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MATE CHOICE AND CONSEQUENCES FOR CONSANGUINEOUS MATING IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

The ability to discriminate between prospective mates is potentially important not only for the avoidance of unsuccessful or incompatible matings, but also for the selection of higher “quality” mates. Inbreeding, reproduction between closely related individuals, has long enjoyed the attention of biologists, and has classically been associated with fitness consequences that are primarily negative, termed “inbreeding depression”. Indeed, the costs of mating with a relative are well documented across a wide variety of species. If individuals are capable of kin recognition, it is expected that species should evolve to avoid consanguineous matings. However, a number of recent models suggest circumstances in which inbreeding might be favoured or at least not result in substantial fitness penalties. In support of these models, several recent studies have reported that in the model species *Drosophila melanogaster*, males and females are either indifferent with respect to the degree of relatedness of their mates, or favour closely related mates when exercising mate choice. Other work has suggested that relatedness might also serve to reduce sexual conflict. However, these studies are not without their limitations. In some cases, relatedness has been confounded with developmental environment, and it is not possible to determine if relatives are preferred as mates, or if there is a positive association between individuals who are developmentally familiar. In a number of these studies, the fitness consequences associated with such matings have not been quantified. Overwhelmingly, all studies have addressed intra- and inter-sexual relatedness in isolation from each other, and have neglected to assess the interplay between these factors. In chapter one, I assess intra- and inter-sexual relatedness and the short- and long-term fitness consequences of inbreeding and interacting with kin. In chapter two, I focus on the reproductive behaviour of males, and how kinship might modulate conflict and ultimately fitness over the lifespan of an individual. The results of each of these studies suggest that, in our study populations, individuals hold no bias towards kin. Inbreeding depression does occur, but the magnitude of this cost is small, and is balanced by the benefits to inclusive fitness. Ultimately, outcomes and preferences for inbreeding are likely highly context-dependent.

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CHAPTER 1

THE EVOLUTIONARY BASIS FOR KIN-DSCRIMINATION

Introduction

The process of adaptive evolution is rooted in the economy of life; heritable traits that confer either a cost or benefit to an individual are subject to the selective pressures of natural and sexual selection, either in concert or independently (Darwin, 1859; Andersson, 1994). Darwin (1859) was the first to implicate variation in individual success as the driving force in the change of species over time. In many species, individuals – generally the female sex (Fisher, 1930; Andersson, 1994; but see also Edward & Chapman, 2011) – have the opportunity to select from a variety of potential mates, each of which may differ in the specific phenotypic expression of such traits. Given this opportunity, it should be in the best interest of the female to exercise some form of mate choice if her selection of mate(s) will have fitness implications for herself or her offspring (Bateson, 1983; Jennions & Petrie, 1997). Mate choice, a component of sexual selection, is the phenomenon by which one sex mates non-randomly based on the value or expression of certain traits in the opposite sex (Fisher, 1915; Bateson, 1983; Heisler *et al*., 1987). Preferences may be based on the provision or implication of direct or indirect benefits (Kirkpatrick & Ryan, 1991; Andersson, 1994; Kokko *et al*., 2003; Kotiaho & Puurtinen, 2007); a male may directly improve a female's fitness by providing better resources or parental care, enhancing female and offspring survivorship (Reynolds & Gross, 1990; Wagner, 1991), or may indirectly improve her fitness by transmitting better or more compatible alleles, resulting in the production of higher quality offspring (Landry *et al*., 2001; though see Puurtinen *et al*., 2009).

The traits upon which preferences are exerted are wide-ranging, the most conspicuous of which are elaborate features and courtship displays (see Amundsen & Forsgren, 2001; Drăgănoiu *et al*., 2002; Hebets *et al*., 2011), though other, subtler traits are certainly subject to preferences as well (see Johnstone, 1994; Muller *et al*., 2006). Mate choice may also be shaped by the degree of relatedness between potential mates (Smith, 1979; Waser *et al*., 1986; Charlesworth & Charlesworth, 1987; Blouin & Blouin, 1988), which is a topic that has recently received renewed attention in both theoretical models and empirical experiments (*theoretical*: Kokko & Ots, 2006; Cheptou & Donohue, 2011; Puurtinen, 2011; Duthie & Reid, 2015; *empirical*: Ala-Honkola *et al*., 2011; Loyau *et al*., 2012; Robinson *et al*., 2012a; Robinson *et al*., 2012b; Tan *et al*., 2012). Inbreeding (the mating of closely-related individuals) has, classically, been primarily associated with negative fitness consequences due to the phenomenon known as "inbreeding depression" (Smith, 1979; Charlesworth & Charlesworth, 1987; O'Grady *et al*., 2006; Charlesworth & Willis, 2009). As such, preferences corresponding to relatedness have primarily been assumed to evolve for the *avoidance* of mating with closely related individuals (Blouin & Blouin, 1988; Simmons, 1991; Thornhill, 1993; Pusey & Wolf, 1996; Szulkin *et al*., 2013). However, the renewed attention focused on relatedness has revealed that, in some cases, a high degree of relatedness between mates may confer selective advantages, for instance, by maintaining co-adapted gene complexes (Lynch, 1991; Clarke, 1993; Burton *et al*., 1999) and improving the inclusive fitness of inbreeding individuals (Kokko & Ots, 2006). Further recent studies suggest that, in addition to contributing to inclusive fitness benefits, shared relatedness between competitors of the *same* sex may serve to reduce competition, and by extension sexual conflict (Carazo *et al*., 2014; Carazo *et al*., 2015). Sexual conflict arises when males and female vary in their reproductive interests; the source of this variation is anisogamy – asymmetry in the contribution of gametes (and thus investment) by males and female during reproduction (Parker, 1979; Arnqvist & Rowe, 2005). In the following sections I will review the details of preference and choosiness as they pertain to mate choice, the workings of sexual conflict, the costs of inbreeding depression, the potential benefits of "optimal inbreeding", and the details of a number of recent studies and models reporting on inbreeding tolerance, preference, and the interactions between kin.

Mate choice: preferences and choosiness

The arena of sexual selection encompasses a number of components, which fall into pre- and post-copulatory categories; these include sexual conflict, mate choice, male-male competition, and sperm competition, among other types of selective phenomena (Andersson & Simmons, 2006). Mate choice is important from an evolutionary perspective, as it contributes to differences in lifetime reproductive success between individuals (Andersson, 1994; Hunt *et al*., 2005). Selective behaviour exhibited towards potential mates by either sex can therefore be an important driving factor in the change of characters in a species (Andersson, 1994). Mate choice is often described as having two components: preferences and choosiness (Jennions &Petrie, 1997). Preference is directly related to how an individual ranks potential mates based on favoured traits; this is a function of the "attractiveness" of potential mates available to the female. Choosiness has to do with the amount of effort an individual allocates to the assessment of and discrimination between potential mates. Both preference and choosiness may vary in their direction and intensity, and may fluctuate based on a number of factors, such as mate density, the level of variability in an expressed trait, and the abiotic environment itself (Kvarnemo & Forsgren, 2000). Variation in the expression of preference and choosiness can occur both within and between populations (Brooks, 2002; Hunt *et al*., 2005). The expression of preferences, while clearly holding the potential for fitness benefits, may also be costly for the choosy sex. To be selective, an individual must invest time and energy into locating suitable mates (Parker, 1983; Real, 1990; Real, 1991; Forsgren, 1997; Parri *et al*., 1997; Etienne *et al*., 2014), and may be at increased risk of injury or predation while engaging in this activity (Godin & Briggs, 1996; Grafe, 1997; Booksmythe *et al*., 2008; Uy *et al*., 2000). Despite these costs, the phenomenon of mate choice is extensively documented in a wide variety of species (e.g. *insects*: Jones *et al*., 1998; *rodents*: Drickamer *et al*., 2000; *fish*: Agbali *et al*., 2010; *amphipods*: Cothran *et al*., 2012).

Sexual conflict

When males and females have different reproductive interests and fitness maximizing strategies, conflict may occur during reproductive interactions. Frequently, what is optimal reproductive behaviour for females is not optimal for males, and vice versa (Parker, 1979; Arnqvist & Rowe, 2005; Chapman, 2006). Typically, as there is often a cost associated with mating that is higher for females than males, females invest more than males in any singular reproductive interaction (Fowler & Partridge, 1989; Arnqvist & Nilsson, 2000; Dukas & Jongsma, 2012), and often invest more energy in caring for any resulting offspring (Barta *et al*., 2002; Chapman, 2006). As a result, females might prefer to mate less frequently than males (Arnqvist & Nilsson, 2000; Chapman *et al*., 2003; Westneat & Fox, 2010), or to produce fewer offspring at any one time (Smith & Härdling, 2000; Chapman, 2006). Conflict may also extend past the time of reproduction itself to factors such as the level of care provided (Požgayová *et al*., 2015) for these offspring and the future mating behaviour of either sex (Ram & Wolfner, 2007). Other conflict may be manifested as differences in optimal phenotypes, for instance, differences in body size (Pitnick & García-González, 2002; Lewis *et al*., 2011), colouration (Svensson *et al*., 2009), and the size or form of specific morphological features (Abbott *et al*., 2010). Phenotypic conflict may further extend to traits such as lifespan, as males may favour intense early-life reproduction while females may benefit from longer lives and intermittent investment in reproduction (Promislow, 2003; Bonduriansky *et al*., 2008). The circumstances in which these conflicts occur can be sorted into two categories, inter-locus conflict and intra-locus conflict. Inter-locus conflict describes scenarios in which males and females have different optimal outcomes for their interactions, controlled by genes at different loci. Intra-locus conflict describes conflict between males and females over shared traits controlled by the same genes. Due in part to the asymmetry of the costs associated with reproduction, females frequently have reason to mate discriminately, choosing mates who will minimize these costs in some way (Gavrilets *et al*., 2001; Moore *et al*., 2001); in doing so, choice may drive further conflict as a means to overcome female preferences (Promislow, 2003; Andersson & Simmons, 2006).

Inbreeding depression

Inbreeding is the occurrence of mating between related individuals, while outbreeding describes matings between very distantly related or unrelated individuals. In many species (particularly those in which “selfing” does not occur), inbreeding is frequently documented as having predominantly negative effects (Charlesworth & Charlesworth, 1987). Inbreeding depression in the phenomenon responsible for these effects, resulting in a reduction of the mean fitness of the offspring produced from consanguineous matings (Lynch & Walsh, 1998; Cheptou & Donohue, 2011). This fitness reduction is due to the increased expression of deleterious recessive alleles and/or a decrease in heterozygosity, and thus the loss of favourable heterozygotic combinations; these changes are brought about by the combination of similar genetic material inherited from parents who are genetically related to each other (Charlesworth & Charlesworth, 1987; van Bergen *et al*., 2013). The negative effects of inbreeding can be manifested at all stages of life (Liu *et al*., 2014), and occur at a level affecting individual fitness as well as at the population level (Charlesworth & Charlesworth, 1987; Loyau *et al*., 2012; Leclaire *et al*., 2013).

As noted by van Bergen *et al*. (2013), inbreeding depression disproportionately affects males, most often by resulting in a reduction in male, but not female, mating success (see also: Brekke *et al*., 2010; Agrawal, 2011). This is most likely due to the direct effects of inbreeding depression on the overall condition of inbred offspring. For males, a reduction in condition can significantly reduce their ability to acquire successful matings, while females are presumably less reliant on condition for mating success (Agrawal, 2011). Additionally, females may also be strongly biased against males exhibiting traits that are suggestive of inbreeding depression. This phenomenon has been observed in the butterfly species *Bicyclus anynana*; females of this species identify males using olfactory cues, and males exhibiting high levels of inbreeding depression are impaired in their ability to produce appropriate pheromone signals and are therefor avoided (van Bergen *et al*., 2013).

Inbreeding may also be responsible for fitness reductions brought about by lower offspring survival, rather than simply reducing the "quality" of offspring that do survive. For instance, a study by Olsson *et al*. (1996) revealed that inbred sand lizards were significantly more likely to be physically malformed and to suffer from reduced survival. Reduced survival appears to be a common cost of inbreeding across multiple species (Pusey & Wolf, 1996). Because of the well-documented (and often quite strong) negative effects of inbreeding depression, it is often hypothesized that selective pressure on mate choice should leading to preferences that are highly biased against kin in order to avoid fitness costs (Blouin & Blouin, 1988; Simmons, 1991; Thornhill, 1993; Le Vin *et al*., 2010; Mateo, 2010; and see Szulkin *et al*., 2013). In support of this, evidence for aversion to kin-matings has indeed been found across a wide variety of taxa (Packer, 1979; Pusey, 1980; Clutton-Brock, 1989; Pusey & Wolf, 1996; Gerlach & Lysiak, 2006; Sherborne *et al*., 2007; van Bergen *et al*., 2013; Fitzpatrick *et al*., 2014).

Inbreeding avoidance

Inbreeding avoidance mechanisms may exist in a number of forms, including evasion of kin through dispersal, extra-pair/extra-group matings, reproductive depression, and pre- and post-copulatory preference for non-kin (Pusey & Wolf, 1996; Szulkin *et al*., 2013). Organisms that are able to disperse from their natal grounds before reaching sexual maturity avoid inbreeding simply by reducing their chances of encountering related individuals; this is a particularly effective strategy for those species where dispersal tendencies differ between the sexes (Charlesworth & Charlesworth, 1987; Blouin &

Blouin 1988; Pusey & Wolf, 1996; Lehmann & Perrin, 2003). In species where dispersal is uncommon or absent, extra-pair and extra-group matings ensure that non-kin males father a higher proportion of offspring, despite females continuing to associate with close relatives. This strategy helps maintain heterozygosity within a population and can prevent the maladaptive combination of genes that are identical by descent (Pusey & Wolf, 1996; Foerster *et al*., 2003). Additionally, when kin remain in close proximity to one another, maturation of offspring may be delayed, thereby reducing the likelihood of parent-offspring matings. Evidence for this can be seen in number of species where removal of the opposite-sex parent reduces the time it takes for offspring to reach sexual maturity (Waldman, 1988; Pusey & Wolf, 1996). Finally, individuals may avoid inbreeding via active discrimination against relatives, either by abstaining from consanguineous mating altogether (Blouin & Blouin, 1988), or via cryptic choice against related-male sperm (Gasparini & Pilastro, 2011). Kin recognition (and therefore discrimination) can occur via direct recognition, such as phenotype matching (Le Vin *et al*., 2010; Mateo, 2010), and via indirect recognitions that is context dependent, such as familiarity or imprinting (Waldman, 1988; Simmons, 1989). These mechanisms might occur in some combination or independently, the presence and intensity of which may be condition-dependent (Pusey & Wolf, 1996; Hunt *et al*., 2005; Cheptou & Donohue, 2011; Billeter *et al*., 2012).

Social context can also play an important role in the variation of reproductive behaviour. Using *D. melanogaster*, Billeter *et al*. (2012) found that female fecundity can be influenced by the presence, absence, or composition of groups of additional males during mating events. It was found that females copulating with males of their own genetic "variety" became infecund at the expense of the males similar to their own phenotypes (therefore, potential relatives) seemingly in favour of novel males presented to her. This phenomenon would seem to promote outbreeding. A similar phenomenon (the Bruce effect) has been observed in rodents, and is proposed as a mechanism of inbreeding avoidance (Billeter *et al*., 2012). In preliminary work conducted for this thesis, I was able to partially replicate these results; females in the presence of related and unrelated males were not found to induce infecundity during egg-laying on the basis of the genetic identity of her selected mate (Appendix 1a), but ultimately eggs fertilized by relatives (phenotypically similar males) did result in fewer offspring (Appendix 1b). However, it is possible that inbreeding depression, rather than female-induced infecundity, may be responsible for the reduced survival of these eggs. Despite this variation in egg survivorship, females did not mate preferentially with unrelated males (Appendix 2). While the high cost of inbreeding is widely documented, circumstances may exist in which these costs are sufficiently low, or benefits are sufficiently high, such that the evolution of tolerance or preference for inbreeding is possible (Cheptou & Donohue, 2011; Robinson *et al*., 2012a; Robinson *et al*., 2012b; Tan *et al*., 2013).

Recent literature: inbreeding tolerance and preference, and the role of relatedness

In contrast to (or despite) the large body of research documenting the negative effects of inbreeding and frequent avoidance of consanguineous matings, a number of recent models have focused on scenarios in which inbreeding might be tolerated or beneficial (Kokko & Ots, 2006; Cheptou & Donohue, 2011; Puurtinen, 2011; Szulkin *et al*., 2013; Duthie & Reid, 2015). In these models, the benefits an individual gains via "inclusive fitness" are sufficient to offset the deleterious effects of inbreeding depression; in these situations, an optimal balance between inbreeding and outbreeding is maintained, termed "optimal inbreeding" (Robinson *et al*., 2012b). Inclusive fitness, as originally described by Hamilton (1964), takes into account the overall fitness achieved by an organism considering additional gene copies passed on by its relatives and by offspring with a higher relatedness coefficient. Therefore, inbreeding might be beneficial if it increases the total number of parental gene copies that are propagated into the next generation (Kokko & Ots, 2006; Szulkin *et al*., 2013). Inbreeding tolerance might also evolve if the costs of avoidance are sufficiently high. That is, by tolerating some degree of relatedness in potential mates, an individual may significantly increase the number of mating opportunities available to it in its lifetime that would otherwise be lost due to rejecting relatives (Widemo & Sæther, 1999). In line with these possibilities, Kokko and Ots (2006) suggested that the prevalence of inbreeding may be much higher than expected, particularly in cases where mating is sequential rather than simultaneous.

Several recent studies have focused specifically on inbreeding in the model species *Drosophila melanogaster*, and have found some empirical evidence to support these theory-based models. In these studies, it has been reported that females may copulate indiscriminately with males regardless of their degree of relatedness (Tan *et al*., 2012; Ala-Honkola *et al*., 2011), but in some cases may demonstrate a significant preference for brothers over non-kin (Loyau *et al*., 2012; Robinson *et al*., 2012a; Robinson *et al*., 2012b). In addition to inbreeding, the link between relatedness and social interactions has also been considered. In some cases, males who were related to one another were found to behave less antagonistically towards each other, and as a result reduced the harmful effects of sexual conflict on a female (Carazo *et al*., 2014; Carazo *et* *al*., 2015). However, these studies have potential limiting factors and are in need of further refinement; I detail these issues below.

