The effect of relatedness on sexual dynamics.

Studies of red junglefowl and fruit flies.

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ABSTRACT

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In this thesis, I explore four different ways in which relatedness affects sexual interactions in the red junglefowl *Gallus gallus* ssp., and the fruit fly *Drosophila melanogaster*. First, I show that in both species, inbreeding depression is sex-specific and modulated by parental age and gametic age. However, the sex that suffers higher inbreeding depression was trait- and species-dependent. Second, I examined patterns of inbreeding avoidance. I found no evidence of inbreeding avoidance in the fruit fly, but in the red junglefowl both males and females avoided mating with relatives, independently from sex-ratio of the social group. Third, I investigated whether relatedness amongst members of one sex affects mate choice in members of the opposite sex. Male fruit flies preferentially courted females unrelated to females with whom they had previously mated, while female flies displayed a weak preference for males related to their previous mates. In the red junglefowl, females exposed to male trios of two males related to each other and one unrelated male, displayed a marked preference for mating with the male unrelated to the other two males, and might also bias postcopulatory sperm utilization in favour of the unrelated male. Fourth, I explored the implications of male relatedness on the intensity of male-male competition. Male red junglefowl were less aggressive towards related competitors, but invested more sperm in females that had previously mated with a related male rather than with an unrelated male. In fruit flies, male relatedness had a strong impact on female life-history and offspring viability, although I found no evidence that these effects were modulated by changes in male-male competition. Collectively, the findings of these studies demonstrate the complex relationship between relatedness and other important biological phenomena as such senescence and sexual conflict.

Photos courtesy of Rebecca Dean

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Authors contributions

Listed in the table below are the contributions of various authors to the different data chapters. '-', '*', '**' and '***' denote no, minor, major and substantial contributions respectively. Shaded cells mean that the individual was not a co-author of that chapter

	Authors									
	Tan C	Bell L	Bagshaw E	Doyle P	Burrell S	Greenway E	Goodwin SF	Løvlie H	Wigby S	Pizzari T
2. Parental age, gametic age and inbreeding interact to modulate offspring viability in *Drosophila melanogaster*										
Designed project	**								**	**
Collected data	***								*	*
Analysed data	***								*	*
Funded project	**								**	*
Wrote/ Edited manuscript	***								*	*
3. Sex-specific inbreeding depression in the red junglefowl: The role of parental age and sperm age										
Designed project	**		-	*	*				-	**
Collected data	**		*	*	**				-	-
Analysed data	**		*	**	*				*	*
Funded project	-		-	-	-				-	***
Wrote/ Edited manuscript	**		-	*	*				*	**
4. No evidence for precopulatory inbreeding avoidance in *Drosophila melanogaster*										
Designed project	**							*	**	*
Collected data	***							*	*	*
Analysed data	***							*	*	-
Funded project	-							-	-	***
Wrote/ Edited manuscript	***							*	*	*
5. Group sex-ratio and male inbreeding avoidance in the red junglefowl										
Designed project	**	**							-	*
Collected data	**	**							-	-
Analysed data	**	**							-	*
Funded project	-	-							-	***
Wrote/ Edited manuscript	**	*							*	*

Chapters	Authors									
	Tan C	Bell L	Bagshaw E	Doyle P	Burrell S	Greenway E	Goodwin SF	Løvlie H	Wigby S	Pizzari T
6. Sex-specific responses to genetically novel mates, and the role of olfaction in the fruit fly										
Designed project	**					*	*	**	*	*
Collected data	**					*	-	*	**	*
Analysed data	**					*	-	*	*	*
Funded project	-					-	*	*	*	*
Wrote/ Edited manuscript	**					-	*	*	**	**
7. Pre- and postcopulatory sex-specific responses to male-male relatedness in the red junglefowl										
Designed project	**		*	*					-	**
Collected data	**		**	*					-	-
Analysed data	**		**	*					-	*
Funded project	-		-	-					-	***
Wrote/ Edited manuscript	***		*	-					*	*
8. The impact of inter-male relatedness on female life-history and female fitness in the fruit fly										
Designed project	**								**	**
Collected data	**								**	-
Analysed data	**								*	*
Funded project	-								**	**
Wrote/ Edited manuscript	**								*	**

Chapter 1 Introduction

1.1. Overview

1.1.1. Sexual dynamics

Reproductive success is fundamental to the fitness of an individual and it depends on the outcome of intricate interactions within and between the sexes, in which individuals attempt to pursue their own fitness interests. These interactions can be socially complex and are often characterised by continuous change in time and space. The way that the frequency, intensity and direction of sexual interactions vary temporally and spatially, as well as the factors which cause variations, are described by sexual dynamics. Patterns of sexual dynamics are to a large extent driven by both sexual selection and evolutionary sexual conflicts within and between sexes.

Sexual selection, a concept first coined by Charles Darwin (1859; 1871), arises from variation in individual ability to compete for reproductive opportunities with members of the same sex and species, and it is one of the most powerful and least understood evolutionary forces (Andersson 1994; Shuster & Wade 2003). Sexual selection takes two major forms: intersexual selection, where members of the limiting sex (usually females) choose members of the opposite sex; and intrasexual selection in which individuals of the same sex (usually males) compete among themselves for access to the limiting sex. Because males and females have divergent interest in reproduction, sexual selection is often sex-specific and promotes traits, which – while adaptive in the carrier sex – can impose costs on the opposite sex with whom the carrier interacts over reproduction. This generates a conflict between the evolutionary interests of individuals of the two sexes (sexual conflict, Parker 1979; Arnqvist & Rowe 2005; Parker 2006). Therefore, the evolution of a trait that imposes harm on one sex can lead to the

evolution of a counter adaptation to reduce the harm imposed on the affected sex (Parker 1979; Chapman et al. 2003; Parker 2006).

Sexual interactions between individuals can be highly dynamic and vary with time and space, in frequency (e.g. how many times an individual copulates with another), intensity (e.g. how much sperm is invested by a male in a female) and direction (e.g. whether male A acts aggressively to male B or vice versa). Importantly, there is a multitude of factors that mediate such changes, for example, an individual's age (e.g. Dean et al. 2010), fluctuation in environmental conditions (e.g. seasonality in breeding) and relatedness between individuals (e.g. Kokko & Ots 2006). This thesis focuses on testing how genetic relatedness affects intersexual, intrasexual interactions and sexual conflict.

1.1.2. Relatedness

Classical (Darwinian) fitness describes how successful an individual is, relative to other competitors, in passing on its own genes through offspring to future generations. Individuals can sacrifice their own direct fitness for the benefit of others, a concept often referred to as altruism. Axelrod and Hamilton (1982) suggest that there are two explanations for the evolution of altruism: when the relatives benefit from acts of altruism, altruism can evolve by inclusive fitness (Hamilton, 1964a); when non-relatives benefit, altruism can evolve by reciprocal altruism (Trivers 1971). Reciprocal altruism describes a behaviour whereby an individual makes sacrifices for another individual, with the expectation that the other individual will act in a similar manner at a later time (Trivers 1971). Hamilton's inclusive fitness theory (1964a) offers an alternative mechanism for the evolution of altruism. The inclusive fitness of an

individual is the sum of its classical fitness and the number of equivalents of its own offspring it can add to the population by benefitting others at a cost to oneself. Hamilton's rule describes mathematically when such a costly action should be performed:

$$r * b > c$$

where

- r is the probability of the actor and recipient sharing an altruistic gene, often referred to as the degree of relatedness.
- b is the reproductive benefit to the recipient of the altruistic behaviour, and
- c is the reproductive cost to the actor,

Hence, an apparently altruistic act toward a related individual may in fact enhance the inclusive fitness of the altruistic individual. Kin selection theory predicts that social behaviours which reduce the fitness of an actor can evolve when direct costs are compensated by the inclusive fitness benefits derived by a related recipient (Hamilton 1964b).

Relatedness here is defined as the probability of sharing genes identical by descent. Genetically, two related individuals differ from two unrelated members of the population in their probability of carrying replica alleles which they have both inherited from one or more common ancestors (Hamilton 1964a). There are three main mechanisms that would increase the probability and frequency of an individual interacting with genetically related individuals: population viscosity, kin recognition and greenbeard effects (Pizzari & Gardner 2012). First, populations often exhibit a degree of viscosity because individuals displace from their place of origin at a

relatively slow pace (Hamilton 1964a, b; Lion & Baalen 2008). Thus, the genetic relatedness of individuals interacting locally can differ substantially from the average relatedness of the population as a whole. Second, by recognizing kin, individuals can maximise their inclusive fitness benefits associated with kin-biased social behaviour. For example, imagine a situation in which a female is given an opportunity to mate with a related male and assume that this mating involves no cost to the male or female. By mating with her brother, the female gains direct fitness through an increased in her number of offspring as well as gains indirect fitness by improving her brother's reproductive success (Kokko & Ots 2006). Of course, the assumption that inbreeding involves no costs may not be valid (e.g. inbreeding depression) and this will be discussed further in the later section (1.2.2. Inbreeding avoidance). Third, the Fisherian runaway process, an example of a greenbeard effect, occurs when a single (or multiple) gene encoding a male sexual ornament is in linkage disequilibrium with gene(s) encoding a female preference for that ornament (Pizzari & Gardner 2012). Therefore, the gene is directly recognising itself and causes its carriers to favour one another so as to improve each other's fitness (Gardner & West 2010). This leads to increased interactions between related individuals owing to genetic relatedness at the greenbeard locus and also results in replication and an increase in frequency of the gene. These three mechanisms may generate non-zero genetic relatedness between social individuals and individuals may therefore have a higher rate of interactions with closer relatives than with distantly related individuals. These social interactions among relatives can potentially impact upon the fitness of the actor directly and indirectly through inclusive fitness.

1.1.3. The role of relatedness in sexual dynamics

Relatedness plays a fundamentally important role in socio-sexual biology. Much attention has been paid to using relatedness and the theory of kin selection to explain the evolution of particular sexual behaviours. First, kin selection theory provides the framework for understanding the evolution of eusociality (e.g. Cronin 1991; Quellar & Strassmann 1998) characterised by cooperative brood care, overlapping adult generations and division of labour between reproductive and sterile individuals. Previous theoretical and empirical studies have demonstrated that ancestral monogamy is a fundamental condition for evolution of eusociality (Boomsma 2007, 2009; Hughes et al. 2008). This is because monandry maximises relatedness and thus the inclusive fitness of helpers exceeds that the benefits of a solitary lifestyle. Second, kin selection has implications for our understanding of sex allocation theory. The haplodiploid sex-determination system (with males arising from unfertilised eggs and females from diploid eggs) in social Hymenoptera results in relatedness between alloparents and brood being higher than that between parents and brood. Full-sib sisters are three times more related to each other than they are to their brothers. Thus, potential conflict over sex allocation is predicted to occur as alloparents (which are females) would favour a female-biased sex-ratio while the parents would favour a 1:1 population sex investment ratio (Trivers & Hare 1976).

Relatedness can also potentially influence sexual conflict. In polygamous systems where individuals are unlikely to remate with each other, males have no evolutionary interest in the survival or future reproduction of females after females have produced offspring from the male. Females, on the other hand, retain interest in future reproduction as they can gain fitness from future reproductive events (Chapman 2006).

This creates the potential for sexual conflict. In particular, males are selected to increase their fitness in the current mating even at a cost to the female's long-term reproduction and survival. However, relatedness between mating males and females (inbreeding) should reduce the potential for sexual conflict because a focal partner's harming of its related mate could potentially reduce its inclusive fitness (Bourke 2009; Rankin 2011). Male-male relatedness is also predicted to help resolve sexual conflict over mating as male harm of females may reduce inclusive fitness benefits due to reductions in reproductive success of male relatives (see below; Rankin 2011; Wild et al. 2011). Wild et al. (2011) also considered the element of postcopulatory sperm competition between males in the evolution of sexual conflict. Specifically, this study took into account differences in the timing of dispersal between males and females. Consider a scenario in which both males and females disperse prior to mating, and in which precopulatory and postcopulatory competition will occur locally. Theory predicts that relatedness would have no effect on the evolution of female resistance to male harm as the inclusive fitness benefits balance the costs. In another situation when females disperse after mating and males disperse before mating, precopulatory competition between males will be local while postcopulatory competition will occur globally. Females would, as always, prefer the minimal costs of mating with these males. Males, however, would be selected to attain fitness benefits for themselves and as a side-effect induce harm to females, with the magnitude of that harm being negatively correlated with the local inter-male relatedness (Wild et al. 2011).

Despite numerous studies on the interactions between relatedness and sexual dynamics, there are still gaps in our understanding. In my thesis, I focus on three main ways in which genetic relatedness affects reproductive strategies, which are briefly outlined in

Figure 1. First, the relatedness between an interacting male and a female, in terms of inbreeding depression, and sexual conflict over inbreeding. Second, male-male relatedness, in terms of how kin selection can modulate the intensity of intrasexual competition and intersexual conflict. Third, relatedness between prospective mates, whereby individuals decide between potential mates that are either related or unrelated to their previous mates (Figure 1).

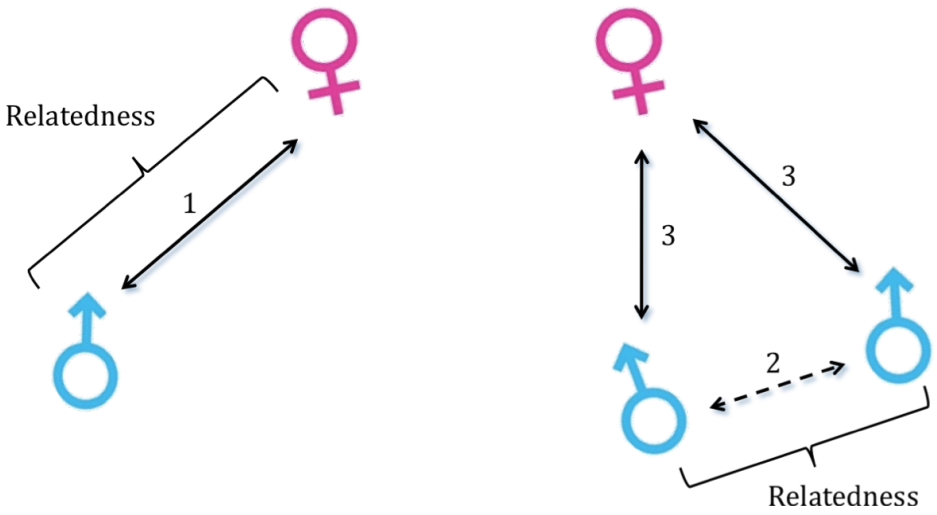

Figure 1. The role of relatedness in sexual dynamics. (1) Relatedness between male and female – inbreeding and kin selection; (2) Relatedness between males – competition and kin selection, intersexual conflict; (3) Interaction between the female and two related prospective partners.

My research addresses four integrated themes. First, I quantify inbreeding depression and test predictions that inbreeding depression is sex-specific and modulated by parental and gametic age. Second, I use information on inbreeding to generate predictions about sex-specific propensity to inbreed or avoid inbreeding and social factors modulating intersexual dynamics over inbreeding. Third, I investigate the manner in which relatedness among prospective mates influences mating decisions and

preferences in actors of the opposite sex. Fourth, I explore the role of male-male relatedness on the intensity of intrasexual competition, female fitness and sexual conflict.

1.2. Themes

1.2.1. Inbreeding depression

Inbreeding can depress offspring fitness through the expression of deleterious recessive alleles and loss of heterozygous advantage (Charlesworth & Charlesworth 1987; Greenwood 1987; Lynch & Walsh 1998). Because of the importance of inbreeding depression in many fields of biology, from the dynamics and evolution of natural populations (Keller & Waller 2002; Andersson 2012; Dierkes et al. 2012) to the cultivation of domestic plants and animals (Meghji et al. 1984; Miglior et al. 1992), there have been numerous studies on the factors modulating inbreeding depression. The magnitude of inbreeding depression has been found to vary with environmental conditions (e.g. Armbruster & Reed, 2005), genetic architecture (e.g. Fox et al. 2006), and life-history stages (e.g. Charlesworth & Hughes, 1996). However, surprisingly few studies have investigated the importance of offspring sex and parental reproductive senescence as potential modulators of inbreeding depression.

Inbreeding is predicted to affect male and female offspring differentially due to genetic asymmetries between the sexes. Two hypotheses have been proposed to explain sex-specific patterns of inbreeding depression. The first hypothesis, 'unguarded X' hypothesis, predicts that the heterogametic sex, in the absence of inbreeding, should suffer reduced fitness because X-linked recessive deleterious mutations will be unconditionally expressed in the heterogametic sex (Trivers 1985). However, under

inbreeding, the ‘unguarded X’-hypothesis predicts that the homogametic sex suffers higher inbreeding depression because of the increased probability of expression of recessive alleles on the X/Z chromosomes (James & Ballard 2003; Fox et al. 2006; Bilde et al. 2009). The second hypothesis is based on sex-specific selection on resources allocated into current and future reproduction (Trivers 1972). Males and females have different optimal reproductive strategies involving sex-specific investments in current and future reproduction (Bonduriansky et al. 2008; Clutton-Brock & Isvaran 2007). Optimally, males should adopt a 'live fast, die young' strategy and females should maximise their reproductive output by conserving energy, thus prolonging reproductive lifespan. Bilde et al. (2009) offered three plausible scenarios of how this hypothesis can explain sex-specific patterns in inbreeding depression. First, via the reduced likelihood of inbred males engaging in energy-demanding reproductive behaviours such as male-male competition, thereby prolonging male lifespan. Second, via genes that code for resource allocation undergoing opposing directional selection in males and females. Directional selection might lead to directional dominance, in which the selected trait shows dominance in the direction of the preferred phenotype. The genes coding for resource allocation into current and future reproduction are predicted to show directional dominance in opposite directions in the sexes. Males would therefore be selected to use resources for intrasexual competition and rapid reproduction at a cost to lifespan whilst females are selected to optimise their reproductive output, thereby extending their lifespan. However, inbreeding would reverse the effects of such genes in the non-preferred direction, that is, to decrease female lifespan while increasing male lifespan. Third, via traits mediating resource allocation into current *versus* future reproduction, which are encoded by autosomal genes. These genes are maintained if male-beneficial alleles are dominant in males and

recessive in females, while female beneficial alleles are dominant in females and recessive in males. By increasing the homozygosity of such genes, inbreeding could then adjust the trait expression to an opposite and maladaptive direction in both sexes, i.e. to increase male lifespan while decreasing female lifespan. A potentially powerful experimental approach to disentangle these hypotheses ('unguarded X' *versus* 'sex-specific resource allocation') would be to compare sex-specific patterns of inbreeding depression in species where males are heterogametic (e.g. XY) and species where females are heterogametic (e.g. ZW). The 'unguarded X' hypothesis predicts a reversal of sex-specific fitness costs across flies (homogametic females) and birds (homogametic males) whereas 'sex-specific resource allocation' hypothesis predicts no reversal in sex-specific inbreeding depression and that females should always suffer a higher magnitude of inbreeding depression.

In addition to its sex-specificity, the magnitude of inbreeding depression might also be modulated by parental effects, including reproductive senescence. Senescence occurs at two levels. At the organismal level, senescence refers to the ageing of the individual's diploid genome, resulting in a decline in survival and reproductive ability with advancing age (Rose, 1991; Finch & Kirkwood, 2000). This could be due to mutation accumulation or antagonistic pleiotropy, both of which predict the persistence of mutations that cause harm late in life (Medawar 1952; Williams 1957), or due to costs of reproduction (the disposable soma theory, Kirkwood 1977). The mutation accumulation theory proposes that late-acting deleterious mutations tend to accumulate, because of their minimal effects on fitness early in life (Medawar 1952), whereas the antagonistic pleiotropy theory postulates that genes with beneficial effects early in life are at a selective advantage despite their pleiotropic deleterious effects on late

performance (Williams 1957). The disposable soma theory suggests that senescence arises from an optimal balancing of resources between current reproduction and somatic repair processes required for future reproduction (Kirkwood 1977). At the gametic level, senescence refers to reductions in fertilizing efficiency and zygote fitness due to DNA damage of their haploid genome during the postmeiotic events of individual gametes, independent of parental ageing. Studies have demonstrated detrimental consequences for offspring fitness due to organismal and gametic senescence (Siva-Jothy 2000; Tarin et al. 2000). It is therefore plausible that the offspring of aged parents and/or gametes might be more vulnerable to the expression of recessive alleles and loss of heterozygosity imposed by inbreeding. However, this hypothesis has received surprisingly little consideration. In this thesis, I address whether inbreeding depression is sex-specific and how this sex-specificity is modulated by parental age and gametic age.

1.2.2. Inbreeding avoidance

Because inbreeding can depress offspring fitness, individuals that avoid mating with kin may accrue fitness benefits over conspecifics that mate randomly (Bateson 1982, 1983). Direct selection to avoid inbreeding should therefore be proportional to magnitude of inbreeding depression. However, mating with a related partner also affects the inclusive fitness of a focal individual (Kokko & Ots 2006; Parker 2006), which generates indirect selection on inbreeding avoidance. For example, when a female chooses to mate with her brother, assuming that he does not forfeit mating with other females, she may pay a direct cost (inbreeding depression), but she gains indirect fitness by improving her brother's reproductive success. Only when the direct cost of inbreeding depression exceeds kin-selected benefits are inbreeding avoidance

mechanisms expected to evolve. These theoretical arguments suggest that the degree of inbreeding avoidance should be plastic and influenced by socio-sexual mechanisms. Importantly, we expect inbreeding avoidance to be sex-specific. Because females typically invest more in reproduction than males, inbreeding costs are suggested to be higher for females and therefore they are more likely to exhibit inbreeding avoidance strategies (Parker 1979; Waser et al. 1986; Pizzari et al. 2004; Kokko & Ots 2006). Therefore, when mating is relatively cheap for males and inbreeding depression is intermediate, we expect conflict between male and female propensity to inbreed (Parker 1979; 2006). In addition, when the sexes have differential reproductive investment, tolerance towards inbreeding would be expected to change with mate availability but in different ways in males and females (Parker 1979). If maternal investment is higher than paternal investment, females are expected to be choosier, and inbreeding tolerance should decrease more rapidly with increased mate availability.

1.2.3. Relatedness between prospective mates

Factors that increase the probability and frequency of interacting with related individuals will also increase the probability that focal individuals will have repeated encounters with new potential mates that are genetically related to previous mates. Therefore, both males and females will likely have to make mating decisions with respect to potential mates that are either related or unrelated to previous mates. Differential response to prospective mates that are either related or unrelated to each other can be explained at different levels. The 'Coolidge effect' describes a male's elevated interest in sexually novel over sexually familiar females (Brown 1974; Dewsbury 1981). Similarly, males might also avoid relatives of previous mates to avoid

risk of mating with same female, particularly if recognition of closely related mates is subject to error.

In addition, both males and females can benefit by mating with multiple partners through the acquisition of genetically diverse offspring (Brown 1974; Dewsbury 1981), predicting a preference for prospective mates unrelated to past mates. Because of anisogamy, this preference is expected to be more pronounced in females than in males (e.g. Ivy et al. 2005; Gershman & Sakaluk 2009). In contrast, mating with genetically different mates might be more costly to females. For example, mating with genetically varied males down regulates haemocyte load, lytic activity and encapsulation in female crickets (Fedorka & Zuk 2005). When such costs are substantial, individuals might prefer mates that are genetically similar to each other. Mate selection with respect to genetic similarity between past and future mates has received little or no consideration and is thus explored in this project.

1.2.4. Kin selection and male-male competition

A higher average relatedness between interacting males may affect the intensity of intrasexual competition through kin selection. Kin selection plays a role in explaining cooperative breeding in vertebrates (Komdeur 1994; Clutton-Brock 2002; Nam et al. 2010) and the evolution of altruism in eusocial insects (Queller & Strassmann 1998; Foster et al. 2006). Kin-selected benefits may also be mediated through sexual selection. The direct interplay between male-male relatedness and kin selection has been demonstrated in lekking behaviour which is characterised by males displaying in groups to attract females and secure matings (Höglund & Alatalo 1995). Males usually compete and defend small territories within leks (Wiley 1991; Droney 1992). Mating

success is typically highly skewed and unsuccessful males appear to be increasing the mating success of their more successful neighbours. However, related males can display together and cooperate to indirectly gain inclusive fitness (e.g. Petrie et al. 1999; Shorey et al. 2000; Krakauer 2005).

The way in which kin selection modulates the intensity of intrasexual selection might also have important repercussions for female fitness. Under intense male-male competition, sexual selection promotes male traits that convey a competitive advantage even if this imposes fitness costs on the females mated, generating sexual conflict. However, in structured populations, male relatedness might relax selection on harmful male traits, because harming a female might indirectly reduce the reproductive success of male relatives (Rankin 2010; Wild et al. 2011), and thus reduce inclusive fitness. Therefore, the life-history and reproductive fitness of females are expected to change in response to the relatedness between males. For example, females might be predicted to live longer and produce more offspring under situations of reduced male-male competition between related male rivals. In this project, I explore the role of male-male relatedness on male-male competition and its effects on female behaviour, female fitness and life history traits.

1.3. Study systems

My project adopts a two-pronged approach using two model systems, the red junglefowl *Gallus gallus* ssp. and the fruit fly *Drosophila melanogaster*. Both model organisms have their own strengths that complement each other. The red junglefowl allows for detailed study of behavioural processes down to the level of gametes. Fruit flies, on the other hand, offer a platform for investigating fitness components of sexual

behaviour because they are relatively short-lived, and also offer genetic tools for investigating molecular mechanisms underlying behaviour. These species represent particularly good model systems for this project for two main reasons. First, sexual selection on genotypic and phenotypic traits is well characterised for both species, and a range of tools are available to experimentally test pre- and post-insemination mechanisms of sexual selection (e.g. Bangham et al. 2002; Pizzari 2007). Second, population viscosity displayed in both the red junglefowl and fruit fly is likely to increase the relatedness between interacting individuals and, potentially making this an important factor determining the evolution of inbreeding avoidance, kin selection, male-male competition and intersexual conflict. Finally, previous studies on these species have demonstrated potential for sexual conflict over mating decisions (Pizzari et al. 2002; Chapman et al. 2003; Pizzari et al. 2004) and evidence of kin discrimination (Averhoff & Richardson 1974, 1976; Pizzari et al. 2004).

1.3.1. Red junglefowl

The red junglefowl is the ancestor of the domestic chicken (*Gallus gallus domesticus*; Siegel et al. 1992). Males and females are sexually dimorphic: males are larger in size and elaborately ornamented both with colourful plumage and with fleshy comb and wattles on the head, whereas females are drab and cryptic. The red junglefowl and its domestic subspecies, the domestic chicken, live in socially structured groups consisting of several males and females (Collias & Collias 1996). Socially dominant males typically monopolise access to mating by interrupting the mating attempts of subdominant males and by performing more courtship feeding than subdominant males (Collias & Collias 1996, Pizzari et al. 2002). Females are often polyandrous and demonstrate a preference for the socially dominant males through higher frequency of

solicitation and reduced resistance towards to the mating attempts of the dominant male (Pizzari 2001). Although mating success is highly skewed towards the dominant male, most subdominant males are able to secure some copulations. As such, the risk and intensity of sperm competition is high, whereby ejaculates from multiple males compete to fertilise the eggs (Pizzari et al. 2002). Female fowl can also store viable sperm for approximately 2 weeks after insemination (Etches 1996) and are able to eject ejaculates through cloacal contractions immediately following an insemination (Pizzari et al. 2004). These behaviours create potential for episodes of precopulatory and postcopulatory sexual selection.

The red junglefowl have been shown to be phylopatric with limited dispersal in natural habitats (Collias et al. 1966; Collias & Collias 1996). Even though individuals may switch flock, 90% of these individuals transfer to an adjacent flock, reflecting the limited dispersal tendencies of these birds. Importantly, around 4% of observed copulations in these groups take place between siblings and between mothers and sons (Collias & Collias 1996). In addition, the apparent lack of sex-biased dispersal in this species suggests that there is an increased likelihood of same-sexed relatives interacting with each other and with individuals of the opposite sex. Furthermore, studies on the domestic chicken revealed that inbreeding imposes fitness costs on the offspring. These include reduced mating frequency (Cheng et al. 1984), decreased social competitiveness (Craig and Baruth 1965), poor egg hatchability (Shoffner et al. 1953; Cheng et al. 1984) and lowered chick survivability (Shoffner et al. 1953). Studies on the red junglefowl demonstrate that males and females exhibit sex-specific responses to inbreeding. Specifically, females avoid inbreeding both before and after copulation, even when only one male is available (Pizzari et al. 2004; Løvlie 2007). However,

males do not avoid inbreeding when one female is available (Pizzari et al. 2004) but avoid inbreeding when exposed to two related and two unrelated females (Løvlie 2007). These results suggest that both sexes of the red junglefowl are able to recognise kin.

The red junglefowl utilised in experiments in this thesis were derived from an individually marked population established at the Oxford University John Krebs Field Station in Wytham, Oxfordshire, in 2006. In 2009, when my project started, there were about 50 males and 30 females. These individuals were genotyped at 31 variable microsatellite loci (Worley et al. 2010) and female-male as well as male-male relatedness were determined by calculating pairwise relatedness based on microsatellite data (Queller & Goodnight 1989). Pairs considered related had coefficient of relationship (r) $0.45<r<0.6$ and those considered unrelated had a coefficient of relationship (r) <0.05 and >-0.05. Related birds were full-siblings as a previous study has shown that $r = 0.46 \pm 0.22$ (mean ± SE) for full-siblings in our red junglefowl population (Løvlie et al. in prep).

1.3.2. Fruit fly

The fruit fly originated in the hot and humid tropical regions of the Old World (Asia, Africa and Europe). Males are typically 2.3 mm long and have dark coloured patches at the rear of their bodies; females are slightly larger and have a lighter abdomen than males. Precopulatory sexual selection in this species typically involves competition between males and females exercising choice. Male fights are characterised by a series of offensive (e.g. chasing or lunging) and defensive actions (e.g. running away; Chen et al. 2002). In addition, males perform a sequence of courtship behaviour towards

females (chasing, singing, tapping, licking of genitals and attempting mounting; Bastock & Manning 1955). Females reject the courtship advances of males via flicking of their wings, kicking, curling of the abdomen, extruding of the ovipositor, extending of the legs at the side of the body nearest the male or running and flying away from the chasing male (Connolly & Cook 1973). Postcopulatory sexual selection is also important in this species. Females mate multiply (Harshman & Clark 1998; Imhof et al. 1998) and can store sperm for up to 2 weeks post insemination (Neubaum & Wolfner 1999). A male's sperm is therefore subjected to competition with other male rivals. As a defensive action, males have to displace the sperm of a previous male from the female storage organs and prevent his own sperm from being displaced during subsequent copulations by the female. These mechanisms are strongly modulated by ejaculate proteins produced in the male's accessory glands (Gilchrist & Partridge 1995; Chapman et al. 2000), which in turn influence postcopulatory mating success. Sperm selection by females can also occur (Birkhead 1998), for example, through the evolution of reproductive tracts that bias paternity towards males with longer sperm (Miller & Pitnick 2002; Clark et al. 1999).

Although past studies on wild populations of the fruit fly indicate that flies are capable of long-distance movements (up to 10 km; Yamazaki et al. 1986; Coyne & Milstead 1987), natural populations exhibit a tendency towards aggregation in particular localities, which increases the probability of related individuals interacting with each other and with members of the opposite sex (Wallace 1970; McInnis et al. 1982). Indeed, recent evidence demonstrates that males in large naturally occurring populations were more likely to be mate with a related female than by random (Robinson et al. 2012). Inbreeding in this species shortens lifespan (Swindell & Bouzat

2006), increases mortality (Vermeulen & Bijlsma 2004), reduces the ability of males to mate and court (Miller et al. 1993) and lowers egg-to-adult viability (Mack et al. 2002).

Here I use a lab-adapted Dahomey wild-type stock of the fruit fly at the Department of Zoology, Oxford University. The stock has been maintained since 1970 in four large (several thousand flies), outbred population cages. To obtain parents of the experimental flies, eggs were collected and raised at standard density (ca. 100 flies per bottle; Clancy & Kennington 2001). Virgin adults single males and females were then paired in vials to produce families for one generation. Individuals were considered related when they were full-siblings and unrelated when they belonged to different families.

1.4. Thesis overview

My research investigates the role of relatedness in sexual dynamics at four different levels, summarised in Table 1 below.

1) The role of parental age, gametic age and offspring sex in inbreeding depression

In chapters 2 and 3, I measure the extent to which full-sib mating, offspring sex, parental age and gametic age interact to determine variation in offspring traits in the fruit fly and red junglefowl respectively. In addition, I quantify sex-specific differences in inbreeding depression. The comparison of a XY system (fruit fly) with a ZW system (red junglefowl) helps distinguish the relative influence of the 'unguarded X hypothesis' and 'sex-specific selection on resource allocation' on sex-specific inbreeding depression.

2) The modulation of inbreeding avoidance mechanisms

In chapter 4, I explore factors modulating premating inbreeding avoidance in the fruit fly, such as sex, mating history of focal individuals and mate availability. Parallel to this study, chapter 5 investigates the role of mate availability in patterns of male inbreeding avoidance of the red junglefowl.

3) The role of relatedness between prospective mates in mating decisions of a focal individual

I use the fruit fly (chapter 6) to explore sex-specific mating responses to the relatedness between past and new partners and the role of olfactory cues. I also examine the role of male-male relatedness on pre- and post- sexual selection by females in the red junglefowl (chapter 7).

4) The influence of male-male relatedness on intrasexual competition and sexual conflict

I examine whether inter-male relatedness increases or decreased male-male competition in chapter 8 using the fruit fly and chapter 7 using the red junglefowl. Also, I investigate the impact of inter-male relatedness on female life-history and fitness in fruit fly (chapter 8) to test the prediction that kin selection might inhibit sexual conflict by lessening male harm to females and modulating female fitness.

Table 1. Summary table of chapters

Theme	**Fruit fly**	**Red junglefowl**
1. Inbreeding depression	Chapter 2	Chapter 3
2. Inbreeding avoidance	Chapter 4	Chapter 5
3. Relatedness of prospective mates	Chapter 6	Chapter 7
4. Relatedness, male-male competition and sexual conflict	Chapter 8	Chapter 7

THEME I

INBREEDING DEPRESSION

Chapter 2

Parental age, gametic age and inbreeding interact to modulate offspring viability in the fruit fly

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ABSTRACT

In principle, parental relatedness, parental age and the age of parental gametes can all influence offspring fitness through inbreeding depression and the parental effects of organismal and post-meiotic gametic senescence. However, little is known about the extent to which these factors interact and contribute to fitness variation. Here, we show that in the fruit fly *Drosophila melanogaster*, offspring viability is strongly affected by a three-way interaction between parental relatedness, parental age and gametic age at successive developmental stages. Overall egg-to-adult viability was lowest for offspring produced with old gametes of related, young parents. This overall effect was largely determined at the pupa-adult stage, although three-way interactions between parental relatedness, age and gametic age also explained variation in egg hatchability and larva-pupa survival. Controlling for the influence of parental and gametic age, we show that inbreeding depression is negligible for egg-hatchability but significant at the larva-pupa and pupa-adult stages. At the pupa-adult stage, where offspring could be sexed, parental relatedness, parental age and gametic age interacted differently in male and female offspring, with females suffering higher inbreeding depression than males. Collectively, our results demonstrate that the architecture of offspring fitness is strongly influenced by a complex interaction between parental effects, inbreeding depression and offspring gender.

INTRODUCTION

Inbreeding, the mating of related individuals, can depress offspring fitness through the expression of deleterious recessive alleles and loss of heterozygous advantage (Charlesworth & Charlesworth 1987). Recent work has identified inbreeding depression as a fundamental determinant of the dynamics (Keller & Waller 2002; O'Grady et al. 2006; Walling et al. 2011) and evolution of natural populations (Andersson 2012; Dierkes et al. 2012). The magnitude of inbreeding depression varies with environmental conditions (Armbruster & Reed 2005; Fox & Reed 2011), genetic architecture (Fox et al. 2006), and life-history stages (Charlesworth & Hughes 1996). For example, Charlesworth and Hughes (1996) demonstrated that inbred male fruit flies, suffer increasing fitness costs as they age, indicating that effects of inbreeding depression can be age-dependent. It is similarly likely that mechanisms of parental senescence might also act as important modulators of the magnitude of inbreeding depression in the offspring, however this hypothesis has received surprisingly little consideration.

Senescence can occur at two distinct levels. At the organismal level, senescence refers to a decline in survival and reproductive ability with advancing age (Rose 1991; Finch & Kirkwood 2000) due to mutation accumulation or antagonistic pleiotropy mutations that cause harm late in life (Medawar 1952; Williams 1957). The mutation accumulation theory proposes that late-acting deleterious mutations tend to accumulate, because of their minimal effects on fitness early in life (Medawar 1952), whereas the antagonistic pleiotropy theory postulates that genes with beneficial effects early in life have pleiotropic deleterious effects on late performance (Williams 1957). At the gametic level, senescence refers to reductions in the fertilizing efficiency and zygote

fitness over the postmeiotic lifespan of individual gametes, due to DNA damage of their haploid genome, independently of the ageing of the parental diploid parent. Both organismal and gametic senescence can have profound consequences for offspring fitness (Siva-Jothy 2000; Tarin et al. 2000). It is therefore plausible that the offspring of ageing parents and/or ageing gametes might be more sensitive to the expression of recessive alleles and loss of heterozygosity imposed by inbreeding.

Here, we experimentally test this hypothesis by measuring the extent to which full-sib mating, parental age and gametic age interact to determine variation in offspring viability in a laboratory-adapted outbred population of the fruit fly *D. melanogaster*. We measure offspring viability as overall egg-to-adult survival and at individual developmental stages: (i) egg hatchability, (ii) larva-to-pupa survival, and (iii) pupa-to-adult survival. Because inbreeding depression may be sex-specific (Saccheri et al. 2005; Bilde et al. 2009), we measured survival separately for sons and daughters at the pupa-adult stage, when phenotypic sexing was possible.

MATERIAL AND METHODS

(a) Experimental population and culturing

We used a lab-adapted Dahomey wild-type stock of the fruit fly. Flies were maintained at 25°C, in a non-humidified room, on a 12:12 hours light:dark cycle, and fed standard sugar-yeast-maize-molasses medium with excess live yeast granules (Lewis 1960). The stock has been maintained since 1970 in four large (several thousand flies), outbred population cages (Partridge & Farquhar 1983) of dimensions 30 cm x 15 cm x 20 cm. Each population was fed with three bottles of food medium per week. These four

populations were mixed into one single large population approximately one year prior to experiments to promote genetic variability in our experimental flies. This stock exhibits substantial levels of genetic variation (Wilkinson et al. 1990; Whitlock et al. 1996; Fowler et al. 1997), contains selectable variation for a range of life-history, behavioural and physiological traits (Wigby & Chapman 2004; Wigby et al. 2009), and displays some levels of inbreeding depression after full-sib mating (Fowler & Whitlock 1999; Mooers et al. 1999; Tan et al. 2012). The Dahomey stock is maintained with overlapping generations to minimise selection on replication rate and life span.

To obtain parents of the experimental flies, eggs were collected and raised at standard density (~100 flies per bottle) (Clancy & Kennington 2001). Parental flies were collected as virgins within eight hours of eclosion using ice anaesthesia, aged for 1 week before single males and females were paired in vials to produce families. The parental pair was discarded after 24 hours and the eggs left to develop. Virgin adults emerging from these vials were used for experimental trials.

(b) Experimental procedure

1-day post-eclosion (young) females were paired with, and mated to, single males of the same age. Males were either full-siblings (related; R) (n = 40) or non-siblings (unrelated; U) (n = 40) of the female. Males were then discarded and females allowed to oviposit for 24 hours in individual vials (1-day post copulation; young). To investigate the effect of gametic senescence on offspring viability, females were transferred to vials where they were deprived of an egg-laying substrate (sugar-yeast-maize-molasses medium) for 15 days. We ensured the survival of the females by providing a smear of Baker's yeast dried onto the side the vial, and 3 ml of saturated

sucrose solution placed onto cotton wool inserted at the bottom of the vial (Partridge et al. 1987). The cotton wool was kept moist all the time by re-supplying saturated sucrose solution on alternate days. These conditions discourage females from ovipositing (Partridge et al. 1987), thereby reducing sperm utilization rates (Trevitt et al. 1988). Therefore, sperm and eggs were retained, and aged, in the female reproductive organs. After 15 days, females were transferred to fresh vials with egg-laying substrate and allowed to oviposit for 24 hours before they were discarded (15-days post-copulation; old). To examine the potential effects of parental age on offspring mortality, we repeated the above protocol with 15-days post-eclosion (old) virgin females and males from the same set of families (n = 40 related pairs; n = 40 unrelated pairs). Namely, females were kept in vials with egg-laying substrate prior to mating on day 15 post-eclosion. Males were removed post-copulation and females allowed to oviposit in individual vials for 24 hours before they were transferred to vials without oviposition substrate for 15 days. Then, 30-days after eclosion and 15-days after copulation, females were placed in vials with egg-laying substrate for 24 hours (see Table 1). Because the first mating of virgin females causes the initial release of mature eggs (Chapman et al. 2001), eggs laid by females on day 15 post-copulation would be, at most, 15-days-old. Altogether, we conducted 320 trials (i.e. 80 pairs x two gametic age treatments x two parental age treatments). Throughout the article, we use the terms ‘young parents’ for individuals that copulated 1-day post-eclosion; ‘old parents’ for individuals that copulated on day 15 post-eclosion; ‘related’ for full-sib matings; ‘unrelated’ for non-sib matings; ‘young gametes’ for gametes contributing to zygotes produced 1-day post-copulation; and ‘old gametes’ for gametes contributing to zygotes produced 15-days post-copulation (Table 1).

Table 1. Parental organismal age across experimental treatments

Treatments	Age at copulation	Age at egg-laying
Young parents, young gametes	1-day-old	1-day-old
Young parents, old gametes	1-day-old	15-days-old
Old parents, young gametes	15-days-old	15-days-old
Old parents, old gametes	15-days-old	30-days-old

To quantify offspring viability at different development stages, we monitored the number of eggs, larvae, pupae and adults produced by individual females. We counted the number of eggs after the females were removed from the vials. 24 hours later, after the viable eggs had hatched, we counted the number of unhatched eggs. We also quantified the number of non-eclosed pupae and adults 12 days after the oviposition period. Because the majority of the flies eclosed 10 days after oviposition, allowing 12 days before counting provided ample time for development. To quantify sex-specific differences in mortality, we also determined the sex of individuals from the pupae and adult stage (but not at the egg and larval stages). Pupae were sexed based on the presence/absence of male-specific foreleg sex combs after opening pupal cases with fine forceps.

For ease of interpreting the results, we also calculated the coefficient of inbreeding depression δ (Lande & Schemske 1985) at each developmental stage:

$$\delta = 1 - (X_I/X_O),$$

where X_I = inbred viability and X_O = outbred viability. Therefore, a higher inbreeding depression coefficient indicates a higher magnitude of inbreeding depression.

(c) Statistical analysis

To analyse the effects of parental relatedness, parental organismal age, and gametic age

on offspring viability, we first considered variation in egg-adult viability, and then considered variation in viability at specific developmental stages: egg-hatchability (proportion of eggs that hatched), larva-pupa viability (proportion of larvae that developed to pupae), and pupa-adult viability (proportion of pupae that developed to adults). The proportion of individuals that developed into the next stage in each vial was thus a unit of replication. Egg-adult viability is widely used as an indication of overall viability in inbreeding studies (Mack et al. 2002; Lopez-Fanjul & Villaverde 1989), while the other response variables were used to better understand the ontogenetic mechanisms through which parental relatedness, parental age and gametic age might affect offspring viability.

There was no effect of parental relatedness, parental age and gametic age on the number of eggs laid by females (Supplementary Table 1). Also, we found no overall difference in variance between the inbred and outbred treatment (F test, $F_{39,39} = 1.07$, $P = 0.416$), suggesting that variability in environmental conditions between treatments was small. Therefore, we did not adjust viability measures for egg numbers or variance. We analysed variation in (i) overall egg-adult viability, (ii) egg-hatchability, (iii) and larva-pupa viability with three separate Generalised Linear Mixed Models (GLMMs) with Binomial error distribution, parental age, parental relatedness and gametic age, and their two- and three-way interactions as fixed factors, and family identity as a random variable. Finally, we analysed variation in (iv) pupa-adult viability through a GLMM with Binomial error distribution, pupa-adult viability as the response variable, parental relatedness, parental organismal age, gametic age , offspring gender, and their interactions as fixed factors, and family identity as a random variable. The significance of the fixed factors was assessed using the likelihood-ratio test on models with and

without the fixed factor (Valdar et al. 2006; Ockinger et al. 2010). For each test (i) – (iv) we used an information theoretic approach with corrected Akaike's information criterion (AIC) to select models. Models in which $\Delta AIC \leq 2$ were retained (i.e. there is no strong indication that one model has the best fit, see Supplementary Table 2). R version 2.15.0 was used for all analyses.

RESULTS

(a) Egg-adult viability

We identified two models that were equally effective ($\Delta AIC = 1$) at explaining variation in egg-to-adult viability, one with a significant interaction between paternal relatedness and parental age and with a significant independent effect of gametic age, and the other with a significant three-way interaction between parental relatedness, parental organismal age and parental gametic age (Table 2A; Supplementary Table 2). The latter model indicates that the viability of outbred offspring produced with young gametes was higher than the viability of outbred offspring produced with old gametes, particularly so in young rather than old parents (Figure 1A), resulting in the offspring of young parents and old gametes attaining the highest inbreeding depression coefficient (Figure 1B).

(b) Egg hatchability

The patterns of variation in egg-adult viability (above) were largely mirrored by patterns of variation in egg hatchability. We identified two models that were equally effective at explaining variation in egg hatchability, one detecting a significant interaction between parental relatedness and parental age and the effect of parental gametic age, and the other detecting a significant three-way interaction between

parental relatedness, parental organismal age and gametic age (Table 2B; Supplementary Table 2), whereby hatchability was higher in zygotes produced with young gametes, particularly so in young unrelated parents (Figure 1C). As a result, evidence of inbreeding depression was limited to the eggs of young parents (Figure 1D).

(c) Larva-pupa viability

Again, the best model detected a significant three-way interaction on variation in larva-pupa viability between parental relatedness, parental organismal age and gametic age (Table 2C; Supplementary Table 2). Inbred offspring generally suffered lower viability than outbred offspring (Figure 1E). However, in this case inbreeding depression was largest in the offspring of old parents and young gametes, and smallest in the offspring of old parents and old gametes (Figure 1F).

(d) Pupa-adult viability and sex-specific effects

The best model detected a significant four-way interaction between parental relatedness, parental organismal age, parental gametic age and offspring gender (Table 2D; Supplementary Table 2). The magnitude of inbreeding depression was substantially higher for old gametes than for young gametes amongst young parents, and slightly lower for old gametes than for young gametes amongst old parents group (Figure 1G and 1H). Consistent with the idea that the magnitude of inbreeding depression differs with offspring gender, the coefficient of inbreeding depression of daughters was more than twice that of sons (Parental relatedness*Sex, P =0.019, Daughters δ = 0.070, Sons δ = 0.032) (Figure 1H). This resulted in a significant four-way interaction between offspring gender, parental relatedness, parental organismal age

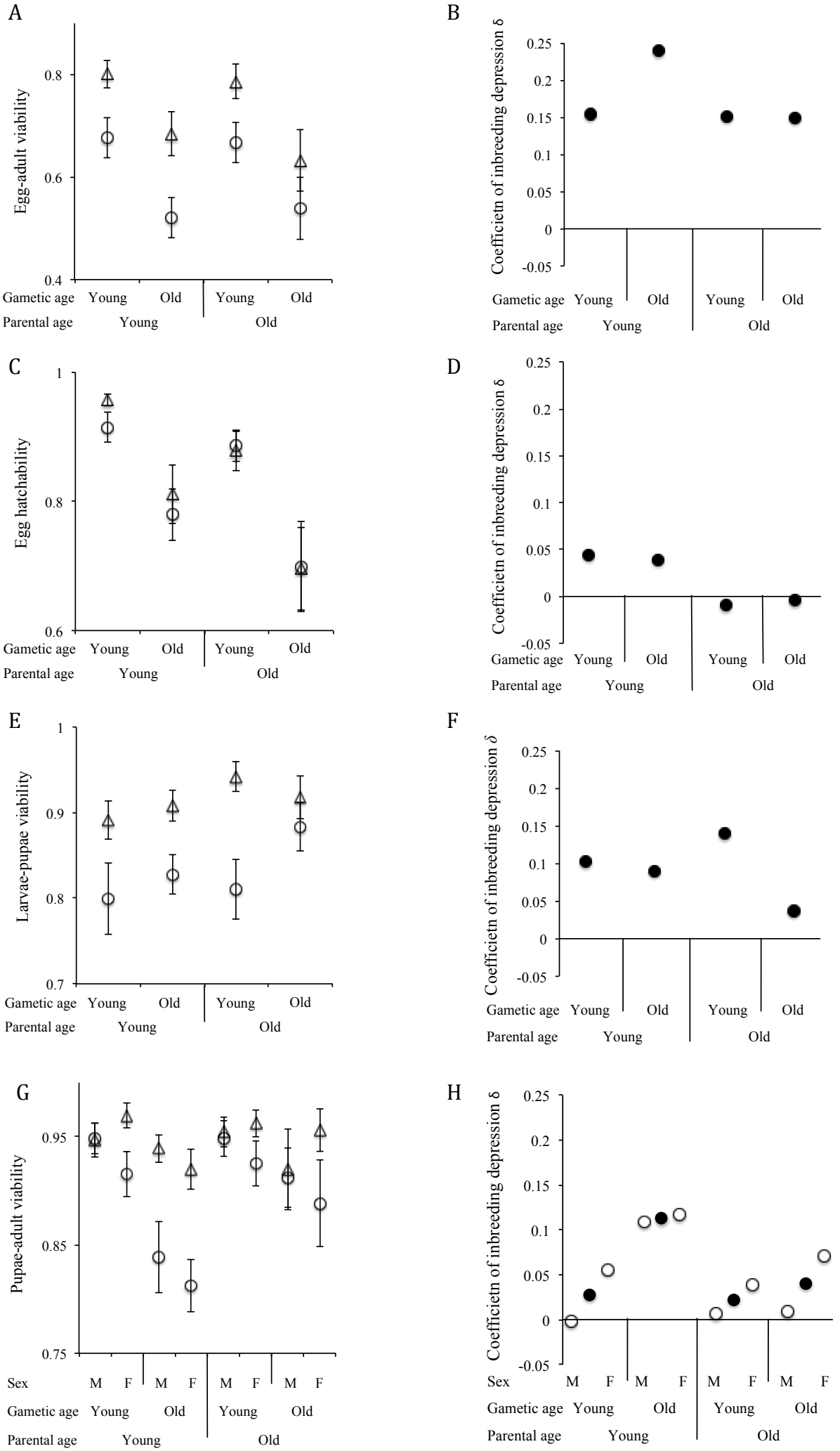
and gametic age (Table 2D; Figures 1G and 1H).

