Family and familiarity in flies

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Thesis submitted for the degree of Doctor of Philosophy
Trinity Term 2018

ABSTRACT

The concept of gene-centred evolution and subsequent inclusive fitness theory provided a formal framework in which to study the adaptation of social behaviours. It highlighted the role of relatedness between individuals in mediating both cooperation and conflict. However, social behaviours can be difficult to study, particularly among animals in the wild. *Drosophila melanogaster* has been studied as a model organism for over a century, and we have a deep understanding of its genetics, development and physiology. Furthermore, its short lifespan, small size and fast reproduction rate make it an ideal laboratory animal. Yet we currently know very little about how relatedness affects social behaviours in this species. In this thesis, I aim to contribute to our knowledge of kin selection and recognition in *Drosophila melanogaster*, developing its use as a model organism for studying inclusive fitness.

In Chapter 2, I examine the role of relatedness on adult sexual behaviours, namely how male-male relatedness mediates sexual harm to females. I distinguish the roles of genetic relatedness and larval social familiarity, and find that familiarity alone is not sufficient and genetic relatedness is required to reduce sexual conflict. However, male intrasexual interactions are important, as in Chapter 3, I find no effect of relatedness when males are presented to the female sequentially rather than simultaneously.

In Chapters 4 and 5, I consider the effect of relatedness on larval social behaviours, which have thus far been understudied compared to their adult counterparts. Contrary to predictions from inclusive fitness theory, in Chapter 4 I find that larvae benefit from developing in unrelated, rather than related, groups. This is possibly due to the increased genetic diversity and therefore behavioural diversity in unrelated groups, reducing direct competition. In Chapter 5, I additionally show that larvae prefer to cannibalise unrelated and unfamiliar conspecific victims, which may have strong fitness consequences for both adult and larval behaviour.

This thesis provides new evidence that relatedness, over and above social familiarity, mediates both adult and larval behaviours in *Drosophila melanogaster*, allowing us further to develop this species as a model organism for kin selection.

ACKNOWLEDGEMENTS

This thesis, and the many curve balls that life has thrown at me in the five years it took to complete, has been the most challenging thing I have ever accomplished and so it goes without saying that I have relied on the help and support of many people along the way.

Firstly, to my number one supervisor, Stu Wigby. It may be a cliché, but you really have been the best supervisor I could ever have asked for. From your knowledge and experience to your support and encouragement, you have cared not only about my work but also about me as a person. To my co-supervisor Stu West, thank you for being a reassuring presence whenever I needed you.

Thank you to the whole fly lab for always being willing to help with my massive experiments and for being such great company through the years, particularly when we weren't in the most favourable of circumstances. Juliano, you made me feel like I belonged from the very start, and for that you are awesome.

Thank you to a group of people I will likely never meet, but nevertheless whose presence has made my experience more enjoyable through your tweets and comments. Thank you for allowing me to follow my dream career.

A huge thank you to my wonderful friends from St John's and Zoology. I have been incredibly fortunate to have such a strong support network, and I honestly could not have reached this point without all of you. In particular, Annet, Jasper, John-Mark, Laura, Lucy, Mikayla, Shana and Suzie; you have shared the ups and the downs of this PhD with me, you have consoled me over hot chocolates and you have kept me grounded as I have focussed on my Internet career.

Ellie, ever since we met eight years ago, you have been my rock at Oxford. More than anyone else, you have been, and will always be, there for me. You know when to tell me what I want to hear and when to tell me what I need to hear. You have learned more about ladybirds and flies and biology than you ever wanted to learn. I really can't imagine my time in Oxford without you.

And finally, thank you to my family. To Lydia, for showing me that a PhD is something that normal people can actually achieve. And to my parents, for igniting my passion for the natural world, for letting me spend my summers catching whatever unsuspecting frog or insect hopped my way and for encouraging me in all my endeavours. Thank you.

Many flies were harmed in the making of this thesis.
I thank them for their sacrifice.

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DECLARATION AND AUTHOR CONTRIBUTIONS

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated below. This work has not been submitted for any degree or professional qualification except as specified below.

Chapter 2

The following published paper arose from this thesis and is included in its published format in Appendix 1.

Le Page S, Sepil I, Flintham E, Pizzari T, Carazo P, Wigby S (2017) Male relatedness and familiarity are required to modulate male-induced harm to females in *Drosophila*, *Proceedings of the Royal Society B.*, 284

Stuart Wigby, Pau Carazo and I conceived of the study. Pau Carazo, Stuart Wigby and I designed the experiment. Ewan Flintham and I generated the experimental flies. Irem Sepil and I collected behavioural data. I collected offspring data and carried out the statistical analyses. Stuart Wigby, Pau Carazo, Tom Pizzari and I drafted the manuscript. Florian Klimm wrote the computer program to randomly assign families and Dan Lunn and George Nicholson assisted with the statistical analysis.

Chapter 3 is my own work, supervised by Stuart Wigby and Pau Carazo. Stuart Wigby, Pau Carazo and I conceived of the study, Stuart Wigby, Pau Carazo and I designed the experiment, I collected all the data, performed the statistical analyses and wrote the chapter. Pau Carazo, Stuart Wigby, Juliano Morimoto, Eleanor Bath and Jen Perry assisted with behavioural observations.

Chapter 4 is my own work, supervised by Stuart Wigby. I conceived the study, Stu Wigby and I designed the experiment, I collected the data, performed the statistical analyses and wrote the chapter. Charlotte Griffiths assisted with the preliminary tests, John-Mark Allen wrote the computer program to randomly assign families and Eleanor Bath, Ben Hopkins, Stuart Wigby and Jen Perry assisted with collecting eggs.

Chapter 5 is my own work, supervised by Stuart Wigby. I conceived the study and designed the experiment, I collected the data, performed the statistical analyses and wrote the chapter. Jessica Norman, Ben Swanson, and Lucy Harper assisted with collecting flies, Jessica Norman assisted with pinning victims, and George Nicholson assisted with the statistical analysis.

CHAPTER 1:

INTRODUCTION

The concept of gene-centred evolution (Hamilton 1964) has arguably had the greatest influence on our understanding of evolution since the concept of evolution by natural selection a century earlier (Darwin 1859). It has formalised our study of social interactions - those behaviours that affect the fitness of not only the acting individual but also the recipients of the action - spawning the field of social evolution. Meanwhile, *Drosophila melanogaster* has been used as a model organism long before we conceptualised inclusive fitness (Morgan et al. 1915) yet we have only recently begun to discover how indirect fitness effects shape its behaviour.

In this thesis, I will introduce each chapter with a review of the relevant literature, so I shall not provide details here that are covered in the individual chapters. However, for clarity of communication and a broader perspective on the common topics, I introduce the key concepts that will play a recurring role throughout this thesis.

Key concepts

Inclusive fitness

Inclusive fitness is defined as "the production of adult offspring…stripped of all components which can be considered as due to the individual's social environment …augmented by certain fractions of the quantities of harm and benefit which the individual himself causes to the fitnesses of his neighbours" (Hamilton, 1964). Hamilton's key insight was that adaptations at the level of the individual serve to selfishly propagate the genes responsible, be that through personal reproduction (**direct fitness**) or through the reproduction of related individuals that also share copies of the gene (**indirect fitness**). Famously summarised in Hamilton's rule, $rb\text{-}c > 0$, it highlights the importance of relatedness in promoting cooperation and altruism.

We often think of selection as a fitness optimisation programme (Grafen, 2007), whereby individuals act as if to maximise their inclusive fitness or contribution to the gene pool. Individuals 'choose' one strategy over another because it confers higher fitness, 'choose' to fight a relative less because it increases their indirect fitness, or 'choose' to cannibalise a relative when the benefits outweigh the costs. It is important to remember that this language is teleological and assigns purpose or agency where there often is none, though I will continue to use it in this thesis for the sake of brevity.

Relatedness

Relatedness is a term we all intuitively understand but has many subtly different definitions (Bourke, 2011). Broadly speaking, relatedness is the fraction of genes that two individuals share, over and above the population average (Bourke, 2011). Two genetically

identical individuals will have $r = 1$ and an actor will be related to an average member of the population as $r = 0$ (by definition). Negative relatednesses, where the actor is less related to the recipient than they are to the population average (Grafen, 1985), are possible but are unusual and will not feature in this thesis.

I will frequently use full siblings and random members of the population to compare the effects of related and unrelated groups on behaviour. In diploid species such as *Drosophila melanogaster*, full siblings have a relatedness of 0.5, that is, they share 50% of their genes over and above the population average. This is an average coefficient for all members of that class. By chance, two siblings may inherit more similar genes from their parents and therefore have more than 50% of their genes in common above the population average. However, an individual does not have access to modern genetic testing techniques (though see 'green beard genes', Dawkins 1976) and does not have perfect knowledge of the fraction of genes they have in common. They must therefore rely on the average coefficient for members of that class (0.5 for full siblings, parents and offspring, 0.25 for half-siblings and cousins etc.).

Kin recognition

Kin recognition is defined by Holmes and Sherman (1983) as "the differential treatment of conspecifics as a function of their genetic relatedness". There has been much debate surrounding the topic, both semantic and biological. This controversy largely revolves around whether individuals need to be able to respond to differences at the genetic level, or whether behaviours that functionally differentiate between kin and non-kin but without a genetic basis should also be included under the term 'kin recognition' (Grafen, 1990, 1991; Stuart, 1991).

In some species, individuals are able to detect genetic similarity using phenotypic markers of highly variable genetic regions, such as the use of major urinary proteins in house mice (*Mus musculus domesticus,* Green *et al*. 2015). Other behaviours have the functional effect of discriminating between kin and non-kin but rely on a proxy of relatedness other than genetic similarity. The classic example of this is nestmate cues, where based on probability alone, nestling birds can reasonably assume that other birds in the same nest are from the same family. They are using a shared rearing environment, or **familiarity**, as a proxy for sibship. The reason why some challenge the inclusion of such behaviours within kin recognition (Grafen, 1990, 1991) is that if genetically unrelated individuals share the same proxy cues used for relatedness, for example a cuckoo chick in a reed warbler's nest, the proxy provides inaccurate information, and the warbler cannot recognise the cuckoo as being unrelated.