A study by Loyau *et al*. (2012) found preference for brothers over non-kin males in *D. melanogaster*. In this study, females mated more readily with brothers than with unrelated males, and showed no biases against copulating with their fathers, while males were "indifferent" to the degree of relatedness of their mates. Overall, this study seems to indicate that in this model species, both tolerance and preference exist for consanguineous matings. However, their experimental design potentially confounds relatedness with developmental environment, in that females may simply have been positively associating with males raised in a shared larval environment. Lyons *et al*. (2014) report that in swordtail fish, developmental environment can play a significant role in mate choice, affecting the strength of preference for various traits. Because developmental environment (and therefore familiarity) were not controlled in the Loyau *et al*. (2012) study, preference for siblings cannot be definitively confirmed. Additionally, the fitness outcomes of these mating were not determined; the consequences of inbreeding, such as egg number, egg-to-adult survivorship, and the fitness of inbred offspring should be further explored.

A study by Robinson *et al*. (2012a) has also found evidence for inbreeding preference in *D. melanogaster*. They reported that increases in the degree of relatedness between mates correlated with lower mating latency times (the time from courtship onset to copulation), a parameter commonly used to measure female preference for more closely related males (Pischedda *et al*., 2012). It is possible that related males may court relatives more intensely, resulting in lower mating latencies, but lack of increased courtship duration suggest that in this case, latency is primarily due to variation in female preference for relatedness (Robinson *et al*., 2012a). The fitness consequences of these mating preferences, again, were unexplored in this experiment. Previous work by the same authors (Robinson *et al*., 2012b) found that in wild populations, *D. melanogaster* also court and copulate preferentially with relatives over non-kin. However, in this case, the degree of relatedness between mates was found to have significant negative effects on offspring production. Because of the apparent conflict between female preferences and fitness here, as well as the lack of fitness quantification under lab conditions, the inbreeding preferences reported here need to be replicated and examined under greater scrutiny.

In a study by Tan *et al*. (2012), aspects of both male and female choice were quantified based on interactions with potential mates of varying relatedness and familiarity. Neither males nor females could be found to demonstrate any bias against courting or mating with kin. However, low but significant levels of inbreeding depression were detected, specifically in egg-to-adult viability. Ala-Honkola *et al*. (2011) also found that *D. melanogaster* females did not appear to avoid consanguineous mating. In this case, sibling males were not found to have lower fertilization success than non-kin. However, in another study by the same authors (Ala-Honkola *et al*., 2013), inbreeding was found to affect both the attractiveness and sperm success of male offspring. Female *D. melanogaster* are known to mediate the post-copulatory process, varying their internal environment in which sperm compete (Lüpold *et al*., 2011). Therefore, it is expected that, should there exist sufficiently high costs to inbreeding, females would evolve to utilize non-kin sperm via cryptic choice; these costs are known to exist but, despite this, kin-sperm-discrimination was absent. Both these studies, while not demonstrating preferential inbreeding, do suggest inbreeding tolerance. In the study by Ala-Honkola *et al*. (2011), development environment may also have been a confounding factor. While Tan *et al*. (2012) did control for this, their experimental population of lab-maintained Dahomey line of flies, which was first collected in 1970 (Clarke *et al*., 2012), may have significant levels of pre-existing inbreeding present due to its age and the potential for overlapping generations in its culture protocol (allowing parent-offspring breeding). Despite maintenance of large populations, captive lab populations of flies may rapidly lose genetic variation over even a short amount of time (Briscoe *et al*., 1992). For inbreeding depression to occur, there must exist a certain level of standing genetic variation within a population (Long *et al*., 2013). If prior bottlenecking of these lab stocks had occurred, deleterious mutations may have been purged from this population, reducing the consequences, and perhaps weakening the expression of inbreeding avoidance (Glémin, 2003).

Two experiments, carried out by Carazo *et al*. (2014) and Carazo *et al*. (2015), examined relatedness in the context of intra-sexual interactions. In the first experiment, the antagonistic behaviour of related and unrelated males was assessed, as was the effect of this behaviour on females. The authors found that when some or all of males in a group of three were related, these groups fought amongst themselves significantly less, and the frequency of courtship behaviour directed at females was significantly reduced. Additionally, females housed with males who were related to each other were found to live longer and produce more offspring. These results have been interpreted as an indication of behaviour modulation driven by relatedness, resulting in the reduction of both intra-sexual competition and sexual conflict. However, criticism of this study, initially raised by Hollis *et al*. (2015), is similar to that of previous studies—developmental and social familiarity were not properly controlled. Similar experiments conducted by Hollis *et al*. (2015) were able to partially replicate the findings of Carazo *et al*. (2014), finding that females had higher reproductive success when housed with males related to each other; however, this was dependent on related males sharing a pre-trial developmental environment. A second experiment by Carazo *et al*. (2015) again found evidence for reduced fighting between males when those male were siblings *and* reared in the same environment when contrasted with males that were both unrelated and unfamiliar with each other. However, unlike their initial experiment, they did not observe reduced courtship intensity. Chippindale *et al*. (2015) recently conducted an experiment inspired by the findings of Carazo *et al*. (2014), focusing more specifically on the fitness results of these scenarios. In this case, the intra-sexual relatedness of males did not affect any measured metric of fitness, suggesting that shared relatedness does not universally act as a modulator of sexual conflict in this species. Evidently, the role of relatedness in shaping intra-sexual interactions is far from clear. Additionally, there is a lack of understanding as to how relatedness between potential mates might affect those interactions and the fitness outcomes of such pairings.

Due to the limitations of these studies, it may be that the effects of relatedness are not being properly detected due to confounding factors, or perhaps that laboratory circumstances are not accurately replicating natural settings. Thus, the patterns observed in these experiments may not be indicative of the same scenarios occurring in the wild. The environments in which individuals develop need to be controlled, and an appropriately large, outbred population of *D. melanogaster* used. Additionally, the work of Carazo *et al*. (2014; 2015) can be expanded to include inter-sexual relatedness. Because of these limitations, follow-up research is required to better elucidate details on the phenomenon of inbreeding.

Significance and objectives

A review of the literature reveals a conflict in our understanding of the importance and consequences of inbreeding avoidance in relation to sexual selection, sexual conflict, and population structure, as well as in the ways in which mate choice influences the presence of inbreeding or outbreeding. The circumstances dictating the direction of biases towards inbreeding are not yet well understood, nor are the fitness consequences of such matings. The results of these studies must be further explored.

I examined inbreeding biases and fitness outcomes under varying circumstances and timeframes. I have considered the effects of both social and developmental environment in my experiments, and have studied both *inter*- and *intra*-sexual relatedness as potential modulators of social and copulatory interactions. I have primarily focused on female mate choice as an indicator of biases for or against kin, though have also assessed how relatedness affects the way in which males treat both each other and females. Specifically, I have considered the phenomenon of inbreeding avoidance from two perspectives:

1. What (if any) degree of bias exists for mating with kin; that is, can inbreeding preference or avoidance be demonstrated?

2. What are the fitness outcomes of inbreeding; do consequences exist that are sufficiently costly or beneficial so as to account for the presence or absence of kin-modulated behaviour?

I have used *Drosophila melanogaster* for this research, as the common fruit fly is a model organism for many genetic models, and is the most widely utilized species for genetic research (Flagg, 1977). This species is easily cultured, reproduces quickly, and requires minimal space and resources, making it well suited for use in the study of evolutionary mechanisms and population genetics (Brookes, 2001).

Hypotheses

I had two mutually exclusive hypotheses for the occurrence and consequences of non-random mating with respect to inbreeding: (a) *D. melanogaster* avoid mating with relatives, and (b) *D. melanogaster* are unbiased in respect to shared kinship with potential mates. These relate to my questions as follows:

1. (a) If inbreeding avoidance exists, female aversion will be detected in the form of longer mating latencies when mating with kin.

   (b) If inbreeding is tolerated, mating latency will not differ significantly between inbred and outbred matings; variation in time to onset of mating for each female will be random.
2. (a) If inbreeding is avoided, we will observe fitness consequences when inbreeding is allowed to occur.

   (b) If inbreeding is tolerated, the fitness consequences of consanguineous matings will be either positive (due to optimal inbreeding) or non-existent.

The following chapters detail the two major experiments, which build and expand upon existing knowledge to provide a more complete characterization of the role of relatedness.

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CHAPTER 2

THE COSTS AND BENEFITS OF INBREEDING: A MULTIGENERATIONAL ANALYSIS

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Preamble

The following chapter was written as a manuscript in the style of *The Journal of Evolutionary Biology*, where it will be submitted for consideration shortly. Table and figure numbering has been modified slightly to differentiate between chapters.

Abstract

The consequences of inbreeding have been classically associated with a reduction of fitness, known as inbreeding depression. However, recent models have indicated that in some cases, inbreeding should be preferred over outbreeding due to the potential benefits of inclusive fitness. Empirical studies have since demonstrated a wide range of inbreeding preferences and consequences in the model organism *Drosophila melanogaster*, spanning the spectrum from positive to negative. We set out to discover more about how this species interacts with kin in varying social environments, and to document the immediate and future consequences of mating with a close relative in these different contexts. We housed females with brothers or unrelated males, and created environments in which choice was allowed between three equivalent males, or choice was not allowed at all. Fitness was quantified for the focal generation as well as for the subsequent generation. We did not observe any differences in the copulatory behaviour based on the relatedness of mates/suitors, nor any meaningful penalties or rewards to fitness when accounting for inclusive fitness. Our results indicate, at least in our study population, that the net costs of inbreeding are balanced by the benefits of heightened inclusive fitness, and suggests that even in the face of inbreeding depression, an indifference or preference for inbreeding may still evolve and be maintained when one considers all outcomes of shared kinship between mates.

Keywords: Inbreeding, inbreeding depression, sexual conflict

Introduction

In many species, females exhibit preferences for some males over others. These preferences may be based on female perception of male "quality", and may be expressed for traits directly and indirectly affecting fitness (Darwin, 1859; Bateson, 1983; Andersson, 1994). These preferences can be costly to females (Parker, 1983; Pomiankowski, 1987; Real, 1990; Real, 1991; Reynolds & Gross, 1990), as they require energy expenditure (Parker, 1983; Real, 1990; Real, 1991; Forsgren, 1997; Parri *et al*., 1997; Etienne *et al*., 2014) and can put choosy females at increased risk of injury or mortality (Godin & Briggs, 1996; Grafe, 1997; Booksmythe *et al*., 2008; Uy *et al*., 2000). However despite these costs, female preferences are well-documented and widespread (e.g. *insects*: Jones *et al*., 1998; *rodents*: Drickamer *et al*., 2000; *fish*: Agbali *et al*., 2010; *amphipods*: Cothran *et al*., 2012), which has led to the inference that they must be of net benefit, or at least of neutral effect, in order for search costs, energy costs, and increased risks to be worthwhile from an evolutionary perspective (for modeling see Real, 1990). There are a number of ways non-random mating might provide benefits to a female. Females may gain directly through the selection of males who are better parents, are more defensive, or contribute better resources (Bateson, 1983; Andersson, 1994), or by avoiding males who provide fewer benefits or inflict greater harm (Borgia, 1981; Holland & Rice, 1998; Gavrilets *et al*., 2001; Eberhard, 2004; Lew *et al*., 2006). Alternatively, females may choose males that provide the most indirect fitness benefits via the contribution of alleles more likely to produce offspring of superior fitness (Bateson, 1983; Andersson, 1994). Understanding variation in the costs and benefits of mate choice ultimately informs us about the factors that have shaped and continue to shape the evolution of this important agent of sexual selection. Here we explore the phenomenon of mate choice with particular attention paid to relatedness and its potential fitness consequences.

One way in which the benefits resulting from mating preferences may differ between prospective mates is when kinship exists between females and some of these males. Although many studies of mating preferences make the implicit or explicit assumption of unstructured populations (in which encounters occur between individuals no more related to each other than individuals selected from the population at random), natural populations are frequently structured into genetic “neighbourhoods” containing individuals of shared relatedness (Pizzari *et al*., 2015). In these situations, individuals of both sexes might choose whether or not to mate with prospective mates on the basis of their degree of relatedness. Classically, mating with a close relative (hereafter “inbreeding”) has been primarily associated with predominantly negative fitness consequences (O’Grady *et al*., 2006; Charlesworth & Willis, 2009), mainly due to the phenomenon of inbreeding depression, whereby offspring of consanguineous matings have lower fitness than outbred individuals (Charlesworth & Charlesworth, 1987). This fitness reduction arises as a result of either the increased expression of deleterious recessive alleles due to increased homozygosity, or the decrease of genetically superior heterozygotic genotypes is the offspring whose parents are related (Tantawy, 1956; Charlesworth & Charlesworth, 1987; Pusey & Wolf, 1996). Because of the costs associated with inbreeding, bias against mating with relatives (“inbreeding avoidance”) is often thought to be favoured by selection (Blouin & Blouin, 1988; Clutton-Brock, 1989; Pusey & Wolf, 1996; Cheptou & Donohue, 2011), and avoidance of reproduction with close kin has indeed been documented in many species (*baboons*: Packer, 1979; *chimpanzees*: Pusey, 1980; *social mammals*: Clutton-Brock, 1989; *Townsend's voles*: Lambin, 1994; *zebrafish*: Gerlach & Lysiak, 2006; *mice*: Sherborne *et al*., 2007). However, inbreeding avoidance is far from universal, as both inbreeding tolerance and in some cases inbreeding *preference* have been observed (*song sparrows*: Keller & Arcese, 1998; *bighorn sheep*: Rioux-Paquette *et al*., 2010; *cichlids*: Thünken *et al*., 2011; *fruit flies*: Ala-Honkola *et al*., 2011; Loyau *et al*., 2012; Robinson *et al*., 2012a; Robinson *et al*., 2012b; Tan *et al*., 2012). Modeling carried out by Kokko and Ots (2006), Cheptou and Donohue (2011), Puurtinen (2011), and Duthie & Reid (2015) suggests that, in certain contexts and environments, some level of inbreeding can in fact be adaptive, lending theoretical support to these empirical observations.

When an individual mates with a close relative, it is possible to maintain beneficial co-adapted gene complexes, resulting in offspring that are better suited to their environment (Kokko and Ots, 2006). Should an individual decide not to mate with a relative, it must invest more energy into searching for an alternative mate if one is not immediately available, potentially reducing the number of mating opportunities available to it within its lifetime (Real, 1990; Kokko & Ots, 2006). Additionally, by mating with a relative, an individual benefits not only via the direct contribution of its own alleles to next generation, but also from increased inclusive fitness via genes identical by descent transmitted by its relative (Hamilton, 1964). Recent work by Carazo *et al*. (2014; 2015) has further suggested that the shared indirect fitness of relatives results in the amplification of common reproductive interests, and therefore might also serve to reduce the negative fitness consequences of sexual conflict (see below). Therefore, as long as the negative effects associated with inbreeding are outweighed by heightened direct and indirect fitness, inbreeding should be tolerated or preferred (Kokko and Ots, 2006).

Sexual conflict results from the division of reproductive roles performed by opposite sexes, and as an outcome causes divergence, and often incompatibility, in the reproductive interests of males and females (Parker, 1979). These differences are ultimately rooted in anisogamy, the asymmetric contribution of gametes (and resources) by males and female in sexual reproduction. In promiscuous mating systems, such as polyandry or polygyny, the dimorphism in fitness strategies of males and females inevitably results in a corresponding asymmetry in the level of reproductive investment that is optimal for either sex (Parker, 1979; Arnqvist & Rowe, 2005). Sexual conflict therefore arises, and is perpetuated, due to the differing costs of reproduction born by opposing sexes (Chapman *et al*., 2003; Arnqvist & Rowe, 2005; Chapman, 2006). This conflict exists in two arenas: inter-locus conflict occurs when individual traits of the sexes, controlled by different loci, have different optimal outcomes for reproductive interactions, and intra-locus conflict, which occurs when the sexes differ in the optimal expression of shared alleles at the same loci. In both cases, selection for phenotypic benefits in one sex comes at the cost of reduced fitness in the opposing sex (Parker, 1979; Holland & Rice, 1998; Arnqvist & Rowe, 2005). For instance, in many species, sexual conflict arises over mating frequency: males generally benefit from more matings due to low costs of reproduction, while females typically achieve optimal fitness with fewer matings and can afford to be more selective in choosing mating opportunities (Trivers, 1972; Westneat & Fox, 2010). Thus, conflict over mating frequency is likely to occur (Holland & Rice, 1998; Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005), which may result in the evolution of traits that are beneficial to the fitness of members of one sex but may be detrimental to the lifetime fitness of their mate (Arnqvist & Rowe, 2002; Wigby & Chapman, 2004).

Harm resulting from sexual conflict has been thoroughly documented in a variety of species (Johnstone & Keller, 2000; Hosken *et al*., 2003; Arnqvist & Rowe, 2005). In *D. melanogaster*, females are harmed before, during, *and* after copulation as a result of traits that are believed to have evolved to enhance male fitness. Prior to copulation, females often endure persistent male “harassment” in the form of vigorous courtship and mating attempts (Partridge & Fowler, 1990; O’Dell, 2003; Lasbleiz *et al*., 2006; Long *et al*., 2009). Upon copulation males transfer accessory gland proteins (ACPs) to females in their seminal fluids, which further harm females due to their toxic properties, and have lasting effects on female fitness (Chapman *et al*., 1995; Wolfner, 2002; Ram & Wolfner, 2007). While these aggressive interactions and ACPs are detrimental to the female fitness, they provide fitness advantages to the males. More aggressive courtship and copulatory behaviour may allow for increased acquisition of mates over other male competitors (Dow & von Schilicher, 1975; Pitnick & García-González, 2002), while ACPs manipulate the post-mating physiology and behaviour of the female, reducing her propensity to remate and increasing her ovulation rate, which comes at a cost of reduced female lifespan (Fowler & Partridge, 1989; Wolfner, 2002; Fiumera *et al*., 2006; Ram & Wolfner, 2007). It has been suggested that the magnitude of sexual antagonism between males and females will differ depending on the relatedness of interacting individuals (Carazo *et al*., 2014; Carazo *et al*., 2015; Pizzari *et al*., 2015; but see also: Hollis *et al*., 2015; Martin & Long, *In Press*). Males, when unrelated to their mates, gain the most fitness by maximizing their share of a female's paternity, which occurs at a cost to the female's lifetime fitness (Chapman *et al*., 1995; Wolfner, 2002; Ram & Wolfner, 2007). If males are related to their mates, in addition to benefiting directly from mating, they will also experience inclusive fitness benefits when their relatives produce offspring sired by other males. In the latter case, males have been predicted to act less antagonistically/be less harmful to their related mates than those in the former group in order to reap the greatest fitness rewards. Despite the potential for reduced conflict, theory does still predict differences between males and females over the level of inbreeding that is optimal (Lehmann & Perrin, 2003; Fitzpatrick *et al*., 2014; Duthie & Reid, 2015). Females (or the "choosy" sex) have traditionally been expected bear the brunt of the initial costs of inbreeding due to their typically greater investment in reproduction and thus greater loss of future fitness-enhancing opportunities (Parker, 1979; Smith, 1979). However, for offspring resulting from consanguineous matings, males may be more affected by inbreeding depression. In contrast to female mating success, male mating success is more reliant on overall body condition, and as such a greater proportion of the male genome is acted upon by sexual selection (Agrawal, 2011). Due to increased selective pressure on males, it has been proposed that inbred sons will likely be more sensitive to the genetic perturbations of inbreeding than inbred daughters (Whitlock & Agrawal, 2009). In support of this, a number of studies have found that the fitness consequences of inbreeding disproportionately affect males (Brekke *et al*. 2010; Agrawal, 2011; Mallet & Chippindale, 2011). We therefore require a clearer picture of the intersection of relatedness, mate-choice, and sexual conflict.