Table 2. Effects of parental relatedness, parental age and gametic age on: A egg-adult viability; B egg hatchability; C larva-pupa viability; D sex-specific pupa-adult viability. Gametic age: young = 1-day post-copulation and old = 15-days post-copulation. Parental age at copulation: young = 1-day post-eclosion and old = 15-days post-eclosion.

Response variable	**Factors**	**Mean ± SE**	**df**	χ^2	***P***
A. Egg-adult viability	Parental relatedness	related: 0.596±0.022; unrelated: 0.733±0.021	1	20.17	**<0.001**
	Parental age	young: 0.662±0.020; old: 0.669±0.024	1	6.35	**0.012**
	Gametic age	young: 0.724±0.018; old: 0.591±0.025	1	206.01	**<0.001**
	Parental relatedness*Parental age		1	19.21	**<0.001**
	Parental relatedness*Gametic age		1	1.72	0.190
	Parental age*Gametic age		1	0.704	0.401
	Parental age*Gametic age*Parental relatedness		1	4.36	**0.037**
B..Egg hatchability	Parental relatedness	related: 0.824±0.020; unrelated: 0.851±0.021	1	3.60	0.058
	Parental age	young: 0.865±0.017; old: 0.806±0.024	1	111.19	**<0.001**
	Gametic age	young: 0.904±0.012; old: 0.754±0.027	1	396.94	**<0.001**
	Parental relatedness*Parental age		1	27.79	**<0.001**
	Parental relatedness*Gametic age		1	0.38	0.539
	Parental age*Gametic age		1	1.50	0.220
	Parental age*Gametic age*Parental relatedness		1	2.05	**0.044**

Response variable	Factors	Mean ± SE	df	χ^2	*P*
C. Larva-pupa viability	Parental relatedness	related: 0.826±0.017; unrelated: 0.915±0.010	1	17.44	**<0.001**
	Parental age	young: 0.856±0.014; old: 0.886±0.015	1	5.38	**0.020**
	Gametic age	young: 0.861±0.016; old: 0.882±0.012	1	3.89	**0.048**
	Parental relatedness*Parental age		1	2.26	0.133
	Parental relatedness*Gametic age		1	0.18	0.671
	Parental age*Gametic age		1	2.81	0.094
	Parental age*Gametic age*Parental relatedness		1	6.71	**0.010**
D. Pupa-adult viability	Parental relatedness	related: 0.898±0.009; unrelated: 0.946±0.006	1	32.76	**<0.001**
	Parental age	young: 0.909±0.008; old: 0.936±0.008	1	28.75	**<0.001**
	Gametic age	young: 0.947±0.006; old: 0.892±0.010	1	14.97	**<0.001**
	Sex	male: 0.925±0.008; female: 0.919±0.008	1	8.24	**0.004**
	Parental relatedness*Parental age		1	1.51	0.219
	Parental relatedness*Gametic age		1	0.02	0.897
	Parental relatedness*Sex		1	15.18	**<0.001**
	Parental age*Gametic age		1	0.01	0.903
	Parental age*Sex		1	0.56	0.453
	Gametic age*Sex		1	0.67	0.412
	Parental relatedness*Parental age*Gametic age		1	16.83	**<0.001**
	Parental relatedness*Parental age*Sex		1	1.71	0.192
	Parental relatedness*Sex*Gametic age		1	0.09	0.761
	Parental age*Gametic age*Sex		1	0.62	0.433
	Parental relatedness*Parental age*Gametic age*Sex		1	4.81	**0.028**

Figure 1. Viability of offspring produced by young and old, related and unrelated parents at different developmental stages (left column) and coefficient of inbreeding depression (δ) (right column). A & B: Cumulative egg-adult viability; C & D: Egg-hatchability; E & F: Larva-pupa viability; G & H: Pupa-adult viability. For figures A, C, E and G, triangular symbols represent viability when parents were unrelated; circular symbols represent viability when parents were related. Unfilled symbols for figure H denote δ for the separate sexes while filled symbol denote mean δ for both sexes. Error bars denote S.E. Results are presented in Table 2.


A
Egg-adult viability
0.8
0.6
0.4
Gametic age Young Old Young Old
Parental age Young Old
B
Coefficietn of inbreeding depression δ
0.25
0.2
0.15
0.1
0.05
0
-0.05
Gametic age Young Old Young Old
Parental age Young Old
C
Egg hatchability
1
0.8
0.6
Gametic age Young Old Young Old
Parental age Young Old
D
Coefficietn of inbreeding depression δ
0.25
0.2
0.15
0.1
0.05
0
-0.05
Gametic age Young Old Young Old
Parental age Young Old
E
Larvae-pupae viability
1
0.9
0.8
0.7
Gametic age Young Old Young Old
Parental age Young Old
F
Coefficietn of inbreeding depression δ
0.25
0.2
0.15
0.1
0.05
0
-0.05
Gametic age Young Old Young Old
Parental age Young Old
G
Pupae-adult viability
0.95
0.85
0.75
Sex M F M F M F M F
Gametic age Young Old Young Old
Parental age Young Old
H
Coefficietn of inbreeding depression δ
0.25
0.2
0.15
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0.05
0
-0.05
Sex M F M F M F M F
Gametic age Young Old Young Old
Parental age Young Old

DISCUSSION

(a) Effects of ageing on inbreeding depression

Previous studies of the fruit fly have demonstrated the independent effects of inbreeding depression, e.g. in terms of decreased offspring fertility and viability (Vermeulen & Bijlsma 2004; Partridge et al. 1985), and parental ageing, in terms of reduced offspring fertility (David et al. 1975; Economos et al. 1979). The aim of our study was to test the extent to which inbreeding depression is modulated by organismal and gametic mechanisms of parental ageing. We found that variation in offspring mortality at all developmental stages and cumulative egg-adult viability is strongly influenced by a three-way interaction between parental relatedness, parental organismal age and gametic age. In terms of overall egg-adult viability, the magnitude of inbreeding depression suffered by the offspring of senesced gametes and young parents is 1.6 times that of the offspring of young gametes and young parents, and those of gametes of both ages of old parents. Thus, gametic ageing appears to exacerbate the effects of inbreeding in young parents but not in old parents. One potential reason for this dichotomy might be that older parents increase investment in offspring, for example by increasing egg resources, to offset the effects of inbreeding. However, this hypothesis remains to be tested. It is also important to note that the relationship between viability and the interaction among parental age, parental relatedness and gametic age, varies with developmental stage. The deleterious mutations that cause inbreeding depression and senescence may manifest at different development stages (Schupbach & Wieschaus 1986; Hurd & Saxton 1996), potentially modifying the relative effects of parental age and gametic age on offspring viability.

The influence of parental gametic age was strong across all developmental stages. Our

experimental design measured the effect of gametic age by comparing the viability of inbred and outbred offspring produced on day 1 and day 15 post-copulation. The egg deposition rates of females were suppressed by deprivation of suitable oviposition sites, subsequently reducing sperm depletion rate Trevitt et al. 1988). Therefore, this design captures the effect of sperm senescence occurring during female sperm storage. The effect of post-meiotic sperm senescence is beginning to emerge as a significant determinant in offspring viability in natural populations (White et al. 2008). Because females were prevented from remating and from ovipositing between day 1 and day 15 post-insemination, it is likely that a proportion of these eggs may have been ovulated days in advance of oviposition. The retention of mature eggs in the ovary, which can be induced by dietary restriction (Drummond-Barbosa & Spradling 2001), inhibits the development of younger oocytes in the fruit fly (Meola & Lea 1972). It has been suggested that these oocytes would therefore undergo cell death to recycle macromolecules and prevent blockage of the ovarioles (Chao & Nagoshi 1999; Buszczak & Cooley 2000). Therefore, we cannot rule out the possibility that eggs laid on day 15 will be a mixture of newly-produced eggs and old eggs, suggesting that egg senescence may also contribute to explain the effect of gametic age. Further studies should aim to disentangle the effects of egg and sperm age on inbreeding depression.

To our knowledge, only two other studies of humans and seed beetles, *Callosobruchus maculatus*, have examined how inbreeding depression in offspring fitness traits change with maternal age (Yaqoob et al. 1998; Fox & Reed 2010). In these studies, the magnitude of inbreeding depression increased with maternal age, contrary to our study. In our study, the reduced inbreeding depression in older parents is largely caused by a decreased difference in egg hatchability between related and unrelated parents. One

possibility is that this result may have been due to the selective disappearance of families with reduced egg hatchability in older parents. However, we found no evidence of this as none of the parental flies died during the experiment and thus all family lines were represented across all age treatments (data not shown). A more plausible explanation might be that the large effects of parental senescence on fertility and/or pre-hatching mortality might dominate that of inbreeding, and potentially mask differences between inbred and outbred offspring. Declines in sperm production and genetic quality in old males (Dean et al. 2010; Pizzari et al. 2008), as well as an increase in the levels of oxidatively-damaged proteins in the eggs (Fredriksson et al. 2012), can result in lower fertilizing efficiency and/or embryonic viability. Such mechanistic effects may occur independently of the expression of recessive mutations during inbreeding and mask the effects of increased homozygosity, potentially explaining the decreased magnitude of inbreeding depression in old parents. These results highlight the potential importance of interactions between inbreeding and parental age effects.

(b) Variability of inbreeding effects at different developmental stages

Although there was an overall decrease in egg-adult viability due to inbreeding, this was mainly mediated by larva-pupa and pupa-adult viability, not by egg hatchability. This is consistent with a previous study of the fruit fly, which found that egg hatchability was only slightly affected by inbreeding (< 2%), but relative larval-adult survival was reduced by 20% (Enders & Nunney 2010). The delayed detrimental effect of inbreeding suggests that the genes involved in early development may acquire mutations that are not fully detrimental, limiting selection against these alleles. In addition, we found an overall decline in mortality, in both inbred and outbred offspring,

from the egg to adult stage, consistent with the evolutionary demography of ontogenesis in which the death rate of each cohort tends to decrease with increasing age between conception and maturity (Levitis 2011). High mortality during egg hatchability may have masked the ability to detect the effects of inbreeding.

(c) Inbreeding depression and sex-specific effects

Our results indicate that the effects of inbreeding vary with offspring sex and developmental stages. The 'unguarded X' hypothesis posits that the heterogametic sex should suffer less from inbreeding compared to the homogametic sex (i.e. females here), since inbreeding will increase the risk of expression of any X-linked recessive deleterious allele only in the heterogametic sex. We found that inbreeding depression, was higher for the homogametic sex, which is in accordance with this theory and consistent with previous studies on inbreeding depression in the longevity of insects (Saccheri et al. 2005; Bilde et al. 2009), birds (Keller et al. 2008) and mammals (Rioux-Paquette et al. 2011; Coulson et al. 1999). Interestingly however, the reversed effect, where the heterogametic sex suffers more from inbreeding depression, is often observed in reproductive traits such as reproductive success in the fruit fly (Millers et al. 1993; Enders & Nunney 2010), fertility in the butterfly *Bicyclus anynana* (Saccheri et al. 2005), fledging success in Takahes, *Porphyrio hochstetteri* (Jamieson et al. 2003) and hatching rate in song sparrows, *Melospiza melodia* (Keller 1998). Why the difference in sex-differential effects of inbreeding depression in survival and reproduction is unknown. Because we could not determine the sex of dead embryos or larvae, nor measure the adult reproductive success of the inbred *versus* outbred offspring, we cannot determine sex-specific mortality prior to the pupae stage, or during adulthood, meaning that we can make only limited conclusions about sex-

specific inbreeding load at different life-history stages.

Conclusions

Our study indicates that inbreeding depression is sex-specific and modulated by parental effects such as parental organismal age and parental post-meiotic gametic age. The variation in offspring viability at different development stages with these factors demonstrates the importance of considering the various life-history stages when studying the fitness costs of inbreeding. Ultimately, further elucidating the genetic underpinnings of such variations in fertility and mortality will provide important insights into the relationship between inbreeding, senescence and gender.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Effects of parental relatedness, parental age and gametic age on egg numbers. Gametic age: young = 1-day post-copulation and old = 15-days post-copulation. Parental age at copulation: young = 1-day post-eclosion and old = 15-days post-eclosion.

Factors	**Mean ± SE**	**df**	χ^2	***P***
Parental relatedness	related: 35.67±1.21; unrelated: 34.38±1.10	1	0.625	0.429
Parental age	young: 35.02±1.19; old: 35.03±1.10	1	<0.001	0.985
Gametic age	young: 35.37±0.71; old: 34.60±1.60	1	0.227	0.634
Parental relatedness*Parental age		1	0.118	0.730
Parental relatedness*Gametic age		1	1.008	0.315
Parental age*Gametic age		1	0.139	0.709
Parental age*Gametic age*Parental relatedness		1	0.246	0.620

Supplementary Table 2. Akaike's information criterion (AIC) of Generalised Linear Mixed Models for the different response variables. A egg-adult viability; B egg hatchability; C larva-pupa viability; D sex-specific pupa-adult viability. Gametic age: young = 1-day post-copulation and old = 15-days post-copulation. Parental age at copulation: young = 1-day post-eclosion and old = 15-days post-eclosion. Models are presented sequentially, starting with lowest AIC.

Response variable	**Model**	**AIC**
A. Egg-adult viability	Parental relatedness * Parental age + Gametic age	1837.2
	Parental age * Gametic age * Parental relatedness	1838.2
	Parental relatedness + Parental age + Gametic age	1854.4
	Parental relatedness * Gametic age + Parental age	1854.7
	Parental relatedness + Parental age * Gametic age	1855.7
B..Egg hatchability	Parental age * Gametic age * Parental relatedness	2107.3
	Parental relatedness * Parental age + Gametic age	2107.4
	Parental relatedness + Parental age + Gametic age	2133.2
	Parental relatedness + Parental age * Gametic age	2133.7
	Parental relatedness * Gametic age + Parental age	2134.8
C. Larva-pupa viability	Parental age * Gametic age * Parental relatedness	1129.9
	Parental relatedness + Parental age * Gametic age	1133.0
	Parental relatedness * Parental age + Gametic age	1133.6
	Parental relatedness + Parental age + Gametic age	1133.8
	Parental relatedness * Gametic age + Parental age	1135.6
D. Pupa-adult viability	Parental relatedness * Parental age * Gametic age * Sex	797.73
	Gametic age + Parental age + Sex * Parental relatedness	804.21
	Parental relatedness * Parental age * Sex + Gametic age	806.49
	Parental relatedness * Parental age * Gametic age + Sex	806.86
	Parental relatedness * Gametic age * Sex + Parental age	809.56
	Parental relatedness + Parental age + Gametic age + Sex	817.39
	Parental relatedness * Parental age + Gametic age + Sex	817.88
	Parental relatedness + Parental age + Gametic age * Sex	818.71
	Parental relatedness + Gametic age + Sex * Parental age	818.82
	Parental relatedness * Gametic age + Parental age + Sex	819.37
	Parental relatedness + Parental age * Gametic age + Sex	819.37
	Parental relatedness + Parental age * Sex * Gametic age	823.77

Chapter 3

Sex-specific inbreeding depression in the red junglefowl: The role of parental age and sperm age

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ABSTRACT

The cost of inbreeding depression is expected to differ between sons and daughters due to sex-specific selection and genetic asymmetries between the sexes. In addition, organismal and gametic senescence of the parents might also modulate the magnitude of inbreeding depression. However, little is known about the extent to which mechanisms of parental senescence affect inbreeding depression differentially in male and female offspring. Here, we show that in the red junglefowl *Gallus gallus* ssp., inbreeding depression is sex-specific, with the sex that suffers the higher cost being trait-dependent. The effects of inbreeding depression on growth rate and tarsus length were greater in sons than in daughters, but for post-hatch survival the effects were greater in daughters than in sons. We also found that sex-specific inbreeding depression in social status, embryonic viability and comb size were modulated by parental age. Parental age also interacted with sperm age and parental relatedness to affect variation in growth rate and tarsus length. Overall, our results demonstrate that important fitness traits in offspring are influenced by a complex interaction between parental effects, inbreeding depression and offspring sex.

INTRODUCTION

Inbreeding, reproduction between genetically related individuals, is well known to depress offspring fitness (inbreeding depression) through the expression of deleterious recessive alleles and loss of heterozygous advantage (Charlesworth & Charlesworth, 1987). Evidence is emerging that patterns of inbreeding depression might be sex-specific (i.e. affect male and female offspring to a different extent). Recently, several studies have reported sex-specific effects of inbreeding depression across a variety of taxa: insects (Fox et al. 2006; Saccheri et al. 2005; Bilde et al. 2009), birds (Jamieson et al. 2003; Keller et al. 2008) and mammals (Rioux-Paquette et al. 2011). However, the sex suffering higher inbreeding depression varies across species and populations, and sometimes is contingent on the trait and context considered. For example, some studies have shown that inbreeding depression affects the survival of homogametic sex more than that of the heterogametic sex in insects (Wilkinson et al. 1990; Fox et al. 2006), birds (Keller et al. 2008) and mammals (Rioux-Paquette et al. 2011); others however have shown that the heterogametic sex suffers a more drastic reduction in reproductive fitness compared to the homogametic sex as a result of inbreeding (Jamieson et al. 2003; Enders & Nunney 2010).

Two hypotheses have been proposed to explain sex-specific patterns of inbreeding depression. First, the 'unguarded X' hypothesis predicts that the heterogametic sex, in general, should suffer reduced fitness because X/Z-linked recessive deleterious mutations will be unconditionally expressed in the heterogametic sex (Trivers 1985). However, the 'unguarded X' hypothesis also predicts that the homogametic sex will suffer higher fitness costs as a result of inbreeding because of the increased opportunity for the expression of recessive alleles. In contrast, the heterogametic sex will not suffer

from the increased probability of expression of X/Z-linked recessive deleterious mutations brought about by inbreeding. The 'unguarded X' hypothesis therefore has the potential to explain sex-specific inbreeding depression. The alternative hypothesis is based on sex-specific selection on resources allocated into current and future reproduction (Trivers 1972). Males and females have different optimal reproductive strategies involving sex-specific investments in current and future reproduction (Bonduriansky et al. 2008; Clutton-Brock and Isvaran 2007). Males are predicted to adopt a 'live fast, die young' strategy and females are predicted to maximise their reproductive output by conserving energy and prolonging reproductive lifespan. There are three plausible scenarios of how this hypothesis can explain sex-specific patterns in inbreeding depression (Bilde et al. 2009). First, via the reduced likelihood of inbred males engaging in energy-demanding reproductive behaviours such as male-male competition, thereby prolonging male lifespan. Second, via genes that code for resource allocation undergoing opposing directional selection. Directional selection typically leads to directional dominance in opposite directions in the sexes. However, inbreeding would reverse the effects of such genes in the non-preferred direction, that is, to decrease female lifespan while increasing male lifespan. Finally, via traits mediating resource allocation into current *versus* future reproduction, which are encoded by autosomal genes and are maintained if male-beneficial alleles are dominant in males and recessive in females, while female-beneficial alleles are dominant in females and recessive in males. By increasing the homozygosity of such genes, inbreeding could then adjust the trait expression to an opposite and maladaptive direction in both sexes. Although the 'sex-specific resource allocation' hypothesis is specific to competition and resource allocation, the general mechanism of sex-specific optimal phenotype being reversed by the expression of recessive alleles during inbreeding, could still

apply independently of resource allocation.

An additional complication is that the magnitude of inbreeding depression might be labile and change in response to a number of factors such as environmental quality (Armbruster & Reed 2005) and offspring age (Charlesworth & Hughes 1996). In particular, mechanisms of parental ageing have been shown to influence offspring fitness through a number of epigenetic effects and reduced parental investment (Fox 1993; Opit & Throne 2007), suggesting that inbreeding depression might also be modulated by patterns of parental senescence.

Senescence can occur at two distinct levels. Senescence at the organismal level refers to a decline in survival and reproductive ability with advancing age (Rose 1991; Finch & Kirkwood 2000) due to mutations that cause harm late in life (Medawar 1952; Williams 1957), or due to costs of reproduction (the disposable soma theory, Kirkwood 1977). Parental organismal senescence can have profound consequences for offspring fitness. For example, older mothers typically allocate fewer resources to eggs than younger mothers (Fox 1993; Opit & Throne 2007) resulting in higher offspring mortality. At the gametic level, senescence refers to reductions in the fertilizing efficiency and zygote fitness due to DNA damage of the haploid genome over the postmeiotic lifespan of individual gametes, independent of parental age (Pizzari et al. 2008; Tatone 2008). One form of gametic senescence is sperm ageing which can occur after insemination and during the storage in the female reproductive tract. This would be applicable to both young and old males. Considering the negative consequences of senescence, it is plausible that the offspring of ageing parents and/or gametes might be more sensitive to the expression of recessive alleles and loss of heterozygosity imposed

by inbreeding. However, the idea that inbreeding depression is modulated by parental and gametic senescence remains untested.

Here, we experimentally test whether inbreeding depression is sex-specific and if so, how this sex-specificity is modulated by parental age and sperm age in a captive population of the red junglefowl *Gallus gallus* ssp. Several fitness costs due to inbreeding have been documented in the domestic chicken *Gallus gallus domesticus* (Shoffner et al. 1953; Craig & Baruth 1965; Cheng et al. 1984; Cheng & Burns 1988). In addition, recent studies in the red junglefowl have demonstrated male organismal senescence in a number of reproductive traits (Dean et al. 2010; Noguera et al. 2012). Specifically, old males accumulated more oxidative damage in their sperm DNA (Noguera et al. 2012) and suffered reduced mating and fertilisation success (Dean et al. 2010). At the gametic level, earlier studies have also shown a significant increase in offspring embryonic death and retarded development with increasing age of spermatozoa, suggesting detrimental effects of sperm senescence on offspring (Dharmarajan 1950; Lodge et al. 1971). These age-dependent declines in reproductive performance and sperm quality might thus interact with inbreeding. We measure inbreeding depression in relation to parental and sperm age in multiple fitness-related traits of the offspring: (i) growth rate and mortality, (ii) comb size, comb redness and tarsus length, (iii) gametic quality and (iv) social status.

MATERIAL AND METHODS

(a) Study population and breeding procedure

The study was conducted on an individually marked population of the red junglefowl, from March to September in 2010, 2011 and 2012 at the Oxford University John Krebs

Field Station in Wytham, Oxfordshire. The relatedness of the experimental population (n_{males} = 49, $n_{females}$ = 37) was determined by calculating pairwise relatedness based on microsatellite data (Queller & Goodnight, 1989). Therefore, male-female pairs considered related had a coefficient of relationship (r) 0.45<r<0.6 and those considered unrelated had a coefficient of relationship (r) >-0.05. Individuals were also divided into two age groups: young (1-3-years-old) and old (4-6-years-old). The categorization of the two age groups was based on patterns of senescence in a domestic population, which showed reproductive traits declining at approximately age of 4 (Dean et al. 2010).

To investigate the effect of parental age on inbreeding depression, we bred offspring from four different parental groups: (a) young, related; (b) young, unrelated; (c) old, related and; (d) old, unrelated. Females were segregated from males for two weeks to deplete their reproductive system of sperm from previous copulations. To control for maternal effects resulting from responses to individual males, females were artificially inseminated. Semen was collected from males by abdominal massage (Etches 1996) and subsequently inseminated into the female's cloaca using a pipette. Females were then reproductively isolated for 11 days to allow for egg collection. Eggs were subsequently transferred to a cooler (14 to 16°C and 65% relative humidity) before incubation. Because previous studies have shown detrimental effects of egg storage time on embryonic viability (Walsh et al. 1995; Lapao et al. 1999), we minimised egg storage time in the cooler (1-7 days) prior to incubation. We performed preliminary analyses to confirm that variation in egg storage time did not have any effect on embryonic viability and post-hatch survival (Generalised linear mixed model, χ^2_1 = 2.12, P = 0.146; Cox survival, χ^2_1 = 1.15, P = 0.283). Nevertheless, we entered egg

storage time as a covariate in all our statistical models to control for the potential effect of this variable. Sperm age here is defined as the number of days between insemination and egg collection, independent of male parental age.

Eggs were incubated at 37.5°C and 45% relative humidity for 18 days and subsequently incubated at 37.5°C and 55% relative humidity from day 18 to 21. Each year, five sets of artificial insemination trials (5-12 male-female pairs per set) were conducted and five batches of chicks (10-40 chicks per batch) were bred. Hatched chicks were individually ringed and vaccinated against Marek's disease to minimise high death rates due to the disease experienced in years prior to 2010. The sex of the chicks was determined at six weeks of age, when sexual plumage dichromatism became unambiguous.

(b) Mortality and growth rate

Chick mass was recorded just after hatch, and at one week (n = 163), two weeks (n = 163), three weeks (n = 163), one month (n = 163), two months (n = 150) and three months after hatch (n = 141). We analysed the effect of parental and sperm age on inbreeding depression using a Generalised Linear Mixed Model (GLMM) with 'growth' as a response variable, 'parental relatedness', 'parental age', 'offspring sex' and 'sperm age' as fixed factors, 'year' (2010, 2011, 2012), 'offspring age' and 'batch' as covariates and 'offspring identity' nested within 'mother identity' nested within 'father identity' as a random factor. We also entered selected two-way and three-way interactions as fixed factors to address specific *a priori* predictions (Table 1). Unless otherwise stated, all models had fixed factors stated in Table 1. 'Lifespan' was entered as a covariate to account for between-individual variation in mortality (Dean et al.

2010).

Table 1. Hypotheses tested and the corresponding fixed factors and/or interactions entered into the statistical model.

Fixed factors	Test
Parental relatedness	Is the trait subjected to inbreeding depression?
Parental age	Does parental age affect traits?
Sex	Does the trait differ with offspring sex?
Sperm age	Does sperm age affect traits?
Parental relatedness*Parental age	Is inbreeding depression dependent on parental age?
Parental relatedness*Sex	Is inbreeding depression sex-specific?
Parental relatedness*Sperm age	Does inbreeding depression depend on sperm age?
Parental age*Sex	Do the effects of parental age differ with offspring sex?
Parental age*Sperm age	Does parental age interact with sperm age ?
Parental age*Sex*Parental relatedness	Is sex-specific inbreeding depression modulated by parental age?
Sperm age*Sex*Parental relatedness	Is sex-specific inbreeding depression modulated by sperm age?
Parental age*Sperm age*Parental relatedness	Is inbreeding depression modulated by an interaction between parental age and sperm age?

We monitored the mortality of all individuals and unhatched embryos (n = 183). Tissue samples were collected from individuals that died before hatching or before six weeks of age when sexing by phenotypic means might be inaccurate. Unhatched eggs were opened to determine if they were fertilised and embryonic tissue present in fertilised eggs were extracted and stored in 70% alcohol for subsequent sexing using genetic tools. Brain tissue was extracted from chicks younger than six weeks. These samples were subsequently sexed by a PCR-based protocol (Fridolfsson & Ellegren 1999).

We analysed variation in mortality using Cox proportional hazards model (Cox 1972)

with 'offspring age' as the censoring time, 'mortality' (1 for death; 0 for survived) as the event indicator, 'year' and 'batch' as covariates and 'mother identity' nested within 'father identity' as a random factor.

(c) Comb size, redness and tarsus length

Under mate choice trial conditions, female red junglefowl often prefer to mate with males with larger and redder combs (Zuk et al. 1990; Parker et al. 2006). Similarly, males allocate more sperm to females with larger combs (Pizzari et al. 2003), although no studies have investigated the correlation between female comb redness and male choice. The methods used to measure comb size and redness are described in previous studies (Cornwallis & Birkhead 2006; Alonso-Alvarez et al. 2008). To measure comb size and redness, we took digital photographs of the combs of 1-year-old offspring against a standard red-coloured paper and a ruler ($n_{males} = 41$, $n_{females} = 36$). In order to standardise and allow for valid comparisons across years, photos were taken using a standard camera (Toshiba Camileo X400) in the same room with constant light levels each year. Both the left and right side of the combs of each bird were photographed. We then used Photoshop version 11.0 to highlight the comb area using the magnetic lasso tool and subsequently recorded the comb size (in pixels) and RGB parameters. As references, we recorded the length of 1cm (in pixels) on the ruler and RGB parameters of the standard red-coloured paper. We then calculated the comb size in cm^2, and comb redness using the following equation:

(Comb Red - Comb Blue - Comb Green) / (Paper Red - Paper Blue - Paper Green)

We tested the effect of parental and sperm age on sex-specific inbreeding depression in comb size and redness using GLMM. Two separate models were used to analyse

variation in average comb size and redness, i.e. average of the left and right sides of the comb. 'Year', 'batch' and 'offspring age' were covariates and 'offspring identity' nested within 'mother identity' nested within 'father identity' was a random factor.

In birds, tarsus length is positively correlated with skeletal size (Senar & Pascual 1997), which is an indicator of true body size (Rising & Somers 1989). A wire was used to trace the mid-line of the tarsus and subsequently straightened and aligned against a ruler to find the length. Both left and right tarsi of 1-year-old offspring were measured (n_{males} = 51, $n_{females}$ = 53). We found significant differences in left and right tarsi length for females but not for males (paired t-tests, t_{52} = 2.81, P = 0.006; t_{50} = -1.17, P = 0.246 respectively). Therefore, we did not take the average of the left and right tarsus length but entered in a GLMM, 'tarsus length' as the response variable, 'individual identity' as a random effect and 'tarsus side' as a covariate in our analysis. Similarly to our analyses on comb size and redness, 'year', 'batch' and 'offspring age' were covariates and 'offspring identity' nested within 'mother identity' nested within 'father identity' was a random factor.

(d) Gametic quality of offspring

Previous studies have demonstrated a positive correlation between egg mass and hatchling body mass (Wilson 1991; Göth & Evans 2004). Egg number is a good indicator of female fecundity. To quantify female egg size and mass, females were fed with coloured dyes (Sudan black or Sudan red or a mixture of both dyes) and kept with females fed with other coloured dyes. Their eggs were collected for 14 days. Eggs were then opened and identified as belonging to either female using the colour of the yolks. The number of eggs laid and the egg mass of individual females were thus quantified (n

= 34). We analysed variation in egg number using a GLMM with Poisson error distribution, 'egg number' as the response, 'average egg mass' (by that particular female) and 'offspring age' as covariates and 'mother identity' nested within 'father identity' as a random factor. Similarly, we analysed variation in egg mass using another GLMM with Poisson error distribution, 'egg mass' as the response, 'egg number' and 'female age' as covariates and 'female identity' nested within 'mother identity' nested within 'father identity' as a random factor. In both models on egg mass and egg number, we entered all the fixed factors listed in Table 1 except those factors with 'sex'.

A positive correlation between sperm motility and the likelihood of fertilisation has been demonstrated in the domestic chicken (Wishart & Palmer 1986). To examine sperm quality of sons, semen samples were collected via abdominal massage (Etches 1996) and placed in eppendorf tubes (n = 51). Prior to analysis under the microscope, the eppendorf tube was place in a water bath of 41°C, which simulates the body temperature of the red junglefowl. We then diluted 2.5µl of semen sample with 197.5µl of Dulbecco's modified Eagle's growth medium (DMEM) and transferred onto a heated stage (41°C) placed under the microscope (Wilson-Leedy & Ingermann 2006). Sperm swimming pathways were recorded with a Basler A312fc digital camera at 50 frames per second at x100 magnification connected to a Nikon E200 microscope and analysed using the Sperm Class Analyzer, SCA v. 3.0.3 (Dean et al. 2010). Four aspects of sperm swimming pathways were used as indicators of sperm quality: velocity curvilinear (VCL), velocity straight line (VSL), average path velocity (VAP) and linearity (LIN = VSL/VCL) (Wilson-Leedy and Ingermann, 2006). The curvilinear velocity (VCL, µm/s) describes velocity over the actual sperm track, which includes all

deviations of sperm head movement, Angular path velocity (VAP, μm/s) is the velocity over a calculated, smoothed path, straight-line velocity (VSL, μm/s) is the velocity over the straight line distance between the beginning and end points of the sperm track and linearity (LIN, %) is the ratio of net distance moved to total path distance (King et al. 2000). To examine the effect of parental age on inbreeding depression in sperm quality, we entered in four separate GLMMs the four parameters of sperm quality as response variables, 'offspring age' as a covariate and 'mother identity' nested within 'father identity' as a random factor. In these models, we entered all the fixed factors listed in Table 1 except those factors with 'sex'. To account for multiple testing (four parameters of sperm quality), we used Bonferroni correction. Thus, *P* values were considered significant only when $P < 0.05/4 = 0.013$.

The number of sperm holes on the periviteline layer of eggs is positively correlated with fertilisation potential of the sperm (Wishart 1997). We might therefore expect sperm of inbred males to attain fewer hydrolysis points on the PVL than sperm of outbred males if inbreeding imposes a cost on sperm quality. For females, inbred individuals also attained a lower fertility rate than outbred females (Cheng et al. 1984). However, the mechanism for this observation is unclear and could be either due to reduced mating rate of these females or poor quality of eggs produced by these females. In this experiment, we tested the idea that gametes of inbred males and females attained lower fertility than outbred males and females respectively *in vivo*. Semen samples were obtained from two males: one inbred and one outbred, by abdominal massage (Etches 1996). The sperm from each sperm donor was then standardised to have the same number of sperm. This was done by first estimating the number of sperm from each male, by multiplying the absorbance value (linearly

positively correlated with sperm concentration; Ciereszko & Dabrowski 1993; Donoghue et al. 1996) obtained using a spectrometer by the volume measured with a pipette. The limiting sperm sample had the lower 'absorbance multiplied by volume' value. We then calculated the volume of the other sperm sample to have the same number of sperm, i.e. volume of other sperm sample = absorbance * volume of limiting sperm sample / absorbance of other sperm sample. Each standardised sperm sample was then divided into two equal halves and inseminated artificially into an inbred and an outbred female (n_{set} of four females each = 6). Due to a limitation in the number of individuals, all individuals used had young parents to control for any effects of parental age. Eggs were collected for 6 days and the number of hydrolysis points on the outer perivitelline layer (PVL) was quantified (Pizzari et al. 2004). We analysed variation in PVL hydrolysis points using a GLMM with Poisson error distribution, 'inbreeding status of males' (inbred or outbred), 'inbreeding status of females', 'lay-date' and 'parental sperm age' as fixed factors, 'male inbreeding status*lay-date' and 'female inbreeding status*lay-date', 'male inbreeding status*parental sperm age' and 'female inbreeding status*parental sperm age' as interaction terms, 'ejaculate amount' as a covariate and 'male identity' nested within 'female identity' as a random factor.

(e) Social status

Social status is important for both males and females of the red junglefowl since dominant males gain higher mating success (Collias & Collias 1996; Pizzari & Birkhead 2000; Collet et al. 2012) and dominant females are able to obtain more food and resources, indirectly increasing their reproductive fitness (Collias et al. 1994). Males and females were kept visually separated for two weeks before trials. Trials were conducted in a 3m by 3m pen visually isolated from other pens to minimise distraction

to the experimental birds by other birds. One inbred and one outbred individual of the same sex and from parents of the same age were released simultaneously into the pen (n_{males} trials = 72, $n_{females}$ trials = 54). Male-male pairs were observed for 10min and female-female pairs for were observed 15 min as females were generally less confrontational than males. An individual was considered dominant if it won a fight, chased the other individual or if the other individual constantly avoided his/her approaches (Johnsen et al. 2001; Froman et al. 2002). To control for any effects of size on social status, we paired inbred and outbred individuals with similar mass. We analysed variation in dominance using a GLMM with Binomial error distribution, 'dominance' (1 for dominant; 0 for subdominant) as the response, 'year' as a covariate and both 'individual identities' a random factors. We entered all the fixed factors listed in Table 1 except 'sex', 'parental age' and the interaction 'sex*parental age' because these factors were experimentally controlled for.

(f) Standardised inbreeding coefficient

To facilitate the interpretation of the results, we also calculated the standardised coefficient of inbreeding depression δ (Hedrick & Kalinowski 2000) at each developmental stage:

$$\delta = (X_O - X_I) / X_O F,$$

where X_I = trait value of inbred offspring, X_O = trait value of outbred offspring and F = is the inbreeding coefficient. F is 0.25 in our study as related individuals were full-siblings (Løvlie et al. in prep). Therefore, a higher inbreeding depression coefficient indicates a higher magnitude of inbreeding depression. Sperm age was also categorised into two groups: young sperm (eggs that were laid days 1-5 post-insemination) and old sperm (eggs that were laid days 6-11 post-insemination) to simplify the calculation and

interpretation of δ.

RESULTS

(a) Mortality

There was a significant three-way interaction between parental relatedness, parental age and offspring sex for embryonic mortality (Table 2). Inbred daughters suffered lower viability than outbred daughters amongst old parents group whereas the outbred individuals suffered a marginally lower viability than inbred individuals for sons of both parental age groups and for daughters of young parents (Figure 1A).

We detected two significant interactions on variation in post-hatch survival. A significant interaction between parental relatedness and offspring sex indicates that inbred daughters suffered substantially higher mortality rates than outbred daughters while outbred sons suffered marginally higher mortality rates than inbred sons (Figure 1B; Tables 2 and 3). In addition, offspring of young parents suffered significantly higher mortality rates than the offspring of old parents, particularly so in sons (Figure 1C; Table 3).

(b) Growth rate

Consistent with the idea that the magnitude of inbreeding depression differs with offspring sex, inbred sons grew at a significantly slower rate than outbred sons, whereas outbred daughters grew at a marginally slower rate than inbred daughters (Figure 1D; Tables 2 and 3). We also identified a significant interaction between paternal relatedness, parental age and sperm age, whereby the growth rate of inbred

offspring produced with old sperm from old parents was lower than that of other types of offspring produced by old parents (Figures 1E and 1F; Tables 2 and 3).

Table 2. Effects of parental age and parental relatedness on offspring (1) Growth and mortality; (2) Morphological traits; (3) Gametic quality and; (4) Social status. 'Sex' refers to offspring sex, i.e. sons or daughters. Values given for growth are slopes and those given for mortality are the hazard ratios, i.e. the hazard rate relative to the other group. Test statistic values were based on the χ^2-distribution except for sperm quality where the test statistic is based on the F-distribution. $0.10>P>0.05$ are underlined and $P < 0.05$ are in bold. The effect of parental relatedness on sperm linearity (LIN) was not significant after Bonferroni correction.

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(1) Mortality and growth					
(a) Embryonic mortality	Parental relatedness	related: 0.720±0.045; unrelated: 0.703±0.039	1	0.17	0.679
	Parental age	young: 0.736±0.043; old: 0.689±0.043	1	0.02	0.891
	Sex	sons: 0.672±0.044; daughters: 0.746±0.040	1	32.05	**<0.001**
	Sperm age		1	0.11	0.742
	Parental relatedness*Parental age		1	0.87	0.352
	Parental relatedness*Sex		1	1.10	0.293
	Parental relatedness*Sperm age		1	0.78	0.379
	Parental age*Sex		1	1.14	0.285
	Parental age*Sperm age		1	3.60	0.058
	Parental age*Sex*Parental relatedness		1	5.42	**0.020**
	Sperm age*Sex*Parental relatedness		1	0.78	0.378
	Parental age*Sperm age*Parental relatedness		1	1.17	0.279
(b) Posthatch survival	Parental relatedness	unrelated: 72.2%	1	0.32	0.570
	Parental age	young: 236.5%	1	0.89	0.346
	Sex	sons: 59.5%	1	3.62	0.057
	Sperm age		1	0.63	0.427
	Parental relatedness*Parental age		1	3.18	0.074
	Parental relatedness*Sex		1	6.69	**0.010**
	Parental relatedness*Sperm age		1	0.49	0.482
	Parental age*Sex		1	4.18	**0.041**
	Parental age*Sperm age		1	0.27	0.606
	Parental age*Sex*Parental relatedness		1	0.08	0.772
	Sperm age*Sex*Parental relatedness		1	0.42	0.518
	Parental age*Sperm age*Parental relatedness		1	2.40	0.121

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(c) Growth relative to hatch mass, g	Parental relatedness	related: 6.60±0.19; unrelated: 6.82±0.16	1	3.37	0.067
	Parental age	young: 6.71±0.17; old: 6.75±0.17	1	0.05	0.829
	Sex	sons: 7.43±0.18; daughters: 6.13±0.14	1	125.91	**<0.001**
	Sperm age		1	3.48	0.062
	Parental relatedness*Parental age		1	2.19	0.139
	Parental relatedness*Sex		1	11.19	**<0.001**
	Parental relatedness*Sperm age		1	0.02	0.888
	Parental age*Sex		1	0.35	0.552
	Parental age*Sperm age		1	0.06	0.800
	Parental age*Sex*Parental relatedness		1	2.95	0.086
	Sperm age*Sex*Parental relatedness		1	0.08	0.776
	Parental age*Sperm age*Parental relatedness		1	4.67	**0.031**
(2) Morphological traits					
(a) Mean comb size, cm^2	Parental relatedness	related: 5.53±0.76; unrelated: 5.53±0.67	1	0.75	0.387
	Parental age	young: 5.06±0.97; old: 5.14±0.53	1	1.63	0.202
	Sex	sons: 7.80±0.74; daughters: 2.63±0.15	1	32.70	**<0.001**
	Sperm age		1	0.20	0.654
	Parental relatedness*Parental age		1	3.15	0.076
	Parental relatedness*Sex		1	5.38	**0.020**
	Parental relatedness*Sperm age		1	4.02	**0.045**
	Parental age*Sex		1	0.194	0.659
	Parental age*Sperm age		1	6.06	**0.014**
	Parental age*Sex*Parental relatedness		1	13.65	**<0.001**
	Sperm age*Sex*Parental relatedness		1	0.26	0.612
	Parental age*Sperm age*Parental relatedness		1	0.06	0.767

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(b) Mean relative comb redness	Parental relatedness	related: 0.231±0.057; unrelated: 0.311±0.069	1	7.92	**0.005**
	Parental age	young: 0.185±0.074; old: 0.329±0.052	1	3.30	0.069
	Sex	sons: 0.494±0.036; daughters: -0.025±0.057	1	42.62	**<0.001**
	Sperm age		1	0.00	0.998
	Parental relatedness*Parental age		1	4.88	**0.027**
	Parental relatedness*Sex		1	0.47	0.495
	Parental relatedness*Sperm age		1	1.05	0.306
	Parental age*Sex		1	6.76	**0.009**
	Parental age*Sperm age		1	0.10	0.749
	Parental age*Sex*Parental relatedness		1	0.24	0.624
	Sperm age*Sex*Parental relatedness		1	0.01	0.932
	Parental age*Sperm age*Parental relatedness		1	0.00	0.989
(c) Mean tarsus length, mm	Parental relatedness	related: 65.38±1.04; unrelated: 63.68±0.82	1	0.05	0.824
	Parental age	young: 65.38±0.84; old: 63.85±0.87	1	4.07	**0.044**
	Sex	sons: 69.98±0.91; daughters: 58.51±0.41	1	62.9	**<0.001**
	Sperm age		1	1.76	0.184
	Parental relatedness*Parental age		1	0.77	0.379
	Parental relatedness*Sex		1	8.83	**0.003**
	Parental relatedness*Sperm age		1	1.70	0.192
	Parental age*Sex		1	3.32	*0.069*
	Parental age*Sperm age		1	0.68	0.410
	Parental age*Sex*Parental relatedness		1	0.57	0.450
	Sperm age*Sex*Parental relatedness		1	1.21	0.272
	Parental age*Sperm age*Parental relatedness		1	6.03	**0.014**

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(3) Gametic quality					
(a) Sperm quality					
(i) VCL, μm/s	Parental relatedness	related: 42.99±2.80; unrelated: 53.68±2.74	1	1.00	0.384
	Parental age	young: 39.52±1.96; old: 54.41±2.69	1	2.15	0.156
	Sperm age		1	0.33	0.572
	Parental relatedness*Parental age		1	0.02	0.895
	Parental relatedness*Sperm age		1	0.78	0.387
	Parental age*Sperm age		1	1.43	0.244
	Parental age*Sperm age*Parental relatedness		1	2.32	0.144
(ii) VSL, μm/s	Parental relatedness	related: 24.89±2.08; unrelated: 33.48±2.01	1	3.16	0.089
	Parental age	young: 22.65±1.36; old: 33.74±2.02	1	1.09	0.307
	Sperm age		1	0.38	0.546
	Parental relatedness*Parental age		1	0.20	0.657
	Parental relatedness*Sperm age		1	0.76	0.392
	Parental age*Sperm age		1	2.05	0.166
	Parental age*Sperm age*Parental relatedness		1	0.89	0.357
(iii) VAP, μm/s	Parental relatedness	related: 33.14±2.44; unrelated: 42.50±2.32	1	1.34	0.282
	Parental age	young: 30.55±1.70; old: 42.87±2.33	1	1.43	0.245
	Sperm age		1	0.43	0.521
	Parental relatedness*Parental age		1	0.09	0.770
	Parental relatedness*Sperm age		1	0.80	0.380
	Parental age*Sperm age		1	1.55	0.225
	Parental age*Sperm age*Parental relatedness		1	1.55	0.228
(iv) LIN, %	Parental relatedness	related: 56.96±1.67; unrelated: 61.99±1.52	1	4.62	**0.042**
	Parental age	young: 57.11±1.79; old: 61.28±2.69	1	0.02	0.897
	Sperm age		1	0.05	0.824
	Parental relatedness*Parental age		1	0.34	0.547
	Parental relatedness*Sperm age		1	0.26	0.613

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
	Parental age*Sperm age		1	1.35	0.257
	Parental age*Sperm age*Parental relatedness		1	0.84	0.371
(iv) *In vivo* hydrolysis points	Parental relatedness	related: 26.29±5.14; unrelated: 21.41±6.37	1	0.02	0.879
	Sperm age		1	0.73	0.392
	Parental relatedness*Sperm age		1	1.81	0.179
	Parental relatedness*Day		1	17.06	**<0.001**
(b) Egg mass and number					
(i) Mean egg mass, g	Parental relatedness	related: 37.56±0.62; unrelated: 37.14±0.46	1	0.10	0.757
	Parental age	young: 35.62±0.51; old: 39.00±0.51	1	8.35	**0.004**
	Sperm age		1	4.09	**0.043**
	Parental relatedness*Parental age		1	0.93	0.335
	Parental relatedness*Sperm age		1	0.00	0.970
	Parental age*Sperm age		1	0.31	0.578
	Parental age*Sperm age*Parental relatedness		1	3.66	0.056
(i1) Mean number of eggs	Parental relatedness	related: 4.53±0.53; unrelated: 6.57±0.74	1	5.92	**0.015**
	Parental age	young: 4.42±0.54; old: 6.71±0.69	1	10.02	**0.002**
	Sperm age		1	1.52	0.218
	Parental relatedness*Parental age		1	0.10	0.755
	Parental relatedness*Sperm age		1	1.68	0.195
	Parental age*Sperm age		1	1.65	0.199
	Parental age*Sperm age*Parental relatedness		1	0.39	0.532
(iv) *In vivo* hydrolysis points	Parental relatedness	related: 16.87±4.41; unrelated: 28.25±5.74	1	3.41	0.065
	Sperm age		1	11.65	**<0.001**
	Parental relatedness*Sperm age		1	0.00	0.978
	Parental relatedness*Day		1	92.63	**<0.001**

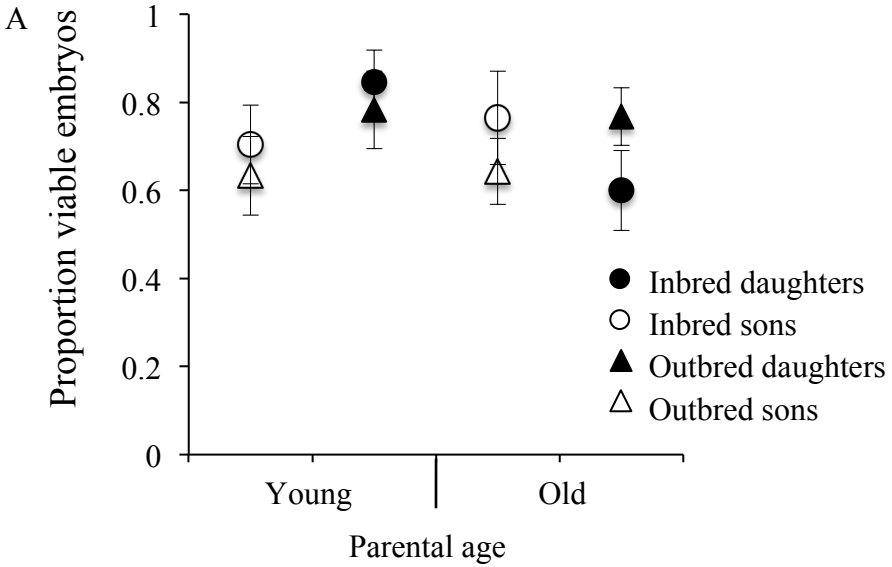
Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(4) Social status					
(a) Social status	Parental relatedness	related: 0.422±0.049; unrelated: 0.581±0.050	1	3.73	0.054
	Sperm age		1	0.21	0.646
	Parental relatedness*Parental age		1	0.00	0.999
	Parental relatedness*Sex		1	3.98	**0.046**
	Parental relatedness*Sperm age		1	0.16	0.688
	Parental age*Sperm age		1	1.10	0.294
	Parental age*Sex*Parental relatedness		1	4.11	**0.043**
	Sperm age*Sex*Parental relatedness		1	3.17	0.078
	Parental age*Sperm age*Parental relatedness		1	0.23	0.629

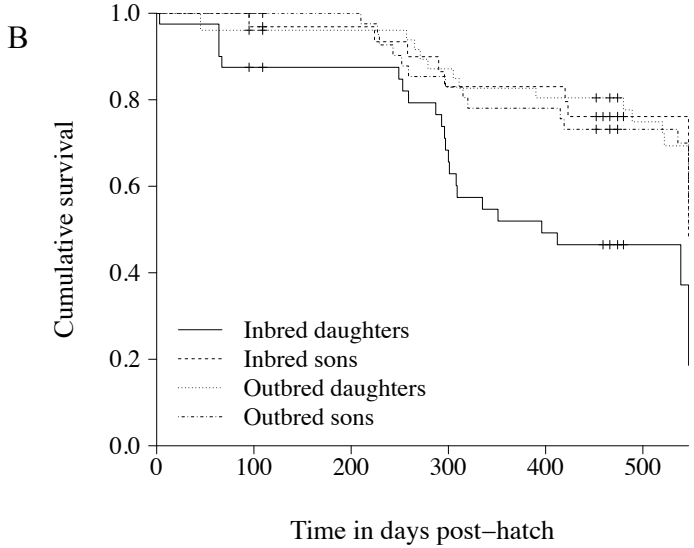
Table 3. Standardised inbreeding depression coefficient δ for the different traits measured. Young parents: 1-3 years-old; old parents: 4-6 years-old. Sperm age was also categorised into two groups: young sperm (eggs that were laid days 1-5 post-insemination) and old sperm (eggs that were laid days 6-11 post-insemination). We calculated standardised inbreeding depression coefficient for survival and growth with the hazard ratio and regression slope respectively.

