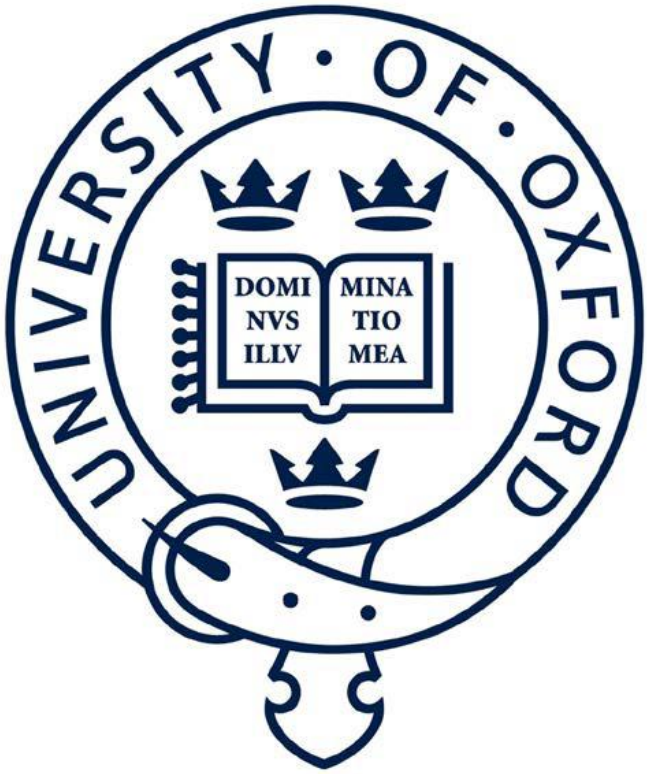
Temporal dynamics of sexual selection in the red junglefowl (*Gallus gallus*)



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CREATOR ineffabilis, qui de thesauris sapientiae tuae tres Angelorum hierarchias designasti et eas super caelum empyreum miro ordine collocasti atque universi partes elegantissime distribuisti: Tu, inquam, qui verus fons luminis et sapientiae diceris ac supereminens principium, infundere digneris super intellectus mei tenebras tuae radium claritatis, duplices, in quibus natus sum, a me removens tenebras, peccatum scilicet et ignorantiam. Tu, qui linguas infantium facis disertas, linguam meam erudias atque in labiis meis gratiam tuae benedictionis infundas. Da mihi intelligendi acumen, retinendi capacitatem, addiscendi modum et facilitatem, interpretandi subtilitatem, loquendi gratiam copiosam. Ingressum instruas, progressum dirigas, egressum compleas. Tu, qui es verus Deus et homo, qui vivis et regnas in saecula saeculorum. Amen.

AVE, augustissima Regina pacis, sanctissima Mater Dei, per sacratissimum Cor Iesu Filii tui Principis pacis, fac ut quiescat ira ipsius et regnet super nos in pace. Memorare, o piissima Virgo Maria, non esse auditum a saeculo quemquam tua petentem suffragia esse derelictum. Ego tali animatus confidentia, ad te venio. Noli, Mater Verbi, verba mea despicere, sed audi propitia et exaudi. O clemens, o pia o dulcis Virgo Maria. Amen.

CREDO quod sis angelus sanctus, a Deo omnipotente ad custodiam mei deputatus. Propterea peto, et per illum qui te ad hoc ordinavit, humiliter imploro, ut me miseram fragilem atque indignam semper et ubique in hac vita custodias, protegas a malis omnibus atque defendas, et cum Deus hinc animam meam migrare iusserit, nullam in eam potestatem daemonibus habere permittas, sed tu eam leniter a corpore suscipias, et in sinu Habrae1 suaviter usque perducas iubente ac iuvante creatore ac salvatore Deo nostro, qui est benedictus in saecula saeculorum. Amen.

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ABSTRACT

Temporal dynamics of sexual selection in red junglefowl (*Gallus gallus*)

DPhil thesis by Rômulo Carleial, St. Catherine's College, submitted Michaelmas Term 2020

Sexual selection has been evoked as a contributing cause of many morphological and behavioural adaptations aimed at increasing an individual's reproductive success. Recent research has begun to unveil the roles of environmental variation, changes in the operational sex ratio, and behavioural plasticity as potential drivers of fluctuating selection in natural populations. Nevertheless, we have just started scratching the surface of the tempo of evolutionary change, and while the mechanisms underpinning different episodes of sexual selection have been increasingly elucidated in the past decades, very little remains known about how these mechanisms are modulated over time. The overarching goal of this thesis therefore, was to explore temporal dynamics in both pre- and post-copulatory episodes of sexual selection by analysing data of socio-sexual interactions and molecular parentage data across replicate flocks of red junglefowl (*Gallus gallus*), an established research model. Specifically, my thesis had four objectives: (i) characterise circadian patterns in mating and courtship behaviour, (ii) quantify phenotypic changes associated with mating and social status over the course of a mating trial, (iii) predict the probability of fertilization success across individual eggs laid over successive days, and (iv) quantify changes in the opportunity for selection over a mating trial. By addressing these objectives, I seek to demonstrate how temporally dependent expression of mating behaviour and post-copulatory biases in fertilization can lead to changes in sexual selection over short-time scales.

The findings reported in my thesis demonstrate potential costs associated with pre-copulatory sexual selection. Males that mate more often and with more females, males that are chased more often by other males, and females that experience more coerced copulations, all tend to lose more body condition over a mating trial. I demonstrate female resistance to mating, and male courtship effort show clear circadian trends, differing between the morning and evening. I found evidence for temporal changes in post-copulatory sexual selection which are partially driven by variation in the relative importance of certain predictors of male fertilization success over successive days of a mating trial. Males that mate last and maintain consistent higher levels of remating with the same female than his competitors, increase the probability they will fertilize eggs laid by this female at the end of a laying sequence. Lastly, I observed sharp temporal changes in the opportunity for pre- and post-copulatory episodes of sexual selection over a mating trial, which demonstrates the importance of accounting for the cumulative sequence of copulations to properly estimate the strength of sexual selection. Moreover, I show that stochastic mechanisms also contribute to changes in variance in reproductive success over time, reinforcing the need for the incorporation of null models in sexual selection research.

In conclusion, this thesis contributes to refine our understanding of how different pre- and post-copulatory mechanisms may lead to temporal changes in sexual selection. Future work should attempt to increase the resolution of data collection by employing modern tracking technologies, and explore the generality of my results by comparisons with species with different mating systems.

Declaration and author contributions

The work presented in this thesis is my own with the following acknowledgements.

Chapter 2 – Circadian patterns in mating behaviour in red junglefowl (*Gallus gallus*)

Authors: Rômulo Carleial*, Grant C. McDonald*, Tommaso Pizzari
Contributions: RC, GCM, and TP conceived the study. GCM collected the data. RC analysed the data and wrote the manuscript. GCM and TP provided comments.
Status: In preparation

Chapter 3 - Dynamic phenotypic correlates of social status and mating effort in male and female red junglefowl, *Gallus gallus*
Authors: Rômulo Carleial*, Grant C. McDonald*, Tommaso Pizzari
Contributions: RC, GCM, and TP conceived the study. GCM collected the data. RC analysed the data. All authors contributed to writing the manuscript.
Status: Published in Journal of Evolutionary Biology

Chapter 4 - Temporal dynamics of competitive fertilisation in social groups of red junglefowl (Gallus gallus) shed new light on avian sperm competition
Authors: Rômulo Carleial*, Grant C. McDonald*, Lewis G. Spurgin, Eleanor A. Fairfield, Yunke Wang, David S. Richardson, Tommaso Pizzari
Contributions: RC, GCM, and TP conceived the study. GCM collected the data. RC analysed the data. LGS, EAF and DSR performed molecular analyses for parentage assignment. YW provided unpublished data for Figure S1. RC, GCM, and TP wrote the manuscript and DSR provided comments.
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Chapter 5 – Towards an appreciation of short-term dynamics of sexual selection in polyandrous populations
Authors: Rômulo Carleial*, Tommaso Pizzari*, Lewis G. Spurgin, Eleanor A. Fairfield, David S. Richardson, Grant C. McDonald
Contributions: RC, GCM, and TP conceived the study. GCM collected the data. RC analysed the data. LGS, EAF and DSR performed molecular analyses for parentage assignment. RC, GCM, and TP wrote the manuscript.
Status: In preparation

CHAPTER 1

General Introduction

SEXUAL SELECTION

Sexual selection is the process by which variation in traits associated with competition between members of one sex to gain access to gametes of the other sex leads to differential mating and reproductive success (Darwin, 1871; Andersson, 1994). The occurrence of extravagant male secondary sexual traits in nature that seem detrimental to survival led Darwin (1871) to suggest they had evolved by competition for access to females (intra-sexual selection), or by female preference for the most ornamented male (inter-sexual selection). While Darwin was aware that in some species females competed among themselves for males, such examples were rare in his time so females were usually considered "coy" (Darwin, 1871). Under this framework, sexual selection was thought to be more common and stronger in males, as females were seen as one of the causes, rather than a target, of sexual selection. The diversity and exuberance of male weaponry and ornaments, leading to striking differences between the sexes across the animal kingdom, became paradigmatic examples of how male-male competition and female choice could drive evolutionary change (Andersson, 1994; Berglund et al., 1996; Andersson & Simmons, 2006). For example, pioneering work by Andersson (1982) showed that female long-tail widowbirds (*Euplectes progne*) preferred males with experimentally elongated tails, suggesting that the evolution and maintenance of extreme male ornaments can be caused by female choice.

The paradigm of sexual selection being stronger in males was reinforced after Bateman's (1948) seminal study of *Drosophila* in which he postulated that variance in reproductive success should be greater in males than in females, mainly as a consequence of anisogamy. Since males produce a large number of small, and therefore less energetically demanding gametes, their reproductive success would be more strongly constrained by the number of eggs available for fertilization and thus by the number of females with whom they are able to mate (i.e., high potential reproductive rate [PRR], see

Clutton-Brock, 2017). Females, on the other hand, by producing fewer and expensive gametes would have their reproductive success constrained by the number of gametes they could produce. Trivers (1972) expanded this general idea by suggesting that differences in parental investment between males and females, with the latter generally spending more time and energy raising the offspring, would further constrain their access to other potential mates. Males, on the other hand, would increase their reproductive success by deserting parental care in order to seek additional mating partners. While some of Trivers' ideas on the evolution of parental care based on PRRs have been questioned, since very few males ever actualize their full reproductive potential in male-biased sex ratios (Kokko et al., 2006), the generality of anisogamy as a fundamental driver of conflicts between the sexes remain largely confirmed (Kokko et al., 2006; Parker, 2014). Moreover, additional studies on other aspects of sexual conflict led to the conclusion that individuals of one sex – usually males – can increase their relative fitness by employing "tools" to manipulate the other sex – usually females – (Kokko & Jennions, 2014), reducing the female reproductive output below its optimum (i.e., increased lag load, Smith, 1976; Kokko & Jennions, 2014). Collectively, these studies helped cementing the Darwin-Bateman paradigm (Dewsbury, 2005).

While the Darwin-Bateman paradigm continues to accurately describe the overall sexual selection pattern in many sexually reproducing species (Fritzsche & Arnqvist, 2013; Janicke et al., 2016), the realisation that male gametes are not cheap to produce (reviewed in Wedell et al., 2002), and that females mate multiply – henceforward polyandry – opened a novel field of inquiry which has exploded in the past decades (Birkhead, 2010; Parker & Birkhead, 2013; Pizzari & Wedell, 2013). Female polyandry allows for intra-sexual selection (i.e., male-male competition) to continue after mating since the sperm from different males may compete to fertilize the female eggs (sperm competition, Parker, 1970). It also allows for further inter-sexual selection (i.e., female choice) since females may be able to bias the retention and utilization of sperm from different males (cryptic female choice, Thornhill, 1983; Eberhard, 1996). An explosive interest in post-copulatory sexual selection led

to an accumulation of empirical evidence for these two selective episodes in the past decades (Parker & Birkhead, 2013; Pizzari & Wedell, 2013). However, the difficulty in detecting cryptic female choice, and disentangling its effects from sperm competition, has meant evidence for its occurrence remains less forthcoming. Nevertheless, multiple experimental studies have demonstrated sperm adaptations shaped by sperm competition and, to a lesser extent, female-driven sperm choice mechanisms (Pizzari & Parker, 2009; Parker & Pizzari, 2010; Firman et al., 2017). For example, variation in sperm motility has been identified as a common target of selection since there seems to be a barrier in the female reproductive tract against immotile sperm (e.g., Bakst et al., 1994), and because more motile sperm should reach the egg faster providing a fertilizing advantage in sperm competition (Snook, 2005). Differential sperm ejection mediated by male and sperm phenotypes has also been a common emerging mechanism among studies investigating the role of female control in post-copulatory sexual selection (reviewed in Firman et al., 2017). Moreover, females of many species are able to store sperm for long periods, adding an additional layer of complexity to post-copulatory sexual selection (Birkhead & Møller, 1993; Orr & Brennan, 2015). For example, females from different species of crickets preferentially store sperm from unrelated males to reduce inbreeding depression, leading to a bias in paternity share in their favour during sperm competition (Bretman et al., 2009; Tuni et al., 2013). While the drivers of polyandry are still debated, potential benefits to females can in principle be either non-genetic or genetic (Simmons, 2005; Slatyer et al., 2012). On the one hand, polyandrous females can secure non-genetic resources such as an adequate sperm supply or the acquisition of food (e.g., nuptial gifts, reviewed in Eberhard, 1996), which in some species can increase the life-time fecundity of a female (Arnqvist & Nilsson, 2000; Simmons, 2005). On the other hand, polyandry could increase the genetic diversity of a set of offspring, or cryptic female choice could potentially increase offspring fitness by biasing the success of sperm from males with favourable genotypes (reviewed in Slatyer et al., 2012).

Since its first exposition in the works of Darwin (1859, 1871), sexual selection theory has therefore been expanded and invoked as a contributing cause for a range of broader biological phenomena, such as sexual conflict between the sexes (Chapman et al., 2003; Hosken & Stockley, 2004; Arnqvist & Rowe, 2005; Parker, 2006), the evolution of mating systems (Clutton-Brock & Parker, 1992; Rowe et al., 1994), and as a driver of speciation (Panhuis et al., 2001; Kirkpatrick & Ravigne, 2002).

TEMPORAL DYNAMICS IN SEXUAL SELECTION

Despite the vast and growing literature in sexual selection, most of this work has ultimately been targeted at understanding variation across species and across populations (Andersson 1994). Studies that have explored within population patterns in sexual selection have either (1) experimentally tested for causal relationships between specific traits and mate choice or mating success (Cotton et al., 2004; Andersson & Simmons, 2006; Tobias et al., 2012), or (2) measured present variation in mating or reproductive success as an estimate of total sexual selection in natural populations (Jones, 2009; Krakauer et al., 2011; Evans & Garcia-Gonzalez, 2016). While these approaches have led to a comprehensive understanding of the way sexual selection operates, there remains an outstanding gap in knowledge in relation to the tempo of sexually selected mechanisms (Cornwallis & Uller, 2010). Extrapolating the overall strength and direction of sexual selection either based on isolated causal relationships or on discrete data samples of a population, invariably assumes that selection remains constant over time. This assumption is in accordance with a tenet in evolutionary theory that directional selection must be a common feature in natural populations in order to account for the vast diversity of life features (Endler, 1986). However, although both empirical and theoretical work have demonstrated strong directional selection as an important mechanism of evolutionary change (Hoekstra et al., 2001; Hereford et al., 2004; but see Kingsolver et al., 2001), a growing number of studies have identified fluctuating selection as a common feature of natural populations (Estes & Arnold, 2007; Robinson et

al., 2008; Siepielski et al., 2009; Bell, 2010; Siepielski et al., 2011), implying that current selection does not necessarily reflect future selection. Therefore, understanding temporal changes in the strength and direction of selection is important because, ultimately, adaptive evolution reflects the cumulative outcome of selection (Siepielski et al., 2009).

Increasing appreciation for the necessity in accounting for temporal variation in sexual selection has led to a number of cross-sectional studies, usually comparing yearly differences in selection on a given trait (reviewed in Siepielski et al., 2009) or comparing standardized variances in reproductive success early versus late in a mating period (e.g., Reichard et al., 2008; Wacker et al., 2014). These studies have identified a role for environmental variation (Lindström et al., 2009; Janicke et al., 2015; Morimoto et al., 2016; Cattelan et al., 2020), and changes in the operational sex-ratio (OSR, Emlen & Oring, 1977; Forsgren et al., 2004; Liker et al., 2013; Wacker et al., 2013, 2014; Janicke & Morrow, 2018) as potential causes of dynamic changes in sexual selection. For example, male-biased OSR earlier in the breeding season increases variation in male mating and reproductive success in spotted gobies (*Gobiusculus flavescens*), as a larger proportion of smaller males fail to secure females at this time (Wacker et al., 2014). However, studies collecting longitudinal data are scarce (Blanckenhorn et al., 1999; Jann et al., 2000; Preston et al., 2001; Grant & Grant, 2002), despite recognition that quantifying life-time reproductive success is important to correctly estimate the strength and direction of sexual selection (Brommer et al., 2004; Hunt & Hodgson, 2010). However, monitoring the performance of individuals over their entire life-span is methodologically unrealistic for many species, and discerning causal relationships between scattered events is no trivial matter. Moreover, many studies of sexual selection using either wild or captive populations rely solely on genetic parentage data to estimate mating success leading to bias in proxies of sexual selection (Collet et al., 2014; Anthes et al., 2016). For example, variance in mating success can be overestimated if males that successfully mated but never sired offspring are assumed to have never mated through genetic parentage assignment (Collet et al., 2014). Moreover, studies rarely quantify the relative contribution of pre- and post-

copulatory episodes of selection (i.e., male-male competition and female mate choice versus sperm competition and cryptic female choice, Evans & Garcia-Gonzalez, 2016) and whether these vary over time. For example, increases in polyandry can saturate sexual networks and erode variance in male mating success, thus reducing opportunity for pre-copulatory sexual selection (Jones et al., 2001; Collet et al., 2012; Pélissié et al., 2014; Turnell & Shaw, 2015; McDonald et al., 2017). The lack of temporal resolution in sexual selection studies, limited efforts to account for the contribution of different episodes, and the lack of null models to which compare observed estimates of sexual selection (Prum, 2010; Klug et al., 2010) hamper our current understanding of the implications of temporal dynamics in sexual selection in natural populations. Therefore, understanding temporal changes in sexual selection requires collecting both fine-grained mating behaviour and parentage data, incorporating the cumulative sequence of copulations, length of sperm storage, and null expectations arising from this data.

Despite the advantage of long-term studies to the understanding of cumulative sexual selection, even characterization of annual or short-term changes in mating behaviour and paternity and their consequences for sexual selection should be informative for different study systems. In birds, the combination of female sperm storage and polyandry, together with variation in male remating rates and timing of copulations, leads to complex dynamics in male paternity share across a single clutch of eggs. For example, in different species of birds offspring from extra-pair matings tend to be disproportionally represented in eggs laid earlier in a laying sequence, probably due to maternal effects (e.g., collared flycatcher: Krist et al., 2005; western bluebirds: Ferree et al., 2010; blue tit: Vedder et al., 2012). Moreover, temporal studies evaluating female mate choice have shown that preferences for secondary male sexual characters can vary over relatively short periods (e.g., days) suggesting that phenotypic variation may be maintained by oscillation in mating preferences (e.g., Rios-Cardenas et al., 2007; Chaine & Lyon, 2008; Lehtonen et al., 2010; Clark & Backwell, 2015). Similarly, male courtship behaviour has been shown to change dynamically over time (e.g., Svensson et al., 2010; Jordan &

Brooks, 2012; Kahn et al., 2013), perhaps as a function of female mating preferences. For example, female fiddler crab (*Uca mjoebergi*) shift from preferring males sporting a large claw to males sporting a small claw over the mating period (i.e., 7 days), so small males increase their courting effort over time to match female preferences (Kahn et al., 2013). Similarly, in some bird species, female preference for individuals that sing early in the morning can increase their reproductive success (Poesel et al., 2006; Kempenaers et al., 2010; Greives et al., 2015). Despite the growing number of temporal studies on sexual selection, such fine-grained variation in mating behaviour over short time periods, particularly within-day (i.e., circadian), have seldom being characterized.

Another potential source of dynamic variation in sexual selection results from within-individual trade-offs in the expression of condition-dependent traits (e.g., Emlen, 2001; Joseph et al., 2018; Somjee et al., 2018), or across-individual variation in mating strategies (Shuster, 2010; Neff & Svensson, 2013). Because intra-sexual competition and mating effort can be costly for males, variation in sexually selected traits may indicate condition dependence and capture additive genetic variance associated with sustaining costly phenotypes (Hamilton & Zuk, 1982; Vehrencamp et al., 1989; Barske et al., 2011; Gasparini et al., 2013). If costs associated with sexual selection are felt differently across individuals over time, loss in mating performance associated with these costs can potentially lead to dynamic changes in mating or reproductive success. For example, in a feral population of sheep (*Ovis aries*), where males lack clearly defined alternative mating strategies, dominant males benefit from early reproductive success due to frequent mating, but sperm depletion leads to loss in paternity to subordinate males later in the breeding season (Preston et al., 2001). More generally, negative covariances between pre-copulatory traits and fertilization success have been demonstrated in several taxa (e.g., Froman et al., 2002; Lüpold et al., 2014; Dines et al., 2015; see Kvarnemo & Simmons, 2013 for a review), suggesting that an increase in mating success through investment in weapons, ornaments or social status can be costly to a male and hamper his success in sperm competition. However inconclusive these trade-offs may be (Mautz et al., 2013; Simmons et al., 2017), these studies suggest

that costs associated with differential allocation to resources can lead to dynamic changes in reproductive success across individuals. In females, costs of reproduction are usually associated with investment in gametes and offspring (Qvarnström & Forsgren, 1998; Bakker et al., 1999; Wong & Candolin, 2005; Clutton-Brock, 2009), but mating and male harassment can also be energetically costly or even physically damaging to females (Jormalainen et al., 2001; Perry et al., 2009). Moreover, males are expected to target high quality females (e.g., more fecund) differentially (Clutton-Brock, 2009; Edward & Chapman, 2011), and time their attempts in relation to the female reproductive period to maximize their reproductive success (Løvlie & Pizzari, 2007; Hau et al., 2017). This suggests that loss in female condition may vary as a function of female phenotype and time of the day. However, most studies measuring costs of mating effort and male harassment to females have done so indirectly (Fox et al., 2019), so direct quantification of these costs are lacking.

Finally, as it is clear from the above paragraphs, organisms are not a combination of discrete traits but a dynamic whole. Therefore, while experimentally controlling a predictor to investigate its relationship with reproductive success is of primary importance to infer causality in sexual selection processes (Klug et al., 2010; Anthes et al., 2016), in nature the outcome of selection will depend upon the combination of multiple traits and how they interact over time. This can be particularly pronounced in species where females are able to store sperm for multiple days, or even years, as the time window for sperm competition will increase (Birkhead & Møller, 1993; Orr & Brennan, 2015). This sharp separation of insemination and fertilization events may lead to dynamic patterns of reproductive success driven by the interaction of multiple mechanisms. For example, in the fruit fly (*Drosophila melanogaster*) the majority of eggs (~80%) are fertilized by the second male to mate with a female, and this advantage is caused by the combination of passive sperm release from the female storage organs, active sperm ejection by females, and active displacement of resident sperm by the sperm of the second male (Manier et al., 2010, 2013). Therefore, isolated causal relations demonstrated in controlled experiments may not accurately capture this dynamism found in nature (Simmons et al., 2007; Calisi &

Bentley, 2009), so future research should seek to estimate how the relative importance of different predictors change over time and how their combination ultimately determines an individual's reproductive success.

The overarching goal of this DPhil thesis is to contribute to the understanding of unexplored temporal aspects of sexual selection, by studying dynamic patterns of reproductive behaviour and paternity share at multiple temporal scales using an extensive dataset on replicate groups of a bird species, characterised by intense pre- and post-copulatory sexual selection on males, the red junglefowl (*Gallus gallus*).

THE RED JUNGLEFOWL

The red junglefowl is the main ancestor of the domestic chicken (*G. domesticus*, Fumihito et al., 1996; Eriksson et al., 2008; Lawal et al., 2020) and an established research model in sexual selection research (Zuk et al., 1990; Pizzari & McDonald, 2019). In the wild, this species forms polygynandrous groups, which are socially structured in sex-specific hierarchies (Collias et al., 1966; Collias & Collias, 1967, 1996). Red junglefowl are sexually dimorphic, with males being larger and more ornamented than females, although both sexes have a bright comb, a fleshy head ornament. Dominant males red junglefowl and feral fowl often display redder and larger combs than subordinates (Ligon et al., 1990; Cornwallis & Birkhead, 2008), which may function as honest signals of immunocompetence (Zuk et al., 1995; Zuk & Johnsen, 1998, 2000). There is some evidence that comb size, and also body mass, may be condition-dependent traits. Subordinate males experience comb shrinkage in the presence of dominant males (Zuk & Johnsen, 2000; Cornwallis & Birkhead, 2008), and both subordinate and dominant individuals lose body mass after spending time with females (Zuk & Johnsen, 2000). In females, comb size has been shown to be positively correlated with reproductive investment (Pizzari et al., 2003; Cornwallis & Birkhead, 2007; Wright et al., 2008), and dominant males may use female comb size as a cue during mating decisions (Pizzari et al., 2003; Cornwallis & Birkhead, 2007).

Studies of domestic fowl and red junglefowl indicate that mating patterns are highly contingent on the social environment. While in small, strongly female-biased groups mating is largely driven by females, in larger or male-biased groups, matings are often initiated by males (reviewed in Pizzari & McDonald, 2019). Socially dominant, more aggressive males are typically favoured in sexual selection, through both intra-sexual and inter-sexual mechanisms (Pizzari & McDonald, 2019). For example, dominant males are more likely to perform courtship displays (i.e., waltzes, Figure 1) (Zuk et al., 1995) and provide courtship feeding to females, which may increase their mating success (Pizzari, 2003; Wilson et al., 2008). Dominant males also tend to monopolise the majority of females by suppressing subordinates from mating, although the latter often achieve some mating success through coerced copulations (Cheng & Burns, 1988; Pizzari & Birkhead, 2000; Pizzari, 2001). However, differential female behaviour plays an important role in determining the outcome of male mating attempts. For example, females can influence the success of an insemination by differentially ejecting semen after inseminations (Pizzari & Birkhead, 2000; Dean et al., 2011), resisting copulation attempts (Løvlie & Pizzari, 2005), or by manipulating male-male competition and copulation interference (e.g., by uttering distress calls, Pizzari & Birkhead, 2000; Pizzari, 2001; Løvlie et al., 2014). Females have also been shown to alter their mating behaviour in male-biased groups by preferentially soliciting copulations in the morning to avoid male harassment (Løvlie & Pizzari, 2007), which tends to be higher in the evening due to increased mating activity (Cheng & Burns, 1988; Pizzari & Birkhead, 2001). This circadian pattern in male mating behaviour is considered to be an adaptation to maximise his probability of fertilizing eggs, since shortly before and after oviposition the fertilization success of an ejaculate is reduced due to oviduct contractions associated with egg laying which tends to occur in the late morning and afternoon (reviewed in Birkhead, 1998).

Female junglefowl ovulate and lay once a day for a mean clutch size of around 8 eggs (Collias et al., 1994), and can store sperm in specialized invaginations of the oviduct epithelium (i.e., sperm storage tubules [SSTs], Bakst et al., 1994; Sasanami et al., 2013) for up to roughly two weeks (Etches,

1996). Sperm from competing ejaculates mix randomly within the female reproductive tract before being stored in the SSTs, but are thought to be passively lost over time at a near constant rate (Lessells & Birkhead, 1990; Birkhead et al., 1995; Birkhead & Biggins, 1998). The mechanisms of random sperm mixing and passive sperm loss have been used in mathematical models which predict that—all else being equal—the last male to copulate with a female should retain a fertilization advantage for subsequent eggs, which is proportional to the time elapsed between competing inseminations (Lessells & Birkhead, 1990; Birkhead & Biggins, 1998). Therefore, the combination of female polyandry and sperm storage leads to strong sperm competition, which has been demonstrated by findings showing that variance in reproductive success in male red junglefowl is largely driven by variance in paternity share (Collet et al., 2012; McDonald et al., 2017). Studies using artificial insemination in domestic populations have shown that variance in paternity share can be driven by relative differences between two competitors in sperm numbers (Martin et al., 1974) or measures of sperm motile performance, such as mobility (Birkhead et al., 1999; Froman et al., 2002). The importance of these two factors have been shown to vary temporally, with differences in sperm numbers predicting the paternity of eggs produced shortly following insemination, while differences in mobility predicting paternity later in the laying sequence (Pizzari et al., 2008). Moreover, sperm competition outcomes can be modulated by both male and female characteristics. For example, females preferentially retain sperm from dominant males after coerced copulations (Pizzari & Birkhead, 2000; Dean et al., 2011). However, in some domestic populations subordinates males have been shown to have more motile sperm (Froman et al., 2002; Pizzari et al., 2008), suggesting a possible trade-off between male investment in pre- vs. post-copulatory intra-sexual competition. Similarly, old males tend to inseminate fewer sperm than young males (Dean et al., 2011; Cornwallis et al., 2014), and their sperm tend to swim more slowly, possibly due to lower levels of seminal antioxidants (Noguera et al., 2012). Similarly, older females have been shown to be less fecund, and that sperm stored in their SSTs are lost at a faster rate than in young females (Brillard, 2003). Finally, genetic relatedness between a male and a female may influence

fertilization 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 (Pizzari et al., 2004; Løvlie et al., 2013).



Figure 1. A red junglefowl (*Gallus gallus*) male (right) performing a courtship display (i.e., waltz) to a female (left) at the John Krebs Field Station of the University of Oxford. Circadian patterns in courtship behaviour were analysed in **Chapter 2** of this thesis.

THESIS STRUCTURE AND AIMS

The overall goal of this thesis is to explore temporal variation in multiple mechanisms driving sexual selection. To achieve this, I use a previously published and fine-grained dataset on mating behaviour and parentage data of freely mating replicate groups of a captive population of red junglefowl at the John Krebs Field Station of the University of Oxford. Data were collected by my supervisor Grant

McDonald during 20 mating trials conducted in 2011–2013, with each group being observed in an outdoor arena (5.8 × 16.5m) for at least 6 hours a day, for a period of 13 days. Groups were composed of 12 females and 10 males, which is within the range of group size and sex ratio observed in free-ranging populations (Pizzari & McDonald, 2019). The dataset comprises male-male and female-female agonistic interactions, male-female sexual interactions, and parentage data for 849 eggs collected during the trials. The high resolution and longitudinal aspect of this dataset is therefore ideal to explore questions related to temporal dynamics in sexual selection. This dataset has been previously analysed to investigate the relationship between male phenotypes and overall male reproductive success (McDonald et al., 2017), and the role of female sociality on the structure of socio-sexual networks (McDonald et al., 2019), although neither of these previous studies accounted for temporal variation. I therefore defined the aims of this thesis together with my supervisors to prevent significant overlaps with these previous publications. Moreover, in **Chapter 3** of this thesis, I supplement this dataset to include a further four additional replicate trials collected from the same population, which have not been previously analysed. The following chapters of this thesis are defined as follows:

In **Chapter 2** I start by characterising circadian patterns in pre-copulatory sexually selected behaviours in males and females, a poorly explored time window in sexual selection studies. I then test for consistent overall trends in these behaviours over time, whether they significantly differ over the two sessions (morning vs evening), and how they compare to earlier studies in the fowl. I further investigate the possibility that individuals of different social status may engage in alternative mating tactics by expressing different chronotypes i.e., that individuals of different phenotypes shift their active time period to maximise their mating success. For example, whether subordinate males are more active earlier in the morning in order to cuckhold dominant males, who usually prevent their access to females at busier hours of the day.

In **Chapter 3** I investigate dynamic changes in social status and condition-dependent traits over the course of mating trials to test for possible costs associated with mating activity and social status in

both sexes. To do this, I test for a relationship between male and female social status and two well characterized condition-dependent traits: comb size and body mass. I then explore the possibility that changes in body mass and comb size may be driven by mating effort, represented by mating success and number of copulations, and social status. Additionally, I explore the possibility that further loss in condition could be caused, in males, by being frequently chased by other males, and in females by experiencing a higher frequency of coerced copulations (i.e., male harassment).

In **Chapter 4** I move to post-copulatory episodes of selection and explore outcomes of sperm competition in semi-natural conditions. I first characterise, per each male in a mating group, a diverse set of traits previously associated with outcomes in sperm competition in controlled conditions (e.g., relatedness to a female, age, social status). This required that I developed a novel quantitative approach to estimate both the sperm depletion of a male's extra-gonadal sperm stores after successive copulations, and the passive loss of sperm from a female's sperm storage tubules over time. I then develop a methodological platform to disentangle the relative contribution of these multiple predictors to sperm competition by attempting to predict a male's probability of fertilization of individual eggs laid over successive days of a mating trial.

In **Chapter 5** I combine insights from the previous chapters and assess, more generally, how variance in both pre- and post-copulatory episodes of sexual selection varies over the relatively short sampling period of a mating trial, and how differences in sampling methods and the absence of null models may affect broader conclusions on sexual selection. I start by characterising temporal changes in the opportunity for selection on males and females using cumulative measures of mating and reproductive success. Specifically for males, I further decompose variance in reproductive success to access whether the relative contribution of pre- and post-copulatory sexual selection episodes and their covariances change over time. I then compare cumulative estimates of selection with estimates generated by random mating simulations in order to access how null expectations of variance in reproductive success differ from actual estimates. Finally, I assess how overlooking the cumulative

sequence of copulations when estimating variance in mating and reproductive success may bias estimates of sexual selection. I achieve this by comparing opportunity estimates calculated using cumulative values of reproductive success, with values calculated independently on each day of the trial.

Finally, in **Chapter 6,** I summarise key findings, identify emerging patterns and discuss caveats and avenues of future research arising from this work.

CHAPTER 2

Circadian patterns in mating behaviour in red junglefowl (*Gallus gallus*)

Circadian patterns in mating behaviour in red junglefowl (*Gallus gallus*)

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Despite evidence of heritability and across-individual variation in traits underpinning circadian phenotypes, few studies have explored circadian rhythms in mating behaviour within the context of sexual selection. Here we characterise circadian patterns in female receptivity to mating, and male mating and courtship effort across 24 replicate groups of red junglefowl (*Gallus gallus*). We show that circadian patterns in these behaviours are highly consistent over a 10 day mating trial, and that trends can differ between the morning and the evening. Females tended to reduce the level of resistance to male mating attempts and increase the number of solicitations in the morning, while they did the opposite in the evening. Circadian patterns in male mating behaviour and courtship were consistent with previous studies of domestic fowl (*G. domesticus*). Males attempted to mate for most of the morning and evening, but tended to perform more courtship displays and food calls in the morning. We did not find strong evidence that dominant and subordinate males express different chronotypes, with differences in timing being more likely driven by dominant males suppressing subordinates by constantly initiating agonistic interactions. These results demonstrate consistent circadian patterns in multiple mating behaviours in red junglefowl, which can have important consequences for sexual selection in this species and welfare management in domestic populations.

1. Introduction

Characterising temporal patterns in mating behaviour is important to better understand the outcome of sexual selection episodes such as female choice and sperm competition, and patterns of cooperation and conflict between the sexes (Cornwallis & Uller, 2010). For example, conflict over mating may have a strong temporal component since males may attempt to delay – or prevent – females with whom they have already mated from remating with other males. In some Arthropods, the first male to mate usually sires most of the offspring by supressing or delaying female remating, e.g., through inhibitory accessory gland products in the ejaculate, or by covering the female genitalia with copulatory plugs (Birkhead & Hunter, 1990; Simmons, 2001). Alternatively, in species where females store sperm for longer periods, mechanisms of second male sperm precedence are more common (Birkhead & Møller, 1993; Orr & Brennan, 2015). For example, in birds the second male to mate has a fertilization

advantage over previous males, since the sperm from earlier rival inseminations will have been partially lost from the female's storage organs (Lessells & Birkhead, 1990; Birkhead & Biggins, 1998; Carleial et al., 2020a). Moreover, different individuals may employ alternative mating tactics whereby success is dependent upon timing. For example, satellite or subordinate males may have to time their sneaky mating attempts in order to increase the likelihood of success, so they monitor their surroundings for the right opportunity (e.g., Berard et al., 1994; Taborsky, 1998; Iwata et al., 2005; Crockford et al., 2007). In females, resistance to mating is expected to vary over time. Female *Drosophila* have a refractory period after mating, in which they tend to refuse further male mating attempts (Sirot et al., 2011; Hurtado & Hasson, 2013). Conversely, some models predict that females should reduce choosiness when opportunities for sampling new mates decrease over a mating season, but strategies will depend on the costs associated with the sampling effort (Johnstone, 1997). In species where reproduction is confined within a short period, cooperation, rather than conflict between the sexes, is crucial for synchronisation and optimisation of reproductive events. For example, in some explosive breeders mating opportunities lasts for just a few days, and courtship is coordinated and triggered by environmental events such as rainfall (Ulloa et al., 2011). Therefore, the timing associated with expression of traits that boost mating attraction or increase the odds of winning in intra-sexual competition can itself be under sexual selection (Hau et al., 2017).