With emerging evidence for relatedness as a potential modulator of reproductive interactions (Carazo *et al*. 2014; Carazo *et al*., 2015; Pizzari *et al*., 2015), we designed a study to test the consequences of inter-sexual relatedness and mate choice over the course of two generations. We exposed female *Drosophila melanogaster* to four possible treatments varying in the number (one or three) and degree of relatedness (siblings or unrelated) to potential mates. We examined female fitness as well as the fitness of her offspring in order to quantify the associated direct and indirect fitness consequences of these different mating environments. We also assessed the courtship and copulatory behaviour of males. This work builds on a growing body of research on these topics (Robinson *et al*., 2009; Ala-Honkola *et al*., 2011; Loyau *et al*., 2012; Tan *et al*., 2012; Carazo *et al*. 2014; Carazo *et al*., 2015), while adding much-needed elaboration on the extent of inbreeding depression under varying social and environmental contexts.

Methods

(a) Experimental Populations

We used fruit flies (*Drosophila melanogaster*) from two distinct but related populations, each derived from the *Ives* (henceforth "IV") stock, which itself was created from a sample of 200 inseminated, wild-caught females collected in South Amherst, MA, USA, in 1975. The IV population has been maintained as a large, outbred population under standardized culture conditions since 1980 (Rose, 1984). Flies from the IV line have wild-type eyes, while flies form the IV-*bw* line, express a recessive brown-eyed phenotype. IV-*bw* was created by repeatedly backcrossing of the bw^{-1} allele into the IV genetic background over 10 generations (Chippindale *et al*., 2015). Regular backcrossing has since been carried out to ensure that the IV and the IV-*bw* populations remain otherwise genetically similar. Each of these populations is maintained in large (>7000 adults), non-overlapping generations at 25°C and 60% humidity on a 12h:12h diurnal light:dark cycle, and are cultured every 14 days via mixing *en masse* under light CO_2 anaesthesia (Tennant *et al*., 2014). The density at the start of each culture cycle is standardized to ~100 eggs vial^{-1}.

(b) Treatments

To initiate the experiment, we set up 30 "*families*": each was created by randomly mating a single virgin male with a single virgin female (where all flies were collected within 2h of eclosion from pupae), drawn from the IV population. These pairs of flies were housed together in vials for 24h, allowing sufficient time for mating to occur and egg-laying to begin. At that time the flies were removed, the number of laid eggs were counted, and a sufficient number of IV-*bw* eggs were added to each vial to reach a final density of 100 eggs vial^{-1}. Vials were then returned to the incubator, and, beginning 9 days later, wild-type males and females were collected as virgins (within 8h of their eclosion from their pupae). From each of the 30 families, four "focal" females were selected and held individually in vials. Males were assigned to one of four treatments as they were collected, in which we experimentally varied both intersexual relatedness (siblings or unrelated) and the potential for sexual selection (mate-choice or no-choice). These treatments were as follows: 1) "*related – no choice*", a single, full-sibling male taken from the same family as the female; 2) "*unrelated – no choice*", a single, unrelated male taken from a randomly selected family differing from that of the female; 3) "*related – choice*", three full sibling males from the female's family; and 4) "*unrelated – choice*", three randomly selected males unrelated to either each other or the female. All treatment flies were held for a maximum of 72h prior to initiation of the mating assay.

(c) Mating Assays

Mating trials began at 9:00AM, at which time males were transferred without anaesthesia into the appropriate female vial by lightly tapping the vials together. All vials were immediately mounted horizontally, and were scanned continuously by observers for a period of 90-min. The time until the onset of mating (*mating latency*), and the time at which mating ceased (later used to calculate *mating duration*) were recorded to the closest second. Mating latency is frequently used as a proxy for male sexual attractiveness because "attractive" males achieve successful mating more quickly (Barth *et al*., 1997; Ritchie *et al*., 1999; Taylor *et al*., 2008), while longer mating duration has been linked to increased intra-sexual competition and perceived sperm competition between males (Friberg, 2006; Taylor *et al*., 2008; Taylor *et al*., 2013). Once copulations were complete (or upon the end at the trial) flies were anaesthetized and the males were removed. Females were retained in their "*focal*" vials for a period of 18h before also being discarded; the eggs laid during this period were counted, and, as above, a sufficient number of IV-*bw* eggs were added to the vial to achieve a density of ~100 eggs per vial. At the same time, 120 "*companion*" vials containing ~100 IV-*bw* eggs of the same age each were established. All vials were subsequently maintained under standard lab conditions for 14 days to allow for the development and eclosion of all viable adult flies. Upon eclosion, all flies were allowed to mate freely. The inclusion of brown-eyed eggs in *focal* vials provided non-sibling mating opportunities for focal male and female offspring; *companion* vials were set aside until the fitness quantification stage.

(d) Fitness Quantification

All flies were removed from each of the focal vials, and the number and sex of wild-type flies (the offspring of our focal females) present were recorded. Egg-to-adult survivorship for each vial was calculated from these values. Next, to measure son fitness, wild-type male offspring in each *focal* vial were divided into four equal groups (where numbers were high enough to allow this), each of which was transferred to a new "*sons*" vial; all brown-eyed flies (male and female) within each *focal* vial were divided into four groups as well and were transferred alongside corresponding groups of sons to these new vials (Figure 2.1(a)). To measure daughter fitness, the remaining wild-type female offspring in each *focal* vial were also divided into four groups, as were flies within each *companion* vial; one of each group were combined and transferred to a new "*daughters*" vial, creating an environment consistent with that of the *sons* vials (Figure 2.1(b)). Flies were retained, undisturbed, in these new vials until the egg density in each reached 100 eggs vial^{-1} (generally <2-h), at which point all adult flies were discarded. Sex-specific fitness of sons and daughters was obtained by counting the number of wild-type offspring (grandchildren) that eclosed 14 days later as adults in the *sons* and *daughters* vials; individual fitness of offspring (their individual reproductive success, or IRS) was measured by dividing the number of grandchildren by the number of sons or daughters originally counted in the associated focal vial.

(e) Statistical analyses

Statistical analyses were performed using R (version 3.2.0, R Core Team 2015), and data are available online at the data Dryad (*upload in progress*). All data were initially assessed for normality using Shapiro-Wilk tests and for homogeneity of variance across treatments using Levene's Tests. We created generalized linear models (GLMs) in which relatedness, sexual selection, and their interaction were set as independent factors, with mating behaviours (latency and duration) and fitness measures as dependent factors; error distributions were specified as quasipoisson for count data, Gaussian for continuous data, and quasibinomial for egg-to-adult survivorship data. The statistical significance of these factors was determined by conducting log-likelihood χ^2 tests, via the *Anova* function, available in the *car* package (Fox & Weisberg, 2011), in which the fit of the full/maximal models was compared to those that did not include male-female relatedness and/or the potential for mate choice as factors. Where groups were found to significantly differ, means and standard errors are reported. Effect size for significant variation arising from either factor was calculated using Cohen's d estimate, or Cliff's δ for non-parametric conditions, with the functions *cohen.d* and *cliff.delta* respectively in the *effsize* package (Torchiano, 2015). As offspring produced via inbreeding will possess a greater proportion of a focal female's alleles (on average) than those produced via outbreeding (a coefficient of relatedness of 0.75 for inbred offspring and 0.5 for outbred offspring, see Falconer, 1981), we multiplied counts of eggs and offspring by the appropriate coefficient of relatedness to compare the relative number of allele copies resulting from females in each treatment. We also did this for the grandchildren generation, again multiplying counts of inbred and outbred grandchildren by their coefficient of relatedness (0.375 and 0.25 respectively) to the original focal female. In this way we assess fitness in the "currency of alleles", or the "genetic value" of inbred and outbred progeny.

Results

Analysis of mating behaviours in the parent generation revealed that females in treatments that allowed mate choice began mating much more quickly than those in treatments with only a single male (LR $\chi^2 = 24.261$, df = 1, p = 8.41×10^{-7}, choice = 1036.267 (s) ± 142.541, no-choice = 1886.883 (s) ± 169.479, Cliff's $\delta = 0.492$ (large), 95% C.I. = (0.294,0.650)). However, duration of mating did not differ between treatments (all $p > 0.05$; see Table 2.1). We also assessed the data to see if differences in maternal mating latencies were correlated with differences in direct (fecundity) or indirect (offspring IRS) fitness traits, but did not find any link between these elements for either inbreeding or outbreeding under *choice* or *no-choice* maternal environments ($p > 0.05$ for each environment).

Total egg production by focal females did not differ between treatments. However, when the genetic value of eggs was accounted for, we observed that females who mated with a relative produced more allele copies via their eggs than females who mated with an unrelated male (Table 2.2). Eggs produced via inbreeding represented 14.225 ± 1.266 genome copies per female on average, while those eggs produced via outbreeding represented only 9.950 ± 0.847 genome copies per female (Figure 2.2). The magnitude of this effect, however, was small. In our analysis of egg-to-adult survivorship, the main effects of inbreeding and the opportunity for mate choice were not significant (all $p > 0.05$). However, the interaction of these factors did affect egg-to-adult survivorship (Figure 2.3; Table 2.2). *Post hoc* Tukey analysis revealed that egg-to-adult survivorship differed between the *related – no choice* and *unrelated – no choice* treatments, and also between the *unrelated choice* and *unrelated no-choice* treatments

(Table 2.3). Eggs laid by females in the *unrelated – no choice* treatments were more likely to survive to adulthood than eggs from *related – no choice*; this was of medium effect size (Table 2.4). Females in *unrelated – no choice* treatments also produced eggs with higher survivorship than females in *unrelated – choice* treatments; the size of this effect was of similar (medium) magnitude (Table 2.4). We did not observe any differences between treatments in total offspring production however, nor were any differences apparent when accounting for the increased genetic value of inbred offspring (all $p > 0.05$; Table 2.5). The sex-ratio of these offspring was not affected by relatedness or mate-choice (all $p > 0.05$; Table 2.5).

In the offspring generation, inbred sons produced fewer offspring than outbred sons (LR $\chi^2 = 8.196$, df = 1, p = 0.004, inbred son IRS = 7.609 ± 0.885, outbred son IRS = 12.338 ± 1.422, Cliff's $\delta = 0.273$ (small), 95% CI = (0.073, 0.452); Table 2.6; Figure 2.4). When we accounted for the inclusive fitness value of inbred grandchildren, however, inbred sons were not found to differ from outbred sons in the reproductive success ($p > 0.05$). Daughters did not differ in their IRS across treatments (LR $\chi^2 = 3.106$, df = 1, p = 0.078; Table 2.6); even when we accounted for the inclusive fitness value of inbred offspring produced by daughters, no differences were detected ($p > 0.05$). In comparing the *total* amount of all resulting grandchildren for each focal female (that is, not taking into account the individual reproductive success of her offspring), we observed a significant effect of relatedness (Figure 5; LR $\chi^2 = 5.936$, df = 1, p = 0.015; Table 6), though to a slightly lesser extent (effect size: Cliff's $\delta = 0.234$ (small), 95% CI = (0.029, 0.421)) than in the IRS of sons. The average number of grandchildren produced by all inbred offspring was 119.283 ± 15.834, while outbred offspring produced an average of

182.267 ± 20.100 grandchildren. However, when the inclusive fitness value of these grandchildren was considered, we did not observe any effect of relatedness or mate choice on parental genome representation in the grandchildren generation ($p > 0.05$; Table 2.6; Figure 2.6).

Discussion

Inbreeding and its resulting fitness consequences have been extensively studied, and classically, studies have focused primarily on negative fitness outcomes (See Hedrick & Kalinoski, 2000). However, recent studies, both empirical (Ala-Honkola *et al*., 2011; Loyau *et al*., 2012; Tan *et al*., 2012; Long *et al*., 2013; Tan *et al*., 2013; Carazo *et al*., 2014; Hollis *et al*., 2015; Carazo *et al*., 2015) and theoretical (Lehmann & Perrin, 2003; Kokko & Ots; 2006; Puurtinen, 2011; Duthie & Reid, 2015), have indicated that receptivity to kin and inbreeding depression may be highly context-dependent, and that below certain thresholds of inbreeding depression, mating with a relative can provide substantial benefits to inclusive fitness. While the costs of inbreeding are largely evident and extensively documented (DeRose & Roff, 1999), few studies have focused on the factors that might shape or mitigate these costs. Furthermore, the potential benefits of inbreeding are even less well understood. In this experiment, we have investigated variation in the costs and benefits of inbreeding in the context of mate choice using the model species *D. melanogaster*. Our treatments allowed us to assess both immediate and latent (fitness of offspring) effects of inbreeding under choice and no-choice conditions. The opportunity for mate choice/competition was associated with alteration to mating latency, and in one instance shaped the effects of inbreeding, but otherwise did not affect the fitness outcome of mating with a relative. Inbreeding was found to negatively affect some aspects of fitness, though many fitness traits showed no reduction under inbreeding, while others were enhanced

Despite evidence for fitness variation associated with inbreeding, the mating behaviours we assessed were entirely unaffected by the degree of relatedness between mates. Mating latency, a factor largely under female control (Markow & Hanson, 1981; Lasbleiz *et al*., 2006) and a proxy for male attractiveness (Barth *et al*., 1997; Ritchie *et al*., 1999; Taylor *et al*., 2008), did not differ between treatments using siblings or unrelated groups. The lack of differences suggests that female *D. melanogaster* do not actively prefer or avoid mating with their kin, and is consistent with some previous findings of inbreeding indifference (Ala-Honkola *et al.,* 2011; Tan *et al*., 2012), though contrary to others that found inbreeding preference (Loyau *et al*., 2012). However, mating latency was not uniform across all treatments, and was significantly reduced when females were exposed to three potential mates rather than one; there are several explanations for this. The observed reduction in time to copulation when mate choice was possible may have resulted from the more immediate fulfillment of mate-sampling requirements. For instance, females who might have ideally used strategies resembling either best-of-*n*/simultaneous or sequential sampling (see Wiegmann *et al*., 1996; Kokko & Ots, 2006) may have delayed mating after assessing only one male, and resisted copulation until it became apparent that the single male present was the only immediate mating opportunity. Given the opportunity to sample from multiple males, females may have begun mating more quickly having assessed multiple males and satisfying some minimum quality threshold, or reaching an acceptable sample size. However, we found no correlation between mating latency and fitness traits indicative of higher quality males, suggesting that lower mating latencies were not the result of "attractive" males being selected more quickly in scenarios offering choice. It is possible that increased male courtship pressure, rather than variation in male quality, prompted reductions in mating latency. Under heightened male pressure, females may respond with acquiescence to mating sooner, thereby reducing the harm brought about by intense male courtship (see Long *et al*., 2009). Mating duration is thought to be primarily under male control (Gilchrist & Partridge, 2000; Snook & Hosken, 2004; Friberg, 2006; Tayler *et al*., 2012; Bretman *et al*., 2013; Crickmore & Vosshall, 2013; Kim *et al*., 2013) though is influenced by females to some extent (Edward *et al*., 2014). Previous studies have shown that males will mate longer with females they perceive to be non-virgins in order to improve fertilization success (Friberg, 2006), possibly via the mechanism of inducing sperm dumping, a process that appears to require standard copulation durations (Snook & Hosken, 2004). It is possible that the virgin-status of all females in our experiment was sufficient to reduce variation in mating duration. However, variation might still be expected if males were investing asymmetrically in kin versus non-kin. The fact that duration was consistent across all treatments suggests that males, like females, do not actively discriminate against or prefer kin, investing equally in all successful matings.

Our assessment of the immediate fitness consequences of inbreeding revealed evidence for both costs and benefits to fitness, though more frequently no differences were detected between inbred and outbred treatments. Total egg and offspring numbers, as well as the sex-ratio of those offspring, did not differ between any of our treatments, suggesting that inbreeding depression does not act strongly at this stage of life, and affects each sex equally. This finding is consistent with studies that find heighted vulnerability to mutation load tends to occur after sexual maturity (Mallet *et al*., 2011). Slight differences in parental allele representation in eggs (in favour of inbreeding) did not translate to differing allele representation in offspring – likely the result of the small magnitude of this difference in combination with dissimilar variation observed in egg-to-adult survivorship. Variation in egg-to-adult survivorship was brought about by the interactions of relatedness and the opportunity for mate choice. In single-male treatments, a greater proportion of eggs laid by outbreeding females went on to survive to adulthood, though this difference was not evident in the triple male treatments (Figure 2.3). These results suggest that by selecting from three different siblings, females were able to reduce or avoid the effects inbreeding depression on egg-to-adult survivorship entirely. Interestingly, other studies have found that *D. melanogaster* egg viability (which encompasses egg-to-adult survivorship) is fairly sensitive to inbreeding, as genetic variation in this trait is largely non-additive (Lopez-Fanjul & Villaverde, 1989; Garcia *et al*., 1994). It is possible that allowing a female to select from three of her brothers reduced the magnitude of inbreeding depression via the selection of the most genetically compatible relative – potentially facilitating optimal inbreeding (see Lehmann & Perrin, 2003; Puurtinen, 2011). However, selecting from three unrelated males provided no overall benefit, but instead resulted in eggs with lower survivorship when compared to *unrelated – no choice* treatments and equivalent to *related – choice* treatments. It is possible that groups of unrelated males were more harmful to females, and that this resulted in eggs/larvae of lower quality. Previous studies do suggest that male groups that are more heterogeneous (for instance, groups of non-relatives) fight amongst themselves more frequently, while also increasing their courtship effort, and thereby amplify direct and collateral harm sustained by females (Carazo *et al*., 2014; Carazo *et al*., 2015; Hollis *et al*., 2015; Martin & Long, *In Press*). In a number of species, maternal condition has been shown to influence the quality of eggs. For instance, in some fish species, female condition is shown to affect a variety of egg characteristics (Solemdal, 1997; Marteinsdotter & Steinarsson, 1998); in beetles, female nutritional status has been linked to the number of eggs laid, and their rate of survival to adulthood (Steiger *et al*., 2007). Our experiment, in conjunction with these studies, offers further support to suggest that heterogeneity (and potentially relatedness) among males may indeed be associated with reductions to female fitness and subsequent offspring fitness as well. In addition to persistent courtship, male-caused harm may also be imposed via the toxic effects of ACPs in their seminal fluids (Lung *et al*., 2002; Wolfner, 2002; Wigby & Chapman, 2005; Ram & Wolfner, 2007). Though we did enforce single matings, male *D. melanogaster* have been observed to tailor their seminal products to transfer more harmful ACPs when competition is *perceived* to be higher (Price *et al*., 2012; Bretman *et al*., 2009). Thus, despite the restriction of females to a single mating in our experiment, males who mated in the presence of other males may have invested more in the allocation of sperm and competitive, non-sperm compounds to optimize their paternity (Sirot *et* al., 2011; Garbaczewska *et al*., 2013) in the face of greater perceived competition, ultimately to the detriment of female condition (Wolfner, 2002) and by extension egg-to-adult survivorship.