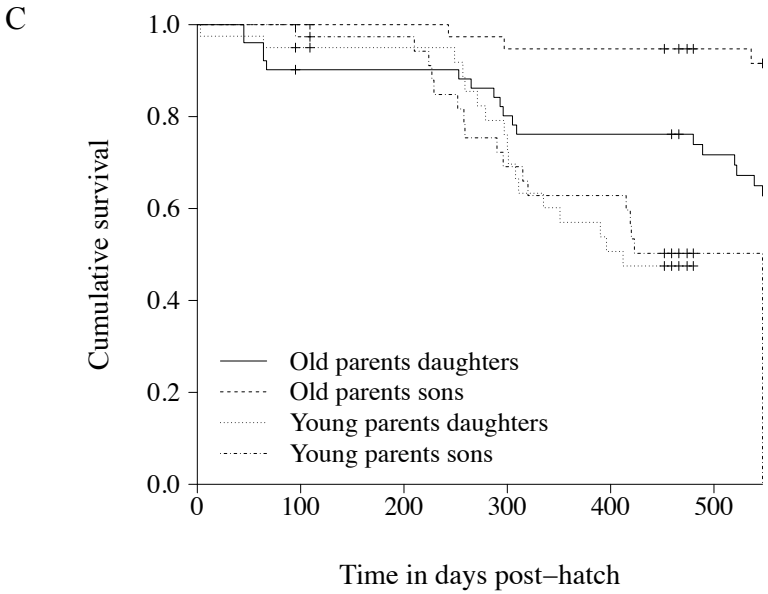
Trait	**Offspring sex**	**Young parents, young sperm**	**Young parents, old sperm**	**Old parents, young sperm**	**Old parents, old sperm**
Embryonic viability	Sons	-0.53	-0.40	-1.00	-0.65
	Daughters	-0.74	-0.12	-0.05	1.57
Posthatch survival	Sons	0.06	-1.18	0.47	-0.29
	Daughters	0.22	0.70	0.74	0.42
Growth	Sons	0.20	0.00	0.70	0.59
	Daughters	-0.37	-0.02	-0.39	0.05
Comb size	Sons	0.37	-5.71	-0.55	1.67
	Daughters	1.20	0.93	0.67	0.50
Comb redness	Sons	0.53	1.13	-0.01	1.52
	Daughters	0.02	-0.67	-3.74	2.34
Tarsus length	Sons	-0.28	0.07	0.15	0.20
	Daughters	-0.27	0.15	-0.32	0.04
Sperm quality VCL	Sons	-0.21	0.62	1.79	0.37
Sperm quality VSL	Sons	-0.15	0.50	2.11	0.63
Sperm quality VAP	Sons	-0.18	0.56	1.86	0.50
Sperm quality LIN	Sons	0.16	-0.17	0.46	0.34
Egg mass	Daughters	-0.08	0.21	-0.05	0.02
Number of eggs	Daughters	1.10	1.60	0.80	1.67
In vivo hydrolysis points	Sons	Young sperm: -0.21		Old sperm: 0.31	
	Daughters	Young sperm: 1.55		Old sperm: 2.19	
Social status	Sons	-0.45	1.96	2.44	2.43
	Daughters	0.00	3.20	0.34	-5.33

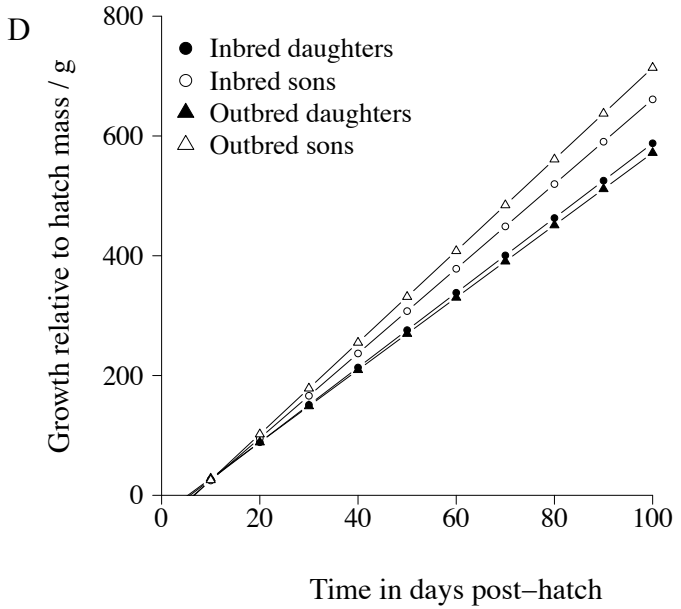
Chapter 3 Parental and gametic age alter inbreeding depression in the red junglefowl

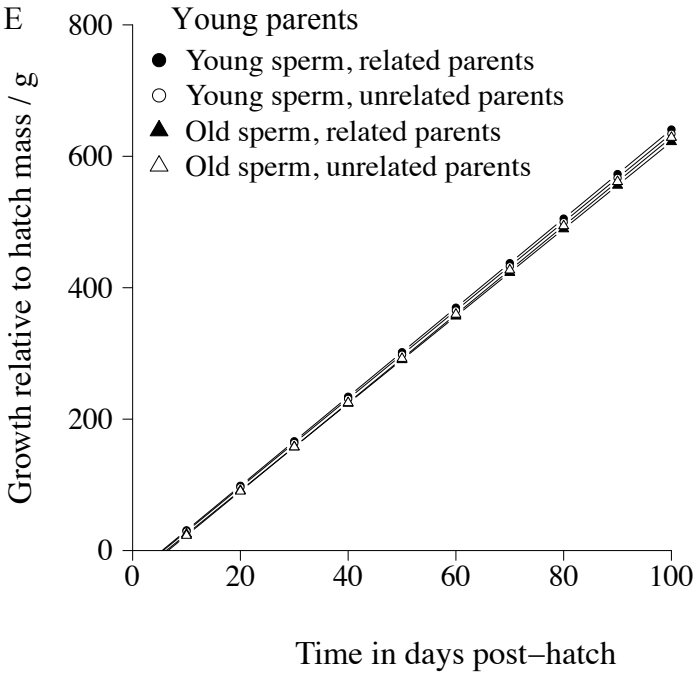
Figure 1. Mortality and growth of inbred sons, inbred daughters, outbred sons and outbred daughters from young or old parents. A. Embryonic viability of sons and daughters sired by young or old, related or unrelated parents. This figure show a significant interaction between parental age, parental relatedness and sex. B. Post-hatch mortality of offspring showing parental relatedness by offspring sex interaction; C. Post-hatch mortality of offspring showing parental age by offspring sex interaction. In Figures B and C, the crosses represent right-censored data where subjects are still alive when the study ended. D. Growth relative to hatch mass, showing significant interaction between parental relatedness and offspring sex; E and F. Growth rate, showing significant interaction between parental age, sperm age and parental relatedness. Sperm age was classified as young (1-5 days) or old (6-11 days). Figure E is for young parents and figure F is for old parents. The lines drawn for growth are the least squares regression line. Error bars denote S.E. Results are represented in Table 2.

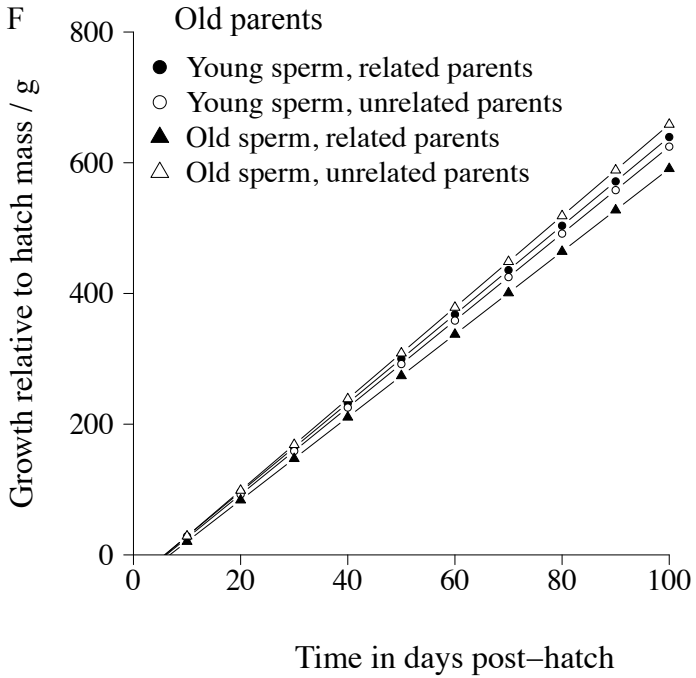

A
Proportion viable embryos
1
0.8
0.6
0.4
0.2
0
Young
Old
Parental age
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons


B
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
Time in days post–hatch
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons


C
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
Time in days post–hatch
Old parents daughters
Old parents sons
Young parents daughters
Young parents sons


D
Growth relative to hatch mass / g
800
600
400
200
0
0
20
40
60
80
100
Time in days post–hatch
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons


E
Young parents
Young sperm, related parents
Young sperm, unrelated parents
Old sperm, related parents
Old sperm, unrelated parents
Growth relative to hatch mass / g
800
600
400
200
0
0
20
40
60
80
100
Time in days post–hatch


F
Old parents
Young sperm, related parents
Young sperm, unrelated parents
Old sperm, related parents
Old sperm, unrelated parents
Growth relative to hatch mass / g
800
600
400
200
0
0
20
40
60
80
100
Time in days post–hatch

(c) Comb size and redness

We detected sex-specific differences in inbreeding depression in comb size: controlling for parental age, inbred daughters had small comb sizes than outbred daughters but the reverse was true for sons (Figure 2A; Table 2). This sex-specific inbreeding depression was modulated by parental age, as indicated by a significant three-way interaction between parental relatedness, parental age and offspring sex. Inbreeding depression was detected for sons of old parents and daughters of both young and old parents but not for sons of young parents where inbred sons had marginally larger comb sizes than outbred sons (Figure 2A; Tables 2 and 3). In addition, a significant interaction between parental age and sperm age demonstrates that offspring comb size increased with increasing sperm age for young parents but remained fairly constant with increasing sperm age for old parents (Figure 2B; Table 2).

There was a significant interaction between parental age and offspring sex on variation in comb redness (Table 2). Daughters from young parents had on average redder comb than daughters from old parents (Figure 2C). We also found a significant interaction between parental relatedness and parental age: the magnitude of inbreeding depression was higher for offspring sired by old parents than by young parents (Figure 2C; Table 2).

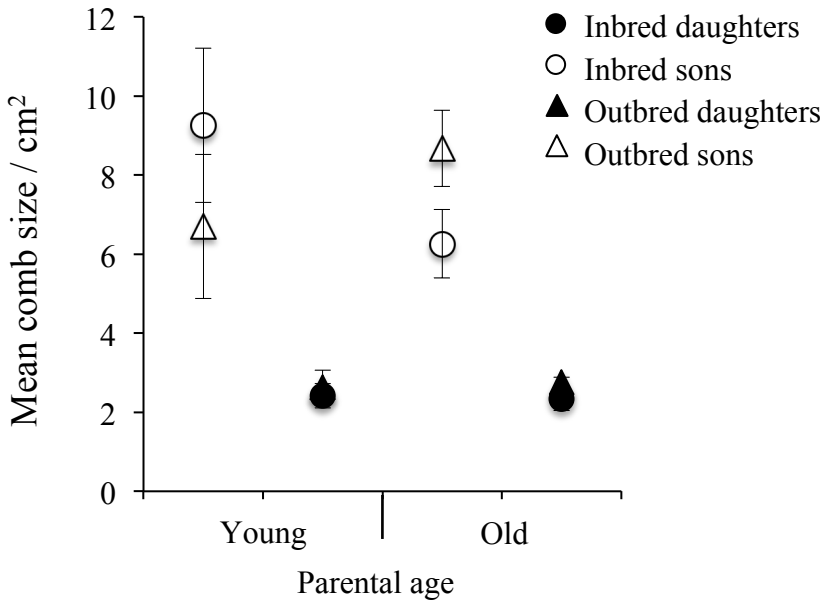
(d) Adult tarsus length

Again, inbreeding depression in tarsus length was sex-specific. Controlling for parental age, inbreeding depression was only detected in sons but not daughters (Figure 2D; Table 2). We also identified a significant three-way interaction between parental age, sperm age and parental relatedness, whereby tarsus length of inbred offspring decreases

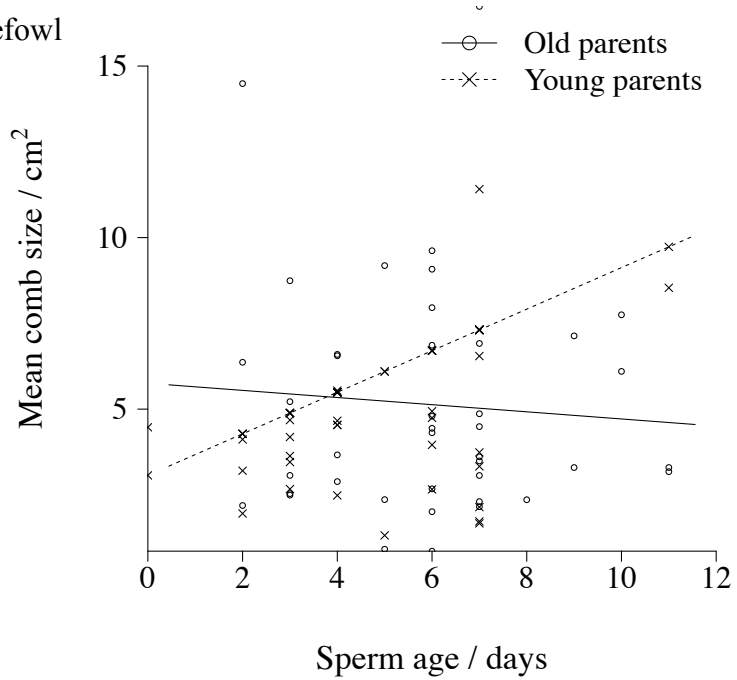
with increasing paternal sperm age, particularly so in young parents (Figures 2E and 2F; Tables 2 and 3).

Figure 2. Morphological traits of sons and daughters sired by young or old, related or unrelated parents. ‘Sperm age’ labelled on the x-axis refers to paternal sperm age not the sperm age of the sons. A. Comb size showing parental age by offspring sex by parental relatedness interaction; B. Comb size showing parental age by sperm age interaction. C. Comb redness showing parental age by offspring sex interaction as well as parental relatedness by parental age interaction; D. Tarsus length showing parental relatedness by sex interaction; E and F. Tarsus length, showing interaction between parental age, sperm age and parental relatedness. Figure E is for young parents and figure F is for old parents. Error bars denote S.E.

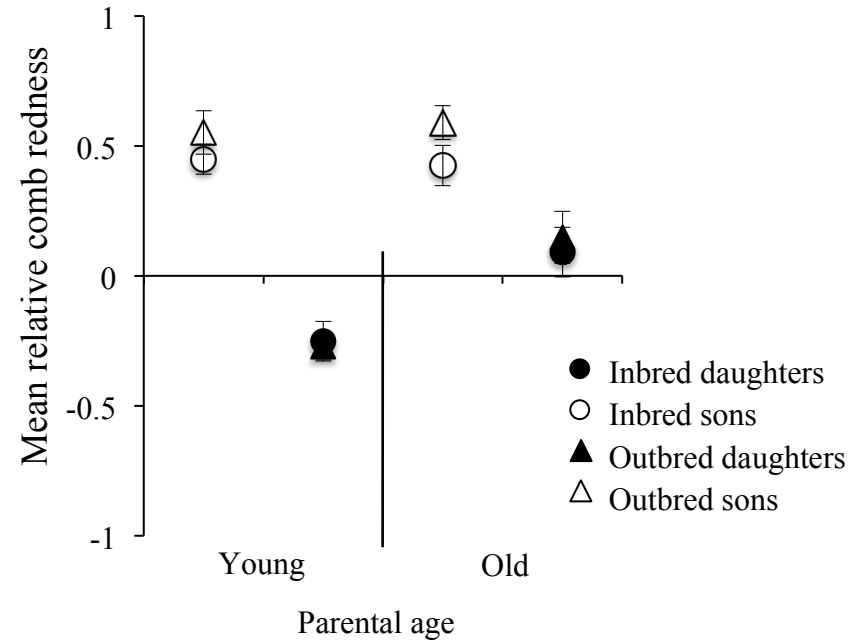
A


Mean comb size / cm2
12
10
8
6
4
2
0
Young
Old
Parental age
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons

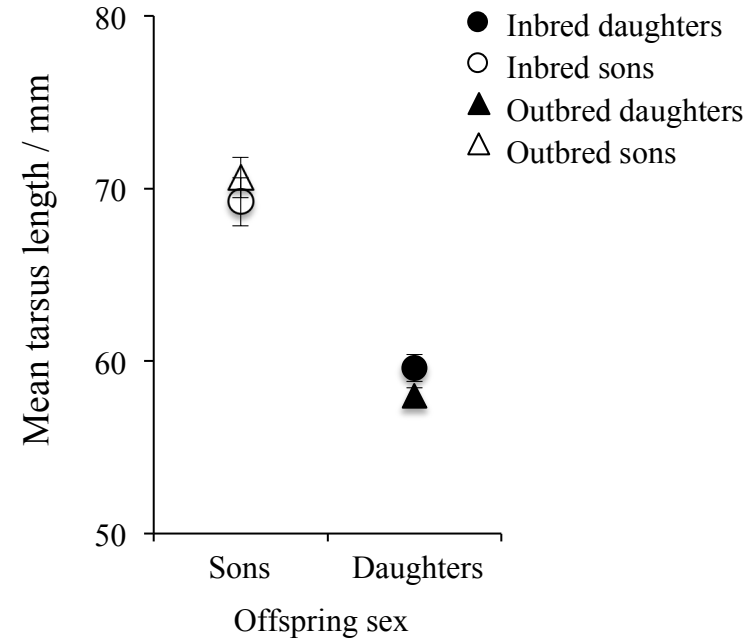
B

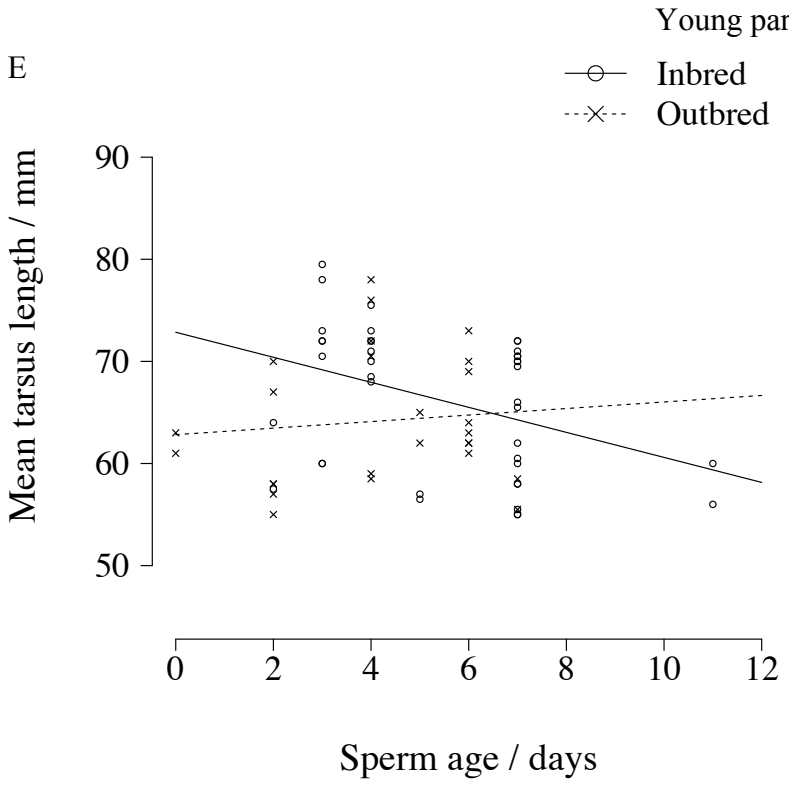

Mean comb size / cm2
15
10
5
0
2
4
6
8
10
12
Sperm age / days
Old parents
Young parents

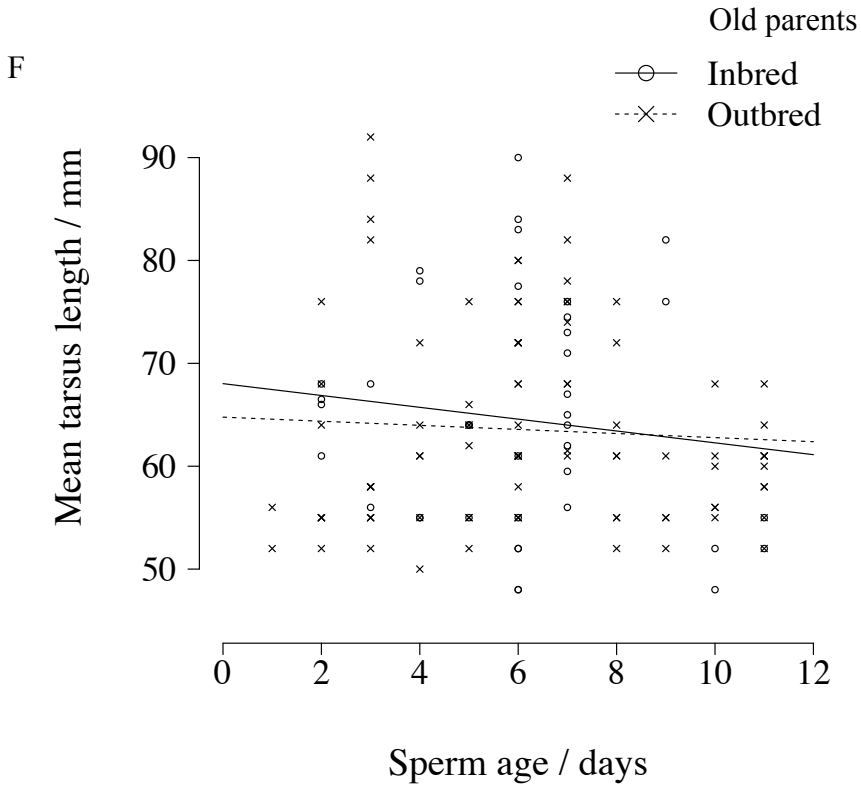
C


Mean relative comb redness
1
0.5
0
-0.5
-1
Young
Old
Parental age
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons

D


Mean tarsus length / mm
80
70
60
50
Sons
Daughters
Offspring sex
Inbred daughters
Inbred sons
Outbred daughters
Outbred sons


E
Young parents
Inbred
Outbred
Mean tarsus length / mm
90
80
70
60
50
0
2
4
6
8
10
12
Sperm age / days


F
Old parents
Inbred
Outbred
Mean tarsus length / mm
90
80
70
60
50
0
2
4
6
8
10
12
Sperm age / days

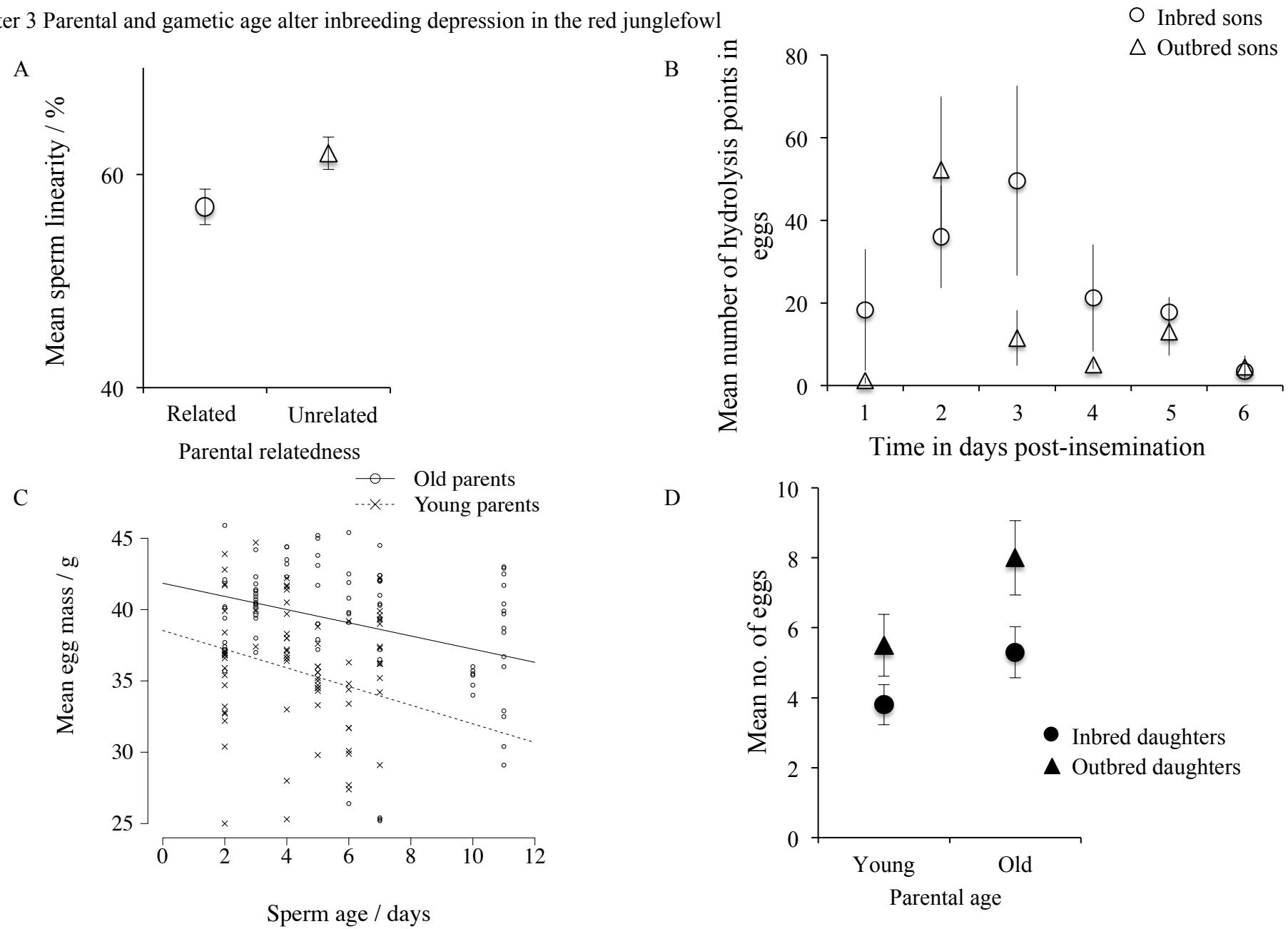
(e) Sperm

We did not find inbreeding depression in VAP nor in other measures of sperm motility (Table 2). However, inbred sons produced sperm with significantly lower linearity (LIN = VSL/VCL) than outbred sons but this effect was not significant after Bonferroni correction (Figure 3A; Table 2). The initial rate of increase in hydrolysis points on the perivitelline layer of the eggs following artificial insemination was faster for outbred males than for inbred males whereas the rate of decline after day 2 was faster for outbred males than for inbred males (Figure 3B; Tables 2 and 3).

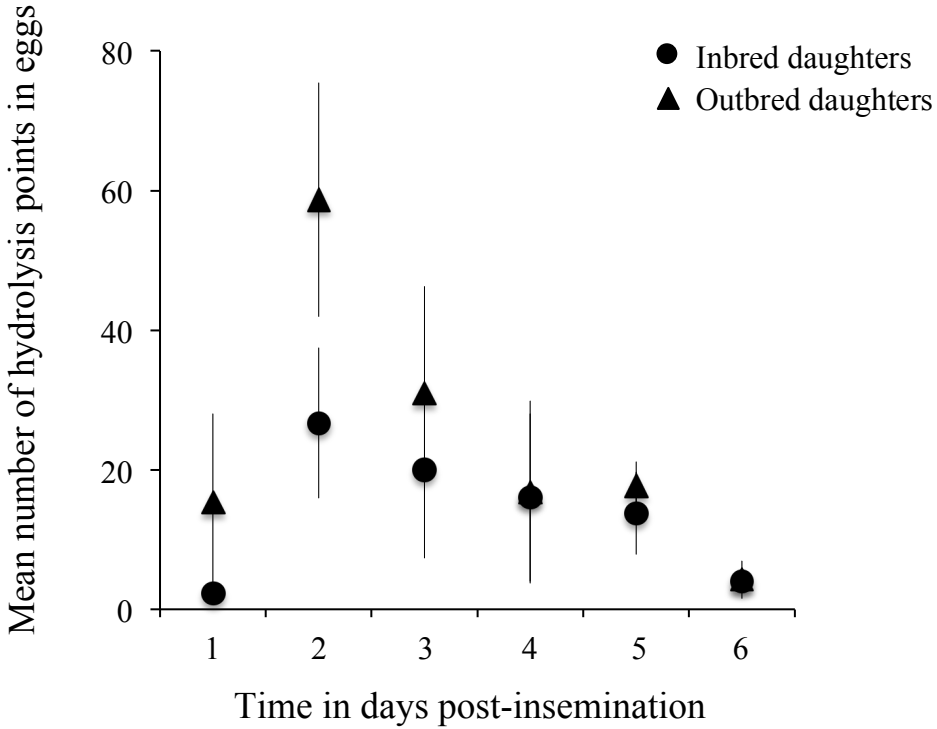
(f) Eggs

Daughters of old parents laid significantly heavier eggs than daughters of young parents (Figure 3C; Table 2). Also, the egg mass of daughters decreased with increasing paternal sperm age (Figure 3C; Table 2). However, there was no effect of parental relatedness on the egg mass of daughters. Inbred daughters laid significantly fewer eggs than outbred daughters (Figure 3D; Table 2). We found that in daughters, there was no effect of inbreeding on the number of sperm that reached the perivitelline layer of the eggs (i.e. eggs of inbred *versus* outbred daughters). Nevertheless, the rate of decline in hydrolysis points was faster for inbred females than for outbred females (Figure 3E; Table 2).

Figure 3. Sexual traits of sons and daughters sired by young or old, related or unrelated parents. ‘Sperm age’ labelled on the x-axis refers to paternal sperm age not the sperm age of the sons. A. Inbred sons exhibited significantly lower sperm linearity than outbred sons. B. More sperm reached the perivitelline layer of the eggs at day 2 post-insemination by an outbred male than by an inbred male. The rate of decline in hydrolysis points was faster for outbred males than for inbred males. C. Daughters of old parents laid significantly heavier eggs than daughters of young parents. There was also a negative correlation between mean egg mass and sperm age. D. Inbred daughters laid significantly fewer eggs than outbred daughters. Daughters of young parents also laid significantly fewer eggs than daughters of old parents. E. The rate of decline in hydrolysis points was faster for inbred males than for outbred males. Error bars denote S.E. Results are displayed in Table 2.


A
Mean sperm linearity / %
60
40
Related
Unrelated
Parental relatedness
B
Inbred sons
Outbred sons
Mean number of hydrolysis points in eggs
80
60
40
20
0
1
2
3
4
5
6
Time in days post-insemination
C
Old parents
Young parents
Mean egg mass / g
45
40
35
30
25
0
2
4
6
8
10
12
Sperm age / days
D
Mean no. of eggs
10
8
6
4
2
0
Inbred daughters
Outbred daughters
Young
Old
Parental age

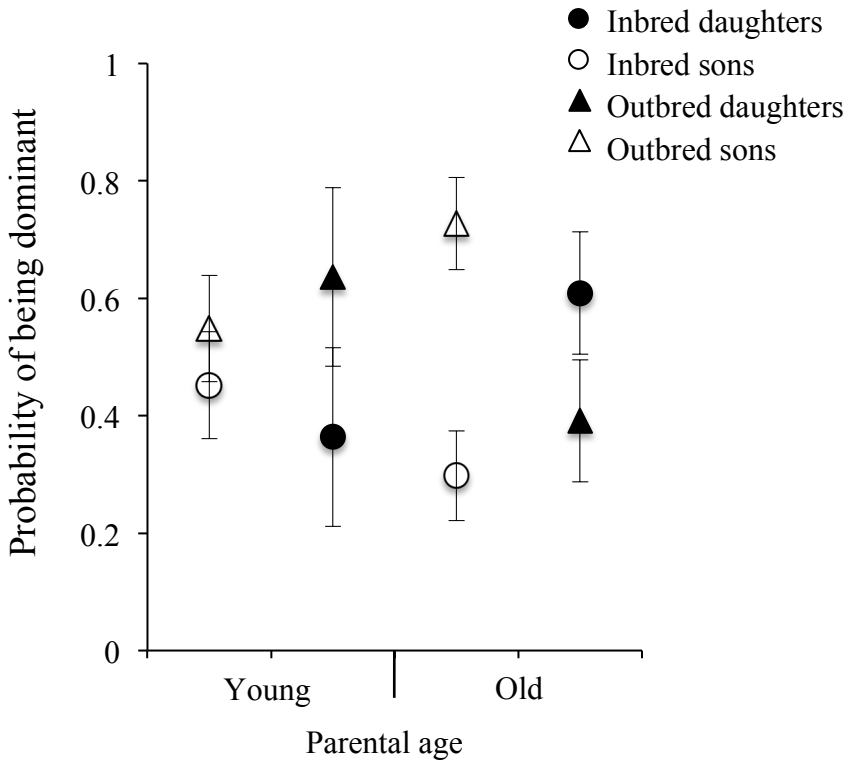
E


Mean number of hydrolysis points in eggs
80
60
40
20
0
1
2
3
4
5
6
Time in days post-insemination
Inbred daughters
Outbred daughters

(g) ***Social status***

We again found that inbreeding depression in social status was sex-specific: inbred sons were less likely to dominate outbred sons than inbred daughters to dominate outbred daughters (Table 2). In addition, there was a significant three-way interaction between parental relatedness, parental age and offspring sex (Table 2), whereby inbred sons suffered more when parents were old whereas the effect in daughters was marginal (Figure 4; Table 3).

Figure 4. Social status of sons and daughters sired by young or old, related or unrelated parents. Pairs of same-sexed individuals were placed together and observed for dominance status. Error bars denote S.E.



DISCUSSION

The aims of this study were to test whether inbreeding depression differs with offspring sex as well as to examine the extent to which inbreeding depression is modulated by organismal and gametic mechanisms of parental ageing. Controlling for other factors, we found that the magnitude of inbreeding depression in growth rate, post-hatch survival and tarsus length differs with offspring sex. In addition, variation in embryonic mortality, comb size and social status was strongly affected by a three-way interaction between parental relatedness, parental age and offspring sex. We detected a significant interaction between parental age, sperm age and parental relatedness in patterns of growth rate and tarsus length. Inbreeding depression in comb redness was also affected by parental age.

(a) Sex-specific inbreeding depression and the effects of parental age

The 'unguarded X' hypothesis predicts a reversal of sex-specific fitness costs across insects (homogametic females) and birds (homogametic males) whereas 'sex-specific resource allocation' hypothesis predicts no reversal in sex-specific inbreeding depression and that daughters in both systems should always suffer a higher magnitude of inbreeding depression. Our experiment in the red junglefowl indicates that inbreeding depression in embryonic mortality and post-hatch death rates is higher for daughters than for sons. This is consistent with predictions from the 'sex-specific resource allocation' hypothesis. A previous study in the seed beetle *Callosobruchus maculatus* (Fox et al. 2006; Bilde et al. 2009) also demonstrated that daughters suffer a higher magnitude of inbreeding depression than sons. There are three plausible explanations based on sex-specific selection on resources allocated into current and future reproduction to explain why inbreeding affects females more than males, which

we have briefly described in the introduction. The three possible scenarios predicts that inbreeding reverses the male optimal life-history strategy of 'live fast, die young' and female optimal life-history strategy of prolonging reproductive lifespan (Bilde et al. 2009). Male-beneficial alleles that prime males for sexual competition and early reproduction (and hence early death) might not be expressed in homozygous recessive inbred males. This results in prolonged lifespan in inbred males, consistent with our observations that inbred males exhibited a marginally higher embryonic viability and similar lifespan compared to outbred males. Similarly, alleles that cause outbred females to conserve energy during early ages for future reproductive events would not be expressed in homozygous recessive individuals, resulting in reduced lifespan consistent with the observed data. Sex-specific patterns in embryonic viability were more prevalent when offspring were bred from old parents (Figure 1D), as indicated by the three-way interaction between parental relatedness, parental age and offspring sex. This suggests that sex-specific inbreeding depression in embryonic viability could be further exacerbated by parental ageing.

Patterns in mortality might explain why inbreeding has negative consequences for growth rates of sons but not of daughters. High mortality rates of poor-quality inbred daughters prior to hatch and at early days post-hatching (Figure 1D) might explain the similar growth rates between inbred and outbred daughters. The opposite trend was observed in sons whereby a higher proportion of outbred sons died during the embryonic stage (Figure 1A), probably leaving better-condition outbred sons that grew faster than inbred sons (Figure 1D). In addition, patterns in tarsus length are similar to patterns in growth rate in which we detected inbreeding depression in sons but not in daughters. This is not surprising as tarsus length is directly correlated with skeletal size

and hence body size (Rising & Somers 1989; Senar & Pascual 1997). Alternatively, the general mechanism proposed by the 'sex-specific resource allocation' hypothesis might also explain these results. Males and females have optimal phenotypes, which could be reversed in maladaptive directions by inbreeding. We see here that the growth rate and tarsus length of inbred males are lower relative to outbred males whilst inbred females posses higher growth rate and tarsus length than outbred females. This is assuming that the optimal phenotype of red junglefowl males (outbred) is fast growth rate and a larger body size (and hence tarsus length). Indeed, body size in males is positively correlated to dominance status (Ligon et al. 1990) and mating success (Johnsen et al. 2001). In contrast, a smaller body size might be more adaptive for females if this promotes camouflage and escape from predators.

In terms of comb size, sons suffered higher inbreeding depression than daughters, but only when individuals were bred from the old parents. The 'unguarded X' hypothesis posits that the heterogametic sex should suffer less from inbreeding compared to the homogametic sex (i.e. males here), since the heterogametic sex will not suffer from an increased probability of expression of X-linked (Z-linked here) recessive deleterious mutations. We did find that sons suffered more from inbreeding than daughters but only if they were from old parents. Given that old individuals might acquire an increase in germ-line mutational load (Crow 2000), we would expect an increased in the probability of expression of recessive alleles in the heterogametic sex, if parents were not related. In contrast, when parents were related, there is an increased possibility of germ-line mutations occurring at the same loci, resulting in an increased recessive allele accumulation in homozygous individuals, thus causing sons to suffer a higher inbreeding depression. Parental senescence can thus increase the potential for the

'unguarded X' mechanism to act, revealing sex-specific effects of inbreeding on male comb size. However, this idea is speculative and remains to be tested.

Sex-differential inbreeding depression in social status depended on parental age: when parents were old, inbred sons attained a lower social status than outbred sons whereas inbred daughters attained a marginally higher social status than outbred daughters. Again, this provides evidence that parental age can exacerbate sex-specificity inbreeding depression. Patterns in comb size and dominance status are similar, which is not surprisingly given that comb size can be positively related to dominance rank (Parker et al. 2002).

The impact of parental age on sex-specific inbreeding depression thus appears to vary with traits. When examining only old parents, inbreeding depression in embryonic viability was higher for daughters than for sons, whereas inbreeding depression in comb size and social status were higher for sons than for daughters. This observation has important implications for our understanding of sex-specific patterns of inbreeding depression and how it is modulated by parental ageing. First, parental senescence could increase the potential for mechanisms underlying sexual dimorphism in inbreeding depression ('unguarded X' and 'sex-specific resource allocation') to act. This could, in turn aggravate or reveal sex-differential inbreeding depression. Second, different mechanisms could be acting at different life-history stages: 'sex-specific resource allocation' in embryonic development and survival *versus* 'unguarded X hypothesis' on mature individuals. Our findings illustrate that sex-specific inbreeding depression is modulated by parental age and trait-dependent, and thus could explain the incongruence in literature on which sex suffers more from inbreeding.

We also found an effect of parental relatedness on gametic quality in both males and females. More sperm from outbred sons reached the perivitelline layer of eggs on the 2nd day post-insemination. However, we found no effects or marginally non-significant effects of inbreeding on sperm motility and path character parameters (VCP, VAP, VSL, LIN). Previous studies have demonstrated a decrease in the percentage of morphologically normal and motile spermatozoa with inbreeding (Roldan et al. 1998; Gomendio et al. 2000; Gage et al. 2006). However, we do not know to what extent these percentages translate into average motility and path character (as in our study). Thus, our study reveals non-significant effects of inbreeding, namely sperm of inbred males were not faster in velocity nor exhibit less linearity in their travel paths. We detected higher numbers of sperm hydrolysis points by outbred males than by inbred males on day 2 post-insemination, followed by a rapid reduction in sperm hole numbers (Figure 3B). This indicates that, perhaps, fertilisation potential by outbred males compared to that of inbred males could be higher in the early days post-insemination. This could be an important determinant in fertility during episodes of sperm competition. Future studies should aim at determining the temporal variation in reproductive success post-insemination.

The effects of inbreeding on egg quality of daughters were also generally negative. Consistent with previous studies on the Japanese quail (Sittmann et al. 1966) and the domestic chicken (Abplanalp et al. 1992), female fecundity was reduced by inbreeding. However, contrary to other studies that have shown reductions in egg mass by inbreeding in the domestic chicken (Roberts et al. 1952, Shultz 1953), our study demonstrated that parental relatedness had no effect on egg mass, Because the domestic chicken is larger in size than the red junglefowl, and thus lays larger eggs, the

difference in egg mass from inbred *versus* outbred female might be more readily detected in the domestic chicken.

We found a faster decline in sperm hydrolysis hole numbers in inbred daughters as compared to outbred daughters, which might translate to lower fertilisation success found in another study (Cheng et al. 1984). Daughters from young parents also laid fewer and lighter eggs than daughters of old parents. Because eggs laid by young hens are typically smaller than eggs laid by mature hens, it is likely that hatchlings from young hens would be smaller than those from old hens (Wilson 1991). Therefore, the fewer and lighter eggs laid by daughters of young parents may be a result of their smaller sizes.

(b) Effects of parental age and sperm age on inbreeding depression

Our results reveal a significant interaction between parental age, sperm age and parental relatedness on variation in growth rate and tarsus length. The slowest growth rate was found for inbred offspring produced with old sperm and from old parents. This suggests that older males produce sperm that are more vulnerable to ageing and thus suffer higher rates of senescence than the sperm produced by younger males (Zubkova & Robaire 2006). This could in turn exacerbate the degree of inbreeding depression. However, we also found that the synergistic effect of inbreeding and sperm age in tarsus length was more pronounced in the offspring of young –rather than old- parents: tarsus length of inbred offspring decreases with increasing sperm age, particularly so in young parents. Perhaps this could be an artefact of how the data points were distributed. As seen in figure 2E, most of the data points were distributed within sperm ages of 1-7 days and there were two data points from the inbred line at day 11 which

seemed to have a strong effect on the slope of the line. This could be partly due to low fertility of eggs collected on days 8-11 post-insemination and therefore fewer offspring to represent data points at these sperm ages.

Our measurements of comb redness indicate that the effects of inbreeding vary with parental age. Parental senescence might increase germ-line mutational load (Crow 2000) and related parental might have increased probability of gaining mutations at the same loci. It is therefore possible that the offspring of related parents might be more sensitive to the expression of recessive alleles imposed by senescence. Therefore, we observed inbreeding depression in comb redness of offspring of old parents but not that of offspring of young parents.

We found that sperm ageing had negative effects on egg mass of daughters. Given that female comb size is an important predictor of mean egg mass (Pizzari et al. 2003), we might expect daughters of both old and young parents to possess smaller combs with increased sperm ageing. Indeed we found a marginally negative correlation between comb size and sperm age when parents were old. However, when parents were young, we found that comb size was positively correlated with sperm age after controlling for offspring sex. This might be due to opposite sex (sons) displaying an opposite trend, in other words, sons of young parents possess combs with increased sperm ageing. Consistent with this idea, we found a significant interaction between offspring sex, sperm age and parentage age on variation in comb size (GLMM, $\chi^2_1 = 5.11$, $P = 0.024$), thus explaining a positive relationship between sperm age and comb size when parents were young (Figure 2B). The reason for this pattern is currently unknown. Nevertheless, our study provides first evidence that sperm ageing can have detrimental

downstream effects on the reproductive output of daughters.

Early experimental studies have shown that sperm ageing increases embryonic mortality in domestic chickens (Lodge et al. 1971) and negatively affects four sequential stages of reproduction, namely fertilisation potential, rate of embryonic development, embryonic mortality and chick condition at hatching in Kittiwakes, *Rissa tridactyla* (White et al. 2008). However, we did not find effects of sperm ageing on embryonic mortality or survival in our study. Domestic chickens have been artificially selected for thousands of generations and their response to sperm ageing might not be representative of the non-domestic red junglefowl. The study on Kittiwakes was conducted on wild populations and therefore the negative effects of sperm ageing found might be due to the harsh conditions faced by the embryos and offspring. In contrast, the chicks reared in our study were artificially incubated and kept with warmers until 4 months of age. Such conditions are milder than the wild and might have mitigated the effects of sperm ageing.

Conclusions

Our study indicates that inbreeding depression is sex-specific but the sex which suffers more is trait-dependent and modulated by parental age. The two different hypotheses explaining sexual dimorphism in inbreeding depression might be acting at different life-history traits. First, the 'unguarded X' hypothesis could explain why sons (homogametic sex) suffer higher inbreeding depression in comb size and dominance status. Second, the 'sex-specific resource allocation' hypothesis might explain the higher magnitude of inbreeding depression in embryonic viability and post-hatch survival for daughters. In addition, parental organismal senescence might reveal and

amplify sex-specific inbreeding depression in embryonic viability, comb size and social status. We also show that inbreeding depression is affected by sperm age, parental age and the interaction between these two mechanisms of reproductive senescence. However, these patterns were inconsistent across traits and difficult to explain with our current knowledge of senescence mechanisms. Further elucidation of the genetic underpinnings of such variations in inbreeding costs would provide important insights into the relationship between inbreeding, senescence and offspring sex.