A more biological side of the kin recognition debate asks not how individuals are recognising kin, but why (Hollis, Kawecki and Keller, 2015). Individuals may appear to be discriminating between kin, even based on genetic similarity, but this kin discrimination is a by-product of another behaviour, such as species recognition or sensory bias. If there is a benefit to associating with conspecifics, an individual may use the 'armpit effect' (Dawkins, 1982), comparing the genetic similarity of others to itself to identify the species. They may therefore be more attracted to genetically similar individuals, not because they are attracted to relatedness, but because the signal of conspecificity is stronger. This is why it is important to understand the mechanisms underpinning kin recognition behaviours before attributing them to kin selection.

Sexual selection

Sexual selection is a subset of natural selection, where traits are favoured if they increase an individual's reproductive success. Within the sexes, the traditional paradigm is that of males selfishly competing with each other over access to limited reproductive opportunities with choosy females (Bateman, 1948; Parker, 1979). We would describe males as having steeper Bateman gradients, benefiting more from each subsequent mating (Bateman, 1948) and therefore males experience stronger sexual selection than females.

Reproductive competition can be split into two episodes; **pre-copulatory competition**, where males compete over access to mating opportunities (Darwin, 1871), and **post-copulatory competition**, where sperm compete over access to fertilisation opportunities (Parker, 1970). Each favours different male traits. The showy ornaments and armaments that initially puzzled Darwin (1871) result from pre-copulatory competition, whereas the products of post-copulatory competition are usually more subtle, such as sperm length or ejaculate chemical composition, but also testis size and penile spines.

Within the 'choosy female' paradigm, females may choose to mate with a male because of the immediate benefits he can bring to her through mating, such as food and water through a nuptial gift, or because of the delayed benefits he can bring to her by providing her with high quality offspring (Andersson, 1994). In a decision that has long obfuscated the flow of ideas between the fields of sexual selection and kin selection, these two benefits are termed **direct effects** (immediate benefits) and **indirect effects** (genetic benefits). Thankfully, I am not comparing direct and indirect mating effects in this thesis, so any reference to direct and indirect effects are referring to direct fitness and indirect fitness effects (see **Inclusive Fitness**).

Sexual conflict

Males and females may differ in how they optimise their fitness during reproduction. Any behaviour that moves the reproductive optimum away from the optimum of one sex and towards that of the other will generate sexual conflict (Chapman *et al.*, 2003; Parker, 2006; Pizzari and Gardner, 2012; Pizzari, Biernaskie and Carazo, 2015). This sexual conflict can be so strong that Wilson (1975) went so far as to describe sex as an "antisocial force". In polyandrous systems, males may compete so fiercely to meet their own fitness optimum that they harm the female in the process. One example from *Drosophila melanogaster* is that females exposed to larger groups of males, thus experiencing more male harassment, show faster **reproductive ageing**; the decline in fertility over time (Wigby and Chapman, 2005).

Sexual conflict can generate a '**tragedy of the commons**' scenario (Rankin and Kokko, 2006; Rankin, Dieckmann and Kokko, 2011). In a tragedy of the commons, a group all share a common resource (e.g. males share access to fecund females). Although the benefits of exploiting the resource (e.g. males harassing females for increased matings) lie solely with the individual, the costs (e.g. reduced fecundity in females due to harassment) are shared among the whole group. Therefore, it is in each individual's interest to selfishly exploit the shared resource (Hardin, 1968). Female harm caused by self-interested males can be reduced when competitors are related, for example brothers competing over access to a single female, so long as Hamilton's rule is still satisfied. When competing among brothers, a male not only gains fitness through his own offspring, but also in part through the offspring of his brothers, thus, being selfish at the genetic level may mean being cooperative at the individual level and being less sexually aggressive.

An unnatural history of *Drosophila melanogaster*

The fruit fly, *Drosophila melanogaster*, is one of over 3750 species of small (3mm long) fly in the Drosophilidae family (Ashburner *et al.*, 2005). It is originally native to the tropics of Africa and South and South East Asia but has benefitted from a synanthropic relationship with humans and has since expanded its range to every continent except Antarctica (Ashburner *et al.*, 2005). *D. melanogaster* have been studied in laboratory settings from as early as 1909 (Bellen and Yamamoto, 2015) and have since become a model organism for research into biochemistry, genetics, development, physiology, neuroscience, and behaviour. This is in part due to the ease of animal husbandry involved; flies have a short lifespan, reproduce rapidly and profusely, can be housed in small, dense cages and feed on a cheap, easy-to-produce substrate as both larvae and adults. As a result, we are fortunate to have a well characterised genome, and an in depth understanding of the mechanisms behind many of its behaviours. *D. melanogaster* have been taken from the wild for use in laboratory research multiple times from habitats across the globe, meaning laboratory populations can have diverse origins as well as diverse rearing conditions.

After hatching from an egg to a larva, *Drosophila melanogaster* gain almost all their final body mass from feeding on the bacteria and fungi growing on decomposing fruits. Larvae breathe air through spiracles at their posterior end, restricting the depth to which they can tunnel and feed. After two moults dividing the three larval instars, the third instar ceases feeding and starts wandering to find a suitable pupation site. Through prepupation and pupation, the fly is completely immobile as it undergoes metamorphosis into a winged adult. On eclosion, flies take several hours to inflate and harden their exoskeleton and become sexually mature. Males are easily characterised by their smaller size and black rear

end (Ancient Greek, *mélas*: black, *gaster*: belly), whereas females are approximately twice the size of males and have a prominent white ventral abdomen. Fruit flies are most active at dawn and dusk (Ancient Greek, *drosos*: dew, *phila*: to love), at which times we see an increase in their mating and oviposition behaviours.

A single female can typically lay up to 2,500 eggs in her lifetime depending on temperature, humidity and population density, with an optimum of 24˚C. A single male may sire up to 14,000 offspring in his lifetime, although males can become sterile if they mate too many times in quick succession (Ashburner *et al.*, 2005). There is strong last-male sperm precedence, with roughly 80% of stored sperm from previous matings being displaced by sperm from the most recent mating (Manier, Belote and Berben, 2010). As both males and females mate multiple times (Imhof *et al.*, 1998) and females can preferentially store and allocate sperm (Amitin and Pitnick, 2007), there is strong post-copulatory selection in males. Males produce accessory gland proteins in the ejaculate that influence female behaviour, increasing her investment in his offspring and reducing her life expectancy (Wigby and Chapman, 2005; Fricke *et al.*, 2009).

The wild-type population of *D. melanogaster* I use in this thesis was collected from Dahomey (present-day Benin, Africa) in 1970, and has since been maintained in laboratory conditions in a large, outbred population (Partridge and Farquhar, 1983). This Dahomey population is maintained in dense cages at 25°C with a 12:12 light-dark cycle and overlapping generations. For experiments, flies are reared in either polystyrene vials or polyethylene bottles containing standard Lewis medium (Lewis, 1960), which is a medium-firmness agar substrate containing yeast, cornmeal, sugars and an antifungal agent. Adults feed and oviposit on this medium, and larvae are able to burrow into it during the third instar stage, liquefying and acidifying the medium.

Family and the fruit fly

Since the turn of the millennium, we have begun to consider the role of relatedness and kin recognition on the behaviours of *Drosophila melanogaster*, and here I will briefly summarise the literature on this topic to date. Initially, work on kin recognition focussed on optimal inbreeding and inbreeding avoidance, i.e. whether the relatedness between the male and female influenced sexual behaviour. Males that were full siblings to the female were less successful in sperm competition than less related males, suggesting females may cryptically choose the sperm of less related males (Mack, Hammock and Promislow, 2002). However, this result could not be replicated (Ala-Honkola *et al.*, 2011). Moving from post-copulatory to pre-copulatory preferences, Loyau *et al*. (2012) found females preferred mating with their brothers than with unrelated males, but found no such relatedness preference among males. Experiments in a second laboratory population similarly found females preferred to mate with related males (Robinson, Kennington and Simmons, 2012b), though there was no such effect of relatedness on either male or female choice in a third laboratory population (Tan *et al.*, 2012). Microsatellite analysis of flies caught in the wild showed that mating pairs were more related to each other than we would expect by chance (Robinson, Kennington and Simmons, 2012a), suggesting some degree of preference for inbreeding in wild populations.

Inbreeding, or the relatedness between the male and female, is not the only opportunity for kin recognition in sexual behaviours. Later work has focussed on intrasexual competition and relatedness, or the relatedness between males competing over a (unrelated) female and *vice versa*. Males that had previously mated preferred to court a female unrelated (and from an unfamiliar larval rearing environment) to his first mate than a related (and from a familiar larval rearing environment) female to his first

mate, though this increased courtship did not result in more matings (Tan *et al.*, 2013). In the same study, females remated faster with a male related (and familiar) to her first mate than to a male unrelated (and unfamiliar) to her first mate. Both of these preferences disappeared when using *Orco* mutants that had no olfactory sense.

One notable study compared male-male competitive behaviours and female fitness between triplets of brothers and an unrelated female and triplets of unrelated males and an unrelated female (Carazo *et al.*, 2014). They found males in the related groups fought less, courted females less and survived for longer than males in unrelated groups. Furthermore, females mated to the related triplets had higher lifetime reproductive success and slower reproductive ageing than females mated to unrelated triplets of males. A follow-up experiment from the same group replicated the reduction in male aggression rates among related triplets and also found trans-generational effects of male-male relatedness, where daughters of females mated to related triplets lived longer than daughters of females mated to unrelated triplets, with no effect seen in sons (Carazo *et al.*, 2015).

Related males in the original study (Carazo *et al.*, 2014) had been reared in the same larval environment, whereas unrelated males had been reared in different larval environments, thereby conflating relatedness with familiarity. This sparked several subsequent experiments in different laboratory populations. Hollis *et al*. (2015) tested related familiar, unrelated unfamiliar and a third, related unfamiliar, treatment and found no difference in female lifetime reproductive success or reproductive ageing between the related unfamiliar and unrelated unfamiliar treatments, concluding that there was no effect of relatedness. However, as they only tested three of the four possible combinations, their study could not conclude that relatedness was not necessary, only that

it was not sufficient. Chippindale *et al*. (2015) replicated these findings among the three treatments. Martin and Long (2015) found triplets of related familiar males fought and courted less, as per Carazo *et al*. (2014), but found no effect between related familiar and unrelated unfamiliar males on female harm.