Our assessment of the fitness of inbred and outbred offspring, *post*-sexual maturation, revealed that the effects of inbreeding at the reproductive stage differed between males and females. Inbred sons were found to have lower individual reproductive success than outbred sons, while inbred daughters performed as well as their outbred counterparts. This is consistent with other evidence for higher male sensitivity to inbreeding depression (Brekke *et al*. 2010; Agrawal, 2011; Mallet & Chippindale, 2011). As noted by Agrawal (2011), male mating success often depends more heavily on overall body condition than does female mating success because males typically must invest more energy in searching and courtship to successfully gain mating opportunities. Due to the nature of this experiment, the exact mechanism by which inbred son individual reproductive success (IRS) was reduced cannot be conclusively identified. In the offspring generation, fitness was quantified exclusively by measuring IRS, as we were unable to measure other traits such as mating success, or egg-to-adult survivorship. It may be that inbred males do suffer from reduced mating success stemming from a reduction in condition (Agrawal, 2011; van Bergen *et al*., 2013). It is also possible that inbred males may have fewer or lower quality sperm, and thus fertilize fewer eggs than males not suffering from inbreeding depression. In a study by Saccheri *et al*. (2005), inbred male butterflies were frequently infertile, while female fertility was unaffected by inbreeding. However, while the reduction of the average IRS of inbred sons was statistically significant in our experiment, the difference between inbred and outbred sons was of small magnitude. Furthermore, this effect was absent when we assessed the numerical representation of the focal female's alleles in the offspring of sons, which allowed us to properly evaluate the inclusive fitness value of these grandchildren. Overall, the diminished reproductive performance of inbred sons appears to have been sufficient to have a small, negative effect on the total number of grandchildren linked to inbreeding focal females. Inbreeding females had fewer grandchildren than outbreeding females; however this difference, again, was entirely negated via the increased inclusive fitness offered by inbred progeny.

This experiment, along with others (Ala-Honkola *et al*., 2011; Tan *et al*., 2012; Martin & Long, *In Press*), suggests that inbreeding does not substantially impact the direct and indirect fitness of *Drosophila melanogaster*, which manifests as indifference towards mating with kin. This species mates often and multiply (Orchando *et al*., 1996; Imhof *et al*., 1998; Kuijper & Morrow, 2009), has been noted to have significant dispersal ability (Coyne & Milstead, 1987), and is therefore unlikely to have evolved under the selective pressure required to develop inbreeding avoidance mechanisms (Ala-Honkola *et al*., 2011; Chippindale *et al*., 2015). The patterns of inbreeding that we did observe were largely consistent with expectations: (1) egg-to-adult survivorship, an important component of fitness, was sensitive to inbreeding (though only in no-choice environments), and (2) inbreeding depression was most evident following the maturation of inbred offspring, and was negatively male-biased. Though we expected to detect some level of sexual conflict over inbreeding preferences within the focal generation, neither male nor female behaviour indicated any aversion to (or preference for) inbreeding. Mate choice appears to offer some protection from inbreeding depression during juvenile life stages, but is otherwise inconsequential. Future assessment of the multigenerational effects of inbreeding would benefit from measuring a greater variety of offspring fitness traits (particularly their mating success and the egg-to-adult survivorship of their eggs) to better capture the mechanisms by which inbreeding depression acts.

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Tables and figures

Table 2.1. Results of log-likelihood χ^2 tests on generalized linear models (GLM) for the copulatory behaviour of focal generation flies. GLMs included male-female relatedness and the opportunity for mate choice as factors. These models were compared to models not including those factors to assess the significance of each factor. Mating latency was measured as the time (in seconds) from the initial combination of males and females to the time at which copulation began. Mating duration describes the length (in seconds) of each courtship event. Latency was found to be significantly influenced by the opportunity for mate choice.

	LR χ^2	df	*p*	
Mating latency				
relatedness	0.085	1	0.770	
mate choice	24.262	1	$8.41\text{x}10^{-7}$	***
relatedness:mate choice	0.925	1	0.336	
Mating duration				
relatedness	0.129	1	0.720	
mate choice	0.027	1	0.871	
relatedness:mate choice	<0.001	1	0.992	

Table 2.2. Reproductive success of the parent generation in terms of egg-laying metrics. As previously, GLMs were fit with relatedness and mate choice as factors, and then compared to models without these factors using log-likelihood χ^2 tests. Egg number was not affected by either factor, though the number of female alleles present in these eggs was. Egg-to-adult survivorship was affected by the interaction of these factors.

	LR χ^2	df	p	
Total eggs				
relatedness	0.151	1	0.698	
mate choice	0.461	1	0.497	
relatedness:mate choice	0.745	1	0.388	
Total genetic value of eggs				
relatedness	8.147	1	0.004	**
mate choice	0.721	1	0.396	
relatedness:mate choice	0.717	1	0.397	
Egg-to-adult survivorship				
relatedness	2.945	1	0.086	
mate choice	1.481	1	0.224	
relatedness:mate choice	4.864	1	0.027	**

Table 2.3. Tukey *post hoc* results for differences in egg-to-adult survivorship. Treatment codes are as follows: (A) *related – no choice*, (B) *unrelated – no choice*, (C) *related – choice*, and (D) *unrelated – choice*. The "*no choice*" treatments differed significantly from each other, as did the "*unrelated"* treatments.

Treatment comparison	Estimate	S.E.	z value	*p*	
B – A	0.218	0.068	3.184	0.007	**
C – A	0.090	0.068	1.326	0.547	
D – A	0.013	0.068	0.189	0.998	
C – B	-0.128	0.068	-1.870	0.241	
D – B	-0.205	0.069	-2.969	0.016	*
D – C	-0.077	0.068	-1.125	0.674	

Table 2.4. Mean values for fitness traits of treatments (or groups of treatments) found to differ significantly, and the effect size of the differences between these means.

Fitness trait	Group/treatment	Mean	S.E.	Effect size	95% C.I.	
Genetic value of eggs	Inbreeding	14.225	1.266	Cliff’s δ = 0.240	0.030	0.430
	Outbreeding	9.950	0.847			
Egg-to-adult survivorship	*unrelated – no choice*	74.6%	3.8%	Cliff’s δ = 0.409	0.106	0.642
	related – no choice	52.8%	4.7%			
	unrelated – no choice	74.6%	3.8%	Cliff’s δ = 0.357	0.049	0.603
	unrelated – choice	54.1%	6.4%			

Table 2.5. Reproductive success of the parent generation in terms of offspring production. Full/maximal models were compared to models not including relatedness or mate choice as factors using log-likelihood χ^2 tests. No significant differences were found, though there was a marginal effect of the interaction of these factors on both total offspring number and the inclusive fitness value (calculated based on inbred and outbred coefficients of relatedness) of these offspring.

	LR χ^2	df	p	
Total offspring				
relatedness	1.483	1	0.223	
mate choice	0.007	1	0.933	
relatedness:mate choice	3.090	1	0.079	.
Total genetic value of offspring				
relatedness	2.317	1	0.128	
mate choice	0.074	1	0.785	
relatedness:mate choice	3.075	1	0.080	.
Offspring sex-ratio				
relatedness	1.272	1	0.259	
mate choice	0.114	1	0.736	
relatedness:mate choice	0.889	1	0.346	

Table 2.6. Assessment of fitness in the offspring generation; models created for son and daughter fitness using per-individual output of grandchildren (IRS) as a dependent term and inter-sexual relatedness and mate-choice opportunity in parent generation as independent factors. These models were compared to models not including these variables as factors. Sons were negatively impacted by inbreeding in the previous generation, while daughters were unaffected. The reduction of the fitness of sons appears to have been sufficient to reduce over all production of grandchildren, though if the inclusive fitness values of inbred versus outbred grandchildren are considered, inbreeding does not reduce the transmission of ancestral alleles.

	LR χ^2	df	*p*	
Fitness of sons				
relatedness	8.196	1	0.004	**
mate choice	2.479	1	0.115	
relatedness:mate choice	0.007	1	0.381	
Fitness of daughters				
relatedness	3.106	1	0.078	.
mate choice	0.186	1	0.666	
relatedness:mate choice	0.696	1	0.404	
Total grandchildren				
relatedness	5.936	1	0.015	*
mate choice	0.213	1	0.644	
relatedness:mate choice	0.407	1	0.523	
Total grandchildren genetic value				
relatedness	0.011	1	0.916	
mate choice	0.106	1	0.745	
relatedness:mate choice	0.411	1	0.522	

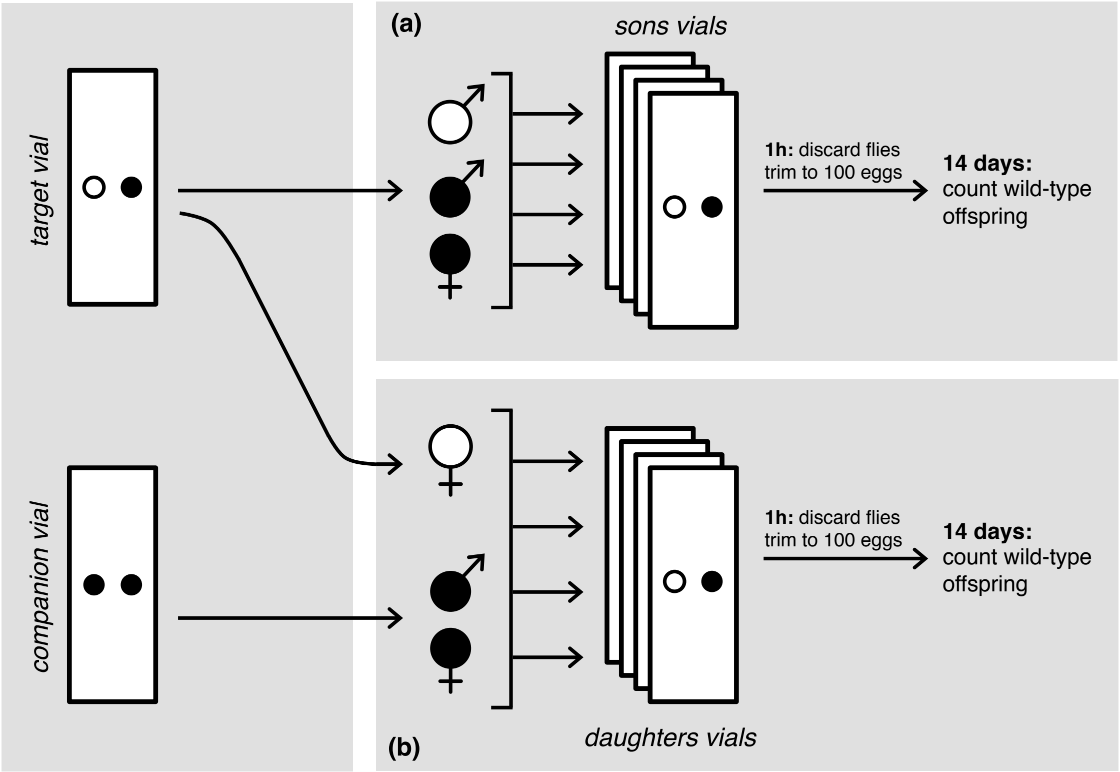

Figure 2.1. Schematic of experimental design for the creation of offspring vials of each sex. **(a)** Male wild-type offspring were collected from each target vial; these flies were counted and divided into four, roughly equal, new groups. All brown-eyed flies from the same target vials were divided in the same manner, and transferred along side groups of sons. **(b)** Female wild-type offspring were counted from each target vial and divided in the same manner as male offspring. Each companion vial, containing exclusively brown-eyed flies, was roughly divided into four groups as well, each of which was transferred with a group of daughters to a new vial.

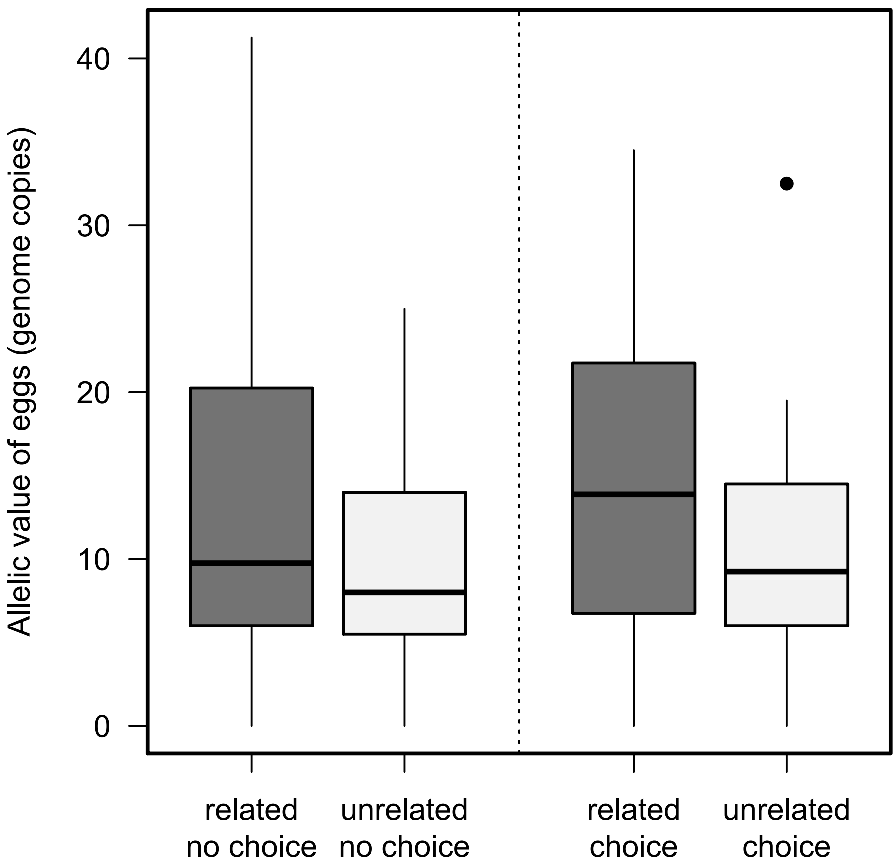

Figure 2.2. Boxplots showing the distribution of total inclusive fitness values for eggs laid by focal females. Inbred eggs share more alleles with focal females than do outbred eggs; this was calculated via the coefficients of relatedness for inbred (r = 0.75) and outbred eggs(r = 0.50). In both "*no choice*" and "*choice*" environments, females who mated with a brother shared more alleles with the resulting eggs than did females mating with a non-relative (LR χ^2 = 8.147, df = 1, p = 0.004).

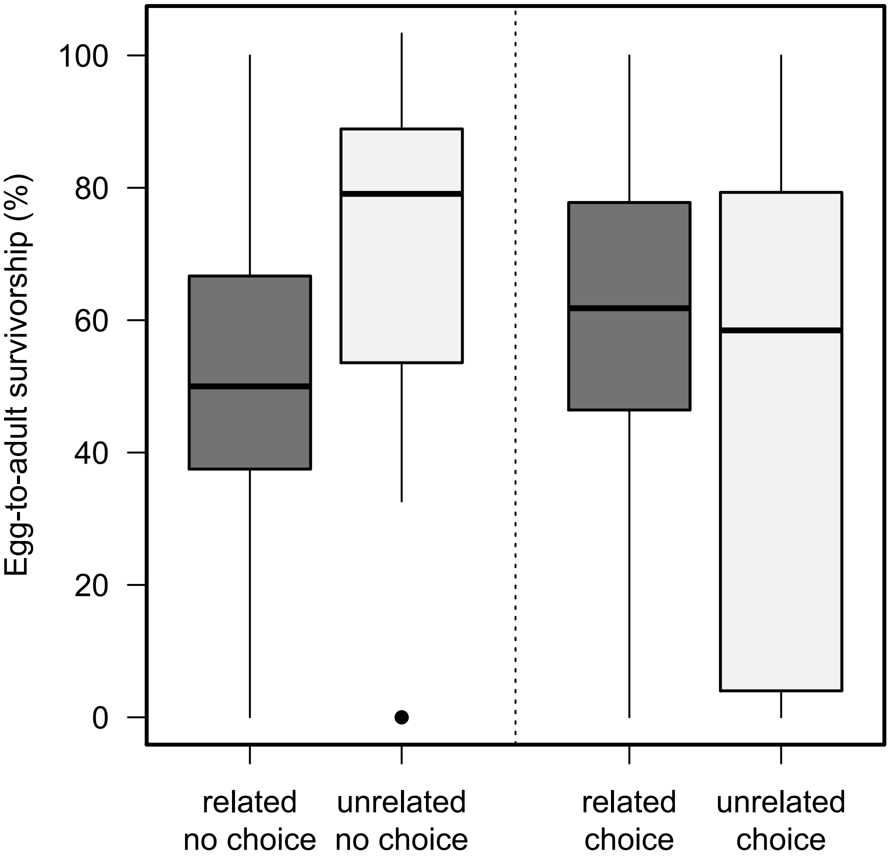

Figure 2.3. Boxplot showing variation in egg-to-adult survivorship across all treatments. This fitness variable was affected by the interaction of relatedness and mate choice. Tukey *post hoc* testing revealed that in *no choice* treatments, inbreeding resulted in eggs that more frequently failed to reach adulthood (est ± s.e. = 0.218 ± 0.068, z = 3.184, *p* = 0.007), and that when mating with an *unrelated* male, the presence of multiple males negatively impacted egg-to-adult survivorship (est ± s.e. = -0.205 ± 0.069, z = -2.969, *p* = 0.016).