Circadian periods are poorly explored temporal windows in sexual selection studies, despite evidence of heritability and across-individual variation in traits underpinning circadian phenotypes (e.g., Daan & Beersma, 2002; Michael et al., 2003; Schöfl et al., 2009, Helm & Visser, 2010; Matsumura et al., 2020). Variation in circadian patterns in sexual behaviour can be modulated by environmental cues (i.e., zeigebers), so they tend to be contingent on specificities of the animal group. For example, while cooler temperatures modulates fighting behaviour in field crickets (*Gryllus firmus*), as it allows for longer fights (Nguyen & Stahlschmidt, 2019), in Emei music frogs (*Babina daunchina*)

and oyster toadfishes (*Opsanus tau*) higher temperatures in the late afternoon are associated with an increase in mating vocalizations (Cui et al., 2011; Van Wert & Mensinger, 2019). Similarly, changes in light conditions can affect the expression of a variety of traits associated with mate attraction across different taxa (e.g., birds: Kempenaers et al., 2010; crustaceans: Takahashi et al., 2015; insects: Lu et al., 2017; fishes: O'Naill et al., 2019). These traits can be expressed differently among individuals, which can lead to alternative mating tactics with distinct circadian patterns. For example, studies have shown that in some socially monogamous bird species, males that start singing earlier in the morning, sometimes due to the presence of artificial light (Kempenaers et al., 2010), increase their reproductive success by cuckolding other males who start singing later in the dawn (Poesel et al., 2006; Kempenaers et al., 2010; Greives et al., 2015). Besides environmental cues, differences in circadian rhythms can be associated with endogenous circadian clocks. Variation in singing behaviour in great tits (*Parus major*) can occur as a function of different free-running period (FRP) lengths, which have been shown to be heritable (Helm & Visser, 2010). Similarly, whether mutant lines of *Drosophila melanogaster* expressing different circadian rhythms will outcompete wild-type lines over food and mating opportunities will depend on the light conditions in the lab, suggesting a selective role for circadian clocks (Horn et al., 2019). Finally, circadian rhythms can differ between the sexes (e.g., Gershman et al., 2016; Huffeldt & Merkel, 2016; Broadhead et al., 2017). For example, in some species of moth, female locomotor activity peaks several hours before males, who become active only when females stop moving and become sexually receptive (Broadhead et al., 2017). To date, most studies on circadian patterns have evaluated the association between environmental cues and circadian clocks, or differences in patterns across populations or species (Helm et al., 2017). However, we have less information on whether variation in circadian patterns in mating behaviour across individuals (i.e., chronotypes, Schwartz et al., 2017) and between the sexes will be favoured by sexual selection.

Here, we use replicate mixed-sex groups of red junglefowl (*Gallus gallus*) to investigate circadian patterns in mating behaviour in both males and females, and a possible role for differences in chronotypes between dominant and subordinate males. The red junglefowl is the main ancestor of the domestic chicken (*G. domesticus*), and in nature live in fission-fusion, socially structured flocks comprising one dominant male, some subordinate males, and multiple females (Pizzari & McDonald, 2019). Dominant males tend to monopolise access to females by actively interrupting copulation attempts from other males, but the mating system can be described as polygynandrous, since females usually mate with multiple males (Pizzari & McDonald, 2019). Polyandry is achieved by females soliciting copulations from different males, usually dominants, or by males coercing females into mating due to their larger size (Cheng & Burns, 1988; Pizzari & Birkhead, 2000; Pizzari, 2001). Males also perform courtship displays (i.e., waltzes) aimed at attracting females (Zuk et al., 1995), and provide courtship feeding, which has been shown to be associated with higher mating success (Pizzari, 2003; Wilson et al., 2008), although the effectiveness of these displays is not well understood (Wilson et al., 2008). Females usually lay daily with a mean clutch of around 8 eggs (Collias et al., 1994), and can store sperm from an insemination for up to around two weeks (Etches, 1996), which, in addition to high mating rates, contributes to the intense sperm competition that can characterise fowl flocks (Pizzari & McDonald, 2019). Sexual conflict occurs as females usually attempt to resist male mating attempts, which can bias fertilization success in favour of certain males or mating events (Løvlie & Pizzari, 2005; Carleial et al., 2020a), but it is physically costly to females (Pizzari, 2001; Carleial et al., 2020b). Females can also eject sperm after mating, and the volume ejected varies in relation to the male social status (Pizzari & Birkhead, 2000; Dean et al., 2011).

Studies of the related domestic chicken suggest that circadian patterns in mating behaviour may play important roles in sexual selection and sexual conflict. During a laying cycle, females ovulate once daily in an asynchronous pattern in which the first egg of a sequence is oviposited about 2h after

dawn, with each subsequent egg being laid later in the day than the previous (Etches, 1996). Therefore, the majority of eggs tend to be laid late in the morning and early afternoon. Copulations performed shortly before and after egg laying are less likely to lead to fertilization due to oviductal contractions and changes in pH associated with egg laying (Etches, 1996; Brillard, 2003). This likely contributes to explain circadian patterns of sexual activity, which typically shows a peak in the early morning and one in the late afternoon, at times when fertilization success is thought to be higher (Cheng & Burns, 1988; Pizzari & Birkhead, 2001). Because male harassment tends to be higher in the evening, females have been shown to alter their mating behaviour by preferentially soliciting copulations in the morning rather than in the evening in male sex-biased groups (Løvlie & Pizzari, 2007). At least one male behaviour, crowing, shows consistent circadian patterns which have been shown to be regulated by circadian clocks (Shimmura & Yoshimura, 2006; Ito et al., 2017), although social dominance determines who crows first (Shimmura et al., 2015). Similarly, dominant males have been shown to waltz to females more often than subordinates, particularly in the morning (Cheng & Burns, 1988) and tend to interrupt mating attempts from subordinates more often in the evening than at other times of day (Cheng & Burns, 1988). Collectively, these studies demonstrate the importance of within-day adjustments in behaviour aimed at minimising costs associated with sexual conflict in case of females, and maximising reproductive success in case of males. Little however is known about circadian patterns in the red junglefowl, and how such patterns may vary over time and across phenotypes.

In this study, we expand on these previous findings by addressing three aims. First, we establish whether mating behaviours display daily cyclical patterns over the course of the 10 days of a mating trial. Second, we characterise and compare patterns in mating behaviour between the morning and evening. Third, we test whether males of different social ranks express different chronotypes (i.e., perform the same mating behaviour at different times of the day) and whether these patterns are in synchrony with circadian patterns in female responses. For aim (i) we expect that, if circadian patterns

in mating and courtship behaviour are heavily modulated by the social environment, then they should vary across days as group structure changes. On contrary, if mating and courtship behaviour is at least partially entrained by environmental cues (i.e., zeitgebers) or by endogenous circadian clocks, then we would expect more consistent patterns across days. For aim (ii) we predicted a peak in mating activity in the evening and higher courtship effort from males in the morning, as reported by previous studies of domestic chickens (Cheng & Burns, 1988; Pizzari & Birkhead, 2001; Løvlie & Pizzari, 2007). For aim (iii), we predicted that more subordinate males would attempt to mate and court females at suboptimal times of day when fertilization success is thought to be lower, and when dominant males are possibly less active, e.g., early morning and late evening, and that females would increase mating resistance and reduce solicitations at these times. On the other hand, we expected more dominant males to be more active at the most optimal times of the day, particularly during the evening, and that they will increase the frequency of aggression towards other males at these times (Cheng & Burns, 1988).

2. Methods

2.1 Behavioural observations

We used a previously published dataset on mating and agonistic behaviour from 20 replicate male-female red junglefowl groups (McDonald et al., 2017, 2019; Carleial et al., 2020a, Carleial et al., 2020b), plus data from four novel groups collected following an identical observation protocol. All mating groups were assembled using individuals from a captive population at the John Krebs Field Station of the University of Oxford. Individuals of both sexes belonged to different cohorts and were sexually mature ranging from 1 to 7 years of age (McDonald et al., 2017, 2019). Protocols of the mating trials have been extensively described elsewhere (McDonald et al., 2017). Briefly, each group comprised 10 males and 12 females, and was observed by one of us (GCM) twice a day, 3 hours in the morning (05:00-08:00h) and 3 hours in the evening (18:00-21:00h) for 10 consecutive days, which represented a trial. Trials were conducted over four years (2011-2013, 2015) in the same outdoor pen,

and birds had food and water *ad libitum*. We recorded all male mating attempts, both unsuccessful and successful (i.e., when we observed cloacal contact, or the male tail bending towards, and obscuring, the female cloaca), male courtship (i.e., waltzing), male food calls (i.e., calls uttered by males when they courtship feed females, Pizzari, 2003), and female solicitations (i.e., when a female crouched in front of a male and solicited a copulation). We also recorded the intensity of female resistance to each male mating attempt following the score provided in Løvlie & Pizzari (2005), such that: 1= solicitation; 2 = the male approaches the female from behind with raised hackles, and the female responds by crouching; 3 = the male approaches the female, and the female walks away; 4 = the male grabs the female, the female passively accepts the copulation; 5 = the male runs after and grabs the female, and the female initially resists before accepting the copulation; and 6 = the female resists the whole copulation. All agonistic interactions between males (i.e., pecks, aggressive waltzes, and fights) were recorded during the 10 days of the mating trials. Individuals that retreated at least one body length from the other after an agonistic interaction were considered the losers of the interaction (Johnsen et al., 2001; Froman et al., 2002; Wilson et al., 2009). Agonistic interactions among males were used to calculate scores following the randomised Elo rating method (Sánchez-Tójar et al., 2018) as in Carleial et al., (2020b), which were then converted to discrete social ranks ranging from 1 (more dominant) to 10 (more subordinate). All instances of mating and agonistic behaviour were recorded to the nearest minute.

2.2 Analytical approach

2.2.1 Cycles in mating behaviour

First, we investigated whether circadian patterns of mating behaviour in the morning and evening were consistent over the 10 days of the mating trial. We grouped observations in 15-minute intervals across the observation session (i.e. morning vs evening) and each day of the trial (i.e., 1-10). Within each 15-minute interval we calculated: i) mean resistance score to male mating attempts (scores 2-6, Løvlie &

Pizzari, 2005) across females, ii) the mean number of mating attempts across males, iii) the mean number of waltzes across males, iv) the mean number of initiated agonistic interactions across males, and v) the mean number of uttered food calls across males. We also analysed temporal variation in female solicitations (score 1). However, because female solicitations were rare (n = 447 across the 24 groups) we used a larger time interval of 1-hour rather than 15-minutes. To test whether circadian patterns in mating behaviour were cyclical, we ran linear mixed models (LMMs) with each variable (female resistance, female solicitations, mating attempts, male waltzes, male initiated agonistic interactions, or male food calls) as the response variable and the time intervals (1-hour intervals for female solicitations, and 15-minute intervals for the others) and its interaction with the day of the mating trial (a factor with 10 levels) as the explanatory variable (i.e., Time interval*Day). The identity of the mating group was entered as a random effect to allow for the intercept of each group to vary. To facilitate comparisons between the slopes across different days, models were run separately for morning and evening observations.

2.2.2 Circadian patterns in mating behaviour

After confirming that circadian patterns were mostly cyclical (see Results), we evaluated morning and evening trends in mating behaviour in more detail. We used the grand mean across the 10-day of the mating trial for each of the behavioural variables (i)-(v) as above. Circadian patterns in mating behaviour were tested with LMMs, but this time the session (morning vs evening) was entered in the same model to compare differences between these two periods. Models therefore, had the different mating behaviours as response variables, with the time intervals and its interaction with the session as the explanatory variable (i.e., Time interval*Session). The identity of the mating group was entered as the random effect.

2.2.3 Differences between socially dominant and subordinate males

We investigated whether the social status of a male modulates circadian patterns in mating behaviour. To do this, we compared males of different social ranks (1-10) by testing whether they express different chronotypes e.g., whether higher ranking males would be more active at different times of the day than lower ranking males. We also investigated whether female propensity to mate with males of different ranks varied over the day. Within each 15-minute interval we calculated per each male within a discrete social rank (1-10) and across the 10 days of the trial: i) mean female resistance score across his mating attempts, ii) his mean number of mating attempts, iii) his mean number of waltzes, iv) the mean number of agonistic interactions initiated by this male, and v) his mean number of food calls. As in the previous sections, female solicitations to each male were calculated within 1-hour intervals, rather than 15-minute intervals due to their rarity. To test whether males from different social ranks were more active at different times of the day, we ran LMMs separately per each time period (morning vs evening) with each mating behaviour as the response variable and social status (a factor with 10 levels) and their interaction with the time interval (1-hour or 15-minute) as explanatory variables (i.e., Status*Time interval). Here, models were run separately per each session (morning and evening), and the time interval was entered as a factor with 12 levels, rather than numeric as above, in order to compare males at finer-scales within the day. We entered the identity of the group, and the identity of the males as random effects to control for pseudo-replication, as some males were re-used across groups (McDonald et al., 2017). Comparisons between male mean values within each time interval (15 minutes or 1h) were performed with *post-hoc* Tukey tests.

2.2.4 Statistical analyses

All LMMs were conducted using the software R v3.6.2 (R Core Team, 2019) using the package "lme4" v1.1-21 (Bates et al., 2015), with *post-hoc* Tukey tests being implemented in the package "emmeans" v1.4.4 (Lenth et al., 2020). Model residuals were explored for any violation of assumptions, and variables were squared-root transformed when violations occurred. *p* values and denominator degrees

of freedom for fixed effects were calculated using *F* tests with Satterthwaite's approximation implemented in the package "lmerTest" v3.1-1 (Kuznetsova et al., 2017).

3. Results

3.1 Circadian patterns in mating behaviour across days

Circadian patterns on most mating behaviours were highly consistent across days (Figure 1). Because some of the relationships looked quadratic, we included a quadratic terms in the models to explore this possibility (i.e., Time interval^2) There was no strong evidence that patterns in female solicitations varied across the 10 days of the mating trial in the morning, and we found that there was a negative quadratic effect in this relationship such that solicitations ceased to increase after the second hour of observation (Time interval*Day: $F_{1,676} = 0.24$, $p = 0.988$; Time interval^2: $F_{1,676} = 8.75$, $p = 0.003$, Figure 1a). Female solicitations also did not vary significantly over the 10 days in evening (Time interval*Day: $F_{1,677} = 0.52$, $p = 0.863$, Figure 1b). Similarly, patterns in female resistance were consistent across days in both morning (Time interval*Day: $F_{1,2334} = 0.76$, $p = 0.657$, Figure 1c) and evening (Time interval*Day: $F_{1,2089} = 0.9$, $p = 0.524$, Figure 1d). Male mating attempts varied over the 10 days in the morning, and there was a negative quadratic effect, such that mating attempts stopped increasing after 2 hours of observation (Time interval*Day: $F_{1,2836} = 2.10$, $p = 0.026$; Time interval^2: $F_{1,2836} = 39.19$, $p < 0.001$, Figure 1e). The significant effect of the day order was caused by higher mating frequency in the first day of the trial, when males and females were first introduced after days of sexual rest (see Løvlie & Pizzari, 2007 for a similar pattern). After removing the first day of the trial, the effect of day order was no longer significant (Time interval*Day: $F_{1,2550} = 0.67$, $p = 0.719$; Time interval^2: $F_{1,2550} = 48.07$, $p < 0.001$). Mating attempts did not vary significantly over the 10 days in the evening, although there was a significant and negative quadratic effect, such that attempts started to decrease after 19:15 (Time interval*Day: $F_{1,2836} = 0.53$, $p = 0.855$; Time interval^2: $F_{1,2836} = 107.25$, $p < 0.001$, Figure 1f). Trends in male waltzing tended to vary over the 10 days of the trial in the morning

(Time interval*Day: $F_{1,2836} = 3.11$, $p = 0.001$; Time interval^2: $F_{1,2836} = 45.58$, $p < 0.001$, Figure 1g) and in the evening (Time interval*Day: $F_{1,2837} = 4.56$, $p < 0.001$, Figure 1h), and a negative quadratic effect was only found in the morning, such that waltzes stopped to increase over the last hour of observation. As with male mating attempts, removing the first day of the trial eliminated the effect of day order in patterns of male waltzes in the morning (Time interval*Day: $F_{1,2550} = 0.67$, $p = 0.717$; Time interval^2: $F_{1,2550} = 40.29$, $p < 0.001$), but not in the evening (Time interval*Day: $F_{1,2550} = 4.47$, $p < 0.001$). Upon inspecting the marginal effects of this relationship (Figure 1h) however, we found that the significant effect of day order was caused by shallower slopes towards the later days of the trial, rather than changes in direction in daily patterns. We found no strong evidence that trends in male agonistic interactions changed over the trial in the morning, but we found a negative significant effect on this relationship (Time interval*Day: $F_{1,2836} = 0.53$, $p = 0.852$; Time interval^2: $F_{1,2836} = 9.22$, $p = 0.002$, Figure 1i). However, we found a significant effect of day order and a negative quadratic effect in patterns of male aggression in the evening (Time interval*Day: $F_{1,2836} = 2.12$, $p = 0.025$; Time interval^2: $F_{1,2836} = 106.84$, $p < 0.001$, Figure 1j). Once again, this pattern was caused by higher male aggression on the first day of the trial, so removing the first day eliminated the effect of day order (Time interval*Day: $F_{1,2550} = 1.19$, $p = 0.299$; Time interval^2: $F_{1,2550} = 86.39$, $p < 0.001$). Finally, we found no evidence that trends in male food calls changed over the 10 days of the mating trial in the morning (Time interval*Day: $F_{1,2836} = 0.75$, $p = 0.659$; Time interval^2: $F_{1,2836} = 8.55$, $p = 0.003$, Figure 1k) and in the evening (Time interval*Day: $F_{1,2836} = 1.10$, $p = 0.363$; Time interval^2: $F_{1,2836} = 38.28$, $p < 0.001$, Figure 1l), but both showed negative quadratic effects.

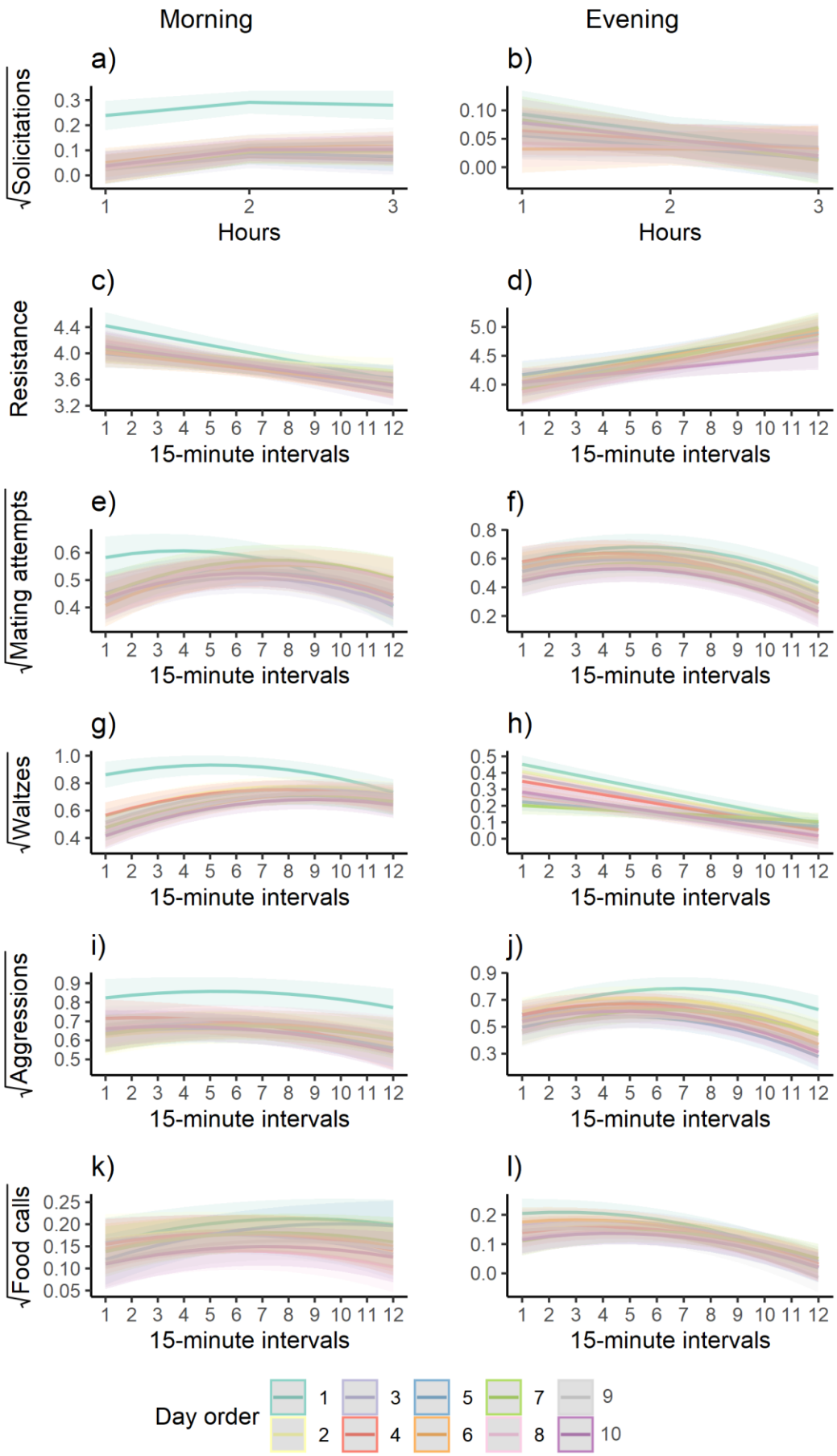

Morning
Evening
a)
b)
√Solicitations
Hours
c)
d)
Resistance
15-minute intervals
e)
f)
√Mating attempts
15-minute intervals
g)
h)
√Waltzes
15-minute intervals
i)
j)
√Aggressions
15-minute intervals
k)
l)
√Food calls
15-minute intervals
Day order
1
2
3
4
5
6
7
8
9
10

Figure 1 – Relationship between patterns in mating behaviours and different days of a mating trial in male and female red junglefowl (*Gallus gallus*) in the morning and in the evening. Panels represent the marginal effects of a linear mixed model (LMM). a-b) overall female resistance to male mating attempts, c-d) number of female mating solicitations, e-f) number of mating attempts, g-h) number of male courtship waltzes, i-j) number of aggressions initiated by males, and h-i) the number of food calls uttered by males. We used average values across birds within each time unit and day of the trial. All behaviours were measured continuously over a period of three hours in the morning (05:00-08:00) and in the evening (18:00-21:00), but were grouped within 15-minute intervals for analyses. Graphs are colour coded in relation to the observation day in a mating trial (1-10) and shaded areas represent the 95% confidence intervals.

3.2 Behavioural patterns in the morning and evening

Similar to the previous section, temporal trends in some mating behaviours looked quadratic, and seemed to differ between the morning and the evening. Therefore we explored the interaction between a quadratic term and the observation session (i.e., Time interval^2*Session). We found that morning and evening trends in female solicitations followed opposite directions (Time interval*Session: $F_{1,116} = 22.77$, $p < 0.001$; Time interval^2: $F_{1,116} = 4.87$, $p = 0.029$, Figure 2a, Table 1). In the morning females tended to increase the number of solicitations from 5am to 6am, but solicitations remained constant in the third hour. In the evening females tended to reduce the number of solicitations from 6pm to 9pm. Similarly, trends in the level of female resistance to male mating attempts also followed opposite directions in the morning and evening (Time interval*Session: $F_{1,536} = 282.79$, $p < 0.001$; Time interval^2: $F_{1,536} = 5.00$, $p = 0.026$, Figure 2b, Table 1). While females tended to decrease overall resistance over the morning until a certain threshold, they increased resistance steadily over the evening. There was a significant difference between trends in male mating attempts in the morning and in the evening (Time interval*Session: $F_{1,547} = 4.35$, $p = 0.037$; Time interval^2*Session: $F_{1,547} = 7.94$, $p = 0.005$, Figure 2c, Table 1). Males increased the number of mating attempts slightly from 5am to around 6:30am, but they tended to mate slightly less until 8am. In the evening, males increased more sharply the number of mating attempts until it peaked at around 7:30pm, but afterwards they substantially reduced the number of attempts until 9pm. Trends in male courtship differed between the morning and evening (Time interval*Session: $F_{1,547} = 25.50$, $p < 0.001$; Time interval^2*Session: $F_{1,547} = 6.63$, $p = 0.010$, Figure 2d, Table 1). Males tended to increase the number of waltzes over the morning

until it reached plateau at around 7am, after which they reduced the number of waltzes slightly. Males waltzed much less in the evening, and they tended to progressively waltz less from 6pm to 9pm. Males tended to initiate agonistic interactions with other males differently over the morning and the evening (Time interval*Session: $F_{1,547} = 6.70$, $p = 0.010$, Time interval^2*Session: $F_{1,547} = 7.83$, $p = 0.005$, Figure 2e, Table 1). In the morning males initiated aggressive interactions constantly from 5am to 8pm. However, in the evening they tended to increase the number of aggressive interactions until 7pm, but then tended to initiate less agonistic interactions until 9pm. Finally, trends in male food calls to females showed different trends in the morning and in the evening (Time interval*Session: $F_{1,548} = 54.27$, $p < 0.001$; Time interval^2: $F_{1,548} = 37.74$, $p < 0.001$, Figure 2f, Table 1). In the morning, males tended to increase the number of food calls from 5am to 7am, which started to decrease slightly until 8am. In the evening, males sharply reduced the number of food calls after 7pm.

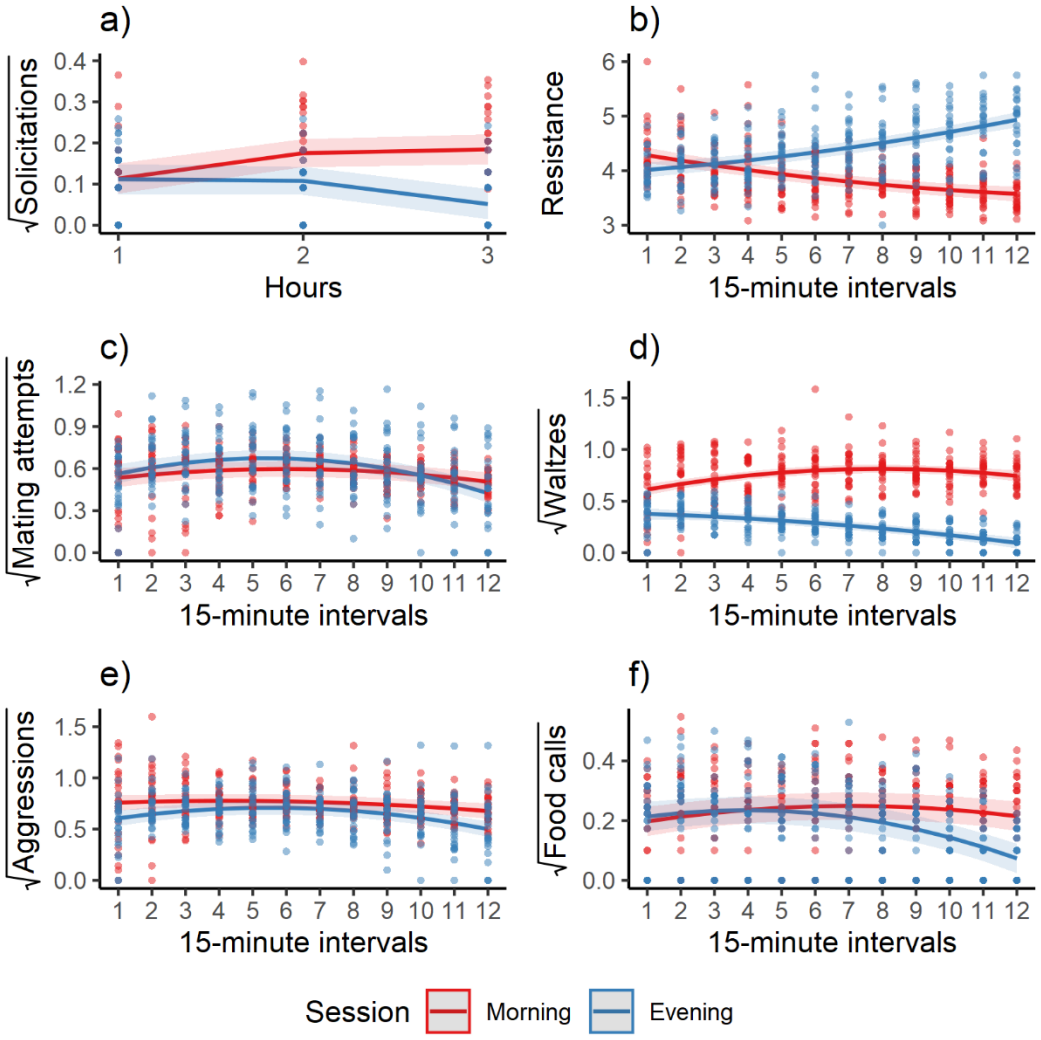


Figure 2 – Relationship between different mating behaviours and time of the day in male and female red junglefowl (*Gallus gallus*). Panels represent the marginal effects of a linear mixed model (LMM). a) overall female resistance to male mating attempts, b) number of female mating solicitations, c) number of male mating attempts, d) number of male courtship waltzes, e) number of aggressions initiated by males, and f) number of food calls uttered by males. We used average values across birds and over the 10 days of the mating trial. All behaviours were measured continuously over a period of three hours in the morning (05:00-08:00) and in the evening (18:00-21:00), but were grouped within 15-minute intervals for analyses. Graphs are colour coded in relation to the time of the day (morning and evening) and shaded areas represent the 95% confidence intervals.

Table 1. Linear mixed-effect models (LMMs) results for the relationship between multiple behaviours and time of the day in red junglefowl (*Gallus gallus*).

	sqrt(Solicitations)					**Resistance**					**sqrt(Attempts)**				
Predictors	*Estimates*	*CI*	*F*	*p*	*ddf*	*Estimates*	*CI*	*F*	*p*	*ddf*	*Estimates*	*CI*	*F*	*p*	*ddf*
(Intercept)	<0.01	-0.09 – 0.09				4.38	4.22 – 4.55				0.46	0.39 – 0.53			
Time interval	0.14	0.05 – 0.24				-0.11	-0.14 – -0.07				0.05	0.04 – 0.07			
Session [Evening]	0.06	0.01 – 0.12				-0.42	-0.54 – -0.29				0.1	0.05 – 0.15			
Time interval^2	-0.03	-0.05 – -0.00	4.87	**0.029**	116	<0.01	<0.01	4.99	**0.026**	536	<0.01	<0.01			
Time interval * Session [Evening]	-0.07	-0.09 – -0.04	22.77	**<0.001**	116	0.15	0.13 – 0.17	282.79	**<0.001**	536	-0.01	-0.02 – -0.00	4.35	**0.037**	547
Time interval^2 * Session [Evening]											<0.01	-0.01 – -0.00	7.94	**0.005**	547
Random Effects															
σ^2	0					0.13					0.02				
$\tau_{00\ group\ ID}$	0.00					0.06					0.02				
Observations	564					144					576				

	sqrt(Waltzes)					**sqrt(Aggressions)**					**sqrt(Food calls)**				
Predictors	*Estimates*	*CI*	*F*	*p*	*ddf*	*Estimates*	*CI*	*F*	*p*	*ddf*	*Estimates*	*CI*	*F*	*p*	*ddf*
(Intercept)	0.59	0.54 – 0.65				0.8	0.72 – 0.87				0.16	0.11 – 0.21			
Time interval	0.05	0.03 – 0.06				-0.01	-0.01 – -0.00				0.03	0.02 – 0.04			
Session [Evening]	-0.25	-0.30 – -0.20				-0.09	-0.14 – -0.03				0.05	0.02 – 0.08			
Time interval^2	<0.01	<0.01									<0.01	<0.01	37.74	**<0.001**	548
Time interval * Session [Evening]	-0.04	-0.04 – -0.03	24.5	**<0.001**	547	<0.01	-0.01 – 0.01	6.7	**0.01**	547	-0.01	-0.02 – -0.01	54.27	**<0.001**	548
Time interval^2 * Session [Evening]	<0.01	0.00 – 0.01	6.63	**0.01**	547	<0.01	-0.01 – -0.00	7.83	**0.005**	547					
Random Effects															
σ^2	0.02					0.02					0.01				
$\tau_{00\ group\ ID}$	0.01					0.02					0.01				
Observations	576					576					576				

Note: p values of fixed effects are based on *F* tests with Satterthwaite's approximation and provided only for higher-order terms. Results are highlighted in bold when statistically significant ($p < .05$). Abbreviations: CI, confidence intervals; F, F statistics; ddf, denominator degrees of freedom; sqrt = square-root; σ2, residual variance; τ00, random intercept variance.

3.2 Differences in circadian patterns between socially dominant and subordinate males

We found that the distribution of mating activity within the day was similar across the different social ranks, and that differences occurred in terms of frequency, rather than individuals from different social ranks being active at different times of the day (supplementary material, Figure S1). Therefore, for simplicity we report comparisons between the top- (rank 1, dominant) and bottom-ranking (rank 10, subordinate) males, as they accurately represent both extremes of the behaviour spectrum. Results from the Tukey *post-hoc* tests are provided in Figure 3. Females solicited more often to dominant than subordinate males although this difference was only significant during the second hour of observations (6am-7am) in the morning (Figure 3a), and the first hour of observations (6pm-7pm) in the evening (Figure 3b). Females resisted mating attempts from subordinates more strongly than attempts from dominant males both in the morning and evening, although this difference was not entirely consistent over the evening (Figure 3c, d). Dominant males attempted to mate as often as subordinate males over all time periods in the morning and in the evening (Figure 3e, f), but they waltzed to females more often than subordinates during most of the morning, with the exception of the last 15 minutes of the observations (Figure 3g). Similarly, dominant males waltzed to females more often than subordinate males during most of the evening, but they tended to converge during the last 45 minutes of the observations (Figure 3h). Dominant males initiated more agonistic interactions than subordinate males during all time periods in the morning (Figure 3i) and evening (Figure 3j). Finally, dominant males tended to courtship females with food calls more often than subordinate males for one hour (6am-7am) during mid-morning (Figure 3k) and for one hour (6:30pm-7:30pm) mid-evening (Figure 3l).

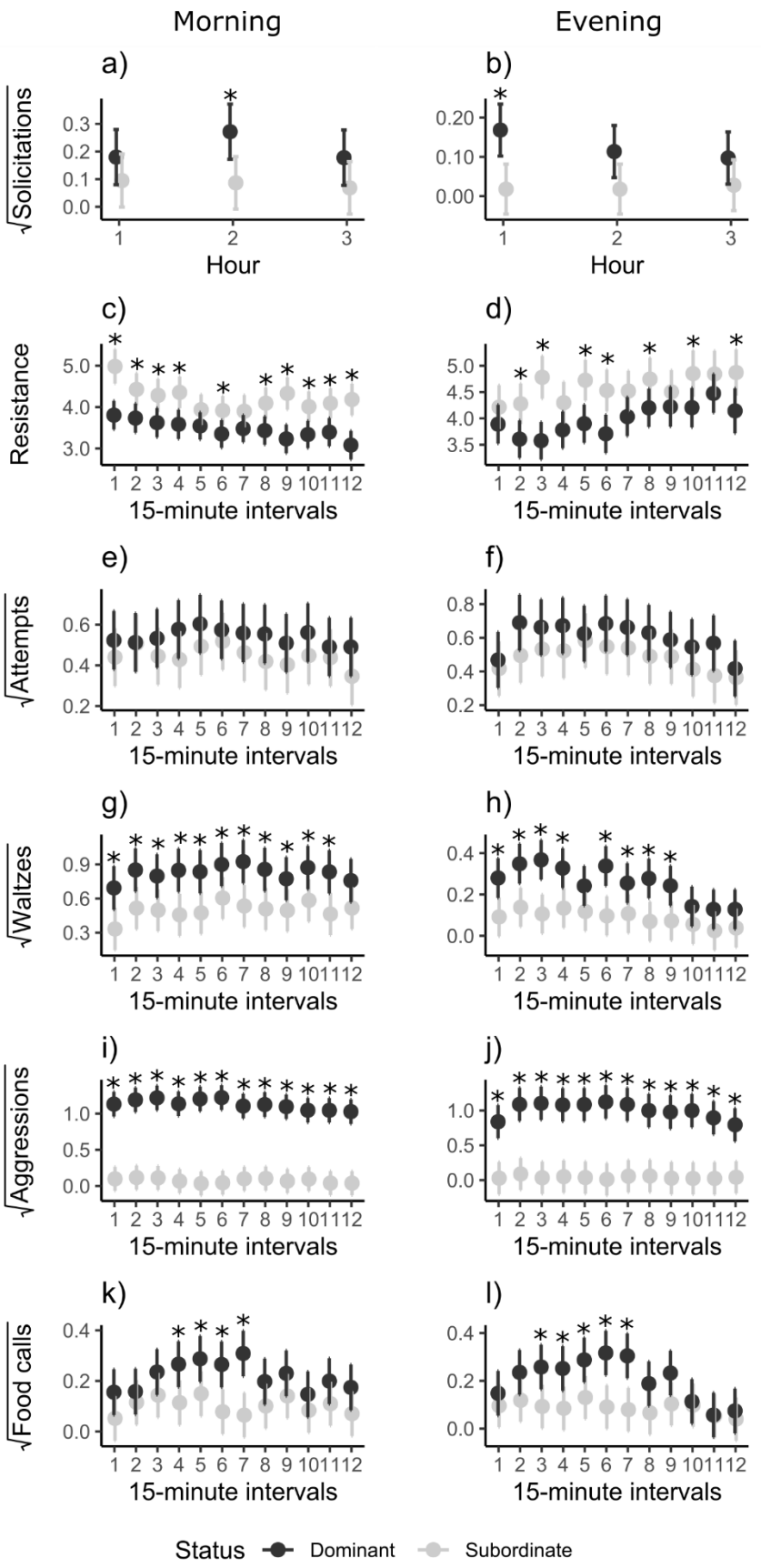

Morning
Evening
a)
b)
√Solicitations
Hour
c)
d)
Resistance
15-minute intervals
e)
f)
√Attempts
g)
h)
√Waltzes
i)
j)
√Aggressions
k)
l)
√Food calls
Status
Dominant
Subordinate

Figure 3 – Comparison of patterns in mating behaviours in the morning and in the evening between dominant (top-ranking) and subordinate (bottom-ranking) males red junglefowl (*Gallus gallus*). Panels represent the marginal effects of a linear mixed model (LMM). a-b) overall female resistance to male mating attempts, c-d) number of female mating solicitations, e-f) number of mating attempts, g-h) number of male courtship waltzes, i-j) number of aggressions initiated by males, and h-i) the number of food calls uttered by males. We used average values across days per each individual male. All behaviours were measured continuously over a period of three hours in the morning (05:00-08:00) and in the evening (18:00-21:00), but were grouped and used as discrete factors contemplating 15-minute intervals for analyses. Graphs are colour coded in relation to male social rank, bars represent the 95% confidence intervals and asterisks the level of significance of *post-hoc* Tukey tests (<0.05).