Thesis overview

In my thesis, I will contribute to developing the use of *Drosophila melanogaster* as a model organism for studying inclusive fitness. Across my chapters, I identify behaviours where flies behave differently towards kin and non-kin and begin to unravel the underlying mechanisms. I study not only well-characterised adult sexual behaviours, but also social behaviours among larvae, which have been thus far understudied.

In Chapter 2, I isolate the contributing effects of genetic relatedness and larval social familiarity on male sexual harm to females using a fully factorial design. I test triplets of males that are full siblings or non-siblings and reared in the same vial or reared in different vials, and mate them to unrelated females. I find that larval familiarity alone is not sufficient to reduce sexual conflict, and genetic relatedness is necessary to mediate male harm.

In Chapter 3, I test whether the reproductive advantage we see when an unrelated male competes simultaneously against two brothers holds in a sequential mating system. I found no differences between females sequentially mated to two brothers and a focal male and two unrelated males and a focal male.

In Chapter 4, I study the fitness consequences of relatedness on larval crowding. I examine the well-known responses of larval fitness to increasing density, comparing larvae raised in groups of full siblings versus larvae raised in unrelated groups. Larvae do not

benefit from rearing in a related group, as might be expected from kin selection, but instead benefit from rearing in an unrelated group. This is possibly to be due to the increased genetic variation providing greater variation in feeding and foraging strategies, reducing the level of direct competition within the group.

In Chapter 5, I further examine the recently described behaviour of cannibalism among larval *D. melanogaster*. I study whether relatedness and familiarity affect the cannibals' decision as to which victim to favour in a direct choice test. I find that larvae prefer to cannibalise unrelated and unfamiliar victims, as predicted by kin selection theory, but are not influenced by the relatedness of other cannibals present on a victim. Larvae also show a strong and consistent preference for more-cannibalised victims.

CHAPTER 2:

MALE RELATEDNESS AND FAMILIARITY ARE REQUIRED TO MODULATE MALE-INDUCED HARM TO FEMALES

Abstract

Males compete over mating and fertilisation, and often harm females in the process. Inclusive fitness theory predicts that increasing relatedness within groups of males may relax competition and discourage male harm of females as males gain indirect benefits. Recent studies in *Drosophila melanogaster* are consistent with these predictions and have found that within-group male relatedness increases female fitness, though others have found no effects. Importantly, these studies did not fully disentangle male genetic relatedness from larval familiarity, so the extent to which modulation of harm to females is explained by male familiarity remains unclear.

Here we performed a fully factorial design, isolating the effects of male relatedness and larval familiarity on female harm. Whilst we found no differences in male courtship or aggression, genetic relatedness and familiarity among males had interacting effects on female reproduction and survival. Relatedness amongst males increased female lifespan, reproductive lifespan and overall reproductive success, but only when males were familiar. By showing that both male relatedness and larval familiarity are required to modulate female harm, these findings reconcile previous studies, shedding light on the potential role of indirect fitness effects on sexual conflict and the mechanisms underpinning kin recognition in fly populations.

Note: This chapter has been published as

Le Page S, Sepil I, Flintham E, Pizzari T, Carazo P, Wigby S (2017) Male relatedness and familiarity are required to modulate male-induced harm to females in *Drosophila*, *Proceedings of the Royal Society B.*, **284**

and is reproduced as published in Appendix 1.

Introduction

The evolutionary strategies that maximise female fitness may simultaneously hamper male fitness and *vice versa*, generating sexual conflict over reproductive decisions (Parker, 1979; Arnqvist *et al.*, 2005; Parker, 2006). This conflict often arises because intense competition among males over access to mating and fertilisation opportunities can harm females (i.e. reduce their fitness). Such harm has been likened to a tragedy of the commons (Hardin, 1968; Rankin and Kokko, 2006; Rankin, 2011; Rankin, Dieckmann and Kokko, 2011), in which selfish exploitation results in the depletion, or even destruction, of a shared resource. Male harm of females may occur through a number of pathways including sexual harassment, sexual coercion, traumatic insemination, male accessory gland products, pathological polyspermy, and infanticide (Arnqvist *et al.*, 2005). In all these cases, sexual selection promotes male strategies even if they happen to harm females in the process (i.e. collateral female harm), or precisely because they harm females (e.g. Lessells 2005). Male harm of females is emerging as an important factor in population ecology and evolution, as increasing evidence indicates its role in a number of fundamental processes, such as dispersal (Eldakar *et al.*, 2009), population extinction (Le Galliard *et al.*, 2005), and intersexual coevolution (Arnqvist *et al.*, 2005). However, the mechanisms underpinning the variation in the severity of female harm observed across species and populations remain little understood.

Recent theoretical work has suggested that indirect fitness effects might play a key role in modulating male harm to females (Rankin, 2011; Wild, Pizzari and West, 2011; Pizzari and Gardner, 2012; Faria, Varela and Gardner, 2015; Pizzari, Biernaskie and Carazo, 2015). This happens whenever males tend to compete with males to whom they are more

genetically related than the population average, for example when population viscosity limits dispersal, and competition is not exclusively local (Taylor, 1992; Faria, Varela and Gardner, 2015). In this context, a male may indirectly reduce his own inclusive fitness by harming females that could also reproduce with his male relatives, and this is expected to relax male-male competition and selection for male traits leading to female harm (Rankin, 2011; Wild, Pizzari and West, 2011; Pizzari and Gardner, 2012; Faria, Varela and Gardner, 2015; Pizzari, Biernaskie and Carazo, 2015).

While the expectation that, under some circumstances, within-group male relatedness reduces the intensity of intra-sexual competition has received empirical support (e.g. Díaz-Muñoz *et al*. 2014; Kapranas *et al*. 2016; Rosher *et al*. 2017; Tan *et al*. 2017), the notion that within-group male relatedness might also reduce female harm is only beginning to be investigated. Consistent with this idea, female least killifish, *Heterandria formosa,* died younger and produced progressively smaller offspring when experimentally mated to males that are unrelated to each other, compared with females mated to males highly related to each other (but always unrelated to the female; Ala-Honkola, Friman, *et al*. 2011). Similarly, female bulb mites, *Rhizoglyphus robini*, laid more eggs over a two day period when paired for five days with males that had experimentally evolved in populations comprising their full siblings than when paired with stock males (Lukasiewicz *et al.*, 2017).

The influence of male relatedness on female harm has also been explored in the fruit fly, *Drosophila melanogaster*. Carazo *et al*. (2014) found that females had higher lifetime reproductive success and slower reproductive ageing (a more gradual decline in fecundity and fertility with age) when exposed to a triplet of brothers that were unrelated to the female but had been raised together as larvae than when exposed to a triplet of

males that were unrelated to each other and had been raised separately as larvae. These patterns have now been explored by different research groups, and in different *D. melanogaster* populations (Carazo *et al.*, 2015; Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015; Martin and Long, 2015), resulting in some studies reporting results consistent with Carazo *et al*.'s findings and others reporting no effects (summarised in Supplementary Table 2.1), suggesting that these effects are not entirely consistent and that they might be modulated by other mechanisms.

One such mechanism might be familiarity. Hollis *et al*. (2015) identified larval familiarity among males as a requirement for reduced harm to females. By introducing a new treatment in which females were exposed to males that were related to each other but raised apart as larvae, this study showed that males were only benign to females when they were related and raised together as larvae. These results are consistent with larval familiarity acting as a kin-recognition mechanism, as demonstrated in other taxa (Hepper, 1986; Stuart, 1991; Komdeur and Hatchwell, 1999; Hatchwell *et al.*, 2001; Sharp *et al.*, 2005; Aquiloni and Tricarico, 2015). In principle, these results may also indicate that male flies might have evolved to reduce female harm strategically in response to male familiarity *per se*, independently of relatedness, through direct (rather than indirect) fitness effects (Hollis, Kawecki and Keller, 2015). For example, mechanisms such as reciprocity might reduce competition among familiar males, and this may in turn reduce female harm.

A scenario in which variation in male harm is entirely predicted by relatedness, not familiarity, would suggest that flies use genetic cues to recognise kin and reduce harm in the presence of relatives to gain indirect fitness benefits. A scenario in which variation in male harm is entirely predicted by male familiarity, not relatedness, would be consistent both with the idea that direct benefits associated with familiarity drive changes in female

harm, and the idea that female harm is driven by indirect effects, whereby flies may rely entirely on familiarity clues to recognise kin. Finally, variation in male harm may be predicted by the interaction between relatedness and familiarity cues. For example, indirect fitness effects may reduce male harm to females when males are related, but male flies may only be able to recognise relatives under familiarity (Holmes and Sherman, 1983). However, because no study has tested the fully factorial combination of relatedness and familiarity (Carazo *et al.*, 2014, 2015; Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015; Martin and Long, 2015), the relative roles of these factors remain unresolved.

In this study, we conducted an experiment using a novel, fully factorial design to isolate the separate effects of relatedness, familiarity (shared larval environment) and their interaction on male sexual behaviour (as measured through assays of male-male aggression, courtship and mating rates) and female harm (as measured through female lifetime reproductive success, reproductive ageing, lifespan and reproductive lifespan) in *D. melanogaster*. We used four different social environments in which males were: (i) related and familiar, (ii) related and unfamiliar, (iii) unrelated and familiar, and (iv) unrelated and unfamiliar. While we found no effect on male behaviours, we did observe an interaction between male relatedness and larval familiarity, thereby showing that larval familiarity alone is insufficient to reduce harm to females. Male relatedness increased female reproductive success, lifespan and reproductive lifespan, and slowed reproductive ageing, but only when males were familiar.

Methods

Stock cultures

We used a laboratory-adapted, wild-type Dahomey stock of *Drosophila melanogaster*, maintained in large outbred populations since 1970 (Partridge and Farquhar, 1983; Clancy and Kennington, 2001) at 25˚C in a non-humidified room and a 12:12h light:dark cycle. This is the same stock used by Carazo *et al.* (2014, 2015). All flies were maintained in cages containing bottles of Lewis medium (Lewis, 1960) with overlapping generations.