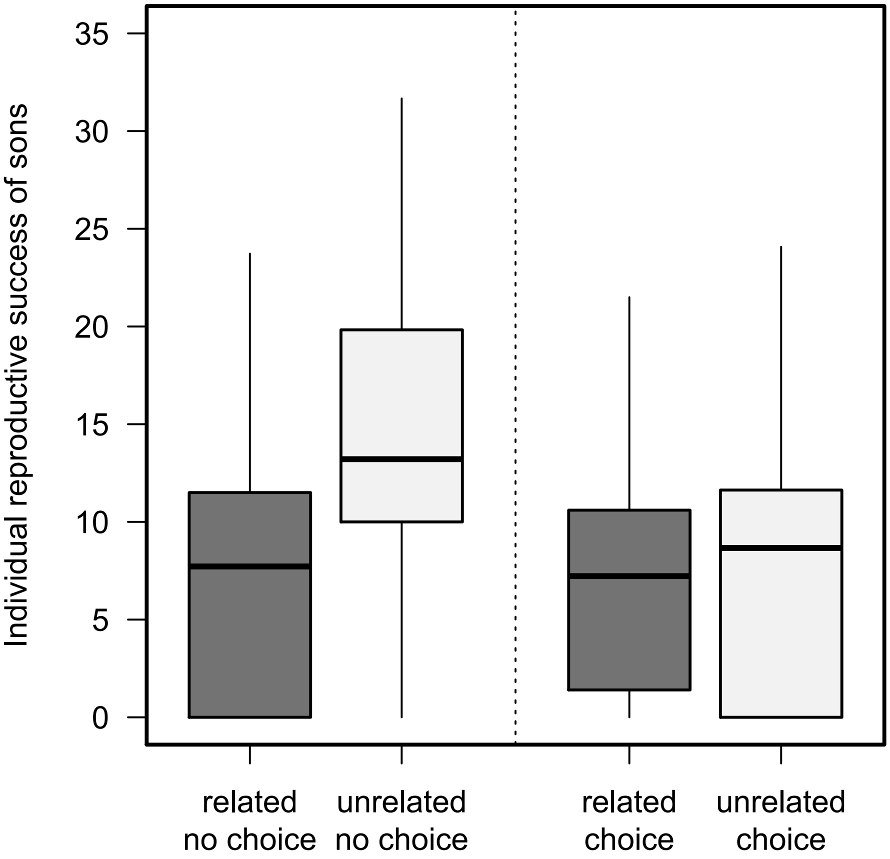

Figure 2.4. Boxplot showing variation in the individual reproductive success of sons. Analysis of our model revealed that inbreeding significantly impacted the reproductive success of sons (LR $\chi^2 = 8.196$, df = 1, p = 0.004).

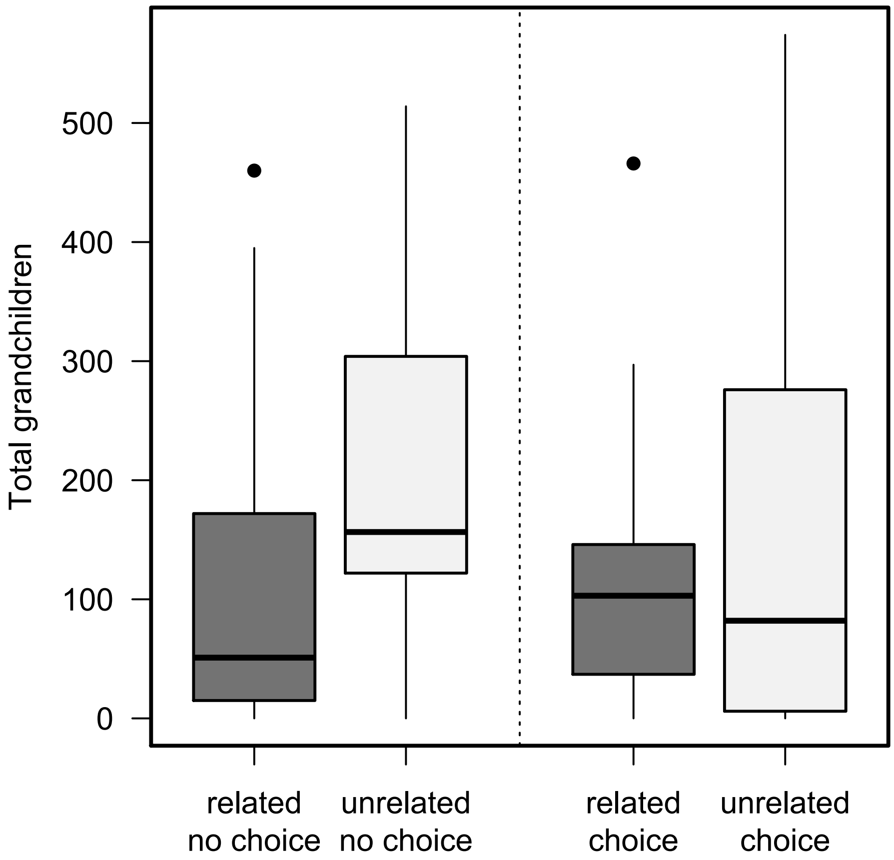

Figure 2.5. Variation in the total number of grandchildren produced by the offspring of focal females. Inbreeding in the parent generation resulted in a depression in the total number of grandchildren produced by offspring (LR $\chi^2 = 5.936$, df = 1, p = 0.015).

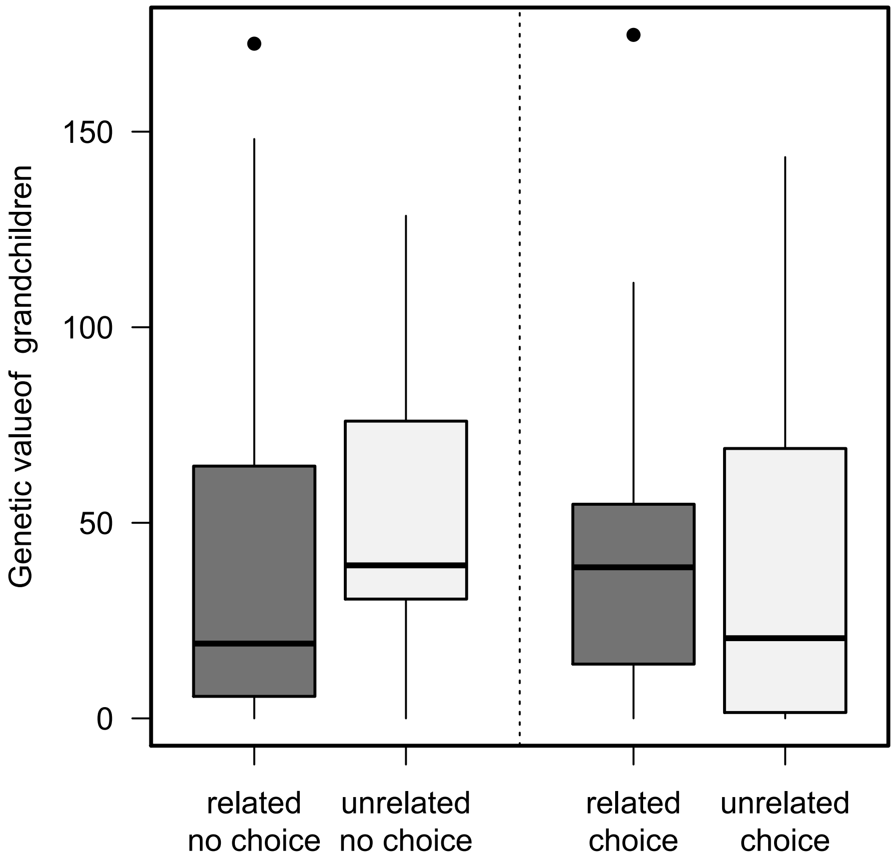

Figure 2.6. Variation in the inclusive fitness value of the total number of grandchildren produced by offspring of each focal female. Relatedness coefficients for inbred and outbred grandchildren ($r_{inbred} = 0.375$ and $r_{outbred} = 0.25$) were used to calculate these values. Inter-sexual relatedness between mates in the focal generation did not affect the inclusive fitness value of total grandchildren.

CHAPTER 3

ARE FLIES KIND TO KIN? THE ROLE OF INTRA- AND INTER-SEXUAL RELATEDNESS IN MEDIATING REPRODUCTIVE CONFLICT

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Preamble

The following chapter was written as a manuscript in the style of *Proceedings of the Royal Society B*, where it has been accepted and is in the process of publication. Table and figure numbering has been modified slightly to differentiate between chapters.

Abstract

As individual success often comes at the expense of others, interactions between the members of a species are frequently antagonistic, especially in the context of reproduction. In theory, this conflict may be reduced in magnitude when kin interact, as co-operative behaviour between relatives can result in increased inclusive fitness. Recent tests of the potential role of co-operative behaviour between brothers in *D. melanogaster* have proven to be both exciting and controversial. We set out to replicate these experiments, which have profound implications for the study of kin selection and sexual conflict, and to expand upon them by also examining the potential role of kinship between males and females in reproductive interactions. While we did observe reduced fighting and courtship effort between competing brothers, contrary to previous studies we did not detect any fitness benefit to females as a result of the modification of male antagonistic behaviours. Furthermore, we did not observe any differential treatment of females by their brothers, as would be expected if the intensity of sexual conflict was mediated by kin selection. In light of these results, we propose an alternative explanation for observed differences in male-male conflict, and provide preliminary empirical support for this hypothesis.

Keywords: sexual conflict, kin selection, inclusive fitness, sexual selection, aggression, social behaviour

Introduction

An individual's fitness is defined by the size of their contribution of alleles to the next generation [1]. As such, there is often strong selection on individuals for traits and/or behaviours that maximize fitness via outcompeting rivals [2–4]. Such "selfish" strategies are favoured by selection when an individual is surrounded by unrelated conspecifics, because fitness is strongly associated with the "direct" transmission of genetic material from parent to offspring [5,6]. However, when the competitors also include relatives, it may be beneficial (and thus adaptive) to act less antagonistically towards those individuals. This is because the reproductive success of one's relatives also results in "indirect" fitness benefits due to the increased transmission of alleles inherited from a common ancestor [7]. Thus, according to kin selection theory, it is hypothesized that individuals should act more altruistically towards their relatives to maximize their inclusive fitness, as long as the net benefit(s) outweigh costs [7,8].

Several theoretical studies have attempted to identify how the predictions of kin selection theory may interact with those of sexual conflict theory [8–10]. According to these models, there are circumstances in which altruistic behaviour towards relatives should occur, both in intra-sexual interactions [9,10] and in inter-sexual interactions [8]. When competing against same-sex relatives, one adaptive strategy is to reduce the amount of harm inflicted on these "rivals", thereby maximizing potential inclusive fitness benefits via the enhanced success of relatives and the shared reproductive resource [10]. When the potential for mating exists *between* relatives, it is further hypothesized that there exists an optimal level for inbreeding, where it can be beneficial to mate with a relative [8]. In these situations, where an individual benefits from the increased transmission of alleles due to shared ancestry with a related mate, a reduction in the level of *inter*-sexual harm on this relative and thus a direct gain (due to lifetime reproductive success of a related mate) is also predicted to occur [8].

Inspired by these predictions, Carazo *et al*. [11] attempted to empirically determine whether the presence of familial bonds between potential male rivals modulated the expression of antagonistic behaviour and subsequently influenced fitness outcomes using the model species *Drosophila melanogaster*. In *D. melanogaster*, males often fight with each other for pre-copulatory access to females [12–14], and competition continues in the post-copulatory realm via the effects of the sperm and accessory gland products (ACPs) of rival males, which are transferred in their ejaculates [15]. Females are often harmed by males, either as a direct result of harassment by courting males [16–20], the physical harm associated with mating [16,21,22], and/or the toxic side effects of the ACPs [23–25; and see 26,27]. With a wide variety of well-documented intra- and inter-sexual interactions, this species is well suited to the study of sexual conflict. In a series of assays, Carazo *et al*. [11] experimentally housed a single adult female fly with three males (all unrelated to her) where the relatedness between these rivals was experimentally manipulated. Groups were comprised of 1) full-sibling males, 2) unrelated males, or 3) two brothers and a third unrelated male. Male behavioural traits, such as the frequency of male-male fighting, the intensity of courtship, and mating rate were measured, as were a number of male fitness variables. The longevity, reproductive lifespan, and lifetime reproductive success of the females were measured. They observed that when some (or all) males in a group were related, fighting frequencies and courtship intensities were significantly lower than in groups of unrelated males. Furthermore, females housed with groups of brothers lived longer, and produced more offspring than females housed with groups of unrelated males, a finding that Carazo *et al*. [11] attributed to differences in harm associated with copulatory behaviours. Overall, Carazo *et al.* [11] concluded that male flies modulated their intrasexual behaviour to act less selfishly towards relatives in order to benefit via indirect fitness gains in a manner theoretically consistent with the predictions of kin selection and inclusive fitness models [see 8,10].

The results of Carazo *et al*. [11] – which have potentially broad implications for the understanding of sexual selection and the evolution of fitness-maximizing strategies [28] – have been received with great interest [28] and some scepticism. Hollis *et al*. [29] argued that by failing to properly control for developmental and social familiarity between relatives, the results of Carazo *et al*. [11] cannot be clearly interpreted as arising due to kin selection alone. Specifically, Hollis *et al*. [29] argued that increased male-male aggression in the unrelated-males treatment could be an artefact of the pre-trial developmental conditions. Whereas the relatives all developed in the same vial, the unrelated individuals were all obtained from different vials. Thus the difference ascribed to perception of kinship might be due to social/developmental familiarity. In order to test this theory, Hollis *et al*. [29] conducted their own set of experiments where relatedness and developmental familiarity were independently manipulated. They found that females housed with sets of brothers had greater lifetime reproductive success (LRS), but that this effect was only manifested if those brothers had also shared a pre-trial developmental environment. Brothers reared in separate vials had the same effect on female LRS as sets of unrelated (and unfamiliar) males. As Hollis *et al*. [29] did not measure male-male aggression or courtship intensity, the functional changes responsible for the differences in female LRS could only be inferred. A follow-up experiment by Carazo *et al.* [30] has also reported higher rates of male-male fighting (but not courtship intensity) when males are unrelated and unfamiliar to each other (reared in separate vials) compared to groups of brothers raised in the same environment. Most recently, a study by Chippindale *et al*. [31] that used a similar protocol to that of Carazo *et al*. [11] failed to find any significant differences in the longevity, reproductive lifespan, or LRS of females housed with three brothers or three unrelated males.

Together, these studies offer an intriguing (albeit controversial) preliminary perspective on the role for relatedness/kin selection in behaviour modulation, particularly because their findings are in some cases at odds with one another. Moreover, many aspects of the role of kin selection and its relation to the study of sexual conflict remain unknown and untested. Of considerable importance is the role of inter-sexual relatedness: what happens when males encounter their sisters as potential mates? The logic invoked by Carazo *et al.* [11] and Pizzari *et al.* [10] for behavioural modification driven by kin selection in *intra*-sexual interactions should also apply for *inter*-sexual interactions. In a species such as *D. melanogaster* where there is considerable inter-locus sexual conflict [15-21], small changes in the intensity of inter-sexual interactions have the potential to dramatically affect male and female lifetime reproductive success. Thus, determining how kinship and social dynamics might influence sexual conflict has important implications for understanding sexual co-evolution in this species, as well as for social evolution in general [28].

In order to more fully understand the role of inter- and intra-sexual relatedness on reproductive behaviour and fitness, we conducted a series of experiments designed to replicate and elaborate upon the first Carazo *et al*. [11] study. Using *Drosophila melanogaster*, we examined variation in behaviour and fitness under a number of possible mating and social settings that differed in the potential for male-male competition, the degree of relatedness between males, and the relatedness of male(s) to a target female. We measured the effects of these combinations in a number of ways: by quantifying male fighting frequencies, courtship intensities, female longevities, female reproductive lifespans, and lifetime reproductive success. We designed this study to provide our own assessment for the role (if any) for relatedness and kin selection in inter- and intra-sexual selection.

Materials and methods

(a) Experimental population and culturing protocol

All flies used in this study originated from the large, outbred, wild-type *Ives* (hereafter "IV") population of *Drosophila melanogaster*, which was created using a sample of wild-caught flies from South Amherst, MA, in 1975 and has been maintained under standardized culture conditions since 1980 [32]. Our population was obtained from the lab of Adam Chippindale (Queen's University, Kingston) in 2011. These flies are housed in vials containing a standard banana/agar/killed-yeast medium, and develop at a controlled density of ~100 eggs per vial. Flies are raised at 25°C and 60% humidity, on a 12L:12D diurnal light cycle, and this population is maintained on a discrete (non-overlapping) 14-day generation culture cycle; flies are cultured *en masse* using light CO_2 anaesthesia [33].

From the IV population, we created 45 familial lineages for use in our assays. Each lineage was created by first mating a virgin female to a single, randomly selected, unrelated male. In each of the following 12 generations, a single virgin brother and sister were mated to propagate the lineage. Subsequently, the size of each lineage was increased to 100 adults per generation and cultured every 14 days on non-overlapping generations. Before the start of the assay, a replicate culture of each lineage, temporally offset by 7 days, was established, which permitted continuous access to young adult males (<4 days post eclosion) from each lineage throughout the course of the experiment. The protocol for the creation of familial lineages resulted in individuals in each familial lineage having a high degree of relatedness (at least r = 0.9255, as per Falconer [1]). We used these highly related familial lineages in our experiment in order to maximize the probability that any kin-related changes to sexual interactions would be detected. Furthermore, if there exists preference for mating with relatives, as a number of past studies have suggested (33,34), and if this preference is driven by genetic factors, inbreeding should enhance these preferences. The use of highly inbred lines, however, does come with the caveat that fitness-associated traits often exhibit directional dominance (35,36,37), with inbreeding resulting in reduced fitness in offspring. If kin recognition is such a trait, our inbreeding procedure may have (ironically) resulted in flies with reduced ability to detect their kin.

(b) Mating treatments and fly handling

We began by collecting five adult females as virgins (<2h of eclosion) from each of the 45 familial lineages, which were held individually in vials containing standard medium. At the same time, adult males were also collected as virgins (<2h of eclosion) from all familial lineages. Males were assigned to one of five different experimental treatments (Figure S3.1) compiled in replicate for each family (*n* = 45), and were held in these groups for a maximum of 72-h post-eclosion until the start of the experiment. While we did not control for larval social familiarity, all triple-male groups received comparable adult socialization prior to introduction to the female, regardless of their treatment. Additionally, if larval familiarity is required for kin recognition, allowing brothers to develop in the same environment should increase their ability to modulate their behaviour when encountering each other.