DISCUSSION

Despite a broad range of studies evaluating the relationship between environmental cues and circadian rhythms responses, much less focus has been given to its role on mating behaviour and sexual selection. Here we show that circadian patterns in different mating behaviours are highly consistent over a 10 day mating trial. The few variations in these trends were mostly driven by animals being keener to mate on the first day of the trial after being sexually rested for multiple days (Løvlie & Pizzari, 2007). Circadian patterns in female behaviour showed a remarkable consistency, but went in opposite directions in the morning and in the evening. Females tended to increase their receptivity to mating over the morning, as shown by their reducing the level of resistance to male mating attempts and by their increasing the number of mating solicitations. In the evening however, they progressively increased the level of resistance to mating attempts and reduced the number of solicitations. Circadian patterns in male mating behaviour and courtship were similar to previous studies with domestic chicken. Male mating attempts peaked at around 6:30 in the morning and showed a higher peak at 19:30 in the evening, and males increased courtship displays and food calls for most of the morning, but decreased these two behaviours over the evening. We did not find strong evidence that dominant and subordinate males attempted to peak mating activity at different hours of the day, but dominant males displayed and uttered food calls to females more often than subordinates for most of the morning and evening. Lack of temporally consistent opportunistic behaviour on the part of subordinates may be due to dominant males maintaining a consistent level of aggression towards other males throughout the morning and evening.

Females became increasingly less resistant to mating and more likely to solicit over the morning, but they behave in the opposite way over the evening. One intuitive explanation for this pattern is that females respond to increases in male mating attempts by elevating resistance. However, our results do not support this. In the morning, levels of resistance continued to decrease over the whole observation period, despite a slight increase in male mating attempts over the first 2h of observations (5:00-7:00). Similarly, levels of resistance continued to increase in the evening even at the time when male mating attempts were at their lowest levels (20:45-20:59). An alternative explanation is that females become more receptive to mating in the morning in order to top up sperm reserves in their sperm storage tubules (e.g., Bisazza et al., 2001), but become less receptive to mating after securing sperm from desirable males later in the day. This is supported by our finding that females were more likely to solicit to dominant males mid-morning and beginning of the evening, and that resistance against mating attempts from subordinate males never went below a certain threshold in the morning, while it continued to decrease in relation to dominant males. In the evening however, female resistance tended to increase, even against dominant males. This is in agreement with a previous study with feral fowl, which showed that inseminated females solicit less and resist further mating attempts more strongly than unmated females (Løvlie & Pizzari, 2005). Similarly, in many Arthropoda, recently mated females have a refractory period in which they resist more matings (e.g., Simmons & Gwaynne, 1991; Brent, 2010; Hurtado & Hasson, 2013), although sometimes this refraction is modulated by the male seminal fluid or sperm (Cook & Wedell, 1999; Perry et al., 2013). Because female red junglefowl are able to store sperm for multiple days, we expected resistance to mating would remain consistently high in later days of the trial, but females seem to reset their propensity to mate on a daily basis, suggesting a tentative role for an endogenous period length of 24h in mating receptivity. Further studies monitoring female activity for longer than 6 hours will be necessary to confirm this suggestion. However, females are still able to adjust their level of resistance in relation to male social status (Cheng & Burns, 1988; Pizzari & Birkhead, 2000; Pizzari, 2001; this study), and are able to shift receptivity to

mating in response to changes in sex-ratio (Løvlie & Pizzari, 2007), suggesting plasticity in female behaviour can be triggered by the social environment. This could be an example of masking (Schwartz et al., 2017), in which male harassment represses females from manifesting their natural mating pattern (Helm et al., 2017). Future studies could explore these ideas by testing how circadian patterns in female red junglefowl change as a function of different group densities and sex ratios (see Løvlie & Pizzari, 2007 for an example with feral fowl). These studies could have important implications for management welfare in domestic populations, as understanding circadian patterns of harassment and female receptivity may lead to measures that increase the well-being and productivity of females.

Males peaked the number of mating attempts half-way through the observations in the morning (6:30) and evening (19:30), with the evening peak being higher as previously reported in a study with domestic chickens (Cheng & Burns, 1988). More interestingly, males did not attempt to mate more often at times when females were more receptive to mating, suggesting that male and female behaviour are not well synchronized in this species. Female red junglefowl advertise her receptivity to mate by crouching in front of a male and soliciting a copulation (Pizzari & McDonald, 2019), and the hue and size of her comb can be used by males to gather information on her fecundity (Pizzari et al., 2003; Cornwallis & Birkhead, 2007). However, solicitations are rare, and mating is mostly initiated by males, so sexual conflict is bound to occur (Pizzari & McDonald, 2019). Similarly, female *Drosophila* have a clear circadian gate of mating receptivity, but males consistently court females throughout the day (Sakai & Ishida, 2001). In other groups, females advertise receptivity in a more conspicuous way, so male and female activity usually coincides. For example, in the noctuid moth (*Spodoptera littoralis*) females release pheromones at specific times in the day, and males' response to these pheromones are matched by a corresponding circadian rhythm (Silvegren et al., 2005). Therefore, whether male and female mating activity are controlled by circadian gates, and whether they are in or out of phase, will contribute to whether conflict or collaboration over mating will occur. Additionally, we found no

evidence that subordinate males attempt to mate at different times than dominant males, which does not support our hypothesis that subordinates express a different chronotype. Most mating attempts performed by subordinate red junglefowl have to be sneaky (Cheng & Burns, 1988; Pizzari & Birkhead, 2000; Pizzari, 2001), since dominant males tend to punish them by chases or beatings, which can lead to physical costs to subordinates (Carleial et al., 2020b). In other taxa, subordinate males have to correctly time their mating attempts in order to achieve some mating success (e.g., Berard et al., 1994; Taborsky, 1998; Iwata et al., 2005; Crockford et al., 2007). For example, subordinate baboons use copulation calls as a cue to access whether female pair bounding with dominant males has ended so they can attempt to mate (Crockford et al., 2007). Therefore, expressing a different chronotype could be potentially adaptive to subordinate male red junglefowl, but here they attempted to mate as often and at the same time as dominant males. One possible explanation is that the consistent level of aggressiveness maintained by dominant males throughout the observation period leaves no specific time window for subordinates to consistently exploit. However, given that we observed birds for only 6 hours, we cannot rule out the possibility that dominant males become less aggressive or vigilant during the afternoon period when females usually lay, since inseminations closer to egg laying are more inefficient then than at other times (Brillard, 2003). Future studies could investigate whether subordinate males are more active at this suboptimal time. Another possibility is that mating activity in red junglefowl can be partially synchronized through social facilitation (Davidson & Menaker, 2003). For example, exposing colonies of royal penguins (*Eudyptes schlegeli*) to continuous playbacks of conspecific vocalizations increases the number of sexual activities within the group (Waas et al., 2000). Similarly, we observed in our groups that a male mating attempt can stimulate other males to mate (GCM, personal observation), but further studies would be necessary to investigate this hypothesis.

We found that peaks in courtship activity (i.e., waltzes and food calls) tended to coincide with the time period when females were more receptive to mating (second part of the morning, and

beginning of the evening), but a causal role between the two could not be determined in this study. Previous studies with fowl have found mixed results on whether courtship activity can lead to higher mating success. While some studies have found a positive effect of courtship (Zuk et al., 1995), others have suggested that courtship increases mating success insofar as it correlates with social status (Pizzari, 2003; Wilson et al., 2008). In accordance with this, we found that dominant males tended to courtship females with waltzes and food calls more often than subordinates for most of the morning and evening. In species where male mating success is more reliant on courtship behaviour, synchronization of male courtship effort should better match female receptivity. For example, in some species of crickets, males synchronize singing activity with the phase in which female mating activity is higher (Bertram et al., 2013). Moreover, we confirmed previous studies with domestic chickens which have shown that males tend to courtship more often in the morning, rather than in the evening (Cheng & Burns, 1988). Circadian patterns in courtship activity can be driven by environmental cues such as changes in daylight. For example, in different species of passerines, males that start singing earlier at dawn achieve more extra-pair copulations, presumably because females prefer males that sing early (Poesel et al., 2006; Kempenaers et al., 2010; Greives et al., 2015). Similarly, in guppies artificially extending the light period over the night leads to longer nocturnal displays (O'Neill et al., 2019), while a similar treatment in *Drosophila* delayed the onset of courtship in the morning (McLay et al., 2018). Studies have identified a role for melatonin – a hormone involved in circadian rhythms – underpinning these patterns, as birds administered with melatonin take longer to join the dawn chorus (Greives et al., 2015; Harpole et al., 2020), with similar effects being found in some fish (Feng & Bass, 2016). To our knowledge, the role of melatonin on courtship behaviour in galliforms has not been explored, but at least one behaviour, the predawn crowing, seems to be modulated by an endogenous circadian clock in both the domestic rooster and red junglefowl (Shimmura & Yoshimura, 2006; Ito et al., 2017). Courtship display in red junglefowl is simple and fast, and males can advertise food items to females without uttering a food call, with both signals apparently being redundant (Smith & Evans,

2008). Our finding that courtship effort is more frequent in the morning, and that it decreases steadily throughout the evening, seems to suggest a role for daylight as a cue for this behaviour. This is intuitive, as females must be able to see either the courtship display itself or the food being presented, and it is also in agreement with findings that show that day light can modulate some behaviours in the fowl, for example the initiation of roosting (e.g., Kent et al., 1997). Future studies should seek to clarify the role of circadian gates in red junglefowl, as multiple modulators of photoperiodism have been shown to respond well to experimental changes in day lengths in this species (Ono et al., 2009).

In conclusion, we show that circadian patterns in mating behaviour in the red junglefowl are cyclical but that trends in behaviour may show clear divergent directions in the morning and in the evening. Females tend to increase receptivity to mating over the morning, but become more reluctant to mate over the evening, while males tend increase courtship over the morning and decrease over the evening. Males attempt to mate consistently over the morning and evening, and we did not find significant differences in chronotypes between dominant and subordinate males. Future studies would benefit from exploring whether these circadian patterns are more strongly modulated by the social environment, environmental cues, or endogenous circadian clocks. Such characterizations may not only improve our understanding of the role of behavioural timing in sexual selection, but also lead to developments in welfare and productivity in domestic chicken flocks.

Suplementary material for:
Circadian patterns in mating behaviour in red junglefowl (*Gallus gallus*)

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1. FIGURES

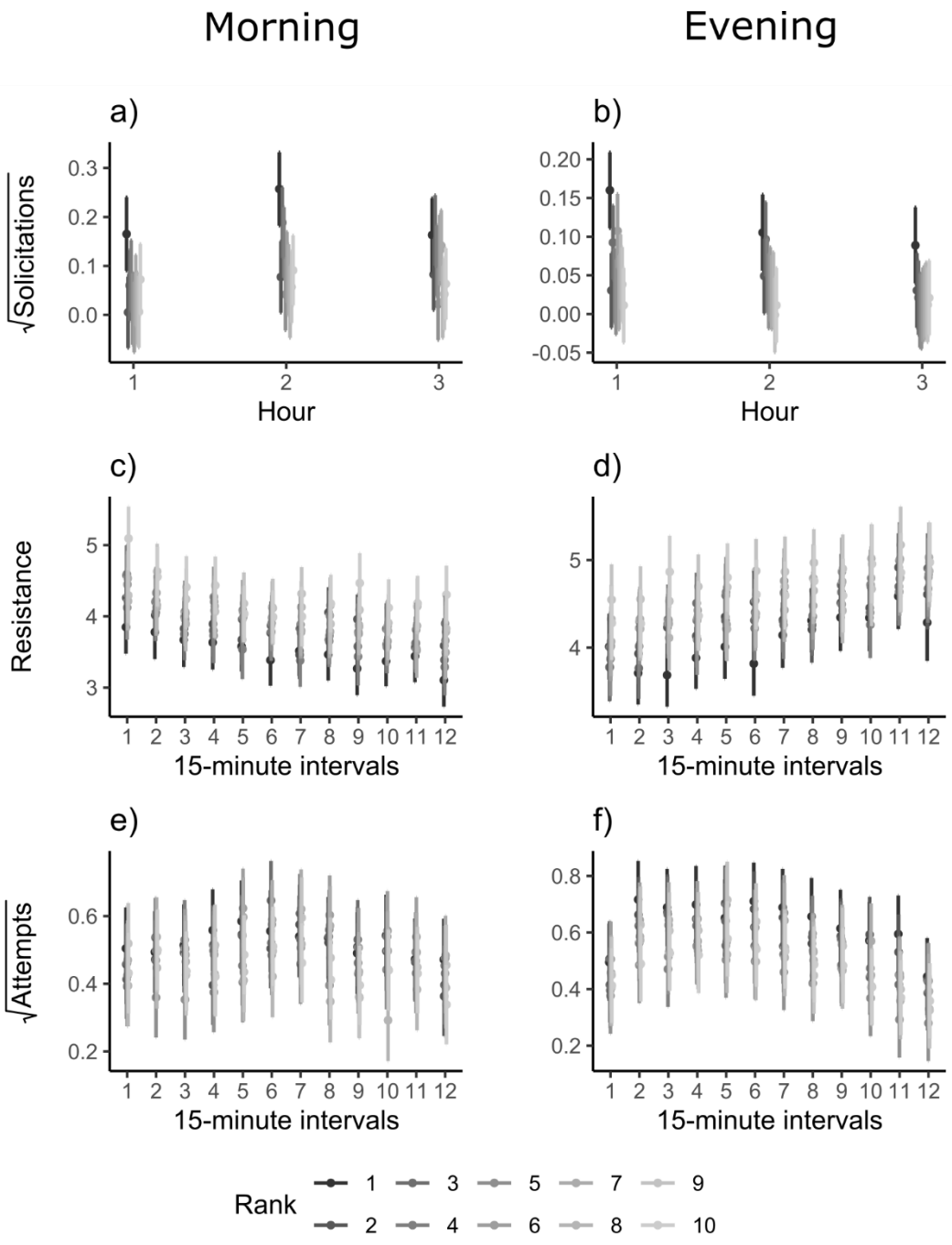


Figure S1 – Comparison of patterns in mating behaviours in the morning and in the evening between males of different social ranks (1-10) in red junglefowl (*Gallus gallus*). Panels represent the marginal effects of a linear mixed model (LMM). a-b) overall female resistance to male mating attempts, c-d) number of female mating solicitations, e-f) number of mating attempts. We used average values across days per each individual male. All behaviours were measured continuously over a period of three hours in the morning (05:00-08:00) and in the evening (18:00-21:00), but were grouped and used as discrete factors contemplating 15-minute intervals for analyses. Graphs are colour coded in relation to male social rank, bars represent the 95% confidence intervals.

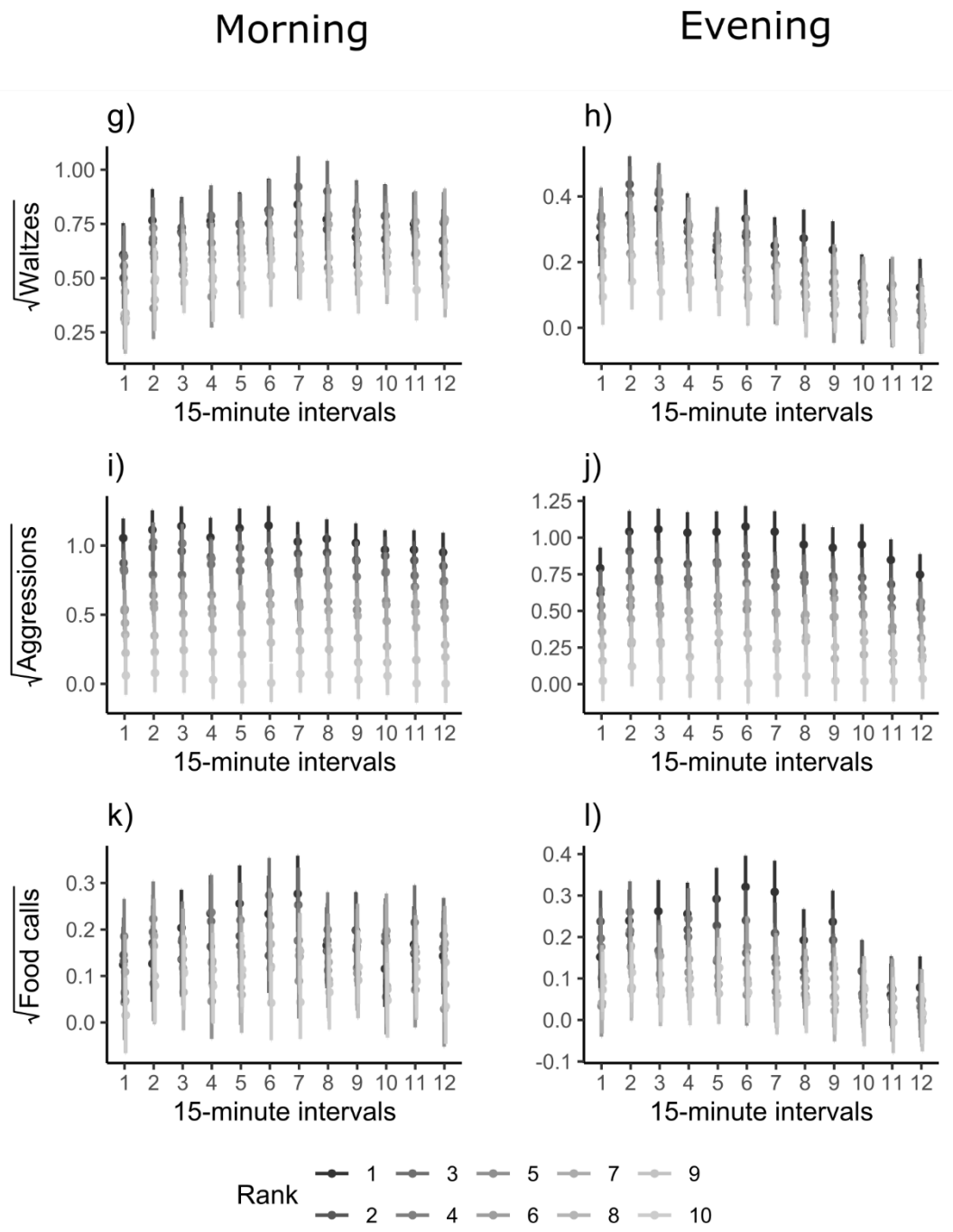


Figure S2 – Comparison of patterns in mating behaviours in the morning and in the evening between males of different social ranks (1-10) in red junglefowl (*Gallus gallus*). Panels represent the marginal effects of a linear mixed model (LMM). g-h) number of male courtship waltzes, i-j) number of aggressions initiated by males, and h-i) the number of food calls uttered by males. We used average values across days per each individual male. All behaviours were measured continuously over a period of three hours in the morning (05:00-08:00) and in the evening (18:00-21:00), but were grouped and used as discrete factors contemplating 15-minute intervals for analyses. Graphs are colour coded in relation to male social rank, bars represent the 95% confidence intervals.

CHAPTER 3

Dynamic phenotypic correlates of social status and mating effort in male and female red junglefowl, *Gallus gallus*

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Dynamic phenotypic correlates of social status and mating effort in male and female red junglefowl, *Gallus gallus*

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Abstract

Despite widespread evidence that mating and intra-sexual competition are costly, relatively little is known about how these costs dynamically change male and female phenotypes. Here, we test multiple hypotheses addressing this question in replicate flocks of red junglefowl (*Gallus gallus*). First, we test the inter-relationships between social status, comb size (a fleshy ornament) and body mass at the onset of a mating trial. While comb size covaried positively with body mass across individuals of both sexes, comb size was positively related to social status in females but not in males. Second, we test for changes within individuals in body mass and comb size throughout the mating trial. Both body mass and comb size declined at the end of a trial in both sexes, suggesting that mating effort and exposure to the opposite sex are generally costly. Males lost more body mass if they: i) were socially subordinate, ii) were chased by other males, or iii) mated frequently, indicating that subordinate status and mating are independently costly. Conversely, females lost more body mass if they were exposed to a higher frequency of coerced matings, suggesting costs associated with male sexual harassment and female resistance, although costs of mating *per se* could not be completely ruled out. Neither competitive nor mating interactions predicted comb size change in either sex. Collectively, these results support the notion that sex-specific costs associated with social status and mating effort result in differential, sex-specific dynamics of phenotypic change.

KEYWORDS

Sexual selection, social status, comb, sexual coercion, cost of mating, condition dependence, badge of status

1 | INTRODUCTION

Sexual reproduction is typically associated with costs of intra-sexual competition over access to mating and fertilizations, mating and mate choice (Foley et al., 2018; Lehtonen, Jennions & Kokko, 2012). These costs can be substantial and have been indicated as key drivers of phenotypic variation among individuals of both sexes (e.g., Andersson, 1994; Biernaskie, Grafen, & Perry, 2014; Hare & Simmons, 2019). In males, particularly in non-monogamous species, reproductive costs are largely associated with intra-sexual competition and sexual selection, promoting phenotypes that confer a competitive advantage through direct intra-sexual competition (e.g., male-male combat) and/or inter-sexual mechanisms of mate selection (Andersson, 1994; Andersson & Simmons, 2006; Darwin, 1871). Differential costs associated with mechanisms of intra-sexual competition can in principle explain variation in male competitive ability. In social species, intra-sexual competition is often regulated by social hierarchies (Drews, 1993), with dominant males having privileged access to reproductive opportunities (e.g., Cowlishaw & Dunbar, 1991; Eason & Sherman, 1995; Klinkova, Hodges, Fuhrmann, Jong, & Heistermann, 2005; Majolo, Lehmann, Vizioli, Schino, & Sapienza, 2012; McElligott et al., 2001). However, the differential costs associated with being dominant and subordinate are unclear. Some studies have shown that retaining high status is particularly stressful, while others have indicated that low status is associated with relatively high levels of physiological stress (Abbott et al., 2003; Creel, 2001; Gesquiere et al., 2011; Goymann & Wingfield, 2004; see Habig, Doellman, Woods, Olansen, & Archie, 2018 for a recent meta-analysis). Similarly, variation in attractiveness to females may be driven by differential costs associated with the expression of male ornaments or courtship (Barske, Schlinger, Wikelski, & Fusani, 2011; Hamilton & Zuk, 1982; Vehrencamp, Bradbury, & Gibson, 1989). Individual variation in sexually-selected male phenotypes may thus reflect condition-dependence and

capture additive genetic variance in the ability of an individual to sustain costly investments (Lorch, Proulx, Rowe, & Day, 2003; Rowe & Houle, 1996; Tomkins, Radwan, Kotiaho, & Tregenza, 2004). For example, recent experimental studies in invertebrates suggest that the costs of maintaining sexually-selected weaponry are felt differentially across individuals, phenotypes, and are subject to dynamic changes (Joseph, Emberts, Sasson, & Miller, 2017; Somjee, Woods, Duell, & Miller, 2018). In females on the other hand, phenotypic variation is thought to reflect mainly differential investment in fecundity and the energetic requirements associated with mating, and mate discrimination (Bakker, Künzler, & Mazzi, 1999; Clutton-Brock, 2009; Qvarnstrom & Forsgren, 1998; Wong & Candolin, 2005). Evidence that these female behaviors incur immediate costs however, remains ambiguous. For example, female resistance to male mating advances (sexual harassment) is thought to be energetically costly (Jormalainen, Merilaita, & Riihimaki, 2001; Perry, Sharpe, & Rowe, 2009), although measurements of these energetic costs are often indirect (reviewed in Fox, Head, & Jennions, 2019). Establishing how male and female phenotypes change within and among individuals in relation to intra-sexual competition and mating effort is therefore important to understand mate preferences, the maintenance of variation in sexually-selected traits, and sexual conflict (Andersson & Simmons, 2006; Arnqvist & Rowe, 2013; Dale, Delhey, Kempenaers, & Valcu, 2015; Foley et al., 2018; Joseph et al., 2018; Kokko, Brooks, Jennions, & Morley, 2003; Sánchez-Tójar et al., 2018a; Somjee et al., 2018).

Here, we characterize male and female patterns of inter-individual variation and dynamic within-individual changes associated with intra-sexual competition and mating interactions in replicate groups of red junglefowl (*Gallus gallus*). In the wild, this sexually dimorphic species forms polygynandrous social groups, characterized by sex-specific social hierarchies (Collias & Collias, 1967, 1996; Collias, Collias, Hunsaker, & Minning, 1966). Captive populations of red junglefowl and populations of the related domestic fowl (*G. domesticus*) have been extensively

studied in sexual selection research (e.g., Parker & Ligon, 2002; Pizzari & McDonald, 2019; Zuk, Thornhill, Ligon, & Johnson, 1990a). Unless populations are heavily female-biased, matings are often initiated by males, and females tend to resist the majority of these attempts, leading to mating struggles and sexual coercion (reviewed in Pizzari & McDonald, 2019). While such mating effort appears to involve considerable energetic cost for both males and females, little is known about the association between mating effort and phenotypic variation.

Socially dominant, more aggressive males are typically favored in sexual selection, through both intra-sexual and inter-sexual mechanisms (Pizzari & McDonald, 2019). There is some evidence that male body mass is positively related to the outcome of dyadic competitive contests, suggesting that achieving high social status might require condition (Ligon, Thornhill, Zuk, & Johnson, 1990). It is however unclear whether attaining high social status incurs more costs than remaining at the bottom of the hierarchy. On the one hand, low-ranking males may suffer from limited access to food and other resources; on the other, high-ranking males may face higher energetic costs to maintain dominant status via aggressive interactions. Dominant males also invest less time feeding and resting than subordinate males (Pizzari & McDonald, 2019), suggesting that over time, high-ranking males may be prone to lose weight. In addition, several studies have shown a female preference to mate with males sporting a large comb (a fleshy head ornament; reviewed in Parker & Ligon, 2002; Pizzari & McDonald, 2019). Comb expression is characterized by high phenotypic plasticity, and the significance of male comb size variation has received considerable focus. Increasing evidence indicates that comb size captures condition through sensitivity to immune challenges (reviewed in Parker & Ligon, 2007; Pizzari & McDonald, 2019). First, comb size is dependent on testosterone plasma levels (Allee, Collias, & Lutherman, 1939; Hardesty, 1931), which may depress the ability of an organism to respond to immune challenges (Folstad & Karter 1992). Consistent with this idea, male comb size responds to parasitic infections (Zuk et al., 1990a; but see Chappell, Zuk,

Johnsen, & Kwan, 1997) and domestic chicken lines artificially selected for strong immunological responses tend to display both low plasma levels of testosterone and small combs (Verhulst, Dieleman, & Parmentier, 1999). Second, comb size may also reflect the effect of free radicals released by phagocytic activation triggered by parasitic infections (von Schantz, Bensch, Grahn, Hasselquist, & Wittzell, 1999). This has led researchers to suggest that comb size can function as an honest signal of individual quality, and particularly of immunocompetence (Zuk et al., 1990a, 1995; Zuk & Johnsen, 1998, 2000). Additionally, testosterone control of male comb size has led to the suggestion that comb size may function as a badge of status (Ligon et al., 1990). Such badges are expected to evolve to signal individual quality and competitive ability, allowing individuals to minimize the risks of physical contests based on their predicted outcome (Santos, Scheck, & Nakagawa, 2011; Sánchez-Tójar et al., 2018a). Consistent with this idea, male comb size appears to be sensitive to social challenges. For example, subordinate males experience comb shrinkage in the presence of dominant males (Cornwallis & Birkhead, 2008; Zuk & Johnsen, 2000), and individuals isolated from dominance interactions experience faster comb growth than individuals that remain in flocks (Parker, Knapp, & Rosenfield, 2002). Comb size was also positively correlated with social status among males in some studies (Graves, Hable, & Jenkins, 1985; Johnsen, Zuk, & Fessler, 2001; Ligon et al., 1990; Parker et al., 2002; Zuk et al., 1990b, 1990c; reviewed in Pizzari & McDonald, 2019). Evidence for a relationship between male comb size and social status however, remains inconsistent. For example, Johnsen et al. (2001) found that comb size and, to a lesser extent, body size and body mass were associated with male social status in the first year of their study, but failed to find an association in the following year. Similarly, Ligon et al. (1990) found that comb size predicted the outcome of competitive dyadic contests between yearling red junglefowl males that had been socially isolated, but not the outcome of dyadic contests between socially familiar yearling and older males. More recently however,

comprehensive studies have produced less consistent evidence for badges of status in other avian systems. While some studies have provided evidence consistent with badges of status reliably signalling individual quality in both sexes (e.g., López-Idiáquez, Vergara, Fargallo, & Martínez-Padilla, 2016; Santos et al., 2011), others have failed to support this hypothesis (e.g., Sánchez-Tójar et al., 2018a).

As in males, social status modulates access to resources in females in both red junglefowl and domestic fowl populations (Collias, Collias, & Jennrich, 1994; Sanctuary, 1932; Shimmura et al., 2008), and evidence suggests that female comb size is associated with female social status in both taxa (Bradshaw, 1992; Collias, 1943; Forkman & Haskell, 2004; Guhl & Ortman, 1953; Martin, Beaugrand, & Lague, 1997), although body mass may also play a role (Cloutier & Newberry 2000; Zuk, Kim, Robinson, & Johnsen, 1998). Moreover, as in males, female comb size correlates with plasma levels of steroid hormones, which in turn are good predictors of female aggressiveness (Allee et al., 1939; Collias, 1943). Large-combed females usually initiate and win dominance contests or are promptly avoided by other females, suggesting that large combs are associated with a competitive advantage and might be used for social signalling and individual recognition (Guhl & Ortman, 1953). Additionally, female comb size has been shown to be positively correlated with reproductive investment (Cornwallis & Birkhead, 2007; Pizzari, Cornwallis, Løvlie, Jakobsson, & Birkhead, 2003; Wright et al., 2008), and dominant males may use female comb size as a cue during mating decisions (Cornwallis & Birkhead, 2007; Pizzari et al., 2003).

Here, we studied replicate mating groups of red junglefowl (*Gallus gallus*) to characterize variation in traits associated with intra-sexual competition and mating interactions. We first characterize variation among individuals of each sex, and then investigate dynamic changes within individual males and females over time.

2 | MATERIALS AND METHODS

2.1 | Behavioral observations

Behavioral observations were performed by a single observer (GCM) in a captive population of red junglefowl at the University of Oxford field station in Wytham, UK. We observed 20 mixed-sex groups (12 females and 10 males per group) in an outdoor arena (5.8 × 16.5m) over three breeding seasons (April–October, 2011–2013). The relatively small group size, its social cohesiveness and the size of the outdoor arena allowed for observation of the whole group at all times during the trial. Birds used in the study were sexually mature adults ranging from 1 to 7 years old. Each group was exposed to a 13-day trial. In the first three days of a trial, males and females were housed in sex-specific pens in order to allow social familiarization with the other members of the same sex and the formation of sex-specific hierarchies. We then assembled the mixed-sex mating group by placing these male and female groups together in the same experimental pen on the morning of the fourth day (Figure 1a), and conducted behavioral observations for 10 consecutive days (Figure 1b), from 5am to 9am and from 6pm to 9pm (see McDonald, Spurgin, Fairfield, Richardson, & Pizzari, 2017 for details). In total, 127 unique males and 78 unique females were used in this study, with 61 males and 48 females being reused across groups due to limitation of new unique birds (see Table S1 for a summary). In particular, the limited number of females available for the study meant that we assembled 10 unique female groups, which were used multiple times with different male groups to form 20 unique mixed-sex mating groups (Figure 1). On six occasions, a female became sick or died during a trial and was replaced immediately with a new female to maintain a constant sex-ratio among groups. Replacement females were randomly selected using the “sample” function in R to select the identity of an individual from the pool of available reproductively mature females. All agonistic interactions between males (pecks, fights, waltzes, chases and avoidances) were recorded during the 10 days of mixed-sex observations and were posteriorly used to establish individual social status (see below). We considered the loser of these interactions the male that retreated at least one body length from the other male (Froman, Pizzari, Feltmann, Castillo-Juarez, & Birkhead, 2002; Johnsen et al., 2001; Wilson, Nelson, & Evans, 2009). When it was not obvious which individual had won the interaction, the outcome was defined as a draw. Agonistic interactions between females were recorded during three days immediately before the 10 days of mixed-sex observations (i.e., day 1-3), when females were still isolated from males. Observations lasted for 3h between 11am to 6pm. We found this to be necessary given the more subtle nature of female behavior, which are harder to detect when females interact

with males. Male courtship (i.e., waltzing to a female), copulation attempts, and successful copulations (i.e., when we observed direct cloacal contact or the male tail bending towards the female cloaca) were also recorded during the 10 days of mixed-sex observations (i.e., day 4-13). Additionally, we used published methods to score the level of female resistance during copulation attempts, which ranged from 1 (female solicitation) to 6 (female was chased by the male, grabbed and sexually coerced; Løvlie, Cornwallis, & Pizzari, 2005; Løvlie & Pizzari, 2007). Starting on the second day of the trial, eggs were collected each morning up to, and including, one day post-trial. Eggs were assigned to females using parentage analyses that are described elsewhere (McDonald et al., 2017). In short, eggs were incubated artificially for at least five days, and embryo tissue samples were then collected and stored in absolute ethanol at 4^{o}C prior to parentage assignment. We disregarded any egg for which parentage was not assigned molecularly with at least 95% confidence (see McDonald et al., 2017) and the total number of identifiable eggs per female was used as the measure of her fecundity.

2.2 | Phenotypic measurements

Body mass and comb size were measured on two occasions for each bird: on the third day of a trial before the male and female groups were assembled together as a single mixed-sex mating group, and one day post-trial. On each occasion, birds were weighed to the nearest 10g with a digital hanging scale, and their combs were photographed using a Canon Ixus 107 with 12.1mb/p resolution, holding the bird at a fixed distance and in the same position in relation to the camera, with a scale attached to the wall behind the bird which allowed comb measurements to be performed digitally. To do so, we drew a polygon around the comb (Figure 1c) using the software ImageJ v1.51k, and the comb area was calculated using the scale in the photo as the reference. The pictures were chosen at random. In order to access the repeatability of comb measurements, a single observer (RC) measured the comb of a random subset of 44 individuals (24 females and 20 males) on two separate occasions (i.e., at least a week apart), and measurements were compared using a repeatability analysis implemented in the R software (R Core Team 2017, version 3.4.2) package “rptR” v0.9.2 (Stoffel, Nakagawa, & Schielzeth, 2017). The different measurements were highly repeatable ($r = 0.982$, $p < 0.001$), giving us confidence of detecting biological differences in comb size within and between individuals. Due to methodological constraints, we were unable to get estimates of skeletal body size (e.g., tarsus length) and thus the first weighting of a bird captures both skeletal size and

residual mass for size. Changes in body weight within individual birds, between the first and second weighing on the other hand, are taken to represent changes in residual body mass.