Male treatments

We produced triplets of males belonging to one of four treatments generated from a fully factorial cross of relatedness and familiarity of the larval environment; related and familiar, related and unfamiliar, unrelated and familiar, and unrelated and unfamiliar (Figure 2.1).

To generate each experimental male triplet, we created families using parents that were two days post-eclosion, and had been collected as eggs from the stock population and reared at standard larval density at 25˚C (Clancy and Kennington, 2001). We paired a single virgin male and female for 12 hours in individual larval collection chambers containing a Petri-dish filled with hard grape agar (550ml water, 25g agar, 300ml grape juice concentrate and 21.25ml 10%w/v Nipagin) with a smear of live yeast paste, before discarding the males. Twenty-four to thirty-six hours after egg laying, we picked larvae with a mounted needle into 36ml vials containing 8ml of Lewis medium, collecting 60 larvae in

total per family over a period of three days. Any families that failed to produce 60 larvae were excluded.

From each of 135 families, 45 larvae were divided equally among three "single family" vials and 15 larvae were distributed individually among each of 15 "mixed family" vials. Thus, each "single family" vial contained 15 larvae from a single family, and each "mixed family" vial contained 15 larvae from 15 randomly allocated families (Figure 2.1). These vials were kept at 18˚C and adult virgin males were collected within 16h of eclosion.

Virgin males were immediately aspirated and housed in vials of Lewis medium and excess live yeast grains at 18˚C in their experimental triplets; "related familiar", "related unfamiliar", "unrelated familiar" and "unrelated unfamiliar". "Related familiar" comprises three males collected from the same "single family" vial. "Related unfamiliar" comprises one male taken from each of the three "single family" vials of the same family. "Unrelated familiar" comprises three males taken from the same "mixed family" vial. "Unrelated unfamiliar" comprises one male taken from each of three "single family" vials of three different families. No family contributed to more than one vial of each related familiar and related unfamiliar treatments, and families were randomly assigned so that each had an equal contribution to the unrelated familiar and unrelated unfamiliar treatments. Two days before the introduction of females, males were transferred to fresh vials and kept at 25˚C. To produce virgin females, we reared eggs from the cage population at 18˚C at standard density (~250 flies per 75ml bottle containing 45ml of Lewis medium) in parallel with male collection, collected adult females under ice anaesthesia and aged them at 25˚C in individual yeasted vials for three days before the start of the experiment.

We performed the experiment across two blocks, producing a combined total of 95 related familiar triplets (39 in Block 1, 56 in Block 2), 86 related unfamiliar triplets (37 in

Block 1, 49 in Block 2), 96 unrelated familiar triplets (22 in Block 1, 74 in Block 2) and 86 unrelated unfamiliar triplets (33 in Block 1, 53 in Block 2). Differences in sample sizes across treatments are due to some flies escaping and stochastic variation in the number of adult males emerging in each family vial within the short collection period.

Behavioural observations

On day 1 we added a single virgin female to each male triplet in a randomly numbered vial to blind the observer to the treatment throughout data collection. On days 2, 3, 4, 5, 8, 9, 10, 11 and 12 we observed the vials during eight scans in the morning (only seven scans on day 2, block 1), 10-20 minutes apart and recorded whether any males were displaying aggression (Chen *et al.*, 2002), courtship (Bastock and Manning, 1955) and mating behaviours. Note that in Carazo *et al.* (2014), triplets of males were replaced at regular intervals to prevent males co-ageing with the female. The setup we used to generate unrelated familiar males prevented us from replacing males during the experiment, therefore males were allowed to age with the female in this study, and as such, we did not expect a similarly strong level of female harm as reported in (Carazo *et al.*, 2014).

Flies were transferred to fresh vials under light CO_2 anaesthesia on days 3, 5, 8 and 11 in both blocks and additionally on day 15 in block 2, and the vials were retained to collect adult offspring (see Fitness Measures). Vials were discontinued upon the female's death, and we recorded the day of death up to day 15 in block 1 and up to day 19 in block 2 after which time any remaining females (6% across both blocks) were censored. We also censored vials in the event of male death (4 related familiar vials, 1 related unfamiliar vial,

1 unrelated familiar vial, 1 unrelated unfamiliar vial) or flies escaping during handling (1 related familiar vial, 1 related unfamiliar vial).

Fitness measures

Vials containing the offspring of experimental groups were reared at 25˚C for 16 days, allowing sufficient time for offspring to develop to pupal or adult stage, when they were then frozen. To account for different egg-to-adult development times between vials, we counted adult flies and pupae that had reached the P13 phanerocephalic pupal phase (Bainbridge and Bownes, 1981), identified by the black wing colour, and included both in offspring counts.

Statistical analysis

Survival models were performed using JMP (Cary, 2012). All other models were performed using the MASS package (Venables and Ripley, 2002) in R (R Core Team, 2014) using type III sums of squares to calculate P-values. For all analyses, we included block and all interaction terms that include block as fixed effects. In all cases, the interaction terms that include block were not significant (Supplementary Table 2.2), so we removed these terms from the models and kept block as a fixed main effect. Whilst we know which families contributed to the related familiar, related unfamiliar and unrelated unfamiliar treatments, our experimental design makes it impossible to know the family identities of flies in the unrelated familiar treatment. Since our knowledge of family identity is confounded with treatment, we were not able to include family identity in the full model analysis.

For aggression and courtship, we analysed the number of scans per day in which the behaviour was observed with a binomial penalised quasi-likelihood GLMM (Bolker *et al.*, 2009), with relatedness, familiarity and their interaction, and block as fixed effects and day within vial ID as nested random effects. For mating rate, we analysed whether or not a mating was observed for each day using a binomial penalised quasi-likelihood GLMM with relatedness, familiarity and their interaction, and block as fixed effects, and vial as a nested random effect.

For female reproductive success, we analysed the total number of offspring produced during the experiment. Only 27 of the 357 females were still reproducing at the end of the experiment, and short-term reproductive success is known to be a strong predictor of long-term reproductive success in this species (Nguyen and Moehring, 2015). Therefore, our measurements of reproductive success during the experiment can be considered a very close estimate of lifetime reproductive success. Vials where a male died before the death of the female were excluded from this analysis. We analysed lifetime reproductive success with a quasi-Poisson GLM with relatedness, familiarity and their interaction, and block as fixed effects. For female reproductive ageing, we divided the number of offspring laid in each time period by the number of days in that period to create an estimated daily offspring measure that accounts for the differing number of days in each time period. Vials where a male died before the death of the female were right censored in this analysis. We analysed daily offspring estimates with a Poisson penalised quasi-likelihood GLMM with relatedness, familiarity and day and their interactions, and block as fixed effects, and the vial ID as a random effect.

To estimate female reproductive lifespan, we used the last day of the last time period in which the female reproduced. We fitted proportional hazards models for female

lifespan and female reproductive lifespan, with relatedness, familiarity and their interaction and block as fixed effects. Vials were right-censored in the analysis when male death, male escape or the end of the experiment preceded female death or the end of reproduction.

Results

Male behaviour

The frequency of male-male aggression, courtship and mating were not significantly affected by male relatedness, larval environmental familiarity, or their interaction (Supplementary Tables 2.3 & 2.4)

Female harm

Female lifetime reproductive success was significantly increased by relatedness among male triplets (t_{349}=-2.1 P=0.034), but there was no significant effect of familiarity (t_{349}=-0.97, P=0.33), and no significant interaction (t_{349}=1.40, P=0.16; Figure 2.2). To further investigate the possibility of an interaction, we ran the same model on the familiar and unfamiliar halves of the dataset separately. Females had a higher lifetime reproductive success when housed with related familiar males than unrelated familiar males, but this effect was marginally non-significant (t_{184}=-1.9, P=0.053). However, there was no effect of relatedness when comparing the lifetime reproductive success of females exposed to related unfamiliar and unrelated unfamiliar males (t_{168}=-0.123, P=0.90).

There was a significant effect of the interaction between relatedness and day on female reproductive ageing (t_{895}=-3.14, P=0.0017) whereby the age-specific offspring production of females housed with unrelated male triplets declined faster than that of females housed with related male triplets. There was no significant interaction between familiarity and day (t_{895}=-0.74, P=0.46), nor between relatedness, familiarity and day (t_{895}=1.04, P=0.30), on daily offspring production (Supplementary Figure 2.1). Again, we ran the model on the familiar and unfamiliar datasets separately. When comparing familiar

treatments, the interaction between relatedness and day remained significant (t_{463}=-3.23, P=0.0013), with females ageing faster when housed with unrelated triplets. When comparing unfamiliar treatments, there was no significant interaction between relatedness and day (t_{432}=-1.53, P=0.13).

There was a significant interaction between relatedness and familiarity on female reproductive lifespan (Wald X^2=5.34, P=0.021; Figure 2.3), whereby females housed with related familiar males reproduced for longer than females housed with unrelated familiar males (Risk ratio = 1.37, P=0.041; Supplementary Table 2.5). The interaction between relatedness and familiarity also had a significant effect on female lifespan (X^2_1=4.76, P=0.029; Figure 2.3), with a marginally non-significant trend for females housed with related familiar males to live longer than those housed with unrelated familiar males (Risk ratio = 1.322, P=0.069; Supplementary Table 2.5).

Taken together, these results indicate that female harm is minimised when females are exposed to triplets of males that are both related and familiar to each other.