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THEME II

INBREEDING AVOIDANCE

Chapter 4

No evidence for precopulatory inbreeding avoidance in *Drosophila melanogaster*

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No evidence for precopulatory inbreeding avoidance in *Drosophila melanogaster*

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Inbreeding depression can lead to the evolution of inbreeding avoidance before or after mating. However, despite widespread evidence of inbreeding depression, studies of inbreeding avoidance have generated different results across populations or species. These differences could potentially reflect the confounding effects of factors such as magnitude of inbreeding depression, sex, social familiarity, state of primary sexual receptivity and mating history. We examined the influence of these proximate factors on precopulatory inbreeding avoidance in a laboratory-adapted, outbred population of *Drosophila melanogaster*. We found a significant but low coefficient of inbreeding depression based on egg–adult viability measures. Controlling for sex-specific responses, familiarity, sexual receptivity and mating history, we found no evidence of precopulatory inbreeding avoidance. Mate choice of virgins was random with respect to relatedness and measurements of courtship frequency, mating latency and mating duration did not indicate any preference for unrelated partners. In fact, the only evidence for differential sexual behaviour in response to relatedness was that males first mated to unrelated females were significantly faster to remate with related females than with unrelated females. These results suggest that inbreeding avoidance may be limited in outbred populations of *D. melanogaster*, and fit theoretical predictions that inbreeding is not selected against in either sex when the coefficient of inbreeding depression is relatively low.

Theory predicts that inbreeding depression, caused by the expression of deleterious recessive alleles or loss of overdominance effects suffered by the offspring of closely related parents, can promote the evolution of mechanisms of inbreeding avoidance (Charlesworth & Charlesworth 1987; Marr et al. 2002). In structured populations in which dispersal is limited or occurs after mating, inbreeding can be avoided before copulation through the recognition and avoidance of kin as mating partners. Evidence of precopulatory inbreeding avoidance, however, varies across and even within species. Negative assortative mating with respect to relatedness (i.e. inbreeding avoidance) has been documented in several studies of both vertebrates (e.g. Dewsbury 1982; Bateson 1983; Penn & Potts 1999) and invertebrates (e.g. Smith & Ayasse 1987; Simmons 1991; Stuart & Herbers 2000). Other studies, however, have failed to demonstrate precopulatory inbreeding avoidance (e.g. Keller & Arcese 1998; Guevara-Fiore et al. 2010), while some have reported preferences for mating with kin, both in invertebrates (e.g. Schjørring & Jäger 2007; Schjørring 2009) and in vertebrates (e.g. Thünken et al. 2007, 2011).

There are several explanations that could potentially account for this incongruence. First, variation in the magnitude of inbreeding depression is predicted to influence the intensity of selection on inbreeding avoidance across species or populations (Frommen & Bakker 2006; Kokko & Ots 2006). Second, theory predicts that inbreeding avoidance is sex specific (Parker 1979; Kokko & Ots 2006; Puurtinen 2011). Because females typically invest more in a reproductive event than males, for intermediate levels of inbreeding depression, females might be selected to avoid inbreeding while males are selected to inbreed (Parker 1979; Pizzari et al. 2004; Facon et al. 2006; Kokko & Ots 2006). Therefore, it is important to tease apart male- from female-specific sexual responses to kin. Third, lack of consideration for the proximate mechanisms causing inbreeding avoidance may undermine the power of a study to test inbreeding avoidance. Common proximate mechanisms that mediate precopulatory kin recognition and avoidance include prior association (where kin discrimination is based on social familiarity) and phenotype matching (where recognition is based on self-referent cues; Holmes & Sherman 1982; Holmes 1986). The relative influence of familiarity and phenotypic similarity in kin recognition has been investigated in vertebrates (Tang-Martinez 2001) but relatively little is known about the mechanisms of kin recognition in invertebrates. In addition, female *Drosophila* might be more likely to mate with

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strangers than familiar males, suggesting that individual recognition and mate choice are modulated by social familiarity (Ödeen & Moray 2008). Therefore, it is important to examine the effect of social familiarity on kin recognition when investigating inbreeding avoidance. Fourth, variation in a female's state of primary sexual receptivity (initial receptivity before first mating) is also associated with female choosiness (e.g. Lynch et al. 2005). This might influence the degree of inbreeding avoidance as a virgin female that is kept sexually isolated for a longer period might discriminate less between related and unrelated males. Consistent with this, in *Drosophila melanogaster*, recently eclosed female virgins (1-day posteclosion) display lower sexual receptivity than virgins that are given more time to mature (2-day posteclosion; Manning 1967) and this difference presents a convenient method of investigating the effect of the female's receptivity on inbreeding avoidance. Finally, in many insects, including *D. melanogaster*, the male seminal proteins transferred during mating cause dramatic changes in females that alter subsequent reproductive behaviour (reviewed in Wolfner 2002; Chapman & Davies 2004). However, little is known about inbreeding avoidance in nonvirgins and how it is adjusted by previous mating experiences. Seldom have all these factors been considered by inbreeding avoidance studies, making it difficult to interpret the evolutionary significance of variation in inbreeding avoidance.

Although *D. melanogaster* is capable of long-distance movements (up to 10 km; Yamazaki et al. 1986; Coyne & Milstead 1987), natural populations are characterized by limited dispersal and a tendency towards aggregations in particular localities (Wallace 1970; McInnis et al. 1982), which can increase the probability of related individuals interacting and the risk of inbreeding. Although direct information on inbreeding in wild *D. melanogaster* is scarce, estimates of genetic load suggest there is some inbreeding caused by patchy distribution of resources and population substructuring (Danieli & Costa 1977; Nielsen et al. 1985; Alonso-Moraga et al. 1988). Early work suggested potential for inbreeding avoidance in laboratory strains of *D. melanogaster*, by showing that in inbred isofemale lines flies avoid mating with individuals from the same line (Averhoff & Richardson 1974, 1976). However, a subsequent study failed to replicate these early results (van den Berg et al. 1984). In addition, studies investigating the role of relatedness in sexual behaviour in *D. melanogaster* have exclusively used virgin individuals (Averhoff & Richardson 1974, 1976; Mack et al. 2002), raising questions as to what extent these findings can be extrapolated to mated individuals: an important issue given that in most sexually reproducing organisms, including *D. melanogaster*, males and females typically mate multiply. Resolving patterns of inbreeding avoidance in *D. melanogaster* is therefore important in order to characterize a key aspect of the biology of the model organism, and more generally, to gain insight into the mechanisms underpinning precopulatory inbreeding strategies in invertebrates.

We addressed these goals in a laboratory-adapted outbred population of *D. melanogaster*. We first examined whether inbreeding depression occurred in our population. We then tested for evidence of sex-specific inbreeding avoidance behaviours via no-choice and mate choice assays and examined the proximate influence of social familiarity, primary receptivity and mating history.

METHODS

Experimental Population and Culturing

For all experiments we used a laboratory-adapted Dahomey wild-type stock of *D. melanogaster*. Flies were maintained at 25 °C, in a nonhumidified room, on a 12:12 h light:dark cycle, and fed standard sugar–yeast–maize–molasses medium with excess live yeast granules (Lewis 1960). The stock has been maintained since 1970 in four large (several thousand flies), outbred population cages (Partridge & Farquhar 1983) of dimensions 30 × 15 cm and 20 cm high. Each population was fed with three bottles of food medium per week. These four populations were mixed into one single large population approximately 1 year prior to experiments to promote genetic variability in our experimental flies. Previous studies have also shown that this stock exhibits substantial levels of genetic variation (Wilkinson et al. 1990; Whitlock & Fowler 1996; Gardner et al. 2005), and experimental evolution studies show that this population contains selectable variation for a range of life history, behavioural and physiological traits (e.g. Sgrò et al. 1998, 2000; Sgrò & Partridge 1999; Wigby & Chapman 2004; Wigby et al. 2009). The Dahomey stock is maintained with overlapping generations to minimize selection on replication rate and life span. Therefore, related individuals can interact (and mate) in the Dahomey population both within and across generations.

Virgins were collected within 8 h of eclosion using ice anaesthesia. To obtain parents of the experimental flies, eggs were collected and raised at standard density (ca. 100 flies per bottle; Clancy & Kennington 2001). Virgin adults were placed in same-sex vials and aged for 1 week before single males and females were paired in vials to produce families. The parental pair was removed after 24 h and the eggs left to develop. To create individuals that were related and familiar to one another (R_f) siblings were raised from egg to adult together in the same vial. To generate individuals that were unrelated and unfamiliar to one another (U_u) nonsiblings were raised in separate vials. Virgin adults emerging from these vials were used for experimental trials.

To investigate the potential effects of social familiarity on inbreeding avoidance, we raised siblings in separate vials to create individuals that were related but unfamiliar (R_u). This emulates natural situations in which adult females lay eggs in spatially separated substrates. By comparing mating responses to R_f and to R_u individuals, we investigated the effects of familiarity while controlling for relatedness. Similarly, by comparing mating responses to R_u and to U_u flies, we examined the effects of relatedness while controlling for social familiarity. To create R_u individuals, the food medium containing unhatched eggs was split in half after the removal of the parental flies: half was transferred into a separate vial and all vials were supplemented with fresh medium. We standardized average egg density across treatments by discarding half of the medium in the R_f and U_u treatments and replacing it with fresh yeast medium. This procedure removed any potential familiarity effects caused by shared larval environment since none of the larvae hatched prior to separation of the eggs. All experiments were conducted blind with respect to relatedness and familiarity between individuals.

Inbreeding Depression

To investigate the cost of inbreeding, we quantified egg–adult viability of 1-day posteclosion (24–36 h posteclosion) females mated to either a same-aged R_f ($N = 40$) or U_u ($N = 40$) male. Males were removed and females allowed to oviposit in individual vials for 24 h postmating before they too were removed. We measured egg–adult viability as the ratio of eclosed adults to oviposited eggs in the vials 12 days after the oviposition period. Because the majority of the flies eclosed 10 days after oviposition, allowing 12 days before fly collection provided ample time for development.

Inbreeding Avoidance

Theory predicts that inbreeding avoidance is sex specific and changes with the availability of unrelated partners (e.g. Kokko & Ots

2006; Puurtinen 2011). To disentangle the roles of male and female behaviour in inbreeding avoidance, we used three types of assays: (1) an individual female was exposed to two males (female–male–male; FMM), (2) an individual male was exposed to two females (male–female–female; MFF), and (3) single females were placed with single males (male–female; MF). In the MF assay, effects of relatedness on the mating behaviour of the flies could be an outcome of female or male preference. In MFF and FMM, there is also a possibility for intrasexual interactions and an opportunity for the single male or female, respectively, to compare the two opposite-sex individuals and thus choose between them.

In the FMM and MFF assays, the two same-sex flies were marked on the thorax with either red or orange acrylic paint (Nilsen et al. 2004) in a randomized balanced design with respect to their relatedness to the fly of the opposite sex, to allow recognition. Males for FMM and MF assays as well as females for MFF were aspirated into individual vials 12–14 h prior to the trial. At lights-on the following morning, a single, same-aged individual of the opposite sex was aspirated into the vial, and mating behaviour was observed until mating (in the 'mate choice', 'social familiarity' and 'primary receptivity' experiments, see below) or remating (in the 'mating history' experiment, see below) had been observed in at least 85% of the vials. FMM, MFF and MF assays were used to answer specific questions in the different experiments outlined below. Assays within each experiment were conducted at the same time using the same batch of flies.

Mate choice

Using FMM and MFF assays, we examined whether focal individuals avoided inbreeding when given a choice of two opposite-sex individuals (Fig. 1a). Each of 45 focal females and 47 focal males were aspirated individually into vials with two virgin members of the opposite sex, of which one was R_f and the other U_u to the focal individual. All flies in this experiment were 1-day posteclosion virgins.

Social familiarity

To examine the effects of social familiarity on inbreeding avoidance, we conducted two sets of FMM assays, exposing females to two male types in the following combinations: (1) U_u and R_u ($N = 52$) and (2) R_f and R_u ($N = 50$). In addition, we included one set of MF observations of R_u pairs ($N = 45$), R_f pairs ($N = 45$) and U_u pairs ($N = 45$; Fig. 1b). All flies in these experiments were 1-day posteclosion virgins.

Primary receptivity

We examined the response of 1-day posteclosion (24–36 h posteclosion) virgin females ($N = 44$) and 2-day posteclosion (48–54 h posteclosion) virgin females ($N = 49$), each presented with two males, one R_f and one U_u, of similar time posteclosion as the focal female (Fig. 1c).

Mating history

To assay the effect of first mating on subsequent inbreeding avoidance, we initially mated females to either an R_f ($N = 41$) or a U_u ($N = 43$) male. Immediately following the first mating, females were placed with two novel males (FMM): an R_f and a U_u male (Fig. 1d). In treatments in which females were first mated to R_f males and thereafter presented with both a U_u and an R_f male, the second R_f male was the full sib brother of the first male. Therefore, to control for between-male relatedness, females that had first mated with a U_u male were presented subsequently with a new U_u male that was the full sib brother of the first U_u male.

Similarly, males were initially mated with an R_f female ($N = 42$) or a U_u female ($N = 44$) and thereafter presented with a choice of one R_f and one U_u female (Fig. 1d). The first and second U_u female used in a single trial were full siblings. All flies were 1-day post-eclosion at the time of the first mating.

We measured four aspects of sexual behaviour (Pekkala et al. 2009).

(1) Courtship behaviour before mating (orienting, tapping, wing vibration, licking and attempting copulation) in which we recorded the occurrence of courtship events in 1 min spot-checks until mating occurred. Male *Drosophila* must perform courtship before mating (Spieth 1952) and courtship of *D. melanogaster* males typically consists of periods of courting and not courting. The number of courtship counts directed at a particular female would be indicative of male preference whereas the amount of courtship required by the female to mate would reflect the female's receptivity to mate with either mate type. The number of spot-checks corresponded to the number of minutes between the start of the trial and the start of mating, which was measured as the latency to mating.

(2) Latency to mating. This is the time from the start of a trial to the start of mating. Latency to mating could be determined by female receptivity and male activity.

(3) Duration of mating. This is the time between the start and end of mating. Although traditionally thought to be mainly under male control (Wigby et al. 2009), mating duration can be modulated by female genotype (Goodwin et al. 2010).

(4) Type of partner that mated with the focal individual (i.e. R_f, U_u or R_u). This could be influenced by female choice and activity of the male.

Statistical Analysis

All statistical analyses were conducted using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, U.S.A.). To test for the effect of relatedness on egg–adult viability, we used a generalized linear model with binomial error distribution. Egg–adult viability was entered as the response variable and parental relatedness as the fixed factor. We also calculated the coefficient of inbreeding depression δ (Lande & Schemske 1985):

$$\delta = 1 - (X_I/X_O),$$

where X_I = inbred egg–adult viability and X_O = outbred egg–adult viability.

In the 'inbreeding avoidance' experiments, we used chi-square tests to test whether the focal individuals mated preferentially with either mate type. For FMM and MMF assays, we analysed variation in mating latency using analysis of covariance (ANCOVA). Mating latency was the dependent variable, and mate type (R_f, U_u or R_u) was added as the fixed factor. As a covariate, we included courtship counts (sum of frequencies of all different courtship behaviours) by the mated male relative to total number of courtship events by both males, or courtship counts directed towards the mated female relative to total courtship counts to both females. For the MF assay, we entered courtship counts in our ANCOVA model. Courtship counts were included as a covariate in the above analyses because courtship effort has a strong stimulating effect on females' receptivity (Kowalski et al. 2004). Thus, courtship effort was entered in the statistical models to account for some of the variance in mating latency. We used two-tailed t tests to compare mating duration with either mate type in FMM and MFF assays and we used ANOVA to compare mating duration between the three mate types in MF assays. Post hoc Tukey's tests were carried out to identify which of the three groups differed from each other. To achieve homogeneous variances and normality of residuals, we log transformed mating latency and mating duration data prior to analysis.

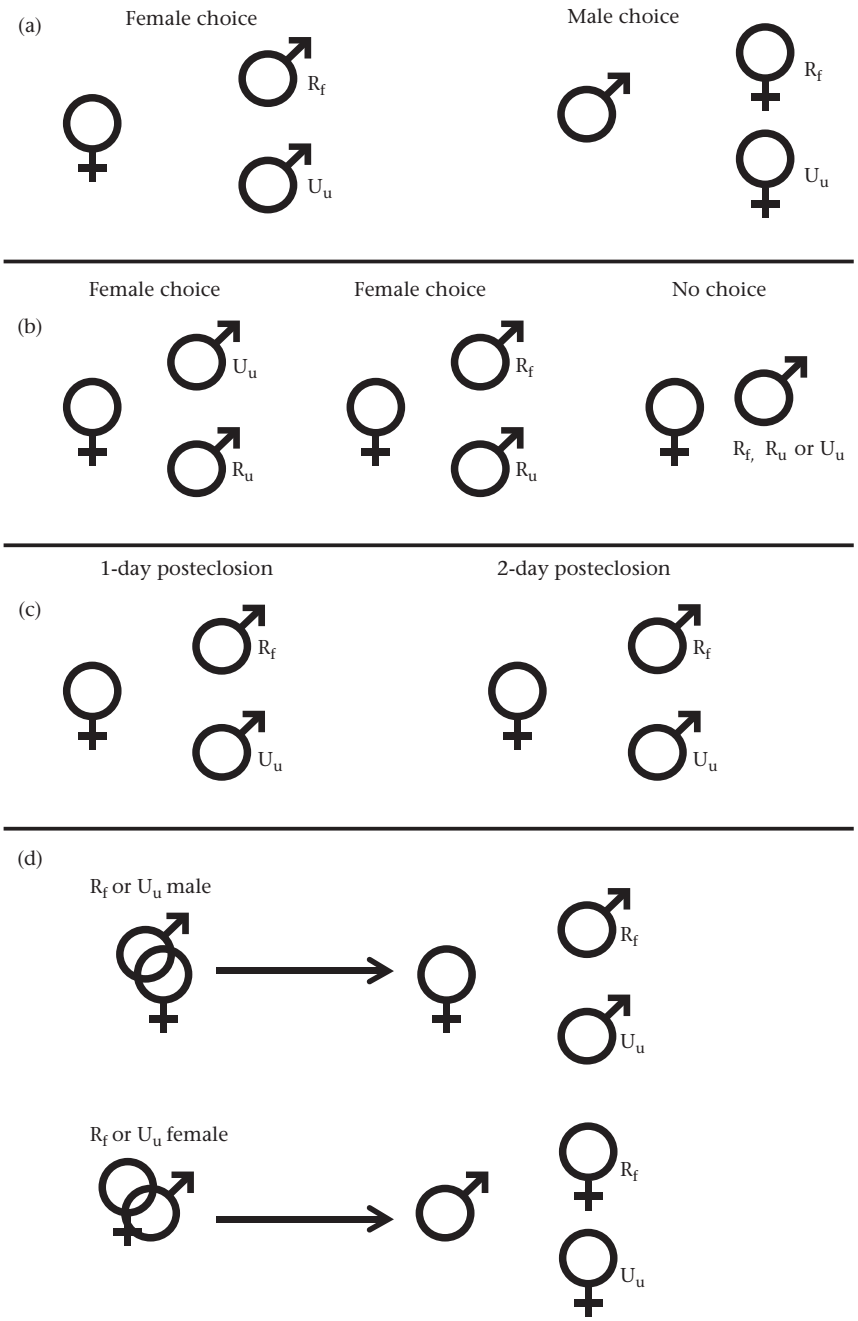

Figure 1. Experiments used to investigate inbreeding avoidance. R_f = related/familiar, U_u = unrelated/unfamiliar and R_u = related/unfamiliar. (a) 'Mate choice' experiments in which single females or males were introduced to two opposite-sex individuals. (b) 'Social familiarity' experiments in which single females were placed with two males or single males. (c) 'Primary receptivity' experiments in which either 1-day posteclosion or 2-day posteclosion virgin females were presented with both an R_f and a U_u male. (d) 'Mating history' experiments in which focal individuals were mated with either an R_f (related/familiar) or U_u (unrelated/unfamiliar) partner and subsequently presented with both an R_f and a U_u individual.

To investigate variation in female choice in the FMM assay, we compared the amount of courtship before mating with either the related or unrelated male, using *t* tests. Amount of courtship was the sum of counts of the various courtship behaviours. For the analysis of courtship effort in the MFF assays, we compared courtship counts directed by the male to the related versus unrelated female using paired *t* tests. We used a one-way ANOVA to compare the courtship effort of males from different treatment groups (R_f, R_u or U_u) in the MF assays. To account for multiple testing (four variables measured per assay), we used Bonferroni correction. Thus, *P* values were considered significant only when $P < 0.05/4 = 0.013$. In our study, the minimum sample size WAS 38. This means that we had a high (0.67) probability of detecting a large effect (Cohen's $d = 0.8$) and a relatively low probability (0.09) of detecting a small effect size (Cohen's $d = 0.2$).

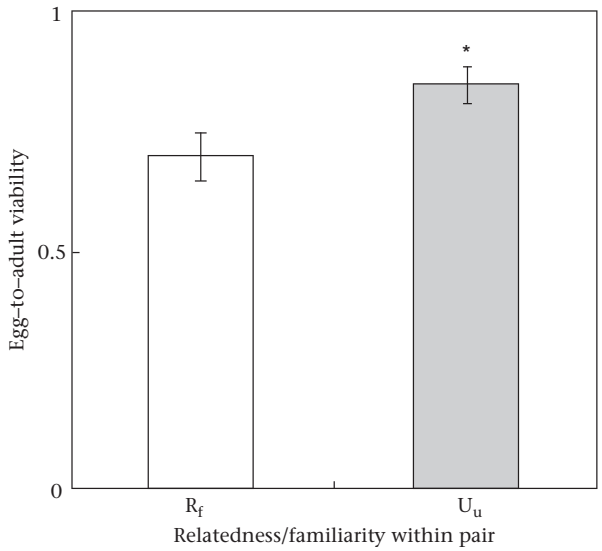

Figure 2. Egg–adult viability of offspring sired by R_f = related/familiar or U_u = unrelated/unfamiliar parents. Error bars denote SE. *$P < 0.05$.

RESULTS

Inbreeding Depression

There was no difference in the number of eggs laid by females mated to either an R_f (mean ± SE = 21.75 ± 0.96) or a U_u male (21.30 ± 0.96; *t* test: $t_{78} = 0.33$, $P = 0.74$). Therefore, we did not adjust egg–adult viability for egg numbers. We observed significantly lower egg–adult viability of offspring sired by brothers (mean ± SE = 0.70 ± 0.05) than by unrelated males (0.85 ± 0.03; generalized linear model: $Z = 7.01$, $N = 78$, $P < 0.001$). Consistent with inbreeding depression, the offspring of full sib mating suffered from on average 17.6% lower egg–adult viability than the offspring of unrelated parents (Fig. 2). The coefficient of inbreeding depression is therefore relatively low ($\delta = 0.176$).

Inbreeding Avoidance

Mate choice

There was no evidence of inbreeding avoidance in either the FMM or the MFF assays (Table 1 'Mate choice'). Males in the MFF assay did not preferentially court unrelated females and in the FMM, there was no difference in the amount of courtship required by the female to mate with either a related or an unrelated male (Table 1 'Mate choice').

Social familiarity

There was no evidence that social familiarity influenced responses to related and unrelated mates in the FMM assay (Table 1 'Social familiarity'). The amount of courtship required by females did not differ with male type (R_u, R_f or U_u). In the MF assay, latency to mating, probability of mating and courtship frequency did not differ between the three groups (Table 1 'Social familiarity'); R_u individuals mated for longer than R_f individuals (Tukey's test: $P = 0.015$), but at a marginally nonsignificant level after Bonferroni correction (Table 1 'Social familiarity').

Primary receptivity

As expected, latency to mating was significantly shorter for 2-day posteclosion virgins than for 1-day posteclosion virgins (1 day: 92.10 ± 7.55 min; 2 days: 15.45 ± 7.16 min; $F_{1,91} = 88.70$, $P < 0.001$), indicative of the difference in primary receptivity. However, we detected no evidence of inbreeding avoidance in either 1-day posteclosion or 2-day posteclosion virgin females of the FMM assays (Table 1 'Primary receptivity').

Mating history

Females first mated to U_u or R_f males also did not show a difference in amount of courtship required, remating probability, latency and duration with either male type (Table 1 'Mating history'). Females that had first mated to R_f males showed a nonsignificant trend towards mating faster with U_u males (Table 1 'Mating history'). In the MFF assays, males that were first mated to U_u females were slower to remate with U_u females then they were to remate with R_f females (Table 1 'Mating history'; Fig. 3). However, remating duration, probability of remating with R_f or U_u females and proportion of courtship directed to each female type did not differ (Table 1 'Mating history'). Males first mated to R_f females showed no difference in the latency to remating, duration of remating and proportion of courtship to either mate type (Table 1 'Mating history').

DISCUSSION

We found significant but low inbreeding depression and little evidence of precopulatory inbreeding avoidance, after controlling for sex-specific behaviours, social familiarity, primary receptivity and mating history. Our results contrast with some previous studies showing inbreeding avoidance in invertebrates using virgin individuals (e.g. Simmons 1991; Lihoreau et al. 2008) and, in particular, in *D. melanogaster* (Averhoff & Richardson 1974, 1976; Tompkins & Hall 1984; but see van den Berg et al. 1984). It is worth noting that previous studies on *D. melanogaster* imposed pair mating between full siblings for several generations. This may result in selection for highly inbred flies that exhibit mating behaviour not representative of less inbred populations (Miller et al. 1993; Miller & Hedrick 1993, 2001). The different inbreeding protocols used could have resulted in directional selection for or against inbreeding avoidance mechanisms. The preference for unrelated partners in Averhoff & Richardson's (1974, 1976) studies could be a consequence of the importance of restoring female fitness when inbreeding depression is substantially high. Conversely, the inbreeding regime used by van den Berg et al. (1984) might have purged deleterious alleles in the population, eliminating the need for inbreeding avoidance. Flies used in our study were not inbred prior to experimentation and thus might not show inbreeding avoidance because the cost of inbreeding is relatively low compared to that of inbred lines. Theory predicts that for low or no male parental investment, both males and females should mate with a full sibling if the coefficient of inbreeding depression is lower than 1/3 (Parker 1979, 2006). The coefficient of inbreeding depression calculated from egg–adult viability in our study was 0.176. While this corresponds to a relatively large amount of inbreeding depression (DeRose & Roff 1999), it would appear not to be sufficiently high to generate strong selection for inbreeding avoidance. Therefore, the lack of inbreeding avoidance mechanisms observed in this study appears broadly consistent with theoretical predictions, particularly in light of limited male parental investment in this species. However, our study did not quantify the effects of inbreeding on adult survival and reproductive success, and thus might have underestimated inbreeding depression somewhat. Nevertheless, the proportion of inbreeding depression missed by our study would have to be substantial to very large (relative to inbreeding depression for life history traits, DeRose & Roff 1999) for inbreeding avoidance to evolve (i.e. at least 0.16 for inbreeding avoidance to be selected in

Table 1
Mating responses of *D. melanogaster* in inbreeding avoidance experiments

Response variable	Factors	Values (mean±SE)	*df*	Test statistic	*P*
Mate choice					
FMM					
Courtship counts	Relatedness	R_f: 2.09±0.49; U_u: 2.14±0.50	42	0.097	0.923
Mating latency	Relatedness	R_f: 31.58±10.88; U_u: 40.52±8.35	41	1.200	0.280
Mating duration	Relatedness	R_f: 23.95±1.52; U_u: 20.96±1.04	42	0.529	0.102
Mating probability	Relatedness	R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.818	0.366
MFF					
Courtship counts	Relatedness	R_f: 2.19±0.21; U_u: 1.83±0.20	45	1.258	0.799
Mating latency	Relatedness	R_f: 36.54±4.83; U_u: 34.57±6.00	44	0.288	0.739
Mating duration	Relatedness	R_f: 22.81±0.64; U_u: 23.95±1.09	45	0.416	0.679
Mating probability	Relatedness	R_f: 0.55±0.07; U_u: 0.45±0.07	1	0.532	0.466
Social familiarity					
FMM					
Courtship counts	U_u vs R_u	U_u: 2.49±0.95; R_u: 1.43±0.41	48	1.153	0.255
	R_f vs R_u	R_f: 2.21±0.53; R_u: 2.21±0.80	46	<0.001	1.000
Mating latency	U_u vs R_u	U_u: 40.23±8.80; R_u: 29.44±8.62	47	0.145	0.705
	R_f vs R_u	R_f: 32.95±6.76; R_u: 22.61±7.86	45	0.343	0.561
Mating duration	U_u vs R_u	U_u: 15.96±1.27; R_u: 16.50±1.20	48	0.055	0.954
	R_f vs R_u	R_f: 16.96±1.30; R_u: 19.50±1.48	46	1.012	0.932
Mating probability	U_u vs R_u	U_u: 0.49±0.07; R_u: 0.51±0.07	1	0.020	0.889
	R_f vs R_u	R_f: 0.57±0.07; R_u: 0.43±0.07	1	1.042	0.307
MF					
Courtship counts	Relatedness/familiarity	R_f: 3.95±0.62; U_u: 4.66±0.77; R_u: 3.03±0.65	103	1.407	0.250
Mating latency	Relatedness/familiarity	R_f: 49.70±6.73; U_u: 47.82±8.13; R_u: 64.69±9.24	102	1.140	0.324
Mating duration	Relatedness/familiarity	R_f: 16.32±0.87; U_u: 15.48±0.84; R_u: 18.97±1.06	103	4.459	0.014
Mating probability	Relatedness/familiarity	R_f: 0.82±0.06; U_u: 0.67±0.07; R_u: 0.77±0.06	2	3.093	0.213
Primary receptivity					
FMM					
Courtship counts	Relatedness (1-day posteclosion)	R_f: 3.80±0.76; U_u: 3.70±9.82	43	0.251	0.803
	Relatedness (2-day posteclosion)	R_f: 2.37±0.53; U_u: 2.73±0.61	48	0.701	0.487
Mating latency	Relatedness (1-day posteclosion)	R_f: 89.47±11.48; U_u: 94.73±9.82	42	0.014	0.908
	Relatedness (2-day posteclosion)	R_f: 19.78±10.44; U_u: 11.12±9.82	47	3.628	0.063
Mating duration	Relatedness (1-day posteclosion)	R_f: 17.90±0.89; U_u: 18.04±0.76	43	0.071	0.908
	Relatedness (2-day posteclosion)	R_f: 17.52±0.80; U_u: 17.19±0.76	48	0.327	0.745
Mating probability	Relatedness (1-day posteclosion)	R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.083	0.943
	Relatedness (2-day posteclosion)	R_f: 0.47±0.07; U_u: 0.53±0.07	1	0.818	0.366
Mating history					
FMM					
Courtship counts	First mating R_f	Second mating R_f: 6.36±0.73; U_u: 6.41±1.10	38	0.048	0.962
	First mating U_u	Second mating R_f: 6.12±0.67; U_u: 5.12±0.65	40	1.533	0.133
Remating latency	First mating R_f	Second mating R_f: 351.4±42.64; U_u: 250.1±42.64	37	4.471	0.041
	First mating U_u	Second mating R_f: 286.48±37.35; U_u: 289.16±41.09	39	0.010	0.919
Remating duration	First mating R_f	Second mating R_f: 17.75±1.37; U_u: 15.65±1.37	38	0.897	0.286
	First mating U_u	Second mating R_f: 16.13±1.34; U_u: 16.16±1.47	40	0.014	0.989
Remating probability	First mating R_f	Second mating R_f: 0.50±0.08; U_u: 0.50±0.08	1	0.000	1.000
	First mating U_u	Second mating R_f: 0.55±0.08; U_u: 0.45±0.08	1	0.381	0.537
MFF					
Courtship counts	First mating R_f	Second mating R_f: 2,14±0.20; U_u: 1.86±0.20	40	0.543	0.590
	First mating U_u	Second mating R_f: 2.50±0.28; U_u: 3.50±0.43	42	1.504	0.140
Remating latency	First mating R_f	Second mating R_f: 49.33±15.45; U_u: 39.86±15.45	39	0.916	0.344
	First mating U_u	Second mating R_f: 55.37±26.52; U_u: 130.20±23.12	41	6.870	**0.012**
Remating duration	First mating R_f	Second mating R_f: 18.62±1.44; U_u: 18.48±1.44	40	0.228	0.821
	First mating U_u	Second mating R_f: 18.68±1.21; U_u: 18.52±1.05	42	0.520	0.606

Table 1 (*continued*)

Response variable	Factors	Values (mean±SE)	*df*	Test statistic	*P*
Remating probability	First mating R_f	Second mating R_f: 0.50±0.08; U_u: 0.50±0.08	1	0.000	1.000
	First mating U_u	Second mating R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.818	0.366

R_f = related/familiar, U_u = unrelated/unfamiliar and R_u = related/unfamiliar partners. 1-day posteclosion, $N = 24–36$ h posteclosion and 2-day posteclosion, $N = 48–54$ h posteclosion. Values for mating latency and mating duration are in minutes and nontransformed values are presented for the purpose of visualization. Values for mate choice represent the mating probability. Test statistic values were based on the *t* distribution except for 'mating/remating probability' where the test statistic is based on the chi-square distribution and 'mating latency' where the test statistic is based on the *F* distribution. *P* values that are significant after sequential Bonferroni correction ($P < 0.013$) are highlighted in bold.

females, and at least 0.50 for inbreeding avoidance to be selected in males). It is difficult to estimate the magnitude of inbreeding depression arising after offspring reach adulthood because few studies have attempted to quantify this component of inbreeding depression, and because the amount of inbreeding depression is strongly contingent on the breeding regimes, population parameters and environmental conditions of different populations (Sharp 1984; Mackay 1985). However, there is some evidence suggesting that inbreeding depression arising from adult reproductive success might be modest for one generation of inbreeding. For example, Tantawy & Reeve (1956) found no reduction in net fertility following one generation of sib–sib mating (but substantial inbreeding depression in fertility following successive generations of inbreeding). Similarly, Hughes (1996) found no inbreeding depression in male fertility. Swindell & Bouzat (2006) measured inbreeding depression in lineages of *D. melanogaster* maintained under different levels of ancestral inbreeding, as the 72 h production of individual females, a measure that takes into consideration both egg–adult survival and some reproductive success. Mean inbreeding depression ranged from 0.27 to 0.09 across different ancestral inbreeding treatments. In addition to demonstrating the variability of inbreeding depression, these results show that even when a more inclusive measure of offspring fitness is used, inbreeding depression in *D. melanogaster* is unlikely to exceed the 0.33 threshold. Inbreeding depression caused by reproductive performance alone was also low in a population of prairie voles, *Microtus ochrogaster* (Bixler & Tang-Martinez 2006), indicating that this pattern is more broadly plausible.

The use of the Dahomey population in our study provides advantages and potential caveats. An advantage is that flies were assayed in an environment to which they are adapted (since 1970) and is thus likely to represent behaviour that occurs in the laboratory cages. However, the behaviour may not be representative of wild populations of *D. melanogaster*. It is possible that the maintenance of large, well-mixed, population cages might have relaxed selection on inbreeding avoidance mechanisms compared to the wild. For example, the cage environment may reduce the probability of mating with a relative owing to the large number of flies within close proximity. Using freshly extracted flies from the field would be more reflective of wild populations. However, this approach would also not be without caveat. First, wild flies might not behave naturally when placed in the laboratory, a novel environment to which they are not adapted. Second, adaptation to the laboratory is rapid in *D. melanogaster* (e.g. Frankham & Loebel 1992) and thus recently caught flies are likely to be assayed while undergoing a process of intense selection. Males of the Dahomey stock have recently been shown to exhibit strategic copulation behaviour in response to the presence or absence of rival males (Wigby et al. 2009; Bretman et al. 2009, 2010): a behaviour that would have evolved in the wild and would be predicted to undergo relaxed selection in the laboratory where numerous rivals are always present. Thus, there is reason to expect that if inbreeding avoidance behaviour was present in the wild ancestral population, we would be able to detect it in our present study. No one approach is ideal, but the respective limitations of using recently caught versus laboratory-adapted populations should be considered when drawing conclusions.

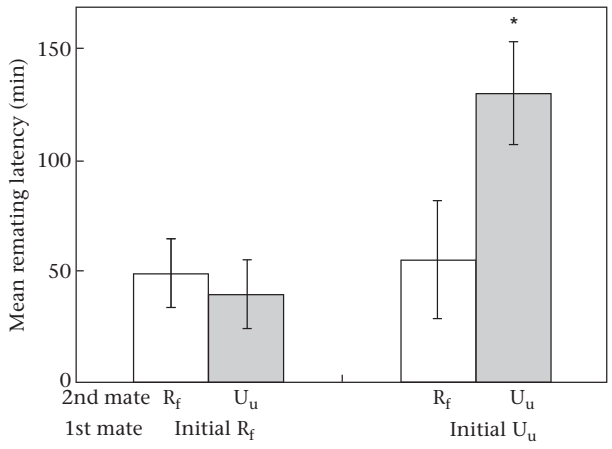

Figure 3. Remating latency in the 'mating history male choice' experiment (Fig. 1). R_f = related/familiar, U_u = unrelated/unfamiliar. Error bars denote SE. *P values that are significant after sequential Bonferroni correction ($P < 0.013$).

In the FMM and MFF assays, although focal individuals were presented with a choice, it was impossible to rule out potential effects of intrasexual competition. We observed no female–female interactions during our MFF trials, and so it is unlikely that such interactions confound the results of these assays. Observations of contact between the males in most of the FMM mating trials and reduced courting of the males in the presence of another male indicates interaction between the males (Table 1). Male–male interactions could potentially mask female inbreeding avoidance if the related male was particularly successful in competing with the unrelated male or that the former was relatively more intense in courting the female, for example, if one male was larger or in better condition than the other. However, all males were reared under identical conditions and thus variation in body size and condition was minimized and random across treatments. Moreover, it is unlikely that related males courted females more intensely because we detected no difference in the amount of courtship required by the female to mate with either the related or unrelated male. Therefore, although we cannot unequivocally exclude male–male interactions as a potential explanation for the results in our FMM and MMF assays, all the available evidence suggests that they were unlikely to mask female inbreeding avoidance.

In contrast to a previous study demonstrating female's preference to mate with unfamiliar males (Ödeen & Moray 2008), we detected no effect of social familiarity on female mate choice in our

'Social familiarity' experiment. Females were equally likely to mate with related/familiar males and with related/unfamiliar males. This is probably due to the difference in designs between our study and that of Ödeen & Moray (2008). We did not house females with males behind a piece of netting to allow for 'familiarization' prior to trials. This treatment might have been necessary for females to distinguish between previously encountered and not previously encountered males.

Most of our experiments involved virgin flies. Virgin *D. melanogaster* of both sexes are typically eager to mate, which could potentially mask important behaviours that are present only in previously mated individuals. Given that, like most animals, male and female *D. melanogaster* typically mate multiply (Harshman & Clark 1998; Imhof et al. 1998) the mating behaviour of nonvirgin individuals needs to be addressed in more studies. In our experiments we found that males first mated to unrelated females took significantly longer to remate with another unrelated female than with a novel related female, suggesting that mating history could potentially play an important role in mediating inbreeding likelihood.

Despite little evidence of precopulatory inbreeding avoidance in our study, it is possible that other mechanisms exist in this species to minimize mating with kin. First, polyandry might reduce the number of inbred offspring produced by a female where a brood is sired by multiple males (Harshman & Clark 1998; Imhof et al. 1998; Michalczyk et al. 2011; but see Hosken & Blanckenhorn 1999). Second, postcopulatory inbreeding avoidance might occur in *D. melanogaster*, where sperm competitive ability is shown to be negatively correlated with relatedness (Mack et al. 2002; Panhuis & Nunney 2007). However, a recent experimental test found no evidence of this (Ala-Honkola et al. 2011). Moreover, Ala-Honkola et al. (2011) also found no evidence of precopulatory inbreeding avoidance in no-choice experiments between related and familiar virgin flies, which is consistent with the findings of our study. In conclusion, our results suggest that, although there was significant inbreeding depression, precopulatory inbreeding avoidance is absent in our study population. This is not surprising as the magnitude of inbreeding depression in our population is less than the threshold estimated by theoretical models.

Acknowledgments

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Chapter 5

Group sex-ratio and male inbreeding avoidance in the red junglefowl

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ABSTRACT

Theory predicts that the propensity of an individual to avoid inbreeding changes with opportunity costs associated with mating. In males, which are predicted to have lower inbreeding avoidance thresholds than females, opportunity costs can reflect the availability of unrelated partners and intensity of intrasexual competition. We tested this idea by studying patterns of male inbreeding avoidance in response to experimentally manipulated sex-ratios in mating groups of the red junglefowl *Gallus gallus* ssp. First, we set up groups of different male-female ratios: 3:1, 2:1, 1:1, 1:2 and 1:3 to investigate precopulatory responses. We found that, controlling for female inbreeding avoidance, males courted unrelated females more frequently than related females, independently of group sex-ratio. As a result, matings were significantly more likely to occur between unrelated partners than between related partners. Second, we investigated male sperm investment under different sex-ratio treatments and found that while males increased sperm investment in the presence of male rivals, there was no evidence that males invested preferentially in copulations with unrelated females under any sex-ratio. These results indicate precopulatory but limited postcopulatory male inbreeding avoidance, and challenge the hypothesis that inbreeding avoidance is influenced by variation in mate availability and intensity of intrasexual competition.

INTRODUCTION

Inbreeding depression, caused by the expression of deleterious recessive alleles or loss of overdominance effects, reduces the fitness of progeny of closely related parents (Charlesworth & Charlesworth 1987). This in turn promotes the evolution of inbreeding avoidance mechanisms (Marr et al. 2002). Theory predicts that inbreeding avoidance is sex-specific (Parker 1979; Kokko & Ots 2006; Puurtinen 2011). At intermediate levels of inbreeding depression, males – which typically invest less in a reproductive event than females – may gain from inbreeding while females are more likely to evolve inbreeding avoidance tactics (Parker 1979; Pizzari et al. 2004; Facon et al. 2006; Kokko & Ots 2006). Thus, we might expect conflict between male and female propensity to inbreed (Parker 1979; 2006). However, opportunity costs associated with mating are predicted to modulate the intensity of sexual conflict over inbreeding (Parker 1979, 2006). Opportunity cost describes the cost of the next best alternative that is not chosen, for example, the cost of missing a mating with another available female when multiple female mates are present. Propensity to inbreed is expected to change with mate availability (Parker 1979; Kokko & Ots 2006): a male's propensity to mate with related females should decline with the availability of unrelated females particularly when male sperm reserves are depleted over successive mating (e.g. Wedell et al. 2002).

Males have also been shown to adjust their ejaculates according to the relatedness of the female (Pizzari et al. 2004; Lewis & Wedell, 2009) and are also known to tailor their ejaculates in response to sperm competition risk and intensity (Ball & Parker 1996, 1997; Parker et al. 1996; Parker et al. 1997; Parker 1998; Wedell et al. 2002; Parker & Pizzari 2010; Kelly & Jennions 2011). Individual males should respond to

higher risk of sperm competition (from zero to one rival) by increasing their ejaculate expenditure (Wedell et al. 2002; Gage & Baker 2008; Parker & Pizzari 2010; Kelly & Jennions 2011). Under high sperm competition intensity, when ejaculates of more than two males compete, males are often predicted to decrease their ejaculate allocation (Pilastro et al. 2002; Parker & Pizzari 2010). However, ejaculate size adjustment according to sperm competition intensity is has received less support from empirical studies (Kelly & Jennions 2011). Kelly & Jennions (2011) conducted a meta-analysis and revealed no evidence for ejaculate size decreasing as the number of rivals increases from one to several males. Nevertheless, the number of competitors in a group can simultaneously influence male ejaculate investment and the propensity to invest in inbreeding.

In the red junglefowl *Gallus gallus* ssp., dispersal is limited under natural unconfined settings (Collias et al. 1966; Collias & Collias 1996), meaning that inbreeding can occur. Even though individuals may switch flock, 90% of these individuals transfer to an adjacent flock, and around 4% of observed copulations in these groups takes place between siblings and between mothers and sons (Collias & Collias 1996). In addition, studies on domestic chickens demonstrated that inbreeding imposes fitness costs on the offspring (Cheng et al. 1984; Craig & Baruth 1965; Shoffner et al. 1953). Evidence of inbreeding depression has also been found in our study population of red junglefowl (See chapter 3). Therefore, it should be expected that inbreeding avoidance mechanisms will be employed in this species, particularly in females because of their higher investment in offspring.

Recent empirical work supports the expectation that the red junglefowl exhibits sex-

specific responses to inbreeding. Females have been shown to avoid inbreeding both before and after copulation, even when only one male is available, when inbreeding propensity should be the highest (Pizzari et al. 2004; Løvlie 2007). Therefore, females are expected to avoid inbreeding over the range of opportunity costs, that is, when more males are available. However, males were found to be less discriminating than females, and only avoided inbreeding in certain circumstances. There was no evidence of male inbreeding avoidance when only one female was available (Pizzari et al. 2004) but males avoided inbreeding when exposed to two related and two unrelated females (Løvlie 2007). This suggests that male inbreeding avoidance changes in response to mate availability and sex-ratio. Male fowl have also been shown to differentially invest their sperm in females in response to different levels of rival male competition (Pizzari et al. 2003). In the absence of additional competitors, males minimise their sperm investment, but as the number of competitors increases, sperm investment also increases. Therefore, males might be expected to modulate sperm allocation when both relatedness and the degree of rival male competition vary.

Here, we experimentally manipulate the sex-ratio of replicate red junglefowl mating groups to investigate whether male inbreeding propensity is modulated by mate availability and number of same-sex competitors, First, we examined precopulatory male and female responses to inbreeding in groups of different male:female ratios. Second, we tested whether male copulation propensity and sperm investment varies on the basis of female relatedness, male-male competition and female availability.

MATERIAL AND METHODS

(a) Study Population

The study was conducted on an individually marked captive population of the red junglefowl in July 2010 at the Oxford University John Krebs Field Station in Wytham, Oxfordshire. Individuals were genotyped at 31 microsatellite loci (Worley et al. 2010). The relatedness of the experimental population (n_{males} = 32, $n_{females}$= 21) was determined by calculating pairwise relatedness based on this microsatellite data (Queller and Goodnight 1989). Therefore, pairs considered related had coefficient of relationship (r) $0.45<r<0.6$ and those considered unrelated had a coefficient of relationship (r) <0.05 and >-0.05. We avoided using male pairs that had $0.05<r<0.45$ to ensure that related and unrelated males differed in their degree of relatedness. Males and females were separated two weeks prior to the study in March 2010.

(b) 'Sex-ratio groups'

To study the effects of mate availability and male-male competition on inbreeding propensity, individuals were subjected to one of the following sex-ratio treatments: (a) 1 male: 1 female (related); (b) 1 male: 1 female (unrelated); (c) 1 male: 2 females; (d) 1 male: 3 females; (e) 2 males: 1 female; (f) 3 males: 1 female (n_{trials} = 72; 12 trials per treatment). In treatments c, d, e and f, there was only one related male-female pair within the group. When a single male was presented with a single female (1:1 ratio; treatments a and b), it simulated a sequential mate choice scenario (Gillingham et al, 2008), whereas a single male that was presented with more than one female (treatments c and d) could choose between them simultaneously. Treatments where a male was exposed to a single female in the presence of other males (i.e. treatments e and f) incorporated the additional variable of male-male competition.

Trials started on the afternoon of day 1: individuals were released simultaneously into a pen and observed for three hours. Thereafter, observations were made on the morning and afternoon of day 2 as well as on the morning and afternoon of day 3, for periods of three hours each. The number of male-initiated copulation attempts, female solicitation behaviours and level of female resistance behaviours were recorded as in Løvlie (2007). Male-initiated attempts were defined as events in which the males attempted to mate with the female and excluded events in which the female solicited. Male and female identity was recorded in all courtship actions and copulation attempts. In addition, we recorded male dominance status and the number of male courtship events. We assessed the dominance status of males based on the number of times one male avoided the other. A male was regarded as being dominant over another when it is more likely to be avoided (Froman et al. 2002; Gulh et al. 1945). Courtship behaviour consisted of waltzing (the male lowers one wing and circles hen) and 'tidbitting' (courtship feeding) (Zuk et al. 1990). The latter was only recorded when it was clear which particular female the male was directing the feeding towards. After each observation, females were removed from the pen and sexually isolated from the males until the next observation.

(c) 'Controlled copulation'

This experiment examined the effect of sex-ratio on a male's sperm investment in related *versus* unrelated females. Similar to the 'sex-ratio groups' experiment, individuals were exposed to all six treatments mentioned above (n_{trials}=113). To quantify male sperm investment, females were fitted with a harness covering their cloaca, allowing for effective ejaculate collection (Pizzari et al. 2003). Females were

presented facing the males for one minute to enable the males to inspect the female. Thereafter, females were held in a soliciting position, thus enabling males to observably copulate with the female. Males may sometimes mount a female without delivering any sperm (aspermic copulation, Løvlie et al. 2005). Therefore, in each trial, a male was given a maximum of 20 minutes to perform one spermic copulation. We measured latency to first spermic copulation and whether spermic copulations occurred. Ejaculates were collected after each copulation, and the volume measured to the nearest 0.5 µl using a Gilson pipette (Pizzari et al. 2003). When multiple males were used, we ensured that the males were separated visually for at least 10 days prior to trials. During the trial itself, males were held together by one person and thus prevented from interacting with one another to establish dominance. All experiments were conducted blind with respect to relatedness between individual birds. When males were used for more than one time, they were sexually rested for two days to allow replenishment of sperm reserves (Parker et al. 1942; Pizzari et al. 2003).

Ejaculates were homogenised by gentle shaking, 5 µl of semen was then extracted and mixed with 195 µl of phosphate buffer saline solution (PBS) before obtaining the absorbance value at 595 nm using a spectrometer (Scientific Laboratory Supplies, UV 1101). If the semen sample was too diluted or concentrated, we added more semen or phosphate buffer saline solution respectively and thereafter adjusted the absorbance value accordingly. Because sperm absorbance is directly correlated with sperm concentration (Ciereszko & Dabrowski 1993; Donoghue et al. 1996), we were able to calculate standardised set of values for the number of sperm in an ejaculate by multiplying the absorbance value with the ejaculate volume. All absorbance values were corrected for the dilution factors.