2.3 | Social status

Social status was estimated using the randomized Elo-rating method implemented in the R package "aniDom" v.0.1.4 (Farine & Sánchez-Tójar 2017; Sánchez-Tójar, Schroeder, & Farine, 2018). In the original Elo-rating method (Elo, 1978; Neumann et al., 2011), individuals start out with equal ratings, which are updated after each dyadic agonistic interaction, with winners gaining points and loser losing points. The amount of points gained or lost in each interaction is dependent upon the scores of the two individuals at the moment of the interaction: a higher-rated individual receives fewer points if it wins against a lower-rated individual, whereas the latter receives more points if it wins against a higher-rated individual. The randomized method expands on this by generating replicate datasets, which randomize the order of the dyadic interactions, yielding more robust estimates of Elo scores (Sánchez-Tójar et al., 2018b). We assessed the repeatability of 1,000 replicates for each of the 20 male groups, and the individual's mean cumulative Elo score (i.e., the score held at the last day of the trial) calculated from these randomizations was used as the measurement of social status. Because draws were rare (1.18% of males' agonistic interactions), they were removed from calculations of Elo scores, following McDonald & Shizuka (2012). We followed a similar protocol with females, however in this case, we calculated mean cumulative Elo scores using randomizations of the dyadic agonistic interactions that took place during the first three days when the female group was isolated from the males. No draws were recorded among females, so all agonistic interactions were used to calculate Elo scores. Additionally, we calculated the transitivity (i.e., linearity) of the dominance hierarchies of each sex using the triangle transitivity method (t_{tri}, *sensu* Shizuka & McDonald 2012) and the stability (i.e., how often individuals swap ranks) of these hierarchies using the modified stability index (S_t, Neumann et al., 2011; McDonald & Shizuka 2012), both of which are implemented in the R package "EloRating" v0.46.8 (Neumann et al., 2011).

On average, we recorded 231.69 (SD = 141.97) agonistic interactions per individual male over the 10 days of a trial, and 41.27 (SD = 28.04) agonistic interactions per female over the three days of observation preceding the trial, i.e., prior to the introduction of males. These numbers of interactions are well above the sampling effort recommended by a previous study (i.e., n = 20; Sánchez-Tójar et al., 2018b), suggesting that hierarchies were reliably estimated.

The mean repeatability of randomized Elo scores was 0.93 (SD = 0.06) in males, and 0.95 (SD = 0.04) in females. Male dominance hierarchies (Figure S1) were relatively stable ($\overline{S_t}$ = 0.91; SD = 0.06) and linear ($\overline{t_{tri}}$ = 0.79, SD = 0.16). Female dominance hierarchies (Figure S2) were on average slightly less stable ($\overline{S_t}$ = 0.83; SD = 0.09) and more linear ($\overline{t_{tri}}$ = 0.86, SD = 0.16) than male dominance hierarchies.

2.4 | Statistical analyses

We used linear mixed effects models implemented in the R packages "lme4" 1.1-21 (Bates, Mächler, Bolker, & Walker, 2015) and "lmerTest" 3.1-0 (Kuznetsova, Brockhoff & Christensen 2017). All models included individual identity, female group identity (i.e., the identity of the unique group of 12 females used in each trial, Figure 1a) and mixed-sex mating group identity as random effects to control for the pseudo-replication arising from the use of repeated individuals and to allow the intercept of each group to vary. Model residuals were explored for any violation of assumptions (e.g., heteroscedasticity and non-normality) and collinearities among covariates were tested with the variance inflation factor (VIF) method implemented in the package "car" v3.0-3 (Fox & Weisberg 2019). We followed Zuur, Leno, & Elphick (2010) by considering any VIF ≥ 3 as a sign of collinearity, in which case the covariate with higher VIF was removed. *P* values and denominator degrees of freedom for fixed effects were calculated using *F* tests with Satterthwaite's approximation. In presenting statistical inference, we attempt to follow recent recommendations (Amrhein, Greenland & McShane 2019) within the journal's format.

2.4.1 | Inter-individual relationships between status, comb size and body mass.

We first characterized among individual males and among individual females, the relationship between body mass and comb size, between body mass and social status, and between social status and comb size, and female traits. We investigated whether body mass or comb size were related to social status by modelling the individual Elo score as the response variable and body mass and comb area as explanatory variables. The individual's age (in years) was modelled as a covariate in these models to control for any confounding effect age may have on social status (e.g., via greater social experience). For females, we also modelled fecundity as a covariate, since previous work indicates that measures of female fecundity in fowl populations are correlated with female social status (Collias et al., 1994; Sanctuary, 1932)

and comb size (Cornwallis & Birkhead, 2007; Pizzari et al., 2003; Wright et al., 2008). The inclusion of female fecundity as a covariate controlled for any potential confounding effect of fecundity on status and comb size variation. Finally, there is evidence that relationships between female comb size and other phenotypic traits tend to show patterns of non-linearity (Pizzari et al., 2003; Wright et al., 2008), so we explored this possibility by including a quadratic component in the model.

2.4.2 | Intra-individual temporal changes

We tested whether body mass and comb size showed consistent changes within individual males and within individual females across time. We compared the first measurement, taken the day before birds were moved to the experimental mixed-sex mating group, with the second measurement, taken on the day after the trials were complete. Body mass or comb size was used as the response variable and treatment (before/after) as the independent variable. For logistical reasons (i.e., single observer), trials were run sequentially within a breeding season, leading to variation in the time of year each trial was conducted e.g., early *versus* late in the breeding season (variation in seasonality). To exclude the possibility of variation in our data being driven by such seasonality alone, we investigated whether changes in body mass or comb size varied consistently over the breeding season. To do so, the first day of the earliest trial across the three years of study was set as 0 and later groups were numbered according to the number of days elapsed from day 0 to the day that trial began. This continuous variable therefore indicates the stage of the breeding season in which each group was initiated, and was then used as a proxy of seasonality. Changes in phenotype were calculated by subtracting the measurement of initial body mass or comb size from the second measurement. Following Kelly & Price (2005), we calculated the amount of change between two measurements that is expected solely due to the regression to the mean effect (i.e., when due to measurement error, individuals that are far away from the mean tend to approximate the mean in a posterior measurement), and discounted this amount from the original differences of phenotypic measurements. We only used individuals for which we had information on both phenotypic measurements (i.e., initial and final comb size or body mass), for example, birds that were swapped during the mating trial or that were incorrectly measured were not included (see sample sizes in table results).

We investigated whether the rate of phenotypic change within males and females were dependent on a bird's social status. We used the mean cumulative Elo score (i.e., across all

randomizations) held by the individual following his last interaction over the course of the trials (3 days in the case of females and 10 days in the case of males) as the measure of social status. This cumulative value was modelled as the independent variable and used to predict changes in body mass and comb sizes in both sexes. Because we had information on male hierarchies over the course of the 10-day mating trial (as opposed to only 3 days for females), we tested whether the stability of a male's position in the dominance hierarchy was related to consistent changes in body mass and comb size. We did this in two ways. First, stability was represented by the number of days, over the 10-day mating trial, in which a male changed social rank (i.e., ended the day in a different rank than he had started). Stability ranged from 0, if the male never changed rank, to 9, for a male that finished each day in a different rank. Therefore, regardless of how many positions the male climbed or lost in the hierarchy during a single day, we regarded it as a single change (i.e., going from 2nd to 9th or 6th to 7th in the hierarchy were both expressed as one change), with these discrete ranks being derived from daily measurements of Elo scores. We hypothesized that frequent changes in rank would be stressful to males and have a negative impact in their body mass and comb size. However, changes in the social rank of a focal male might also arise as indirect consequence of changes in the rank of other males, without the focal male being necessarily involved in agonistic interactions (see Strauss & Holekamp, 2019, for similar argument about 'passive' processes). To avoid the potentially confounding effect of these indirect processes and capture the potential costs of agonistic interactions associated with changes in social rank, we also considered the total number of agonistic interactions of a focal male as an alternative proxy of social stress. We hypothesized that a higher number of interactions would reflect a higher cost for the focal male. Additionally, we investigated whether the number of times a male chased a rival, or was chased by him, impacted his body mass and comb size.

Finally, we investigated potential costs of mating effort in driving dynamic changes in the expression of male and female phenotypes. For mating behavior, we tested whether courtship (i.e., number of waltzes performed by a male or received by a female), mating success (i.e., number of unique copulation partners) and the total number of copulation attempts (both successful and unsuccessful) are associated with changes in body mass or comb size in either sex. We hypothesized that the energetic investment by more sexually active individuals would lead to losses in body mass and reduction in comb size. Additionally, we tested whether the number of coerced copulation attempts suffered by females (scores from 4 to 6 as described in Løvlie et al., 2005) are associated with changes in female body mass and comb size. Coerced

copulations represent a subset of the total number of copulation attempts. We predicted the stress of harassment by males would lead to a decrease in body mass and comb size, and that females that were exposed to higher levels of sexual harassment would lose more body mass and suffer a greater reduction in comb size. We also investigated whether a female's fecundity (number of eggs laid during a trial) was associated with changes in her body mass and comb size. Given that more fecund females tend to be more sexually active (e.g., Løvlie & Pizzari, 2007; McDonald, Spurgin, Fairfield, Richardson, & Pizzari, unpublished), we predicted that higher fecundity would expose females to more intense sexual harassment, leading to greater loss in body mass and reduction in comb size.

3 | RESULTS

3.1 | Inter-individual relationships between status, comb size and body mass

Body mass and comb size were positively inter-correlated in both males (LMM: $F_{1,192.7} = 29.23$, $p < 0.001$, estimate ± SE: 0.68 ± 0.13) and females (LMM: $F_{1,208.6} = 21.86$, $p < 0.001$, estimate ± SE: 0.21 ± 0.04), so both variables were entered as covariates when we analysed their relationship with social status. We found that across groups, when age was controlled for, there was a statistically significant and positive association between body mass and social status in males (LMM: $F_{1,133.5} = 29.94$, $p < 0.001$, estimate ± SE: 1.25 ± 0.23, Figure 2a) but not in females (LMM: $F_{1,187.4} = 0.02$, $p = 0.896$, estimate ± SE: 0.02 ± 0.18, Figure 2c). Conversely, there was no statistically significant association between comb size and status in males (LMM: $F_{1,124.7} = 0.02$, $p = 0.901$, estimate ± SE: 0.01 ± 0.10, Figure 2b), but in females there was a statistically significant and positive linear relationship between comb size and social status (LMM: $F_{1,143.3} = 10.23$, $p = 0.002$, estimate ± SE: 1.98 ± 0.62), and evidence for a statistically weaker negative quadratic relationship (LMM: $F_{1,137.3} = 3.97$, $p = 0.048$, estimate ± SE: <-0.01 ± <0.01, Figure 2d). Despite these overall patterns, there was considerable variation among groups in the relationships between comb size and status, and between body mass and status (Figure S3).

3.2 | Intra-individual temporal changes

We found a statistically significant decrease in body mass (males: LMM: $F_{1,245.5} = 103.78$, $p < 0.001$, estimate ± SE: 60.1 ± 5.9; females: LMM: $F_{1,376.7} = 83.15$, $p < 0.001$, estimate ± SE: 39.41 ± 4.32) and comb size (males: LMM: $F_{1,249.4} = 7.99$, $p = 0.005$, estimate ± SE: 28.5 ± 10.1; females: LMM: $F_{1,378.6} = 36.69$, $p < 0.001$, estimate ± SE: 14.6 ± 2.41) in both sexes during the 10 days of a trial in which males and females mixed and interacted freely. We found no evidence that the decline in body mass varied consistently over the breeding season in neither males (LMM: $F_{1,17} = 2.90$, $p = 0.107$, estimate ± SE: 0.25 ± 0.15, Figure S4a) or females (LMM: $F_{1,16.1} = 1.72$, $p = 0.209$, estimate ± SE: -0.19 ± 0.15, Figure S4c). Similarly, there was no evidence that the decline in female comb size was consistently affected by seasonality (LMM: $F_{1,17} = 2.94$, $p = 0.105$, estimate ± SE: -0.14 ± 0.08, Figure S4d). However, there was a statistically significant tendency for the male comb to shrink more as the breeding season progressed (LMM: $F_{1,18.1} = 6.02$, $p = 0.025$, estimate ± SE: -0.73 ± 0.29, Figure S4b). Accounting for this effect in the following analyses did not affect qualitatively our results, so we do not report it here.

After controlling for any regression to the mean, males that were initially heavier lost more body mass than initially lighter males (LMM: $F_{1,134.1} = 35.42$, $p < 0.001$, estimate ± SE: -0.11 ± 0.02), and large-combed males suffered a larger degree of comb shrinking than males with initially smaller combs (LMM: $F_{1,185.4} = 4.64$, $p = 0.033$, estimate ± SE: -0.03 ± 0.01), and these relationships were independent of social status. We therefore entered initial body mass or comb size as a covariate in all statistical models in which body mass change or comb size change (respectively) were used as the response variable. Controlling for initial body mass, we found a statistically significant and positive association between social status and body mass change, such that males of higher status either gained or tended to lose less mass over the course of the 10-day trial (LMM: $F_{1,187.3} = 21.52$, $p < 0.001$, estimate ± SE: 0.03 ± <.01, Figure

3a). On average, each standard deviation (SD) increment in Elo score resulted in a 19.7g increment in body mass. We found that neither the number of times a male swapped rank (LMM: $F_{1,192} = 1.02$, p = 0.314, estimate ± SE: 1.47 ± 1.45), nor the total number of agonistic interactions he engaged (LMM: $F_{1,94.7} = 2.84$, p = 0.095, estimate ± SE: -0.05 ± 0.03), were statistically associated with changes in his body mass. We found a statistically borderline non-significant trend for males that mated with more females to have lost more body mass (LMM: $F_{1,170} = 3.67$, p = 0.057, estimate ± SE: -2.13 ± 1.11), whereas there was no statistically significant association between the total number of copulation attempts and body mass change in males (LMM: $F_{1,154.8} = 1.17$, p = 0.282, estimate ± SE: -0.07 ± 0.06). However, when the positive effect of social status was controlled for, we found a statistically significant association between an increased loss in body mass and the number of females mated (i.e., mating success, LMM: $F_{1,177.8} = 9.40$, p = 0.003, estimate ± SE: -3.31 ± 1.08, Figure 3b), or the total number of copulation attempts (LMM: $F_{1,138} = 4.58$, p = 0.034, estimate ± SE: -0.14 ± 0.06, Figure 3c). One SD increment in mating success translated into 11.9g of body mass loss on average, whilst one SD increment in copulation attempts translated into 8.47g of body mass loss. There was no statistically significant association between the number of times a male courted a female (i.e., number of waltzes) and changes in his body mass (LMM: $F_{1,146.3} < 0.01$, p = 0.972, estimate ± SE: <0.01 ± 0.06), although there was a statistically weak relationship between these variables when social status was controlled for (LMM: $F_{1,159.6} = 3.22$, p = 0.075, estimate ± SE: -0.11 ± 0.06). Males that chased other males more often tended to lose less or gain more body mass (LMM: $F_{1,195.4} = 5.26$, p = 0.023, estimate ± SE: 0.10 ± 0.04), however when the confounding effect of social status was controlled for, this relationship was no longer statistically significant (LMM: $F_{1,179.4} = 0.76$, p = 0.384, estimate ± SE: -0.05 ± 0.05). On the other hand, males that were chased more often by other males tended to lose more body mass (LMM: $F_{1,172.5} = 13.26$, p = 0.001, estimate ± SE: -0.22 ± 0.06), and evidence for this relationship,

albeit statistically weaker, remained after controlling for status (LMM: $F_{1,136.2} = 4.02$, p = 0.047, estimate ± SE: -0.13 ± 0.06, Figure 3d). One SD in the number of chasing episodes translated on average into 8.22g lost in body mass. While male body mass changes were related to social status, aggression and some aspects of mating effort, we found little evidence for similar patterns in changes in male comb size. There was no statistically significant association between male comb size change and social status (LMM: $F_{1,177.4} = 2.58$, p = 0.110, estimate ± SE: 0.02 ± 0.01), chasing other male rivals (LMM: $F_{1,186.1} = 2.79$, p = 0.097, estimate ± SE: 0.11 ± 0.06), or being chased by them (LMM: $F_{1,195.4} = 1.55$, p = 0.215, estimate ± SE: -0.13 ± 0.10). Similarly, there was no evidence for a statistically significant relationship between comb size changes and changes in social rank (LMM: $F_{1,195.9} = 0.40$, p = 0.834, estimate ± SE: 0.54 ± 2.57) or the total number of agonistic interactions (LMM: $F_{1,194.6} = 0.81$, p = 0.369, estimate ± SE: 0.04 ± 0.05). Finally, we did not find any statistically significant association between comb size change and any of the measures of male mating effort (LMMs: mating success: $F_{1,194.3} = 0.71$, p = 0.399, estimate ± SE: -1.52 ± 1.80; copulation attempts: $F_{1,196} = 0.06$, p = 0.803, estimate ± SE: 0.03 ± 0.11; courtship: $F_{1,189.8} = 0.29$, p = 0.591, estimate ± SE: 0.05 ± 0.09).

As with males, females with initially larger combs experienced more comb shrinking (LMM: $F_{1,218.4} = 4.18$, p = 0.042, estimate ± SE: -0.03 ± 0.01), even after accounting for any regression to the mean. Therefore, we entered initial comb size as a covariate in all the statistical models where change in comb size was the response variable. There was no evidence for a statistically significant relationship between female social status and changes in body mass (LMM: $F_{1,198.5} = 0.10$, p = 0.751, estimate ± SE: <-.01 ± <.01, Figure 4a) or comb size (LMM: $F_{1,212.4} = 0.21$, p = 0.644, estimate ± SE: <.01 ± <.01). Similarly, there was no evidence of a statistically significant relationship between female mating success (i.e., polyandry) and changes in body mass (LMM: $F_{1,170.5} = 2.39$, p = 0.124, estimate ± SE: -2.73 ± 1.77, Figure 4b) or between female mating success and comb size change (LMM: $F_{1,213} = 1.06$,

p = 0.305, estimate ± SE: -0.81 ± 0.79). There was no statistically significant association between the total number of copulation attempts experienced by a female and changes in her body mass (LMM: $F_{1,118.9} = 1.96$, p = 0.164, estimate ± SE: -0.13 ± 0.09, Figure 4c), and there was only a statistically weak tendency for the comb of more sexually active females to shrink more than the comb of less sexually active females (LMM: $F_{1,207.5} = 3.24$, p = 0.073, estimate ± SE: -0.08 ± 0.04). When only coerced copulations (i.e., male sexual harassment) were considered, females exposed to more sexual harassment appeared to lose more body mass (LMM: $F_{1,152.3} = 4.31$, p = 0.039, estimate ± SE: -0.29 ± 0.14, Figure 4d). One SD increment in the number of coerced copulations suffered by a female translated on average into an 8.03g body mass loss in females. We further attempted to disentangle a potential confounding effect of mating from male harassment by entering the total number of copulation attempts as a covariate. In this new analysis, coerced copulations remain statistically associated, albeit weakly, with changes in body mass (LMM: $F_{1,208.3} = 3.20$, p = 0.075, estimate ± SE: -2.73 ± 1.77). However, because male harassment is a subset of total copulation attempts, the collinearity between the total number of copulation attempts and coerced copulations is predictably higher than the threshold we had set *a priori* (VIF = 5.16). Therefore, these results must be interpreted with caution. A similar, albeit statistically weaker, pattern was observed when we considered the effect of coerced copulations on comb size changes (LMM: $F_{1,225.3} = 3.44$, p = 0.065, estimate ± SE: -0.11 ± 0.06). There was no statistically significant association between the number of courtship events that a female received and changes in body mass (LMM: $F_{1,202.9} = 1.99$, p = 0.159, estimate ± SE: -0.12 ± 0.09) or comb size (LMM: $F_{1,231.7} < 0.01$, p = 0.982, estimate ± SE: <-.01 ± <.01). Finally, there was no statistically significant relationship between female fecundity and changes in body mass (LMM: $F_{1,211.5} = 1.32$, p = 0.252, estimate ± SE: -1.09 ± 1.65) or comb size (LMM: $F_{1,226.7} = 1.04$, p = 0.308, estimate ± SE: -0.74 ± 0.73).

4 | DISCUSSION

Investigating inter- and intra-individual patterns of variation in phenotypes associated with reproduction is critical to further our understanding of sex-specific reproductive costs, sex-specific selection, sexual conflict over reproductive decisions, condition-dependence and the significance of ornaments and ornament preference. In the present study, we used replicate groups of red junglefowl to explore how inter- and intra-individual variation in body mass and comb size are related to social status and different behaviors related to mating effort in both males and females. First, we showed that body mass, but not comb size was associated with social status across males, while comb size, but not body mass was associated with social status across females. Second, over the course of a trial, both individual males and females on average tended to lose body mass, likely reflecting costs associated with activities related to exposure to the opposite sex, e.g., mating and intra-sexual competition. Importantly, subordinate males lost more body mass than more dominant males, and this was likely due, at least in part, to subordinate males being chased more often by other males. In addition, males that mated more frequently and females that suffered more coerced copulations lost more body mass. Over the course of a trial, combs also became smaller on average in both individual males and females, suggesting that relative comb size reflects these general costs. However, we found no strong evidence that comb size changes within individuals were associated with individual social status or mating effort in either sex.

We show that, for a given comb size, body mass is positively associated with social status across males. This relationship may in part capture an effect of body size, because variation in the first measurement of body weight will reflect both skeletal size and residual body mass. Larger males may have a competitive advantage in social contests and thus be more likely to attain high rank in the hierarchy in fowl populations (Cornwallis & Birkhead, 2008; Graves et al., 1985; Johnsen et al., 2001; Ligon et al., 1990; Wilson et al., 2009). Conversely,

we show that, for a given body mass, comb size is not statistically associated with male social status. This finding is inconsistent with some previous studies that have indicated a relationship between male comb size and social competitive ability (e.g., Graves et al., 1985; Johnsen et al., 2001; Ligon et al., 1990). For example, Ligon et al. (1990) showed that large-combed males tend to be more aggressive and therefore more likely to initiate and win fights. It is however difficult to assess the independent role of comb size given that males with large combs are often heavier (Cornwallis & Birkhead, 2008; Graves et al., 1985; Johnsen et al., 2001; Ligon et al., 1990; this study). Here we attempted to overcome this difficulty by controlling statistically for the effect of body mass when we investigated the association between comb size and social status. Moreover, several studies have used pairs of males with pre-selected differences in comb size, whereas here, comb size and status were analyzed under arguably more natural conditions, as continuous variables within larger groups of freely-interacting males. Our results are consistent with the idea that intra-sexual selection favors larger, heavier males, but do not support the idea that independent variation in comb size is informative of male social status. However, comb size may covary with male status only to the extent to which comb size is associated with male body size/mass. Finally, our finding that male comb is an unreliable indicator of social status complements recent findings in other species challenging the idea that male ornaments in many cases may function as 'badges of status' (Sánchez-Tójar et al., 2018a).

These results have implications for patterns of intra- *versus* inter-sexual episodes of sexual selection, given that females have been shown to prefer males with large combs (Ligon & Zwartjes, 1995a, 1995b; Zuk et al., 1990b, 1990c, 1995). Our results call into question the adaptive significance of female preference for male comb size since, in addition to the lack of association between comb size and social status as described above, we found little evidence that male comb size is particularly sensitive to costs associated with mating effort and intra-

sexual competition. Although males generally experienced comb shrinking over the course of a trial, suggesting that relative comb size can reflect general costs associated with exposure to females, we failed to find consistent changes in comb size in relation to male status or mating effort, suggesting that relative changes within males are unlikely to inform females of male status or condition. An important caveat is that status-dependent comb size differences may require a longer period of time to emerge, e.g., through the reinforcement of hierarchical relationships between males, than that allowed in our study. This does not appear likely however, given that birds were given 3 days to establish sex-specific hierarchies prior exposure to the opposite sex, and the study detected status-dependent variation in body mass. The lack of a relationship between male comb size and social status has implications for the maintenance of variation. If large-combed males were also dominant on average, female choice would align with, and reinforce, intra-sexual selection on dominance and its covariate body mass (Hunt, Breuker, Sadowski, & Moore, 2009). On the other hand, a lack of positive covariance between social status and comb size combined with female preference for large-combed males would contribute to the maintenance of variation. In addition, the extent to which male status and comb size influence female preference may be socially modulated. For example, Johnsen et al. (2001) showed that, in small red junglefowl flocks comprising three females and two males, top- and second-ranking females consistently mated with the dominant male independently of his comb size, while bottom-ranking females tended to mate with the subordinate male if he had a larger comb. However, female preference for large combs may not be actualized if female access to preferred males is curtailed by dominant males (Cheng & Burns, 1988; Dean, Nakagawa, & Pizzari, 2011; Pizzari, 2001).

As with males, female comb tended to shrink over the course of the trial, but we did not find any consistent association between these changes and the female status or mating frequency. However, we did find that across individuals, dominant females tended to have a

larger comb, which is in line with the majority of previous studies (e.g., Bradshaw, 1992; Collias, 1943; Forkman & Haskell, 2004; Guhl & Ortman, 1953; Martin et al., 1997) with few exceptions. Studying an inter-cross of domestic strains, Cloutier and Newberry (2000) found that female status was associated with comb size in one of two trials and with body weight across both trials. Similarly, Zuk et al. (1998) found that in red junglefowl groups, female social status was associated with body weight but not comb size. Differences in the relationship between social status and comb size in females *versus* males in the present study could reflect the social circumstances under which social status was measured in different sexes (e.g., Procter, Moore & Miller, 2012). While male social hierarchies were based on interactions in mixed-sex groups, female social hierarchies were assessed in single sex groups. More generally, the relationship between female status and comb size likely emerges as a function of female aggressiveness, which together with comb size, is modulated by circulating levels of steroid hormones (Allee et., 1939; Collias, 1943; Guhl & Ortman, 1953). Dominant females initiate most of the aggressive interactions with their peers (Kim & Zuk, 2000), and females injected with testosterone climbed their way up the pecking order when reintroduced into their previous flock (Allee et al., 1939), whereas the opposite occurred with females that had their combs surgically removed (Marks, Siegel, & Kramer, 1960). This is interesting since, while female ornamentation is often explained as simply the result of a genetic correlation with male ornamentation (Lande, 1980), recent studies indicated that ornaments may fulfill adaptive functions in females (Amundsen, 2000). For example, female ornaments may increase mate acquisition by signalling fecundity (Domb & Pagel, 2001; Potti, Canal, & Serrano, 2013) or may evolve as a function of female competition for resources (e.g., Heinsohn, Legge, & Endler, 2005). In the fowl, female comb size has been positively associated with egg investment (Cornwallis & Birkhead, 2007; Pizzari et al., 2003; Wright et al., 2008), and evidence suggests that dominant males prefer females with larger combs (Cornwallis & Birkhead, 2007; Pizzari et al., 2003). Therefore, the covariance

between status and comb size suggests that large-combed, dominant females may have preferential access to resources and mating opportunities, likely underpinning the positive female Bateman's gradient described for this species (Collet et al., 2014). Supporting this possibility, Collias et al. (1994) and recent work with this population (McDonald et al., unpublished) have shown that, controlling for age, dominant females tend to have higher reproductive success. The negative quadratic relationship between female comb size and status found in our study is similar to curvilineal relationships between female comb size and both egg number and weight found by Wright et al. (2008). These patterns suggest that, beyond a certain size, comb size is no longer informative of female status and egg investment.

Both sexes tended to lose body mass during the course of a trial. Similarly, Zuk and Johnsen (2000) found that both subordinate and dominant males lost body mass after being moved from male-only pens to experimental pens with females. In both Zuk and Johnsen (2000) and our present study, birds had *ad libitum* food and water, and the main difference from their pre-experiment housing was larger group size and exposure to the opposite sex, suggesting that the overall loss in body mass was at least partially driven by activities associated with courting, mating effort, and intra-sexual competition over access of mating opportunities. Supporting this conclusion, we show that, when holding status constant, males that mated more often tended lose more mass, highlighting a possible cost of mating. This cost may have been exacerbated in our study due to the polyandry of these groups, demanding males to re-mate often with a female to defend paternity in sperm competition (McDonald et al., 2017). An increase in copulation frequency in the presence of competitors has been described in other species of birds (e.g., Crowe et al., 2008; Sax, Hoi, & Birkhead, 1998). While studies have typically focused on cost of mating to females (Daly, 1978) there is increasing evidence that exposure to females and mating can exact substantial costs on males. Costs associated with courting females and mating have been documented in several invertebrate species (Cordts &

Partridge, 1996; Kotiaho, 2000; Mappes, Alatalo, Kotiaho, & Parri, 1996; Paukku & Kotiaho, 2005) and in some vertebrates. For example, in insectivorous marsupials, polyandry and a brief mating season select for male investment in testis size and longer copulation, which leads to immune collapse followed by death (Fisher, Dickman, Jones, & Blomberg, 2013). In a less extreme example, Preston, Stevenson, Pemberton, & Wilson (2001) showed that dominant male Soay sheep (*Ovis aries*) which copulate frequently and earlier in the breeding season, benefit from early reproductive success, but suffer a decline in reproductive performance due to sperm depletion later on the breeding season. Similarly, a recent study with mosquitofish (*Gambusia holbrooki*) showed that males raised with females grew slower, and showed reduced immune response, compared to sexually-naïve males, highlighting possible costs of mating (Iglesias-Carrasco, Fox, Vincent, Head, & Jennions, 2019).

Independent of mating costs, we also find evidence of a cost of social competition in males. Both tenure of high and low social rank have been suggested to be associated with substantial energetic and physiological costs. Here we show that in red junglefowl groups, subordinate males tended to lose more body mass than dominant males, suggesting that subordination is relatively costly. In accordance with this idea, we were able to show that the degree of body mass loss in a male was predicted by the frequency at which he was chased by other males. In fowl, chasing subordinate males, which is sometimes followed by physical aggression, is a frequent behavior that dominant males use to interrupt copulation attempts from subordinates and reinforce their dominance status (Cheng & Burns, 1988; Dean et al., 2011; Pizzari, 2001). The loss in body mass associated with subordinate status and the frequency at which subordinates were chased is in agreement with a vast body of literature that has found an association between stressing factors and subordination in captive populations (Abbott et al., 2003; Creel, 2001; Goymann & Wingfield, 2004). For example, a decrease in body mass is a frequently described effect of social stress in subordinate laboratory rats

(reviewed in Tamashiro et al., 2005). Alternatively, high and low rank may be associated with similar costs but dominant males find these costs easier to bear. This explanation would be consistent with our finding that heavier males are more likely to achieve a higher rank, and could in part be mediated by the privileged access of dominant males to resources such as food, perching and dustbathing spots (Pizzari & McDonald, 2019).

Finally, our study presents new evidence that male sexual coercion may lead to costs to females, as shown by the increased loss in body mass in females that experience frequent male harassment. However, because sexual harassment is conflated with the act of mating, we cannot completely rule out a contribution of mating *per se* to losses in body mass. We find this to be unlikely however, since the total number of copulation attempts was not statistically associated with changes in female body mass. More generally, we show that these costs to females are comparable to the rate of body mass loss suffered by males. For example, the body mass reduction on average associated with each SD increment in male mating success (11.91g) represents approximately 0.85% of a male body mass (average 1392.55g). Similarly, SD increments in copulation attempts or being chased by other males correspond on average to 0.61% and 0.59% of average male body mass, respectively. In comparison, we show that one SD increment in sexual harassment suffered by females corresponds on average to approximately 0.77% loss of female body mass (average 1035.29g). The energetic cost of male harassment is likely one of the selective pressures leading to female strategies of male avoidance (Løvlie & Pizzari, 2007; Pizzari, 2001). This pattern of sexual conflict is expected when intra-sexual competition for mating opportunities is intense among males (Chapman, Arnqvist, Bangham, & Rowe, 2003; Parker, 2006). Female costs associated with mating, sexual coercion and male harassment have been well documented (Arnqvist & Rowe, 2005). This evidence however is often restricted to lab-adapted invertebrate populations, and female costs of mating are rarely empirically quantified in more natural populations, although they are likely

substantial. For example, female water striders (*Aquarius remigis*) struggling to reject attempted copulations from males can spend around 200% more energy carrying the harassing male on their back, compared with normal locomotion (Watson, Arnqvist, & Stallmann, 1998). Extreme cases where females are injured or even killed by males have been documented in several vertebrates (e.g., Chilvers, Robertson, Wilkinson, Duignan, & Gemmell, 2005; Le Galliard, Fitze, Ferriere, & Clobert, 2005; Réale, Boussès, & Chapuis, 1996). In our study females could have lost body mass due to the energetic expenditure of struggling against males and/or due to high stress levels as a consequence of persistent harassment. Alternatively, the loss in body mass could have been a consequence of male disruption of feeding behavior. For example, male sexual harassment substantially decreases female foraging time in both guppies (*Poecilia reticulate*) and Atlantic mollies (*P. mexicana*) (Magurran & Seghers, 1994; Plath, Parzefall, & Schlupp, 2003), whereas females of the solitary bee *Anthophora plumipes* are forced to feed in less rewarding parts of the flower to escape male harassment (Stone, Road, & Ox, 1995). These results suggest a striking potential for the structure of social groups to influence the condition and productivity of groups through costs imposed to females. By reducing female condition, male harassment is likely to increase the male bias of the operational sex ratio of the population, thus exacerbating the intensity of sexual harassment suffered by the few females that are reproductively available, triggering a negative feedback loop which may lead to local population extinction (Le Galliard et al., 2005).

In conclusion, our study helps resolve the relationship between a fleshy ornament (comb size) and social status in fowl. We demonstrate that body mass, rather than comb size, is consistently associated to status in males, which suggests that female choice and male-male competition target different male traits. Importantly, we show that body mass changes dynamically over time in response to social status and mating effort in males, and in response to male sexual harassment in females. These results highlight the sex-specific costs of mating

and their implications for temporal changes in an individual's phenotype over a mating season. Our results also show how such dynamics can have important ramifications for the maintenance of variation in sexually selected traits. Future studies should explore the way in which these patterns are modulated by factors such as seasonality, group size and sex-ratio. This work may also inform better management of productivity and animal welfare in commercial flocks and captive populations (Pizzari, 2016).

4.1 | Data archiving

Data archival statement: Data will be made available on DRYAD.

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TABLES AND FIGURES

Table 1 - Linear mixed effect models (LMMs) results for the relationship between multiple traits and social status in male and female red junglefowl (*Gallus gallus*).

	Male Elo-score				
Fixed effects	***Estimate***	***SE***	***F***	***ddf***	***p***
Intercept	-756.7	251.8	-	137.13	-
Age	-1.8	23.33	0.01	118.09	0.939
Body mass	1.25	0.23	29.94	133.5	**<.001**
Comb size	0.01	0.1	0.02	124.72	0.901
Random Effects					
σ^2	227123.3				
T_{00} Male Identity	35531.5				
T_{00} Group Identity	1.12^{-14}				
T_{00} Female group Identity	2.37^{-15}				
Observations	200				
	Female Elo-score				
Fixed effects	***Estimate***	***SE***	***F***	***ddf***	***p***
Intercept	334.84	183.46	-	167.8	-
Age	90.56	15.88	32.52	79.61	**0.002**
Body mass	0.02	0.18	0.02	187.41	0.897
Comb size	1.98	0.62	10.23	143.29	**<.001**
Comb size^2	-0.002	<0.001	3.98	137.3	**0.048**
Fecundity	15.54	6.39	5.89	207.92	**0.016**
Random Effects					
σ^2	22754.11				
T_{00} Female Identity	35871.47				
T_{00} Group Identity	0.11				
T_{00} Female Group Identity	1441.02				
Observations	226				

Notes: SE = standard error; *F* = *F* statistics; ddf = denominator degrees of freedom; σ^2 = residual variance; τ00 = random intercept variance. P values of fixed effects are based on F tests with Satterthwaite's approximation, and are highlighted in bold when results are statistically significant ($p < 0.05$).

Table 2 - Linear mixed effect models (LMMs) results for the relationship between social status and multiple behaviors on changes in body mass and comb size in male red junglefowl (*Gallus gallus*).