Our treatments were designed to test the following questions: 1) Does the relatedness of a group of males alter *intra*-sexual behavioural interactions and/or their effects on a target female? 2) Does relatedness between the sexes alter the *inter*-sexual interactions between a single male or group of males and a target female, or influence a female's fitness or longevity? The experimental treatments were: 1) *"related-pair"*, a single male from the same familial lineage as the target female; 2) *"unrelated-pair"*, a single male from a different familial lineage than the target female; 3) *"all-related"*, three males related to each other (same familial lineage) and to the target female; 4) *"males-related"*, three males related to each other but from a different familial lineage than the target female; and 5) *"all-unrelated"*, three males unrelated (different familial lineages) to either each other or to the target female. In treatments involving unrelated males (both single- and triple-male treatments) combinations of lineages were assigned randomly and were equally represented. To better study the consequences arising from male-male competition, we ensured that for each set of treatments for individual females of a given familial lineage, the same lineage of males was used in the *unrelated-pair* treatment as in the *males-related* treatment (Figure S3.1).

(c) Longevity and male behaviour experiment

The experiment began by combining unanaesthetized males and females into a single vial by lightly tapping the male(s) into each female's vial. Following the combination of flies, all vials (*n* = 45*5 = 225) were placed horizontally in a quiet, temperature-and-humidity-controlled room. Vials were observed and behaviours scored daily in 5 sessions beginning at 9 A.M. In each session, vials were scanned for a period of 5-s each, during which counts were made of the number of instances of copulation, courtship, or fighting between males. In the case of fighting, fights involving two males were scored as a single event, while cases of all three males fighting with each other were recorded as double events. The numbers of each type of event scored during daily sessions were summed prior to analysis. All vials were observed on a daily basis until the death of the target female, at which time the date of each death (female longevity) was recorded.

In each of the first three days of the experiment all living flies were transferred (after the completion of observations) using light anaesthesia to new vials containing fresh media. The numbers of eggs laid in these three sets of vials were immediately counted. After 14 days, the numbers of eclosed adults in the vials were also counted. Data from egg-to-adult viability yielded from the first three days of the experiment were later used to correct for differences between vials in offspring numbers resulting from inbreeding depression. For the remainder of the experiment, flies were transferred (with light anaesthesia) to new vials every second day. Eggs laid during these time periods were not counted, but the numbers of offspring eclosing from these vials 14 days later were recorded. These numbers were adjusted using the vial-specific egg-to-adult survivorship rates to generate estimated total egg values. Based on the final date of viable offspring production, we were also able to quantify a female's reproductive lifespan. Following the procedure of Carazo *et al.* [11] all males were replaced, on average, every 7 days to ensure male co-aging did not impact female mortality or fecundity. This was accomplished by replacing all old males every 6th or 8th day of the experiment (corresponding to the closest date of transfer of female flies to new vials) with virgin adult males collected from one of the two temporally offset sets of familial lineage populations. This protocol of vial-transfer and male-replacement was continued for each vial until the death of the target female.

(d) Statistical analysis

All statistical analyses were performed using R (version 3.2.0, the R Foundation for Statistical Computing [39]). When analysing cumulative totals for courtship and fighting events, we calculated the daily rate for each of these events in order to control for any potential confounds associated with differences in female longevity; this was done for estimated egg production rates (eggs per day) as well, although estimated lifetime totals were also analysed. Estimates for total egg production and egg production rate were compared across treatments. Total egg estimates were compared for the entire lifespan of the female, and for the first three days of the experiment – a time period that is evolutionarily relevant to the female due to the nature of the IV population culture protocol. Fighting and courtship variables were compared as daily rates, while copulation events were compared as lifetime totals. All data were assessed for normality and homogeneity of variance, using the Shapiro-Wilk test and Levene's Test respectively, in order to determine whether data met parametric assumptions.

To compare behaviour and fitness metrics for the three triple-male treatments (*n* = 45 each), data were analysed using general linear models (GLM) constructed with quasipoisson error distributions. A model was created for each class of behavioural or fitness response, with treatment as an independent factor. The significance of treatment was determined using the *Anova* function (in the *car* package), with type II sums of squares. Following the identification of significant differences between treatments, contrast analysis was used to test *a priori* hypotheses about differences between treatments. Specifically, we contrasted data for: 1) groups of brother (*all-related* and *males-related*) were contrasted against the *all unrelated* treatment; 2) the *all-related* treatment against the *males-related* treatment; and 3) and the *males-related* and the *all-unrelated* treatments against the *all-related* treatment. Respectively, these contrasts allowed us to assess 1) the effects of intra-sexual relatedness, regardless of male relation to a focal female; 2) the effects of inter-sexual relatedness when males are all related to one another; and 3) the effects of inter-sexual relatedness, regardless of male intra-sexual relatedness.

We created GLM models using data from the *related-pair*, *unrelated-pair*, *all-related*, and *males-related* treatments (*n* = 45 each) to assess the effects of both relatedness and exposure to multiple males. In these models we used estimated egg numbers or behaviour traits as the response variable, with intersexual relatedness, the number of males as factors, and their interactions as independent variables, and specified a quasipoisson error distribution. Significance of these terms was determined as noted previously using likelihood ratio tests. To examine whether the presence of male rivals caused equivalent changes to behaviour and fitness outcomes across treatments differing in intersexual relatedness, data from single-male treatments (*related-pair* and *unrelated-pair*) were also compared with their corresponding triple-male treatments (*all-related* and *males-related*). This was accomplished using paired *t*-tests or paired Wilcox signed-rank tests when data did not meet parametric assumptions.

For both comparisons of intra-sexual and inter-sexual relatedness, female longevity and reproductive lifespan were assessed via survivorship analysis modeling (using the *survreg* function in the *survival* package), with number of males, inter-sexual relatedness, and their interactions as independent variables.

Results

(a) Effects of male intra-sexual relatedness

The daily courtship rate of males in the triple-male groups differed significantly between treatments, both over the entire lifespan (henceforth “full-term”) of the female (LR χ^2 = 10.202, df = 2, p = 0.006), as well as within the first three days of the assay (LR χ^2 = 8.141, df = 2, p = 0.017). *Post hoc* contrast analyses revealed that overall, brothers courted females less frequently than did males unrelated to each other (estimate ± s.e. = 0.340 ± 0.106, z = 3.200, p = 0.004). This phenomenon was of medium effect size (Cliff’s delta = 0.343, 95% C.I. = [0.141, 0.518]). No differences arising from variation in inter-sexual relatedness were found for courtship frequency in either the contrast of *all-related* against *males-related* (est. ± s.e. = -0.023 ± 0.065, z = -0.356, p = 0.928), or the contrast of *all-related* against *males-related* and *all-unrelated* (est. ± s.e. = 0.135 ± 0.110, z = 1.226, p = 0.419). Daily fighting rate also differed (Figure 3.1) between treatments (LR χ^2 = 6.941, df = 2, p = 0.031). Contrast analyses of *all-unrelated* against *all-related* and *males-related* revealed that unrelated males fought amongst themselves more frequently than did related males (est. ± s.e. = 0.448 ± 0.172, z = 2.612, *p* = 0.022). Effect size for this difference in fighting rate was small (Cliff's delta = 0.245, 95% C.I. = [0.051, 0.422]. Again treatments did not differ in the contrast of *all-related* against *males-related* (est. ± s.e. = 0.055 ± 0.107, z = 0.520, *p* = 0.853) or the contrast of *all-related* against *males-related* and *all-unrelated* combined (est. ± s.e. = 0.3078 ± 0.183, z = 1.682, *p* = 0.197), indicating that inter-sexual relatedness did not modulate male aggressiveness in the presence or absence of control for male-male relatedness. Table 3.1 summarizes the values of these behaviours and fitness variables across treatments.

Despite the observed differences in courtship and fighting frequencies between treatments, no differences were found between these treatments for mean number of copulations observed (LR χ^2 = 1.142, df = 2, *p* = 0.565). Furthermore, no significant differences were observed between treatments for estimated total egg production either in the full-term (LR χ^2 = 2.336, df = 2, *p* = 0.311) or in the first three days of the assay (LR χ^2 = 0.475, df = 2, *p* = 0.789); egg production rate was also found not to differ between treatments (LR χ^2 = 2.937, df = 2, *p* = 0.230). Finally, using survivorship modeling, we detected no differences between treatments in female longevity (χ^2 = 1.13, df = 2, *p* = 0.57, n = 135) or reproductive lifespan (χ^2 = 0.49, df = 2, *p* = 0.78, n = 135) for triple-male treatments.

(b) Effects of inter-sexual relatedness

The effects of intersexual relatedness were assessed via the paired comparison of the two paired single-male treatments – *related-pair* vs. *unrelated-pair*, and the correspondingly paired three-male treatments – *all-related* vs. *males-related*. In these analyses, treatments were paired according to female lineage. The results of these analyses are presented in

Table S3.1 (available online), and revealed no significant differences between treatments differing in male-female relatedness for any of the variables considered. We also analysed whether the number of males in a vial and inter-sexual relatedness (and the interaction between these two factors) were associated with differences in behaviour or fitness variables. The rate of courtship was significantly lower in single-male vials than in triple-male vials ($F_{1,176} = 171.603, p < 2 \times 10^{-16}$). However, there was no significant effect of the inter-sexual relatedness of flies ($F_{1,176} = 1.150$, $p = 0.285$), or the interaction between these factors ($F_{1,176} = 0.243$, $p = 0.623$). The number of males a female was exposed to significantly affected lifespan ($\chi^2 = 19.409$, df = 1, $p = 1.055 \times 10^{-5}$), with female exposure to trios of male experiencing earlier mortality than those housed with single males. Female longevity did not differ based on inter-sexual relatedness ($\chi^2 = 0.001$, df = 1, $p = 0.976$) or the interaction of relatedness and number of males ($\chi^2 = 0.144$, df = 1, $p = 0.704$). Reproductive lifespan was significantly lower in triple-male groups ($\chi^2 = 7.020$, df = 1, $p = 0.008$) than in single-male groups (Figure 3.2), but there was no significant effect of inter-sexual relatedness ($\chi^2 = 0.024$, df = 1, $p = 0.876$) or the interaction ($\chi^2 = 0.102$, df = 1, $p = 0.749$).

We observed a significant effect of the number of males in a vial on the lifetime production of eggs ($F_{1,176} = 7.250, p = 0.008$). Females housed with three males produced fewer eggs when compared to single-male groups. However, total egg production was not affected by either inter-sexual relatedness ($F_{1,176} < 0.001$, $p = 0.998$) or its interaction with male quantity ($F_{1,176} = 0.247$, $p = 0.620$). When egg production rates were compared, we found no significant effect of the number of males in a vial ($F_{1,176} = 2.265$, $p = 0.134$), the relation of these males to the female ($F_{1,176} = 0.006$, $p = 0.940$), or the interaction of these factors ($F_{1,176} = 0.363$, $p = 0.548$). Similarly, no significant differences were detected for short-term total egg production (number of males: $F_{1,176} = 0.113$, $p = 0.738$; inter-sexual relatedness: $F_{1,176} = 0.034$, $p = 0.855$; interaction: $F_{1,176} = 0.002$, $p = 0.965$).

Discussion

In the quest to maximize fitness, selection has often favoured the evolution of traits by which an individual benefits by acting antagonistically towards others in the population (be they males or females) [12-14,16,19,21,25]. It has been hypothesized that the magnitude of this conflict may be relaxed in the presence of kin, as an individual can benefit indirectly via the reproductive success of relatives [9,10]. A recent high profile paper by Carazo *et al*. [11] attempted to test this prediction using *D. melanogaster* (an important model species for the study of sexual selection and sexual conflict), and found evidence of harm modulation. Their results have been controversial [29,31], and motivated our attempt to replicate and extend the experiments of Carazo *et al*. [11,30]. In our study, we set out to examine the potential role of kinship in *intra*-sexual interactions and fitness consequences, and further investigate *inter*-sexual interactions as this factor that has not previously been considered. Our assays revealed higher fighting and courtship rates in groups of unrelated males compared to groups of brothers, consistent with the observations of Carazo *et al*. [11] and (partially) Carazo *et al*. [30]. Similar results have been interpreted [11,30] as an indicator of altruistic behaviour between kin for the purpose of increasing inclusive fitness. However, we did not observe any decrease in the magnitude of harm inflicted on females when males were related. Females in our triple-male treatments did not differ in their longevity, or in the length of their reproductive lifespan. Furthermore, intra-sexual kinship had no effect on female reproductive output, measured in the currency of egg production, in either the short-term or over a female's entire lifespan.

Additionally, we found no effect of kinship on any of the *inter*-sexual interactions or fitness outcomes in our paired comparisons of single or triple-male treatments (Table S3.1). While the number of males had a profound impact on the rate of *intra*-sexual behaviour and female longevity (Figure 3.2), most fitness-related indices were unaffected by either kinship between male(s) and the female in the vial, or the interaction between kinship and the number of males (Table 3.1). In other words, males acted equally as antagonistically towards their sisters as they did towards unrelated females. In their study, Carazo *et al.* [11] had suggested that males modulate aggression when housed with kin to reduce the magnitude of harm done to brothers and to shared mates, thereby increasing indirect fitness gains. However, our study, like that of Chippindale *et al.* [31], reveals no such fitness benefit. Additionally, the logic invoked to predict reduced intra-sexual aggression between brothers should also theoretically apply for inter-sexual interactions, as a male mating with a relative stands to gain two-fold by acting less aggressively towards his sister – both directly via increased LRS from a mate with greater fecundity, and indirectly via the transmission of shared alleles inherited from the female. Therefore the benefits of preferential treatment towards opposite-sex kin are potentially greater than cooperative behaviour between same-sex kin. However, the differences we see in the fitness of females exposed to a single male versus three males (Figure 3.2) indicates that males are not reducing the magnitude of harm they cause to sisters in any meaningful way.

It is possible fruit flies are unable to recognize female kin. This would account for the equal treatment of all potential mates regardless of shared relation. However, a study by Tan *et al.* [40] suggests that male *D. melanogaster* are capable of differentiating between females of different genetic or environmental backgrounds, which could theoretically include an ability to recognize individuals with whom they share a genetic or environmental background as well. A number of other studies do indeed report that *D. melanogaster* are capable of kin recognition, as individuals were observed to preferentially mate with relatives [33,34], which necessitates the ability to recognize kin. In our assay, we used flies with a high degree of relatedness, to increase the likelihood that differences between individuals would be easier to perceive, and so that the gains from indirect fitness would be maximized. And yet we found no evidence of inter-sexual kin-related modulation of antagonistic behaviour, and little evidence of intra-sexual modulation – save for differences in fighting and courtship rates. But then how can we account for these observations (as well as those of Carazo *et al*. [11,30])? Our results have led us to question the plausibility of the kinship-modulated conflict explanation of Carazo *et al*. [11] and instead consider other reasons why levels of fighting and courtship may vary between treatments, independent of intra-sexual relatedness. Here we propose one potential alternative explanation, and present preliminary data in support of this hypothesis.

When considering groups of unrelated flies, it is almost inevitable that they will be more variable than groups of related flies. If variation in behaviour, such as aggression level or courtship intensity, has a genetic basis – something that has been extensively documented in *D. melanogaster* [13,41–44] – it is worth considering the possibility that the increased rates of fighting and/or courtship by genetically heterogeneous groups of males are the product of combining different genotypes together, and thus novel social environments [see 45,46]. Indeed, Saltz [13] reported that the overall aggressiveness of a group of male *D. melanogaster* is influenced by the genetic composition of that group, or rather, by indirect genetic effects (IGEs). Further evidence for IGEs is provided by Kent *et al*. [47], who report that chemical signaling employed by this species also varies based on the genotypes of neighbours; similar effects of genetic social environment have been documented in mice [48]. Therefore, if genetically determined aggression levels vary within our population, it is possible that social heterogeneity, rather than kinship, may be responsible for the between-treatment variation seen in overall group aggression.

To begin to test this hypothesis, we made use of the fact that for each of our 45 *all-unrelated* vials, we knew the familial identities of the three males that made up each trio, and that we also had made independent measurements of how members of each family acted when housed with others of the same sex and lineage (i.e. the phenotypic values measured from the *males-related* vials). Thus, we were able to create a synthetic data set comprised of a response variable (the fighting rate values observed in the 45 *all-unrelated* vials), and three independent variables (corresponding to the fighting rate values of the three families that made up each specific trio, ranked from most to least aggressive within each trio). This ranking was essential to understand the phenotypic composition of fighting rates in each combination of males, but it resulted in multicollinearity of the variables. Thus, we first performed a principle component analysis (PCA) on these three variables (using the *princomp* function in R) to convert them into a set of orthogonal, linearly uncorrelated variables (see Table 3.2 for PCA loadings). Using the re-scored data, we created a model to determine if we could predict the observed phenotypic variation in the *all-unrelated vials* (response variable). Our original model included all three principle components (and their interactions) as independent factors, and was subsequently simplified by the sequential removal of all non-significant parameters, until we reached the minimum adequate model. Despite the limitations in our dataset, this model (Table 3.3) was quite effective at describing a sizable fraction of the variation in fighting values observed in groups of unrelated males. In our model, we saw that in the synthetic group trios, when the range of "fighting phenotypes" was reduced (i.e., male aggression levels were more similar to each other) (Figure S3.2), and when the male of intermediate aggressiveness displayed a relatively high aggression level within that range (Figure S3.3), there was a correspondingly higher frequency of overall conflict in the *all-unrelated* vial (Table 3.3). In essence, when trios were comprised of males with relatively similar levels of aggression, and the aggression phenotypes of the two most aggressive lineages were even more similar, this was associated with a greater frequency of fighting within the *all-unrelated* trio. This model (Table 3.3) was able to predict group phenotypic variation in the *all-unrelated* fighting values (AIC = 68.439, adjusted $R^2 = 0.\ 214$). This preliminary analysis provides some tentative support for social composition as a driver of the behavioural differences observed in groups of unrelated individuals, but more research on this subject is warranted. Though we did not set out to deliberately create combinations that differed in their aggressive behaviours, our work, like the work of Billeter *et al.* [49], Carazo *et al.* [11,30], and Saltz [13], does highlight the complexities associated with IGEs, and is suggestive of a fruitful avenue for future research.

In summary, our experiment tested and extended the predictions of the kin selection model for conflict modulation. In our population, evidence for any modulation of conflict due to shared relatedness is tenuous. While we did detect differences in intra-sexual interactions between related males, we did not observe any such effect of kinship on inter-sexual interactions, which are theoretically a more likely target for modulation by kin selection. Furthermore, in spite of altered male-male interactions, we did not observe any mediating effect on fitness outcomes for these groups. Because of the success with which our alternative model is able to predict male-male aggression between non-kin, we propose that the dynamics and structures of social groups (IGEs) may be more important factors both in our experiment and those of Carazo *et al*. [11,30] and should be considered in future studies. Our results join the ranks of many other studies documenting the importance of social structure and their effects on conflict and other social behaviours [13,10,48,49].

Competing Interest:

The authors declare no competing interests.