Figures

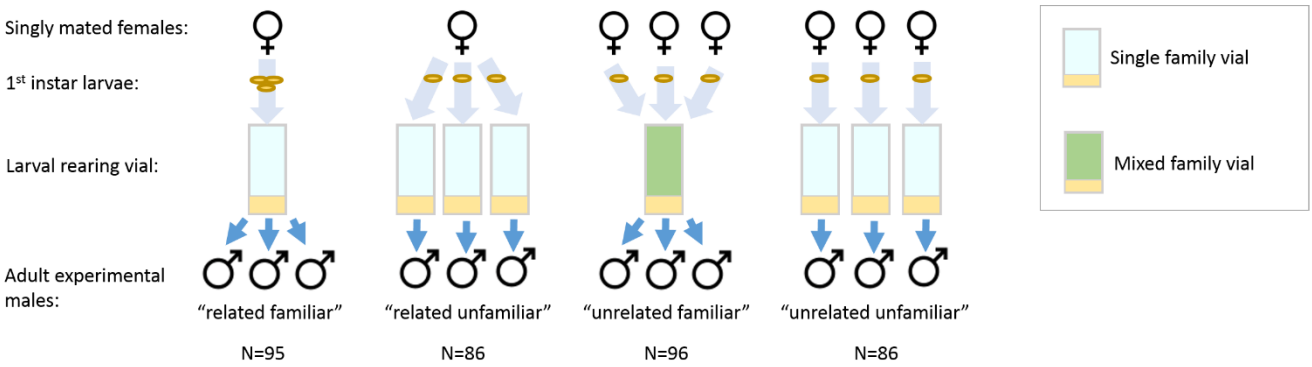


Figure 2.1 *Scheme of how we generated the four male treatments*. Each rearing vial contained 15 larvae, either all 15 from one singly mated female (single family vial) or one larva from each of 15 singly mated females (mixed family vial). We collected adult virgin males from these rearing vials, which were immediately housed in their experimental triplets; "related familiar", "related unfamiliar", "unrelated familiar" or "unrelated unfamiliar".

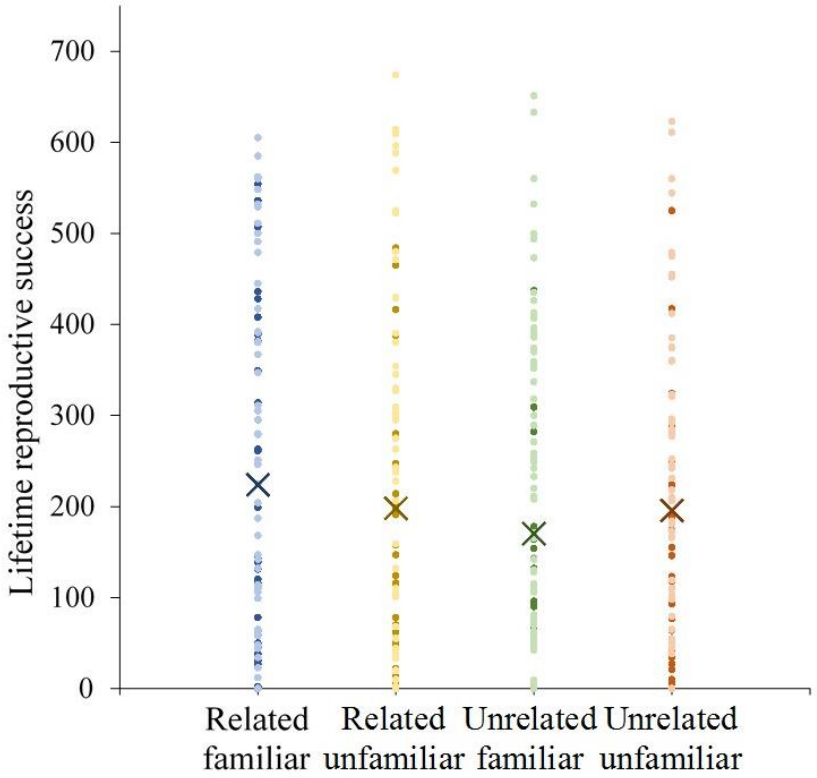


Figure 2.2: *The effect of male relatedness and larval familiarity on female lifetime reproductive success.* Points show the total number of offspring laid by each female during the experimental period that reached adult and P13 pupal stage from the first experimental block (dark points), the second experimental block (light points) and predictions from the generalised linear mixed model (crosses). Females mated to triplets of males that were related produced significantly more offspring than those mated to triplets of unrelated males ($P<0.05$). There was no difference in lifetime reproductive success between females mated to triplets of familiar and unfamiliar males ($P>0.05$)

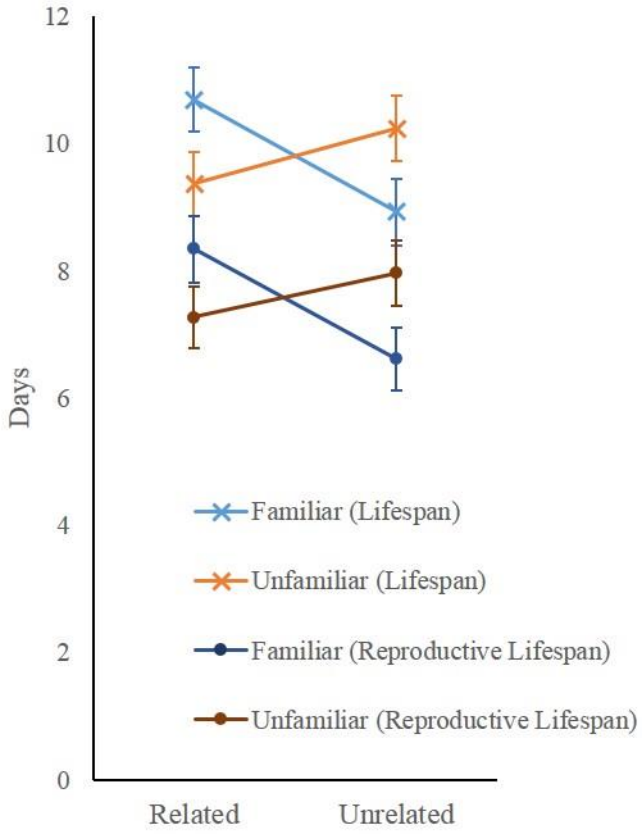


Figure 2.1: *The effect of male relatedness and larval familiarity on female lifespan and reproductive lifespan*. The mean number of days from the start of the experiment until the female died (lifespan; crosses) and the mean last day on which the female reproduced (reproductive lifespan; circles), with error bars representing ± one standard error. The last day of reproduction was estimated as the last day of the last time period in which the female reproduced. The interaction between relatedness and familiarity was significant for both lifespan and reproductive lifespan ($P<0.05$).

Discussion

The role of relatedness in sexual selection and sexual conflict has attracted increasing interest given the potential for indirect fitness effects in structured populations (Rankin, 2011; Faria, Varela and Gardner, 2015, 2017). An important challenge in this context is to disentangle the role of relatedness from that of social familiarity. Our results provide support to previous findings from some *D. melanogaster* populations, indicating that related familiar males are less harmful to females. Importantly, the use of a fully factorial design enables the present study to show that both genetic relatedness and familiarity during development are required for any mediation of male harm to females in our population of *D. melanogaster*.

The present study found that females housed with triplets of full-brothers show a small but significant increase in reproductive success and slower reproductive ageing than females housed with triplets of unrelated males. While there was no significant interaction between male relatedness and familiarity, this reduction in female harm due to relatedness was only apparent when comparing related and unrelated males in socially familiar treatments. Were familiarity not to play any role in mediating lifetime reproductive success, we would expect to see an effect of relatedness in both the familiar and unfamiliar halves of our data. Therefore, these results suggest that, despite the lack of a significant interaction, familiarity may play a role in mediating the effect of male relatedness on female lifetime reproductive success.

Consistent with this, male relatedness interacted with familiarity to affect both female reproductive lifespan and female lifespan: females both reproduced and survived for longer when housed with related familiar males than with unrelated familiar males,

whilst there was no significant difference attributable to relatedness in the unfamiliar treatments. The role of relatedness can be seen clearly in Figure 2.3: without an effect of an interaction between relatedness and familiarity, we would expect the lines to be flat (no effect of relatedness nor an interaction) or parallel (no interaction). The statistical significance of the effects above was generally weak, and thus some caution should be applied in their interpretation.

Similarly, neither male relatedness nor familiarity affected the rates of male-male aggression, courtship, or mating, which seems to contradict previous findings (Carazo *et al.*, 2014, 2015; Martin and Long, 2015). The most likely reason for this is that the experimental design of the present study prevented us from replacing the males at regular intervals as in previous studies (Carazo *et al.*, 2014; Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015; Martin and Long, 2015), and as such, we could not minimise the effects of co-ageing. Male co-ageing with the females is bound to underestimate differences in harm to females across treatments, because males in treatments where they are more harmful to females are also expected to age more quickly (and hence deteriorate faster). Elevated male ageing in high-harm treatments would tend to equalise the levels of female harm across treatments with time, and particularly so towards the end of their lifespan. Thus, our estimates of both general female harm levels and treatment differences are conservative.

Collectively, these results indicate that, at least in the population we studied, within-group male relatedness plays a role in modulating male harm of females, in consort with familiarity. For all measures of female harm, females experienced the least harm when exposed to males that were both related and familiar. This is consistent with the hypothesis that indirect fitness effects contribute to explain reduced female harm when

local male competitors are related to each other. For example, a focal male may be selected to invest less in competition with rival males, and be less harmful to females, if his rivals are more genetically related to him than to the population average and these females are likely to reproduce with his relatives. This is because of the indirect fitness the male would gain via the increased reproductive success of his male relatives, who would experience both less competition for fertilisations and have more fecund mates, and thus gain higher reproductive success. This would reduce both male-male competition and sexual conflict (Pizzari and Gardner, 2012). Males in our study appear capable of discriminating between individuals on the basis on kinship to adopt a less competitive and less harmful strategy with brothers and females respectively. Crucially, however, we now show that male flies can only do this when raised together as larvae.

These new results help reconcile those of previous studies. Specifically, Carazo *et al.* (2014) compared related familiar and unrelated unfamiliar treatments and emphasised the role of male relatedness. Subsequently, Hollis *et al.* (2015) added a related unfamiliar treatment, and by showing that related and unrelated males behave the same when unfamiliar to each other, they suggested that harm to females was driven by male-male familiarity. By using a fully-factorial experiment, we show that both previous studies capture different aspects of a complex social behaviour: male flies do adjust female harm in response to the relatedness of their rivals, but only under conditions of larval familiarity. The results of the present study therefore also shed light onto the proximate mechanisms of kin recognition in *D. melanogaster*. There is some evidence that female *D. melanogaster* preferentially mate with their own relatives (Loyau *et al.*, 2012; Robinson, Kennington and Simmons, 2012b; Tan *et al.*, 2012). In addition, there is evidence that both males and females recognise whether a new partner is related or unrelated to a previous

partner ('genetic familiarity') (Tan *et al.*, 2013). This latter result suggests that kin recognition has the potential to be at least partly based on genetic cues in this species.