(d) Statistical analysis

In order to investigate male inbreeding avoidance, we needed to control for female behaviour. Therefore, we first analysed variation in female inbreeding propensity to understand which behaviour, if any, females exhibit to avoid inbreeding. This was done through Generalised Linear Mixed Models (GLMMs) with 'male identity' nested within 'female identity' nested within 'trial' as a random factor, 'number of male initiated attempts' and 'observation number' as covariates, 'male-female relatedness' (related or unrelated), 'sex-ratio' and 'dominance status' as independent variables, and 'relatedness*sex-ratio' and 'relatedness*dominance' as interaction terms. 'Observation number' accounts for the sequence of observations (1-5) that took place over three days. The three response variables entered in three separate GLMMs were 'average female resistance' using a Normal error distribution, 'proportion of attempts resisted' using a Binomial error distribution and 'probability of solicitation' using a Binomial error distribution.

To examine precopulatory male response, we entered 'male-female relatedness' (related or unrelated), 'sex-ratio' and 'dominance status' as independent variables, and 'relatedness*sex-ratio' and 'relatedness*dominance' as interaction terms. We entered 'number of successful matings' and 'observation number' as covariates in the model. In our previous analyses on female behaviour, we found that – consistent with previous studies (Løvlie 2007) – female exhibited significantly lower resistance scores towards the mating attempts of unrelated males (see results section). Therefore, we entered average female resistance into our models. 'Female identity' nested within 'male identity' nested within 'trial' was entered as a random factor. Two separate GLMMs

analysed variation in two male response variables: 'number of male-initiated attempts per female' and 'number of courtship events per female'. For both analyses, a Poisson error distribution was used. The significance of the fixed factors was assessed using the likelihood-ratio test on models with and without the fixed factor (Valdar et al. 2006). We also tested variation in mating success through a GLMM (similar to above) with Binomial error distribution except that we entered mating success (yes or no) as the response variable.

We tested the effect of male-female relatedness and sex-ratio on male postcopulatory response using General Linear Mixed Models or Generalised Linear Mixed Models (GLMM). To achieve homogeneous variances and normality of residuals, we used a logarithmic transformation 'latency to first copulation' and a squareroot transformation on 'sperm numbers invested' (absorbance units*volume) and entered these as response variables in separate General Linear Mixed Models. We analysed variation in 'sperm numbers invested' with two models, one without trials in which males did not copulate and one including all trials (entering zeros if males did not invest any sperm). 'Propensity to invest sperm' (whether or not sperm was invested) was the response variable in a GLMM using a Binomial error. 'Sex-ratio' and 'male-female relatedness' were fixed factors, 'female identity' nested within 'male identity' was a random factor. Post-hoc Tukey tests were carried out when the effect of a fixed factor was significant.

RESULTS

(a) 'Sex-ratio groups'

Sex-ratio had a significant effect on the number of male-initiated courtship events, whereby courtship counts were highest in the 1:1 sex-ratio trials (Figure 1A). Overall,

males courted unrelated females more frequently than related females (Table 1; Figure 1A). This effect was more prominent in the 2 males: 1 female groups although there was no significant interaction between relatedness and sex-ratio.

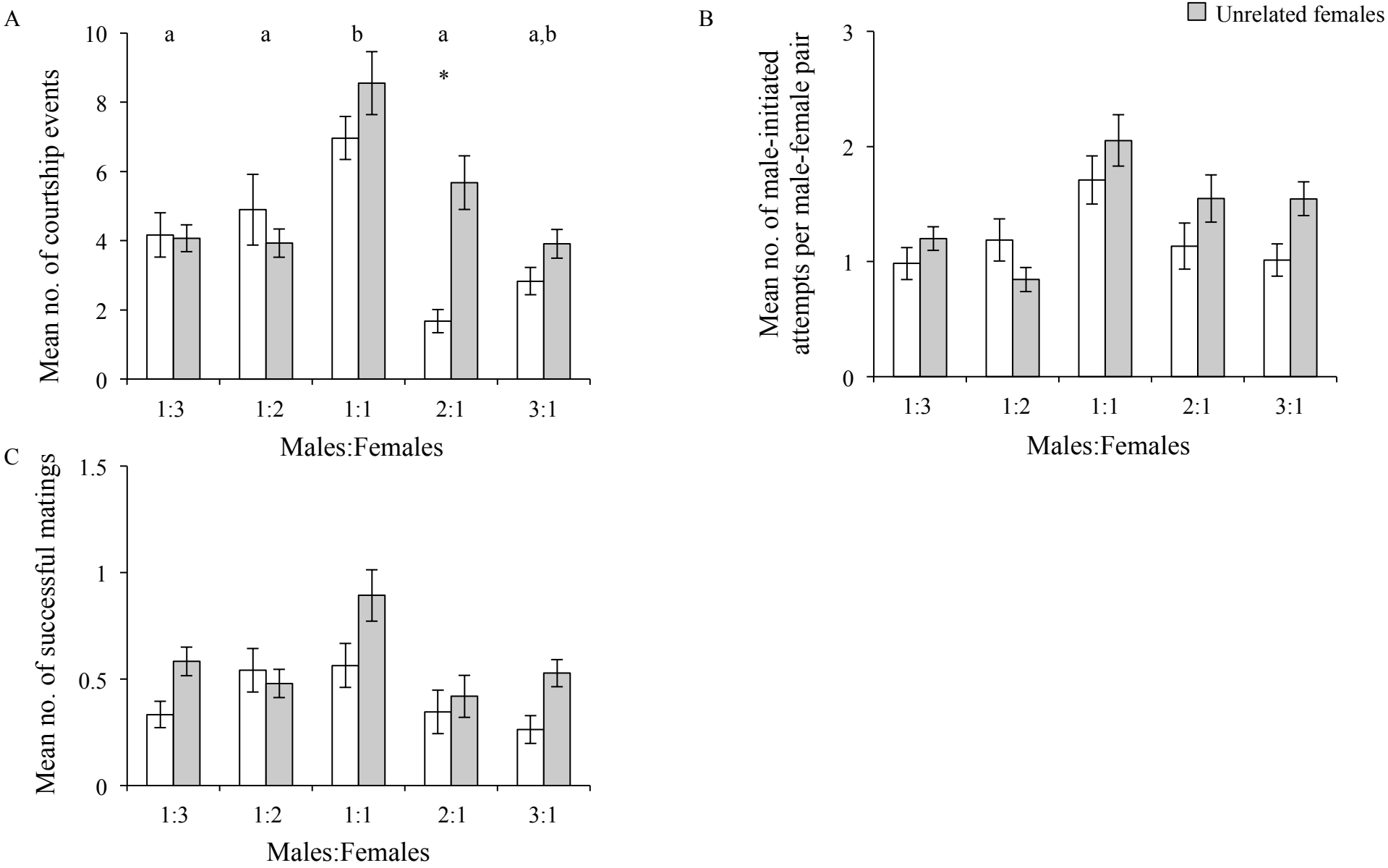
The number of male-initiated attempts did not significantly differ with the relatedness of the female. (Table 1; Figure 1B). There was also no significant effect of sex-ratio on the number of male-initiated attempts (Table 1; Figure 1B) and no significant interaction between male-female relatedness and sex-ratio (Table 1). However, females exhibited significantly lower resistance scores towards the mating attempts of unrelated males ($\chi^2 = 8.66$, $P = 0.003$). Females also resisted a marginally non-significantly lower proportion of attempts ($\chi^2 = 3.74$, $P = 0.053$) but displayed no difference in probability of solicitation ($\chi^2 = 1.01$, $P = 0.315$). The number of successful matings per pair that occurred between unrelated male-female pairs was overall significantly higher than that between related male-female pairs (Table 1; Figure 1C).

Table 1. Results of experiments on (1) 'Sex-ratio groups'; (2) 'Controlled copulations'. 0.10>*P*>0.05 are underlined and $P < 0.05$ are in bold.

Response variable	**Factors**	**Values (mean ± S.E.)**	**df**	**Test statistic**	***P***
(1) 'Sex-ratio groups'					
(a) Courtship counts	Sex-ratio (male:female)	1:3: 4.100±0.335; 1:2: 4.319±0.480; 1:1: 7.766±0.556; 2:1: 3:851±0.486; 3:1: 3.459±0.296	4	23.966	**<0.001**
	Relatedness	related: 4.017±0.288; unrelated: 4.844±0.251	1	4.801	**0.028**
	Sex-ratio*Relatedness		4	7.715	0.103
	Dominance	1: 5.254±0.233; 2: 2.250±0.242; 3: 1:971±0.452	2	28.693	**<0.001**
	Dominance*Relatedness		2	2.030	0.362
(b) Number of male-initiated attempts	Sex-ratio (male:female)	1:3: 1.128±0.083; 1:2: 0.983±0.098; 1:1: 1.883±0.154; 2:1: 1.360±0.145; 3:1: 1.326±0.105	4	4.483	0.345
	Relatedness	related: 1.189±0.078; unrelated: 1.396±0.069	1	1.003	0.317
	Sex-ratio*Relatedness		4	3.451	0.485
	Dominance	1: 1.415±0.061; 2: 1.007±0.106; 3: 0.912±0.200	2	8.012	**0.018**
	Dominance*Relatedness		2	0.559	0.756
(c) Mating success	Sex-ratio (male:female)	1:3: 0.500±0.050; 1:2: 0.302±0.050; 1:1: 0.565±0.032; 2:1: 0.386±0.071; 3:1: 0.418±0.047	4	4.017	0.404
	Relatedness	related: 0.395±0.039; unrelated: 0.568±0.036	1	5.528	**0.019**
	Sex-ratio*Relatedness		4	2.339	0.674
	Dominance	1: 0.564±0.032; 2: 0.302±0.050; 3: 0.235±0.074	2	3.789	0.150
	Dominance*Relatedness		2	1.629	0.443

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(2) 'Controlled copulations'					
(a) Latency to copulation (sec)	Sex-ratio (male:female)	1:3: 237.38±75.60; 1:2: 149.50±71.59; 1:1: 198.36±52.88; 2:1: 258.03±54.04; 3:1: 138.41±20.28	4	3.395	0.494
	Relatedness	related: 235.03±46.25; unrelated: 166.49±25.21	1	0.317	0.573
	Sex-ratio*Relatedness		4	0.689	0.952
(b) Probability of investing sperm	Sex-ratio (male:female)	1:3: 0.529±0.125; 1:2: 0.583±0.103; 1:1: 0.788±0.072; 2:1: 0.878±0.058; 3:1: 0.804±0.059	4	8.700	0.069
	Relatedness	related: 0.707±0.060; unrelated: 0.779±0.043	1	0.442	0.506
	Sex-ratio*Relatedness		4	1.390	0.846
(c) Sperm numbers invested (Absorbance * Volume in µl)	Sex-ratio (male:female)	1:3: 7.495±2.581; 1:2: 7.224±1.705; 1:1: 12.732±7.224; 2:1: 27.091±4.822; 3:1: 27.230±5.176	4	20.149	**<0.001**
	Relatedness	related: 20.522±4.437; unrelated: 18.691±2.498	1	0.372	0.542
	Sex-ratio*Relatedness		4	5.600	0.231

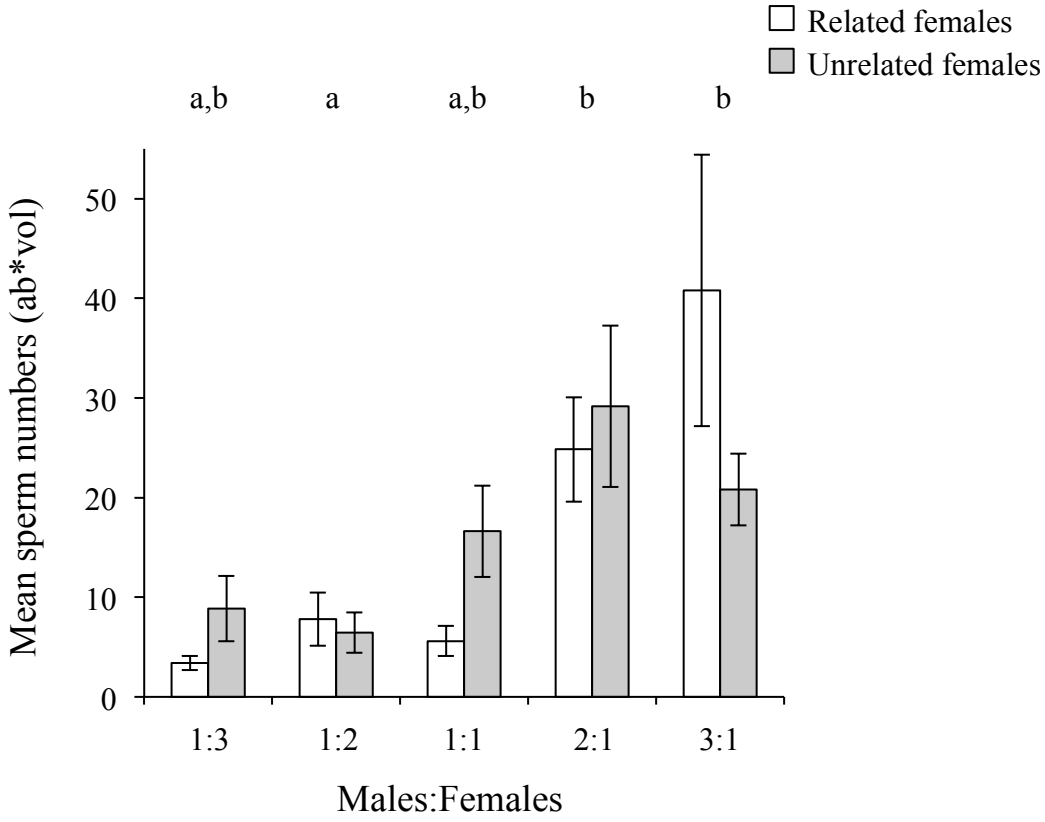
Figure 1. Precopulatory male inbreeding response to mate availability. On the x-axis, the ratio of the number of males to females is listed. Error bars denote S.E. (A) Overall males courted related females significantly less than unrelated females and there was a significant effect of sex-ratio on courtship counts. There was no significant interaction between sex-ratio and relatedness (Table 1). (B) There was no overall effect of relatedness or sex-ratio on the number of male-initiated attempts and there was no significant interaction between sex-ratio and relatedness (Table 1). (C) There was an overall significantly higher number of successful matings that occurred between unrelated partners than between related partners. However, there was no effect of sex-ratio treatment and no significant interaction between sex-ratio and relatedness (Table 1). '*' indicates a significant difference in the response variable between related and unrelated males within that sex-ratio treatment. Sex-ratio treatments that are significantly different (Post-hoc Tukey contrast; $P<0.05$) are denoted by different letters.


A
Mean no. of courtship events
10
8
6
4
2
0
a
a
b
a
a,b
*
1:3
1:2
1:1
2:1
3:1
Males:Females
B
Related females
Unrelated females
Mean no. of male-initiated attempts per male-female pair
3
2
1
0
1:3
1:2
1:1
2:1
3:1
Males:Females
C
Mean no. of successful matings
1.5
1
0.5
0
1:3
1:2
1:1
2:1
3:1
Males:Females

(b) 'Controlled copulation'

There was an effect of sex-ratio treatment on the numbers of sperm invested (Table 1; Figure 2). When excluding trials in which males did not invest sperm, the effect of sex-ratio treatment was the same ($\chi^2 = 13.89$, $P = 0.008$): males invested significantly more sperm in the 2:1 and 3:1 treatments than in the 1:2 treatment. However, there was no effect of female relatedness or no significant interaction between sex-ratio and female relatedness on the number of sperm invested. In addition, neither sex-ratio, relatedness or their interaction had a significant effect on a male's propensity to allocate sperm or his latency to copulation (Table 1).

Figure 2. Postcopulatory male inbreeding response to mate availability: sperm numbers (absorbance value of ejaculate * ejaculate volume) invested in related or unrelated females. On the x-axis, the ratio of the number of males to females is listed. Error bars denote S.E. There was a significant effect of sex-ratio on sperm numbers invested. However, there was no effect of relatedness on the sperm numbers invested and no significant interaction between relatedness and sex-ratio (Table 1). Sex-ratio treatments that are significantly different (Post-hoc Tukey contrast; $P<0.05$) are denoted by different letters.



DISCUSSION

The aim of this study was to examine whether a male's propensity to inbreed is modulated by mate availability and the level of intrasexual competition of a social group. However, we found no evidence that courtship counts, number of male-initiated breeding attempts, and sperm number invested in related *versus* unrelated females, change in response to the group sex-ratio.

(a) Precopulatory response

There was some evidence of precopulatory inbreeding avoidance in both males and females after controlling for sex-ratio treatment. Males courted unrelated females more frequently and females were more likely to resist the mating attempts of related males. This in turn resulted in mating success biased towards unrelated males. Our results are consistent with several studies demonstrating evidence of precopulatory inbreeding avoidance (e.g. Dewsbury 1982; Simmons 1991; Yasuko et al. 2001) and specifically with a study showing that female junglefowl preferentially copulate with unrelated males in female-biased groups (Løvlie 2007). The results of our study thus suggest some form of precopulatory inbreeding avoidance mechanism in the red junglefowl that is mediated by both male and female responses.

Theoretical models predict the degree of inbreeding avoidance should increase with increased mate availability (Kokko & Ots 2006). Interestingly, we found a trend of stronger inbreeding avoidance by males in male-biased sex-ratios where females were rare (i.e. only one female) compared to female-biased populations (i.e. where there was more than one female) (Figures 1A and 1B). In fact, when multiple partners were available, males courted related females as frequently as unrelated females. The

theoretical models developed by Kokko and Ots (2006) distinguish between 'simultaneous choice' and 'sequential choice'. Under the sequential choice scenario, males are predicted to have higher propensity to inbreed than males under the simultaneous choice scenario. The female-limited treatments (male-biased sex-ratios, with only one female) in our study simulate a situation in which choice is sequential, and we would expect lower levels of inbreeding avoidance in these treatments. Our findings therefore contrast with theoretical predictions in these treatments. However, males might tolerate inbreeding when multiple females are available because of their low resource investment in offspring compared to females, and potential inclusive fitness gains from inseminating related females (Parker 1979; Kokko & Ots 2006). To further understand the lack of inbreeding avoidance in males when multiple females were available, future research could determine the fitness consequences of mating with related *versus* unrelated females under scenarios of high female availability. The higher courtship frequency of the unrelated male relative to the related male when the sex-ratio was 2 males:1 female suggests that the intensity of male-male competition could potentially modulate the degree of inbreeding avoidance. Perhaps the related male was reserving resources for future mating opportunities with novel females and therefore reduced his courtship effort in the presence of a rival. This idea, however, is only speculative and warrants further studies. Nevertheless, our results suggest a potentially important role of male-male competition on male inbreeding propensity.

Dominant males may be more likely to avoid inbreeding because they have higher access to females. However, we found no interaction between social status and inbreeding propensity on variation in male courtship counts and number of male-initiated attempts, consistent with findings from a recent study on male bank voles

(Lemaître et al. 2012). These findings thus suggest that social status has limited influence on male inbreeding propensity.

(b) Postcopulatory response

Our results provide no evidence for male postcopulatory inbreeding avoidance tactics: that is, male sperm allocation responded equally to both related and unrelated females after controlling for sex-ratio. A previous study showed that males have a tendency to invest more sperm into sisters than into unrelated females when a single female was presented to a single male (Pizzari et al. 2004). This appears to contrast with our study: we found a non-significant trend for more sperm investment in unrelated females than in related females when the sex-ratio was 1:1. In addition, we found a non-significant tendency for males to copulate faster with unrelated females than with related females (Table 1), while Pizzari et al. (2004), showed that males copulated significantly faster with unrelated females. This difference in the strength of male preference might be explained by the additional variable, familiarity, incorporated in the Pizzari et al. (2004) study. In their study, related females have the same probability of being familiar or unfamiliar with male (and the same with unrelated females) and a male was sometimes exposed to a familiar female and at other times to an unfamiliar female. The effect of relatedness disappeared when variance in latency to first copulation was partitioned into female familiarity and relatedness. Also, the effect of relatedness on sperm numbers invested appeared to be mostly mediated by partner familiarity. In our study, males and females were physically and visually separated from each other two weeks prior to trials and this might have reduced familiarity between individuals. However, we were unable to control for possible interactions prior to separation (e.g. growing up together). Further, we do not know to what extent familiarity is reduced

when individuals were visually but not auditory isolated for two weeks. A previous study suggests that domestic hens discriminate against unfamiliar hens that were visually isolated from them for approximately 10 weeks (Dawkins, 1995). Hence, two weeks of separation might have been too short to remove the effects of familiarity. The duration of isolation and its effects on familiarity and hence inbreeding avoidance could be further investigated.

Gillingham et al. (2009) demonstrated that males allocate more sperm to the most MHC-dissimilar of sequentially presented females. MHC genes refer to major histocompatibility complex genes that code for antigen-presenting molecules essential for immune function. To a certain extent, individuals sharing MHC alleles are likely to be genetically related (Brown & Eklund 1994; Penn & Potts 1999) and therefore their results might be consistent with that of our study: we show that males demonstrate a non-significant trend in investing most sperm in a singly presented unrelated female than in a singly presented related female.

In our study, males invested more sperm in treatments where there were one or two more males than when there were two females available. These results are consistent with a previous work in feral fowl (Pizzari et al. 2003), in which dominant males, when faced with more male competitors (up to three), increased ejaculate expenditure. Therefore, consistent with predictions (Wedell et al. 2002; Parker & Pizzari 2010; Kelly & Jennions 2011), males responded to higher risk of sperm competition by increasing their ejaculate size. However, the sperm competition 'intensity' model predicts a negative relationship between ejaculate size and the number of male rivals when more than one rival is present, contrary to our observations. Empirical support for

the 'intensity' model has so far been inconsistent (Kelly & Jennions 2011) and our study also provides little support for adjustment of ejaculate size according to the intensity of sperm competition.

We failed to detect any interaction between relatedness and intensity of competition on the measured parameters. In response to male-male competition, we hypothesised that related and unrelated males might behave differently because of the prospect of females neutralizing sperm from related males. Previous studies demonstrated that subdominant and dominant males respond differentially to different levels of sperm competition, with the former adopting a strategy predicted under sperm competition intensity and the latter adopting a strategy predicted under sperm competition risk (Pizzari et al. 2003; Pizzari et al. 2004). We might have predicted related males to adopt a strategy similar to that of subdominant males because of the disadvantages imposed by females on their ejaculates. Whether a male plays an advantaged or disadvantaged role in sperm competition can be determined by cryptic female choice (Dean et al. 2011). Theory predicts that when female sperm ejection is imposed on particular males based on phenotypic traits, the disfavoured male phenotype should respond by inseminating larger ejaculates (Ball & Parker 2003). However, differential sperm allocation by related *versus* unrelated males according to varying levels of sperm competition was not detected in our study. We speculate that this might be because females, contrary to our initial predictions, might not preferentially ejected sperm of the related male when unrelated males are available. This would in turn explain why males do not invest differentially in related or unrelated females according to the intensity of male-male competition.

Under conditions of higher female availability, males are expected to decrease their propensity to inbreed (Kokko & Ots 2006). We might therefore predict males to invest more sperm in unrelated females in social situations where there is an increased availability of unrelated females, but this was not found in our study. According to Kokko and Ots (2006), inbreeding avoidance increases with mate encounter rate more rapidly when the choice is sequential than when the choice is simultaneous. Our experiments were designed to investigate inbreeding avoidance when males were presented with one or more females at the same time, that is, when choice was simultaneous. Thus, we might not have been able to detect any changes in inbreeding avoidance with mate availability as the magnitude of increase in inbreeding avoidance with increasing female availability might have been too small for us to detect. Future studies should aim at investigating male propensity to inbreed when females are presented sequentially.

Conclusions

Our study shows that males exhibit some degree of precopulatory inbreeding avoidance but these responses were not modulated by mate availability. Males do not differentially invest sperm in related or unrelated females nor change the degree of postcopulatory inbreeding avoidance according to female promiscuity or female availability.

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THEME III

RELATEDNESS OF

PROSPECTIVE MATES

Chapter 6

Sex-specific responses to genetically novel mates, and the role of olfaction in the fruit fly

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ABSTRACT

The genetic basis of mate choice has been the subject of intense investigation but previous studies have largely neglected the extent to which the mating decisions of an individual are influenced by the genetic similarity between its past and new partners. In viscous populations, an individual might encounter members of the opposite sex with whom it previously mated (sexually familiar) and members of the opposite sex genetically related to its previous mates (genetically familiar). Here, we show that male and female *Drosophila melanogaster* respond to the sexual and genetic novelty of potential future mates in fundamentally different ways. Males preferentially court sexually novel females, and –controlling for sexual novelty- also demonstrate a preference for genetically novel females: when exposed to two sexually novel females, one related and one unrelated to a previous mate, males preferentially court the genetically novel female. In contrast, females display a weak preference for sexually familiar males, and –controlling for sexual novelty- for genetically familiar males. We further show that male preferences only translate into differential mating success when the behaviour of females is experimentally inhibited, suggesting that both sexes influence the outcome of mating interactions. These sex-specific responses to sexual and genetic novelty are significantly weaker or absent in *Orco*^{1} mutants, which lack a co-receptor essential for olfaction, indicating a key role for olfactory cues in individual mate choice. Overall, our results show that olfactory detection of relatedness between previous and future mates plays an important role in sex-specific sexual behaviour.

INTRODUCTION

Establishing the genetic basis of mate choice is an enduring and fundamental challenge in the study of sexual selection, which has attracted intense theoretical and empirical research (Andersson 1994; Kokko et al. 2006). Most of this work has focused on: (i) the genetic covariance between preference and preferred trait (Fisher 1915; Kirkpatrick & Barton 1997), (ii) the genetic covariance between preferred trait and viability (e.g. Rowe & Houle 1996), and (iii) the role of genetic incompatibility between partners, such as inbreeding (Bateson 1982; Kokko & Ots 2006). Another potentially important aspect is the way the genetic similarity between past and future mates affects the mating decisions of an individual. While this aspect has received little or no consideration, it is likely to play a key role in viscous populations.

Natural populations often exhibit a degree of viscosity due to constraints on dispersal (Hamilton 1964; Lion & Baalen 2008; Lion et al. 2011). A neglected consequence of population viscosity is that individuals will not only have repeated encounters with previous (i.e. sexually familiar) mates, but are also likely to encounter new individuals that are genetically related to previous mates (i.e. genetically familiar). Recent work indicates that sexual familiarity can play an important role in mating preferences and the mating system of a diverse range of species. In general, a preference for sexually novel mates is associated with promiscuous mating systems (e.g. Dewsbury 1981; Koene & Ter Maat 2007), while a preference for sexually familiar mates is associated with monogamous pair-bonds (Getz et al. 1981; Black 2001). The fitness payoffs derived by mating with sexually novel mates are also expected to differ between males and females. In males, a preference for sexually novel mates has been observed in several polygynous species, a behaviour known as the Coolidge effect, which has

intuitive adaptive significance (Fox & Rauter 2003). Females on the other hand, might face a more delicate balance between the benefits, such as the increased genetic diversity of the offspring or bet-hedging effects (e.g. Fox & Rauter 2003), and the potential costs of mating with sexually novel males, for example those associated with eliciting a higher immune response against the inseminations of different males (e.g. Nunn 2002).

Mating preferences for or against genetically novel mates are likely to have similarly important, sex-specific fitness consequences. For example, a preference for genetically novel mates (i.e. individuals that are unrelated to previous mates) could increase offspring genetic diversity. Moreover, if recognition of closely related mates is subject to error, then preference for genetically novel mates could enable individuals to reduce the risk of mating repeatedly with the same mate. However, very little is known about the independent roles of sexual familiarity and genetic familiarity in the mating preferences of males and females. This is an important gap in our knowledge considering the profound implications that genetic familiarity can have on the genetic structure of a population, the intensity of local mate competition, as well as intersexual conflict and intrasexual cooperation (Perrin & Mazalov 2000; West et al. 2002; Rankin 2011).

Here, we experimentally test male and female responses to both the sexual and genetic familiarity of mates in the fruit fly *Drosophila melanogaster*. Individuals of this species mate multiply (Imhof et al. 1998) and natural populations are characterised by limited dispersal and a tendency towards aggregations in particular localities (McInnis et al. 1982). These factors are likely to increase the probability of encountering previous

mates, and their relatives, in a local patch. The use of the fruit fly also provides us with the opportunity to utilise genetic tools to explore the proximate mechanisms underpinning differential responses to sexual and genetic familiarity. A key candidate mechanism is olfaction, a sensory system that has a well-known role in species recognition in this taxa (Spiess 1987; Vosshall & Hansson 2011). We address three aims: (i) to characterise male and female behavioural responses to sexual familiarity; (ii) to establish male and female behavioural responses to genetic familiarity; and (iii) to test whether the gene *Orco*[1], which encodes a co-receptor essential for olfaction, is required for the behavioural responses to genetic familiarity. A sexually familiar mate refers to the previous mate of the focal individual while a sexually novel individual refers to a prospective mate that was mated to another opposite-sex individual. A genetically familiar mate refers to a prospective mate that is a full-sibling of the previous mate of the focal individual whereas a genetically novel mate refers to a prospective mate that is a non-sibling of the previous mate.

MATERIAL AND METHODS

(a) Experimental population and culturing

We used a lab-adapted, wild-type, Dahomey stock of the fruit fly maintained since 1970 in large, outbred populations (Partridge & Farquhar 1983). To control for genetic background, a $white^{Dahomey}$ stock (in which flies possess white eyes) was derived by repeated backcrossing w^{1118} into the wild-type (red-eyed) Dahomey background (Broughton et al. 2005). The $Orco^{1}$ loss-of-function allele (Larsson et al. 2004; Vosshall & Hansson 2011) was backcrossed into the $white^{Dahomey}$ genetic background for at least five generations to match the genetic background of the wild-type stock. Prior to experiments, the $white^{Dahomey}$; $Orco^{1}$ stock was backcrossed into the Dahomey

stock to replace the w^{1118} bearing X-chromosome with the wild-type Dahomey X-chromosome to create experimental $Orco^{1}$ flies. Thus all $Orco^{1}$ experimental flies possessed the wild-type (red) eye phenotype. Controls (also possessing the wild-type, red-eye phenotype) were $Orco^{+}$ flies derived from the final generation of backcrossing.

Flies were maintained in a 25°C, non-humidified room, with a 12 hours light: dark cycle, in plastic vials or bottles containing standard sugar-yeast medium with excess live yeast. Virgin flies were collected within 8 hours of eclosion using ice anaesthesia. Larvae were raised at standard density (~100 flies per bottle) (Clancy & Kennington 2001). Virgin adults were placed in same-sex vials for five days before conducting the 'Sexual novelty' experiment. For the 'Genetic novelty' and '$Orco^{1}$' experiments, single virgin males and virgin females were paired in individual vials after one week to produce families. The parental pair was discarded after 24 hours and the eggs left to develop. The families were therefore only maintained for one generation in which the offspring emerging from these vials were used for the 'Genetic novelty' and '$Orco^{1}$' trials. Flies were approximately three days post-eclosion at the time of the first mating.

We adopted two experimental designs. In our first design (vial experiments), live individuals were placed in a plastic vial (10 cm in height and 2 cm width) containing standard sugar-yeast medium with excess live yeast and observable mating parameters (courtship counts, mating latency, mating duration and mating success) were recorded. In our second design, we placed the flies in a mating chamber (2 cm diameter and 1 cm height) to conduct detailed observation of male courtship of beheaded, pinned females (Spieth 1966) or female rejection behaviour to male courtship (Connolly & Cook 1973). A ball of live yeast and a strip of filter paper soaked in distilled water were

placed in the mating chamber to provide food and water for the flies.

(b) Male response to sexual novelty and genetic novelty

We tested whether males prefer novel females by first mating males with a randomly chosen unrelated female and subsequently presenting each male with the female with which he had previously mated (the 'sexually familiar' female) and a novel female that had previously mated with a different male. First matings were performed immediately after lights-on. The timings of the first matings of the two females were tightly matched, such that experimental females finished copulating within 15 minutes of each other, to avoid any potential biases (*e.g.* differences in female receptivity or pheromonal profile) that could be influenced by the time since mating. Both females were randomly chosen from a large population so they were unlikely to be related to the male or to each other. Three separate trials were conducted. In the first and second trial, we adopted the 'vial' experimental design and novel and familiar females were differentiated by different methods, in a randomised balanced design: $white^{Dahomey}$ (white-eyed) *versus* wild-type (red-eyed) Dahomey females ($n_{males} = 58$); marked with acrylic paint on their thorax *versus* unpainted female ($n_{males} = 88$) (Nilsen et al. 2004). In the third trial, females were decapitated and pinned via the thorax at 1 cm apart in a mating chamber ($n_{males} = 28$). This was done to control for potential female influences on male courtship, Decapitated females do not extrude their ovipositor, depress, decamp or twist away from the male, thus displaying a significantly reduced rejection response (Spieth 1966). We recorded the occurrence of courtship events (chasing, singing, genital licking, copulation attempt) (Bastock & Manning 1955) directed at either female type (familiar or novel) in 15-minute spot-checks for the 'vial' experiment until lights-off (12 hours after lights-on) or until a remating occurred, and

in 1-minute spot-checks for the 'decapitated females' for a duration of four hours. The third trial was conducted blind with respect to the female type.

We then examined whether male flies discriminate between potential mates that are either genetically similar (i.e. related) or genetically different (i.e. unrelated) to their previous mates. Focal males were first mated with females unrelated to themselves and thereafter presented with a choice of two virgin females: a full-sibling of the first female and a non-sibling of the first mate. In the 'vial' design, females were marked on their thorax with either red or yellow acrylic paint (Nilsen et al. 2004) in a randomised balanced design ($n_{males} = 36$). We also conducted another trial in which females were decapitated and pinned in a mating chamber ($n_{males} = 79$). We recorded the number of courtship events directed at either female type in one minute spot-checks until remating occurred in the 'vial' experiment or for four hours in the 'decapitated females' experiment. In addition, for the 'vial' experiment, we recorded the remating latency and remating duration with either female type, together with the female type that eventually mated with the male. All trials were conducted blind.

(c) Female response to sexual novelty and genetic novelty

To examine female response to sexual novelty of males, we placed individual females with a previous male mate, and a male that had previously mated to a different female, in a mating chamber ($n_{females} = 43$). We recorded female rejection behaviour towards the courtship of each male type (Connolly & Cook 1973) for four hours.

To investigate female response to genetic novelty of males, focal females ($n_{females} = 40$) were first mated to unrelated males from a randomly chosen family and subsequently

placed with two novel males: a full-sibling of the first male and a non-sibling of the first mate. Males were marked with paint, as described above. As a measure of female response, we recorded the latency to remating with either male type (sibling or non-sibling of first mates) (Finley et al. 1997). We also recorded the duration of remating and the type of male that mated with the female. Focal individuals that did not remate on the first day were separated from the two potential mates before lights-off and replaced into the experimental vials at lights-on the following morning. This procedure was repeated on subsequent days and the trial was concluded when at least 95% of the females remated. In a separate trial to record female rejection behaviour, we placed mated females with two males in a mating chamber and recorded female response to the courtship attempts of each male type for four hours ($n_{females} = 59$).

(d) Orco^1 experiments

The *Orco^1* gene encodes for an olfactory co-receptor essential for olfaction (Larsson et al. 2004; Vosshall & Hansson 2011) and thus focal individuals were unable to use olfaction for discriminating between genetically familiar and genetically novel mates. To explore the potential role of olfactory senses in sex-specific response to genetic novelty, we replicated the experiments on genetic novelty with *Orco^1* as control focal individuals and recorded sex-specific response as outlined above. Non-focal individuals were wild-type Dahomey.

(e) Statistical analysis

We analysed variation in male courtship effort through Generalised Linear Mixed Model (LM) with Poisson error distribution. 'Number of courtship events' was the response variable, 'female type' (novel or familiar; full-sibling or a non-sibling of first

female mate) and 'marked status' (white-eyed or red-eyed; painted or unpainted; yellow or red paint) were fixed factors. Because each male was represented twice in the dataset, that is, once for the courtship directed at the novel female and another for courtship directed at the familiar female, we entered 'male identity' (i.e. male A, male B etc.) as a random factor. The variation in the response variable 'number of courtship events' was assessed using the likelihood-ratio test on models with and without the fixed factor 'female type' (Valdar et al. 2006). The proportion of courtship directed at different female types was weighted by the total courtship counts of that particular male to account for the differential courtship effort between males. We used R version 2.13.0 for these analyses.

We tested the effect of relatedness on female response in the 'vial' experiment using Cox proportional hazards model (Cox 1972) in JMP 9.0.2 with 'latency to remating' as the dependent variable and 'male type' (full-sibling or a non-sibling of first male) as the fixed factor. Because female's latency to remating is likely to be influenced by the duration of the first mating (Bretman et al. 2009) and the courtship intensity of the males (Kowalski et al. 2004), we entered the duration of first mating and courtship proportion by mated male as covariates. The survival curves were compared using the likelihood ratio test.

We analysed variation in female rejection behaviour in the mating chambers using a LM with Binomial error distribution with 'proportion of courtship rejected' as the response variable, 'male type' (novel or familiar; related or unrelated) as fixed factor, 'female identity' as a random factor. We omitted data points in which both males were simultaneously courting the female as it was ambiguous as to which male the female

was responding to. The 'number of courtship rejected' was weighted by the 'total number of courtship events' of that particular male. To test whether there is a difference in courtship intensity between the two male types, we used a LM with Poisson error distribution, 'number of courtship' as the response variable, male type (novel or familiar) as fixed factor and female identity as a random factor. R version 2.13.0 was used for these analyses.

'Remating latency' with either mate type in the male choice 'vial' experiment was analysed using Cox proportional hazards model with 'latency to remating' (time in minutes before 2nd mating) as the dependent variable, 'mate type' as the fixed factor and 'courtship proportion to mated female' (number of courtship events directed to mated female divided by total number of courtship events by that male) as a covariate. In both male choice and female choice trials, difference in remating duration with either mate type was tested with LM. 'Mating success' was analysed with chi-square tests for equal number of matings with either mate type. Where the number of matings was low, i.e. expected value <10, we used a Fisher's exact test. These analyses were conducted using JMP 9.0.2.

RESULTS AND DISCUSSION

(a) Male response to sexual novelty and genetic novelty

When placed with two females: one female to which the male had recently mated, and one female that had mated to a different male, males consistently directed significantly more courtship towards the sexually novel female than towards sexually familiar females (trial 1: $\chi^2_1 = 7.20$, $P = 0.007$; trial 2: $\chi^2_1 = 11.14$, $P < 0.001$; Figure 1A). These results provide the first evidence for a male "Coolidge effect" in the fruit fly: an

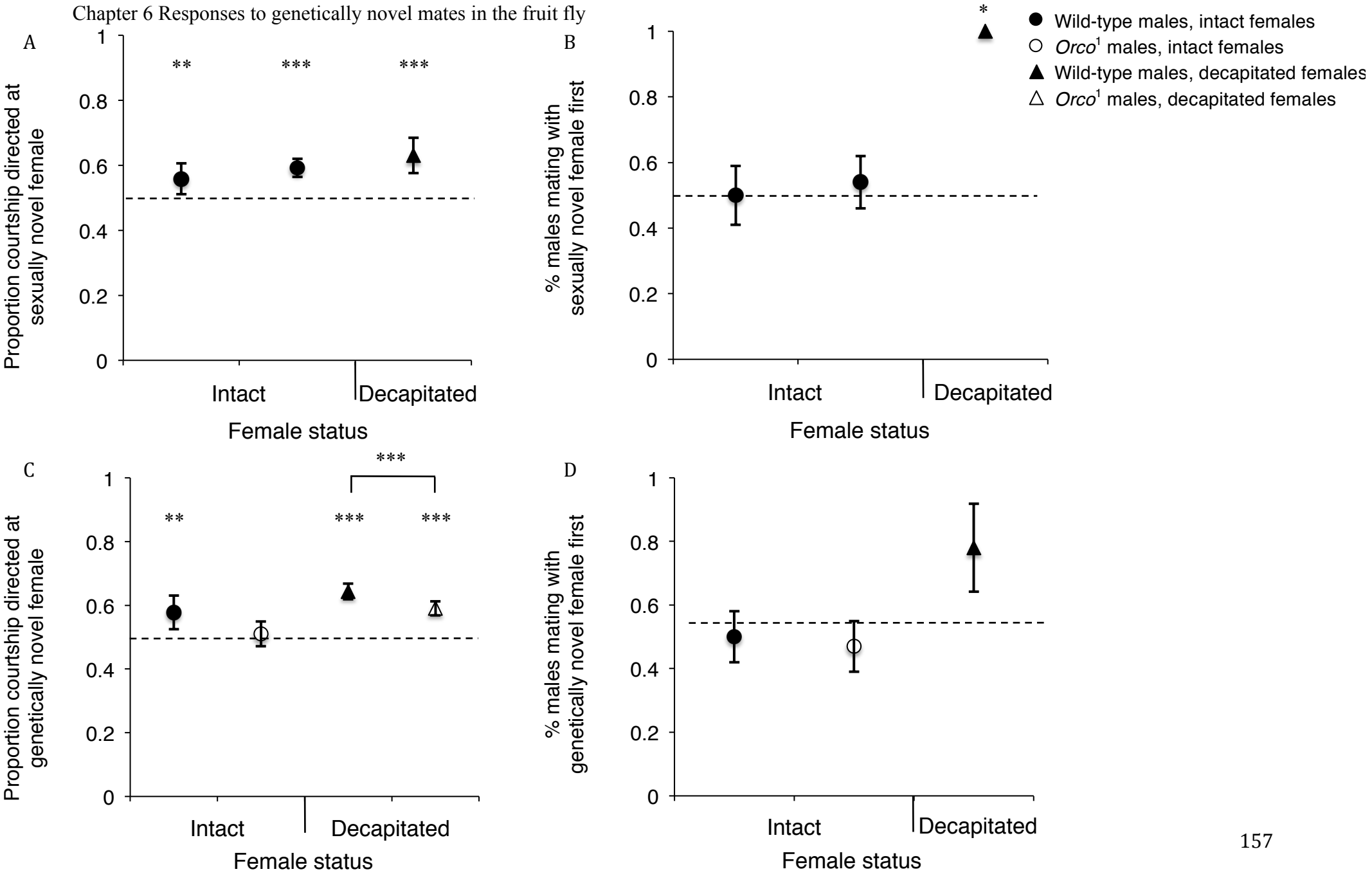
elevated interest in sexually novel over sexually familiar females (Dewsbury 1981). This effect arises in many taxa (e.g. Dewsbury 1981; Pizzari et al. 2003), including another insect (burying beetle, *Nicrophorus vespilloides*; Steiger et al. 2008). However, despite the bias in male courtship effort toward novel females, the likelihood of the first mating to occur with a sexually novel females was not significantly different from with a familiar female (trial 1: $\chi^2_1 = 0$, $P = 1$; trial 2: $\chi^2_1 = 0.23$, $P = 0.63$; Figure 1B) suggesting that male courtship alone does not predict mating success, and that females might play an important role. When females were decapitated to control for their behaviour, we again found that males direct significantly more courtship to sexually novel females ($\chi^2_1 = 20.77$, $P < 0.001$; Figure 1A) confirming the male preference for sexually novel females. Moreover, although few matings occurred with decapitated females because they do not elicit acceptance response (Spieth 1966), all of the forced matings were with the sexually novel female (sexually novel = 6, sexually familiar = 0; Fisher's exact test, $P = 0.023$; Figure 1B). This suggests that only in the absence of female control may males be able to significantly bias mating probability.

Further investigating male responses to genetic novelty, we show that when exposed to a full-sibling of the male's previous mate (i.e. genetically familiar) or a female unrelated to the male's previous mate (i.e. genetically novel), males directed more courtship towards the genetically novel female (i.e. females that are unrelated to the male's first mate; $\chi^2_1 = 6.73$, $P = 0.009$; Figure 1C). We detected no significant difference between female types in the probability of first copulation (Supplementary Table 1; Figure 1D). Experimental control of female behaviour, by decapitation and immobilization, confirmed the finding that males preferentially court the genetically

novel of the two females ($\chi^2_1 = 41.79$, $P < 0.001$; Figure 1C). Matings with decapitated females were again rare but of those that did occur, there was a non-significant trend in the direction predicted by male courtship preferences: more occurred with the unrelated female (unrelated = 7, related = 2; Fisher's exact test, $P = 0.167$; Figure 1D).

The results provide strong evidence that males preferentially court sexually and genetically novel females. However, only when female behaviour is removed do we find any evidence that males are able to impose their preferences on mating probability.

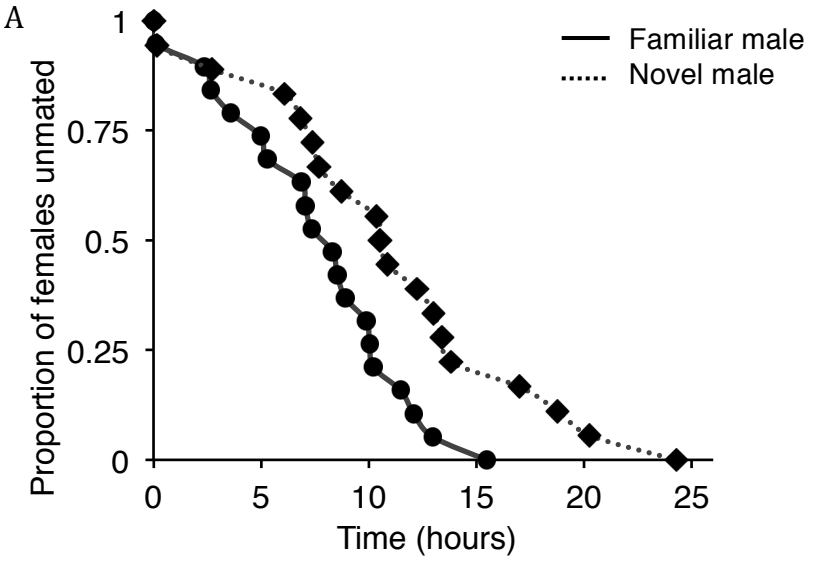
Figure 1. Male responses to sexual novelty and genetic novelty of females. Dotted line drawn at proportion courtship = 0.5. Error bars denote S.E. '*' equals $P < 0.05$, '**' equals $P < 0.01$, '***' equals $P < 0.001$ for proportion significantly greater than 0.5. (A) Proportion of courtship events directed at sexually novel female. From left to right: trial in which intact females differed in eye colour (n = 58); trial in which either intact sexually novel or familiar female was painted (n = 88); trial in which females were decapitated and pinned (n = 28). (B) Percentage males mating with sexually novel female first. Experiments are the same as in '(A)'. From left to right, the total number of males that mated = 28, 40 and 6. There are no standard error bars for wild-type males with decapitated females as all of the males mated with the novel female (see Results) and therefore S.E. = 0. (C) Proportion of courtship events directed at genetically novel female. Focal males were first mated with an unrelated female and subsequently presented with two females, one full-sibling of the first mate (genetically familiar) and one non-sibling of the first mate (genetically novel). There was a significant difference in proportion of courtship between wild-type and *Orco*^{1} males for the 'decapitated females' experiment (denoted by horizontal line). From left to right, n = 36, 35, 79 and 70. (D) Percentage males mating with genetically novel female first. Legend is the same as that of (C). From left to right, the total number of males that mated = 36, 35, 9 and 2. Datum for *Orco*^{1} males with decapitated females is not shown as we were unable to perform statistical test due to the small sample size: only two matings occurred, both with the genetically familiar female.