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Tables and figures

Table 3.1. Summary statistics (mean ± s.e.) from behavioural and life history data collected from our five treatments (n = 45 for each): *related-pair*, a female with a single sibling male; *unrelated-pair*, a female with a single unrelated male; *all-related*, a female with three males related to her and each other; *males-related*, a female with a three males related to each other but not to her; and *all-unrelated*, a female with a group of entirely unrelated males, either to her or each other.

	Variable		***related pair***	***unrelated pair***	***all related***	***males related***	***all unrelated***
Behaviour	Courtships (events/day)		1.08 ± 0.07	0.95 ± 0.06	2.15 ± 0.11	2.10 ± 0.09	2.51 ± 0.10
	Fighting (events/day)		NA	NA	0.91 ± 0.07	0.96 ± 0.06	1.17 ± 0.08
	Copulations (lifetime)		0.42 ± 0.12	0.51 ± 0.13	0.78 ± 0.16	0.84 ± 0.17	0.62 ± 0.13
Fitness	Longevity (days)		29.38 ± 1.03	28.44 ± 1.41	24.20 ± 1.14	25.00 ± 1.00	24.82 ± 1.18
	Reproductive lifespan (days)		23.51 ± 1.44	23.47 ± 1.58	18.64 ± 1.54	18.00 ± 1.48	19.47 ± 1.32
	Egg production	lifetime (total)	154.70 ± 18.12	163.10 ± 20.81	117.90 ± 14.38	109.60 ± 12.69	140.10 ± 16.42
		lifetime (eggs/day)	4.92 ± 0.49	5.25 ± 0.54	4.48 ± 0.48	4.23 ± 0.42	5.36 ± 0.55
		short-term (total)	19.51± 1.93	19.11 ± 1.51	18.84 ± 1.85	18.60 ± 1.70	24.00 ± 1.27

Table 3.2. Loadings of three *related-males* fighting variables on principal components for the 45 *all-unrelated* combinations trios of males in our "synthetic" data set. In PC1 all the loadings are positively correlated and are of similar magnitude, an inevitable result of the ranking of males by their lineage's aggressiveness in the creation of the data set. Variation in PC2 is primarily defined by the range of aggressiveness phenotypes in the trio – large positive values when they are very different, and negative values when they are closer in magnitude. Variation in PC3 is primarily defined by the similarity in the level of aggressiveness of the intermediate and low aggressive lineages. The large positive PC values occur when their phenotypes are closer in magnitude.

Variable	**PC 1**	**PC 2**	**PC 3**
Most aggressive family	0.555	0.797	0.239
Intermediate aggressive family	0.599	-0.184	-0.780
Least aggressive family	0.577	-0.576	0.579
Eigenvalue	2.174	0.489	0.337
% of variance explained	0.725	0.163	0.112

Table 3.3. Minimum adequate model (AIC = 68.439, $F_{3,41}$ = 4.982 , p = 0.005, Adjusted R^2 = 0.214) created to explain phenotypic variation in observed daily fighting rates of trios of males in *all-unrelated* vials.

Variable	**df**	**SS**	***F***	***p***
PC2	1	1.431	6.079	0.018
PC3	1	0.041	0.172	0.681
PC2 x PC3	1	2.048	8.697	0.005
Residuals	41	9.655		

Table S3.1. Results of paired Wilcoxon signed-rank tests; performed using data collected for 45 sets of single-male and triple-male groups.

	Variable and test statistics		***related pair* vs. *unrelated pair***		***all related* vs. *males unrelated***	
Behaviour	Courtships (events/day)		$V_{135} = 657$	$p = 0.117$	$V_{135} = 564$	$p = 0.604$
	Fighting (events/day)		NA	NA	$V_{135} = 448.5$	$p = 0.439$
	Copulations (lifetime)		$V_{135} = 135$	$p = 0.665$	$V_{135} =134.5$	$p = 0.664$
Fitness	Longevity (days)		$V_{135} = 519.5$	$p = 0.578$	$V_{135} = 410$	$p = 1.000$
	Reproductive lifespan (days)		$V_{135} = 429$	$p = 0.990$	$V_{135} = 503$	$p = 0.721$
	Egg production	Lifetime (total)	$V_{135} = 496$	$p = 0.814$	$V_{135} = 527$	$p = 0.713$
		Lifetime (egg/day)	$V_{135} = 408$	$p = 0.221$	$V_{135} = 534$	$p = 0.653$
		Short-term (total)	$V_{135} = 488$	$p = 0.940$	$V_{135} = 482$	$p = 0.707$

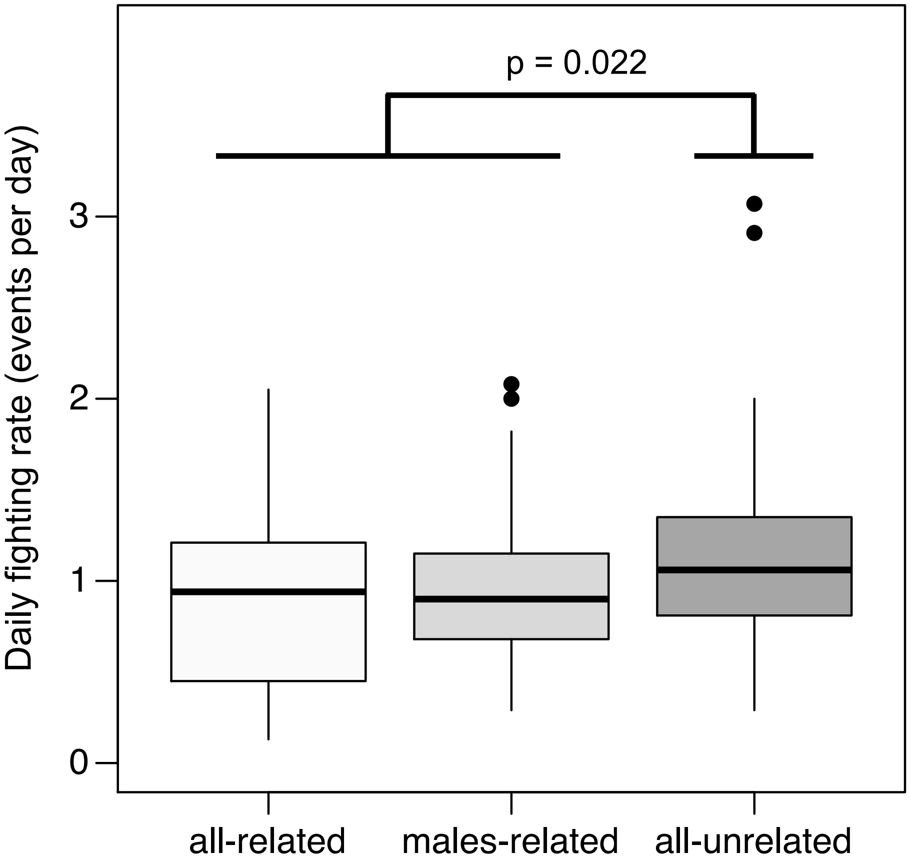

Figure 3.1. Boxplots showing distribution of daily fighting rates observed in vials of *D. melanogaster* containing one female and a trio of males, for treatments differing in the type of inter- and intra-sexual relatedness. In *"all-related"* vials the males are related to each other and to the female, in *"males-related"* vials the males are related to each other but not to the female, and in *"all-unrelated"* vials all flies are from different familial lineages. While statistically significant, male-male relatedness is only a marginal predictor of overall fighting rates between treatments (AIC = 194.841, adjusted R^2 = 0.042). The box encloses values between the first and third quartiles of the data (the inter-quartile range, IQR), while the horizontal bar within the box indicates the median. Whiskers extend from the box to largest/smallest values that are within 1.5 x the IQR of the box. Values outside that range are outliers and are indicated by circles.

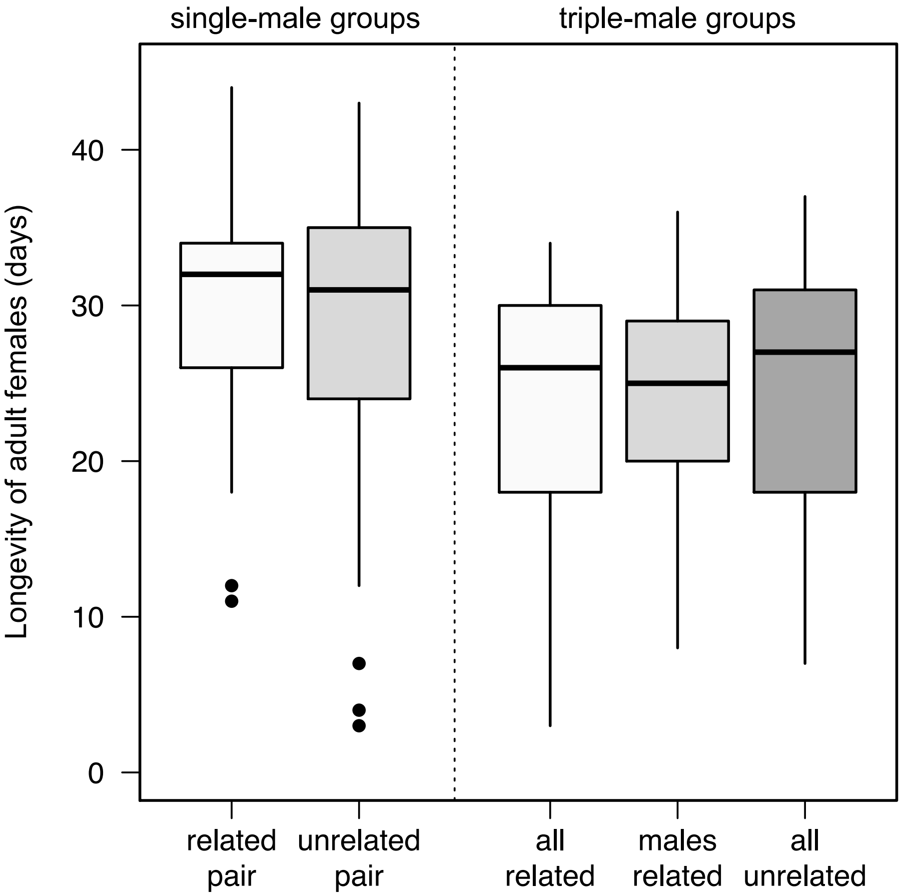

Figure 3.2. Boxplots showing distribution of female longevities (in days) observed in vials of *D. melanogaster* containing one female and male(s), in treatments differing in the number of males and/or the type of inter- and intra-sexual relatedness. Vials in the *"related-pair"* consist of a single male and female from the same familial lineage; while in the *"unrelated-pair"* treatment, the flies are from different lineages. In *"all-related"* vials the males are related to each other and to the female, in *"males-related"* vials the males are related to each other but not to the female, and in *"all-unrelated"* vials the all flies are from different familial lineages. Shading of boxes indicates whether the male(s) in a vial are related to the female (white) or are unrelated (grey). Boxplot components are as described in Figure 1.

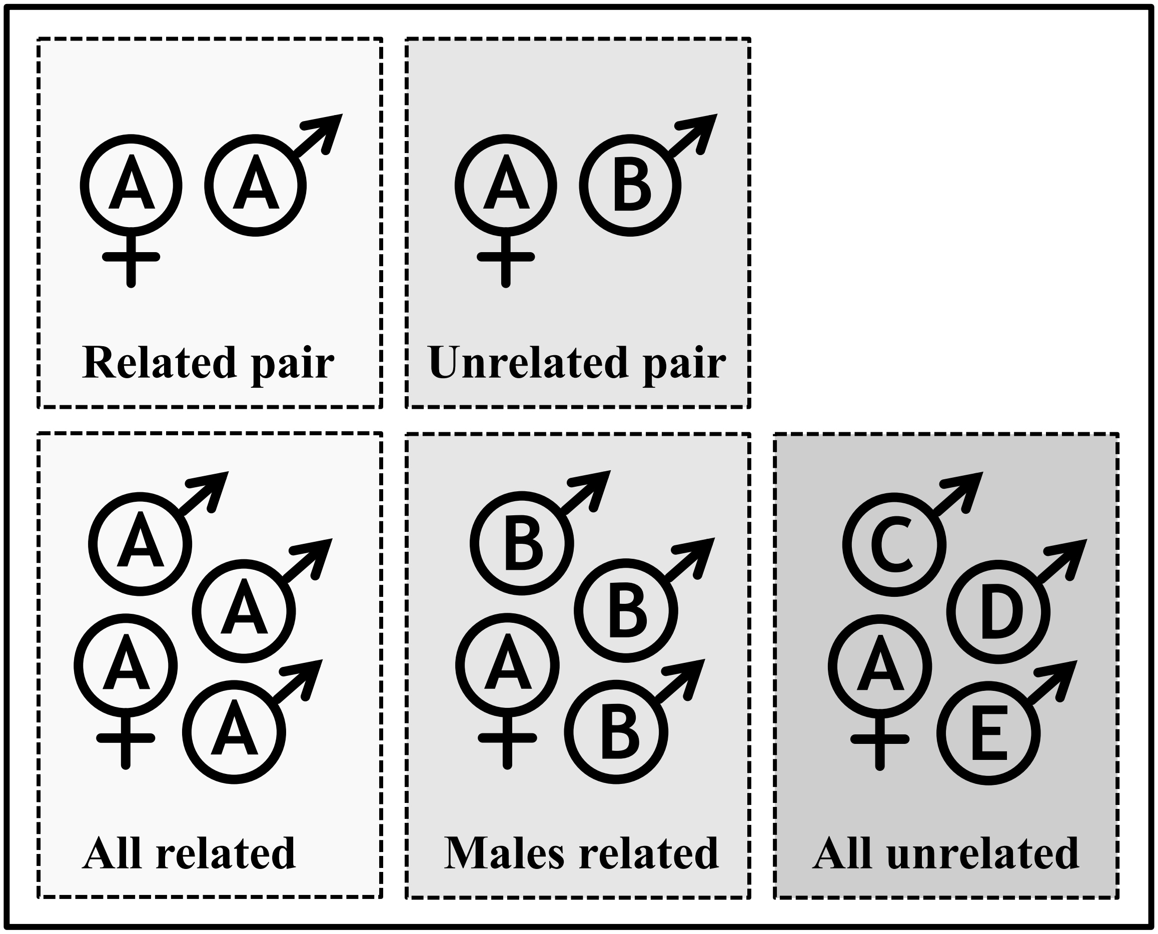

Figure S3.1. A schematic of the 5 different treatments used in this experiment: a target female was housed with male(s) of varying inter-sexual (and in triple-male groups, intra-sexual) relatedness. The *"related pair"* treatment housed females with a single male from the same familial lineage as the female. In the *"unrelated pair"* treatment, the single male was from a randomly selected familial lineage different from that of the female. For the *"all related"* and *"males related"* treatments, three males were selected from the same familial lineages as the corresponding single-male treatments. In the *"all unrelated"* treatment, three males were selected randomly in such a way that they were neither related to each other nor the female. All five treatments were represented for each of the 45 familial lineages.

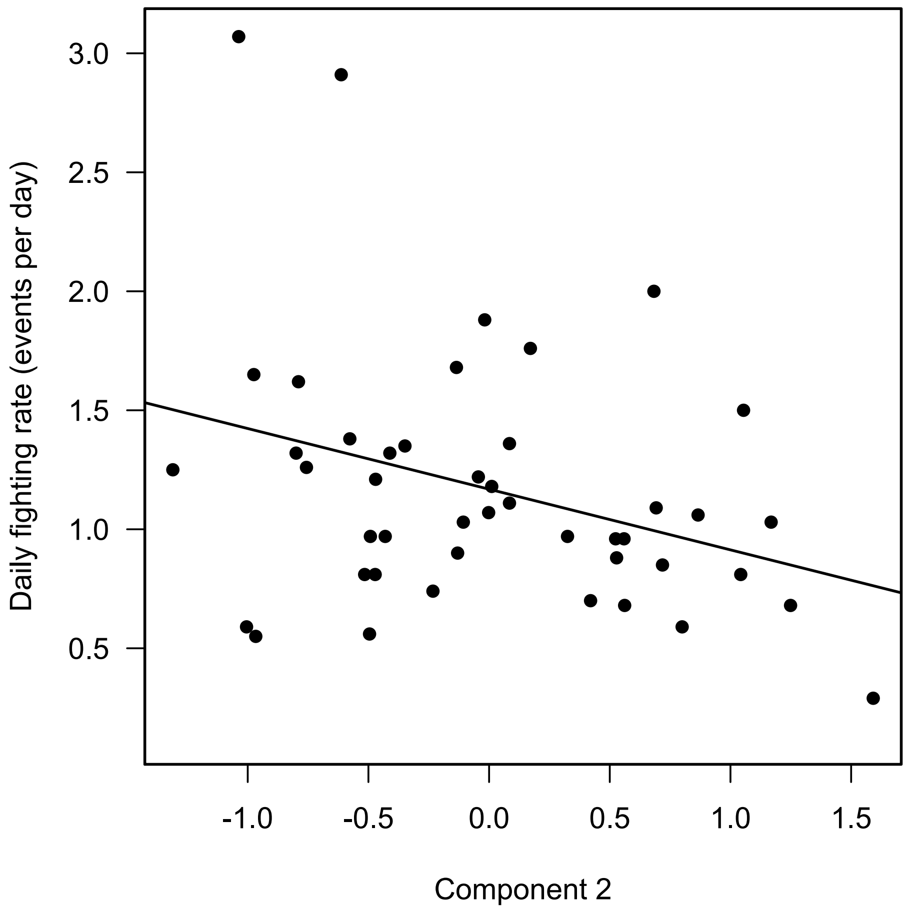

Figure S3.2. Scatterplot and OLS regression depicting the relationship between variation in Principle Component 2 values and daily fighting rates observed between males in the 45 "*all-unrelated*" vials. Variation in PC2 is primarily defined by the range of aggressiveness phenotypes in the trio in the synthetic data set – large positive values when the phenotypic values of the most and least aggressive males are very different, and large negative values when they are closer in magnitude.