Two possible, non-mutually exclusive mechanisms for kin recognition in *D. melanogaster* have been proposed; cuticular hydrocarbons (CHCs) (Ferveur, 2005; Tan *et al.*, 2013) and gut microbiota (Lizé, McKay and Lewis, 2014). CHCs have both a genetic and environmental component, and numerous insect species use CHCs to discriminate between kin (Blomquist and Bagnères, 2010). Furthermore CHCs can be modulated by gut microbiota (Lizé, McKay and Lewis, 2013), which are maternally transmitted to offspring via the egg and are also strongly influenced by diet (Chandler *et al.*, 2011).In our study, we separated larvae after 24-36 hours so it is still possible that individuals are using familiarity cues in this very early period of life, which we would detect as an effect of relatedness in this experimental set-up. This would be particularly true if flies were discriminating based on gut microbiota, as these are largely inherited from mothers via the egg casing (Bakula, 1969; Buchon, Broderick and Lemaitre, 2013). Our experimental design differs in this respect from previous studies (Carazo *et al.*, 2014, 2015; Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015; Martin and Long, 2015), which manipulated males at the egg, rather than larval, stage, thus reducing the possibility for maternal cues.

Kin recognition may be costly (Hepper, 2005), and both its evolution and maintenance require adaptive explanations, albeit these need not be the same. Our study population has been adapted to laboratory conditions of large, dense, confined populations for over 45 years; over 1000 generations. It is hence possible that this population has not been especially structured beyond the microscale; an unlikely scenario for the evolution of kin recognition.

In this context, two mechanisms may contribute to explain mounting evidence for kin recognition in laboratory-adapted *D. melanogaster* populations; one adaptive and one non-adaptive.

First, these responses may reflect a relic of a plastic behaviour evolved in natural populations. Whilst the initial evolution of this behavioural plasticity would have been presumably costly, the cost of maintaining a plastic response to kin under familiarity may be relatively low in laboratory populations where kin structure is expected to be limited. In this scenario, the evolution of kin recognition mechanisms would have been favoured by persistent population viscosity over multiple generations (Chippindale *et al.*, 2015), and natural *D. melanogaster* populations in which recognition would have originally evolved must have been structured such that males could expect to grow up with related and unrelated individuals and compete with familiar individuals as adults.

In the wild, *D. melanogaster* live in orchards, feeding and laying eggs on rotting fruit (Sokolowski, 1985). Little is known about population viscosity and the family-level genetic structure of wild populations. Females co-locate at oviposition sites (Markow and O'Grady 2008, although this is likely due to substrate texture not females actively seeking other larvae; Atkinson 1983; del Solar and Palomino 1966), and larvae disperse (Medina-Muñoz and Godoy-Herrera, 2005), both of which would potentially reduce the likelihood of stable kin interactions. However, larval foraging behaviour, pupation site and adult choice of resting site all have a strong genetic component (Sokolowski, 1985; de Belle and Sokolowski, 1987; Stamps *et al.*, 2005), and early adult habitat experiences shape later habitat preferences (Reaume and Sokolowski, 2006; Stamps and Blozis, 2006). Whilst there is little information on clutch sizes in wild populations, it is inferred from ovariole anatomy that females lay their eggs in small clutches (Atkinson, 1979), and indeed laboratory-reared

females decide on the site quality between each egg (Yang *et al.*, 2008). Small clutch sizes, rather than laying eggs individually, could lead to genetic structure in wild populations by increasing the relatedness among neighbours, increasing the probability that adult males encounter related familiar competitors. There is also evidence of some genetic structure in a wild population, where mating males and females are more related to each other than to the average fly in the population (Robinson, Kennington and Simmons, 2012a).

A particularly important stage for the initial evolution of kin recognition and reduced female harm is likely to be at the colonisation of a new patch. If a small number of females initially populate a new feeding site, the next generation will be small and will contain substantial variation in male relatedness. Any behaviours that increase female fecundity and male fitness at this stage of colonisation may have large, long lasting effects on the genetic distribution of the future population. While less relevant in established populations, which are larger and possibly less structured, kin recognition may be retained at relatively low costs as the relic of a highly successful strategy from the founding of the population. Another possibility is that fly populations may show some structure even in laboratory conditions. Flies are known to form non-random social networks even in small group sizes and small physical environments (Schneider, Dickinson and Levine, 2012), therefore it is possible that some laboratory populations show some degree of relevant micro-structure.

The second, non-adaptive mechanism that has recently been put forward to explain kin-biased sexual behaviour in flies is that, if individual levels of competitiveness (e.g. aggression and courtship) are at least partly heritable, triplets of related males are more likely to have similar levels of competitiveness than triplets of unrelated males. If males with similar levels of competitiveness competed less intensely than males with more

variable levels of competitiveness, this would produce the effect of related males competing less (Martin and Long, 2015). This explanation, however, seems counterintuitive. As expected by contest theory and supported by a wealth of data across different taxa, males tend to invest more in competition with rivals of similar competitive value (Enquist and Leimar, 1983; Hardy and Briffa, 2013), a result also replicated by Martin and Long (Martin and Long, 2015). Also, if female behaviour changes in response to the variability among males, such as being more receptive in the presence of three unrelated (and hence more genetically dissimilar) males (Billeter *et al.*, 2012), this might in turn trigger a proximate increase in male-male fighting and sexual harassment of females, leading to higher female harm.

Another proximate explanation for groups of brothers harming females less than groups of unrelated males might represent a cognitive error. It is possible that if *D. melanogaster* males use variability of smell, be that CHC profiles or gut microbiota, as a measure of how many males they are competing with, they may underestimate the number of competitors when they are related, i.e. smell similar. Thus, if a male is surrounded by brothers, he may assume there is less competition and behave less competitively, harming the female less (Pizzari, Biernaskie and Carazo, 2015).

Whilst our data show that groups of related familiar males are less harmful to females, we do not yet know whether this is mediated through pre- or post-copulatory effects. It is possible that there are post-copulatory differences between treatments for which we did not test. Male *D. melanogaster* are known to adjust the composition of their ejaculate according to the female's previous mating history and perceived competition (Wigby *et al.*, 2009; Sirot, Wolfner and Wigby, 2011; Perry, Sirot and Wigby, 2013). In

particular, we do not currently know if the levels of male accessory proteins transferred to the females differ between treatments.

The present study joins several others looking at the effect of relatedness on sexual behaviour in *D. melanogaster*, with some of the key findings of each study summarised in Supplementary Table 2.1. Each used a very similar experimental design, but different laboratory populations of *D. melanogaster*. The Dahomey population used in Carazo *et al*. (2014, 2015) and this study are the same. The three IV populations used in Hollis *et al*. (2015), Chippindale *et al*. (2015) and Martin and Long (2015), whilst nominally the same, have been reared in separate laboratories for several decades. Aside from genetic differences, the Dahomey and IV populations differ substantially in rearing conditions. The Dahomey population, as used in this study, is maintained in cages with large dense populations and overlapping generations which allows for selection to continue late in life. In contrast, the IV populations are maintained on a discrete 14-day generation culture cycle in vials at a controlled density of approximately 100 eggs per vial, which prevents selection acting beyond that time point. This difference in culturing conditions could potentially alter sexual conflict-mediated selection on female ageing in Dahomey *versus* IV populations. However, there have been no direct tests of this hypothesis. It will be important for future studies to explore, via the fully factorial design applied here, whether relatedness and familiarity among males similarly interact to affect female harm in the IV and other *D. melanogaster* populations.

More generally, one implication of these studies is that local relatedness among male competitors may represent a possible modulator of the 'sexual tragedy of the commons' and population viability. An important avenue of future research, therefore, will be to explore whether the ecology of *D. melanogaster* across different laboratory and wild

populations (e.g. fine-grained population structure) may be more or less conducive to kin-selected sexual cooperation.

Supplementary Table 2.1:

Summary of results from previous studies. The findings from the five previous studies on this topic and the findings from this study on male-male fighting rate, female lifetime reproductive success and female reproductive lifespan. NA means that trait was not observed. The three IV populations, whilst nominally the same, have been reared apart over several decades. The Dahomey populations are all the same population.

Study	*Fly population*	*Fighting rate*	*Lifetime reproductive success*	*Reproductive lifespan*
Carazo et al. *2014*	Dahomey	Lower in related familiar males than unrelated unfamiliar males	Higher in related familiar males than unrelated unfamiliar males	Longer in related familiar males than unrelated unfamiliar males
Hollis et al. *2015*	IV (a)	NA	Higher in related familiar males than both related unfamiliar males and unrelated unfamiliar males	No effect
Carazo et al. *2015*	Dahomey	Lower in related familiar males than unrelated unfamiliar males	NA	NA
Chippindale et al. *2015*	IV (b)	NA	No effect between related familiar males and unrelated unfamiliar males	No effect
Martin et al. *2015*	IV (c)	Lower in related familiar males than unrelated unfamiliar males	No effect between related familiar males and unrelated unfamiliar males	No effect
Le Page et al. *2017 (this study)*	Dahomey	No effect	Higher in related familiar males than unrelated familiar males	Longer in related familiar males than unrelated familiar males

SUPPLEMENTARY TABLE 2.2

Summary of block main effects and interactions including block. The experiment occurred over two blocks. When testing each measure of female harm and male behaviour, we included block and all possible interactions of block with the other main effects as a fixed effects. None of these interactions were significant (P<0.05) so were removed from the final model, and block was retained as a main effect. The values below for the interactions are from the full models including interactions, and the values for block as a main effect are from the final models excluding interactions.