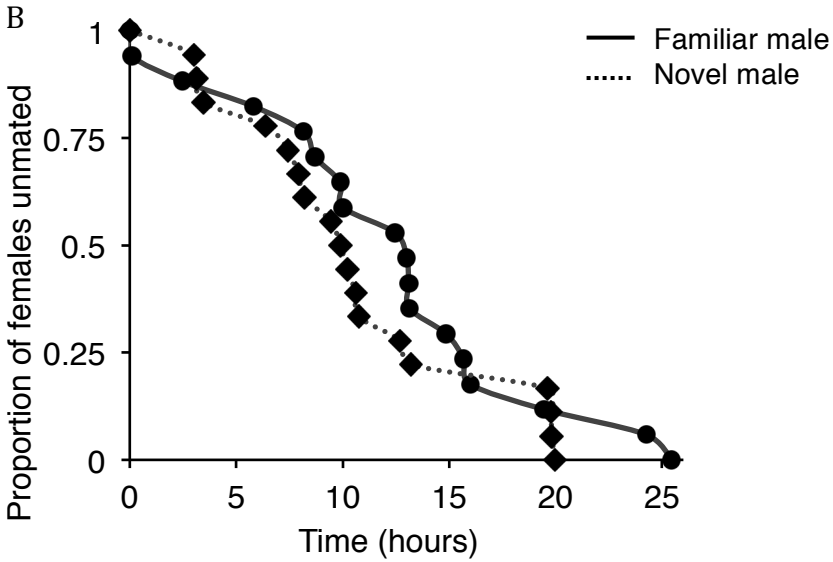

A
Proportion courtship directed at sexually novel female
1
0.8
0.6
0.4
0.2
0
**
***
***
Intact
Decapitated
Female status
B
% males mating with sexually novel female first
*
Wild-type males, intact females
Orco1 males, intact females
Wild-type males, decapitated females
Orco1 males, decapitated females
C
Proportion courtship directed at genetically novel female
***
**
***
***
D
% males mating with genetically novel female first

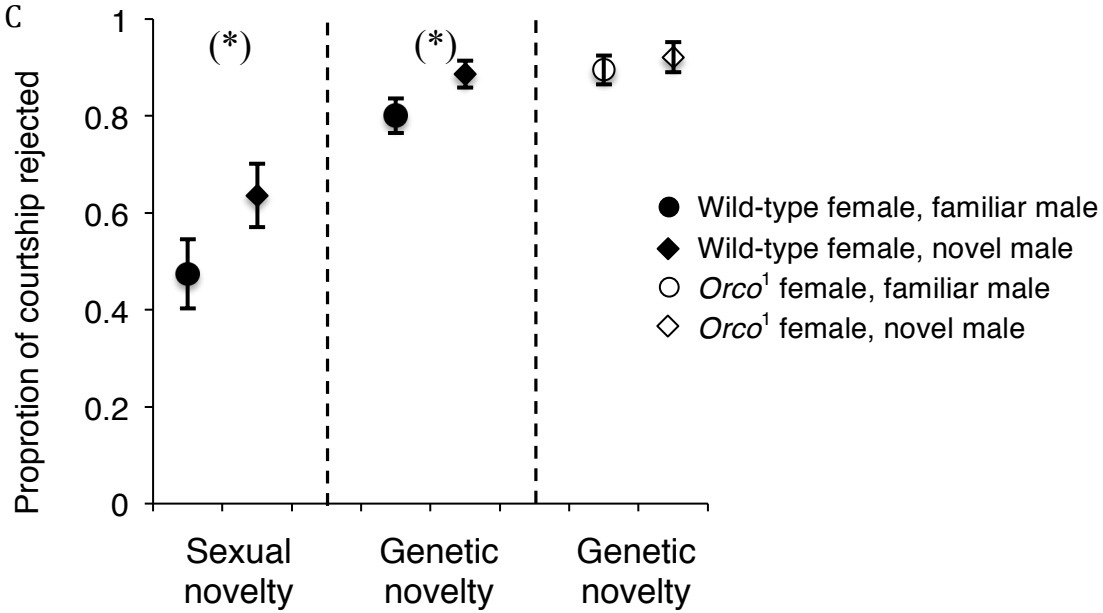
(b) Female response to sexual novelty and genetic novelty

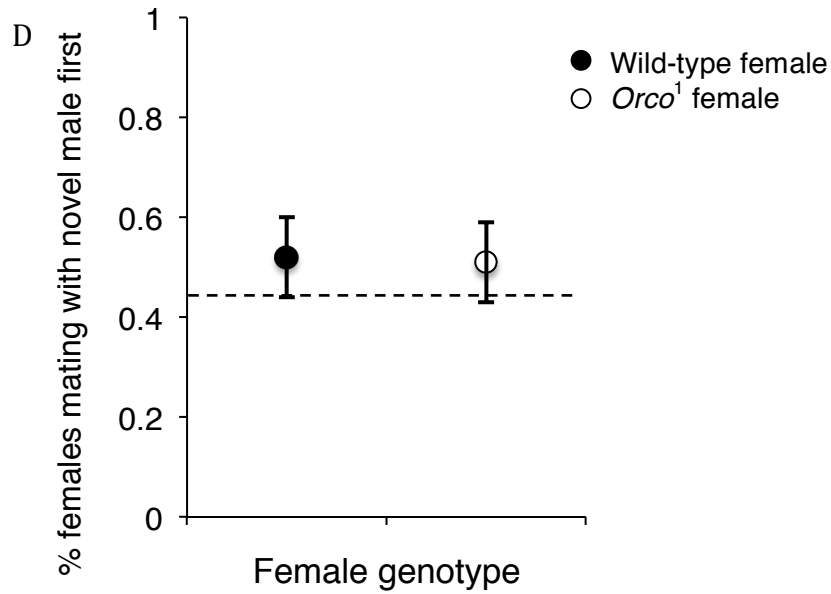
When examining female responses to sexual and genetic novelty, females presented with two males, one sexually novel and one sexually familiar, showed no evidence for a preference for novel males. Instead, there was a marginally non-significant trend in the opposite direction for females to reject the courtship of the novel male more frequently than that of the familiar male ($\chi^2_1 = 2.91$, $P = 0.088$; Figure 2C). This was not due to any difference in courtship intensity between sexually familiar and sexually novel males (GLMM, $\chi^2_1 = 0.26$, $P = 0.610$), Female responses to genetic familiarity were also consistent with a lack of preference for novelty, and females again displayed a trend for neophobia: when exposed to two sexually novel virgin males, one genetically familiar and one genetically novel, females remated faster with the genetically familiar male than with the genetically novel male ($\chi^2_1 = 5.54$, $P = 0.019$; Figure 2A), and there was a marginally non-significant trend for females to reject a higher proportion of courtship by the genetically novel male than that by the genetically familiar male ($\chi^2_1 = 3.29$, $P = 0.082$; Figure 2C). Again, this was not due to any difference in courtship intensity between male types (former experiment, $\chi^2_1 = 0.54$, $P = 0.46$; latter experiment, $\chi^2_1 = 0.01$, $P = 0.94$). Consistent with the assays of male preference, the probability of the first mating with the familiar or novel male did not differ (Supplementary Table 1; Figure 2D).

Figure 2. Female responses to sexual novelty and genetic novelty of males. Cumulative survival functions for (A) wild-type females, and (B) *Orco*^{1} female mutants. Likelihood ratio tests revealed a significant difference in remating latency with sibling and non-sibling of first mates for wild-type females (n = 40, $\chi^2_1 = 5.54$, $P = 0.019$). but not for *Orco*^{1} female mutants (n = 35, $\chi^2_1 = 0.90$, $P = 0.344$). (C) Proportion of courtship rejected by females. From left to right: 'Sexual novelty' experiment in which wild-type females were presented with a previous mate and novel mate (n = 43); 'Genetic novelty' experiment in which wild-type females were presented with a brother of the first mate and a male unrelated to the first mate (n = 59); 'Genetic novelty' experiment in which *Orco*^{1} females were presented with a brother of the first mate and a male unrelated to the first mate (n = 56). Dashed lines separate different experiments. (*) equals $0.05 < P < 0.10$ for tests for difference in proportion of courtship rejected between familiar and novel males. *P*-values from left to right are 0.088, 0.082 and 0.176. (D) Percentage females mating with genetically novel male first. Focal females were either wild-type or *Orco*^{1} and were presented with a brother of the first mate and a male unrelated to the first mate. Error bars denote S.E.


A
Familiar male
Novel male
Proportion of females unmated
Time (hours)
0
0.25
0.5
0.75
1
0
5
10
15
20
25


B
Familiar male
Novel male
Proportion of females unmated
Time (hours)
0
0.25
0.5
0.75
1
0
5
10
15
20
25


C
(*)
(*)
Proprotion of courtship rejected
Wild-type female, familiar male
Wild-type female, novel male
Orco1 female, familiar male
Orco1 female, novel male
Sexual novelty
Genetic novelty
Genetic novelty
0
0.2
0.4
0.6
0.8
1


D
Wild-type female
Orco1 female
% females mating with novel male first
Female genotype
0
0.2
0.4
0.6
0.8
1

(c) Sex-specificity of responses to sexual and genetic novelty

Our results indicate that sexes differ in their response to sexual and genetic familiarity of mates, with males preferring to court sexually and genetically novel females, and females displaying weak preferences for sexually and genetically familiar males. Thus, in both sexes the response to sexual familiarity predicts the response to genetic familiarity. However, evidence for biases in mating success was only apparent when females were immobilised. Due to the rarity of copulations with decapitated females, we had low power for detecting biases in matings when female sexual behaviour was abolished. Nevertheless, our results are broadly consistent with the hypothesis that mating success cannot be attributed exclusively to either male (courtship) or female (rejection) responses (van den Berg et al. 1984; Ödeen & Moray 2008).

The preference for genetically novel females observed in our fruit fly population may reflect a widespread male behaviour. For example, male sweat bees *Lasioglossum zephyrum*, when previously exposed to a female, elicit more mating attempts with a second female if she is less genetically related to the first female (Smith 1983). Because this study prevented mating with the first female, it was not clear as to whether such male preference is linked to mating. Male preferences for genetically novel females might be the consequence of avoidance of previous mates (e.g. as a non-adaptive bi-product of the Coolidge effect). Another, non-mutually exclusive explanation for a male preference for genetically novel females, is that males might benefit by mating with genetically dissimilar females through the higher genetic diversity of their offspring (Brown 1997). However, the benefits of genetically diverse offspring in the fruit fly are still currently unclear (Brown et al. 2004; Slatz et al. 2012) and further studies could be conducted to understand the proximate function (e.g.

minimise risk of mating with previous mates) of this behaviour.

The functional significance of the weak female preferences for sexually and genetically similar mates is currently unclear. In fact, this result appears to contrast with recent studies which show that female fruit flies mate more frequently in groups composed of males from more than one lab strain (Billeter et al. 2012) and discriminate against mating with socially familiarised males (Ödeen & Moray 2008). However, we do not know to what extent differences between individuals from different lab strains (as in reference Billeter et al. 2012) can be compared to differences between individuals within a population (as in our study), or to what extent non-copulatory experiences (as in reference Ödeen & Moray 2008) can be compared to copulatory experiences (our study) in their impact upon future sexual behaviour. Female preferences for genetically similar mates in our study is also in contrast to predictions of the rare male effect where the male of the rare genotype is attains a higher mating success (Partridge 1988). The rare male effect has been reported in many laboratory studies of *Drosophila* species (Knoppien 1985, Partridge 1988). However, the rare male effect may be less common than was actually supposed due to problems with observer bias and lack of repeatability both with experimental design and with data analysis (Knoppien 1985; Partridge 1988). Furthermore, there is much debate as to whether the rare male effect is due to female preferences, male competition (where the rare male competes more vigorously for females) or male activity (where the rare male compensates for its rarity by becoming more sexually active) (Knoppien 1985, Partridge 1988). Our study suggests that female may actually prefer genetically similar males and that choice exert by females might not account for the rare male effect.

Female preferences for genetically familiar males may be to avoid the potential costs of mating with genetically varied males. For example, in some species females may incur costs from seminal diversity (Fedorka & Zuk 2005), or from mating with males that are unrelated to each other (Ala-Honkola 2011). Another intriguing possibility is that female preference for genetically familiar mates might be the result of manipulation of female behaviour by the mating male (e.g. through seminal fluid peptides). This could potentially increase the chances that if the ejaculate of a focal male is to face sperm competition, such competition is restricted to males that are more related to the focal male than the average male in the population. These hypotheses should be explored in future studies.

(d) The role of olfaction

We found that, in contrast to wild-type males, *Orco*^{1} mutant males do not bias courtship toward genetically novel females ($\chi^2_1 = 1.43$, $P = 0.231$; Figure 1C). As expected, the first female that males mate with is equally likely to be genetically familiar or genetically novel (Supplementary Table 1; Figure 1D). When exposed to decapitated females, *Orco*^{1} males court the genetically novel female more frequently than the genetically familiar female ($\chi^2_1 = 30.31$, $P < 0.001$; Figure 1C), but this bias in courtship is significantly weaker than in wild-type males (preference in wild-type males *versus* preference in *Orco*^{1} males; $\chi^2_1 = 29.63$, $P < 0.001$; Figure 1C). Only two matings occurred with decapitated females in the experiments using *Orco*^{1} males, both of which were with genetically familiar females. Similarly, *Orco*^{1} females showed no sexual preferences in relation to genetic familiarity: there was no significant difference in rejection rate directed towards either genetically familiar or genetically novel males, ($\chi^2_1 = 1.83$, $P = 0.176$; Figure 2C), no difference in the latency to remate with either

male type ($\chi^2_1 = 0.90$, $P = 0.344$; Figure 2B), and no difference in the probability of mating (Supplementary Table 1; Figure 2D). As with the experiments on wild-type females, we detected no difference in courtship intensity between male types ('rejection' experiment, *Orco*^{1}: $\chi^2_1 = 2.43$, $P = 0.12$; 'remating latency' experiment, *Orco*^{1}: $\chi^2_1 = 0.20$, $P = 0.65$).

These results show that *Orco* is required for males and females to display full preferences for genetically novel or familiar members of the opposite sex. This suggests that sex-specific responses to genetic novelty are at least in part controlled by olfactory cues, and indicates that olfaction may be key to discrimination between individual potential mates. Olfactory signals are important mediators of species and sex discrimination among many insects (Shorey 1976; Spiess 1987) and since most insects rely on olfaction as the predominant sensory modality, their chemosensory systems have been fine-tuned to high levels of sensitivity and specificity (Hildebrand & Shepherd 1997). Thus, insects have the potential for distinguishing individual differences in pheromonal make-up. In contrast to our knowledge of individual recognition in mammals (Hurst et al. 2001; Johnston & Bullock 2001), we still know little about the mechanism mediating individual discrimination in insects (Barrows et al. 1975; Tibbetts 2002). Our results lay the foundations for future work that can focus on establishing the potential role of specific pheromones in individual mate discrimination in the fruit fly. The fruit fly also use a variety of senses – vision, hearing, touch, taste and smell – to assess individuals and mediate sexual behaviours (Mery et al. 2009; Bretman et al. 2011). The preference for genetically novel mates by *Orco*^{1} males in the beheaded female trials, though significantly weaker than wild-type males, suggests that non-olfactory cues may also play a role in mediating this

behaviour. Future studies should aim to elucidate the relative importance of multiple senses.

Conclusions

Our results show that the fruit fly respond behaviourally to the sexual and genetic novelty of their potential mates. Behavioural responses to the sexual and genetic novelty of mates are likely to evolve in species with limited or sex-biased dispersal prior to mating because this increases the probability of interacting with a previous mate or their relatives. It will be important to determine to what extent these type of responses are shared, or differ, in other taxa, and how this relates to patterns of dispersal and interaction rates. Our findings show that male and female fruit flies have divergent responses to sexual and genetic novelty, indicating that selection may have acted differently on the sexes. Further research is needed to determine the fitness consequences of these behaviours and to uncover the underlying evolutionary dynamics. It will be particularly intriguing to see whether male and female preferences influence mating patterns in larger social groups where more potential mates are available, or whether, as we found, the behaviour of neither sex is able to determine the mating outcome. Finally, our data show that both sexes use olfaction in choosing which individuals to sexually pursue or resist. This opens the door to elucidation of the specific mechanisms underlying individual mate choice in this key genetic model organism.

Acknowledgements

We thank Jennifer Perry, Sozos Michaelides, Grant McDonald for their help during the experiments; and Ada Grobowska and James Sciberras for comments on the manuscript.

SUPPLEMENTARY TABLE

Supplementary Table 1. Sex-specific mating responses of (1) wild-type, (2) *Orco*^{1} fruit flies to genetic novelty of potential mates for the 'vial' experiments. F = genetically familiar, N = genetically novel. Values for mating latency and mating duration are in minutes. Test statistic values were based on the χ^2-distribution except for remating duration where the test statistic is based on the F-distribution. *P*-values that are significant are highlighted in bold.

Response variable	Values (mean ± S.E.)	df	Test statistic	*P*
(1) Wild-type				
(i) Male responses				
(a) Proportion of courtship events	F: 0.42±0.05; N: 0.58±0.05	1	6.729	**0.009**
(b) Remating latency	F: 142.9±23.2; N: 142.7±30.1	1	0.408	0.523
(c) Remating duration	F: 20.0±1.1; N: 20.1±1.1	1,34	0.001	0.973
(d) Remating probability	F: 0.50±0.08; N: 0.50±0.08	1	0.000	1.000
(ii) Female responses				
(a) Remating latency	F: 463.6±49.4; N: 674.3±81.9	1	5.541	**0.019**
(b) Remating duration	F: 20.0±1.4; N: 19.0±1.4	1,38	0.282	0.598
(c) Remating probability	F: 0.48±0.08; N: 0.52±0.08	1	0.100	0.752
(2) *Orco*^{1} mutants				
(i) Male responses				
(a) Proportion of courtship events	F: 0.49±0.04; N: 0.51±0.04	1	1.434	0.231
(b) Mating latency	F: 163.1±26.6; N: 135.6±35.6	1	0.147	0.702
(c) Mating duration	F: 19.3±1.5; N: 19.9±12.4	1,34	0.043	0.836
(d) Mating probability	F: 0.53±0.08; N: 0.47±0.08	1	0.111	0.739
(ii) Female responses				
(a) Mating latency	F: 716.0±83.3; N: 647.6±81.2	1	0.895	0.344
(b) Mating duration	F: 18.4±1.3; N: 18.6±1.2	1,37	0.007	0.942
(c) Mating probability	F: 0.51±0.08; N: 0.49±0.08	1	0.026	0.873

THEME IV

RELATEDNESS,

MALE-MALE COMPETITION

AND SEXUAL CONFLICT

Chapter 7

Pre- and post- copulatory sex-specific responses to male-male relatedness in the red junglefowl

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ABSTRACT

Kin selection theory predicts that males should compete less intensely with closer relatives over reproductive opportunities. While there is some empirical evidence consistent with this expectation, few studies have simultaneously investigated the intensity with which males compete to mate with individual females and the intensity with which their ejaculates compete to fertilise a set of eggs. Importantly, no study has considered the role of pre- and postcopulatory female responses to male relatedness. Here, we experimentally test the effect of male-male relatedness on pre- and postcopulatory intrasexual competition and female preference in social groups of red junglefowl. Consistent with predictions, in groups in which two closely related males and an unrelated male competed to mate with three females (unrelated to the males), males were more aggressive towards unrelated males and interrupted the mating attempts of unrelated males more frequently. Contrary to predictions however, during sperm allocation trials, a male invested more sperm in a female when she had previously mated with a relative of the focal male, compared to when she had mated with a male unrelated to him. Females, on the other hand, when given a choice of three males, showed a marked preference to mate with the single unrelated male than with either of the two related males. As a result, the unrelated male gained a higher proportion of successful mating than each of the two related males. In addition, we found some evidence that females might also bias postcopulatory sperm utilization in favour of the unrelated male. Patterns of female preference suggest that local clusters of related males might play a disadvantaged role in mating and sperm competition, which may explain the seemingly counterintuitive preferential sperm investment in a female following mating by relatives. Our results indicate the importance of considering the active role of females in models of postcopulatory sexual selection.

INTRODUCTION

Kin selection theory predicts that social behaviours which reduce the fitness of an actor can evolve when direct costs are compensated by the inclusive fitness benefits derived by a related recipient (Hamilton 1964a, b). Evidence of kin selection thus far has been largely from cooperative breeding in vertebrates (e.g. Emlen 1997; Russell & Hatchwell 2001) and studies on the evolution of eusociality in insects and vertebrates (e.g. Crozier & Pamilo 1996; Sherman et al. 1991). Indirect benefits of sociality in such systems are mediated through natural selection. Kin selection may also apply when the benefit is mediated through sexual selection, and this is mainly emphasised through examples in lekking species (Kokko & Lindström 1996; HÏglund et al. 1999; Krakauer 2005; Reynolds et al. 2008; but see McDonald & Potts 1994). Leks are the gathering of males for competitive mating display where mating success is typically highly skewed and the majority of males fail to reproduce. Non-preferred males can enhance their inclusive fitness by helping relatives gain a higher mating success.

In addition to effects on precopulatory competition, kin selection has the potential to influence the intensity of postcopulatory competition (Parker 2000). Because the amount of sperm a male can produce is limited and costly, males of many species allocate their sperm strategically (Wedell et al. 2002; Parker & Pizzari 2010; Kelly & Jennions 2011). When the perceived risk of competition is high, males are predicted to invest more sperm in the female (Parker et al. 1997). However, when the relatedness of rivals is known to males, theory predicts that males should actually reduce their sperm expenditure when competing against related rivals than when competing against unrelated males, even when the risk of sperm competition is high (Parker 2000). In large unrelated populations, this strategy would enable males to enhance their relative's

reproductive success as well as benefit themselves through inclusive fitness, while preserving their own sperm reserves for future competition against unrelated rivals.

Despite the importance of kin selection in sexual interactions, there are still several gaps in our current understanding of how kinship can influence sexual dynamics. First, there is limited literature on kin selection mediated by sexual selection in non-lekking species and there is currently no evidence that kinship is a major determinant of the degree of competition between male sexual competitors (Packer & Pusey 1982; Grinnell et al. 1995). Second, there has been little emphasis on the effects of kin selection on male sperm allocation and the two empirical studies that have examined sperm competition games between related males reported no adjustment in ejaculates in response to relatedness of male rivals (Thomas & Simmons 2008; Ramm & Stockley 2009). Third, empirical studies and theoretical models addressing kin selection have often neglected the active role of the opposite sex that might modulate the intensity of male-male competition (Birkhead & Møller 1998; Parker 2000). For example, females' preference for the rare male (unrelated male) in a social group, places any of the relatives in a disadvantaged role and potentially counteract kin selection for altruism. We aim to address these gaps with an experimental approach using a model system, the red junglefowl *Gallus gallus* ssp.

The red junglefowl is phylopatric with limited dispersal in natural unconfined settings (Collias et al. 1966; Collias & Collias 1996). The occurrence of mating between siblings and between mothers and sons in natural groups suggests that relatives interact and no apparent sex-biased dispersal suggests that there is an increased likelihood of interacting with same-sexed relatives. (Collias & Collias 1996). In addition, well-

documented episodes of pre- and postcopulatory sexual selection in this species (Løvlie 2007; Pizzari et al. 2003; Pizzari et al. 2004) allow us to explore proximate mechanisms underpinning sex-specific responses to male-male relatedness. Here, we study the effect of male-male relatedness on intrasexual competition and intersexual conflict using a population of red junglefowl. First, we investigate male-male interactions and female preference in social groups, in which two closely related males and an unrelated male compete to mate with three females (who are unrelated to the males). Second, we examine the ejaculation strategies of focal males upon witnessing his relative or a non-relative mating with the experimental female. Third, we explore postcopulatory female response to the relatedness of the second mate relative to the first mate.

MATERIAL AND METHODS

(a) Study population

The study was conducted on an individually marked population of red junglefowl *Gallus gallus* ssp. ($n_{males} = 25$, $n_{females} = 20$) at the Oxford University John Krebs Field Station in Wytham, Oxfordshire. A pilot study on 'semi-natural populations' (see below) was conducted in August – September 2010. This was followed by a comprehensive study on 'semi-natural populations' and 'controlled copulations' in May – June 2011 and May – June 2012. Individuals were genotyped at 31 variable microsatellite loci (Worley et al. 2010) and male-male relatedness was determined by calculating pairwise relatedness based on microsatellite data (Queller & Goodnight 1989). Therefore, pairs considered related had coefficient of relationship (r) $0.45<r<0.6$ and those considered unrelated had a coefficient of relationship (r) <0.05 and >-0.05. We avoided using male pairs that had $0.05<r<0.45$ to ensure that related and unrelated

males differed in their degree of relatedness. Male and females were raised together and were thus familiar to one another. Prior to the start of each trial, males were kept isolated from the females for at least two days to allow replenishment of sperm reserves (Parker et al. 1942). All experiments were conducted blind with respect to the relatedness between individuals.

(b) Semi-natural populations

We studied precopulatory mating behaviour in social groups of three males and three females in indoor pens (2010: $n_{trials} = 12$; 2011: $n_{trials} = 16$). Therefore, groups had a sex-ratio of 1:1, within the natural range of sex-ratio observed in natural groups (1:1-1:4 males to females; Collias & Collias 1996). In each group, two of the males were related while the third was unrelated to either of the related males. All females were unrelated to one another and unrelated to the males. Trials started on the afternoon of day 1 when the individuals were released simultaneously into the pen and the trials lasted for two hours. Thereafter, observations were made on the morning and afternoon of day 2, each observation lasting two hours each. The times of the morning and afternoon observations were 8am - 10am and 6pm - 8pm respectively. After each observation, females were removed from the pen and sexually isolated from the males until the next observation.

(bi) Male response

Male and female identity were recorded in copulation attempts. To investigate how kinship modulates the intensity of male-male competition, we monitored: (i) any interference of a copulation attempt by other males, (ii) aggression level of the interference, which was scored on a gradient of 1 to 3 (1: male tried to interfere but

with no physical contact with attempting male; 2: male interrupted with physical contact; 3: male interrupted mating attempt and thereafter interacted aggressively with the attempting male), and (iii) number of aggression events between two males: chasing, pecking, pinching of comb, fighting, and male-male waltzing (male waltzing is performed as an act of aggression to other males and as courtship towards females, Wood-Gush, 1956, 1958; Kruijt, 1964). If two actions occurred simultaneously or within five seconds of each other, e.g. a male waltzes and the other initiates a fight, these were recorded as one aggressive event.

We also assessed the social status of males based on the number of times one male avoided the other, i.e. moved away from the approaching male. This excluded the acts of aggression mentioned above. Male A was regarded as being dominant over male B when A was more likely to be avoided by B than vice versa (Froman et al. 2002; Gulh et al. 1945). We also recorded the number of courtship events and number of male-initiated attempts directed by individual males to examine the effect of male type (related or unrelated) on male behaviour (Løvlie & Pizzari 2007; Chapter 5). Male-initiated attempts were defined as events in which the males attempted to mate with the female and excluded events in which the female solicited. Courtship behaviour consisted of the waltzing (the male lowers one wing and circles hen) and 'tidbitting' (courtship feeding) (Zuk et al. 1990) but the latter was only recorded when it was clear which particular female the male was directing the feeding towards.

(bii) Female response

Female response to different male types (related or unrelated; R or U) was measured as the: (i) level of female resistance (1 – 5; 5 being the highest level of resistance, see

Løvlie & Pizzari 2007 for a detailed description of these levels), (ii) proportion of copulation attempts resisted (4 – 5 on the resistance score), and (iii) probability of solicitation to each male (1 on the resistance score) (Løvlie & Pizzari 2007). Copulation was recorded as successful if the male lowered his train over the female's cloaca or if cloacal contact was observed between the male and female.

(c) Controlled copulations

(ci) Male response

This experiment examined the effect of relatedness of a male competitor on a focal male's sperm investment (2011: $n_{trials} = 31$). Females were fitted with a harness covering their cloaca, allowing for effective ejaculate collection. We held the female facing the male for one minute before holding the female in a soliciting position and allowing the male to mate with her (Pizzari et al. 2003). It is known that males may sometimes not release any sperm upon first copulation; therefore each trial was run for 20 minutes or until one spermic copulation had taken place (Pizzari et al. 2003).

Three males (two related and one unrelated) were housed together one day before each trial to enable the establishment of a dominance hierarchy. One of the two related males was randomly chosen as the focal male. On day 1, one of the non-focal males (related or unrelated to the focal male) was allowed to copulate with a female (fitted with a harness) in full view of the other group members. Immediately after this copulation, the focal male was presented with the same female and allowed to copulate with her. After a minimum of two days when his sperm reserves are replenished, the focal male was allowed to copulate with the same female after the other type of male (related or unrelated) has copulated with her. To avoid potential order effect, the order with which the non-focal related male or non-focal unrelated male was allowed to

copulate with the female was alternated in a balanced design.

As indications of the focal male's propensity to copulate and invest sperm, we recorded the time to first mating attempt and the number of sperm allocated to the first spermic copulation. We collected the ejaculate and measured ejaculate volume to the nearest 0.5 µl using a Gilson pipette. To quantify the concentration of sperm, 2.5 µl of the ejaculate was mixed with 197.5 µl of phosphate buffer saline solution and absorbance value was measured at 595 nm wavelength with a spectrometer (Scientific Laboratory Supplies, UV 1101). If the semen sample was too diluted or concentrated, we added more sperm or phosphate buffer saline solution respectively and thereafter adjusted the absorbance value accordingly. Because sperm absorbance is directly correlated with sperm concentration (Ciereszko & Dabrowski 1993; Donoghue et al. 1996), we were able to calculate standardised set of values for the sperm numbers allocated by multiplying the absorbance value with the ejaculate volume. All absorbance values were corrected for the dilution factors.

(cii) Female response

This experiment examined female postcopulatory response to relatedness between past and potential mates (2012: n_{trials} = 22). Three males (two related and one unrelated) were housed together prior each trial and assessed for dominance hierarchy. In each group, the focal male was either one of the related male type or the unrelated male type. Experimental females were sexually isolated from males for two weeks prior to trials in order deplete any sperm reserves in the female (Pizzari et al. 2004). On day 1, a female unrelated to any of the males was fitted with a harness and presented face-to-face to one of the related male types. Thereafter, the female was turned around and mounted

by this male. Immediately after this copulation, the focal male (the other related male type or the unrelated male type) was presented with the same female and allowed to copulate with her without the harness. We recorded the copulation using two Toshiba Camileo X400 camcorders placed at a right angle relative to each other and focusing on the female cloaca. Ejection or acceptance of an ejaculate was determined as in Dean et al. (2011). We also quantified the number of sperm that were ejected, if any. This was done in a similar manner as mentioned above, where number of sperm ejected was estimated by multiplying the absorbance of the sample by its volume. After a minimum of 48 hours to allow for sperm replenishment, the focal male was placed with two other males in which his status was reversed (i.e. related male type if his previous status was unrelated male type and vice versa). The focal male was then allowed to copulate with another female after she was mounted by one of the related male type. To avoid potential order effect, the order with which the focal male was the related or unrelated male type was alternated in a balanced design.

After insemination, females were fed with coloured dyes (Sudan black or Sudan red), kept in pairs and eggs collected for 10 days. Eggs were opened and identified as belonging to either female using the colour of the yolks. We then quantified the number of hydrolysis points on the outer perivitelline layer (PVL) of the egg (Pizzari et al. 2004).

(d) Statistical analysis

(di) Semi-natural populations

We analysed the effect of male-male relatedness on competitive behaviour in the 'semi-natural populations' trials using Generalised Linear Mixed Models (GLMM). Three

separate GLMMs analysed variation in three male response variables: 'probability of interruption' with Binomial error distribution, 'aggression level of interruption' with Normal error distribution and 'number of aggressive interactions' with Poisson error distribution. In all GLMMs, 'male-male relatedness' (related or unrelated) and 'relative dominance' (i.e. whether the status of the interrupting male was higher or lower than the status of the attempting male) were entered as independent variables and 'relatedness*relative dominance' was entered as an interaction. 'Year' (2010 or 2011) was entered as a covariate. For GLMMs that analysed variation in 'probability of interruption' and 'aggression level of interruption', 'female identity' nested within 'attempting male identity' nested within 'interrupting male identity' nested within 'trial' was entered as a random factor. For the GLMM with 'number of aggressive interactions' as the response, 'recipient identity' nested within 'aggressor identity' nested within 'trial' was entered as a random effect. The 'aggressor' was defined as the individual that chased or won in a fight with the 'recipient'. Aggressive events might differ in intensities, for example, waltzing is milder compared to chasing, peaking, fighting and comb-pinching. Therefore we analysed the variation in 'number of aggressive interactions' with and without counts of waltzing. The significance of the fixed factors was assessed using the likelihood-ratio test on models with and without the fixed factor (Valdar et al. 2006; Ockinger et al. 2010).

We also tested the effect of 'male type' (related or unrelated) on the 'number of courtship events' and 'number of male-initiated attempts' using two separate GLMMs. 'Male type' (R or U) and 'male dominance status' (1 – 3; 1 being the most dominant) were entered as independent variables and 'male type*male dominance status' was entered as interaction. 'Year' was entered as a covariate. 'Female identity' nested

within 'attempting male identity' nested within 'trial' was entered as a random factor.

We analysed variation in female response through GLMMs with 'male identity' nested within 'female identity' nested within 'trial' as a random factor, 'year' as a covariate, 'male type' (R or U) and 'male dominance status' (1 – 3; 1 being the most dominant) as fixed factors and 'male type*dominance' as an interaction. The three response variables entered in three separate GLMMs were 'average female resistance' using a Normal error distribution, 'proportion of attempts resisted' using a Binomial error distribution and 'probability of solicitation' using a Binomial error distribution. We also tested variation in mating success through a GLMM with Binomial error distribution with mating success (yes or no) as the response variable.

(dii) Controlled copulations

To test whether males respond differentially to related and unrelated competitor males, we analysed variances through GLMMs, 'male-male relatedness' (relatedness of focal male to first male) and 'relative dominance' (dominance of focal male relative to dominance of non-focal male) as fixed factors, 'relatedness*relative dominance' as an interaction, 'treatment order' (1^{st} or 2^{nd}) as a covariate and 'focal male identity' nested within 'trial' as a random factor. To account for the different number of aspermic attempts made by the focal male, we entered this variable as a covariate. The response variables used in two separate GLMMs were 'probability of spermic copulation' (yes or no) using a Binomial error distribution and 'relative sperm invested' using a Normal error distribution. 'Relative sperm invested' is the sperm numbers (volume multiplied by absorbance) invested in female A in the 1^{st} trial minus that invested in female B in other trial (Pizzari et al. 2003). Similarly, the significance of the fixed factor

'relatedness' was assessed using the likelihood-ratio test on models with and without the fixed factor. We also tested the idea that males modulate their ejaculate composition (seminal fluid *versus* sperm numbers) (e.g. Wigby et al. 2009; Simmons & Beveridge 2011) accordingly to the relatedness of their rivals using a similar GLMM except that we entered ejaculate absorbance (proxy of sperm concentration) as the response variables. For a given ejaculate volume, lower ejaculate concentration would suggest an increased allocation to seminal fluid.

To test the idea that females differentially eject the ejaculate of males related or unrelated to the first male, we used GLMMs with Binomial error distribution, 'male-male relatedness', 'relative dominance' as fixed factors, 'relatedness*relative dominance' as an interaction, 'average sperm numbers of focal male' (from averaging sperm numbers of the focal male when he was the first male in other trials) as a covariate, 'focal male identity' nested within 'female identity' as a random factor. Two separate GLMMs analysed variation in two female response variables: 'probability of ejaculate ejection' and 'proportion of ejaculate ejected'. To calculate the average sperm numbers invested by individual males, we calculated the average of sperm numbers invested by each male in trials where the male was used as the first male. This measurement was used instead of the sperm numbers invested when the male was the second male because we found that males adjusted sperm allocation when they copulated after another male (see Results below). In addition, we found no difference in the sperm numbers invested when the first male was the related or unrelated male type ($\chi^2_1 = 0.084$, $P = 0.772$; data not shown). Therefore, we could average the sperm numbers for individual males across the different trials. The proportion of ejaculate

ejected was calculated using the average sperm numbers as an estimate of the sperm numbers delivered.

We also analysed variation in PVL hydrolysis points using two models: one GLMM with Poisson error distribution on the variation of number of PVL hydrolysis points and another GLMM with Binomial error distribution on the probability of having PVL hydrolysis points (yes *versus* no). 'Male-male relatedness', 'relative dominance' and 'lay-date' were entered as fixed factors, 'relatedness*relative dominance' and 'relatedness*lay-date' as interaction terms, 'average sperm numbers of focal male', 'proportion of ejaculate ejected' as a covariate, 'focal male identity' nested within 'female identity' as a random factor.

RESULTS

(a) Precopulatory response

Consistent with predictions from kin selection theory, males interrupted a significantly higher proportion of mating attempts of the unrelated male than that of the related male (Table 1; Figure 1A). Similarly, males displayed a significantly higher frequency of aggressive interactions to unrelated males than to related males (Table 1; Figure 1B). This result still held when we removed counts of waltzing from the response variable 'number of aggressive interactions' ($\chi^2_1 = 5.23$, $P = 0.022$). Aggression level of the interruption directed at the different male types, however, did not significantly differ (Table 1). When examining male behaviour towards females, we found no significant differences in the number of courtship events or male-initiated attempts directed by the two male types (related or unrelated; Table 1).

Females resisted a significantly lower proportion of- and displayed a significantly lower average resistance towards the mating attempts of the single unrelated male in the group (Table 1; Figures 2A and 2B). In addition, females were significantly more likely to solicit sex to the unrelated male type as compared to either of the other two related males (Table 1; Figure 2C). Consequently, the unrelated male gained a significantly higher proportion of mating success than either of the two relatives (Table 1).

Table 1. Results of experiments on (1) 'Semi-natural populations'; (2) 'Controlled copulations'. R = related, U = unrelated; H (higher) = interrupter or aggressor is more dominant than the attempting or receiving male, L (Lower) = interrupter or aggressor is more subdominant than the attempting or receiving male. 1 = most dominant, 3 = least dominant. Test statistic values were based on the χ^2-distribution. $0.05 < P < 0.10$ are underlined and $P < 0.05$ are highlighted in bold.

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(1) 'Semi-natural populations'					
(i) Male response					
(a) Proportion of attempts interrupted	Relatedness	R: 0.139±0.018; U: 0.187±0.014	1	6.980	**0.008**
	Relative dominance	H: 0.223±0.020; L: 0.139±0.013	1	21.153	**<0.001**
	Relatedness*Relative dominance		1	3.065	0.690
(b) Aggression level of interruption	Relatedness	R: 1.556±0.116; U: 1.657±0.062	1	0.086	0.769
	Relative dominance	H: 1.662±0.081; L: 1.600±0.075	1	2.024	0.846
	Relatedness*Relative dominance		1	10.26	0.068
(c) Number of aggressive interactions	Relatedness	R: 1.066±0.237; U: 1.642±0.270	1	6.020	**0.014**
	Relative dominance	H: 2.689±0.352; L: 0.207±0.091	1	72.573	**<0.001**
	Relatedness*Relative dominance		1	5.313	0.379
(d) Courtship counts	Relatedness	R: 2.341±0.780; U: 2.277±0.285	1	1.768	0.183
	Dominance of male	1: 3.479±0.642; 2: 1.429±0.339; 3: 2.000±0.543	1	6.800	**0.033**
	Relatedness*Dominance		1	2.596	0.273
(e) Number of male-initiated attempts	Relatedness	R: 1.950±0.218; U: 2.431±0.198	1	0.253	0.615
	Dominance of male	1: 2.964±0.307; 2: 2.176±0.209; 3: 1.583±0.221	1	11.466	**0.003**
	Relatedness*Dominance		1	2.292	0.318

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(ii) Female response					
(a) Average resistance	Relatedness	R: 3.625±0.070; U: 3.202±0.131	1	14.391	**<0.001**
	Dominance of male	1: 3.320±0.072; 2: 3.201±0.083; 3: 3.300±0.104	2	2.064	0.356
	Relatedness*Dominance		2	1.505	0.471
(b) Proportion of attempts resisted	Relatedness	R: 0.616±0.026; U: 0.535±0.035	1	7.104	**0.008**
	Dominance of male	1: 0.608±0.031; 2: 0.559±0.037; 3: 0.585±0.043	2	1.119	0.572
	Relatedness*Dominance		2	0.764	0.683
(c) Probability of solicitation	Relatedness	R: 0.012±0.008; U: 0.119±0.036	1	12.356	**<0.001**
	Dominance of male	1: 0.024±0.017; 2: 0.071±0.028; 3: 0.048±0.023	2	2.018	0.365
	Relatedness*Dominance		2	3.790	0.150
(iii) Mating success					
(a) Mating success	Relatedness	R: 0.279±0.045; U: 0.498±0.076	1	4.750	**0.029**
	Dominance of male	1: 0.440±0.075; 2: 0.343 ±0.063; 3: 0.227±0.057	2	1.855	0.396
	Relatedness*Dominance		2	1.596	0.450
(2) 'Controlled copulations'					
(i) Male response					
(a) Relative sperm investment	Relatedness	R: 11.749±3.722; U: -11.749±3.72	1	19.19	**<0.001**
	Relative dominance	H: 0.971±4.579; L: -0.677±4.132	1	0.0614	0.804
	Relatedness*Relative dominance		1	2.3538	0.125
(b) Probability of investing sperm	Relatedness	R: 0.710± 0.083; U: 0.613±0.089	1	1.158	0.282
	Relative dominance	H: 0.630± 0.095; L: 0.686±0.080	1	0.128	0.720
	Relatedness*Relative dominance		1	0.056	0.813

Response variable	Factors	Values (mean ± S.E.)	df	Test statistic	*P*
(ii) Female response					
(a) Probability of sperm ejection	Relatedness	R: 0.143±0.097; U: 0.188±0.101	1	0.536	0.464
	Relative dominance	H: 0.052±0.052; L: 0.364±0.152	1	8.105	**0.004**
	Relatedness*Relative dominance		1	0.816	0.366
(b) Proportion of sperm ejected	Relatedness	R: 0.053± 0.032; U: 0.004±0.002	1	13.639	**<0.001**
	Relative dominance	H: 0.021± 0.017; L: 0.036±0.030	1	3.102	0.078
	Relatedness*Relative dominance		1	1.077	0.300
(c) Probability of having hydrolysis points	Relatedness	R: 4.77±1.10; U: 7.43±2.35	1	7.802	**0.005**
	Relative dominance	H: 8.20± 2.45; L: 3.81±0.59	1	1.260	0.262
	Relatedness*Relative dominance		1	3.530	0.060
	Relatedness*Lay date		1	0.368	0.544
(d) Number of hydrolysis points	Relatedness	R: 4.77±1.10; U: 7.43±2.35	1	2.978	0.084
	Relative dominance	H: 8.20± 2.45; L: 3.81±0.59	1	1.070	0.301
	Relatedness*Relative dominance		1	0.457	0.499
	Relatedness*Lay date		1	18.578	**<0.001**

Figure 1. Precopulatory male response to related (white bars) and unrelated (grey bars) rivals. On the x-axis is the dominance status of the interrupter relative to the dominance status of the attempting male. Error bars denote S.E. (A) Males interrupted a significantly higher proportion of mating attempts by unrelated rivals than by related rivals (Table 1). (B) There was a significantly higher number of aggressive events that occurred between unrelated males than between related males (Table 1).

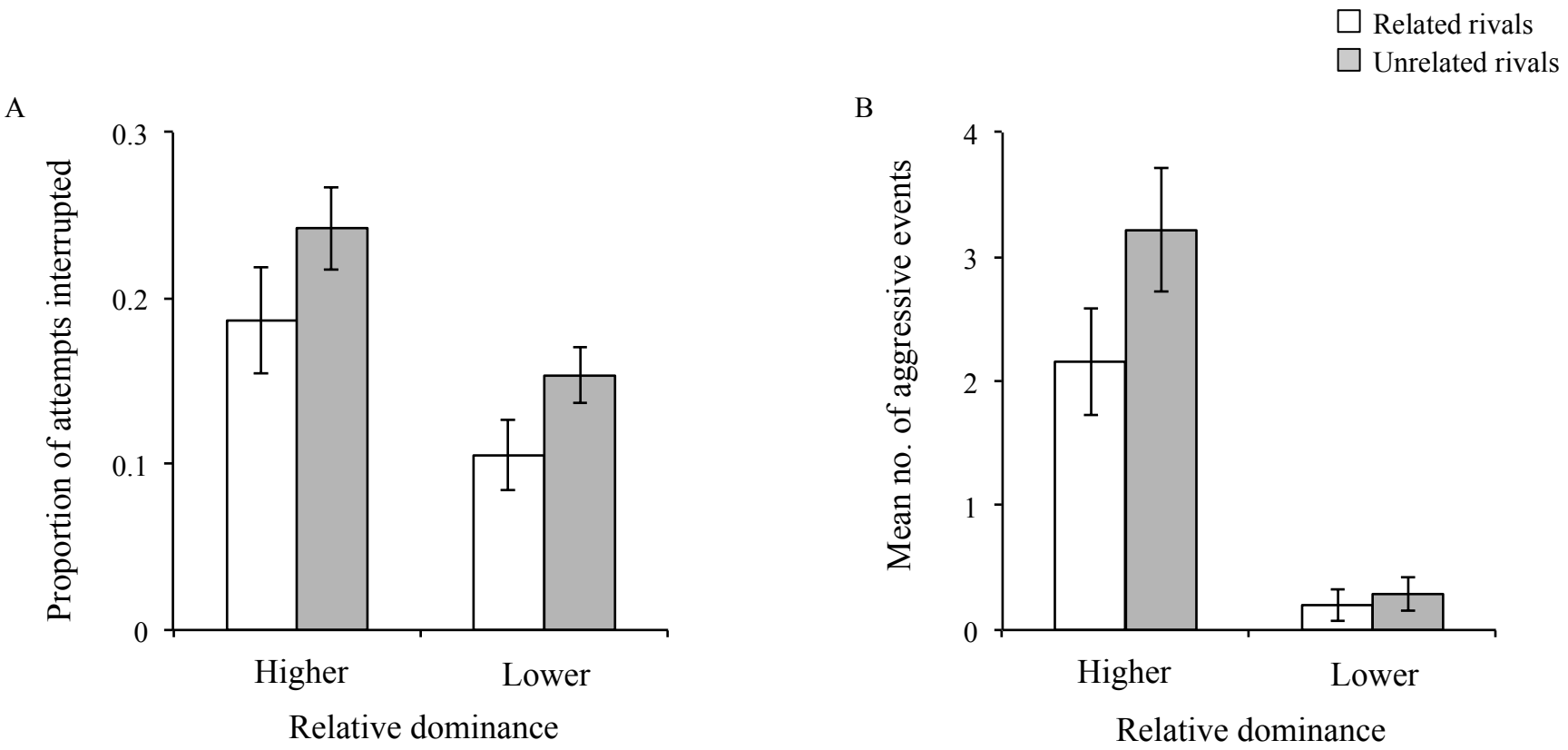
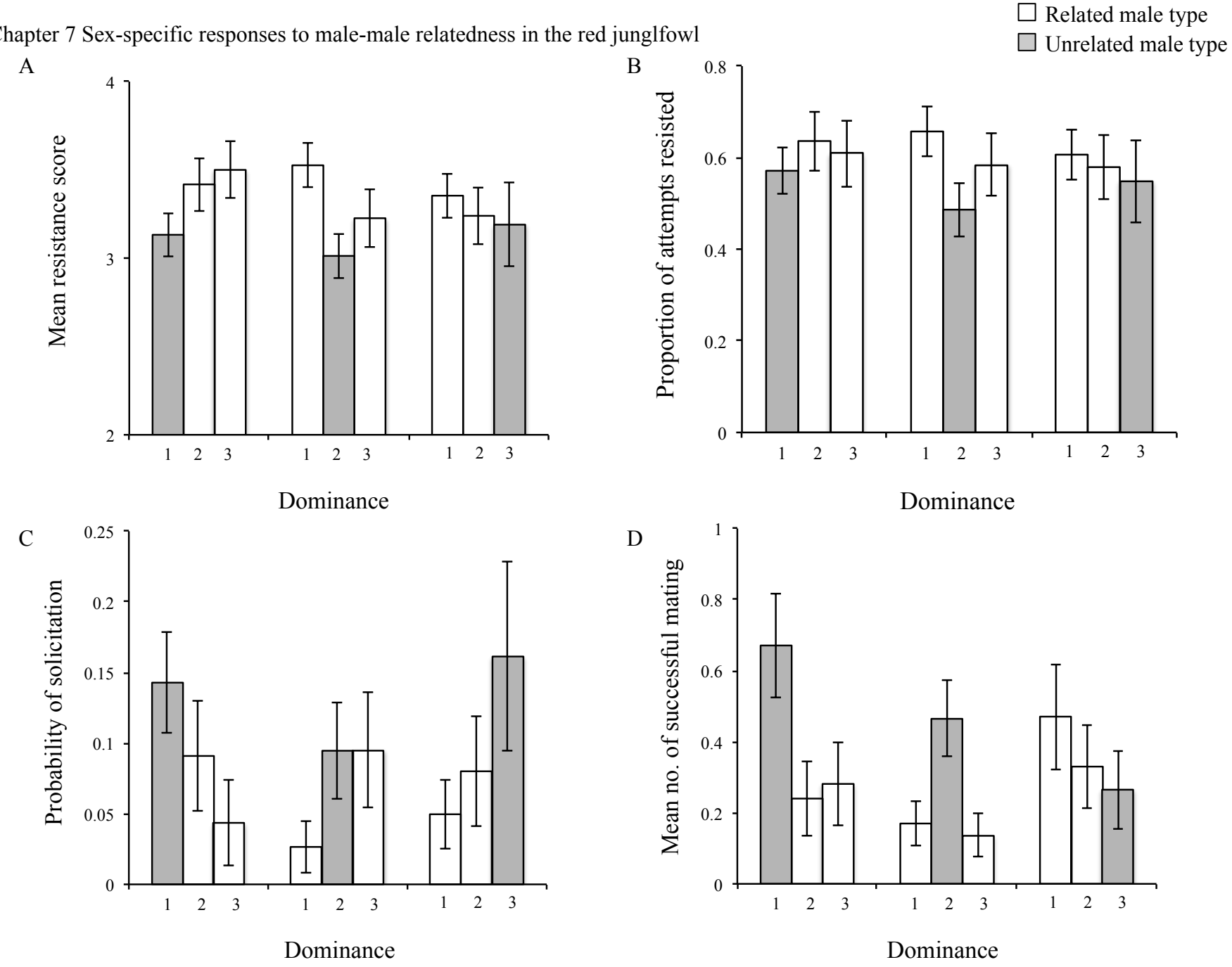

A
Proportion of attempts interrupted
0.3
0.2
0.1
0
Higher
Lower
Relative dominance
B
Related rivals
Unrelated rivals
Mean no. of aggressive events
4
3
2
1
0
Higher
Lower
Relative dominance

Figure 2. Precopulatory female response to related (R) (white bars) and unrelated (U) (grey bars) male types. 1 = most dominant, 3 = least dominant. Trials were grouped according to the dominance status of the U male. Error bars denote S.E. (A) There was a significantly higher female resistance to mating attempts by R males than by the U male. (B) Females resisted a significantly higher proportion of mating attempts by R males than by the U male. (C) Females displayed a significantly higher probability of soliciting to the U male than any of the R males. (D) There was a significantly higher mating success attained by the U male than any of the R males. Results are displayed in Table 1.