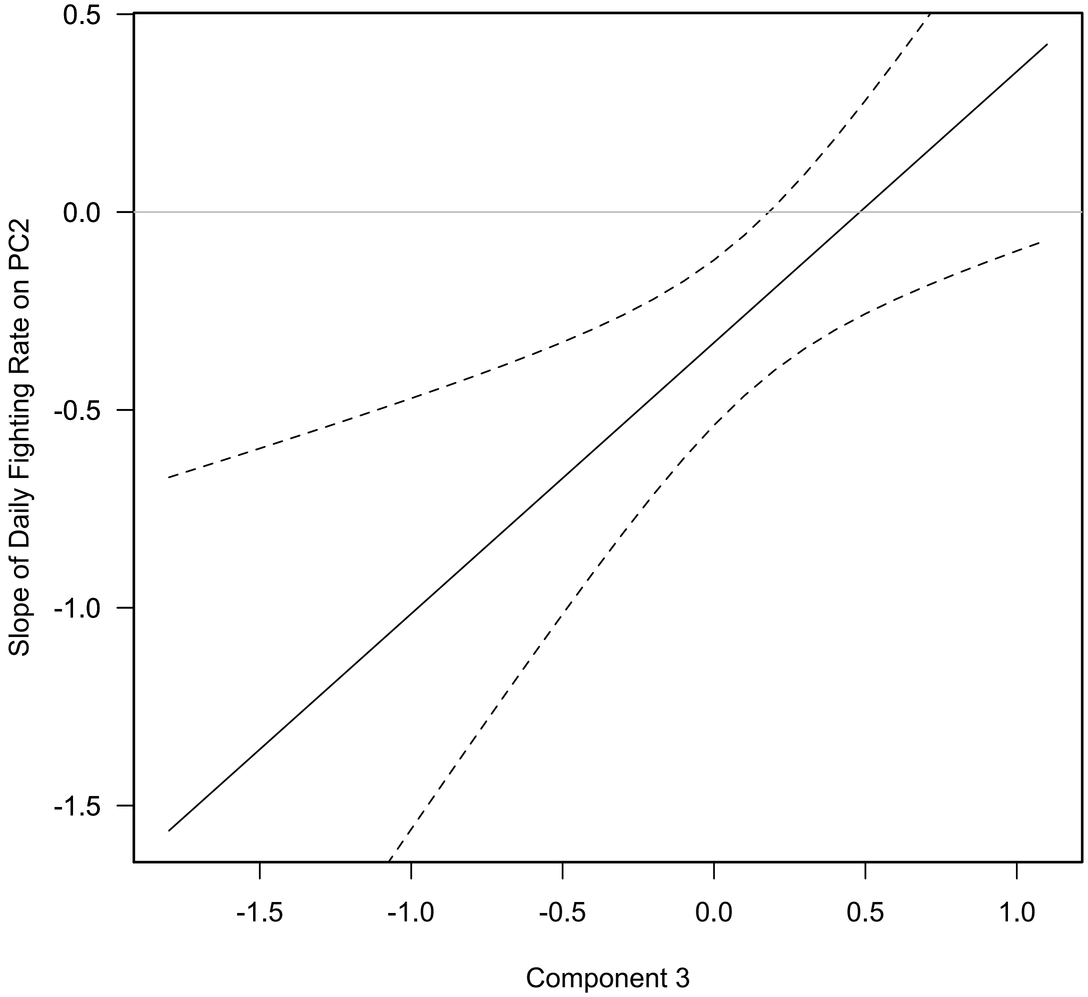

Figure S3.3. Plot indicating the marginal effect of variation in Principle Component 3 on the slope of the regression of Daily Fighting Rate (measured in the "*all-unrelated*" vials) on Principle Component 2. The solid black line indicates the slope, the dashed lines indicate the 95% CI of the slope value, while the horizontal grey line indicates the location of slope = 0. Variation in PC3 is primarily defined by the similarity in the level of aggressiveness of the intermediate and low aggressive lineages. The large positive PC3 values occur when their phenotypes are closer in magnitude, and large negative values occur when their values are more disparate.

CHAPTER 4

THE ROLE OF INBREEDING IN FITNESS OUTCOMES AND SOCIAL DYNAMICS

The purpose of this thesis was to identify how kinship between individuals might shape and influence social interactions, the resulting fitness outcomes of these interactions, and how the opportunity for mate choice might modify any existing links between kinship, behaviour, and fitness. Specifically, projects were designed to see if biases towards relatives could be identified in both reproductive (inter-sexual) and competitive (intra-sexual) interactions by assaying the behaviour of males and females. Additionally, the fitness consequences of inter- and intra-sexual interactions between kin were quantified over short- and long-term time periods. We assessed the immediate fitness consequences of mating with a sibling, the lifetime fitness effects of being housed with opposite sex kin, and how kinship between parents affected the fitness of inbred offspring in the next generation. While inbreeding has long been a topic of study (Charlesworth & Charlesworth, 1987), the study of kinship has recently received elevated attention, with particular focus on the potential benefits of incestuous matings (Kokko & Ots, 2006; Cheptou & Donohue, 2011; Puurtinen, 2011; Szulkin *et al*., 2013), and the potential for kinship to modulate sexual conflict (Duthie & Reid, 2015; Pizzari *et al*. 2015). However, while the results of some empirical studies addressing this topic are consistent with one another (*preference for kin*: Loyau *et al*., 2012; Robinson *et al*., 2012a; Robinson *et al*., 2012b; Carazo *et al*., 2014; Carazo *et al*., 2015), others are in conflict (*indifference to kin*: Tan *et al*., 2012; Ala-Honkola *et al*., 2011). Furthermore, frequently these experiments focused only on one aspect of relatedness, and were restricted to the study of intra-sexual

(Carazo *et al*., 2014; Carazo *et al*., 2015) or inter-sexual (Ala-Honkola *et al*., 2011; Loyau *et al*., 2012; Robinson *et al*., 2012a; Robinson *et al*., 2012b; Tan *et al*., 2012) relatedness in isolation. Often, behaviour and fitness were not assessed together. As a result, the role of relatedness is currently not well understood. My thesis experiments were designed to address the gaps present in these studies to provide a more holistic view of kinship.

In the multigenerational experiment in chapter two, we assessed inter-sexual behaviour associated with reproduction (specifically, mating latency and mating duration), as well as the fitness consequences associated with mating with a relative. In this experiment we manipulated not only relatedness, but also the opportunity for mate choice to occur. From our observations, we found that kinship between males and female neither increased nor decreased the amount of time males spent courting before successfully mating with females. This suggests that females in our study populations of *Drosophila melanogaster* held no biases for or against kin, and mated equally willingly with relatives and non-relatives alike. However, we did find that mating occurred much more quickly when a female was able to select from multiple males, regardless of their relation to her. This may be the result of increased male pressure and thus “convenience” mating, whereby females submit to mating earlier to escape harmful male harassment, or may be the result of satisfying female mate-sampling requirements more quickly, lowering the threshold to mating. In either case, the existence of kinship between males and females in this study did not appear to factor in female willingness to mate. Similarly, we observed no difference in the duration of any of inbred and outbred matings. This suggests that males, like females, do not consider the relatedness of their mates. Surprisingly, durations were not altered by the presence of competitor males (i.e. where environments allowed mate choice), a phenomenon that has been previously observed. It is possible that the use of virgin females was sufficient to elicit maximal male investment regardless of the level of perceived pre- and post-copulatory competition, a topic that is worth considering in future studies.

Despite the lack of bias for relatedness, our multigenerational study did reveal some effects of relatedness on fitness. Immediate consequences were largely absent; the reproductive fitness of inbreeding females differed slightly from outbreeding females in only a small number of situations. Egg-to-adult survivorship was affected by the interaction of relatedness and mate choice, while the inclusive fitness value of eggs was higher for inbred eggs. Ultimately, these different effects resulted in the equal production of offspring across all treatments. Relatedness in the parent generation affected the individual reproductive success of sons, with inbred sons producing fewer offspring of their own than outbred sons. However, when we accounted for the inclusive fitness value of grandchildren produced by inbred sons, the costs of inbreeding depression were fully offset. Daughters were unaffected by inbreeding; this was true for quantification with and without inclusive fitness values considered. Overall, there does not appear to be any significant fitness reduction resulting from inbreeding; outbreeding resulted in slightly more grandchildren, but this difference was not sufficient to negate inclusive fitness gained via the transmission of a greater number of alleles by inbred progeny. These results suggest that, while inbreeding depression may indeed occur, these costs are balanced by the benefits of inclusive fitness gains. It is therefore not surprising that we observed indifference towards inbreeding, as the costs of inbreeding depression appear insufficient to evolve or maintain any preference for unrelated mates.

Our chapter three experiment, the longevity study, again addressed behaviour associated with reproduction, as well as fitness outcomes. However, in this case we primarily focused on male-specific behaviours; these were the frequency of courtship displays, and the frequency of intra-sexual fighting. We also recorded the number of copulations observed. Fitness was quantified via the total production of eggs and female lifespan. In this case, we did find that relatedness appeared to modulate the behaviour of males; when males were related to one another, they reduced both the frequency of their fights with each other and the frequency with which they courted the female. These differences were apparent in the “short-term” time frame (the first three days of the assay) and this trend continued for the duration of the assay. Despite differences in courtship frequency, we did not detect any differences in the total number of copulations achieved. While the differences in courtship and fighting initially appeared to be driven by kinship, the relatedness of the female to those males did not result in any further differences; these results suggest that kinship is important only for intra-sexual interactions. However, we suspected that genetic variability in aggressiveness, rather than the preferential treatment of kin, might have been responsible for the differences observed here. A principal component analysis of this data supported this hypothesis, revealing that, for the unrelated male groups, much of their overall fighting could be predicted by an “aggressiveness phenotype” derived from how these specific male genotypes behaved when in the company of brothers. This could also have occurred in the experiments that inspired our study, and these genetic factors should be considered when designing experiments to study the role of relatedness. The fitness data we gathered from our longevity study was less extensive than that collected during the multigenerational study, but provided similar results. Relatedness, be it inter- or intra-sexual, did not affect total egg production, the rate of egg production, or the lifespan of the female (contrary to other published studies).

Again, the presence of multiple males (i.e. the opportunity for mate choice) had significant effects. Courtship rates were much lower in single male treatments, and females lived far longer and produced more eggs (likely the result of longer lifespans). These results offer further support for the lack of kin-based bias. If males reduced their aggression when competing with brothers, we might expect that this was partially to reduce harm to the female, their shared reproductive resource. Indeed, the reduction of harm to a shared female has been suggested as a possible reason for the "brotherly love" observed in the experiments by Carazo *et al*., (2014 and 2015). However, our results suggest that, even if brothers were reducing their aggressiveness to improve their inclusive fitness, the magnitude of harm directed at females was still significantly higher than what occurred when females were exposed to only a single male. If inclusive fitness benefits were driving these differences, we would expect improvements to female fitness in addition to (and because of) the modulation of male behaviour. The role of genetically determined phenotypes and their control over behaviour is therefore a potentially important factor that has thus far been overlooked.

Overall, our results do provide some support for the hypothesis that inclusive fitness benefits is a driving force for the evolution of inbreeding preferences. However, our studies revealed the presence of inbreeding depression, which could offset these benefits and, could ultimately promote inbreeding avoidance behviours. Understanding the interplay between these costs and benefits is of central importance – not only in these experiments, but also for the general – to understanding of inbreeding. The benefits of increased inclusive fitness were observed during the assessment of egg production. By accounting for the higher degree of relatedness shared between parents and inbred eggs, it was apparent that inbreeding could result in the transmission of a greater number of parentally-derived alleles. This was the only benefit of inbreeding we were able to detect in either of our experiments. If this benefit were to occur without any reduction in fitness associated with inbreeding, it is certainly possible that individuals within such populations would evolve a preference for mating with relatives. However, we did observe costs alongside this benefit, which were manifesting primarily in the offspring generation as a reduction in the reproductive success of sons, though also appeared to influence egg-to-adult survivorship in some circumstance. If inbreeding within a population significantly hampers the ability of inbred offspring to reproduce or eggs to survive, we would then expect inbreeding avoidance to evolve. As both costs and benefits occurred simultaneously in our population, and appeared to be of approximate equal effect, it is not surprising that we were unable to detect any strong bias associated with kinship between mates, with both sexes apparently tolerating inbreeding equally. While our lab-reared study populations are not subject to same stresses or dynamics of a wild populations, and thus may not be the best model for understanding inbreeding-related behaviours in all different species, they do provide insight into the principles at work. Understanding this balance between costs and benefits will always be the key factor in determining the outcomes of encounters with kin, and these experiments demonstrate that well.

Limitations

While considerable thought went in to each of our experiments, hindsight sometimes identifies flaws that might be improved upon or eliminated in future experiments. In our multigenerational study, mating latency was measured as the time from male-female introduction to the onset of mating. However, we did not indicate at what point courtship began, nor its intensity. Variation in male interest may therefore have been obscured, potentially reducing the accuracy of our measures of mating latency and our ability to quantify male preference for relatedness. In the future, recording all components of reproductive behaviour might yield greater insight into the reproductive dynamics of males and females. Additionally, in this experiment we did not include a treatment in which males were all related to each other, but not to the female. While we did subsequently include this treatment in our longevity study, its absence in the multigenerational experiment prevented us from assessing the role of intra-sexual relatedness in isolation from inter-sexual relatedness. While this did not appear to be significant when studied in the longevity experiment, additional data would allow for better comparisons to be drawn between each experiment.

In our longevity study, we used highly inbred lines, allowing us to utilize males virtually identical in their genetic makeup to each other over long periods of time. This was highly convenient for the weekly replacement of males, but may have purged deleterious alleles from the population, artificially reducing the costs of inbreeding depression for those flies that survived this process. In the future, the use of hemiclones lines, which provide flies with consistent levels of relatedness while not causing population bottlenecks, might eliminate this issue. However, it should be noted that in the creation of our inbred lines, complete mortality of individual lines was quite rare, with fewer than 10% of our inbred lines dying off. This suggests that levels of recessive deleterious genetic issues were not substantial in our source population.

Overall, the lack of inbreeding-caused fitness effects seen here may be indicative an underlying issue of populations maintained in lab conditions. It is frequently noted that the costs of inbreeding are far more obvious in natural settings, where environmental conditions are far more variable. Inbreeding depression requires not only variation in the genetics of a population, but also variation in environmental conditions to be fully manifested. By using captive populations housed in highly consistent environments (and in the case of the longevity experiment, highly consistent genetic environments), we may have masked the full extent of inbreeding depression. In the future, studies could be carried out using wild-caught flies and more natural environments.

Future directions

The work conducted here was completed in an effort to link factors previously addressed in isolation from each other. However, the potential for this topic is far from exhausted, and there is certainly great potential for future expansion. Particularly:

1. There may be significant genetic variation determining the level of bias for mating with relatives. To assess the possibility of such a phenomenon, genetically unique fly lines could be studied to determine if variation in preferences concerning kinship are heritable and if the heritability and expression of this trait are influenced by differences in fitness costs and benefits.

2. Our work, like that of many others, primarily focused on female preferences, providing males only no-choice scenarios. In order to better understand how males allocate reproductive effort on the basis of kinship, studies allowing male-choice could be completed to better address the possibility that the sexes might differ in their preferences.
3. In our multigenerational study, we quantified fitness for the parent generation as well as the offspring generation. However, fitness quantification in the offspring generation (count of adult progeny) was less intensive than in the parent generation (egg count, egg-to-adult survivorship, offspring count, offspring sex ratio, and longevity); this was primarily due to the restrictions imposed by limited (time and human) resources. A future study might be designed to direct greater scrutiny towards individual components of offspring fitness, and to potentially continue fitness quantification to even later generations.

My work and the integrative study of biology

During these experiments we considered our biological questions from a number of perspectives. A large part of our work involved the analysis of variation in individual behaviour, and the expansion of these observations to the family and population level. Particularly, in chapter three, our longevity study, we used behavioural phenotypes generated from familial environments to describe trends seen in genetically mixed groups. This allowed us to determine social dynamics based on independent observations taken in entirely different contexts. We also assessed fitness outcomes over an extended period of time, tracking how interactions in one generation shaped the future generations of that population. Fitness was also tracked over the physiological development of this organism: we followed populations from the time they began as eggs, to their eclosion as adults. Ultimately, preferences expressed by males or females have the ability to shape the evolution of a population. With respect to inbreeding, if inbreeding depression occurs, tolerance or preference for inbreeding may still be possible if those costs are balanced by the benefits provided by increased inclusive fitness. In any case, even in the absence of preference, kinship may alter the evolutionary trajectory of a population if that population is partitioned into unique genetic neighbourhoods. These experiments contribute to the growing body of work studying relatedness and its role in the modulation of behaviour and fitness. Importantly, our work considers the inclusive fitness value of inbred progeny, integrating this important factor into the study of optimal inbreeding. Furthermore, we provide information from intra- and inter-sexual perspectives, which are more frequently considered in isolation. Overall, these experiments provide significant insight into the role for relatedness, and address some of the issues apparent in previous studies.

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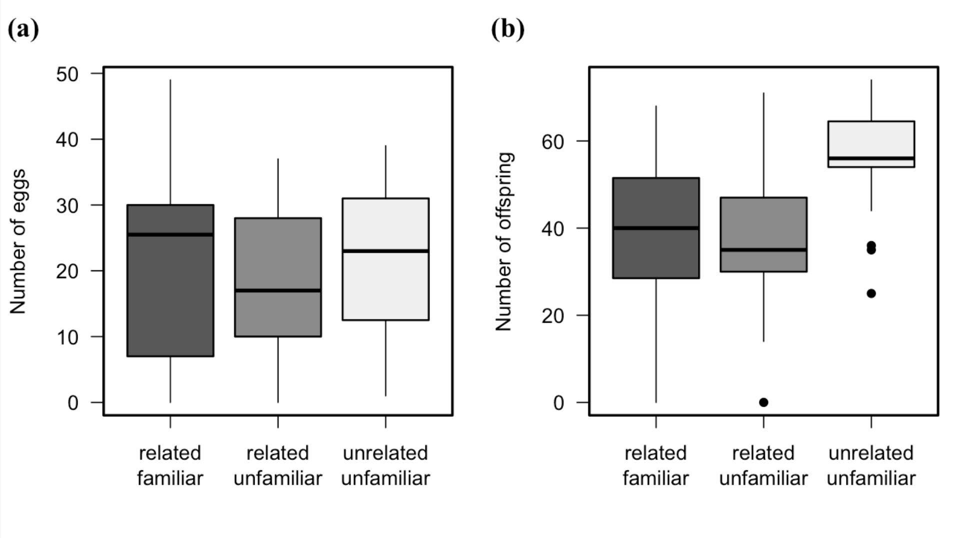
APPENDIX



Appendix 1. Boxplots showing **(a)** the number of resulting eggs and **(b)** offspring from reproductive events with males of each treatment type (related-familiar, related-unfamiliar, and unrelated-unfamiliar males), with each treatments being made up of three males. No difference between these treatments was detected in the number of eggs laid ($F_2 = 0.28$, $p = 0.757$), though mating with unrelated-unfamiliar males did result in more offspring ($F_2 = 7.328$, $p = 0.002$).

Appendix 2. Contingency table showing variation in mating success for three different male treatment types. Analysis reveals that these treatments groups did not vary significantly in their mating success (χ^2 =.0.375, df = 2, $p = 0.829$).

	Familiar brother	Unfamiliar brother	Unfamiliar Unrelated	*Row totals*
Mated	20	17	19	56
Unmated	36	39	37	112
Column totals	56	56	56	168 (Grand total)