Model	*Effect*	*Result*
Lifetime reproductive success	Relatedness*Block	t_{353}= 0.54, P=0.59
	Familiarity*Block	t_{353}= 1.01, P=0.28
	Relatedness*Familiarity*Block	t_{353}=-0.67, P=0.51
	Block	t_{353}= 4.37, P<0.01
Reproductive Ageing	Relatedness*Block	t_{355}=-0.89, P=0.38
	Familiarity*Block	t_{355}=-0.59, P=0.56
	Relatedness*Familiarity*Block	t_{355}= 0.83, P=0.41
	Relatedness*Day*Block	t_{891}= 1.61, P=0.11
	Familiarity*Day*Block	t_{891}= 1.53, P=0.13
	Relatedness*Familiarity*Day*Block	t_{891}=-1.29, P=0.20
	Block	t_{358}= 4.08, P<0.01
Female lifespan	Relatedness*Block	X^2_1= 0.70, P=0.40
	Familiarity*Block	X^2_1= 0.10, P=0.76
	Relatedness*Familiarity*Block	X^2_1= 0.05, P=0.83
	Block	X^2_1= 8.88, P<0.01
Female reproductive lifespan	Relatedness*Block	X^2_1= 1.02, P=0.31
	Familiarity*Block	X^2_1= 0.07, P=0.79
	Relatedness*Familiarity*Block	X^2_1= 0.39, P=0.53
	Block	X^2_1= 0.55, P=0.46
Male courtship	Relatedness*Block	t_{349}= 0.21, P=0.84
	Familiarity*Block	t_{349}=-0.31, P=0.76
	Relatedness*Familiarity*Block	t_{349}=-0.14, P=0.89
	Block	t_{352}= 2.02, P=0.04
Male aggression	Relatedness*Block	t_{349}= 1.66, P=0.10
	Familiarity*Block	t_{349}= 0.75, P=0.45
	Relatedness*Familiarity*Block	t_{349}=-1.43, P=0.15
	Block	t_{352}= 4.71, P<0.01
Mating	Relatedness*Block	t_{349}= 0.04, P=0.97
	Familiarity*Block	t_{349}=-0.77, P=0.44
	Relatedness*Familiarity*Block	t_{349}= 0.77, P=0.44
	Block	t_{352}=-3.16, P<0.01

Supplementary Table 2.3

Summary statistics of the frequency of behavioural observations. Aggression and courtship behaviours were measured as the number of scans per day in which we observed any males performing that behaviour in each vial. Numbers shown are the mean proportion of scans in which that behaviour was shown ± standard error. Mating was measured as whether a mating was observed that day in each vial, with 1 representing mating observed, and 0 representing no mating observed. Numbers shown are the means of these binary values ± standard error.

Male treatment	*Aggression*	*Courtship*	*Mating*
Related familiar	0.197±0.00788	0.497±0.0133	0.0942±0.0122
Related unfamiliar	0.204±0.00854	0.489±0.0148	0.0764±0.0123
Unrelated familiar	0.201±0.00746	0.534±0.0139	0.0866±0.0125
Unrelated unfamiliar	0.189±0.00759	0.529±0.0134	0.0900±0.0127

Supplementary Table 2.4

Summary of statistical results from binomial penalised quasi-likelihood GLMM of the frequency of behavioural observations. Aggression and courtship behaviours were measured as the number of scans per day in which we observed any males performing that behaviour in each vial. Mating was measured as whether a mating was observed that day in each vial.

Behaviour	*Factor*	*t*	*Degrees of freedom*	*P*
Aggression	Relatedness	0.01	352	0.99
	Familiarity	0.75	352	0.45
	Relatedness*Familiarity	-0.99	352	0.32
Courtship	Relatedness	1.26	352	0.21
	Familiarity	-0.35	352	0.73
	Relatedness*Familiarity	0.45	352	0.65
Mating	Relatedness	-0.01	352	0.99
	Familiarity	-0.78	352	0.43
	Relatedness*Familiarity	0.41	352	0.69

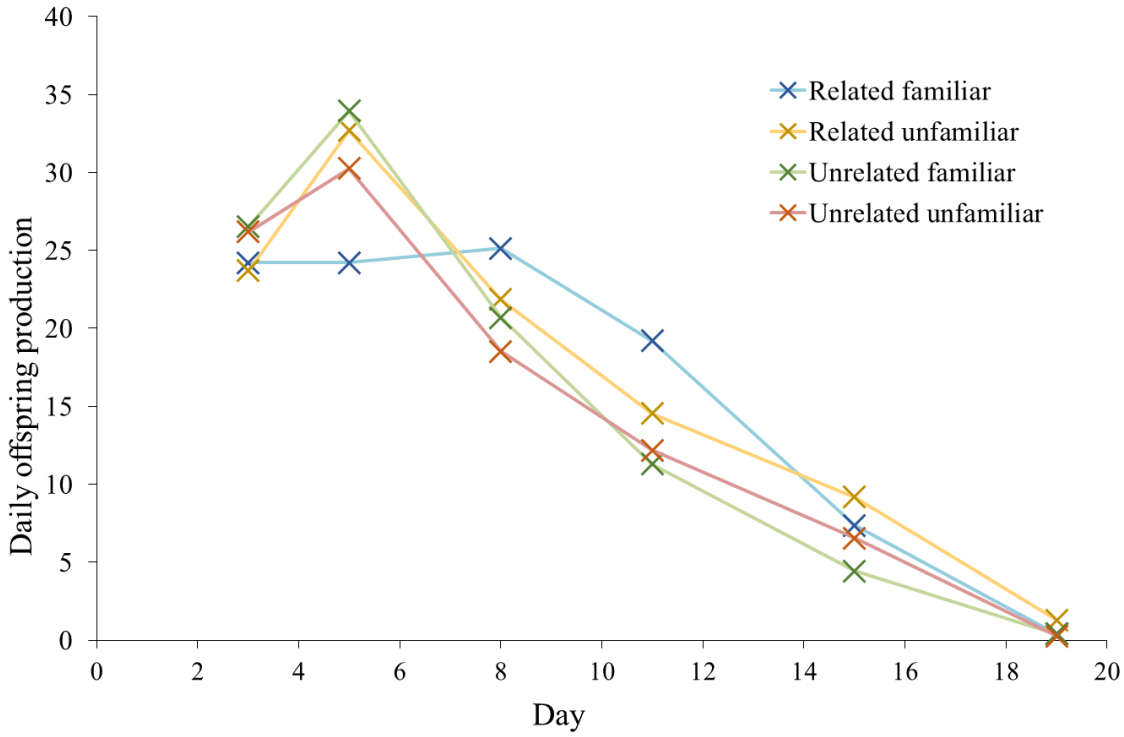
Supplementary Table 2.5

Risk ratios for female reproductive lifespan and lifespan. Reproductive lifespan was measured as the last day of the last time period in which the female reproduced. Numbers shown are the risk ratios between male treatments across both blocks with lower and upper 95% intervals in parentheses.

Ratio	*Reproductive Lifespan*	*P*	*Lifespan*	*P*
Related familiar/Related unfamiliar	0.757 (0.555,1.033)	0.0789	0.750 (0.550, 1.022)	0.0680
Related familiar/Unrelated familiar	0.730 (0.539, 0.988)	0.0414	0.755 (0.558, 1.022)	0.0687
Related familiar/Unrelated unfamiliar	0.912 (0.667, 1.248)	0.563	0.868 (0.637, 1.184)	0.372
Related unfamiliar/Unrelated familiar	0.964 (0.713, 1.301)	0.812	1.007 (0.745, 1.361)	0.962
Related unfamiliar/Unrelated unfamiliar	1.204 (0.881, 1.647)	0.244	1.158 (0.851, 1.576)	0.350
Unrelated familiar/Unrelated unfamiliar	1.249 (0.921, 1.697)	0.153	1.150 (0.850, 1.556)	0.365

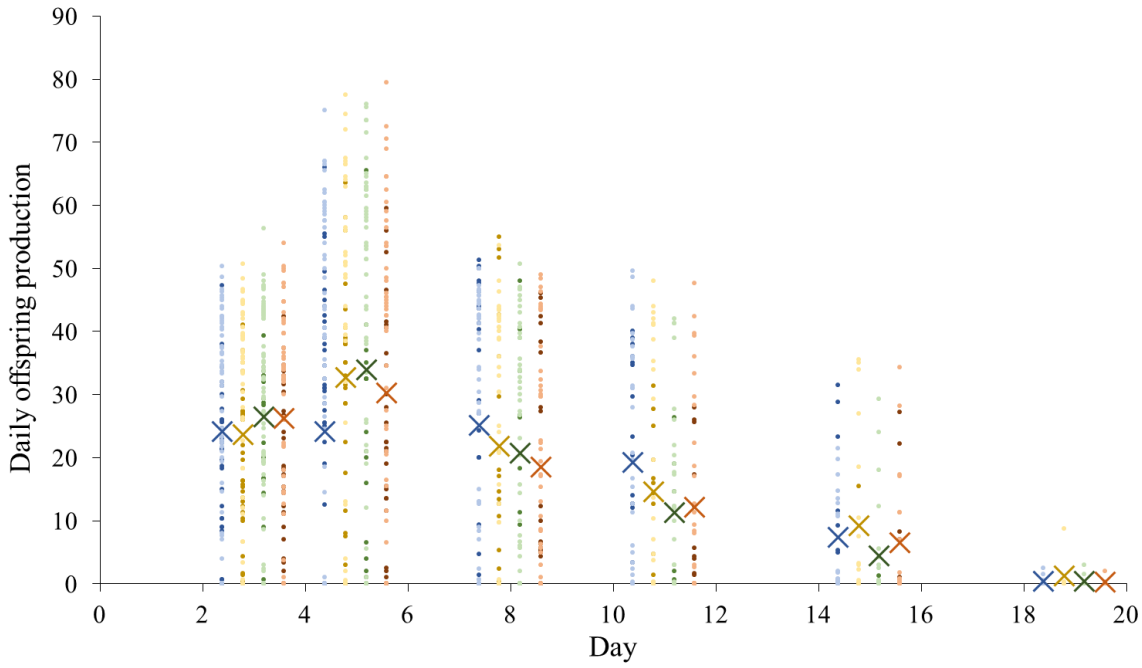
SUPPLEMENTARY FIGURE 2.1

The effect of male relatedness and larval familiarity on female reproduction over time. Points show the average number of offspring that reached adult and P13 pupal stage laid each day by each female during the experimental period from the first experimental block (dark points), the second experimental block (light points) and mean daily offspring production for each male treatment across both blocks (crosses, also shown in Supplementary Figure 2.2). Females mated to triplets of males that were related aged significantly slower than those mated to triplets of unrelated males ($P<0.05$). There was no difference in reproductive ageing between females mated to triplets of familiar and unfamiliar males ($P>0.05$)