Related male type
Unrelated male type
A
Mean resistance score
4
3
2
1 2 3 1 2 3 1 2 3
Dominance
B
Proportion of attempts resisted
0.8
0.6
0.4
0.2
0
1 2 3 1 2 3 1 2 3
Dominance
C
Probability of solicitation
0.25
0.2
0.15
0.1
0.05
0
1 2 3 1 2 3 1 2 3
Dominance
D
Mean no. of successful mating
1
0.8
0.6
0.4
0.2
0
1 2 3 1 2 3 1 2 3
Dominance

(b) Postcopulatory response

There was no significant difference in a male's propensity to copulate with a particular female after she mated with a related or unrelated male (Table 1). Contrary to predictions however, males invested significantly more sperm in a female after she mated with a related male rather than when she had mated with an unrelated male (Table 1; Figure 3). This effect still held when males that did not copulate in either one or both trials were excluded from the analysis ($\chi^2_1 = 17.38$, $P < 0.001$). Controlling for ejaculate volume, we found no significant differences in the ejaculate absorbance, suggesting that males did not differentially modulate their ejaculate composition according to rival relatedness ($\chi^2_1 = 2.44$, $P = 0.118$).

The risk of sperm ejection by females was generally low and similar between related and unrelated males. However, females ejected a significantly lower proportion of sperm numbers by focal males unrelated to the first male (Table 1; Figure 4A). In addition, despite the observation that males invest more sperm in a female when they are related to her previous mate (see above), the probability of sperm presence (detected through the presence of hydrolysis points) on the egg PVL was significantly lower for males related to the first mate (Table 1). Consistent with the idea that females might bias sperm utilization against the sperm of related males, we also found a marginally non-significant tendency for the eggs produced by females following mating with males related to the first male to contain fewer PVL hydrolysis points than the eggs of females mated by males unrelated to the first male (Table 1; Figure 4B). The significant interaction between day and male relatedness indicates that the although males related to first males attained higher number of hydrolysis points on days 1 and 2, males unrelated to first males attained higher number of hydrolysis points

on day 3 and thereafter (Table 1; Figure 4B). There was also a marginally non-significant negative correlation between the proportion of ejaculate ejected and the number of hydrolysis points ($\chi^2_1 = 2.81$, $P = 0.094$), suggesting that female postcopulatory behaviour may have an active role in differential sperm retention.

Figure 3. Postcopulatory male response to related (white bars) and unrelated (grey bars) rivals. Focal males were allowed to copulate with a female fitted with a harness after witnessing a related rival or an unrelated rival copulating with the female. Relative sperm investment = sperm invested in female A – sperm invested in female B. Higher = focal male is more dominant than non-focal male (1st male); Lower = focal male is less dominant than non-focal male. Error bars denote S.E. Focal males invested significantly more sperm in the female that was mated to his relative than to his non-relative (Table 1).

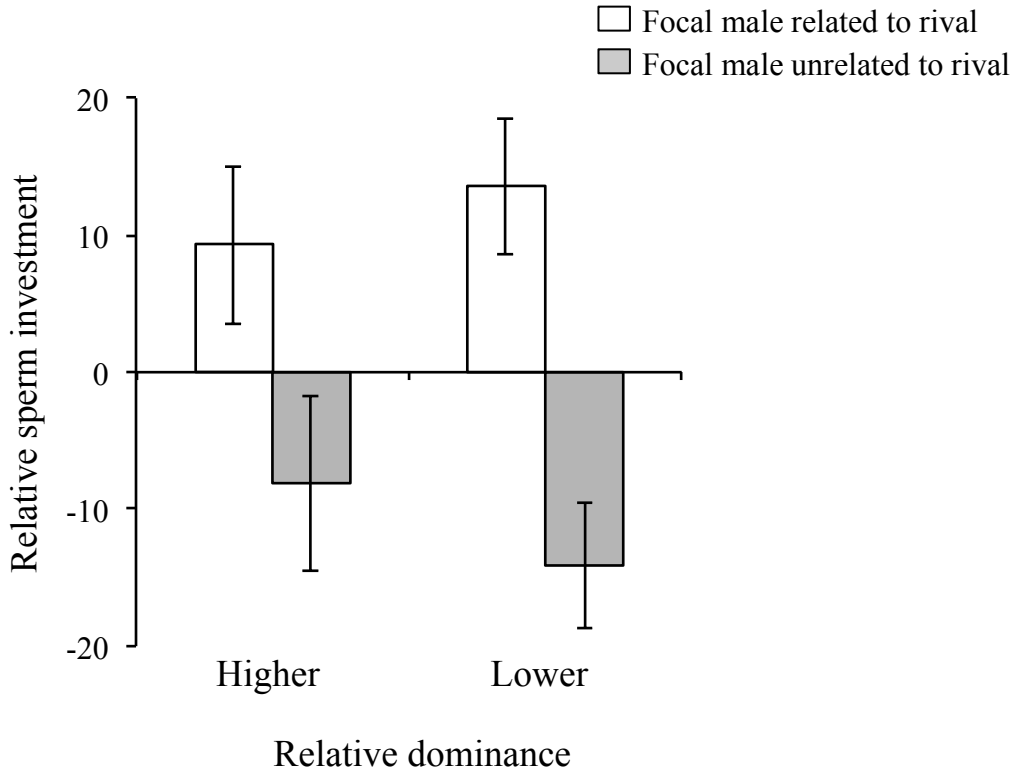
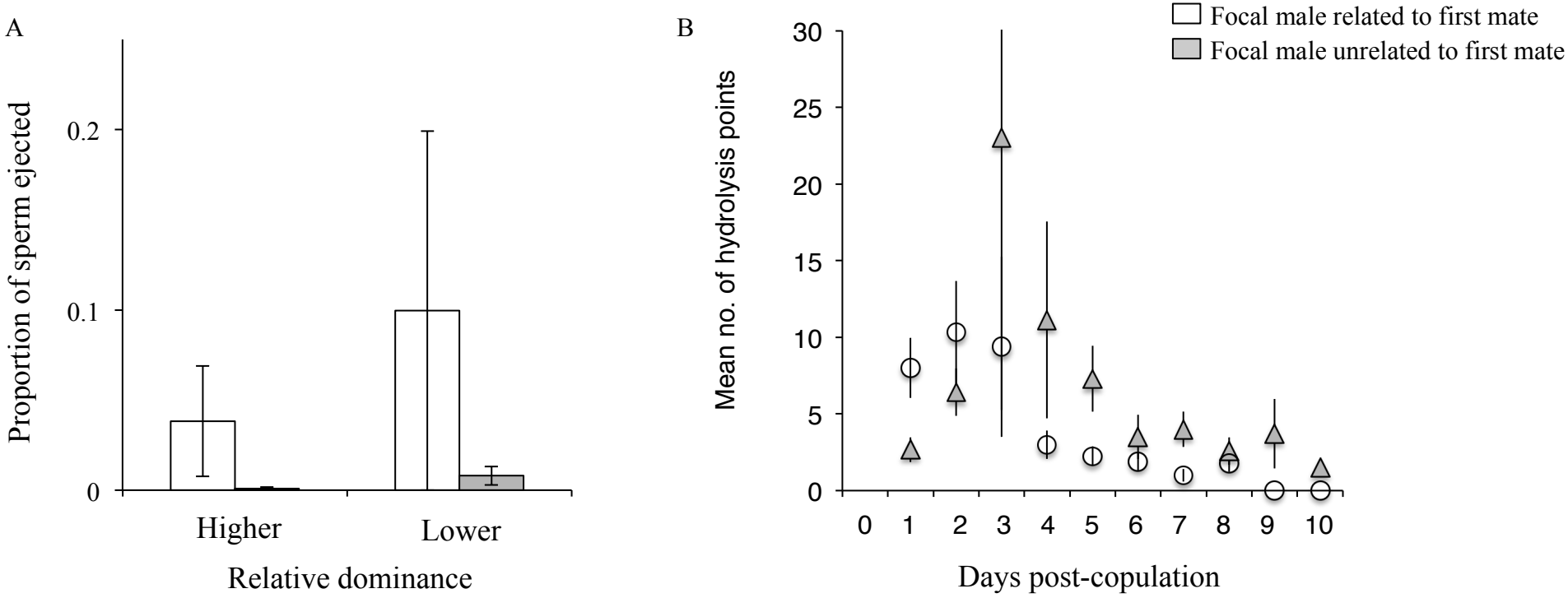


Figure 4. Postcopulatory female response to related (white bars/symbols) and unrelated (grey bars/symbols) male types. Females fitted with harnesses were first mounted by a non-focal male, then allowed to copulate without harness with a focal male that was either related or unrelated to the first male. Higher = focal male is more dominant than non-focal male (1st male); Lower = focal male is less dominant than non-focal male. Error bars denote S.E. (A) Female ejected a marginally non-significantly higher proportion of ejaculate from focal males related to first males than from focal males unrelated to first males. (B) The rate of decline in sperm hydrolysis points on the PVL was significantly slower for males unrelated to the first male than for males related to the first male. Results are displayed in Table 1.



DISCUSSION

Our study demonstrates that the degree of relatedness between rival males modulates the intensity of both precopulatory and postcopulatory male-male competition and female preference. The intensity of male precopulatory competition is reduced between related males, with fewer occurrences of mating interruption and aggressive events. Females, however, displayed a preference for the single unrelated male, indirectly resulting in a higher mating success with this male. When competition is post-insemination, males invest more heavily when their competitor is related. Consistent with their precopulatory response, females appear to exert postcopulatory selection against males that are related to their previous mates.

(a) Precopulatory response

Kin selection theory predicts that social behaviours which reduce the fitness of an actor can evolve when direct costs are compensated by the inclusive fitness benefits derived by a related recipient (Hamilton 1964b). We show that male red junglefowl interrupt mating attempts less often and perform fewer aggressive actions when rivals are related. Such behaviours appear to lower their direct fitness through missed mating opportunities with the same females. However, a male may benefit from an increase in inclusive fitness if the female's eggs are fertilised by a competitor which is more related to himself than the population average rather than by a less related male. The benefit of indirect fitness overriding direct mating success has been used to explain cooperative breeding in vertebrates (Komdeur 1994; Clutton-Brock 2002; Nam et al. 2010), the evolution of altruism in eusocial insects (Queller & Strassmann 1998; Foster et al. 2006) and the evolution of lekking behaviour (Kokko & Lindström 1996). Our results thus provide the evidence that kin selection can influence the mating behaviour

The higher mating success attained by the single unrelated male is consistent with predictions of the rare male effect where the male of the rare genotype enjoys greater mating success (Partridge 1988). The rare male effect has been found to be very widespread in insects, and especially in laboratory studies of *Drosophila* species (Knoppien 1985, Partridge 1988). However, problems with observer bias, lack of repeatability both with experimental design and with data analysis suggest that the rare male effect may be less common than was first supposed (Knoppien 1985; Partridge 1988). In addition, these studies often use mating success as a proxy for rare male advantage and fail to disentangle the role of male and female behaviour. Thus, there is much debate as to whether the rare male effect is due to female preferences, male competition (where the rare male competes more vigorously for females) or male activity (where the rare male compensates for its rarity by becoming more sexually active) (Knoppien 1985, Partridge 1988). Here, we differentiated the relative contribution of these factors and show that the rare male advantage could be a result of both female preference and male-male competition. Male behaviour played a minimal role in the rare male advantage as both related and unrelated male types did not differ in the number of attempts or courtship events. In addition, our study provides first evidence of the rare male advantage in a social group of birds and demonstrates its significant and direct interplay with kin selection in modulating sexual dynamics.

An explanation for a female preference for the genetically rare male is that females might benefit by mating with genetically dissimilar males through the higher genetic diversity of their offspring (Fossøy et al. 2007; Jennions & Petrie 2007). Alternatively,

female behaviour might be the consequence of avoidance of previous mates. The Coolidge effect describes a male's preference for sexually novel females, which has been observed in several polygynous species (Dewsbury 1981). Similarly, females have been shown to prefer sexually novel males in promiscuous mating systems (Lisk & Baron 1982; Bateman 2004). If recognition of closely related mates is subject to error then preference for genetically novel mates could enable individuals to reduce the risk of mating repeatedly with the same male. It will be important to determine the fitness consequences of female preference for rare males in future studies.

(b) Postcopulatory response

Contrary to predictions of previous sperm competition models (Parker 2000), males in the controlled copulation experiments allocated more sperm when competitors were related. We also show that females disfavour the sperm of males related to previous mates by ejecting a higher proportion of semen, resulting in a lower probability of sperm reaching the eggs. Hence, a related male's increased sperm allocation could be a strategy to mitigate the effects of female postcopulatory sexual selection. Consistent with this idea, a recent theoretical model demonstrates that inclusion of female postcopulatory responses that favour unrelated males can lead to males investing more sperm when competing with relatives than with unrelated rivals (Parker, personal communication, Oct 2012).

Nevertheless, females did not show any difference in the probability of sperm ejection between related and unrelated males. This could be due to females being isolated from males for two weeks and being exposed to only two copulations (one without sperm transfer) during trials. Therefore, most females might not be very selective against

sperm given the few mating episodes. Consistent with this idea, the sperm ejection risk was on average low (< 20%), corroborating findings from a previous study (30 - 40% during the female's second mating; Dean et al. 2011). Future studies could therefore aim at increasing the number of male mountings on a female (with harness) to examine if females would then differentially eject sperm from related and unrelated males.

Two alternative mechanisms could potentially explain why males allocate more sperm when competing with relatives. First, males may be attempting to enhance the success of their own sperm and that of relatives by strategically allocating sperm with that of related males. Evidence suggests that sperm viability in a rival male's seminal fluid is lower than that in one's own seminal fluid (Fry & Wilkinson 2004; but see Holman 2009). In addition, a recent study suggests that sperm perform better (although non-significant) in the accessory gland secretions of a related male than that of an unrelated male in polyandrous species of social insects (der Boer et al. 2010). If ejaculates of related males are more similar to each other than those of unrelated males, their seminal fluid components might act cooperatively, or at least less antagonistically. Therefore, it may be beneficial to allocate more sperm with that of relatives to enhance both the focal male's and his relative's probability of fertilisation compared to the unrelated male through synergistic interactions of seminal fluid and sperm. Second, males may simply be less stressed upon seeing a relative copulate with the female because the probability of interruption or aggressive encounters is reduced, therefore males ejaculate more as a non-adaptive side effect. However, we found that the probability of sperm transfer did not differ with male type, suggesting that males were equally keen in copulating with the female after his relative or non-relative.

The higher probability of finding sperm hydrolysis points and a marginally higher number of hydrolysis points reaching the PVL after copulation with males unrelated to first mates could be due to these males allocating more sperm to the female. In our experimental design, we were unable to control for this possibility as we did not investigate sperm allocation strategies of the single unrelated male when he was the second male to copulate with the female. However, two lines of evidence suggest that this possibility is unlikely. First, in the male allocation trials, the sperm numbers invested by the unrelated male when he was the non-focal male did not differ from that of the focal related male (Post-hoc Tukey test, $Z = 1.55$, $P = 0.406$). This suggests that the presence of males related to one another in the group would not modulate the sperm allocation strategy of the unrelated male. Second, a previous study demonstrated that males allocating larger ejaculates suffer from a higher probability of ejection (Dean et al. 2011) but the probability of sperm ejection did not differ between related and unrelated males in our study. Patterns in PVL hydrolysis points vary with day post-copulation whereby males related to first mates attained more hydrolysis points on days 1 and 2 while the reversed was true on days 3 and thereafter. This would suggest a competitive advantage for the rival males related to previous mates during the early days post-copulation. However, whether this trend in hydrolysis points are mediated by female postcopulatory sexual selection or male differential sperm allocation is unclear and warrants further studies.

Our results provide evidence that male red junglefowl are able to recognise relatives and that females are able to distinguish the unrelated male from the related male duo. Previous studies on inbreeding avoidance in this species suggest that males can discriminate between potential mates according to the female's relatedness or genetic

similarity to themselves (Pizzari et al. 2004; Gillingham et al. 2009). Such recognition mechanisms could potentially also apply to intrasexual male-male kin recognition. Recognition of male relatives might confer reproductive fitness advantage through the benefits of kin-selected reduction of aggression and strategic allocation of sperm according to the relatedness of competitors. Two widely-discussed mechanisms that mediate premating kin recognition and avoidance are prior association, where kin discrimination is based on social familiarity, and phenotype matching, where recognition is based on self-referent cues (Holmes & Sherman 1982; Holmes 1986). However, the mechanisms of kin recognition mediating the intensity of intrasexual competition in birds remain unclear and deserve further research. Females could be identifying the single unrelated male type through the sensory habituation to the cue of the common type of male (Ehrman & Spiess 1969). The nature of the cue sexually exciting the female might be different for the common and rare male types, and the rare male type is enabled to break through habituation with the common male types by its different cue (Ehrman & Spiess 1969). The ability of males to discriminate between rivals and females to recognise males according to relatedness may confer a particular advantage in a species like the red junglefowl, where individuals exhibit limited dispersal and social groups comprise of members of varying degrees of relatedness (Collias et al. 1966; Collias & Collias 1996).

Conclusion

Our results show that intrasexual male relatedness can modulate sexual interactions in a sex-specific and counteracting manner. Related males display less aggression towards each other while females prefer the single unrelated male type. Also, male allocate more sperm when competing with relatives but suffer a higher intensity of sperm

ejection by females. This study demonstrates first evidence of sex-specific counteracting responses over male-male relatedness and illustrates the importance of considering female choice alongside sperm competition in models of postcopulatory sexual selection. Further research is needed to determine the fitness consequences of these behaviours and to uncover the underlying mate recognition mechanisms.

Acknowledgements

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Chapter 8

The impact of inter-male relatedness on female life-history and fitness in the fruit fly

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ABSTRACT

Male competition for reproductive opportunities can result in adaptations that harm females. However, the viscosity typical of many natural populations means that local mate competition often occurs between males that are more related to each other than the population average, potentially relaxing competition and the degree of male harm to females. While recent theory predicts patterns of local male relatedness to have important consequences for female fitness, these predictions have not been tested empirically. Here, we experimentally manipulate levels of local male relatedness in the fruit fly *Drosophila melanogaster* and measure the effects on male and female behaviour and fitness. When exposed to groups of full-sib brothers, females lived for longer and reproduced at a slower rate, than when exposed to groups of low male relatedness. This effect was due to the early mortality of females with high reproductive rates under high male relatedness and early mortality of females with low reproductive rates under low male relatedness. However, male relatedness also had a strongly negative impact on offspring viability, most likely due to loss of offspring genetic diversity under low male relatedness. Such impaired female reproductive success counteracted increased female longevity caused by male relatedness, so that overall lifetime female reproductive success did not differ across varying levels of male relatedness. These results show that relatedness between males can have subtle but profound consequences for female life-history and the potential for sexual conflict.

INTRODUCTION

The selfish pursuit of an individual's interest that increase gains relative to other individuals can lead to depletion of common resources, a situation known as tragedy of the commons (Hardin 1968, 1993). Males competing for females can be viewed as analogous to a tragedy of the commons: by competing with one another over a reproductive resource (ova), males reduce the quality of the resource over which they are competing (Rankin & Kokko, 2006) by harming females. Thus, under intense male-male competition, sexual selection will promote male traits that convey a competitive advantage even if this imposes fitness costs on the females mated, generating sexual conflict (e.g. Holland & Rice, 1999; Le Galliard et al. 2005). However, recent theoretical models predict that kin selection might inhibit sexual conflict (Rankin 2010; Wild et al. 2011). Male relatedness might relax selection on harmful male traits, because harming the female partners of male relatives would indirectly reduce the reproductive success of male relatives, and thus reduce inclusive fitness (Rankin 2010).

Male-male relatedness can potentially modulate the intensity of intrasexual competition because of kin selection (Murray & Gerrard 1984; Taylor 1992; Kelly 1994; Queller 1994a; Kokko & Lindström 1996; West et al. 2002). Males that are interacting with both brothers and non-kin are expected to act less aggressively towards their brothers, because the direct costs of missed mating opportunities may, at least in part, be compensated by the inclusive fitness benefits derived by the related recipient (Hamilton 1964a, b). A male might be predicted to compete with a relative, as his fitness would be higher if he monopolises the female. However, in the presence of a non-relative, it is better for the male to lose mating opportunities to a relative than to an unrelated rival as the male gains kin-selected benefits from the successful matings of his relative.

Evidence of kin selection mitigating the intensity of competition has been reported in some eusocial insects (Tsutsui et al. 2000; Tsuisui et al. 2003), cooperative breeders (Gaston 1978; Clutton-Brock 2002) and lekking species (Kokko & Lindström 1996; HÏglund et al. 1999; Shorey et al. 2000; Krakauer 2005; Reynolds et al. 2008; but see McDonald & Potts 1994). In lekking species, males gather for competitive mating displays, mating success is highly skewed, and the majority of the males never reproduce. However, disfavoured males could enhance their inclusive fitness by helping male relatives gain a higher reproductive success. The way in which kin selection modulates the intensity of intrasexual selection might thus have important repercussions for females and the strength of sexual conflict. Despite its theoretical importance however, few empirical studies have considered the influence of relatedness on the intensity of sexual conflict and its interplay with male-male competition (Queller 1994b; Chapman 2006; Bourke 2009; Pizzari & Gardner 2012).

Here, we experimentally investigate the consequences of the genetic relatedness of competing males for female life-history and reproductive success in the fruit fly *Drosophila melanogaster*. Individuals of this species mate multiply (Imhof et al. 1998) and males harm females via actions of the accessory gland proteins (Acps) transferred with sperm during mating (Chapman et al. 1995). These Acps are beneficial to males as they reduce female receptivity and increase egg-laying rate, thereby increasing male fecundity (Chen et al. 1988; Herdon & Wolfner 1995; Chapman et al. 2003). However, increased exposure to these proteins increases female mortality rate (Chapman et al. 1995). These male-induced harm consequently mediated changes in female life-history traits through changes in lifetime reproductive success and lifespan (Edward et al. 2010). Natural populations of the fruit fly are characterised by limited dispersal and a

tendency towards aggregations in particular localities (McInnis et al. 1982; Robinson et al. 2012). Local patches thus comprise of members of varying degree of relatedness, and males that compete locally can do so with related or unrelated rivals. Here, we expose individual females to trios of male competitors of three different levels of inter-male relatedness to address three aims. First, we test whether different aspects of female fitness (lifetime reproductive success, lifespan and reproductive rate) differ across male relatedness treatments. Second, we establish the demographic and life-history patterns underpinning differences across male relatedness treatments, through longitudinal analyses of female variation in longevity and reproductive rates. Third, we investigate the way male behaviour differentially affects female fitness by measuring patterns of male inter- and intrasexual behaviours across male relatedness treatments.

MATERIAL AND METHODS

(a) Experimental Population and Culturing

We used a lab-adapted Dahomey wild-type stock of the fruit fly. Flies were maintained in a 25°C, non-humidified room, with a 12 hours light: dark cycle, in plastic vials or bottles containing standard sugar-yeast medium with excess live yeast (Lewis 1960). The stock has been maintained since 1970 in four large (several thousand flies), outbred population cages (Patridge & Farquhar 1983) of dimensions 30 cm x 15 cm x 20 cm. Each population was fed with three bottles of food medium per week. These four populations were mixed into one single large population approximately one year prior to experiments to promote genetic variability in our experimental flies. This stock exhibits substantial levels of genetic variation (Wilkinson et al. 1990; Whitlock & Fowler 1996; Fowler et al. 1997), and contains selectable variation for a range of life-history, behavioural and physiological traits (Sgro & Partridge 1999; Wigby &

Chapman 2004; Wigby et al. 2009). The Dahomey stock is maintained with overlapping generations to minimise selection on replication rate and life span.

In order to quantify male reproductive success in a replicated manner, we used three distinct phenotypic makers, differentiated based on eye colour (*sepia*, *sparkling* and wild-type red-eyed), to facilitate paternity analysis. *Sepia* and *sparkling* are homozygous recessive mutant eye colours, with the eyes appearing brownish-red and rough in texture respectively (e.g. Anxolabéhère 1976; Fu & Noll 1997). To control for genetic background, the experimental *sepia* (*sep*) and *sparkling* (*spa*) stocks were derived by backcrossing for five generations into the wild-type (red-eyed) Dahomey background. We also crossed 50 virgin *sep* males with 50 virgin *spa* females and subsequently mated the offspring to obtain double homozygous recessive experimental females (*spa*/*sep*).

To obtain parents of the experimental males, eggs were collected separately from the three different strains and raised at standard density (~100 flies per bottle) (Clancy & Kennington 2001). Virgins were collected within 8 hours of eclosion using ice anaesthesia and placed in same-sex vials. Flies were then aged for one week before single males and females were paired in vials to produce three types of families: *sep*, *spa* and wild-type. The parental pair was discarded after 24 hours and the eggs left to develop. Virgin males emerging from these paired crosses were used for experimental trials. All experimental males and females were 48 to 62 hours-post-eclosion.

(b) Experimental Procedure

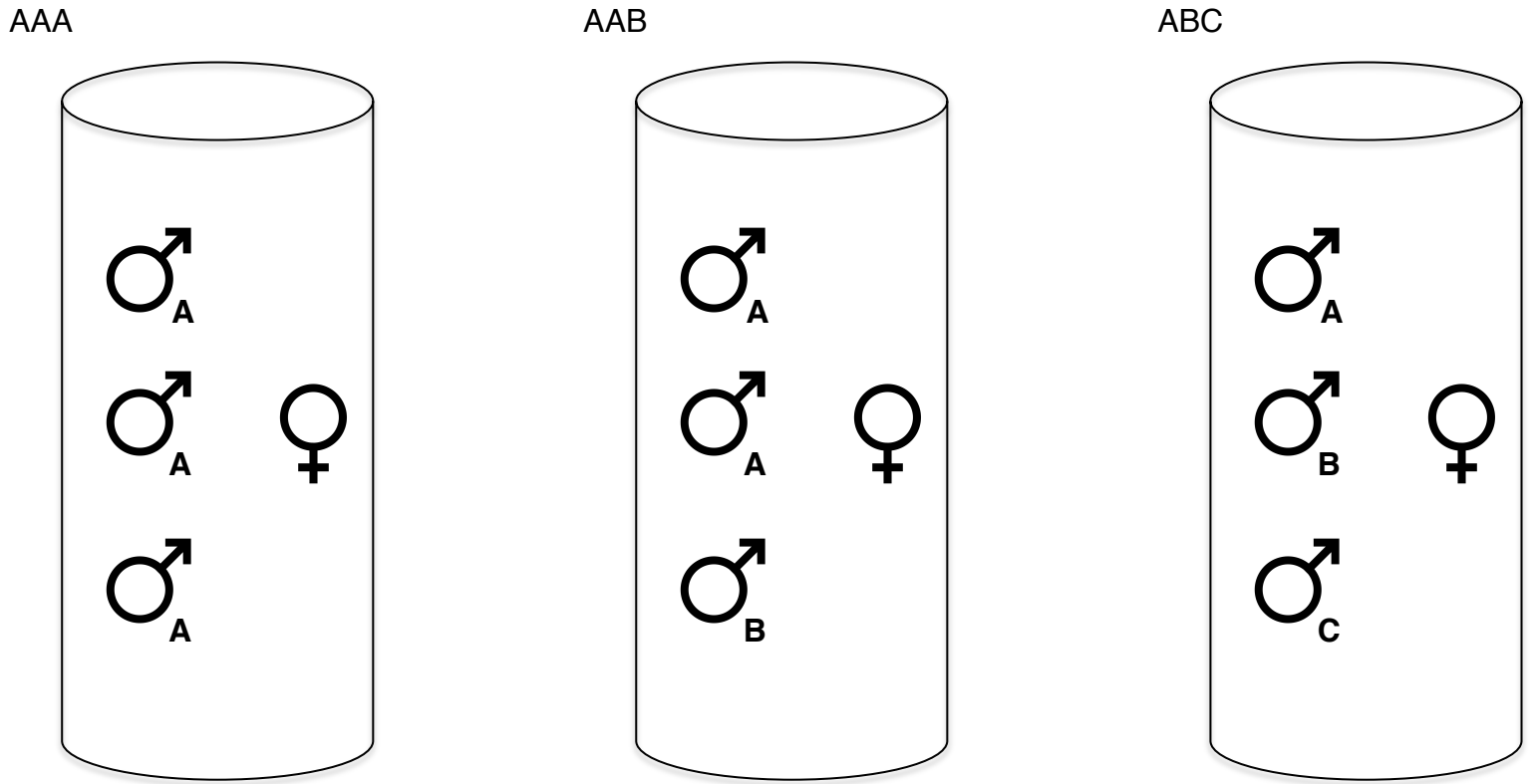
To test whether genetic relatedness between males can mitigate sexual conflict in terms of male harm, we placed a single virgin double homozygous female with three virgin males under three different social treatments: (a) all three males were full-siblings (AAA), (b) two of the males were full-siblings and one male was from another family (AAB), (c) all three males were from different families (ABC). The design was paired: all the 'A' males belonged to the same family and therefore each family of males were represented thrice (AAA, AAB and ABC; one set) (Figure 1). Males from different families also possessed different eye colour to facilitate calculation of paternity estimates (see below). We adopted a randomised balanced design: 54 sets of trios were set up, in which 18 sets had wild-type individuals as 'A' males, 18 sets with *sep* individuals as 'A' males and 18 sets with *spa* individuals as 'A' males. Families used were independent of one another, that is, families used in one set were not used for another.

Flies were transferred to fresh vials every three days to prevent overcrowding of the offspring. Offspring were therefore produced in batches in which the first batch consisted of offspring from days 1-3, second batch from days 4-6 and third batch from days 7-9. We quantified female lifespan, to the nearest day, the number of offspring each female produced per batch (reproductive rate), lifetime reproductive success (total number of offspring) and pupae-adult mortality at 12 days after the oviposition period. Because the majority of the flies eclosed 10 days after oviposition, allowing 12 days before fly collection provided ample time for development. We also conducted observations at lights-on on the first three days and thereafter on alternate days to quantify several important behaviours. Behavioural observations lasted for three hours.

To examine male harassment, we quantified the number of courtship events (chasing, singing, genital licking, copulation attempt) directed at the female in 2-minute spot-checks (Bastock & Manning 1955). We also recorded the frequency of male-male aggressive events to examine the effect of male-male relatedness on intrasexual competition (Chen et al. 2002), and the frequency of mating at each spot-check. We stopped each sample when either a male or the female died within the vial.

In the AAB treatment, males were marked with either red, yellow or green acrylic paint (Nilsen et al. 2004) in a randomised balanced design to allow identification and detailed observations of how male-male relatedness affects intersexual and intrasexual interactions. We predicted that there would be fewer aggressive interactions between related male than between unrelated males. These behavioural parameters were recorded as above. To quantify paternity in treatment AAB, we counted the number of offspring with the different eye colours. We tested if the paternity share by both related males differed from the null hypothesis (i.e. > 2/3).

Figure 1. Experimental design. Three males were placed with a single female. We used three treatments: males from the same family (AAA); two males from the same family and one male from another family and (AAB); three males each from different families (ABC). Males from different families also has different eye colours, i.e. phenotypic markers.



(c) Statistical Analysis

(ci) Female fitness across male relatedness treatments

Several samples were excluded in the analysis of female fitness (except Cox proportional hazard analysis for female lifespan) as an individual male died before the female (10 in treatment AAA, 18 in AAB and 16 in ABC). A chi-squared analysis revealed no significant difference in the probability of a male dying prior to female death among treatments ($\chi^2_2 = 3.25$, $P = 0.197$).

We first analysed the effects of genetic relatedness of males on female fitness attributes using Generalised Linear Mixed Models (GLMM), with genetic relatedness of males (AAA *versus* AAB *versus* ABC) as fixed factor and family identity as a random variable. Four separate GLMMs analysed variation in four response variables: female lifetime reproductive success, reproductive rate (i.e. mean number of offspring per batch), female lifespan and pupae-adult mortality. Error distributions were specified as Normal, Normal, Poisson and Binomial respectively. The variation in the response variable for each analysis was assessed using the likelihood-ratio test on models with and without the fixed factor (Valdar et al. 2006). Post-hoc Tukey test was conducted to identify pairwise difference when the result was significant.

Some males died before the female and we had to exclude these samples from the above analyses as we could not accurately determine the lifespan or lifetime reproductive success of the female in these vials. However, we further analysed variation in female survival using Cox proportional hazards model (Cox 1972) and included these samples. We entered female lifespan as the censoring time, mortality (1 for death; 0 for survived, i.e. when male died before female) as the event indicator and

family identity as a random factor.

We analysed the relationship between reproductive rate, female lifespan and treatment. For each treatment, we entered in separate regression models standardised reproductive rate (individual reproductive rate/mean reproductive rate) as the response and standardised lifespan ((individual lifespan - mean lifespan)/SD lifespan) as the predictor. Standardisation was done to control for inter-treatment variation.

(cii) Longitudinal patterns of female life-history within male relatedness treatments.

To test the idea that females placed in vials with higher male-male relatedness were subjected to a more benign condition, we examined whether the reproductive rate of the female predicted the probability of survival. We conducted logistic regression tests with the reproductive rate of the current batch as the predictor and the probability of survival to reproduce the next batch of offspring as the response. This allowed us to investigate between-individual variation in mortality rate, a phenomena we henceforth term as selective disappearance, (van de Pol & Verhulst 2006; van de Pol & Wright 2008) and how it is related to reproductive rate. We also tested for interaction between treatment and batch on variation in reproductive rate with a GLMM, treatment and batch and their interaction as fixed factors, female identity as a random factor to control for between between-individual variation in mortality rate (i.e. different mortality rates of females with different reproductive rate) and family identity as another random factor.

(ciii) Variation in male behaviour across relatedness treatments.

To investigate whether male mating rate have a negative effect in females, we entered

in a GLMM standardised lifespan as the response, standardised mating rate for the first observation (mating rate - mean mating rate)/SD mating rate) and treatment and their interaction as fixed factors and family identity as a random factor. We only used standardised mating rate for the first three observations to control for any decline in mating rate with lifespan of female.

We analysed variation in behaviour across treatments through General Linear Mixed Models (LM) with genetic relatedness of males as a fixed factor and family identity as a random factor. We assumed that each male in treatments AAA and ABC performed 1/3 of the total number of aggressive events, courtship events and mating frequency recorded and therefore divided the total recorded by three to obtain the mean. Again, we only used behavioural observations from the first three days to minimise variation of these parameters with female lifespan. We took the mean of data recorded over the three days. Three separate LMs analysed variation in the three response variables: mean male courtship frequency, mean no. of male-male aggression events and mean mating frequency. We also analysed variation in an individual A male's reproductive success using a LM with genetic relatedness of males as a fixed factor, family identity as a random factor and total number of offspring sired by an A male as the response variable.

(civ) Variation in male behaviour within AAB treatment.

We were also interested in how the related males performed relative to the single unrelated male within the AAB treatment. Therefore, we tested for any differences in mean male courtship frequency, mean number of male-male aggression events and mean mating frequency between related and unrelated male types using three different

LMs with the three different response variables. Relatedness of male (related *versus* unrelated) was entered s a fixed factor and family identity was entered as a random factor. We took the mean of data recorded over the first three days. To test the idea that percentage paternity gained by the related males was more than 2/3, we used a GLMM with a Binomial error distribution, proportion of offspring sired by both related males as the response, batch as a covariate, family identity and vial as random variables. To control for bias due to phenotype mutation, we entered phenotype of related males and phenotype of the unrelated male as covariates.

We used R version 2.13.0 for all analyses.

RESULTS

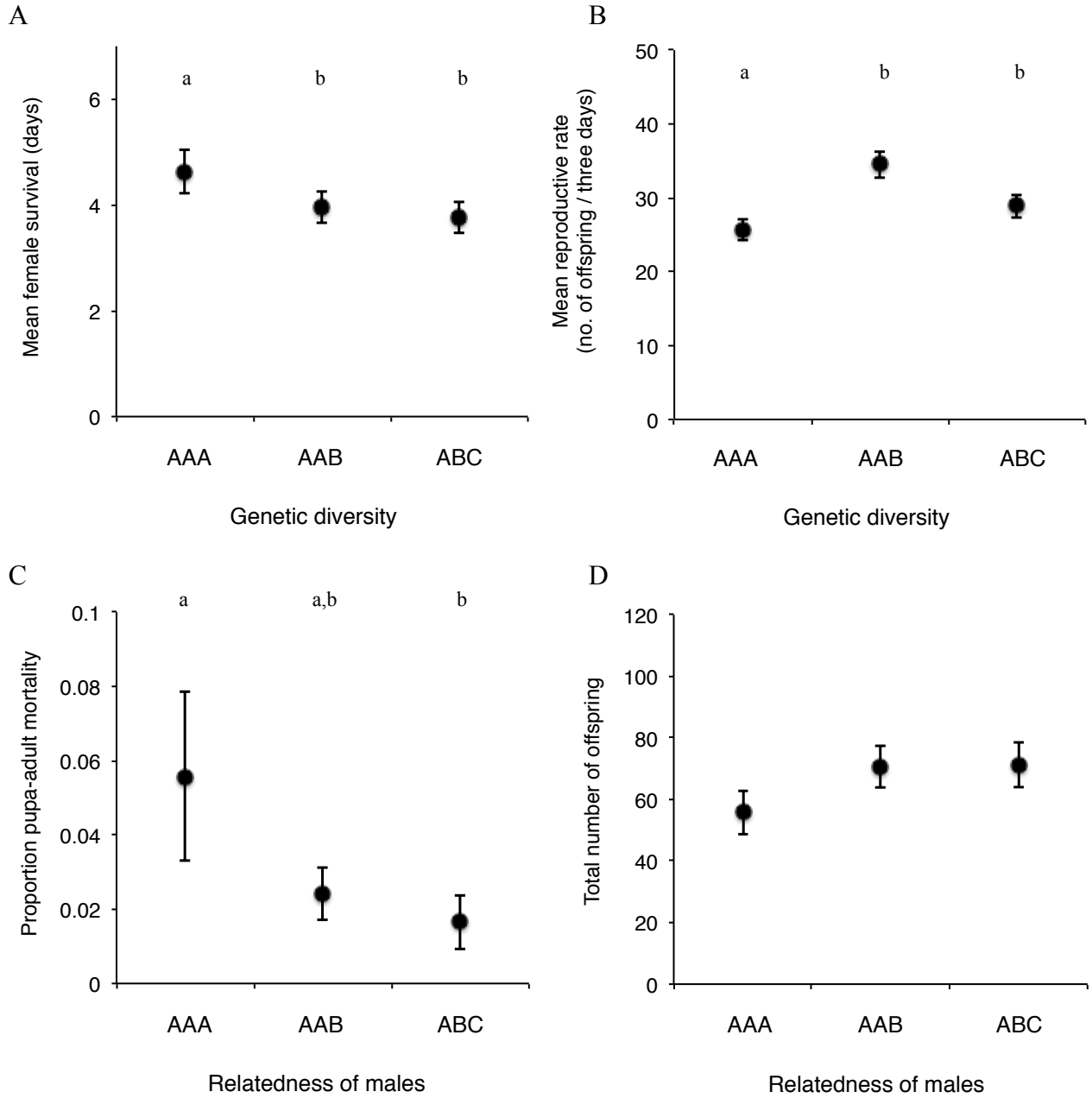
(a) Female fitness across male relatedness treatments

Consistent with the idea that male genetic relatedness mitigates female harm, average female lifespan decreased with decreasing male relatedness ($\chi^2_2 = 6.35$, $P = 0.042$; Figure 2A). Post-hoc Tukey tests revealed that females lived significantly longer under high male relatedness (i.e. AAA treatment) than under low male relatedness (i.e. ABC treatment, where all males are unrelated to one another; Z = 2.38, $P = 0.046$; Figure 2A) while females in the AAB treatment exhibited an intermediate lifespan (AAA *versus* AAB: Z = -1.84, $P = 0.156$; AAB *versus* ABC: Z = -0.54, $P = 0.850$; Figure 2A). A Cox proportional hazard model also revealed a marginally non-significant longer female survival in the AAA treatment than in the ABC treatment (AAA *versus* AAB: Z = 0.87, $P = 0.657$; AAB *versus* ABC: Z = 1.29, $P = 0.403$; AAB *versus* ABC: Z = 2.17, $P = 0.076$)

The reproductive rate (mean number of offspring per batch) also differed among treatments ($\chi^2_2 = 12.40$, $P = 0.002$; Figure 2B), females in the high male relatedness treatment (AAA) reproduced at a significantly slower rate than females exposed to low male relatedness (i.e. AAB and ABC treatments; Tukey test, Z = 3.59, $P < 0.001$; Z = 2.37, $P = 0.046$ respectively; Figure 2B). Consistent with the effect of male relatedness on female lifespan and reproductive rates, we found that females that lived longer exhibited a higher reproductive rate but the strength of the lifespan/reproductive rate relationship is negatively correlated with male-male relatedness (AAA: $t_{43} = -0.10$, $P = 0.920$; AAB: $t_{35} = 2.45$, $P = 0.020$; ABC: $t_{37} = 2.94$, $P = 0.006$; Figure 2E).

Male relatedness also had a strong negative impact on offspring viability. Pupa-adult mortality varied across male relatedness treatments ($\chi^2_2 = 25.06$, $P < 0.001$; Figure 2C). This effect was largely mediated by a significant higher pupa-adult mortality detected in the low male relatedness (AAA) treatment relative to the AAB (Tukey test, $\chi^2_2 = 4.72$, $P < 0.001$; Figure 2C) and ABC treatments (Tukey test, $\chi^2_2 = 3.38$, $P = 0.002$; Figure 2C). The total number of offspring produced did not differ among treatments ($\chi^2_2 = 3.29$, $P = 0.193$; Figure 2D).

Figure 2. Female fitness attributes across different treatments: males from the same family (AAA); two males from the same family and one male from another family and (AAB); three males each from different families (ABC). Significant differences in figures 2A - C are denoted by different letters. (A) Average female lifespan: the lifespan of females decreased with genetic relatedness of males. (B) Reproductive rate: females in the AAB and ABC treatments produced significantly higher number of offspring per three days than females in the AAA treatment. (C) Percentage pupa-adult mortality: the percentage pupa-adult mortality decreased with genetic relatedness of males. (D) Total number of offspring produced: there was no significant differences in the total number of offspring produced. (E) Standardised reproductive rate against standardised survival: there was a significant positive correlation between standardised reproductive rate against standardised survival for treatments AAB and ABC but not AAA. Error bars denote S.E.

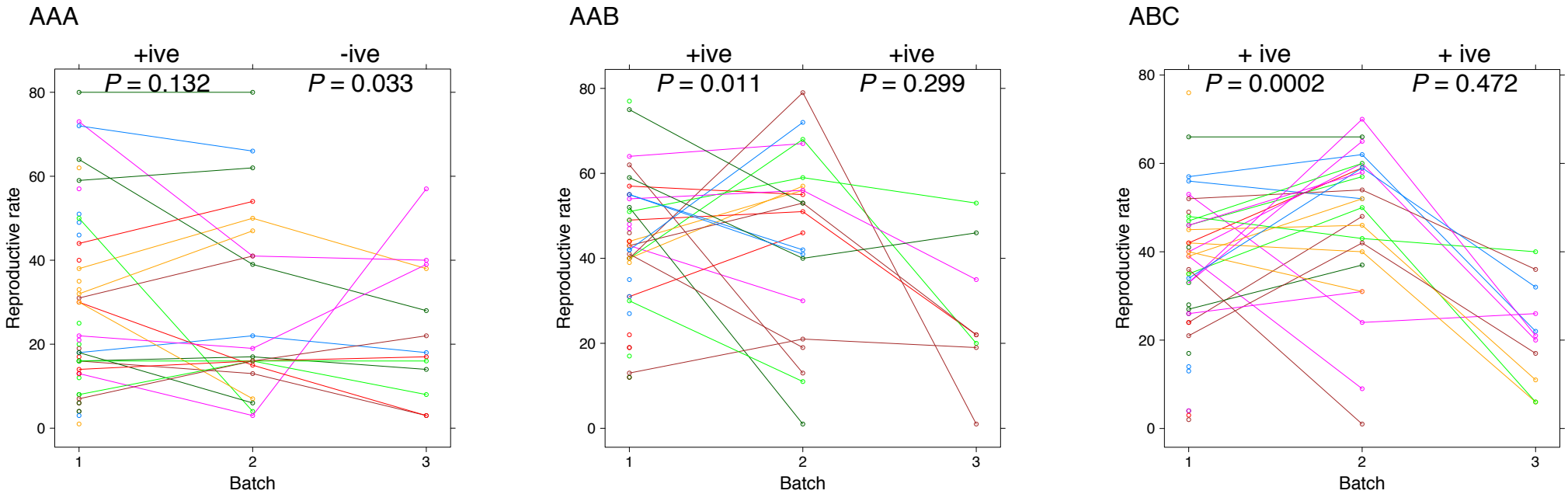

A
Mean female survival (days)
a
b
b
AAA
AAB
ABC
Genetic diversity
B
Mean reproductive rate
(no. of offspring / three days)
a
b
b
AAA
AAB
ABC
Genetic diversity
C
Proportion pupa-adult mortality
a
a,b
b
AAA
AAB
ABC
Relatedness of males
D
Total number of offspring
AAA
AAB
ABC
Relatedness of males
E
Standardised reproductive rate
AAA
AAB
ABC
Standardised survival

(b) Longitudinal patterns of female life-history within male relatedness treatments

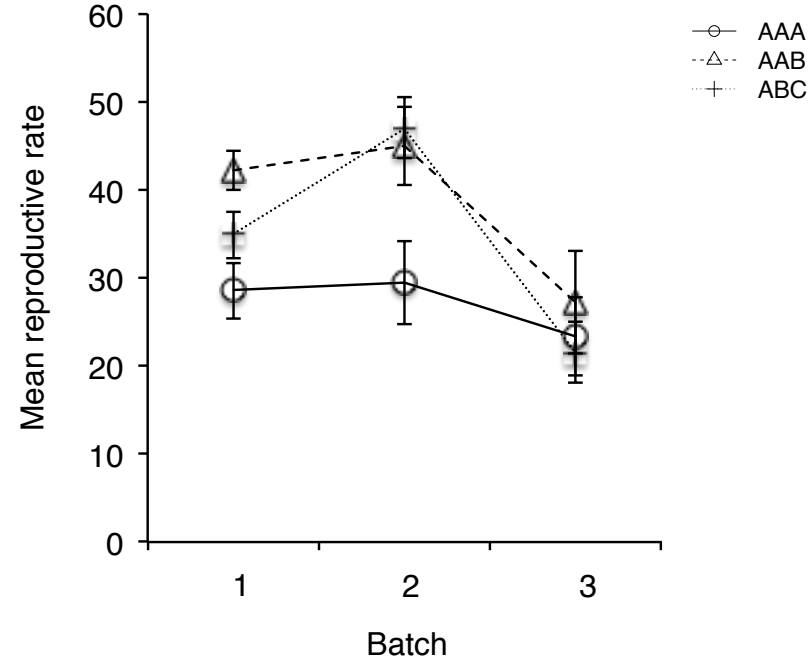
A longitudinal analysis shows that male-male relatedness affects female life-history. Under high male relatedness (AAA treatment), more fecund females had a lower probability of survival from day 3 to day 6, while less fecund females exhibited a longer lifespan (Figure 3A). In low male relatedness treatments (i.e. AAB and ABC), more fecund females were more likely to live longer, but their reproductive rate suffered an age-dependent decline (Figure 3A). Consistent with this, we also detected a significant treatment by batch interaction on variation in reproductive rate ($\chi^2_4 = 13.31$, $P = 0.009$), whereby controlling for between-individual variation in mortality rate, reproductive rate did not change with female age in treatment AAA, but declined with age in treatments AAB and ABC (Figure 3B).

Figure 3. Reproductive rate of females plotted against batch, i.e. days post-copulation. Batch 1 = 1-3 days post-copulation; Batch 2 = 4-6 days post-copulation; Batch 3 = 7-9 days post-copulation. (A) Each point represents an individual female. Treatments are labelled at the top left side of each graph. *P*-values represent the significance of a logistic regression test on whether the reproductive rate of the current batch predicted the probability of survival to reproduce the next batch of offspring. Directions of the correlation are indicated, where +ive means that there is positive correlation between reproductive rate and the probability of surviving to reproduce the next batch of offspring. (B) Mean reproductive rate for each treatment: there was a significant treatment by batch interaction. Error bars denote S.E.