Full model	*n*	*Fixed effects*	*Estimates*	*SE*	*F*	*ddf*	*p*	σ^2	τ00
BMc ~ BM	200	Intercept	152.17	25.88	-	140.34	-	2813	271.5
		Body mass	-0.11	0.02	35.42	134.1	**<.001**		
BMc ~ BM* + Elo	200	Intercept	183.92	25.75	-	143.54	-	2388.63	430.41
		Body mass	-0.16	0.02	60.56	153.22	**0.015**		
		Elo score	0.03	<.01	21.52	187.27	**<.001**		
BMc ~ BM* + Elo* + C^a	200	Intercept	184.39	25.79	-	142.83	-	2385.8	437.95
		Body mass	-0.16	0.02	61.43	154.11	**<.001**		
		Elo score	0.04	0.01	16.63	192.91	**<.001**		
		Chase (actor)	-0.05	0.05	0.76	179.39	0.384		
BMc ~ BM* + Elo* + C^r	200	Intercept	190.65	25.8	-	147.2	-	2332.74	446.75
		Body mass	-0.15	0.02	54.52	154.84	**<.001**		
		Elo score	0.03	0.01	11.87	195.72	**0.001**		
		Chase (receiver)	-0.13	0.06	4.02	136.21	**0.047**		
BMc ~ BM* + Elo* + TC	200	Intercept	211.88	28.27	-	165.18	-	2283	497.62
		Body mass	-0.17	0.02	67.18	166.25	**<.001**		
		Elo score	0.04	0.01	25.43	182.91	**<.001**		
		Total copulations	-0.14	0.06	4.58	138	**0.034**		
BMc ~ BM* + Elo* + Co	200	Intercept	190.79	25.96	-	150.83	-	2309.93	485.64
		Body mass	-0.16	0.02	62.07	157.04	**<.001**		
		Elo score	0.04	0.01	25.2	185.94	**<.001**		
		Courtship	-0.11	0.06	3.22	155.59	0.075		
BMc ~ BM* + Elo* + MS	200	Intercept	213.84	26.99	-	157.25	-	2197.97	526.79
		Body mass	-0.17	0.02	68.13	159.91	**<.001**		
		Elo score	0.04	0.01	27.93	189.07	**<.001**		
		Mating success	-3.31	1.08	9.4	177.77	**0.003**		
BMc ~ BM* + Elo* + Rs	200	Intercept	176.76	26.92	-	153.31	-	2384.91	435.37
		Body mass	-0.16	0.02	59.61	154.69	**<.001**		
		Elo score	0.04	0.01	21.79	194.71	**<.001**		
		Rank stability	1.47	1.45	1.02	192.04	0.314		
CSc ~ CS	199	Intercept	49.73	25.55		171.23		7002	1804.56
		Comb size	-0.03	0.01	4.64	185.39	**0.033**		
CSc ~ CS* + Elo	199	Intercept	41.03	26.06	-	130.77	-	6934	1834.33
		Comb size	-0.04	0.02	6.06	184.6	**0.015**		
		Elo score	0.02	0.01	2.58	177.43	0.11		
CSc ~ CS* + C^a	199	Intercept	44.03	25.73	-	170.52	-	6912.3	1884.1
		Comb size	-0.03	0.01	5.09	184.33	**0.025**		
		Chase (actor)	0.11	0.06	2.79	186.06	0.097		
CSc ~ CS* + C^r	199	Intercept	53.26	25.79	-	120.8	-	6995.86	1770.31
		Comb size	-0.03	0.01	3.47	186.98	0.064		
		Chase (receiver)	-0.13	0.1	1.55	195.4	0.215		
CSc ~ CS* + TC	199	Intercept	47.46	27.2	-	178.11	-	7033.11	1821.46
		Comb size	-0.03	0.01	4.64	184.62	**0.033**		

		Total copulations	0.03	0.11	0.06	195.99	0.803		
CSc ~ CS* + Co	199	Intercept	47.47	25.93	-	173.57	-	7028.52	1806.92
		Comb size	-0.03	0.01	4.88	185.57	**0.028**		
		Courtship	0.05	0.09	0.29	189.82	0.591		
CSc ~ CS* + MS	199	Intercept	57.32	27.1	-	179.82	-	7021.44	1775.63
		Comb size	-0.03	0.01	4.09	185.8	**0.045**		
		Mating success	-1.52	1.8	0.71	194.28	0.399		
CSc ~ CS* + Rs	199	Intercept	47.54	27.66	-	176.73	-	7036.48	1812.14
		Comb size	-0.03	0.01	4.61	184.5	0.033		
		Rank stability	0.54	2.57	0.04	195.91	0.834		

Notes: * = covariates; BM = Body mass; BMc = Body mass change; C^a = Number of chases (actor); C^r = number of chases (receiver); Co = Courtship (i.e., number of waltzes); CS = Comb size; CSc = Comb size change; Elo = Elo score; MS = Mating success; Rs = Rank stability; TC = Total copulations; n = sample size; SE = standard error; F = F statistics; ddf = denominator degrees of freedom; σ^2 = residual variance of the random effects; $\tau 00$ = sum of the random intercept variances of male identity, female group identity and group identity. P values of fixed effects are based on F tests with Satterthwaite's approximation, and are highlighted in bold when results are statistically significant ($p < 0.05$). For more detailed information on predictors see methods.

Table 3 - Linear mixed effect models (LMMs) results for the relationship between social status and multiple behaviors on changes in body mass and comb size in female red junglefowl (*Gallus gallus*).

Full model	*n*	*Fixed effects*	*Estimates*	*SE*	*F*	*ddf*	*p*	σ^2	τ00
BMc ~ BM	220	Intercept	58.36	30.32	-	70.85	-	2866.2	244.65
		Body mass	-0.06	0.03	3.78	69.45	0.056		
BMc ~ Elo	200	Intercept	4.51	12.73	-	207.18	-	2920.81	173.09
		Elo score	<-.01	<.01	0.1	198.49	0.751		
BMc ~ CC	200	Intercept	11.71	7.09	-	52.89	-	2946.97	114.01
		Coerced copulations	-0.29	0.14	4.31	152.25	**0.039**		
BMc ~ TC	200	Intercept	9.79	8.21	-	55.53	-	2967.88	129.35
		Total copulations	-0.13	0.09	1.96	118.92	0.164		
BMc ~ Co	200	Intercept	9.18	7.92	-	72.71	-	2937.58	175.25
		Courtship	-0.12	0.09	1.99	202.9	0.159		
BMc ~ F	200	Intercept	7.19	7.68	-	72.64	-	2951.13	168.07
		Fecundity	-1.09	1.65	1.32	211.48	0.252		
BMc ~ MS	200	Intercept	15.66	10.99	-	104.38	-	2953.96	140.63
		Mating success	-2.76	1.79	2.39	170.58	0.124		
CSc ~ CS	235	Intercept	5.15	4.63	-	34.12	-	540.72	120.4
		Comb size	-0.03	0.01	4.18	218.43	**0.042**		
CSc ~ CS* + Elo	235	Intercept	3.78	6	-	78.82	-	537.23	123.56
		Comb size	-0.03	0.01	4.02	216.06	0.046		
		Elo score	<.01	<.01	0.21	212.35	0.644		
CSc ~ CS* + CC	235	Intercept	10.1	5.26	-	55.27	-	541.19	101.32
		Comb size	-0.03	0.01	4.53	217.38	**0.035**		
		Coerced copulations	-0.11	0.06	3.44	225.32	0.065		
CSc ~ CS* + TC	235	Intercept	11.54	5.8	-	65.83	-	541.54	102.74
		Comb size	-0.03	0.01	5.04	217.59	**0.026**		
		Total copulations	-0.08	0.04	3.24	207.52	0.073		
CSc ~ CS* + Co	235	Intercept	5.2	5.29	-	48.07	-	543.07	120.89
		Comb size	-0.03	0.01	4.13	218.38	**0.044**		
		Courtship	<-.01	<.01	<.01	231.72	0.982		
CSc ~ CS* + F	235	Intercept	8.05	5.41	-	54.8	-	542.61	114
		Comb size	-0.03	0.01	4.37	216.92	**0.038**		
		Fecundity	-0.74	0.73	1.04	226.73	0.308		
CSc ~ CS* + MS	235	Intercept	9.67	6.4	-	88.53	-	543.27	112.48
		Comb size	-0.03	0.01	4.22	217.4	**0.041**		
		Mating success	-0.81	0.79	1.06	213.04	0.305		

Notes: * = covariates; BM = Body mass; BMc = Body mass change; CC = Number of coerced copulations; Co = courtship (i.e., number of waltzes received); CS = Comb size; CSc = Comb size change; Elo = Elo score; F = fecundity; MS = Mating success; TC = Total copulations; n = sample size; SE = standard error; F = F statistics; ddf = denominator degrees of freedom; σ^2 = residual variance of the random effects; τ00 = sum of the random intercept variances of female identity, female group identity and group identity. P values of fixed effects are based on F tests with Satterthwaite's approximation, and are highlighted in bold when results are statistically significant ($p < 0.05$). For more detailed information on predictors see methods.

Suplementary material for:
Dynamic phenotypic correlates of social status and mating effort in male and female red junglefowl (*Gallus gallus*)

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Supplementary table:

Table S1 - Summary of the number of times the same individual was re-used across replicate groups of red junglefowl (*Gallus gallus*)

MaleID	N	MaleID	N	MaleID	N	MaleID	N	FemaleID	N	FemaleID	N	FemaleID	N
1	1	9	2	C14	1	G10	2	108	6	B67	5	E32	3
10	1	A18	1	C2	1	G11	1	19	4	B70	2	E33	3
11	2	B1	1	C3	2	G12	1	23	4	C16	4	E39	3
12	3	B10	1	C4	1	G13	1	25	3	C17	1	E40	3
208	3	B11	1	C5	2	G14	1	26	4	C21	1	E41	2
2907	3	B12	1	C6	2	G15	1	30	3	C22	2	E43	4
3	2	B13	2	C7	2	G16	1	4407	1	D30	3	E46	3
308	2	B15	2	C8	2	G2	2	46	3	D31	5	E48	3
31	3	B17	1	C9	1	G3	1	50	4	D32	2	E49	3
33	3	B18	3	D1	1	G4	1	52	6	D33	3	E51	4
35	3	B19	1	D10	1	G5	1	56	4	D34	3	E52	4
37	3	B2	1	D11	1	G6	1	58	6	D35	3	J32	1
3906	3	B20	1	D12	2	G9	1	60	3	D37	3	J34	3
3907	2	B21	2	D13	2	H24	2	62	6	D38	3	J43	2
4	2	B22	2	D14	2	H25	2	66	6	D39	1		
408	1	B23	1	D18	1	H26	1	70	4	D41	3		
41	1	B24	1	D19	2	H29	2	A27	3	D42	1		
43	2	B25	1	D2	1	H31	1	A29	4	D43	2		
45	3	B26	2	D20	2	H36	1	A31	4	D44	3		
47	2	B29	2	D22	1	H39	1	B33	3	D45	3		
49	1	B3	2	D23	2	H40	1	B34	4	D46	3		
508	2	B30	2	D25	1	J1	1	B35	5	D47	3		
53	1	B31	1	D27	2	J12	1	B37	3	D48	3		
55	2	B4	1	D3	2	J15	2	B39	2	D51	2		
59	1	B5	1	D6	1	J19	1	B42	2	D52	3		
6	1	B8	1	D7	2	J2	2	B44	2	D53	3		
63	2	B9	1	D8	1	J3	1	B46	1	D54	4		
65	3	C1	1	D9	2	J5	1	B49	1	E23	3		
67	3	C10	1	E1	1	J6	1	B55	3	E24	4		
7	2	C11	1	E2	2	J7	2	B56	4	E25	3		
71	2	C12	1	E3	2	J8	2	B59	1	E26	4		
8007	2	C13	1	G1	2			B64	4	E28	4		

Supplementary figures:

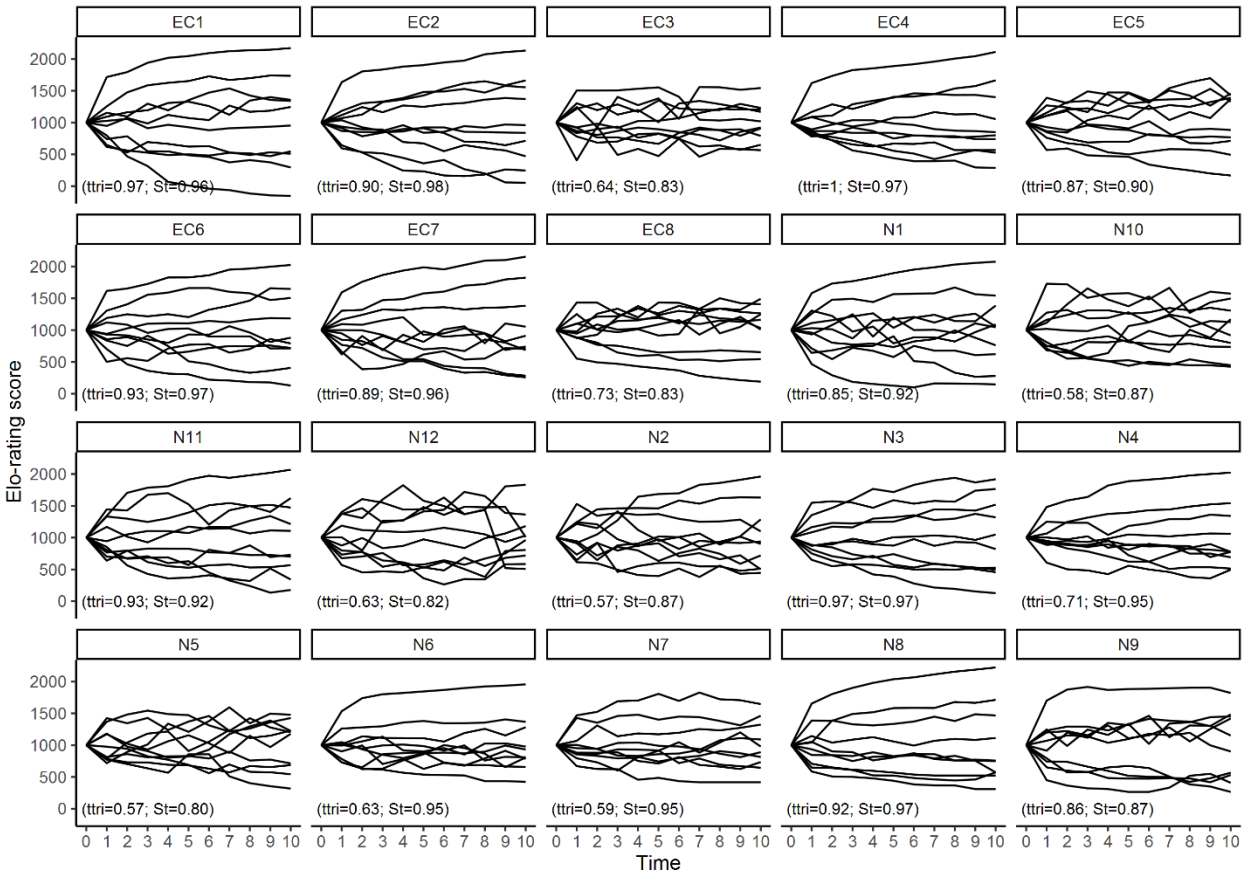


Figure S1 – Trajectories of males' Elo-rating scores over the 10 days of mixed-sex trials. Each panel represents one of the 20 replicate groups, with each individual line representing a male red junglefowl (*Gallus gallus*). In parenthesis we provide the dominance hierarchy transitivity (t_{tri}) and stability (S_t). The former measures the proportion of transitive triads in the hierarchy, and the latter measures how often males swap ranks. Values range from 0 (less transitive or stable) to 1 (more transitive or stable).

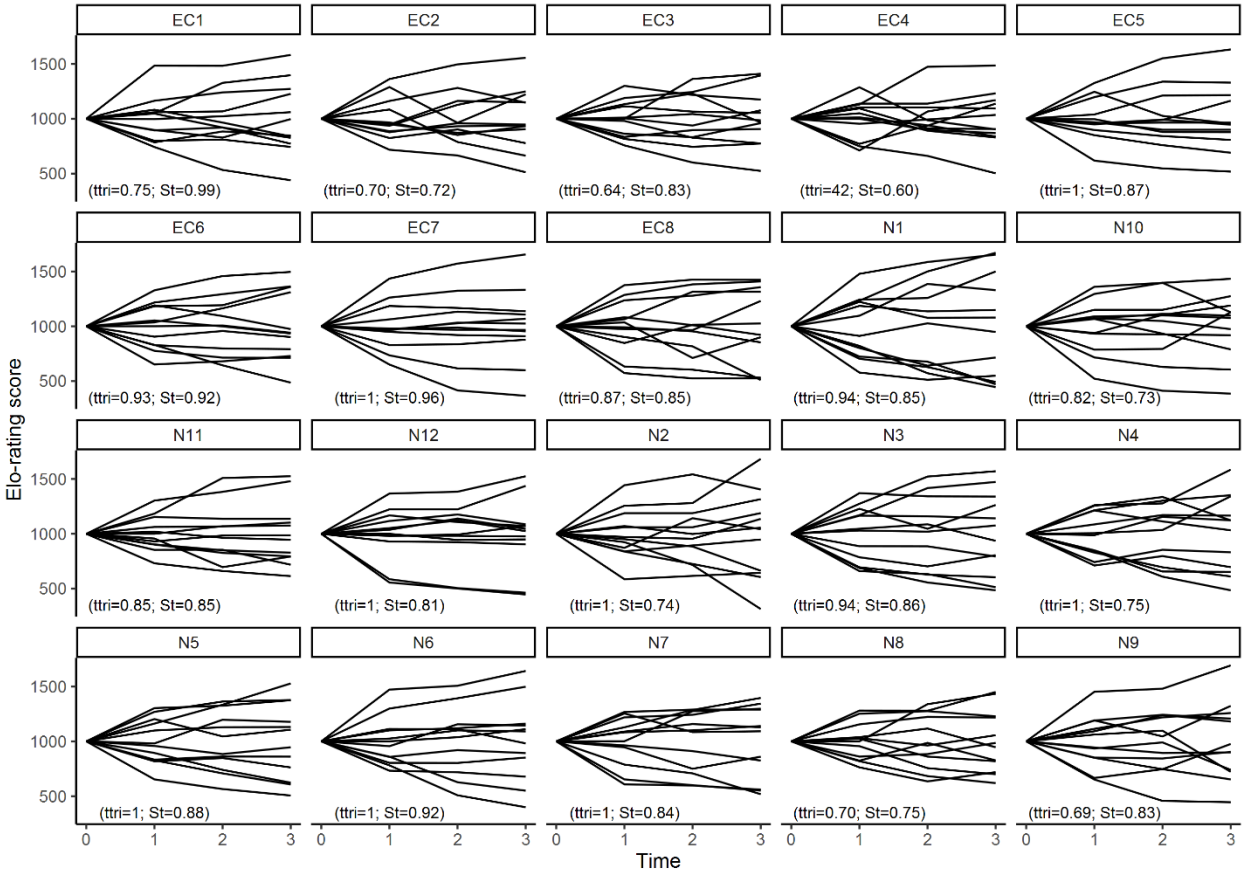


Figure S2 – Trajectories of females' Elo-rating scores over the three days of female-only trials. Each panel represents one of the groups of 12 females used in each mixed-sex trial, with each individual line representing a female red junglefowl (*Gallus gallus*). In parenthesis we provide the dominance hierarchy transitivity (t_{tri}) and stability (S_t). See figure S1.

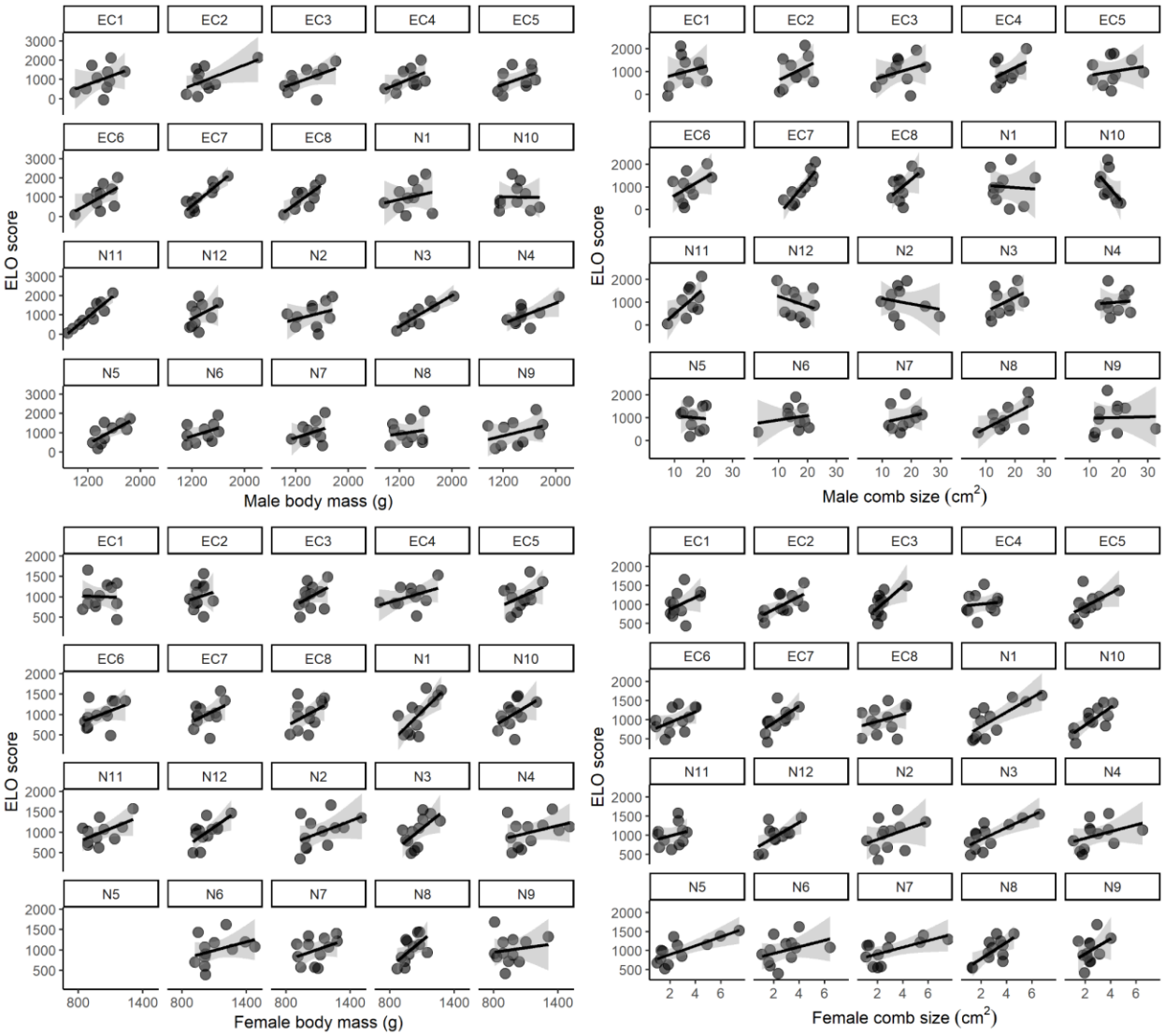


Figure S3 – Relationship between body mass or comb size and social status across the 20 mixed-sex replicate groups of red junglefowl (*Gallus gallus*). Males are represented on the top two graphs and females at the bottom. Data points represent individual birds, with darker regions indicating data point overlaps. Shaded areas around the regression line represent the 95% confidence intervals. We lacked information on female body mass for group N5.

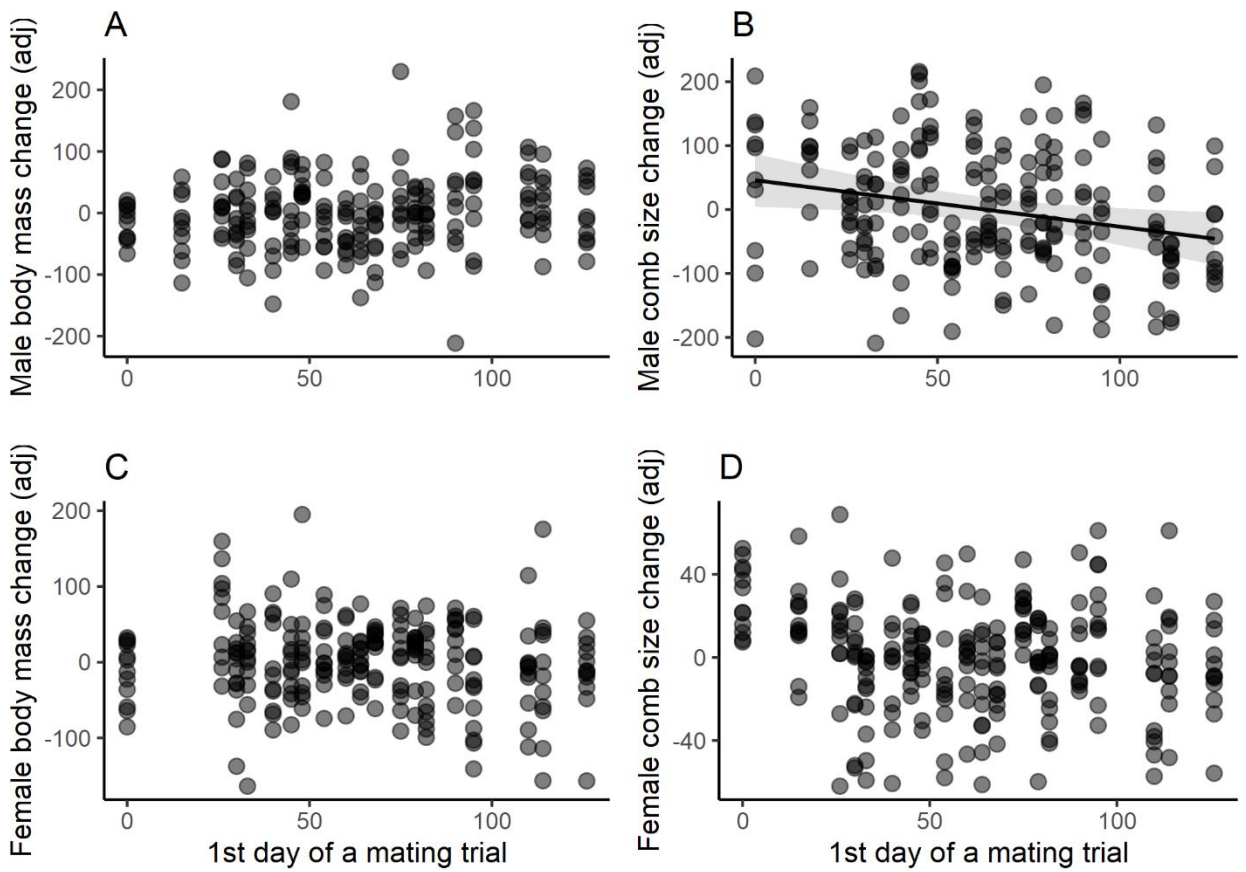


Figure S4 – Variation in body mass and comb size change in relation to seasonality in male and female red junglefowl (*Gallus gallus*). Changes in body mass over the breading season in males (A) and females (C); Changes in comb size over the breading season in males (B) and females (D). Data points represent individual birds, with darker regions indicating data point overlaps. Shaded areas around the regression line represent the 95% confidence intervals.

```
#######R SCRIPT FOR MAIN FINDINGS IN "Dynamic phenotypic correlates of social
#######status and mating effort in male and female red junglefowl, Gallus gallus"

# Clear directory
rm(list=ls())

# load required packages
library(sjPlot)
library(lme4)
library(ggplot2)
library(dplyr)
library(lmerTest)
library(car)

full_male # Male dataset
full_female # Female dataset

###Relationship between male status, body mass comb size and age

mod1<- lmer(meanELO_SCORE ~ Body_Mass_Before + Comb_Area_Before + Age +

              (1|MaleID) + (1|femalegroupID) + (1|groupID), full_male)


plot(mod1) # Check homoscedasticity


qqnorm(resid(mod1)) # Check for non-normality of the residuals
qqline(resid(mod1))


vif(mod1) # Check for collinearity of the predictors
```

```
Body_Mass_Before Comb_Area_Before             Age
        1.761621         1.409007         1.484716


anova(mod1) # Hypothesis testing


Type III Analysis of Variance Table with Satterthwaite's method
                  Sum Sq Mean Sq NumDF  DenDF F value   Pr(>F)
Body_Mass_Before 6799558 6799558     1 133.50 29.9377 2.13e-07 ***
Comb_Area_Before    3568    3568     1 124.72  0.0157   0.9005
Age                 1344    1344     1 118.09  0.0059   0.9388
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


## Plot the marginal effect of body mass on Elo score


plot_model(mod1, terms = "Body_Mass_Before”, type = "pred",
           show.data = T)


###Relationship between female status, body mass comb size,
###age and fecundity


mod2 <- lmer(meanELO_SCORE ~ Comb_Area_Before + I(Comb_Area_Before^2)

             + Age + N_eggs + Body_Mass_Before + (1 | groupID) +

             (1 | FemaleID) + (1|femalegroupID), full_female)


plot(mod2) # Check homoscedasticity


qqnorm(resid(mod2)) # Check for non-normality of the residuals
qqline(resid(mod2))


vif(mod2) # Check for collinearity


     Comb_Area_Before I(Comb_Area_Before^2)                  Age
            11.489889             11.445135             1.294611

               N_eggs      Body_Mass_Before
             1.021512              1.429766   # CS and (CS)² are collinear,
                                              # but this was expected

anova(mod2)


Type III Analysis of Variance Table with Satterthwaite's method
                     Sum Sq Mean Sq NumDF   DenDF F value    Pr(>F)
Comb_Area_Before      232848  232848     1 143.289 10.2343  0.001697 **
I(Comb_Area_Before^2)  90490   90490     1 137.300  3.9773  0.048097 *
Age                   739829  739829     1  79.606 32.5177 1.932e-07 ***
N_eggs                134106  134106     1 207.915  5.8944  0.016041 *
Body_Mass_Before         385     385     1 187.406  0.0169  0.896645
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


# Plot the marginal effect of comb size on elo score


plot_model(mod2, terms = "Comb_Area_Before[all]",
           type = "pred", show.data = T)


###Association between male status and changes in body mass


mod3<- lmer(Corrected_mass ~ Body_Mass_Before + meanELO_SCORE +
```

```
                        (1|MaleID) + (1|femalegroupID) +

                        (1|groupID), full_male)

plot(mod3) # Check homoscedasticity


qqnorm(resid(mod3)) # Check for non-normality of the residuals
qqline(resid(mod3))


vif(mod3) # Check for collinearity

Body_Mass_Before    meanELO_SCORE
        1.269319         1.269319

anova(mod3)


Type III Analysis of Variance Table with Satterthwaite's method
                 Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
Body_Mass_Before 144650  144650     1 153.22  60.558 9.869e-13 ***
meanELO_SCORE     51391   51391     1 187.27  21.515 6.578e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


###Association between times being chased and changes in body mass,
###controlling for social status and initial body mass in males

mod4<- lmer(Corrected_mass ~  Body_Mass_Before + meanELO_SCORE

             + Chased + (1|MaleID) + (1|femalegroupID) +

             (1|groupID), full_male)


plot(mod4) # Check homoscedasticity


qqnorm(resid(mod4)) # Check for non-normality of the residuals
qqline(resid(mod4))


vif(mod4)


Body_Mass_Before    meanELO_SCORE           Chased
        1.307959         1.527056         1.205271


anova(mod4)


Type III Analysis of Variance Table with Satterthwaite's method
                 Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
Body_Mass_Before 127182  127182     1 154.84 54.5205 8.871e-12 ***
meanELO_SCORE     27677   27677     1 195.72 11.8648 0.0006998 ***
Chased             9372    9372     1 136.21  4.0177 0.0470072 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1



###Association between mating success and changes in body mass,
###controlling for social status and initial body mass in males

mod5<- lmer(Corrected_mass ~ Body_Mass_Before + meanELO_SCORE +

            Mating_success + (1|MaleID) + (1|femalegroupID) +

            (1|groupID), full_male)

plot(mod5) # Check homoscedasticity


qqnorm(resid(mod5)) # Check for non-normality of the residuals
qqline(resid(mod5))
```

```
vif(mod5)


Body_Mass_Before    meanELO_SCORE   Mating_success
        1.276993         1.313250         1.039250


anova(mod5)


Type III Analysis of Variance Table with Satterthwaite's method
                 Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
Body_Mass_Before 149756  149756     1 159.91 68.1337 5.384e-14 ***
meanELO_SCORE     61390   61390     1 189.07 27.9302 3.446e-07 ***
Mating_success    20651   20651     1 177.77  9.3954  0.002515 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1



###Association between number of coerced copulation and changes in body mass,
###controlling for social status and initial body mass in females



mod6 <- lmer(Corrected_mass ~ Resisted_cop + (1 | groupID)

             + (1 | FemaleID) + (1|femalegroupID), full_female)


plot(mod5) # Check homoscedasticity


qqnorm(resid(mod5)) # Check for non-normality of the residuals
qqline(resid(mod5))


anova(mod6)


Type III Analysis of Variance Table with Satterthwaite's method
             Sum Sq Mean Sq NumDF  DenDF F value  Pr(>F)
Resisted_cop  12691   12691     1 152.25  4.3064 0.03965 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’
```

CHAPTER 4

Temporal dynamics of competitive fertilization in social groups of red junglefowl (*Gallus gallus*) shed new light on avian sperm competition

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Temporal 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 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]. 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 with 95% confidence for 849 of these eggs [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 convergence 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, $4 \leq \Delta AIC \leq 10$ as a considerable difference of fit, and $\Delta AIC \geq 10$ 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 ($\Delta AIC \geq 6.46$, 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 ($\Delta AIC \leq 2.18$, 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, $\Delta AIC=2.86$, Table S1), but performed considerably better than all other models ($\Delta AIC>4$, 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 (log(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 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 in [97]). 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 infundibulum, 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 or 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. Inbreeding avoidance is typically heterogeneous across wild populations [128], and the role of inbreeding in sperm competition in natural bird populations remains unclear [38]. A meta-analysis found that the incidence of extra-pair paternity increases with the

genetic similarity between social partners [129], suggesting that females are more likely to seek extra-pair copulations when they are closely related to their social partners and/or that post-copulatory sexual selection promotes fertilization by less related males. Others, however, have failed to detect this effect [106]. Long-term studies of some populations, such as some fairy wrens (Maluridae) suggest that the effect of inbreeding avoidance may be non-trivial for small subsets of a population or in some breeding seasons [130]. 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 [131,132] 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 [133,134]. 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., [135]), and we foresee exciting opportunities to understand dynamics of sperm competition and their eco-evolutionary significance in natural populations.

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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
Supporting data are available in Dryad Digital Repository [136] (doi:10.5061/dryad.cz8w9gj1d).

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.

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Table 1. Comparison of selected binomial generalized linear mixed models (GLMMs), predicting the probability that 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; $\boldsymbol{R^2_m}$=Marginal R^2; $\boldsymbol{R^2_c}$=Conditional R^2.

Table 2. Summary statistics and likelihood ratio tests results of the generalized linear mixed-effects model (GLMM) predicting egg fertilization probability in multiple replicate groups of red junglefowl, *Gallus gallus*. Model contains all main effects and statistically significant interactions (mod5, electronic supplementary material, 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			
$\tau_{00\ MaleID}$	0.35			
$\tau_{00\ FemaleID}$	0			
$\tau_{00\ femalegroupID}$	0			
$\tau_{00\ groupID}$	0			
Observations	4134			

Notes: LRT=Likelihood ratio test; σ^2=residual variance; τ_{00}=random intercept variance; *p*-values in bold identify statistically significant effects (<0.05).

Figures

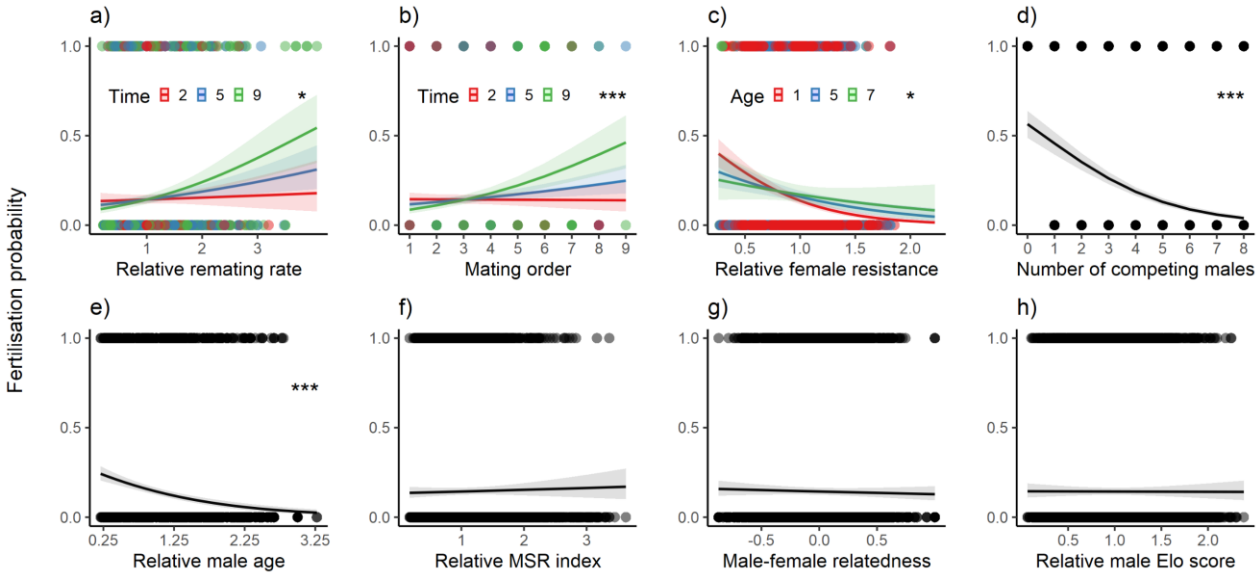


Figure 1. Relationships between different predictors and the probability that competing males fertilise individual eggs in semi-natural groups of red junglefowl, *Gallus gallus*. Panels represent the marginal effects of a predictor based on the generalised linear mixed model (GLMM) in Table 2. a) Relative 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 *** = $p<0.001$.

