Information

Ethics

Research was conducted according to United Kingdom home office legislation (Home office licenses 30/2418 and 30/2931) following approval by the Departmental Animal Welfare Ethical Review Body (AWERB).

Data Accessibility

We will deposit supporting data and R codes in Dryad Digital Repository on acceptance.

Authors' Contributions

R.C., G.C.M. and T.P. conceived the study. G.C.M. conducted the field work, R.C. analysed the data. L.G. S., E.A.F. and D.S.R. performed molecular analyses for parentage assignment. Y.W. provided unpublished data for Figure S1. R.C., T.P. and G.C.M. wrote the manuscript, and D.S.R. provided comments. All other authors gave final approval for publication.

Competing Interests

We have no competing interests.