A



B

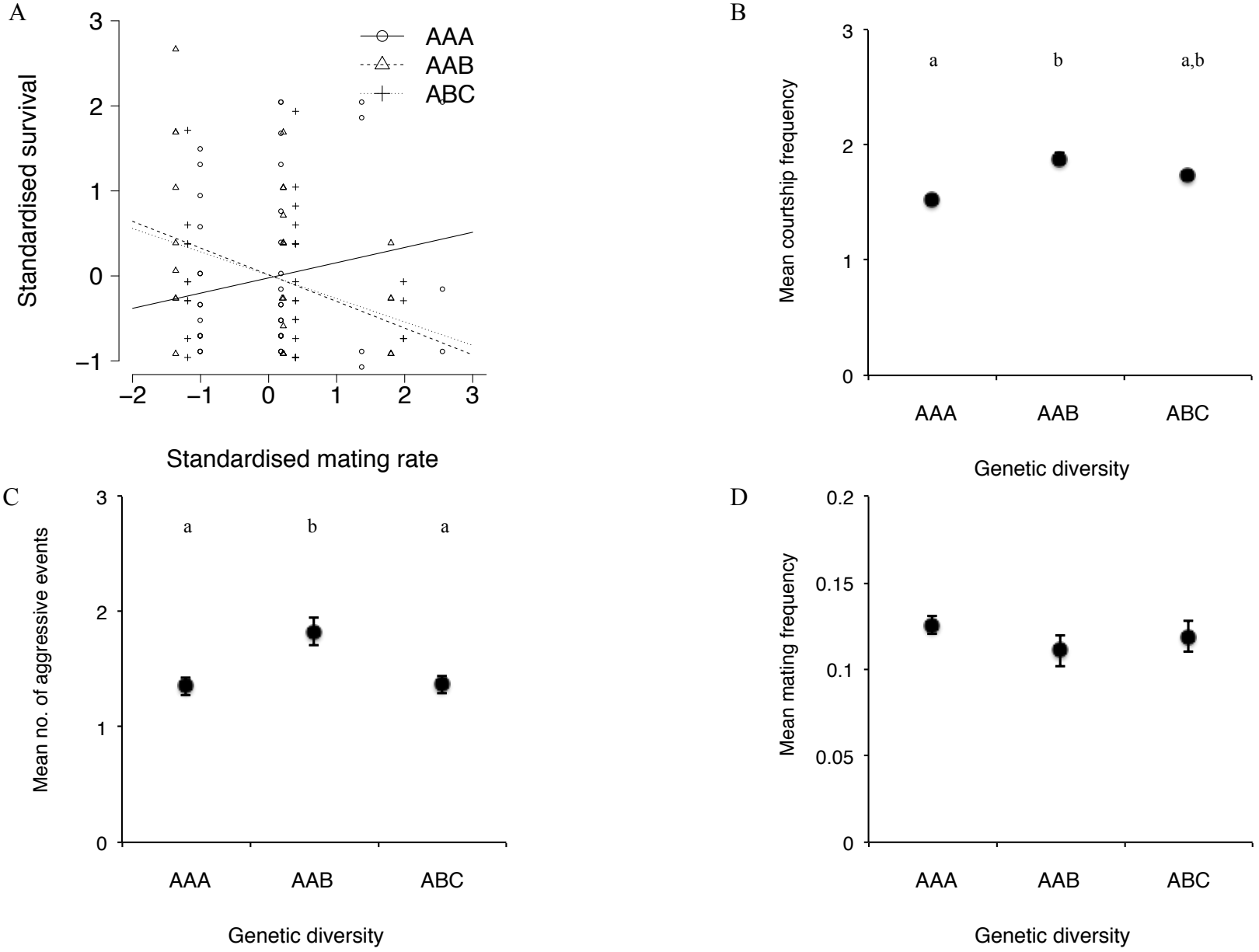

60
50
40
30
20
10
0
Mean reproductive rate
1
2
3
Batch
AAA
AAB
ABC

(c) Variation in male behaviour across relatedness treatments.

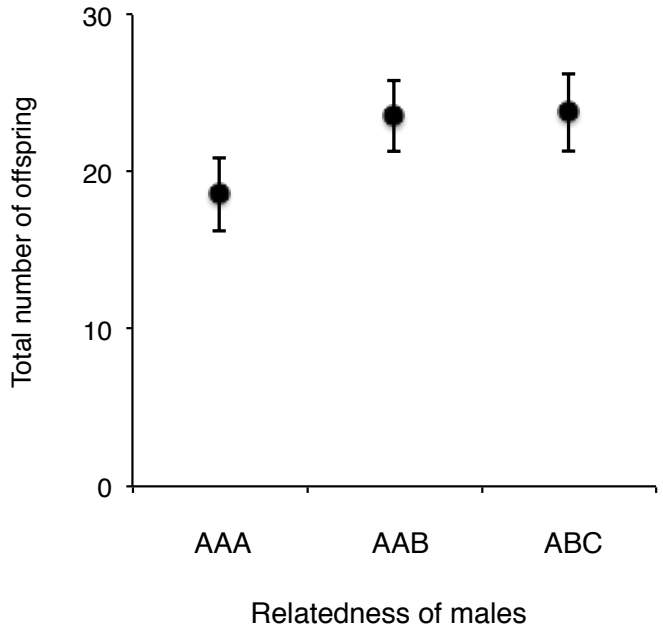
There was a significant interaction between treatment and mating rate on variation in standardised female lifespan ($\chi^2_2 = 6.61$, $P = 0.037$), whereby increased mating rates marginally reduced survival under low male relatedness (treatment AAB: $t_{35} = -2.04$, $P = 0.049$; ABC: $t_{37} = -1.73$, $P = 0.092$) but did not affect survival under high male relatedness (treatment AAA: $t_{43} = 1.21$, $P = 0.235$; Figure 4A).

Courtship frequency and the number of aggressive events differed with relatedness of males ($\chi^2_2 = 7.27$, $P = 0.026$; $\chi^2_2 = 6.24$, $P = 0.044$ respectively). Males in AAB treatment courted the female significantly more often than males in the AAA treatment (Tukey test, AAA *versus* AAB: Z = 2.67, $P = 0.021$; AAA *versus* ABC: Z = 1.66, $P = 0.220$; AAB *versus* ABC: Z = -0.97, $P = 0.595$; Figure 4B) and displayed a higher frequency of aggressive events than males in ABC treatment (Tukey test, AAB *versus* ABC: Z = 2.51, $P = 0.033$; AAA *versus* AAB: Z = 1.02, $P = 0.563$; AAA *versus* ABC: Z = -1.49, $P = 0.298$; Figure 4C). Mating frequency and total number of offspring sired by the A male did not differ between treatments ($\chi^2_2 = 0.080$, $P = 0.961$; $\chi^2_2 = 3.29$, $P = 0.193$ respectively; Figures 4D and 4E respectively).

Figure 4. Behavioural parameters measured in different treatments: males from the same family (AAA); two males from the same family and one male from another family and (AAB); three males each from different families (ABC). Significant differences are denoted by different letters. (A) Standardised survival against standardised mating rate of 1st observation: there was a significant interaction between standardised mating rate and treatment. (B) Courtship frequency: males in the AAB treatment courted the female significantly more frequently than males in the AAA treatment. (C) No. of aggressive events: males in the AAB treatment exhibited a significantly higher number of aggressive counts than males in the ABC treatment. (D) Mating frequency: there was no difference in mating frequency across treatments. (E) Total number of offspring sired by individual A males: there was no difference in the number of offspring between treatments. Error bars denote S.E.


A
Standardised survival
Standardised mating rate
AAA
AAB
ABC
B
Mean courtship frequency
a
b
a,b
AAA
AAB
ABC
Genetic diversity
C
Mean no. of aggressive events
a
b
a
AAA
AAB
ABC
Genetic diversity
D
Mean mating frequency
AAA
AAB
ABC
Genetic diversity

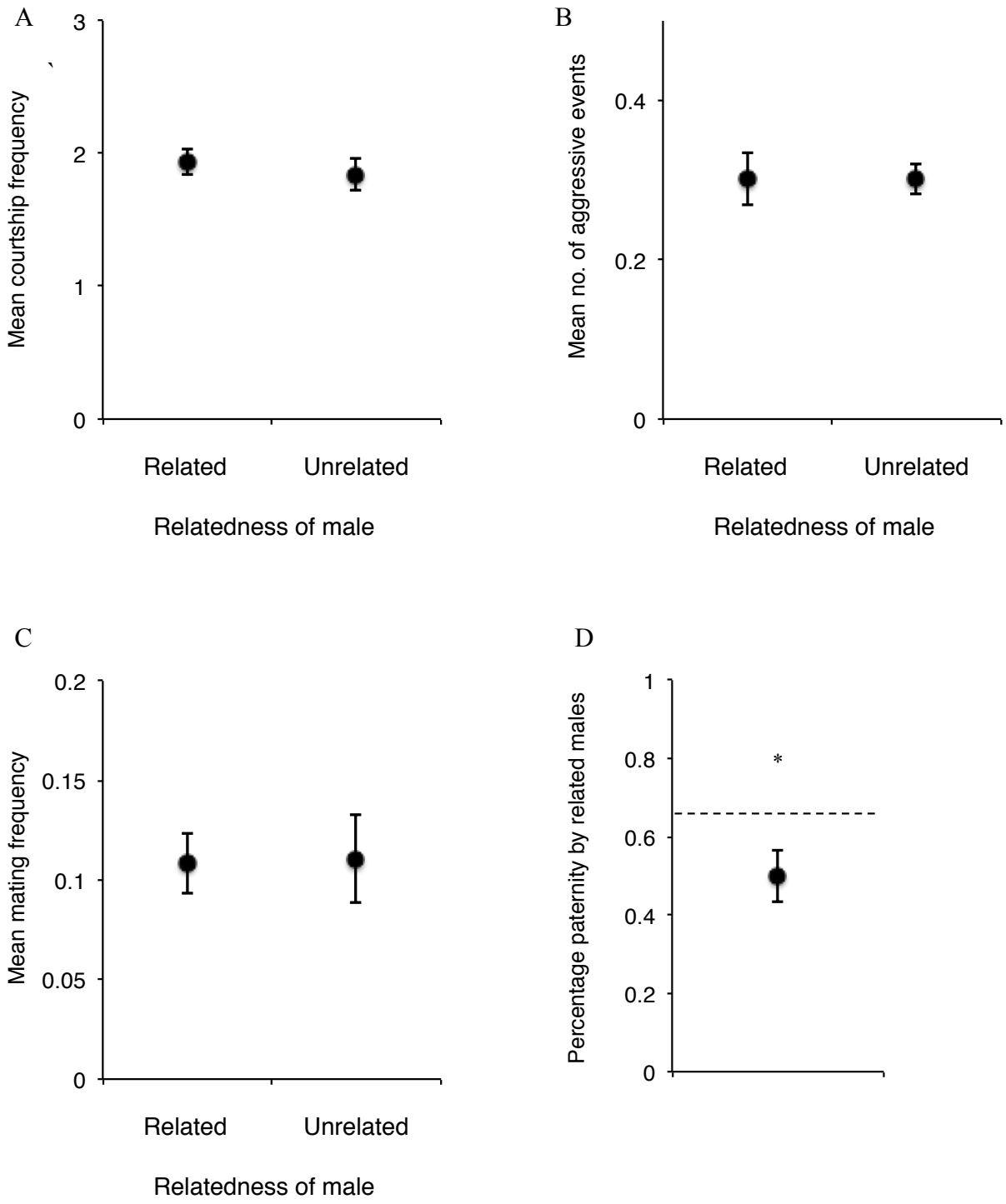
E


30
20
10
0
Total number of offspring
AAA
AAB
ABC
Relatedness of males

(d) Variation in male behaviour within AAB treatment.

We detected no difference in courtship frequency, number of aggressive events and mating frequency between the related and unrelated male types within the AAB treatment ($\chi^2_2 = 0.374$, $P = 0.541$; $\chi^2_2 = 0.042$, $P = 0.838$; $\chi^2_2 = 0.007$, $P = 0.931$ respectively; Figures 5A-C). The total percentage paternity gained by the related males was 50.0%, which was significantly less than the expected 66.7% after statistically controlling for any bias generated by mutations (refer to Statistical Analysis section) (Confidence interval (36.9%, 63.0%) ; Figure 5D).

Figure 5. Behavioural parameters and paternity within the AAB treatment. (A) Courtship frequency: there was no difference in courtship frequency between related and unrelated male types. (B) No. of aggressive events: there was no difference in aggression counts between related and unrelated male types. (C) Mating frequency: there was no difference in mating frequency between related and unrelated male types. (D) Paternity by related males: related males gained a percentage paternity less than 66.7% of the total number of offspring. '*' equals $P < 0.05$ for proportion significantly less than 0.667. Error bars denote S.E.


A
Mean courtship frequency
3
2
1
0
Related
Unrelated
Relatedness of male
B
Mean no. of aggressive events
0.4
0.2
0
Related
Unrelated
Relatedness of male
C
Mean mating frequency
0.2
0.15
0.1
0.05
0
Related
Unrelated
Relatedness of male
D
Percentage paternity by related males
1
0.8
0.6
0.4
0.2
0
*

DISCUSSION

Our study demonstrates that the degree of relatedness between males modulates patterns of female offspring production and survival. We found that high male-male relatedness increased female lifespan and decreased both female reproduction rate and offspring viability, leading to no overall change in the lifetime reproductive success of females exposed to different levels of inter-male relatedness.

(a) Female life-history response to male relatedness

Male relatedness modulates female life-histories: average female lifespan increased with increased male-relatedness and average reproductive rate decreased with increased male-relatedness. Our data suggest that these observations could be to between-individual variation in mortality rate, i.e. selective disappearance. When males are unrelated, females that reproduced at a lower rate suffered early mortality. Consistent with this interpretation, we show a positive relationship between female lifespan and reproductive rate but its strength is negatively correlated with male relatedness. In addition, a longitudinal analysis shows that in AAA, more fecund females died younger than less fecund females which continued to reproduce throughout life at low but constant rate whereas in ABC more fecund females were more likely to live longer, but reproductive rates suffered an age-dependent decline.

Male harm of females is mediated by pre- and postcopulatory mechanisms (e.g. Fowler & Partridge 1989; Long et al. 2009). We show that male precopulatory behaviour (courtship and aggression) is heightened in the AAB treatment, suggesting that male harassment of females can increase with decreasing male relatedness. However, this could not fully explain the decline in female average lifespan as we did not find a

higher courtship rate or frequency of aggressive events in treatment ABC. Alternatively, that the negative correlation between mating rates and female lifespan was more prominent in treatments AAB and ABC suggests that when males were unrelated, the act of mating itself can harm females. This could be mediated via two postcopulatory mechanisms. First, females that mate with several different males, that are unrelated form each other, may elicit a higher immune response against the inseminations of different males (Fedorka & Zuk 2005) and thus suffer fitness costs in terms of reduced lifespan. Second, related males may be adjusting their ejaculates so that they are less harmful for the females. Frequent receipt of ejaculate accessory gland proteins of the fruit fly are associated with an increase in female mortality and a decrease in lifetime reproductive success in the fruit fly (Chapman et al. 1995; Wigby & Chapman 2005). However, it might be beneficial to males to adjust their ejaculate components according to socio-sexual environment (Bretman et al. 2012). For example, by inseminating less sex peptide (which reduces female fitness) (Wigby & Chapman 2005) in the presence of related rivals, male might be able to gain kin-selected reproductive success, or reduce the risk of competing against one's own sperm. The latter is plausible if either or both of the related males erroneously perceive that they have mated with the female before. Consistent with this idea, Chapter 6 provides the first evidence for male fruit flies displaying decreased sexual interest in their previous mates.

We found a lower reproductive rate and longer lifespan in females when the degree of male-male relatedness in the group is higher. This result appear to be consistent with a recent study which show that least killifish females (*Heterandria formosa*, Agassiz; family Poeciliidae) mated to three genetically dissimilar males had a shortened lifespan

compared to those mated to highly related males (Ala-Honkola et al. 2011). However, unlike our study, this study did not quantify reproductive rate or measure overall reproductive success, making it difficult to ascertain the fitness consequences of females being subjected to male of differing relatedness. A previous study on the fruit fly showed that mixing males from different lab strains can affect variance in the reproductive success of females but results vary depending the strain of female and male used (Billeter et al. 2012). Billeter et al. (2012) also found that females mated more frequently over 24 hours in groups composed of males from more than one lab strain, which contrasts with our finding that mating frequency did not vary with genetic diversity of males. However, we did not observe mating frequency over an extended period of time (i.e. 24 hours) and hence only captured at most three matings per observation which might reduce the power to detect small differences in mating frequency. The higher mating frequency with increased male strain diversity found in Billeter et al. (2012) could be consistent with our finding that females in the ABC treatment reproduce at a faster rate. Nevertheless, it is still unclear as to what extent differences between individuals from different lab strains which are likely to be evolutionarily diverged populations (as in their study) can be compared to differences between individuals within a population (as in our study).

Polyandry may enhance offspring fitness through an increased in genetic variance which act as a form of bet hedging against changing environmental conditions or via the reduction in likelihood of gaining sperm from genetically incompatible (e.g. related) males (Jennions & Petrie 2000). Similarly, females can potentially benefit indirectly by mating with genetically dissimilar males *versus* genetically related males through the acquisition of good genes and higher genetic diversity of their offspring,

thereby increasing offspring viability (Fossøy et al. 2007; Jennions & Petrie 2007). Consistent with this idea, when females were placed with three genetically unrelated males, the percentage pupae-adult mortality was lower than that of females mated with genetically familiar males. A previous study in the fruit fly demonstrated that polyandry did not increase female fitness or variance in fitness compared to monogamy (Brown et al. 2004). Polyandry may however be more important in our design then their study because our females were double homozygous recessive and the mutations might have detrimental effects on offspring viability. These deleterious effects could be abated if the recessive mutations were masked by the wild-type gene acquired when mating with genetically different males (some of which are wild-type males).

Fecundity and lifespan are two major parameters used as proxies of female fitness in many sexual conflict studies (Wigby & Chapman 2004; Holland & Rice 1999; Crudgington et al 2005). However, these studies failed to take account between-individual variation in mortality rate that might be mediating the variation in female life-history. For example, we show that the higher mean reproductive rate experienced by females in the AAB and ABC treatments could be potentially explained by the early mortality of low reproductive rate females. In addition, there was a high mortality rate of high reproductive rate females between days 3 and 6 in treatment AAA, contributing to the overall lower reproductive rate recorded in this treatment relative to the other treatments. Moreover, the longer average lifespan of females in the AAA treatment could be due to the retention of low reproductive rate females which might exhibit increased survivorship due to tradeoffs between lifespan and reproductive rate (Snell & King, 1977; Creighton et al. 2009). Changes in overall average fecundity could therefore either mean selective disappearance of females with differential reproductive

rate (as in our study) or an collective change in fecundity of all females. Therefore, by only quantifying fecundity, we might fail to capture the demographic changes associated with changes in average population fecundity. Our results thus demonstrate the importance of considering selective disappearance as a potential mediator of changes in female fitness attributes. In addition, if we only examined lifespan and lifetime reproductive success, we might have missed out the modulation of female reproductive rates with treatment. An alternative approach to ours would be to use rate-sensitive fitness measures (e.g. population growth rate) that take into account the rate of offspring production and between-individual variation in mortality rate (McGraw & Caswell 1996; Edward et al. 2010).

One potential caveat of our experiment is the use of double homozygous recessive females which exhibited shorter lifespan than wild-type females when they were subjected to the same conditions (mean lifespan = 17-21 days) (Carazo, unpublished data). Therefore, the magnitude of male harm may have been more pronounced in our experiment as females with double mutations seemed to be in poorer conditions. Future studies should aim at repeating the experiment with wild-type females to test the dependability of our results.

(b) Male response to male relatedness

Males that are more related to their rivals can potentially gain inclusive fitness benefits from abstaining from female harassment (Rankin, 2010). Also, higher relatedness with other males might reduce the competition for matings (Kokko & Lindström 1996; West et al. 2002). Our results are tentatively consistent with these predictions, but only if we considered the population average relatedness and relatedness symmetry. The

frequency of aggressive courtship events was highest in the AAB treatment, that is, when not all males are equally related to one another compared to the population average relatedness. Each individual male in the AAA and ABC treatment was not more related to one another than the population average. Therefore, individual males are predicted to act for themselves as they are competing against other males that are either all from the same family or all from different families. In contrast, brothers in the AAB treatment might cooperate and compete more intensely with the single unrelated male (but see next paragraph), thereby increasing the overall number of courtship and aggressive events that occurred in that treatment. Indeed, courtship frequency and the number male-male aggressive events were the highest in the AAB treatment, reflecting heightened male-female harassment and intensity of male-male competition when the relatedness of males was asymmetrical. Relatedness asymmetries, with higher relatedness between alloparents and brood than between parents and brood has also been implicated in the evolutionary origins of eusociality and intensity of conflict in Hymenoptera insects (Trivers & Hare 1976). As the relatedness asymmetry increases, through the number of queens or the number of times a queen mates, the degree of conflict between the queen(s) and the workers over sex-ratio is expected to increase (Boomsma & Grafen 1990; Sundström L 1994). An alternative explanation for a higher level of competition in the AAB treatment, is that paint placed on males in this treatment might have caused them to act more intensely to other males and to females. However, paint is unlikely to be a confounding factor given that other studies have shown no effect of paint on the performance of male flies (Krishna et al. 2012; Leftwich et al. 2012).

Nevertheless, when examining the behavioural parameters in the AAB treatment, we

found no evidence that related males were less aggressive to each other or impose a different level of harassment towards the female then the unrelated male. This suggests that although relatedness asymmetry in this treatment might have increased the overall intensity of competition between males compared to treatments with symmetrical relatedness, the level of competition between specific individual males was not dependent on relatedness between those individuals. Our study only quantified the counts of aggression and courtship but not the type of aggressive and courtship behaviour, which would vary in the levels of intensity (Bastock & Manning 1955; Chen et al., 2002). The intensity of aggression and courtship could potentially differ between the relatedness of males. This hypothesis should be explored in future studies.

Paternity biased towards to the single unrelated male in the AAB treatment could be mediated by the rare male effect. The rare male effect predicts that male of the rare genotype enjoys a greater mating success and has been found to be very widespread in insects, especially in laboratory studies of *Drosophila* species (Knoppien 1985, Partridge 1988). One possible explanation for this effect is female preference for the single unrelated male (Knoppien 1985, Partridge 1988). However, we found no significant differences in mating success with related or unrelated male types, suggesting that paternity bias in favour of the rare male may be caused by sperm selection in the female reproductive tract (Zeh & Zeh 1997; Chapter 7). Nevertheless, we might not have enough statistical power to detect small differences in mating frequency due to our short observational periods and hence records of low numbers of mating success. Another explanation for the higher paternity attained by the rare male is that either of the related males invested less ejaculate in the female which has previously mated to himself or his brother. This may be plausible if either or both of

the related males erroneously perceive that they have mated with the female before, as explained above. Further studies should aim at determining the mechanism of this rare male paternity bias.

Conclusion

Our study highlights the role of male-male relatedness in intrasexual and intersexual interactions and female life-history. Such responses to the relatedness of males are likely to evolve in species with limited dispersal because this increases the probability of males interacting with related rivals and females interacting with relatives of previous mates. It will be important to determine to what extent these type of responses are shared, or differ, in other taxa, and how this relates to patterns of dispersal and interaction rates (Wild et al. 2011). Future studies should aim to examine the role of male-male relatedness in the evolution of sexual conflict using selection lines where females are subjected to different treatments of inter-male relatedness and examined for the evolution of female resistance.

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Chapter 9 Discussion

9.1. Overview

The overarching goal of this project was to investigate the role of relatedness on sexual interactions in the red junglefowl, *Gallus gallus* and fruit fly, *Drosophila melanogaster*. I explored four integrated themes: (1) inbreeding depression and the role of offspring sex, parental age and gametic; (2) inbreeding avoidance and the role of socio-sexual factors; (3) how mating decisions are influenced by the relatedness between prospective mates; (4) how kin selection modulates male-male competition.

Using two different species to address this set of questions had its strengths and limitations. One advantage of this approach was that it allowed me to examine whether certain biological phenomena were consistent across species, i.e. whether my observations in species could be further corroborated in another species. The other advantage was that each species has its strengths that are complementary. The fruit fly has a fast generation time, allowing for easy quantification of fitness consequences of its behaviours. It has also many genetic mutants that allowed elucidation of behavioural mechanisms. The red junglefowl is ideal for examining behavioural processes down to the level of gametes. This facilitated better appraisal of the different behavioural processes (e.g. pre- and postcopulatory responses), the effects and the mechanisms underlying these processes. On the other hand, one limitation of this dual system approach was that when differences in results were revealed, there were potentially many reasons that could account for the incongruence, for example, differences in life-history and differences in genetic make-up.

Below, I provide a detailed comparison of the fruit fly and the red junglefowl with regards to the four themes.

9.1.1. Inbreeding depression

In Chapter 2, I found that offspring viability in the fruit fly is strongly affected by a three-way interaction between parental relatedness, parental age and parental gametic age at successive developmental stages. In addition, at the pupa-adult stage, where I determined the sex of the offspring, parental relatedness, parental age and gametic age interact differently in male and female offspring, with females suffering higher inbreeding depression than males. In Chapter 3, I found that inbreeding depression is also sex-specific in the red junglefowl, but which sex suffers a higher cost is contingent on the trait measured. The magnitude of inbreeding depression was greater in sons than daughters with regards to growth rate and tarsus length but the reverse was true for post-hatch survival. Sex-specific inbreeding depression in social status, embryonic viability and comb size were modulated by parental age, but in different ways. Sons suffered a higher inbreeding cost than daughters in social status and comb size but only when parents were old. In terms of embryonic viability, daughters suffered higher inbreeding depression, but only when parents were old. Parental age also interacted with sperm age and parental relatedness on variation in growth rate and tarsus length.

Using the fruit fly and the red junglefowl as study systems allowed for a comparison of sex-specific patterns of inbreeding depression in species where males are heterogametic (e.g. XY in the fruit fly) and species where females are heterogametic (e.g. ZW in the red junglefowl). In general, the 'unguarded X' hypothesis predicts that the heterogametic sex, should suffer reduced fitness because X/Z-linked recessive

deleterious mutations will be unconditionally expressed in the heterogametic sex (Trivers 1985). However, when inbreeding occurs, the 'unguarded X' predicts that the homogametic sex will suffer higher fitness costs because of the increased probability of expression of recessive alleles (Fox et al. 2006; Bilde et al. 2009). Hence, this predicts a reversal of sex-specific fitness costs of inbreeding across flies (homogametic females) and birds (homogametic males). On the other hand, the 'sex-specific resource allocation' hypothesis predicts no reversal in sex-specific inbreeding depression but rather that the sex that invests more resources in competition for mates (often males) should always suffer a lower magnitude of inbreeding depression regardless of the sex determining system (Bilde et al. 2009). I found support for both hypotheses. Patterns of offspring viability were consistent with the 'sex-specific resource allocation hypothesis': daughters suffered higher inbreeding depression than sons in both the fruit fly and the red junglefowl (Chapters 2 and 3). However, inbreeding depression in the red junglefowl was greater in sons than daughters, with regards to growth rate and tarsus length. This could be partly due to high mortality rates of poor-quality inbred daughters prior to hatching and at early days post-hatching, leading to similar growth rates between inbred and outbred daughters. Reversal of optimal phenotypes in the maladaptive directions could also explain these patterns in growth rate and tarsus length (see Chapter 3 for detailed explanation). The 'unguarded X' hypothesis' could also potentially explain the higher magnitude of inbreeding depression (with regards to growth rate and tarsus length) in sons than daughters in the red junglefowl. Different mechanisms could therefore be acting at different life history stages and on different traits, explaining the complicated patterns revealed in my studies.

Chapters 2 and 3 also demonstrated a previously unrecognised modulation of inbreeding depression by parental organismal age and parental gametic age. The patterns of such interactions were complex, and varied between the fruit fly and the red junglefowl, between life-history stages and traits. It is plausible that this complexity is unlikely to be explained by a single genetic mechanism. In Chapters 2 and 3, I proposed opposing mechanistic explanations for these inconsistent effects of senescence on inbreeding depression: acquisition of a higher mutation load with parental age *versus* selective disappearance. The former refers an increased germ-line mutational load with the age of an individual (Crow 2000) which might amplify the magnitude of inbreeding depression. The latter describes the differential mortality rates for individuals with different quality, particularly referring to higher mortality rates of poor-quality (e.g. low fecundity or low offspring viability) individuals. This would retain high-quality individuals which might mitigate the effects of inbreeding and thus decrease the magnitude of inbreeding depression. Therefore, these two mechanisms would predict opposite effects of parental senescence on inbreeding depression, which could be examined in future studies. Below, I briefly elaborate on the several approaches that could be used to disentangle the relative influence of the two hypotheses.

Clearly, there are still current gaps in our knowledge of the genetic architecture of individual fitness and its modulation by evolutionary phenomena such as inbreeding and senescence. Further steps can be taken to progress our understanding in this field. First, a longitudinal study, in which data are collected from the same subjects over a period of time, can be conducted to examine the effects of between-individual variation in mortality rates (selective disappearance) on offspring fitness. For example, one could

breed offspring from a young cohort and then breed offspring from the same cohort after several years/days (depending on species) when individuals have reached old age. Some parents might have died along the way and offspring from these parents could be added and removed from the analyses. Subsequent comparison of the results will provide insights into the effects of selective disappearance on offspring fitness as well as on sex-specific inbreeding depression. Second, available genetic tools can be used to test the underlying mechanisms mediating interactions between inbreeding and parental senescence. While most studies have conducted experiments to understand the genetic mechanism of inbreeding (Charlesworth & Willis 2009) and parental senescence (e.g. Crow 2000) independently, few have sought to examine how the effects of homozygosity imposed by inbreeding changes with the germ-line mutations acquired by senescent parents. Lastly, a meta-analysis of the current literature on sex-specific inbreeding depression will be useful to help us identify common trends in this phenomenon. We could then identify patterns in which sex suffers a higher inbreeding depression according to trait, sex-determining system and species.

9.1.2. Inbreeding avoidance

In Chapter 4, I found no evidence of precopulatory inbreeding avoidance in the fruit fly, after controlling for sex-specific responses, familiarity, sexual receptivity and mating history. In Chapter 5, I found precopulatory inbreeding avoidance in both male and female fowl, after controlling for sex-ratio. Males courted unrelated females more frequently than related females and females exhibited a lower mean resistance score to the mating attempts of unrelated males. However, there was no evidence that male red junglefowl avoid inbreeding after copulation. I also found that male response to inbreeding did not change according to sex-ratio of the social group.

There are several explanations that could account for why inbreeding avoidance was found in the red junglefowl but not the fruit fly. First, the two studies were conducted on experimental individuals that differed in their age relative to lifespan. For the fruit fly, I examined inbreeding avoidance when individuals were relatively young (2-days post-eclosion) compared to their average lifespan (mean lifespan is approximately 35 days) (Luckinbill & Clare 1985). For the red junglefowl, individuals were 4-years-old when the study took place. They can live up to 30 years in captivity and at least up to 5.5 years in semi-natural populations exposed to pathogens and predation (Dean et al. 2010). The difference in age relative to lifespan would mean that individuals of the two species differed in their sexual receptivity and mating history, whereby older individuals might be more selective against kin because of increased mating experience and therefore increased choosiness when exercising mating decisions. Second, the magnitude of inbreeding depression was smaller for the fruit fly (1-day post-eclosion, egg-adult viability, averaged across two sperm age, coefficient of inbreeding depression $\delta = 0.197$) (Chapter 2) than the red junglefowl (4-6 years-old parents, averaged across different traits, $\delta = 0.450$) (Chapter 3). Theory predicts that for when parental investment is low, males and females should avoid inbreeding with their full-siblings when δ is higher than 2/3 (Parker 1979, 2006), whereas males but not females should inbreed if $2/3 < \delta < 1/3$. Indeed, at $\delta = 0.450$, female red junglefowl in my study avoided inbreeding. As for males, although mean δ was not above 2/3, the social context in which the experiment was conducted could have amplified the degree of male inbreeding avoidance. The overall fewer courtship events directed towards related females than towards unrelated females was largely mediated by the treatment where two males (one related and one unrelated) were placed with one female. Males in this

treatment exhibited significantly increased discrimination against related females, potentially due to a high energetic cost of competition and therefore a calculated use of resources that includes the efforts of male-male competitive behaviours. The lack of inbreeding avoidance in fruit flies might be a widespread pattern. Recent work on the fruit fly provided evidence for mating preferences for kin over unrelated males (Loyau et al. 2012; Robinson et al. 2012a, b). This suggests that there may be kin-selected benefits that might compensate for costs of inbreeding. Third, the fruit fly might minimise the costs of inbreeding in other ways. For example, polyandry in the fruit fly could reduce the number of inbred offspring produced by a female, when a brood is sired by multiple males (Harshman & Clark 1998; Imhof et al. 1998; Michalczyk et al. 2011; but see Hosken & Blanckenhorn 1999). In addition, postcopulatory inbreeding avoidance might occur in the fruit fly, where percentage paternity decreases with increasing male-female relatedness (Mack et al. 2002; Panhuis & Nunney 2007; but also see Ala-Honkola et al. 2011).

There are several potential questions that could be addressed in the future. Further studies can be conducted to understand how the degree of inbreeding avoidance varies with the magnitude of inbreeding depression. This could be done in two ways. One, would be to impose inbreeding for varying number of generations so as to vary the magnitude of inbreeding depression while testing for inbreeding avoidance. However this needs to be done carefully. As discussed in Chapter 4, there have been inconsistencies in the evidence for inbreeding avoidance in the fruit fly using inbred isofemale lines, probably due to differences in selection regimes (Averhoff & Richardson 1974, 1976; van den Berg et al. 1984). In addition, inbred individuals might exhibit atypical mating behaviour compared to normal individuals (e.g. Cheng et al.

1984; Miller & Hedrick 1993, 2001). Therefore, this approach has many disadvantages that might confound the interpretation of the results. A better approach might be to conduct a meta-analysis to analyse the relationship between inbreeding depression and the degree of inbreeding avoidance. Another question that could be tested would be how an individual's age (relative to lifespan) and mating history modulate its propensity to inbreed. This could be done using a two-way factorial experimental design manipulating both age (e.g. just reached sexual maturity *versus* mid-age *versus* old age) and mating history (virgin *versus* once-mated *versus* multiply-mated) simultaneously. Such a study would enable us to examine the relative contributions of each factor to inbreeding propensity.

9.1.3. Relatedness between prospective mates

In Chapter 6, I found that individual male and female fruit flies respond to the relatedness between their past and potential future mates in contrasting ways. When exposed to two sexually novel females, one related and one unrelated to a previous mate, males preferentially court the genetically novel (unrelated) female. In contrast, females display a weak preference for genetically familiar males (i.e. males related to previous mates). However, in Chapter 8, where individual females were placed with three males, two related to each other and a single unrelated male, percentage paternity was significantly biased towards the single unrelated male (i.e. > 66.6%) rather than towards the two other males that were related to each other in the group. This suggests that the male of the rare phenotype could be in an advantageous role in sperm competition. In Chapter 7, I found that female red junglefowl show a marked preference to mate with the single unrelated male than with either of the two males related to each other. I also found tentative support that females might also bias

postcopulatory sperm utilization in favour of the unrelated male.

Thus, postcopulatory responses to the relatedness of male mates were similar in both the fruit fly and the red junglefowl, whereby the single unrelated male attained higher reproductive success than any of the related males. In the fruit fly, it is difficult to attribute this bias to female preference as I did not examine the behavioural mechanisms (e.g. sperm dumping; Snook & Hosken 2004) mediating such an observation. Precopulatory preference of female fruit flies for males related to previous mates (Chapter 6) suggests that paternity bias could also be due to male behaviour and not female response. For example, the unrelated lone male might be investing more sperm in the female, resulting in higher paternity. Female fowl, on the other hand, ejected a higher proportion of ejaculate inseminated by males related to their previous mate, suggesting that females are exerting postcopulatory discrimination against such males. Nevertheless, I did not quantify paternity in the experiment on the red junglefowl and thus the fitness consequences of this behaviour remain untested.

I propose several key steps to improve our understanding of these behavioural observations. Firstly, we need to identify the mechanism through which individuals compare the relatedness between two opposite-sexed individuals. I have shown that in the fruit fly, the olfactory sensory system is essential in this behaviour. However, the preference for genetically novel females in *Orco*[1] males, though significantly weaker than in wild-type males, suggests other sensory mechanisms may also be mediating this behaviour. By systematically removing the cues of sound, olfaction, touch, and vision (Bretman et al. 2011), one can examine the relative importance of each cue in this behaviour. Although direct genetic and morphological manipulation in the fowl may

not be directly possible, one could examine the similarity in odour signatures (Karlsson et al. 2010) between related birds, which could provide insights into the recognition of related individuals. Previous studies have shown that the chemical profile of an individual Antarctic prions, *Pachyptila desolata,* was more similar to itself than to that of any other bird (Bonadonna et al. 2007) and that individuals of this species avoided its own odour in the presence of a conspecific odour (Bonadonna & Nevitt 2004). These results indicate that these birds are able to recognise and discriminate against individual odour cues, which might mediate kin recognition and inbreeding avoidance because kin share a higher odour similarity than non-kin (Célérier et al. 2011). Secondly, we could further assay the fitness consequences of these behaviours, for example, by testing whether a female preference for the rare male phenotype leads to higher offspring viability. While future work needs to quantify the fitness consequences of behavioural responses to the relatedness of prospective mates, these issues were explored in part in the last research theme below.

9.1.4. Kin selection, male-male competition and female life-history

In Chapter 8, I examined the role of inter-male relatedness on the intensity of male-male competition in the fruit fly and found that related males were not less aggressive to each other than to the unrelated male. In addition, I also explored the effects of inter-male relatedness on female life-history in Chapter 8. Consistent with predictions that inter-male relatedness mitigates male harm, female fruit flies mated to genetically similar (related) males have a prolonged lifespan compared to those mated to genetically unrelated males. However, these females mated to related males also reproduce at a lower rate, resulting in no difference in overall reproductive success between treatments. In Chapter 7, I found that male red junglefowl competed less

frequently with closer relatives over access to mating opportunities, consistent with predictions of kin selection theory. Therefore, kinship had an effect on the intensity of male-male competition in the red junglefowl but not in the fruit fly.

I propose two reasons that could account for this incongruence. In the study on fruit flies, changes in female life-history in response to inter-male relatedness suggest that other key aspects of male behaviour might be missed in this study. I only quantified the counts of aggression and courtship but not the type of aggressive and courtship behaviour, which could potentially vary in the levels of intensity (Bastock & Manning 1955; Chen et al., 2002). The intensity of aggression and courtship could therefore potentially differ with the relatedness of males. In addition, males might alter their postcopulatory responses accordingly to the relatedness of male competitors. For example, in the presence of related rivals, males might inseminate less sex peptide (which increases egg production and reduces female fitness) into the female (Chapter 8). This idea is consistent with our findings: females mated to related males have a lower reproductive rate and there is also no correlation between mating rates and female lifespan in this treatment. Females mated to unrelated males, however, show a negative correlation between lifespan and mating rates.

The second reason that could explain the difference in effect of inter-male relatedness on male-male competition is the dissimilarity in sex-ratios used in the two studies. In both studies, three experimental males were used. However, in the red junglefowl study, three females were available whereas in the fruit fly experiment, only one female was available. Hence, in order to gain mating success, each male fruit fly would be highly competitive regardless of the relatedness of rivals. Consistent with this idea, I

found no difference in mating success between related and unrelated male fruit flies, whilst in the red junglefowl, the unrelated male gained a higher proportion of mating success as compared to any of the two relatives.

In the future, it will be interesting to manipulate sex-ratio of the group to investigate whether the intensity of competition between related males relative to that between unrelated rivals can be altered when more females are available. It will also be important to determine how related males (especially male red junglefowl) recognise each other. Kin recognition could be mediated through social familiarity or phenotype matching (refer to Chapter 7 for detailed explanation). A simple method to distinguish the two would be to have siblings kept either together or in separate groups (Pizzari et al. 2004; Tan et al. 2012). Thus, four groups of individuals could be created: related and familiar; related and unfamiliar; unrelated and unfamiliar and unrelated and familiar and subsequently used to test the role of familiarity in kin recognition.

9.2. Broader implications

Natural populations often exhibit a degree of viscosity due to constraints on dispersal (Hamilton 1964; Lion & Baalen 2008; Lion et al. 2011). This increases the probability that both males and females will encounter individuals that are related to themselves and/or related to previous mates. Here, I have demonstrated that genetic relatedness can affect sexual dynamics in several important ways, from altering fitness consequences when mating with kin (Chapters 2 and 3), to modulating sex-specific mating decisions when choosing between prospective mates that are related or unrelated to previous mates (Chapters 6 and 7), to abating the intensity of male-male competition (Chapters 7 and 8) and to affecting potential for intrasexual conflict (Chapters 7 and 8).

Importantly, I have shown that the effects of relatedness on sexual interactions are far from one-dimensional. Firstly, relatedness can interact, in complex manners, with other principal biological phenomena such as senescence and sexual dimorphism to modulate fitness. This could potentially explain the inconsistency in results from previous studies examining the magnitude of inbreeding depression as well as sex-specific differences in inbreeding depression. For example, while many studies report evidence of inbreeding depression (Charlesworth & Charlesworth 1987; Crnokrak & Roff 1999), some also indicate no evidence of inbreeding depression (e.g. Keane et al. 1996; Peer & Taborsky 2005). Similarly, some studies have shown that the homogametic sex suffers greater inbreeding depression in survival in insects (Wilkinson et al. 1998; Fox et al. 2006), birds (Fowler et al. 1997) and mammals (Promislow 1992), but others have shown the opposite effect (Whitlock & Fowler 1996; Enders & Nunney 2010). Thus, when comparing among studies or when conducting future work, it is imperative to consider and control for other factors that might be influencing inbreeding effects. I have shown in my studies that the magnitude of inbreeding depression and the sex which suffers a higher inbreeding depression is dependent on the fitness trait measured, parental organismal senescence and gametic senescence. Hence these modulating variables will need to be considered in further studies. Secondly, several mechanisms may operate simultaneously and can either exacerbate or counteract each other. An example is illustrated in Chapter 7 which demonstrated the interplay of kin selection and rare male advantage in the red junglefowl, resulting in sperm competition strategies of related male to be contrary to that of theoretical models (Parker 2000). Another example is seen in Chapter 8, which showed that average female lifespan was a consequence of selective disappearance, male behaviour and genetic diversity of males.

As such, these studies revealed the importance of examining interconnected mechanisms when studying reproductive behaviour.

9.3. Future directions

There are two broad questions that could be examined in the future: the role of epigenetics in fitness architecture and kin recognition mechanisms.

9.3.1. Epigenetics, ageing and inbreeding depression

Effects of parental organismal age and sperm age on offspring traits revealed in my thesis suggest the potential role of epigenetics in fitness architecture of offspring. Epigenetics refers to heritable changes in gene activity and expression without a change in the DNA coding itself. Many studies have explored the relationship between epigenetics and ageing. For example, a pioneer study showed that genomic global DNA methylation decreases with age in spawning humpbacked salmon (Berdyshev et al. 1967) and another study reported the loss of DNA methylation with increasing age of mouse tissue and human bronchial epithelial cells (Wilson et al. 1987).

Epigenetic inheritance is a component of epigenetics that refers to the transmission of epigenetic changes to subsequent generations of cells or organisms. One example of epigenetic modification being transmitted between generations of organisms is found in *agouti* mice. Morgan et al. (1999) showed that the phenotype of a mouse dam with the A^{vy} allele was related to the phenotypes of the offspring, whereby an A^{vy} dam with agouti phenotype would more likely have a agouti phenotype. This maternal effect might have resulted from the persistence of epigenetic modifications through meiosis. In another example, epigenetically silenced germ-line mutations have been implicated

in familial forms of renal (Herman et al. 1994), colon (Estellar et al. 2000a) and breast cancer (Estellar et al. 2000b), suggesting inheritance of epigenetic changes across generations. Therefore, it is possible that the effects of parental organismal and sperm ageing on offspring fitness could act through epigenetic modifications.

There is also a growing appreciation of the impact of epigenetic changes on inbreeding (Biémont 2010). Increasing evidence is provided for the variation of epigenetic patterns among individuals and its effects on natural morphological variation and developmental defects observed in inbreeding (Richard 2006, 2009; Schilling et al. 2009). Therefore, understanding heritable epigenetic processes such DNA methylation, DNA-associated protein modifications and RNA interference can help elucidate underlying genetic causes of inbreeding depression that occur during embryonic development and in adulthood.

Heritable epigenetic processes and epigenetic control mechanisms are important in the study of evolution because they affect both the processes of adaptation and of divergence (Jablonka & Lamb 1995, 2005). Further studies on the relationship between epigenetics, ageing and inbreeding will shed light on the genetic mechanisms underlying fitness architecture and will increase our understanding of the role of these biological phenomena in evolutionary processes.

9.3.2. Kin recognition

My thesis showcases the ability of an individual to recognise relatedness of other individuals to oneself and relatedness between potential mates. Further steps could be taken to understand the mechanisms and cues underlying kin recogition. An important

question to address is the importance of familiarity or prior learning in kin recognition. In birds, discrimination of kin was not dependent on shared prior experience in peacocks (Petrie et al. 1999) and zebra finches (Burley et al. 1990) while other species require learning of the characteristics of relatives in order to identify them (e.g. long-tailed tits, *Aegithalos caudatus*, Sharp et al. 2005; bank swallows, *Riparia riparia,* Beecher & Beecher 2010). Related to this question, it will be important to decipher the type of cues that is used to discriminate kin. Previous studies have shown that birds can discriminate between kin and non-kin based on a variety of cues: individual-specific calls or songs (e.g. long-tailed tits, *Aegithalos caudatus*, Sharp et al. 2005; zebra finch, *Taeniopygia guttata* Miller 1979), olfaction (zebra finch, Krause 2012; European storm petrels, *Hydrobates pelagicus*, Bonadonna & Sanz-Auilar 2012) and vision (Japanese quail, *Coturnix japonica*; Riters & Balthazart 1998). In insects, extensive work has been done on cuticular hydrocarbons which play an essential role in sex and species recognition (Singer 1998). These chemical signatures may also be important in kin recognition in eusocial insects such as wasps and honeybees. Studies on some species of wasps have revealed colony-specific hydrocarbon profiles (Singer et al. 1992; Layton et al. 1994) and studies on the honeybee showed that closely related individuals share similar hydrocarbon composition (Page et al. 1991). Indeed, previous studies on the fruit fly have suggested the potential role of olfactory cues in inbreeding avoidance (Averhoff & Richardson 1974; 1976) but another study failed to replicate the results (van der Berg 1984).

The above mentioned kin recognition mechanisms could also apply to situations in which focal individuals compare the relatedness of potential and past mates. Two related opposite-sexed individuals could smell, look or behave in the same manner,

allowing opposite-sexed individuals to compare between the relatives and an unrelated prospective mate.

Another interesting future study can be undertaken to understand the link between kin recognition, the cues involved and major-histocompatibility (MHC) genes in the red junglefowl. MHC genes code for antigen-presenting molecules that are key to the acquired immune function (Janeway et al. 1999). Previous studies have shown that more MHC diverse individuals may be more likely to cope with a larger repertoire of pathogens than less MHC diverse individuals, probably because of heterozygote advantage or because of an increased probability of carrying MHC alleles that are able to defend against a particular pathogen (Penn & Potts 1999; Milinski 2006). A recent study demonstrated that the male red junglefowl allocates more sperm to the more MHC-dissimilar females (Gillingham et al. 2009). Because genetically related individuals are likely to share MHC alleles (Brown & Eklund 1994; Penn & Potts 1999), results from this study suggest some form of inbreeding avoidance mechanism based on MHC genotype. Genes linked to the MHC genes, such as olfactory receptor genes, may mediate MHC recognition and therefore kin recognition (Penn & Potts 1999). This link between the immune system, olfactory cues and kin recognition could be tackled in the future.

Thus, there remain several questions to be answered for both these species: (1) What are the cues necessary to identify kin? (1) Are multiple cues needed to for kin recognition? (2) Is familiarity important? (4) How is kin recognition related to genes of the immune system and how does this translate to the fitness consequences of mating or avoiding kin?

9.4. Conclusions

To conclude, my thesis has shown that relatedness can have profound effects on sexual interactions of social groups and fitness consequences of offspring. In both the fruit fly and red junglefowl, inbreeding depression is sex-specific and modulated by parental and gametic age. I found no evidence of inbreeding avoidance in the fruit fly and no evidence that inbreeding propensity in the male red junglefowl is modulated by sex-ratio. Relatedness of prospective mates influences the mating decisions of an opposite-sex individual in both species. Lastly, male-male relatedness abates the intensity of intra-male competition in the red junglefowl but not the fruit fly. Further studies can seek to understand the genetic mechanisms involved in the interaction between parental ageing, gametic ageing and inbreeding, elucidate kin recognition mechanisms and quantify the fitness consequences of the behaviours revealed in my studies.

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APPENDIX

Some of these Chapters and other peripheral research findings by fellow colleagues have been communicated to the wider audience through artistic platforms. A choreography on how relatedness affects sexual interactions in the fruit fly (Chapter 6) won the Biology category of the Dance your PhD 2011 competition by *Science* journal (http://news.sciencemag.org/sciencenow/2011/10/dance-your-phd-winner-announced.html). Another dance and music video, based around the theme of changing social groups to increase one's relative attractiveness won the Evolution Video contest 2012 by National Evolutionary Synthesis Center (https://vimeo.com/44808911). The male mating strategy depicted in this video is related to findings in Chapter 7 where it might be beneficial for a male red junglefowl to disperse from a group where he is disadvantaged role (with a relative) to another group where he is the favoured rare genotype (unrelated male). A collaboration with a fellow researcher, Dr Rebecca Dean, showcased her PhD on the evolutionary sexual battle between ageing roosters and hens (http://vimeo.com/14541766). Her findings that old males produce sperm with lower swimming velocity and impose fertility cost on the female are applicable to our results in Chapter 3, which describes the effects of parental age and sperm age on inbreeding depression.

This method of communicating up-to-date research findings has proven very effective and popular as the videos have received considerable media attention (e.g. BBC Oxford radio, *Science* journal, *the Oxford Student* newspaper) and at least 1.5K views for each video. Following the success of this form of science communication, the team at the Edward Grey Institute is currently brewing up new ideas for our new video, illustrating sperm competition and sexual conflict in the red junglefowl (Chapter 7).