Suplementary material for: Temporal dynamics of competitive fertilisation in social groups of red junglefowl (Gallus gallus) shed new light on avian sperm competition

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1. Methods

1.1 Description of predictors

We considered the following predictors of male fertilisation success in the present study.

(i) Number of competitors. This measured the intensity of sperm competition for each ovum, as the number of unique males other than the focal male that copulated with the female up to and including the day before the egg was laid.

(ii) Remating rate. This provided a measure of a male cumulative sperm investment to fertilise an ovum, and was the total number of repeat copulations between a male and female that had the potential to fertilise each of her eggs (i.e., copulations that occurred at least up until the day prior to the laying of each egg).

(iii) Mating order. Mating order ranged from 1 (first male to copulate) to 9 (last male to copulate, as in no occasion all 10 males in a group copulated with the same female). We used the last copulation performed by each male to determine the mating order, irrespective of how many previous copulations they had achieved with the female. In the occasions where two or more males copulated within a minute of each other, they were all assigned the same mating order.

(iv) Male social status. We used the agonistic interactions between males to estimate male social status over each day of the mating trial using the Elo rating method [1,2]. A full description of the method is provided elsewhere [2]. Briefly, ratings of males interacting agonistically are updated according to the outcome of the interaction, with the winner gaining points and the loser losing points. The number of points gained and lost during an interaction depends on the Elo rating of the individuals when the interaction took place, such that a higher rating male receive less points if he wins against a lower rated male and vice versa. For statistical models, we used the male Elo score after his last agonistic interaction on each day of a trial.

(v) Male age. We measured age in years. Males ranged from 1 to 7 years old [3].

(vi) Average female resistance. We scored the level of female resistance during each mating attempt following [4], such that: 1 = the female crouches in front of the male and solicits a copulation, with no resistance displayed; 2 = the male approaches the female from behind with raised hackles, and the female responds by crouching; 3 = the male approaches the female, and the female walks away; 4 = the male grabs the female, the female passively accepts the copulation; 5 = the male runs after and grabs the female, and the female initially resists before accepting the copulation; and 6 = the female resists the whole copulation. We calculated the mean resistance score across all copulations of the focal male that had the potential to fertilise each of her eggs (see above) as the overall resistance score. We predicted that males that were resisted more strongly during copulations (i.e. had a higher average resistance score) would fail to fertilise eggs more often than males that were less resisted.

(vii) Female age. Measured as (v). Females ranged from 1 to 7 years old [5] .

(viii) Male-female relatedness. We estimated the pairwise relatedness coefficient between each individual male-female pair within each replicate group following [6]. Values closer to 1 mean that a male and a female were more closely related to each other than the average group relatedness, while values closer to –1 mean that the pair was less related than average. Relatedness between a male and a female ranged from -0.871 to 1 across all groups ($\bar{x}$=-0.001, sd=0.33).

(ix) Male sperm reserves (MSR). Because mating depletes, and sexual rest replenishes, a male's extragonadal sperm reserves, males that copulate more frequently may have fewer sperm

available for insemination than males that copulate less frequently. To investigate how this may impact fertilisation success we included an estimate of a male's sperm reserves at the time of a copulation. We developed an index of male sperm reserves (MSR), which estimates the proportion of a male's sperm reserves that would have been available to a male throughout the time window when he mated with a female, as follows:

$$MSR = \frac{1 + \sum_i^m \frac{\tau_i}{48}}{n}$$

where n is the total number of times the focal male mated with any female during the trial, until and including his last copulation with the female that could have fertilised a particular egg, and τ_i is the interval in hours between each of the male's consecutive copulations, i.e. between his 1st and 2nd copulation, 2nd and 3rd copulation, and so on, until the m^{th} time interval to his last copulation with the female which laid the egg, i.e. $m = n$-1. MSR is standardized at one when completely replenished, and thus, the first copulation of a male was given a value of 1, as males were all sexually rested at the beginning of a trial. We set $\tau_i/48$ to range from 0 to 1; $\tau_i /48 = 0$ when a male mated twice within the same minute (the smallest unit of time recorded during behavioural observations, i.e. $\tau_i = 0$), and was capped at 1 when $\tau_i \geq 48$, because 48h is the interval required for the complete replenishment of male extragonadal sperm reserves in fowl [8], so a period of sexual rest longer than 48h could not increase the number of sperm available to a male beyond the maximal amount. Thus, a value closer to 1 reflects the potential to deliver larger ejaculates (i.e. larger sperm reserves) due to, on average, lower mating rates, while values closer to 0 reflect smaller sperm reserves due to higher sperm depletion. Comparison with natural red junglefowl ejaculates shows that MSR behaves in a way qualitatively similar to patterns of male sperm depletion in this population, and that males tend to allocate a consistent fraction of MSR to natural ejaculates (Figure S1).

(x) Time. Time was defined as consecutive days of the mating trial in which we collected eggs (i.e., from the 2nd day to the 11th day of the trial, after observations were terminated [3]).

1.2. Alternative analyses

(a) Female sperm reserves (FSR)

We used the rate of passive sperm loss calculated for the domestic fowl [7] to estimate the temporal discounting of an insemination so that it would be completely depleted from the female SSTs 14 days following insemination (i.e. the maximum sperm storage period estimated for the domestic fowl [6]). FSR therefore represents a theoretical quantity that is proportional to the number of sperm from a particular insemination left in a female's SSTs at on a given day of the trial (Figure S2).

(b) Analysing each day of the trial separately

To confirm and further characterise temporal dynamics identified by interactions between predictors ((i)-(ix)) and time (x), we also conducted separate models for each individual day of the trial in which eggs were collected (i.e. day2 to day11 [3]). We show the statistically significant predictors, and the variance explained by models, on each day of the trial in Figure S4a,b. Outputs of full generalised linear mixed models (GLMMs) for each day are provided in Table S3. Overall, results from daily models were consistent with results from the full model which included time (x) as an interaction term, with two exceptions. First, male sperm reserves (MSR) and mating order were negatively and marginally non-significantly associated with fertilisation probability for the first eggs of a trial (2nd day). This suggests that males that mated first and in quick succession during the very first day of the trial, had a slighter advantage in sperm competition for earlier eggs. Second, we found that more dominant males tended to fertilise more eggs on the 8th day of the trial. Note however that caution is required in the interpretation of the results of these analyses given the relatively limited statistical power and number of comparisons. The analysis of individual days is for illustrative purposes only, and does not represent an alternative to the statistical approach presented in the main study.

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2. Tables

Table S1. Comparison of the output of multiple binomial generalized linear mixed models (GLMMs), predicting the probability of the focal male to fertilize individual eggs in multiple replicate groups of red junglefowl, ranked in ascending order of AIC.

Model ID	Fixed-effects	K	AIC	ΔAIC	AICWt	LL	R^2_m	R^2_c
mod1	RR*T + MO*T + C + A + FR*FA	15	3621.33	0	0.6	-1795.61	0.1498	0.1959
mod2	RR*T + MO*T + C + A + FR	13	3624.18	2.86	0.14	-1799.05	0.1473	0.1928
mod3	RR*T + MO*T + C + A +FR + MSR	14	3625.65	4.32	0.07	-1798.77	0.1466	0.1921
mod4	RR*T + MO*T + C + A + FR + SS	14	3626.16	4.83	0.05	-1799.03	0.1471	0.1929
mod5	RR*T + MO*T + C + A + FR*FA + SS + MSR + R	18	3626.18	4.86	0.05	-1795.01	0.1486	0.1942
mod6	NC*T + MO*T + C + A + FR + SS + MSR	15	3627.65	6.33	0.03	-1798.77	0.1466	0.1922
mod7	RR*T + MO*T + C + A + FR + SS + MSR + R	16	3628.06	6.74	0.02	-1797.97	0.1462	0.1911
mod8	RR*FA*T + MO*T + C + A + FR	17	3629.37	8.04	0.01	-1797.61	0.1476	0.1941
mod9	RR*T + MO*T + C*T + A +FR + SS + MSR + R	17	3629.64	8.32	0.01	-1797.75	0.1449	0.1898
mod10	RR*T + MO*T + C*T + A*T + FR*T + SS + MSR + R	19	3630.01	8.69	0.01	-1795.91	0.1460	0.1907
mod11	RR*T + MO*T + C*T + A*T + FR*T + SS + MSR + R	18	3630.07	8.74	0.01	-1796.95	0.1435	0.1884
mod12	RR*T + MO*T + C*T + A*T + FR*T + SS*T + MSR*T + R	20	3631.88	10.55	0	-1795.84	0.1459	0.1906
mod13	RR*T + MO*T + C*T + A*T + FR*FA + SS*T + MSR*T + R*T	23	3633.24	11.92	0	-1793.49	0.1460	0.1917
mod14	RR*T + MO*T + C*T + A*T +FR*T + SS*T + MSR + R*T	21	3633.61	12.28	0	-1795.69	0.1461	0.1907
mod15	RR*T + MO*T + C*T + A*T + FR*T + SS*T + MSR*T + R*T	22	3635.23	13.9	0	-1795.49	0.1461	0.1909
mod16	RR*T + MO + C + A + FR + SS + MSR + R	15	3639.57	18.24	0	-1804.73	0.1434	0.1882
mod17	RR + MO + C + A + FR + T	11	3649.34	28.01	0	-1813.64	0.1395	0.1854
mod18	RR + MO + C + A + FR + T + R	12	3649.57	28.24	0	-1812.75	0.1389	0.1840
mod19	RR + MO + C + A +FR + T + R + MSR	13	3651.08	29.75	0	-1812.5	0.1384	0.1835
mod20	RR + MO + C + A + FR + SS + R + MSR + T	14	3653.09	31.76	0	-1812.49	0.1383	0.1836
mod21	RR + MO + T + C + A	10	3753.4	132.08	0	-1866.68	0.1059	0.1587

(table continues)

Table S1 - (continued)

Model ID	Fixed-effects	K	AIC	ΔAIC	AICWt	LL	R^2_m	R^2_c
mod22	RR + MO + T + C	9	3787.52	166.19	0	-1884.74	0.0793	0.1538
mod23	C*T	8	3846.36	225.03	0	-1915.16	0.0539	0.1307
mod24	C	6	3851.02	229.69	0	-1919.5	0.0550	0.1318
mod25	FR*T	8	3932.33	311	0	-1958.15	0.0313	0.1460
mod26	RR*T	8	3938.6	317.27	0	-1961.28	0.0295	0.1347
mod27	RR + MO + T	8	3951.3	329.98	0	-1967.63	0.0250	0.1268
mod28	RR + T	7	3957.72	336.4	0	-1971.85	0.0225	0.1284
mod29	MO*T	8	3963.22	341.89	0	-1973.59	0.0191	0.1273
mod30	FR	6	3968.72	347.4	0	-1978.35	0.0205	0.1355
mod31	FR*FA	8	3968.80	347.48	0	-1976.38	0.0221	0.1375
mod32	A*T	8	3982.38	361.05	0	-1983.17	0.0288	0.0991
mod33	RR	6	3997.36	376.03	0	-1992.67	0.0112	0.1168
mod34	MSR*T	8	4004.98	383.65	0	-1994.47	0.0115	0.1163
mod35	SS*T	8	4005.09	383.76	0	-1994.53	0.0105	0.1178
mod36	T	6	4005.36	384.03	0	-1996.67	0.0088	0.1177
mod37	R*T	8	4008.83	387.5	0	-1996.4	0.0090	0.1186
mod38	A	6	4016.99	395.66	0	-2002.48	0.0189	0.0907
mod39	SS	6	4040.27	418.95	0	-2014.13	0.0014	0.1097
mod40	Null	5	4040.63	419.31	0	-2015.31	NA	NA
mod41	MO	6	4041.31	419.98	0	-2014.64	0.0004	0.1089
mod42	FA	6	4041.39	420.06	0	-2014.68	0.0004	0.1099
mod43	R	6	4042.1	420.78	0	-2015.04	0.0002	0.1107
mod44	MSR	6	4042.24	420.91	0	-2015.11	0.0002	0.1076

Note: A=Age; C=Number of competitors; FA=Female age; FR=Female resistance; MO=Mating order; MSR=Male sperm reserves index; R=Relatedness; RR=Remating rate; SS=Social status; T=Time; K= Number of parameters; AIC=Akaike information criterion; ΔAIC=Delta AIC; AICWt=Akaike weight; LL=Likelihood; R^2_m=Marginal R^2; R^2_c=Conditional R^2.

Table S2. Difference in AIC scores between generalised linear mixed models (GLMMs) with either remating rates (RR) or estimated Female Sperm Reserves (FSR) as a fixed effect (depicted as X).

Model ID	Fixed effects	AIC_{RR}	AIC_{FSR}	AIC_{RR} - AIC_{FSR}	ΔAIC
mod1	X*T + MO*T + C + A + FR*FA	3621.33	3623.51	-2.18	2.18
mod2	X*T + MO*T + C + A + FR	3624.18	3625.53	-1.35	1.35
mod3	X*T + MO*T + C + A + FR + MSR	3625.65	3627.19	-1.54	1.54
mod4	X*T + MO*T + C + A + FR + SS	3626.16	3627.48	-1.32	1.32
mod5	X*T + MO*T + C + A + FR*FA + SS + MSR + R	3626.18	3624.74	1.44	1.44
mod6	X*T + MO*T + C + A + FR + SS + MSR	3627.65	3629.17	-1.52	1.52
mod7	X*T + MO*T + C + A + FR + SS + MSR + R	3628.06	3626.77	1.29	1.29
mod8	X*FA*T + MO*T + C + A + FR	3629.37	3629.5	-0.13	0.13
mod9	X*T + MO*T + C*T + A + FR + SS + MSR + R	3629.64	3627.58	2.06	2.06
mod10	X*T + MO*T + C*T + A*T + FR*T + SS + MSR + R	3630.01	3628.25	1.76	1.76
mod11	X*T + MO*T + C*T + A*T + FR*T + SS + MSR + R	3630.07	3628.23	1.84	1.84
mod12	X*T + MO*T + C*T + A*T + FR*T + SS*T + MSR*T + R	3631.88	3630.05	1.83	1.83
mod13	X*T + MO*T + C*T + A*T + FR*FA + SS*T + MSR*T + R*T	3633.24	3631.65	1.59	1.59
mod14	X*T + MO*T + C*T + A*T + FR*T + SS*T + MSR + R*T	3633.61	3631.9	1.71	1.71
mod15	X*T + MO*T + C*T + A*T + FR*T + SS*T + MSR*T + R*T	3635.23	3633.91	1.32	1.32
mod16	X*T + MO + C + A + FR + SS + MSR + R	3639.57	3632.75	6.82	6.82
mod17	X + MO + C + A + FR + T	3649.34	3642.2	7.14	7.14
mod18	X + MO + C + A + FR + T + R	3649.57	3639.63	9.94	9.94
mod19	X + MO + C + A + FR + T + R + MSR	3651.08	3640.93	10.15	10.15
mod20	X + MO + C + A + FR + SS + R + MSR + T	3653.09	3642.89	10.2	10.2
mod21	X + MO + T + C + A	3753.4	3746.01	7.39	7.39
mod22	X + MO + T + C	3787.52	3781.06	6.46	6.46
mod26	X*T	3938.6	3928.21	10.39	10.39
mod27	X + MO + T	3951.3	3906.88	44.42	44.42
mod28	X + T	3957.72	3943.28	14.44	14.44
mod33	X	3997.36	3984.37	12.99	12.99

A=Age; C=Number of competitors; MSR=Male sperm reserves; FA=Female age; FR=Female resistance; MO=Mating order; R=Relatedness; SS=Social status; T=Time; AIC=Akaike information criterion; AIC_{RR}=AIC score with Remating Rate as the predictor; AIC_{FSR}=AIC score with estimated Female Sperm Reserves as the predictor. ΔAIC values represent the absolute difference in AIC between models (i.e. $AIC_{RR} - AIC_{FSR}$).

Table S3. Output of generalized linear mixed-effects models (GLMMs) predicting the fertilisation probability of individual eggs by competing males. Models include all predictors (see methods) and were run separately per each individual day of the mating trial.

	Day 2				**Day 3**				**Day 4**				**Day 5**			
Predictors	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*
(Intercept)	81.39	2.01			23.48	1.21			9.58	0.97			5.26	0.98		
Remating rate	1.39	0.94	0.12	0.728	0.57	0.51	1.24	0.265	0.89	0.37	0.10	0.748	1.61	0.32	2.11	0.147
Mating order	0.53	0.34	3.71	0.054	0.96	0.16	0.07	0.789	0.96	0.13	0.08	0.778	1.13	0.12	1.06	0.303
Number of competitors	0.54	0.18	14.04	**<0.001**	0.57	0.13	24.34	**<0.001**	0.69	0.11	12.02	**<0.001**	0.63	0.11	17.62	**<0.001**
Male age	0.9	0.67	0.03	0.868	0.22	0.36	22.57	**<0.001**	0.44	0.28	9.29	**0.002**	0.37	0.3	13.17	**<0.001**
Female resistance	0.24	0.88	2.72	0.099	0.33	0.5	5.13	**0.024**	0.27	0.49	7.77	**0.005**	0.28	0.5	6.75	**0.009**
Elo score	1.25	1.11	0.04	0.837	1.97	0.59	1.31	0.252	0.83	0.47	0.15	0.696	0.84	0.45	0.16	0.693
Relatedness	0.41	1	0.80	0.372	2.22	0.63	1.64	0.201	0.91	0.59	0.03	0.863	0.32	0.6	3.76	0.052
Sperm reserves (MSR)	0.24	0.78	3.84	0.050	1.13	0.35	0.12	0.731	1.33	0.32	0.76	0.383	1.13	0.34	0.12	0.729
Random Effects																
σ^2	3.29				3.29				3.29				3.29			
τ_{00}	0.00 $_{\text{MaleID}}$				0.00 $_{\text{MaleID}}$				0.23 $_{\text{MaleID}}$				0.43 $_{\text{MaleID}}$			
	0.00 $_{\text{FemaleID}}$				0.00 $_{\text{FemaleID}}$				0.00 $_{\text{FemaleID}}$				0.00 $_{\text{FemaleID}}$			
	0.00 $_{\text{groupID}}$				0.00 $_{\text{groupID}}$				0.00 $_{\text{groupID}}$				0.00 $_{\text{groupID}}$			
Sample size	63 $_{\text{MaleID}}$				88 $_{\text{MaleID}}$				104 $_{\text{MaleID}}$				102 $_{\text{MaleID}}$			
	32 $_{\text{FemaleID}}$				47 $_{\text{FemaleID}}$				50 $_{\text{FemaleID}}$				49 $_{\text{FemaleID}}$			
	18 $_{\text{groupID}}$				20 $_{\text{groupID}}$				19 $_{\text{groupID}}$				19 $_{\text{groupID}}$			
Observations	109				256				323				384			
Marginal R^2/ Conditional R^2	0.437 / 0.437				0.274 / 0.274				0.125 / 0.161				0.139 / 0.198			

(table continues)

Table S3 – (table continued)

	Day 6				Day 7				Day 8			
Predictors	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*
(Intercept)	6.72	0.8			0.19	0.16			13.25	0.91		
Remating rate	1.45	0.26	2.01	0.156	1.13	0.14	0.75	0.387	1.02	0.26	0.01	0.945
Mating order	1.09	0.09	0.80	0.370	1.06	0.16	0.15	0.700	1.49	0.1	15.90	**<0.001**
Number of competitors	0.68	0.09	20.73	**<0.001**	0.56	0.14	19.40	**<0.001**	0.58	0.1	33.17	**<0.001**
Male age	0.52	0.24	7.73	**0.005**	0.73	0.15	4.30	**0.038**	0.27	0.29	25.74	**<0.001**
Female resistance	0.26	0.43	10.53	**0.001**	0.63	0.13	13.77	**<0.001**	0.07	0.55	27.01	**<0.001**
Elo score	0.65	0.35	1.50	0.220	0.92	0.15	0.33	0.567	2.2	0.33	5.74	**0.017**
Relatedness	0.48	0.49	2.29	0.130	1.83	0.49	1.54	0.214	0.61	0.55	0.82	0.364
Sperm reserves (MSR)	1	0.28	<.01	0.989	0.93	0.15	0.22	0.638	1.23	0.26	0.62	0.431
Random Effects												
σ^2	3.29				3.29				3.29			
τ_{00}	0.14 $_{\text{MaleID}}$				0.30 $_{\text{MaleID}}$				0.00 $_{\text{MaleID}}$			
	0.00 $_{\text{FemaleID}}$				0.00 $_{\text{FemaleID}}$				0.00 $_{\text{FemaleID}}$			
	0.00 $_{\text{groupID}}$				0.00 $_{\text{groupID}}$				0.00 $_{\text{groupID}}$			
Sample size	112 $_{\text{MaleID}}$				110 $_{\text{MaleID}}$				110 $_{\text{MaleID}}$			
	56 $_{\text{FemaleID}}$				55 $_{\text{FemaleID}}$				51 $_{\text{FemaleID}}$			
	20 $_{\text{groupID}}$				20 $_{\text{groupID}}$				19 $_{\text{groupID}}$			
Observations	500				541				483			
Marginal R^2/ Conditional R^2	0.109 / 0.129				0.086 / 0.126				0.202 /0.202			

(table continues)

Table S3 – (table continued)

	Day 9				Day 10				Day 11			
Predictors	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*	*OR*	*SE*	*LRT*	*p*
(Intercept)	6.97	0.83			21.78	1.05			1.42	1.01		
Remating rate	1.84	0.22	7.53	**0.006**	1.01	0.26	0.01	0.935	2.78	0.25	20.51	**<0.001**
Mating order	1.15	0.09	2.13	0.144	1.46	0.1	15.15	**<0.001**	1.18	0.11	2.30	0.129
Number of competitors	0.68	0.08	24.06	**<0.001**	0.59	0.1	30.57	**<0.001**	0.65	0.1	19.81	**<0.001**
Male age	0.5	0.26	7.77	**0.005**	0.42	0.3	9.24	**0.002**	0.43	0.32	7.61	**0.006**
Female resistance	0.2	0.47	11.96	**0.001**	0.06	0.59	24.94	**<0.001**	0.23	0.6	6.37	**0.012**
Elo score	0.74	0.34	0.81	0.367	1.28	0.36	0.46	0.497	1.24	0.39	0.28	0.599
Relatedness	0.68	0.49	0.64	0.422	2.19	0.57	1.95	0.163	0.61	0.62	0.64	0.424
Sperm reserves (MSR)	0.83	0.3	0.39	0.534	0.75	0.32	0.84	0.358	1.46	0.36	1.13	0.287
Random Effects												
σ^2	3.29				3.29				3.29			
τ_{00}	0.00 $_{MaleID}$				0.77 $_{MaleID}$				0.79 $_{MaleID}$			
	0.00 $_{FemaleID}$				0.00 $_{FemaleID}$				0.00 $_{FemaleID}$			
	0.00 $_{groupID}$				0.00 $_{groupID}$				0.00 $_{groupID}$			
Sample size	115 $_{MaleID}$				115 $_{MaleID}$				113 $_{MaleID}$			
	56 $_{FemaleID}$				52 $_{FemaleID}$				48 $_{FemaleID}$			
	20 $_{groupID}$				20 $_{groupID}$				20 $_{groupID}$			
Observations	514				543				481			
Marginal R^2/ Conditional R^2	0.139 /0.139				0.180 / 0.261				0.166 / 0.251			

OR = odd ratios; SE = standard error; LRT = likelihood ratio test; σ^2 = residual variance; $\tau 00$ = random intercept variance.

3. Figures

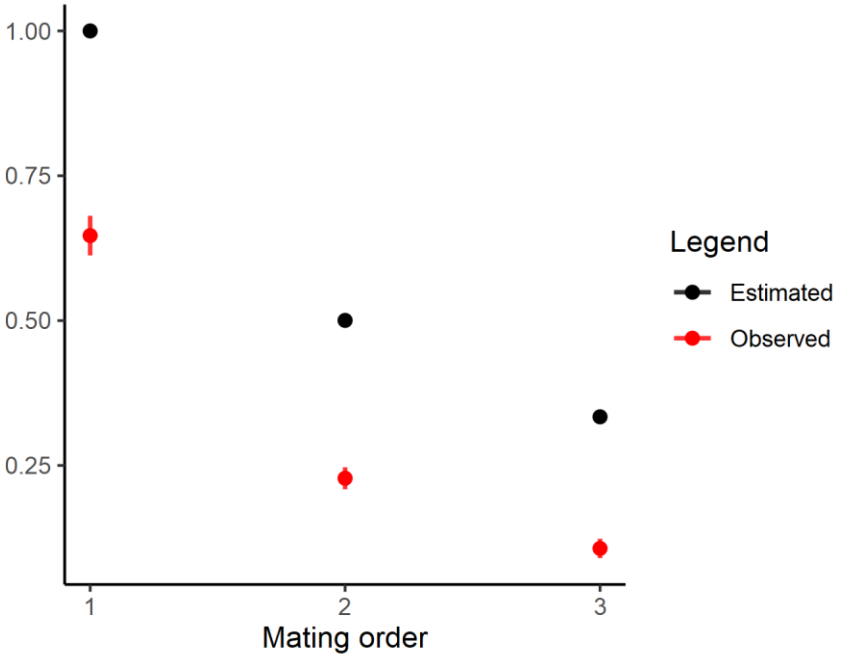


Figure S1. Comparison between mean standardised sperm numbers in a sequence of three consecutive ejaculates obtained in experimental sperm allocation trials in which each male red junglefowl was exposed and allowed to mate with a single female three times in quick succession (the number of sperm in each ejaculate is standardised against the largest ejaculate produced by the same male within the sperm allocation trial), and the male sperm reserves (MSR) parametrised for these same copulations. Data points represent means and bars, standard errors.

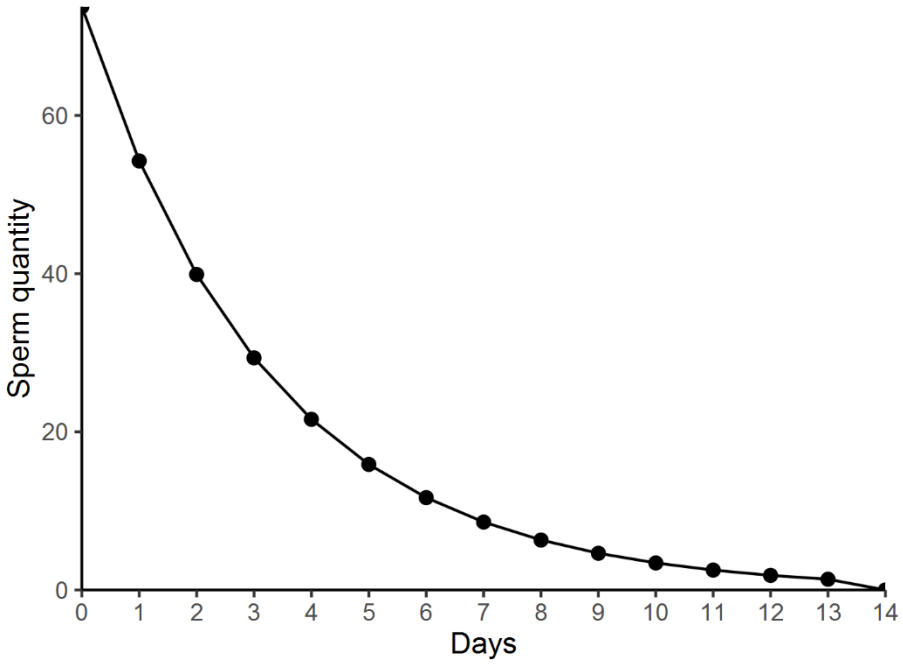


Figure S2. Theoretical sperm quantity in a female's SSTs as function of time elapsed since insemination, estimated using the rate of passive sperm loss (- 1.28 x 10^{-2} ± 1.42 x 10 .3 (s.e.) h^{-1}) for domestic fowl [8], such that sperm are completely depleted on the 14th day following insemination [7].

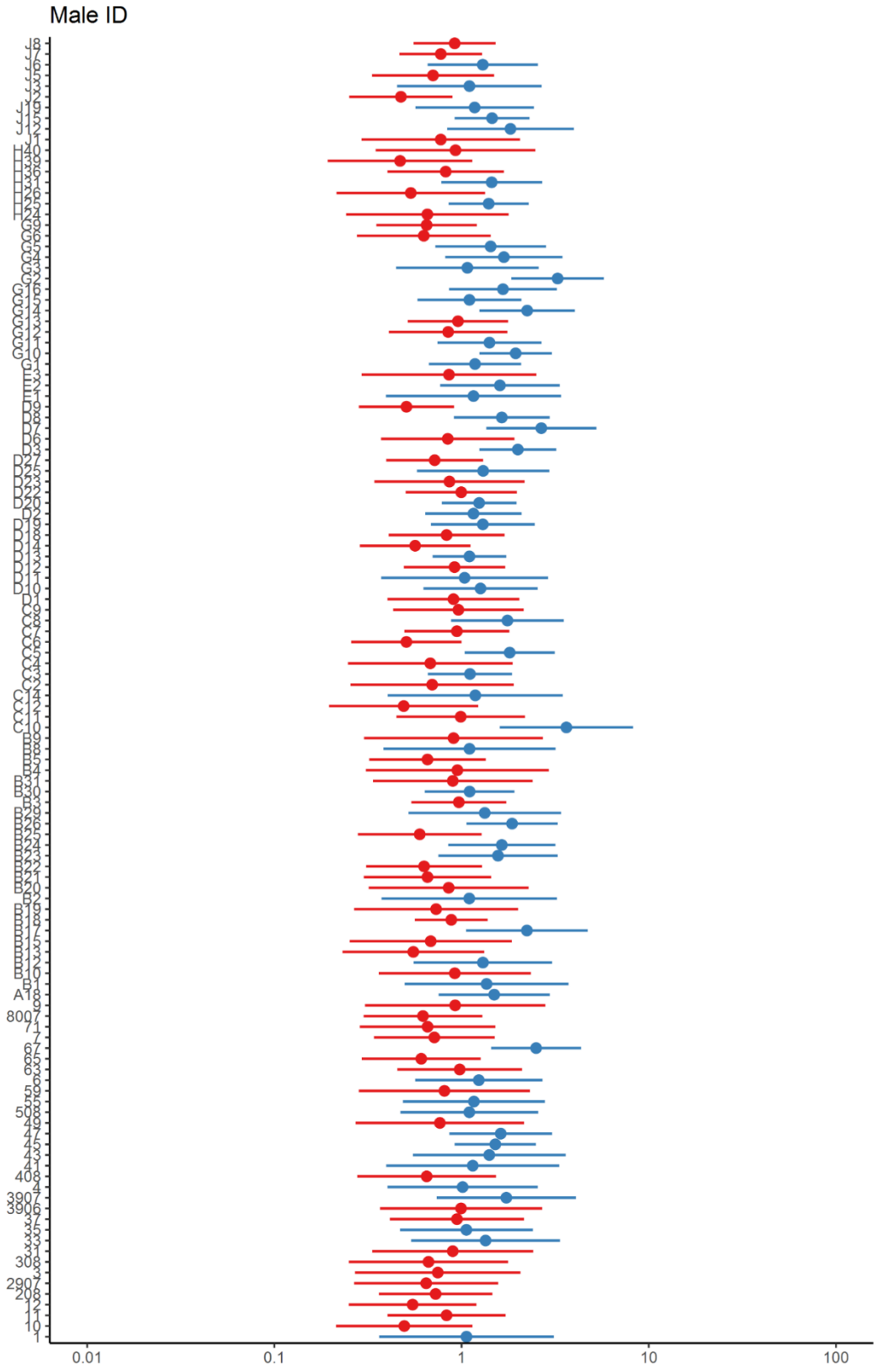
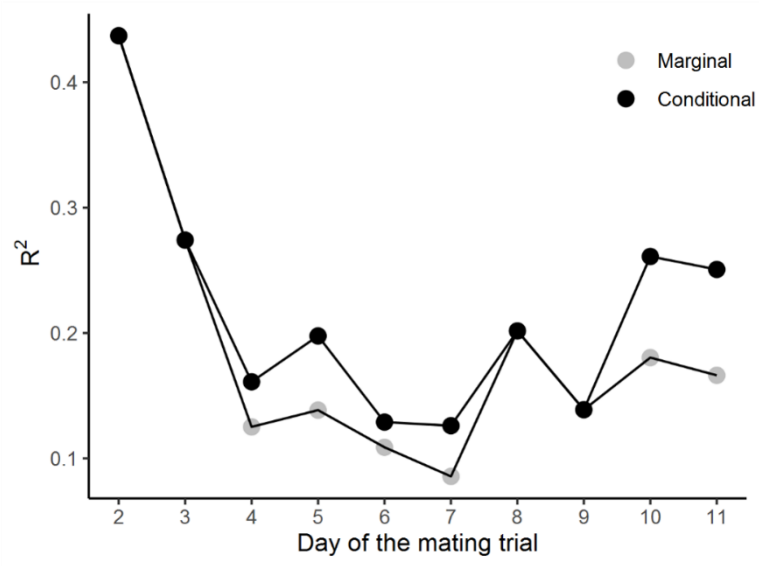


Figure S3. Estimates for the random effect of male identity. The x-axis represents the estimates in odd ratios and the y-axis the identity of each male used in the experiment (n=127). Dots represent the mean and lines the confidence intervals. Estimates above and below 1 are coded in blue and red, respectively.

(a)



(b)

Day of the mating trial	Statistically significant predictors (effect direction)
2	Number of competitors (-)
3	Number of competitors (-) Male age (-) Female resistance (-)
4	Number of competitors (-) Male age (-) Female resistance (-)
5	Number of competitors (-) Male age (-) Female resistance (-)
6	Number of competitors (-) Male age (-) Female resistance (-)
7	Number of competitors (-) Male age (-) Female resistance (-)
8	Number of competitors (-) Male age (-) Female resistance (-) Mating order (+) Elo score (+)
9	Number of competitors (-) Male age (-) Female resistance (-) Remating rate (+)
10	Number of competitors (-) Male age (-) Female resistance (-) Mating order (+)
11	Number of competitors (-) Male age (-) Female resistance (-) Remating rate (+)

Figure S4. Analysis of variation in fertilisation probability for each individual day when eggs were collected (day2-11) in red junglefowl mating trials. (a) Variation in the marginal and conditional R^2 of generalized linear mixed models for each individual day (R^2GLMM(m) and R^2GLMM(c), respectively). (b) The statistically significant predictors and the direction of the effects (+, positive; -, negative).

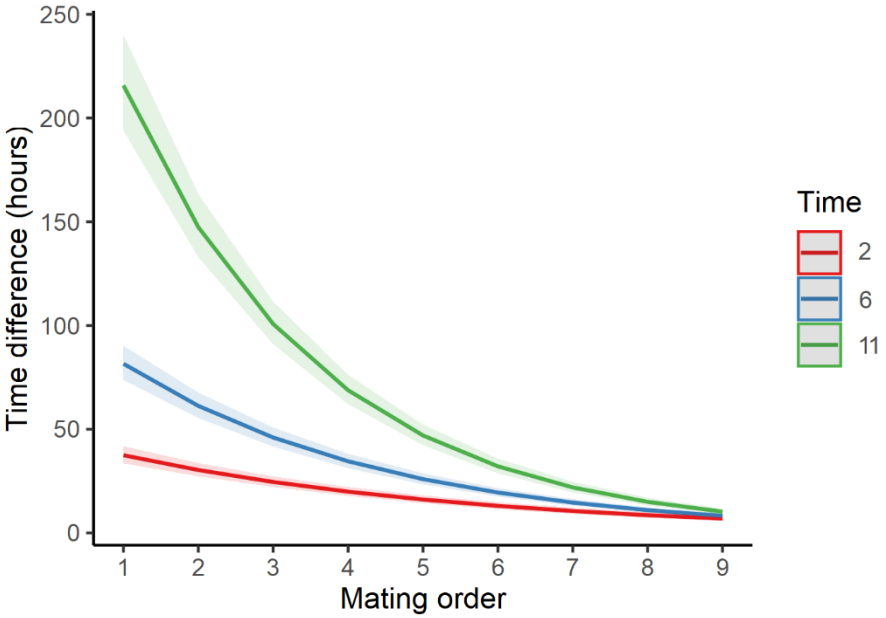


Figure S5. We measured the time elapsed (time difference in hours) between the last copulation by each competitor male with a female and the oviposition of a given egg. The difference in this time interval from the first to the last male to mate with a female was low in the first days of a trial, and large in the last days of

a trial. Plot represents the marginal effects of predictors from a linear mixed model (log(Time gap)~mating order*time: χ^2_1=98.85, p<0.001). Shaded areas represent the 95% confidence intervals, which are coloured in relation to three arbitrary days of the mating trial (i.e., red: day 2, blue: day 6, green: day 11). Time difference was back transformed to its original scale to produce the plot. We assumed eggs were laid at mid-day, and only copulations up to the day before the laying of this egg were considered able to fertilise the ovulum. We used egg laying as the reference point, as opposed to ovulation, because we could not estimate the latter based on our methodology.

CHAPTER 5

Towards an appreciation of short-term dynamics of sexual selection in polyandrous populations

Towards an appreciation of short-term dynamics of sexual selection in polyandrous populations

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The late 20th century has seen a rapid expansion in our understanding of the causes and consequences of variation in sexual selection. Despite this, relatively little is known about the drivers and consequences of dynamic changes in sexual selection, particularly over short time-scales. Here, we use a detailed dataset with fine-grained information on behavioural and paternity data of red junglefowl (*Gallus gallus*) to explore changes in the opportunity for selection over replicated 10-day trials, and use simulations to test whether such temporal patterns arise as a consequence of random mating or require biological explanations. We also explore how the duration of a breeding event or the sampling period may affect studies of sexual selection, by comparing estimates of opportunity calculated from cumulative information on mating and reproductive success against estimates calculated daily. We show that variance in many components of female and male reproductive success tend to erode over time, and that these trends can be partially attributed to random mating or sampling effort, with deterministic processes taking time to generate bias in opportunity, particularly on post-copulatory episodes of selection towards the end of the sampling period. We also show that daily sampling of behavioural data will increasingly overestimate the opportunity for pre-copulatory sexual selection over successive days of the mating trial. We discuss a potential use of combining both cumulative and daily estimates. Collectively, our results suggest that opportunity for selection can vary over short-time scales, and that future studies should compare observed estimates with null models assuming random mating. We also suggest that studies should collect longitudinal data rather than cross-sectional, although the relevant duration of the sampling period will depend on the reproductive biology of the study system, such as the duration of sperm storage in females.

Keywords: opportunity for selection; random mating; pre-copulatory sexual selection; post-copulatory sexual selection

INTRODUCTION

In nature, the direction, strength and targets of selection vary over time, however we still do not fully understand the causes of this dynamism (Hereford et al., 2004; Siepielski et al., 2009; Bell, 2010; Morrissey & Hadfield, 2011; Uyeda et al., 2011). Recent research has revealed radical adaptive evolution over relatively short ecological time scales (Koonin, 2009; Ghalambor et al., 2015; Laland et al., 2015) and thus, even cursory, transient changes in patterns of selection may lead to substantial evolutionary change. Sexual selection, arising from variation in fitness due to intrasexual competition to secure mates and fertilise the gametes of the opposite sex (Darwin, 1871; Andersson, 1994), is a key selective mechanism, which can vary widely between and within populations (Gosden &

Svensson, 2008; Kasumovic et al., 2008; Mobley & Jones, 2009). Research indicates the potential for rapid fluctuations in sexual selection principally as a consequence of temporal of variation in environmental conditions (Wilson et al., 2006; Gosden & Svensson, 2008; Ingleby et al., 2010). For example, studies have demonstrated how changes in resource abundance and dietary stress may reduce courting behaviour, the expression of sexual ornaments, and reproductive performance, leading to drastic changes in sexual selection (Lindström et al., 2009; Janicke et al., 2015; Morimoto et al., 2016; Cattelan et al., 2020). Similarly, other studies have investigated how changes in the ratio of reproductive males to females (operational sex ratio, OSR) can shift patterns of selection within or across breeding seasons (Emlen & Oring, 1977; Forsgren et al., 2004; Liker et al., 2013; Wacker et al., 2013, 2014; Janicke & Morrow, 2018). More generally, rapid changes in local social context may lead to rapid and reversible changes in mating systems (Shuster, 2010; Sih et al., 2017). Collectively, these studies reveal the potential importance of temporal dynamics of sexual selection in shaping phenotypic evolution. However, developing a framework for understanding such dynamics and their role in driving evolutionary change and in maintaining variation within populations hinges on addressing three key challenges.

First, studies often investigate changes in sexual selection over rather coarse scales, e.g., across breeding seasons, years or cohorts (e.g., Gosden & Svensson, 2008; Serbezov et al., 2010; Gerlach et al., 2012; Dubuc et al., 2014), overlooking the potential for more rapid fluctuations in sexual selection. The relatively few studies to investigate dynamics within a reproductive period or breeding season, have shown pronounced changes in patterns of selection (e.g. Blanckenhorn et al., 1999; Jann et al., 2000; Preston et al., 2001; Grant & Grant, 2002). As a result, whether data is collected early or late in a breeding season may deeply alter estimates of variance in reproductive success (e.g., Preston et al., 2001; Reichard et al., 2008; Wacker et al., 2014). In polyandrous species, where males are subjected to pre- and post-copulatory episodes of sexual selection, trade-offs between investment in pre- and post-copulatory sexual selection (Lüpold et al., 2014; Evans & Garcia-Gonzalez, 2016; Simmons et al., 2017) can lead to dynamic variation in reproductive success

over relatively short periods (e.g., Preston et al., 2001). Such changes may be particularly relevant to species where females store sperm and males re-mate with the same females over time, influencing the frequency and intensity of sperm competition. However, substantial fluctuations in sexual selection may also occur over much shorter-term time scales. For example, in many species sexual receptivity, courtship effort, mating propensity and mate choice preferences may change drastically in matters of hours or a few days in both males (e.g., Jordan & Brooks, 2011; Kahn et al., 2013) and females (e.g., Fedina & Lewis, 2007; Clark & Backwell, 2015). Collectively, these insights suggest that tracking fine-scale temporal changes in sexual selection is critical to better understand the way in which sexual selection operates in males and females, and the resulting in dynamics of sexual conflict over reproductive decisions. Doing so however, requires approaches that accurately analyse dynamic changes in individual variation in reproductive success in relation to the sequence of mating events and periods of female sperm storage. Such detailed information is often unavailable or hard to obtain for many populations.

Second, studies on sexual selection have been criticised for the absence of suitable null hypotheses (Prum, 2010). This is important when random mating can also generate individual variation in different components of reproductive success (Klug et al., 2010; Jennions et al., 2012). For example, recent research has indicated that the average polyandry of groups alone can strongly influence covariances between different components of reproductive success and modulate sexual selection (Kvarnemo & Simmons, 2013; McDonald & Pizzari, 2018; Morimoto et al., 2019), and that some estimates of sexual selection based on simulations of random mating can be statistically indistinguishable from the observed data (e.g., Barreto & Avise, 2011; Aronsen et al., 2013). Studies of sexual selection dynamics therefore, require null expectations of temporal variation in individual reproductive success based on random mating and random fertilisation. Understanding the drivers of temporal changes in sexual selection thus require approaches that estimate the extent to which dynamics are driven by random and deterministic processes.

Finally, determining the sampling duration to correctly estimate the strength and direction of

sexual selection is critical for methodological and conceptual purposes (Cockburn et al., 2008; Siepielski et al., 2009). In principle, sexual selection may be measured cumulatively, where the measure of sexual selection at a particular time unit captures the cumulative effects of all preceding time units. Alternatively, sexual selection could be measured instantaneously for each time unit independent of previous values. Ultimately, which approach is best may depend critically on the time window of the study, the specificities of the study system and the difficulty in collecting high resolution behavioural and paternity data. For example, when breeding is completed over extremely short periods of hours or days such as in some Anura (Ulloa et al., 2019), a relatively short instantaneous characterization of mating and paternity data may be sufficient to accurately represent patterns of total sexual selection. On the other hand, in species where females store sperm for long periods of time, short-scale instantaneous estimates may misrepresent actual selection by, for example, failing to account for stored sperm of individual males that successfully mate with females much earlier in the season but do not sire offspring (Klug et al., 2010; Collet et al., 2014; Anthes et al., 2016). Despite these challenges, there has been very little assessment of how altering sampling duration may affect estimates of selection (Baena & Marcías-Ordóñez, 2013; Wacker et al., 2014). For example, a study of coreid bugs (*Margus obscurator*) found that the standardised variance in male mating success was 4 times smaller in the first half of the season if compared to the second half (McLain et al., 1993). Similarly, in a 10-year paternity study with horseshoe bats (*Rhinolophus ferrumequinum*), standardised variance in paternity share was around twice as low in the first and last year compared to the cumulative value over the 10 years (Rossiter et al., 2006). Careful consideration of different approaches, their strengths and limitations is therefore recommended.

Here, we explore these three challenges utilising a previously published high-resolution data on sexual behaviour and molecular parentage collected over a 10-day period (mating trial) across replicate groups from a population of red junglefowl (*Gallus gallus*) (McDonald et al., 2017, 2019; Carleial et al., 2020a, Carleial et al., 2020b). In nature, red junglefowl live in socially-structured fission-fusion flocks characterised by a polygynandrous mating system (reviewed Pizzari &

McDonald, 2019). In such groups, aggressive, dominant males tend to have privileged access to mating opportunities with females (Collet et al., 2012; Pizzari, 2016; McDonald et al., 2017; reviewed Pizzari & McDonald, 2019). However, females mate multiply, and store sperm for up to 14 days (Etches, 1996), inciting often intense sperm competition (Collet et al., 2012; McDonald et al., 2017). Males that remate more often with the same female tend to have an advantage in sperm competition (Collet et al., 2012; McDonald et al., 2017; Carleial et al., 2020a). Females can resist male mating attempts and eject sperm after copulations, which might bias fertilisation success against undesirable males (Pizzari & Birkhead, 2000; Pizzari, 2001; Dean et al., 2011; Carleial et al., 2020a). Our specific aims are three-fold. (i) We characterise temporal changes in the opportunity for sexual selection on males and females across each group, using cumulative measures of mating and reproductive success. For males, we further decompose variance in reproductive success to assess whether the relative contributions of pre- and post-copulatory sexual selection episodes and their covariances change over time. This allows us to assess how variance-based metrics of sexual selection vary over a relatively short but biologically meaningful sampling period (i.e. 10-day mating trial). (ii) We test whether cumulative estimates of sexual selection deviate from temporal changes predicted by simulations of our data assuming random patterns of mating and paternity share. This aim is crucial to provide null expectations for how variance in mating and reproductive success may change through time in the face of consistent temporal increases in polyandry and mean reproductive success alone. (iii) We compare cumulative measures of opportunity indexes against indexes calculated independently on each day of the trial (i.e. instantaneous measures). This enabled us to investigate potential discrepancies between these approaches and their role when estimating temporal dynamics of sexual selection, and the utilities of using both approaches in tandem.

METHODS

Mating trials

We studied 20 replicate groups of sexually mature (1-7 years old) red junglefowl from a captive population at the John Krebs Field Station of Oxford University in Wytham, UK, over three breeding seasons (April–October, 2011–2013). Detailed accounts of the mating trials are described elsewhere (McDonald et al., 2017, 2019; Carleial et al., 2020b). Briefly, one of us (GCM) observed each group twice daily for 10 consecutive days, with each observation period lasting three hours from 5am to 8am, and from 6pm to 9pm. Groups were observed in the same outdoor arena (5.8 × 16.5m) with group size and sex ratio within the range observed in free-ranging populations (10 males and 12 females) (Pizzari & McDonald, 2019). We recorded all successful mating attempts, defined as cases where a male mounted a female and either direct cloacal contact was directly observed, or was assumed to occur when the male tail was lowered over the female cloaca (Pizzari & Birkhead, 2000; Collet et al., 2012). We collected eggs laid successively over 10 days, starting from the second day of a trial until the 11th day, i.e. the day following the end of observations. Eggs were placed in an artificial incubator for 5 to 9 days until embryos were visually identifiable. We collected tissue samples from the embryos through maceration, which were stored in absolute ethanol at 4^{o}C until being sent for parentage assignment (see McDonald et al., 2017). Given that the sperm of an insemination is stored within female sperm storage tubules for up to 14 days (Etches, 1996; Carleial et al., 2020a) and that females produce a clutch of approximately 8 eggs (Collias et al., 1994), ovulating a single egg a day, a 10-day trial period captures a biologically meaningful timeframe suitable for evaluating sexual selection dynamics in this species.

Calculation of reproductive success and its components

We calculated male reproductive success (*RS*) as the total number of embryos sired by a male, and female *RS* as the number of fertilised eggs laid by a female. We also calculated different components of reproductive success. Mating success (*M*) was calculated for each sex as the total number of unique mating partners. Additionally for males, paternity share (*P*) was calculated as the proportion of embryos sired by a male out of all eggs laid by the females with whom he had successfully copulated prior to that day, and average partner's fecundity (*N*) as the mean number of fertilized eggs across all

these females (Webster et al., 1995; Evans & Garcia-Gonzalez, 2016). Mating success was inferred either behaviourally, when a successful copulation was directly observed, or genetically, when a pair was not observed mating but was identified through parentage analysis. Following recent recommendations (Anthes et al., 2016), individuals that were not observed mating and did not sire offspring were given zero *M* and were included in the analyses below. Males that failed to copulate successfully were excluded from calculations of *P*, whereas males that successfully copulated but sired no offspring received zero *P*. Additionally, if a male mated successfully with a female for the first time on the day she laid an egg, this egg did not enter the calculation of his *P* or *N* since the fertilisation of this egg would have occurred on the previous day (Etches, 1996).

Analytical approach

(i) Temporal patterns in cumulative opportunity estimates

First, we investigated temporal patterns in the opportunity for selection (*I*) over the mating trial for males and females. *I* – the standardised variance in reproductive success – is a widely used selection index and measures the maximum potential strength of selection in a population (Crow, 1958; Wade, 1979; Shuster and Wade, 2003). It can be calculated separately for each component of *RS* described above, namely standardised variance in mating success (I_M) for both males and females, and also the standardised variance in partner's fecundity (I_N) and in paternity share (I_P) for males (Collet et al., 2012, 2014; Moorad & Wade, 2013). We calculated opportunity indexes as follows:

(1) $\sigma^2/\overline{X}^2$,

where $\overline{X}^2$ is the square mean of *RS* or one of its components (i.e., *M*, *N*, *P*) and σ^2 its variance. These were calculated per each replicate group, and on each day of the mating trial. Additionally, we investigated whether the relative contribution of each selection episode and their covariances to variance in male *RS* changed over the course of a trial. This allowed us to determine whether pre- or post-copulatory selection episodes increase or decrease in importance over the trial, and whether

trade-offs between these different episodes exist (e.g., Webster et al., 1995; Collet et al., 2012; Devigili et al., 2015; Cattelan et al., 2020). We decomposed variance in male total *RS* following Webster et al., (1995) such that:

(2) var(RS) = var(M) + var(N) + var(P)+ 2cov(M,N) + 2cov(M,P) + 2cov(N,P) + D,

where var and cov denote variance and covariance, respectively, and D is an error term.

To test whether opportunity indexes (1) and relative contributions of selection episodes (2) varied over the mating trial, we used mixed-effects models implemented in the R v.3.6.2 (R Core Team 2019) package 'nlme' v3.1-149 (Pinheiro et al., 2020). Each opportunity index/percentage was entered as the response variable and time (i.e., the day order of a trial) as the explanatory variable. We entered the sampling year (2011-2013) as a 3-level factor to control for any variation across years, since many studies have reported consistent yearly changes in the strength of selection (reviewed in Siepielski et al., 2009). All models included the mating group identity as the random effect to allow the intercept of each group to vary. Model residuals were explored for any violation of assumptions, and values were log transformed when assumptions were violated. Moreover, because values on a given day of a trial are unlikely to be independent from values on previous days, we ran additional models including an autocorrelation structure of order 1 (Box et al., 1994; Pinheiro and Bates 2000). We visually inspected models using the function "plot.ACF" in 'nlme', and competing models (i.e., with and without an autocorrelation structure) were compared using the R package "AICcmodavg" v2.3-1 (Mazerolle 2020). Models with lower AIC scores were retained, and *p* values were calculated using likelihood ratio tests (LRT).

(ii) Testing for deviations from null expectations

We assessed the extent to which temporal changes in I, I_M, I_N, and I_P deviate from expectations generated assuming random mating and random fertilisation. This is particularly important because opportunity indexes have been criticised for being unable to distinguish variance arising from sexually selected and stochastic processes (Klug et al., 2010; Jennions et al., 2012). We generated

simulations of our data in order to compare opportunity indexes calculated from these simulations against the observed values from the 20 replicate groups. We ran 10,000 simulations using custom scripts with the following assumptions:

1) Individuals mate randomly over the 10 days of a trial. Pairs of individuals were randomized from a pool of 12 females and 10 males, emulating the sex ratio and group size in the empirical data.
2) The number of copulations is sampled daily from a normal distribution, using the same range, mean, and standard deviation calculated from the observed dataset. Probabilities were calculated independently for each day of the mating trial for accuracy.
3) The likelihood a female will lay is equal to the probability a female laid a fertilised egg in the empirical data, which was calculated independently per each day of the trial.
4) All males that copulated with a female at least one day before her laying an egg had an equal probability of fertilizing that egg. For simplicity, the number of copulations of a male with the same female did not affect his probability of fertilizing an egg.

We calculated indexes of opportunity as in equation (1) per each of the simulated groups, which were then used as the null expectation for comparisons. Predicted slopes with their associated confidence intervals derived from models in section (i) were plotted within the 95% range of the simulated values. Days in which model confidence intervals fell outside this range were considered to be significantly different from the mean randomised values (see McLain, 1986; McLain et al., 1993 for a similar approach), and could therefore be more confidently attributed to sexual selection. These simulations seek to identify possible deterministic temporal trends that are caused by factors other than sexual selection. For example, I_M should decrease over time as individuals tend to progressively mate with a larger proportion of members of the opposite sex available, thus achieving a similarly high M (i.e., given enough time, all individuals of a population can mate with all available partners exhausting intra-sexual variation in M). Similarly, average P should decline over successive days as females mate with more males. Alternatively, I_P may also decrease over time due to variation in P decreasing

as a function of sample size (i.e., given enough eggs, all males mating with a female ought to sire some embryos). Therefore, testing whether observed cumulative opportunity indexes exceed null expectations elucidates whether variance in some components of reproductive success is higher than expected by chance, consistent with the signature of sexually selected traits or strategies.

(iii) Comparison of cumulative and instantaneous opportunity estimates

We evaluated how opportunity indexes estimated based on a single day (instantaneous) compare with cumulative estimates over the 10-day period. Instantaneous estimates represent a scenario in which researchers are limited to one day of data collection, rather than a continuous sampling over the 10 days of the mating trial. This was done to evaluate the reliability of using opportunity indexes calculated from less complete behavioural and genetic data. We calculated instantaneous opportunity indexes as in equation (1), but this time using daily metrics of *RS*, *M*, *N* and *P* without input from previous days. *RS* was calculated as the number of embryos fertilised by males, or number of fertilised eggs laid by females, on a single day. *M* was calculated as the number of unique partners mated on a single day, irrespectively if they were mated in previous days. *P* was calculated as the percentage of embryos sired by a male across all the eggs laid by females he successfully mated on the previous day, and *N* the average number of eggs laid by these females on a given day. Daily (i.e. instantaneous) and cumulative opportunity indexes were entered as the response variable in a linear mixed model with method (daily or cumulative) and its interaction with the day of the trial (a factor with 10 levels) as the predictors, and the identity of the group as a random effect. The mean opportunity index was compared between methods on each day of the trial using a *post-hoc* Tukey test implemented in the R package "emmeans" v1.4.4 (Lenth et al., 2020)

RESULTS

(i) Temporal patterns in cumulative opportunity estimates

Residuals in all models were autocorrelated, so we included autocorrelation structures of order 1 (Box et al., 1994; Pinheiro and Bates 2000). Moreover, because relationships appeared quadratic, we

included a quadratic term in the models (i.e., Day order^2). In females, the opportunity for selection tended to decrease asymptotically over time until a plateau around the 8th day, represented by the significant and positive quadratic effect (I ~ Day order: χ^2_1 = 131.2, p < 0.001, I ~ Day order^2: χ^2_1= 74.33, p < 0.001, Figure 1a, Table 1). We found a similar negative trend and a positive quadratic effect in the female opportunity for pre-copulatory sexual selection (I_M ~ Day order: χ^2_1 = 91.09, p < 0.001, I_M ~ Day order^2: χ^2_1 = 38.31, p < 0.001, Figure 1b, Table 1).

As in females, we found a negative trend with a positive quadratic effect in male opportunity for selection, such that the curve tended to flatten towards the end of the trial (I ~ Day order: χ^2_1 = 68.46, p < 0.001, I ~ Day order^2: χ^2_1 = 35.71, p < 0.001, Figure 1c, Table 1). There was a similar, but less sharp, decrease in the opportunity for pre-copulatory sexual selection, which also contained a positive quadratic effect (I_M ~ Day order: χ^2_1 = 40.21, p < 0.001, I_M ~ Day order^2: χ^2_1 = 13.08, p < 0.001, Figure 1d, Table 1). Similarly, I_N also decrease over time with a positive quadratic effect (I_N ~ Day order: χ^2_1 = 68.18, p < 0.001, I_N ~ Day order^2: χ^2_1 = 29.3, p < 0.001, Figure 1e, Table 1). Finally, the opportunity for post-copulatory sexual selection in males tended to decrease linearly over time (I_P ~ Day order: χ^2_1 = , p < 0.001, Figure 1f, Table 1), as there was no evidence for a quadratic effect (I_P ~ Day order^2: χ^2_1 = 1.79, p = 0.181).

After controlling for temporal autocorrelations, we found that the contribution of variance in *M* (varM, Table 2) to variance in male *RS* did not change consistently over the trial (χ^2_1 = 2.69, p = 0.101). On the other hand, the contribution of variance in *N* (varN, Table 2) to variance in male *RS* decreased gradually over time, from around 28% on the first day of the trial to 2.92% on the last day (χ^2_1 = 25.58, p < 0.001). This decrease stabilised later in the trial, as we found a positive quadratic effect (χ^2_1 = 8.35, p = 0.004). The contribution of variance in *P* (varP, Table 2) tended to increase over time, from around 36% on the first day, to close to 60% at the end of the trial (χ^2_1 = 6.78, p = 0.009). However, this increase was dependent on the relatively low value on the first day. When the first day was removed from the model, *P* contribution did not increase over time (χ^2_1 = 0.81, p = 0.368). The contribution of the covariance between *M* and *P* (covMP, Table 2) increased over time,

from a negative -0.39% on the first day of the trial, to 14.64% on the last day ($\chi^2_1 = 4.57$, p = 0.033). The contribution of the covariance between *M* and *N* (covMN, Table 2) did not vary significantly over the trial ($\chi^2_1 = 0.80$, p = 0.370). Similarly, the contribution of the covariance between *N* and *P* (covNP, Table 2) to male *RS* was relatively constant over the trial ($\chi^2_1 = 0.06$, p = 0.802).

(ii) Testing for deviations from null expectations

Null expectations of temporal changes in opportunity indexes based on simulations were qualitatively similar to trends found in the empirical data (see (*i*) above). However, empirical estimates tended to be higher than null expectations, mainly towards the end of the trial. In females, observed *I* did not fall outside the 95% range of simulated values until the 9th day of the trial (Figure 1a). On the other hand, observed I_M was significantly higher than null expectations starting from the 6th day of the trial (Figure 1b).

In males, observed values of *I* fell within the 95% range of the simulated values for most of the mating trial, but was significantly higher starting from the 7th day (Figure 1c) and observed I_M was substantially higher than null expectations for most of the trial, starting from the 3rd day (Figure 1d). Conversely, at no point in the trial observed values of I_N fell outside the 95% range of simulated values (Figure 1e). Observed male I_P fell within the 95% range of the simulated values for most of the mating trial, but was significantly higher starting from the 7th day (Figure 1f).

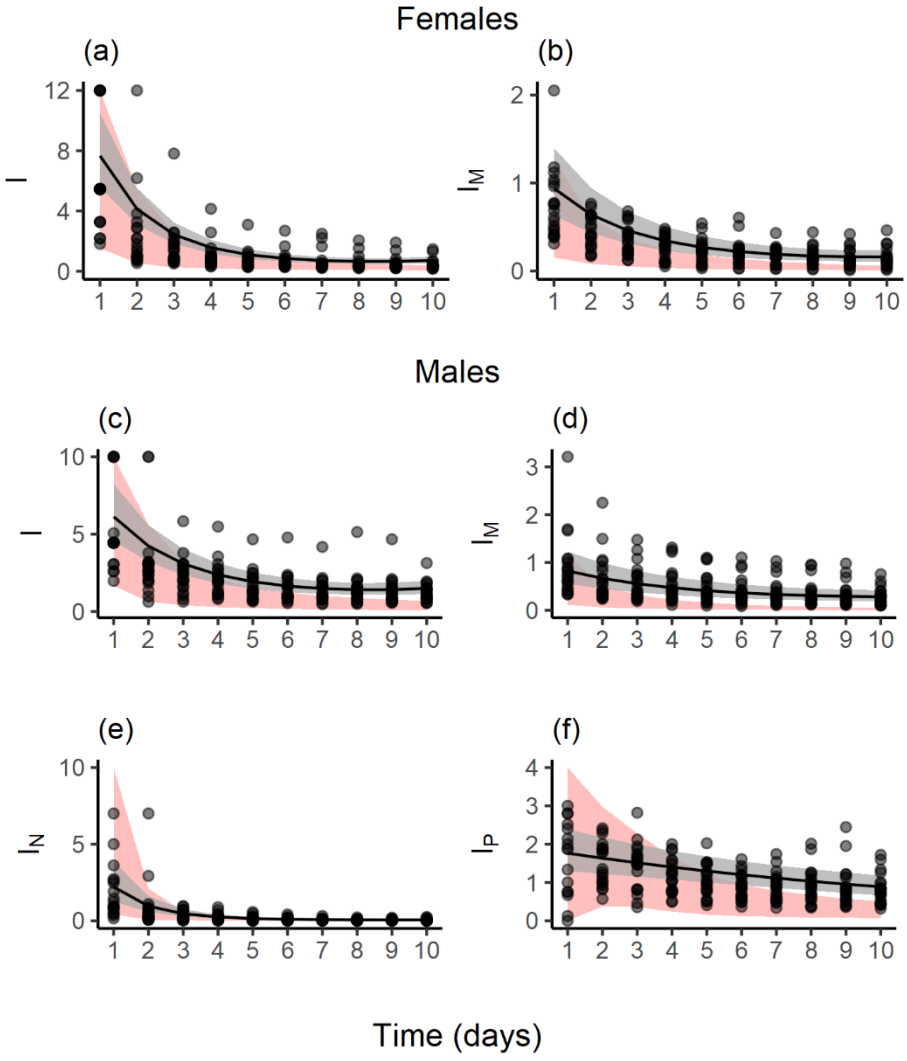


Figure 1 – Patterns of opportunity indexes compared with multiple simulations of random mating in red junglefowl (*Gallus gallus*). Each panel represents the marginal effect of an independent linear mixed model. Females: (a) standardised variance in reproductive success (opportunity for selection, I) (b) standardised variance in mating success (opportunity for pre-copulatory sexual selection, I_M). Males: (c) opportunity for selection (I), (d) opportunity for pre-copulatory sexual selection (I_M), (c) standardised variance in partner's fecundity (I_N); (d) standardised variance in paternity share (oportunity for post-copulatory sexual selection, I_P). Lines represent the predicted slopes and gray shades the confidence intervals. Variables were originally in the log scale, but were back-transformed to produce the figures. Red shaded areas represent the 95% range of 10,000 random mating simulations.

Table 1. Summary statistics and likelihood ratio tests results of multiple linear mixed-effects model (LMM) predicting the opportunity for selection (*I*) and its different variance components multiple replicate groups of red junglefowl, *Gallus gallus* . Notes: LRT, likelihood ratio test; SE, standard error; σ2, residual variance; τ00, random intercept variance.

FEMALES	**log(I)**				**log(I_M)**			
Predictors	*Estimates*	*SE*	*LRT*	*p*	*Estimates*	*SE*	*LRT*	*p*
(Intercept)	2.75	0.185	14.488	**<0.001**	0.36	0.217	6.859	**0.032**
Day_order	-0.75	0.051	131.199	**<0.001**	-0.45	0.042	91.09	**<0.001**
Day_order^2	0.04	0.208	74.326	**<0.001**	0.02	0.283	38.31	**<0.001**
year [2012]	-0.76	0.200			-0.29	0.272		
year [2013]	-0.83	0.004			-0.76	0.004		
Random Effects								
σ^2	0.16				0.64			
$\tau_{00\ groupID}$	0.12				0			
$N_{groupID}$	20				20			
Observations	198				200			
Marginal R^2 /	0.737 /				0.530 /			
Conditional R^2	0.850				0.530			

MALES	**log(I)**				**log(I_M)**				**log(I_N)**				**log(I_P)**			
Predictors	*Estimates*	*SE*	*LRT*	*p*	*Estimates*	*SE*	*LRT*	*p*	*Estimates*	*SE*	*LRT*	*p*	*Estimates*	*CI*	*LRT*	*p*
(Intercept)	2.24	0.176	5.926	0.052	0.040	0.214	0.399	0.819	1.750	0.352	8.456	**0.015**	0.650	0.166	3.937	0.140
Day_order	-0.45	0.050	68.464	**<0.001**	-0.24	0.036	40.206	**<0.001**	-1	0.112	68.178	**<0.001**	-0.08	0.017	18.369	**<0.001**
Day_order^2	0.03	0.191	35.711	**<0.001**	0.01	0.289	13.075	**<0.001**	0.05	0.344	29.298	**<0.001**				
year [2012]	-0.38	0.183			-0.16	0.278			-0.61	0.330			-0.37	0.194		
year [2013]	-0.43	0.004			-0.15	0.003			-1.03	0.010			-0.3	0.186		
Random Effects																
σ^2	0.25				0.36				1				0.28			
$\tau_{00\ groupID}$	0.00				0.03				0				0			
$N_{groupID}$	20				20				20				20			
Observations	198				200				198				194			
Marginal R^2/	0.513 /				0.249 /				0.625 /				0.198 /			
Conditional R^2	0.513				0.304				0.625				0.198			

(iii) Comparison of cumulative versus instantaneous opportunity estimates

The instantaneous (daily) approach led to a significantly overestimation of most opportunity indexes in both sexes. In females, there was a significant overall difference in I ($F_{9,354} = 13.75$, $p < 0.001$, Figure 3a) and I_M ($F_{9,360} = 30.9$, $p < 0.001$, Figure 3b) between daily and cumulative methods. *Post-hoc* Tukey tests showed that daily values of I and I_M were significantly higher than cumulative values starting from the 2th day of the trial.

Similarly, in males there was a significant overall difference in I ($F_{9,354} = 5.3$, $p < 0.001$, Figure 3c), I_M ($F_{9,361} = 10.38$, $p < 0.001$, Figure 3d) and I_N ($F_{9,353} = 10.94$, $p < 0.001$, Figure 3e) between the daily and cumulative method. *Post-hoc* Tukey tests showed that daily values of I and I_M were significantly higher than cumulative values starting from the 2th day of the trial, and daily values of I_N were significantly higher than cumulative values starting from the 4th day of the trial. We found no significant overall difference in I_P between the methods ($F_{9,333} = 0.98$, $p = 0.458$, Figure 3f). A *Post-hoc* Tukey test showed that daily and cumulative methods yielded similar estimates throughout the course of a trial with the exception of the 2nd day, when daily values of I_P were lower than cumulative values.

Table 2 - Percentage of variance in standardised reproductive success (*I*) explained by each selection episode in male red junglefowl (*Gallus gallus*). Data is separated by each day of the mating trial.

	% of *I*						
Day	var(P)	var(M)	var(N)	cov(M,N)	cov(M,P)	cov(N,P)	D
1	36.34	23.69	28.36	-6.66	-0.39	0.73	21.84
2	65.29	24.85	18.24	0.59	4.73	-4.03	-9.48
3	72.77	24.68	15.38	3.25	-1.15	-3.95	-10.97
4	60.10	21.82	11.12	6.31	-0.62	-3.63	4.89
5	70.55	25.01	8.18	6.81	-4.80	-3.77	-1.99
6	63.33	24.88	5.90	3.64	1.46	0.41	0.38
7	65.04	24.44	4.69	2.01	4.99	1.11	-2.28
8	64.15	23.07	3.36	-0.10	7.32	0.74	1.45
9	59.79	22.15	3.43	-0.15	10.07	0.24	4.46
10	58.30	20.75	2.92	-0.70	14.64	-0.62	4.71

Note: cov = covariance, var = variance, D = error, M = mating success, N = average partner's fecundity, P = paternity share. Percentages were calculated from mean values across groups.

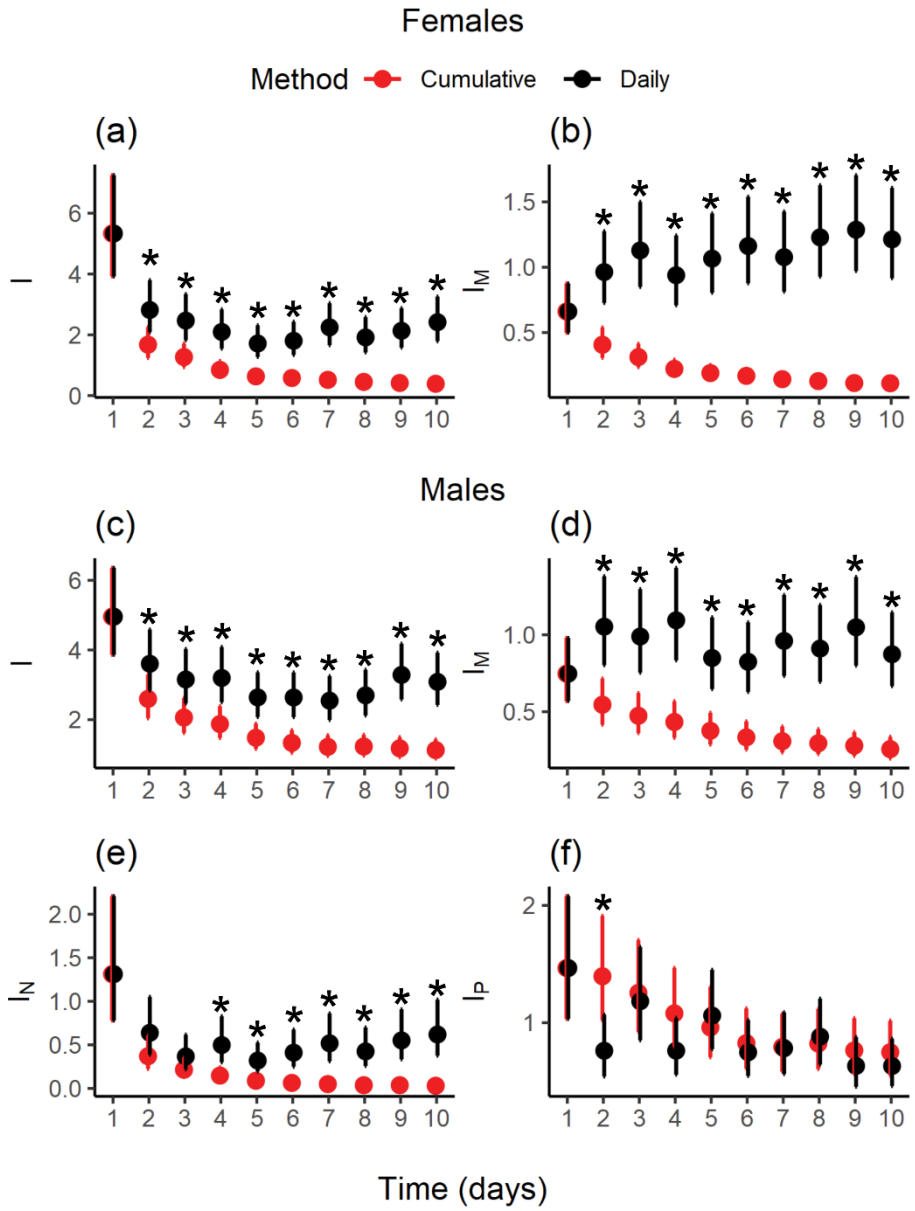


Figure 2 – Comparison between opportunity indexes calculated using cumulative (red) and daily (black) reproductive success in female and male red junglefowl (*Gallus gallus*). Females: (a) Standardised variance in reproductive success (opportunity for selection, *I*), (b) standardised variance in mating success (opportunity for pre-copulatory sexual selection, I_M). Males: (c) Opportunity for selection (*I*); (d) Opportunity for pre-copulatory sexual selection (I_M); (e) standardised variance in partner's fecundity (I_N); (f) standardised variance in paternity share (oportunity for post-copulatory sexual selection, I_P). Panels represent the marginal effects from individual linear mixed models. Dots represent the means, bars the confidence intervals, and asterisks the level of significance of *post-hoc* Tukey tests ($p<0.05$).

DISCUSSION

Understanding the causes and consequences of temporal variation in sexual selection is key to understanding both how phenotypic variation is maintained, and the evolutionary diversity of sexual strategies (Saccheri & Hanski, 2006; Kotiaho et al., 2008; Cornwallis & Uller, 2010). However, progress has been hampered by three key shortcomings. First, current approaches typically explore

seasonal changes in sexual selection, overlooking the potential for fluctuations in selection over much shorter time scales, such as over the course of an individual's mating sequence. Second, the extent to which dynamic changes in variation in components of reproductive success (*RS*) can be explained by random patterns of cumulative mating and fertilisation or non-random mechanisms of selection remains unknown. Third, accurately estimating the strength and direction of sexual selection requires high resolution behavioural and paternity data but these are difficult to obtain. Therefore, we do not fully appreciate how variation in sampling duration will affect estimates of sexual selection. In this study, we attempted to address these issues using a dataset of detailed behaviour and molecular parentage in replicate groups of polyandrous red junglefowl followed over the course of 10-day mating trials. Our results show that standardised variances (i.e., opportunity for selection) in multiple components of *RS* can decrease substantially over the course of just a few days in both females and males, and that the relative contribution of different pre- and post-copulatory sources of variance in male *RS* and their covariances also change dynamically. Additionally, simulations of our empirical data reveal a pronounced temporal trend whereby cumulative opportunity estimates progressively exceed null expectations toward the last days of a trial, highlighting the importance of null models in appraising temporal dynamics of sexual selection in polyandrous groups with extended sperm storage. Finally, we show that variance in most components of *RS* in both sexes are increasingly overestimated over a trial if they are calculated daily rather than cumulatively, although variance in paternity share was not as sensitive to the different approaches. These findings suggest key sensitivity of sexual selection measures to the time over which sampling occurs, so even small differences in sampling periods can influence comparisons across studies or populations.

We found that in both males and females, standardised variances in *RS* and all its components tended to decrease over time in the observed data, but this decrease also occurred in variances calculated from random mating simulations. This suggests that temporal trends in the observed data may be driven by factors other than non-random changes in mating or paternity bias. For example, patterns could be partially attributed to increases in sampling effort over time, as variance in mating

success declines exponentially with the number of observations until an asymptote is reached (McCauley, 1983; McLain et al., 1993). Similarly, we found that opportunity curves tended to flatten later in the trial, as evidenced by significant quadratic effects detected in our models. On one hand, these results support previous criticism that opportunity measures are only estimates of a potential for selection, and that large components of variance may be driven by random mating or sampling issues (Downhower et al., 1987; Klug et al., 2010; Jennions et al., 2012). On the other hand, comparing observed estimates with simulated values allowed us to better disentangle variance arising by random mating alone and possible sexual selection mechanisms. For example, I_M in both sexes tended to be closer to null expectations on the first day, probably due to individuals being more likely to mate when placed together after being sexually rested for multiple days (Løvlie & Pizzari, 2007). However, observed I_M started to deviate from simulated values quite early in the trial, suggesting non-random mating patterns diminished erosion in I_M, which could be driven by social status and mating preferences. In red junglefowl, dominant males are usually preferred by females, and actively interrupt copulation attempts from subordinates (Cheng & Burns, 1988; Pizzari, 2001; Dean et al., 2011). Similarly, females actively solicit copulations from multiple males, and more fecund or more attractive females are usually preferred by males (Pizzari et al., 2003; McDonald et al., 2019). In other species of birds, females also seek extra-pair copulations (EPCs), but while some studies have suggested EPCs increase variance in *M* (Whittingham & Dunn, 2005; Webster et al., 2007; Albrecht et al., 2009), other studies in more polyandrous species have suggested the opposite (Jones et al., 2001; Collet et al., 2012; Pélissié et al., 2014; Turnell & Shaw, 2015; McDonald et al., 2017). It is possible then, that some polyandry might lead to high variation in mating success early in a trial, which posteriorly decreases due to saturation in sexual networks, reducing variance in *M*. Our simulations provide strong evidence this is the case, showing that - given random daily mating patterns - increases in polyandry alone contribute to the erosion in variance in mating success, providing similar results within groups through time as has been observed across groups (McDonald & Pizzari, 2018; Morimoto et al., 2019). Direct comparisons of these mechanisms with other taxa are

difficult due to the scarcity of studies investigating temporal changes in I and I_M without manipulation of environmental conditions or OSR (e.g., McLain et al., 1993; Wacker et al., 2014; Shuster et al., 2019). However, two studies with different species of polygynandrous gobies have found that opportunity for selection decreases over the season (Wacker et al., 2014; Wong et al., 2018), probably due to natural changes in OSR (Wacker et al., 2014). Similarly, a recent 3-year study with the monogamous prairie vole (*Microtus ochrogaster*) showed that differences in variance in *RS* between alternative mating tactics decreased over time, as did the total opportunity for selection in males (Shuster et al., 2019).

As with I_M, observed male I_P tended to decrease over time but only started deviating from randomised values much later in the trial. Additionally, we found no evidence for a positive quadratic effect in decreases in I_P suggesting erosion in variance in paternity share was still continuing when we stopped observations, despite at a lower rate than expected under random mating. These patterns are probably a consequence of the time lag between mating and fertilisation events due to female sperm storage. It is possible that non-random changes in I_M take time to translate into non-random changes in I_P as sperm from earlier inseminations must be either used up or leaked from the female reproductive tract before any consistent bias in paternity share is observed (Lessells & Birkhead, 1990; Carleial et al., 2020a). This result was further supported by decomposition of variance in male *RS*, which showed that the contribution of the covariance between *M* and *P* tended to increase only later in the trial (>7 days). This indicates that while variance in mating success in males decreases, those males with higher *M* are increasingly able to secure a larger share of paternity with their partners. This is in agreement with previous studies using this dataset, which found that dominant males who mate with more females also tended to remate more often than subordinates (McDonald et al., 2017), and that higher remating rates towards the end of the trial increased the fertilisation probability of later eggs (Carleial et al., 2020a). Moreover, our finding that variance in *P* contributed more to variance in male *RS* over the entire trial confirms the higher relative importance of post-

copulatory sexual selection in highly polyandrous populations (Collet et al., 2014; Pélissié et al., 2014; Devigili et al., 2015; Turnell & Shaw, 2015; Morimoto et al., 2019).

Observed opportunity indexes more dependent on female fecundity (i.e., female I and male I_N) barely differed from randomised values over the entire trial. This suggests that the distribution of female fecundity was roughly normal in our trials, and that no male consistently mated with more fecund females. One caveat is that our measure of fecundity was not ideal, as unfertilized eggs could not be assigned to females. Similarly, the sharp decrease in observed female I and male I_N from day 1 to day 2 was probably a function of fertilization failure across multiple eggs at the beginning of the trial (e.g., Collet et al., 2014), due to sperm from earlier copulations not reaching the eggs in time for fertilization (Etches, 1996). This latter result explains why we found that N contributed heavily to variance in male RS early in the trial, but that this contribution tended to decrease over time. Although previous studies with fowl have shown that males prefer females with larger combs – a trait that covariates with fecundity – (Pizzari et al., 2003; Cornwallis & Birkhead, 2007), we know little on whether males are able to identify, and preferentially target, more fecund females. Future studies would benefit from exploring this possibility.

Finally, with the exception of male I_P, we found that in both sexes opportunity values calculated with daily (i.e., instantaneous) measures of RS and its components were increasingly higher than opportunity values calculated with cumulative measures. The discrepancies between cumulative and instantaneous measures was significant after as early as the 2nd day of the trial, suggesting studies relying on instantaneous measures will increasingly overestimate variance in RS even over short time periods. This may explain why many studies found that phenotypic variation is not eroded as quickly as expected given current estimates of directional selection (e.g., Tomkins et al., 2004). It may be the case that actual cumulative sexual selection is these systems is much weaker due to individuals converging to similar RS over time, but it is being overestimated by instantaneous measures of RS. For example, alternative mating strategies may allow subordinate or undesirable males to skew paternity in their favour (Taborsky et al., 2008), or life history trade-offs associated with high early

reproductive success may reduce further success (e.g., Preston et al., 2001). Moreover, while directional sexual selection may be correctly estimated during a sampling period, it may be counter-balanced later by changing environmental conditions (e.g., Ghislandi et al., 2018; Cattelan et al., 2020) or flexible mating preferences (e.g., Chaine & Lyon, 2008; Lehtonen et al., 2010). However, the striking similarity between daily and cumulative I_P is surprising, since we were expecting the instantaneous values would be lower than cumulative values since males that mated successfully prior to observations but failed to sire any eggs would not be included in instantaneous calculations of paternity share. However, the similarity in I_P between both approaches suggests that sampling mating pairs over a single day of behavioural observations prior to collecting eggs, and inferring missing pairs through paternity data is representative of the population of mated individuals. This result is encouraging, as it suggests that estimates of post-copulatory sexual selection based on instantaneous approaches can be reliable. Moreover, our results suggest a potential utility in combining instantaneous and cumulative approaches. While standardised variances in *RS* using cumulative values more accurately represent the total selection in a population, daily estimates of variance in *RS* may help identifying interesting temporal patterns in mating behaviour driving changes in selection (Baena & Marcías-Ordóñez, 2013). For example, imagine a species in which individuals engage in alternative mating strategies which are similarly successful on the long term (e.g., Dowling & Webster, 2017; Shuster et al., 2019). Here, cumulative values of opportunity will decrease over time, but we will be none the wiser as to why, particularly if the behaviour of the species is not well characterised. Comparing daily values with cumulative values may give us some insight however, if for example the alternative mating strategies are clustered at different moments in the sampling period, then we would expect daily opportunities estimates to peak during those times. Therefore, future studies should report values from both approaches when cumulative data is available, particularly when biological information on the study system is lacking. Moreover, studies aimed at quantifying present sexual selection in natural populations should devise carefully the sampling duration, and be aware of the pitfalls arising from estimating overall sexual selection based simply

on snapshots of mating and genetic data, and its implications to broader assumptions related to sexual selection theory.

In summary our results converge to provide three key insights: (1) variance in *RS* can change over short time scales, (2) these changes are partially driven by sampling effort and random mating alone, and (3) deterministic processes take time to generate non-random variance in *RS*, particularly through post-copulatory episodes. Our study therefore, represents a platform for a practical guide to measuring short-term dynamics of sexual election in polyandrous species with sperm storage. It shows that studies estimating variance in *RS* over a short sampling period may confound variance arising by chance with variance arising by sexual selection, or highly overestimate variance in mating success after missing the sequence of copulations. Studies using mating and paternity data should therefore sample over many days (> 5) in order to get more accurate estimates, and should include comparisons with null models to confirm whether temporal trends are driven by sexual selection or by other factors. Moreover, we suggest that the length of sampling should be carefully tailored to the length of sperm storage, and that studies should provide both cumulative and cross-sectional estimates of opportunity for selection in order to gather insights about mating behaviours driving dynamic changes in sexual selection.

Suplementary material for:
Towards an appreciation of short-term dynamics of sexual selection in polyandrous populations

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1. TABLES

Table S1. Summary statistics for components of reproductive success in female red junglefowl (*Gallus* gallus) over 10 days of a mating trial. Main values represent mean across 20 replicate mating groups, and standand erros are within parentheses.

Cumulative	Reproductive success			Mating success		
Time (days)	I	mean	variance	I_M	mean	variance
1	5.98 (0.8)	0.18 (0.03)	0.17 (0.02)	0.74 (0.09)	1.78 (0.11)	2.22 (0.25)
2	2.39 (0.59)	0.5 (0.04)	0.39 (0.04)	0.45 (0.04)	2.66 (0.14)	3.03 (0.31)
3	1.63 (0.36)	0.83 (0.06)	0.82 (0.06)	0.35 (0.04)	3.29 (0.21)	3.35 (0.28)
4	1.05 (0.2)	1.2 (0.09)	1.19 (0.13)	0.26 (0.03)	3.83 (0.24)	3.34 (0.31)
5	0.77 (0.14)	1.62 (0.12)	1.67 (0.17)	0.23 (0.03)	4.28 (0.26)	3.53 (0.33)
6	0.7 (0.13)	2.05 (0.14)	2.35 (0.2)	0.21 (0.03)	4.6 (0.27)	3.65 (0.34)
7	0.68 (0.14)	2.38 (0.17)	2.75 (0.23)	0.18 (0.02)	4.84 (0.28)	3.51 (0.37)
8	0.55 (0.1)	2.78 (0.18)	3.42 (0.31)	0.16 (0.02)	5.14 (0.28)	3.55 (0.36)
9	0.51 (0.1)	3.17 (0.22)	4.05 (0.38)	0.15 (0.02)	5.32 (0.28)	3.47 (0.39)
10	0.46 (0.08)	3.51 (0.22)	4.7 (0.42)	0.15 (0.02)	5.5 (0.28)	3.65 (0.42)
Daily						
1	5.98 (0.8)	0.18 (0.03)	0.17 (0.02)	0.74 (0.09)	1.78 (0.11)	2.22 (0.25)
2	3.78 (0.72)	0.32 (0.03)	0.24 (0.02)	1.08 (0.11)	1.22 (0.09)	1.49 (0.19)
3	2.92 (0.56)	0.33 (0.03)	0.25 (0.02)	1.39 (0.29)	1.16 (0.14)	1.43 (0.19)
4	2.24 (0.26)	0.36 (0.03)	0.25 (0.02)	1.06 (0.11)	1.26 (0.12)	1.53 (0.21)
5	2.18 (0.36)	0.43 (0.03)	0.28 (0.02)	1.44 (0.3)	1.3 (0.12)	1.74 (0.23)
6	2.3 (0.33)	0.43 (0.04)	0.31 (0.05)	1.3 (0.16)	1.25 (0.12)	1.87 (0.23)
7	3.4 (1.07)	0.36 (0.04)	0.25 (0.03)	1.33 (0.21)	1.18 (0.11)	1.55 (0.22)
8	2.54 (0.56)	0.38 (0.03)	0.24 (0.02)	1.38 (0.14)	1.29 (0.1)	2.01 (0.19)
9	3.46 (0.86)	0.39 (0.04)	0.26 (0.03)	1.5 (0.19)	1.09 (0.12)	1.56 (0.23)
10	2.73 (0.31)	0.34 (0.02)	0.26 (0.02)	1.39 (0.18)	1.29 (0.14)	2.02 (0.3)

Note: I = standardised variance in reproductive success; I_M = standardised variance in mating success. Standardised values are calculated as variance/mean^2.

Table S2. Summary statistics for components of reproductive success in male red junglefowl (*Gallus gallus*) over 10 days of a mating trial. Main values represent mean across 20 replicate mating groups, and standand erros are within parentheses.

Cumulative	Reproductive success			Mating success			Average partner's fecundity			Paternity share		
Time (days)	I	mean	variance	I_M	mean	variance	I_N	mean	variance	I_P	mean	variance
1	5.22 (0.62)	0.22 (0.03)	0.24 (0.04)	0.9 (0.15)	2 (0.14)	3.07 (0.38)	1.88 (0.4)	0.26 (0.05)	0.08 (0.02)	1.63 (0.22)	0.52 (0.06)	0.23 (0.03)
2	3.18 (0.55)	0.61 (0.05)	1 (0.15)	0.67 (0.11)	2.97 (0.18)	5.23 (0.71)	0.85 (0.35)	0.62 (0.06)	0.15 (0.04)	1.5 (0.13)	0.36 (0.04)	0.16 (0.02)
3	2.33 (0.26)	1.01 (0.08)	2.33 (0.37)	0.57 (0.08)	3.77 (0.26)	6.94 (0.83)	0.34 (0.07)	0.99 (0.08)	0.28 (0.06)	1.4 (0.14)	0.28 (0.02)	0.11 (0.02)
4	2.11 (0.25)	1.44 (0.1)	4.35 (0.7)	0.53 (0.08)	4.36 (0.3)	8.34 (0.91)	0.24 (0.05)	1.35 (0.11)	0.3 (0.05)	1.17 (0.1)	0.26 (0.03)	0.08 (0.02)
5	1.69 (0.21)	1.95 (0.14)	6.58 (1.11)	0.46 (0.07)	4.95 (0.32)	9.44 (1.01)	0.15 (0.03)	1.77 (0.12)	0.38 (0.07)	1.05 (0.09)	0.22 (0.02)	0.06 (0.01)
6	1.54 (0.21)	2.47 (0.17)	9.61 (1.54)	0.41 (0.06)	5.42 (0.34)	10.05 (1.01)	0.1 (0.02)	2.24 (0.14)	0.43 (0.1)	0.89 (0.08)	0.2 (0.02)	0.04 (0.01)
7	1.4 (0.18)	2.91 (0.2)	11.91 (1.8)	0.37 (0.06)	5.8 (0.35)	10.45 (0.96)	0.07 (0.02)	2.61 (0.16)	0.42 (0.07)	0.87 (0.08)	0.19 (0.01)	0.04 (0.01)
8	1.44 (0.22)	3.37 (0.22)	15.99 (2.4)	0.36 (0.06)	6.18 (0.35)	11.2 (0.91)	0.05 (0.01)	3.04 (0.19)	0.48 (0.11)	0.92 (0.1)	0.17 (0.01)	0.03 (0.01)
9	1.36 (0.2)	3.84 (0.26)	19.82 (3.05)	0.34 (0.05)	6.48 (0.37)	11.61 (0.94)	0.05 (0.01)	3.4 (0.22)	0.58 (0.13)	0.87 (0.12)	0.17 (0.01)	0.03 (0.01)
10	1.25 (0.14)	4.25 (0.27)	22.8 (3.42)	0.3 (0.04)	6.73 (0.36)	11.64 (0.92)	0.05 (0.01)	3.72 (0.23)	0.58 (0.13)	0.83 (0.09)	0.16 (0.01)	0.02 (<0.01)
Daily												
1	5.22 (0.62)	0.22 (0.03)	0.24 (0.04)	0.9 (0.15)	2 (0.14)	3.07 (0.38)	1.88 (0.4)	0.26 (0.05)	0.08 (0.02)	1.63 (0.22)	0.52 (0.06)	0.23 (0.03)
2	4.43 (0.68)	0.39 (0.04)	0.52 (0.08)	1.28 (0.19)	1.51 (0.12)	2.56 (0.42)	1.31 (0.32)	0.45 (0.06)	0.12 (0.03)	0.89 (0.12)	0.59 (0.05)	0.21 (0.03)
3	3.41 (0.32)	0.4 (0.04)	0.57 (0.09)	1.29 (0.25)	1.61 (0.16)	2.55 (0.36)	0.44 (0.09)	0.61 (0.05)	0.15 (0.05)	1.2 (0.16)	0.46 (0.04)	0.19 (0.02)
4	3.59 (0.46)	0.44 (0.04)	0.69 (0.11)	1.35 (0.21)	1.69 (0.14)	3.05 (0.31)	0.78 (0.17)	0.54 (0.07)	0.12 (0.01)	1 (0.15)	0.5 (0.04)	0.2 (0.02)
5	3.05 (0.35)	0.51 (0.04)	0.73 (0.1)	0.94 (0.11)	1.85 (0.16)	2.99 (0.39)	0.5 (0.11)	0.61 (0.05)	0.12 (0.01)	1.15 (0.14)	0.47 (0.04)	0.19 (0.02)
6	3.22 (0.5)	0.52 (0.05)	0.7 (0.1)	1.15 (0.25)	1.82 (0.16)	2.94 (0.39)	0.8 (0.17)	0.63 (0.06)	0.19 (0.04)	0.97 (0.11)	0.51 (0.05)	0.19 (0.02)
7	3.22 (0.6)	0.44 (0.04)	0.52 (0.09)	1.08 (0.11)	1.72 (0.14)	2.82 (0.3)	0.86 (0.26)	0.49 (0.06)	0.11 (0.01)	0.93 (0.14)	0.55 (0.05)	0.18 (0.02)
8	3.33 (0.57)	0.47 (0.04)	0.61 (0.1)	1.07 (0.14)	1.89 (0.12)	3.22 (0.25)	0.86 (0.33)	0.53 (0.05)	0.12 (0.01)	1.08 (0.15)	0.5 (0.04)	0.2 (0.02)
9	3.88 (0.61)	0.47 (0.05)	0.72 (0.13)	1.23 (0.15)	1.63 (0.17)	2.75 (0.35)	1.28 (0.43)	0.55 (0.06)	0.18 (0.04)	0.93 (0.21)	0.57 (0.05)	0.16 (0.02)
10	3.29 (0.26)	0.41 (0.03)	0.53 (0.07)	0.99 (0.13)	1.82 (0.19)	2.93 (0.46)	0.95 (0.17)	0.47 (0.04)	0.15 (0.03)	0.98 (0.2)	0.55 (0.05)	0.18 (0.03)

Note: I = standardised variance in reproductive success; I_M = standardised variance in mating success; I_N = standardised variance in average partner's fecundity; I_P = standardised variance in paternity share. Standardised values are calculated as variance/mean^2.

CHAPTER 6

General Discussion

In this thesis, I explored multiple pre- and post-copulatory mechanisms driving temporal changes in sexual selection in a population of a polygynandrous bird, the red junglefowl (*Gallus gallus*). This investigation was prompted by the pressing need in understanding the tempo of evolution, and from recent suggestions that selection may often change over time (Estes & Arnold, 2007; Robinson et al., 2008; Siepielski et al., 2009; Bell, 2010; Siepielski et al., 2011). Estimates of selection extrapolated from discrete data may therefore misrepresent cumulative selection, and impair our understanding of key paradigms in evolutionary biology such as the persistence of phenotypic variation in populations despite apparent strong sexual selection (Rowe & Houle, 1996). Below, I summarise the main findings of this study.

Mating and courtship activity vary between morning and evening, and can lead to cumulative losses in body condition over a mating trial

Circadian patterns in mating behaviour have received relatively little consideration within the context of sexual selection, despite evidence that the timing a behaviour is expressed can itself be sexually selected (Hau et al., 2017). In accordance with this hypothesis, differences in circadian rhythms across individuals (i.e., chronotypes, Schwartz et al., 2017) can lead to differences in reproductive success (e.g., Kempenaers et al., 2010), and have been shown be inheritable (e.g., Daan & Beersma, 2002; Michael et al., 2003; Schöfl et al., 2009, Helm & Visser, 2010; Matsumura et al., 2020). However, mating and sexually-selected traits and behaviours, such as sexual ornaments and social status, can be costly to express, or can impose costs on the opposite sex (Rowe & Houle, 1996; Lehtonen et al., 2012; Foley et al., 2018; Hare & Simmons, 2019), potentially offsetting benefits of behaviour strategies. In **Chapter 2** I investigated circadian patterns in multiple sexual behaviours in males and females, with particular emphasis on differences between morning and evening. I also tested whether these circadian patterns remain constant across successive days, and whether dominant and subordinate males are active at different times of the

day (i.e., display distinct chronotypes). In **Chapter 3,** I complemented this investigation by exploring potential costs, represented by dynamic changes in two condition-dependent traits (i.e., body mass and comb size), of mating behaviour, social status, and male harassment over the mating trial in both sexes. I showed that male mating and courtship activity, and female responses to this activity, is consistent across days, with behaviours showing clear divergent trends between the morning and the evening. For example, while females tend to increase receptivity to mating over the morning, they become increasingly more resistant to mating over the evening. These changes could be driven by females becoming more resistant to mating after securing enough sperm supplies. Female resistance to mating attempts can reflect sexual conflict over mating decisions, as males usually attempt to mate irrespective of female interest (Pizzari & McDonald, 2019). In accordance with this, I found that male and female behaviours are out of phase, since males do not concentrate mating attempts at times when females are more sexually receptive. In **Chapter 3,** I showed that both mating effort and sexual conflict are associated with loss in condition, as males that mated more often, or with more females, and females who experienced more coerced copulations, lost more body mass over the trial. While costs of mating have been well documented in some insects (e.g., Cordts & Partridge, 1996; Mappes et al., 1996; Kotiaho, 2000; Paukku & Kotiaho, 2005), my thesis is one of the few studies quantifying mating costs in male vertebrates (but see Preston et al., 2001; Fisher et al., 2013). Contrary to previous studies (Zuk & Johnsen, 2000; Cornwallis & Birkhead, 2008), I did not find that changes in comb size were consistently associated with the social environment.

I also showed that male courtship activity varies over the day as they tend to increase courtship effort over the morning, but reduce it over the evening. These changes could be influenced by environmental zeitgebers such as day light, which have been previously shown to affect gonadal development and hormones levels associated with photoperiodism (Ono et al., 2009), and behaviours, such as time of roosting (Kent et al., 1997). Moreover, I found that dominant males courted females more often than subordinates, but that these differences were quantitative, rather

than qualitative i.e., males from different social ranks were active at the same time of the day. These quantitative differences could be explained by dominant males suppressing subordinates by constantly initiating aggressive interactions. In **Chapter 3,** I showed that being on the receiving end of aggressive interactions is associated with loss of body mass in subordinate males. This loss in condition could explain why subordinates were less active at courting females than dominant males, but this is unlikely since I also show that dominant and subordinate males attempt to mate as often and at the same time. Another explanation is that dominant and subordinate males tend to invest in alternative mating strategies. While dominant males may invest more heavily on pre-copulatory strategies such as higher frequency of courtship displays and mate guarding (Cheng & Burns, 1988; Pizzari, 2003; Wilson et al., 2008), subordinate males may invest in post-copulatory traits, such as sperm quality, in an attempt to increase the odds of outcompeting males in sperm competition (Birkhead et al., 1999; Froman et al. 2002).

Collectively, the results of **Chapter 2** and **Chapter 3** of my thesis indicate dynamic within-day, but constant across-days, patterns in mating behaviour, with cumulative effects that can lead to loss in condition over a mating trial. A better understanding of circadian variation in female receptivity to mating, and the causes of male harassment, could have important translational ramifications, by informing better management of animal welfare in commercial poultry flocks and captive populations (Pizzari, 2016).

The relative importance of predictors of male fertilization success vary over time

In **Chapter 4,** I attempted to predict the probability that a male would fertilise individual eggs over successive days of a mating trial, using multiple predictors previously associated with variation in reproductive success in controlled conditions. While earlier experimental work had discovered a role for multiple independent traits and mechanisms determining the outcome of sperm competition between competing males (Birkhead & Pizzari, 2002; Parker & Pizzari, 2010; Simmons &

Fitzpatrick, 2012; Fitzpatrick & Lüpold, 2014), we lack confirmation on whether these factors were important in more natural conditions, where multiple traits interact and influence fertilization probabilities. Testing these relationships is particularly challenging in species with internal fertilization and prolonged female sperm storage, since here there is a clear temporal separation between insemination and fertilisation events, which is tricky to follow without fine-grained data on mating behaviour and paternity (Birkhead & Møller, 1993; Orr & Brennan, 2015). My work therefore, attempts to overcome these challenges, and develops a novel methodological platform to examine the relative importance of different factors to male reproductive success dynamically through time. I found that higher remating rates with the same female, and mating after other competitors increased a male's fertilization success, but only across eggs laid towards the end of the trial. I show that part of this effect is accountable by sperm from his rivals being lost from the female sperm storage organs. I also found that competing against more males, and being older than them, is detrimental to a male's success in sperm competition. Finally, a male's social status, his relatedness to the female and the state of his sperm reserves are not significant predictors of his fertilization success when other factors are taken into account, contradicting some earlier experimental work (e.g., Froman et al., 2002; Pizzari et al., 2004). My work provides three main insights: (1) the relative importance of certain predictors of fertilization success varies over time, demonstrating that temporal components must be included in empirical studies and mathematical models of sperm competition, (2) multiple well-known predictors of male reproductive success in controlled conditions are not necessarily relevant in more natural groups, and (3) most of the variation in male fertilization success (~80%) remains unexplained, confirming the challenge of studying sperm competition in nature, and reminding that more work is required to overcome this difficulty. Nevertheless, my work has hopefully contributed to establish a platform for future studies, and has helped elucidating the temporal complexity of sperm competition in birds.

Opportunity for selection can vary over short time-scales

Despite increasing appreciation for the necessity of accounting for temporal changes in selection, research has suffered from three shortcomings. First, studies have typically explored changes in selection across study years (reviewed in Siepielski et al., 2009), or between the beginning and end of a mating season (e.g., Reichard et al., 2008; Wacker et al., 2014). However, in many species mating can occur at much shorter time scales (e.g., Ulloa et al., 2019), and changes in mating preferences have been shown to oscillate after a few days or even after a single reproductive event (e.g., Fedina & Lewis, 2007). Second, studies on sexual selection often lack null models for comparison (Prum, 2010), despite evidence that random mating alone can lead to variance in reproductive success (Klug et al., 2010; Jennions et al., 2012). For example, the classical study of Sutherland (1985) used simulations to demonstrate how random mating could produce similar patterns in variance in mating success as observed in Bateman's (1948) seminal work with *Drosophila*. Finally, accurate estimates of sexual selection require fine-grained information on mating events and offspring parentage data, which can be difficult to obtain. Therefore, many studies are limited in the data they can collect, and we know little about how sampling effort affects estimates of sexual selection. In **Chapter 5,** I attempted to tackle all these three issues by investigating how standardised variances in multiple components of reproductive success in males and females change over the course of a mating trial. I also developed simulations of random mating to evaluate whether observed patterns significantly deviated from null expectations. Finally, I explored the impact of limited data collection on estimates of selection by comparing cumulative variance in reproductive success with variance calculated independently on each day of the trial. My results showed that standardised variance in all components of reproductive success strongly decrease over the course of just a few days in both sexes, that these decreases can be driven by random mating alone, and that the length of the sampling period can drastically affect estimations of sexual selection in natural populations. These results suggest that studies that do not account for the cumulative sequence of mating events, or fail incorporate null models into their analyses, will possibly overestimate sexual selection and confound variance in different components of

reproductive success arising from random mating and sexual selection mechanisms, even over relatively short time-scales. My work therefore, consolidates and builds upon previous studies which have called attention to the pitfalls of estimating selection without a proper analytical framework (Klug et al., 2010; Jennions et al., 2012).

When and how sexual selection is measured is critical for our understanding of this process in natural populations

The results of this thesis indicate that accounting for dynamics in sexual selection at different temporal scales can be important for a fuller understanding of this process. For example, while I show that sexual selection on male remating rates and mating order increases towards the end of the trial (**Chapter 4**), this increase was not sufficient to prevent erosion in cumulative sexual selection over the entire trial (**Chapter 5**). These results demonstrate how evaluating traits in isolation can be misleading, and the importance of the time window in which selection is measured. For example, my results suggest that stochasticity may contribute to variance in reproductive success at the beginning of a mating trial, but that the cumulative effect of deterministic sexually-selected mechanisms may lead to biases in mating and reproductive success after just a few days. This is not entirely surprising considering that dominance hierarchies take time to stabilise, sampling mates is a continuous process that can be influenced by chance, females can store sperm for multiple days, and costs associated with mating may take time to exert an effect. What is surprising however, is how quickly these different mechanisms can lead to appreciable changes in variance in reproductive success over time. This conclusion raises many questions: how much will these changes impact cumulative selection on the long term (e.g., over a mating season)? How, and for how long, should we sample populations in order to confidently estimate the strength and direction of selection? What constitutes an "episode" of selection? Similar questions have been bothering evolutionary biologists for many decades (Lewin, 1980) but as the appreciation of the tempo of sexually-selected

mechanisms grows, I foresee possible changes in the way we measure and interpret sexual selection. For example, I predict an increase in the number of studies demonstrating that selection in natural populations is not as directional as currently envisioned (Estes & Arnold, 2007; Robinson et al., 2008; Siepielski et al., 2009; Bell, 2010; Siepielski et al., 2011). As I showed in this thesis, estimations of the strength and direction of sexual selection may vary depending on the time window under evaluation, and multiple studies have shown that different phenotypes can be under selection at different times due to fluctuations in the environment (Wilson et al., 2006; Gosden & Svensson, 2008; Bell, 2010; Ingleby et al., 2010) and in mating preferences (Rios-Cardenas et al., 2007; Chaine & Lyon, 2008; Lehtonen et al., 2010; Clark & Backwell, 2015). This suggests that estimations of selection within a short time frame may not be representative of the cumulative selection over longer periods of time e.g., across years (Siepielski et al., 2009). For example, imagine a scenario in which individuals employing a behaviour strategy gain differential reproductive success earlier in a mating period, but are outcompeted by individuals with an alternative strategy later in the season, such that the net differential reproductive success between the two is zero. Conclusions regarding the operation of sexual selection in this population will depend on whether both strategies are examined independently: it could be argued that both strategies are under selection at different times, or that there is no cumulative selection at all. Part of the problem arises from our tendency in confounding past events of selection with present variation in phenotypes and behaviour. In the thought experiment described above, one could easily confound the past disruptive selection which led to the evolution of the alternative strategies, with present causes maintaining these strategies in equilibrium. Therefore, I predict that a more inclusive collaboration between empiricists and theoreticians will be required to clarify the role of time in sexual selection processes. In the next section, I focus on the more modest goal of exploring some of the limitations within my work, and suggest possible avenues for future research.

Limitations and future directions

While this thesis has sought to contribute to our understanding of the operation of sexual selection over time, its empirical approach suffers from a number of limitations. For once, I used a dataset in which birds were observed for a total of 6 hours on each day. While this sampling effort is by no means small, as it required hundreds man-hours of observations, automated technics could facilitate further expansion and resolution of the sampling period. For example, studies could make use of modern biologging, tracking and monitoring devices (Steen, 2017; Ripperger et al., 2020) in order to better characterize the circadian patterns in mating behaviour explored in **Chapter 2**. Tracking individual birds over 24 hours, or continuously for longer periods of time, should allow researchers to employ actograms and time-series analyses to investigate the association between divergent circadian patterns across individuals and their phenotypes (Refinetti, 2012), and whether circadian patterns vary over the season (Halle, 1995; Pita et al., 2011; Pagon et al., 2013). Studies could also manipulate sex ratios and group densities in order to evaluate to what extent consistent circadian patterns in mating behaviour, particularly in females, can be masked or influenced by sociobiological factors such as male harassment. This is important since females should naturally increase receptivity to mating at favourable times in the day to maximize fertilization success, but excessive male harassment may force females to shift receptivity to a less desirable time (Løvlie and Pizzari, 2007). Moreover, since I showed that male harassment can lead to loss in condition in both males and females, these studies could also attempt to identify an optimal group size and sex ratio to minimize harassment without compromising female fertility e.g., too few males could lead to fertilization failure across some eggs due to lack of sufficient sperm supplies (Pizzari, 2016). Therefore, these future studies could inform welfare and productivity measures in the poultry industry, since the red junglefowl is the main ancestor of the domestic chicken (Fumihito et al., 1996; Eriksson et al., 2008; Lawal et al., 2020) and their biology is qualitatively similar.

Second, while **Chapter 4** represents one of the few attempts at evaluating the combined effects of multiple predictors of male reproductive success in natural settings, and over a certain temporal window, much variation remained unexplained. Future work could manipulate group

composition experimentally, for example by assembling animals with respect to age and male-female relatedness, and establish the relative importance of different predictors while still maintaining a natural setting. Moreover, important predictors such as proxies of sperm efficiency (e.g., sperm mobility), which have been shown to affect sperm competition outcomes in different species, were left out of my study (e.g., Birkhead et al., 1999; Gage et al., 2004; Denk et al., 2005; Boschetto et al., 2011). I suggest that, for future work, researchers calculate the mean average path velocity (VAP), a good proxy of sperm efficiency (e.g., Farooq et al., 2018), across multiple sperm samples of each male used in the study. This can be achieved by well-established technics for sperm collection such as abdominal massage (Getachew, 2016), in combination with modern software that accurately and speedily measure VAP. Mean VAP can then be used as a promising predictor of fertilization success in natural conditions.

Third, conclusions reached in my work are derived from one captive population of a single bird species, so studies attempting to replicate my results across distinct populations or across a broader taxonomic range are encouraged. For example, in **Chapter 5,** I showed marked erosion in variance in reproductive success (i.e., opportunity for selection) over time, despite mate guarding being a common behaviour strategy used by dominant males to prevent subordinates from copulating (Pizzari & McDonald, 2019). Because groups studied in this thesis were somewhat large (10 males and 12 females), monopolizing that many females against multiple males attempting to mate simultaneously may be difficult (McDonald et al., 2017). In nature, red junglefowl groups can vary in size (Pizzari & McDonald, 2019), so future studies could investigate whether opportunity for selection decrease as sharply in smaller populations, where dominant males should be better able to control access to females. A similar pattern of mate monopolization is usually found in extreme versions of polygyny such as lek species, where usually a few males mate with most of the females, so it would also be interesting to know whether here, decreases in opportunity for selection over time are less pronounced (e.g., DuVal & Kempenaers, 2008). Similarly, in socially monogamous species, opportunity for selection should remain relatively constant over time if pair

formation is synchronous, and most individuals secure a mate. However, extra-pair copulations (EPCs) are common in monogamous bird species (Brouwer & Griffith, 2019), which could lead to temporal changes in opportunity for selection, particularly if EPCs are consistently influenced by temporal factors such as female "time out" (Clutton-Brock, 2017) during the incubation period. Moreover, female fowl can store sperm for roughly two weeks (Etches, 1996), but in other taxa, such as reptiles and fish, female can store sperm for much longer periods (Orr & Brennan, 2015). Comparing temporal patterns in opportunity across species in which females vary in the length of sperm storage could help elucidate the role of female-driven mechanisms to post-copulatory sexual selection (e.g., Hall et al., 2010; Tuni et al., 2013; Cardozo et al., 2020). Comparative studies across different species within a taxa, or across multiple taxa, have the advantage in that they could be achieved with already published datasets, provided these contain fine-grained mating and parentage information collected over a certain period of time. Studies which have tracked individual life-time reproductive success in wild populations are therefore excellent candidates (e.g., Vanpé et al., 2007; Dubuc et al., 2014; O'Sullivan et al., 2019).

Conclusion

In conclusion, this thesis provides a contribution to our understanding of how different aspects of sexual selection vary at different time scales in *Gallus gallus*, with insights that can hopefully be extended to other study systems. The tempo of evolution and its consequences for our estimates of selection have often been neglected in previous work (Siepielski et al., 2009), so I hope that this thesis will help bringing attention to this fertile and promising area of research.

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