SUPPLEMENTARY FIGURE 2.2

The effect of male relatedness and larval familiarity on female reproduction over time. The mean number of offspring that reached adult and P13 pupal stage laid each day by each female during the experimental period for each male treatment across both blocks. Standard error bars are not shown as the means are an average of two experimental blocks, and the distributions for each treatment and block can be found in the supplementary material. The more negative the gradient of the curve, the faster the reproductive ageing. Females mated to triplets of males that were related aged significantly slower than those mated to triplets of unrelated males ($P<0.05$). There was no difference in reproductive ageing between females mated to triplets of familiar and unfamiliar males ($P>0.05$)



CHAPTER 3:

UNRELATED MALES DO NOT RETAIN THEIR REPRODUCTIVE ADVANTAGE IN A SEQUENTIAL MATING SYSTEM

Abstract

When related males compete over reproductive access to females, sexual conflict can be reduced, increasing the fitness of both sexes, as compared to when competing males are unrelated (Rankin, 2011; Wild, Pizzari and West, 2011; Pizzari and Gardner, 2012). However, evidence suggests that the strategy of only competing amongst brothers may be evolutionarily unstable. For example, in *Drosophila melanogaster*, an unrelated male competing against two brothers sired twice as many offspring as either brother when all three males were simultaneously presented to the female (Carazo *et al.*, 2014). When males simultaneously compete over access to a female, it is difficult to disentangle the effects of male-male competition and cryptic female choice. By examining a sequential mating system, we remove direct male-male competition, and so can observe how female choice affects male fitness. In this study, I sequentially presented females with either two brothers and an unrelated male or three unrelated males in all three possible orders. I found no significant effect of relatedness on paternity share, male fitness, female fecundity or mating duration. Females were not faster to mate with related males as has been found in previous studies (Tan *et al.*, 2013). These results suggest that the unrelated male advantage may be weak or not be entirely under female control, or that males need to be present simultaneously allowing direct comparison or competition to take effect.

Introduction

Males and females differ in how they maximise their fitness during reproduction, generating sexual conflict (Chapman *et al.*, 2003; Parker, 2006). In polyandrous systems, males may potentially exploit females to invest more resources into their offspring to the detriment of the female's ability to invest in future offspring. Combining selfish male competition and sexual conflict creates a 'tragedy of the commons' scenario (Rankin and Kokko, 2006; Rankin, Dieckmann and Kokko, 2011), where male harassment reduces female fitness.

The harm caused by self-interested males can be reduced when competitors are related, for example brothers competing over access to a female. This situation is more likely to arise in viscous populations with low dispersal (Eldakar *et al.*, 2009; Wild, Pizzari and West, 2011; Pizzari and Gardner, 2012), since the average relatedness to a neighbour will be higher than that to a member of the wider population. Here we need to take a gene-centric, Hamiltonian approach and consider not only the direct but also the indirect fitness consequences of social behaviours (Hamilton, 1964). When competing among brothers, a male not only gains fitness through his own offspring, but also in part through the offspring of his brothers, thus being selfish at the genetic level may mean being cooperative at the individual level. A male taking a 'sexual altruist' strategy could therefore be successful when competing against relatives by being less aggressive and losing direct fitness but thereby gaining indirect fitness for a net fitness gain (Pizzari and Gardner, 2012). Males may often not be able to directly choose their competitors, but could alter the time or investment spent courting a particular female depending on the relatedness of the males around him.

In *Drosophila melanogaster*, both males and females mate multiple times (Imhof *et al.*, 1998), and natural populations have limited dispersal (McInnis, Schaffer and Mettler, 1982), creating the opportunity for interactions with relatives (Robinson, Kennington and Simmons, 2012a). Males use accessory gland proteins in the ejaculate to manipulate female behaviour, increasing her investment in his offspring and reducing her life expectancy (Wigby and Chapman, 2005; Fricke *et al.*, 2009). Females store sperm between matings in spermatheca, and can preferentially use sperm for example based on sperm tail length (Amitin and Pitnick, 2007). There is strong last-male sperm precedence, with roughly 80% of stored sperm from previous matings being displaced by sperm from the most recent mating (Manier, Belote and Berben, 2010).

Carazo *et al*. (2014) experimentally altered the relatedness between three male mates of female *D. melanogaster*, all unrelated to the female. They found that, as predicted by theory (Rankin, 2011; Wild, Pizzari and West, 2011; Pizzari and Gardner, 2012), when a female is presented simultaneously with three brothers (AAA), the female had a longer reproductive lifespan and greater lifetime reproductive success than when she is presented simultaneously with three unrelated males (ABC). Furthermore, the males in the AAA treatment fought less with each other and lived longer than the males in the ABC treatment. This supports the prediction that increasing the relatedness in the group reduces the conflict and increases the reproductive success of the individuals within the group.

Carazo *et al*. (2014) then simultaneously presented females with a group of three males unrelated to her, but this time consisting of two brothers and one unrelated male (AAB). In short behavioural assays during the experiment, A males were just as likely to fight with their brother as with the unrelated B male, and there was no difference in

courting or mating rates between males, but the B male sired on average twice as many offspring as either A male. However, the mechanisms of this effect were not elucidated: we do not know whether the differences are driven by female cryptic choice, or male behaviour.

It is important to consider the effect of male relatedness in a sequential mating system, as well as in a simultaneous one. In nature, the AAB scenario is likely to be common in populations with high, but not complete, male viscosity, as is a sequential mating scenario when females are not able to directly compare their mates. In this situation, the males are prevented from directly interacting with each other, removing any differential effect of male fighting or female harassment. The female is unable to choose either with whom she mates or the order in which she mates, but is able to affect the time between matings and differentially adjust which sperm are used.

To determine the evolutionary dynamics of the system and calculate whether sexual altruism is evolutionarily unstable, we must first establish whether the direct fitness advantage to unrelated males holds in a sequential system, and that this advantage is dependent on the relatedness of the competitors, and not other factors such as the 'rare male advantage' (Knoppien, 1985), or a female equivalent of the 'Coolidge effect' (Wilson, Kuehn and Beach, 1963) where males prefer to mate with unfamiliar females. Subsequently, we can determine whether this effect is driven by female choice or by males adopting a sexual altruist strategy.

The goal of the present study was to begin to elucidate the mechanisms by which this unrelated male advantage occurs, specifically by testing whether the effect remains when the female is presented with the three AAB males sequentially rather than simultaneously. The male triplets comprise either two full sibling A males and an unrelated

B male, or two unrelated A males and an unrelated B male. In a sequential system, males are restricted in their ability to detect or respond to their competitive environment. Whilst males use cuticular hydrocarbons as sexual cues with which they show kin discrimination (Robinson, Kennington and Simmons, 2012a, 2012b) it is unlikely, though not impossible, that they can identify cuticular hydrocarbons transferred from previous males onto the female, especially as cuticular signals have a strong environmental component (Tan *et al.*, 2013). Therefore, we can assume that there is little direct male-male competition in a sequential mating environment, and any effects of relatedness are likely due to cryptic female choice.

Predictions

- Since the B male outcompeted A males in a simultaneous system with no observed difference in fighting, courting or mating rates (Carazo *et al.*, 2014), the female may have been preferentially using sperm from the B male. As female effects are maintained in a sequential mating system, I predict that the B male will have higher fitness and paternity share when competing against a pair of brothers (full siblings of each other but unrelated to the focal B male) than against unrelated males.
- I predict females mated to two brothers and an unrelated male will have higher fecundity than females mated to three unrelated males, as kin selection theory predicts increasing male-male relatedness should reduce intrasexual competition, lessening sexual conflict and reducing female harm.
- Based on previous findings by Tan *et al*. (2013), I would predict females to remate faster with males related to her previous mates than with unrelated males.

Methods

Fly stocks and culturing

I used *Drosophila melanogaster* from laboratory-adapted stocks that had been maintained at 25℃ with a 12:12 light-dark cycle and overlapping generations (Partridge and Farquhar, 1983). Flies were from one of two stocks; either a wild-type Dahomey stock (wt, in which flies have a smooth red eye surface) which has been maintained outbred since 1970, or a homozygous recessive mutant, *sparkling poliert* (*spa*, in which flies have a rough red eye surface), backcrossed into the wild-type Dahomey population for at least five generations. I maintained the flies in either plastic vials or bottles containing standard Lewis medium (Lewis, 1960) with excess live yeast.

To produce the parents of the male triplets, I collected eggs from both wt and *spa* stocks and reared them in bottles at a standard density of ~200 eggs per bottle. I collected virgins within eight hours of eclosion under ice anaesthesia, separated them into single sex vials at a density of ten females or 20 males per vial and aged them for 5-8 days.

To produce the experimental males, I randomly paired a male and female of the same genotype in fresh vials for 5-7h, after which the male parents were discarded and the females left to oviposit for a further 17-19h. These eggs were left to develop for 9-10 days, at which point newly eclosed virgin males from each family were collected every six hours for 36h. I reared 6-8 full sibling brothers per vial, which were aged for four days prior to the start of the experiment. This means related males had been reared in a familiar larval environment, whereas unrelated males were unfamiliar, consistent with Carazo *et al*. (2014). I discarded families producing fewer than three males as this suggests the female

had an unusually low fecundity. I put individual males into vials with fresh yeast 12h before their use in the experiment.

To produce the experimental females, I collected eggs from the *spa* stock and pipetted them into bottles at a standard density of ~200 eggs per bottle(Clancy and Kennington, 2001), and reared them for 9-10 days. I collected newly eclosed virgin females every six hours for 36h, and put them in vials at a density of ten females per vial and aged them for four days before start of experiment.

Experimental male triplets

Females were sequentially mated with three males over a six-day period. Each triplet of males contained two males of the same eye genotype ('A'), and one male of a different genotype ('B'). The experimental treatments differed in the relatedness of the two A males; either two full siblings or two unrelated males (of the same genotype).

I randomly assigned male families as generating either A or B males, and either contributing to 'related triplets' (where two of the three males are brothers) or 'control triplets' (where all three males are unrelated). The males were sequentially presented to the females in all three possible orders (AAB, ABA, BAA). To account for any differences

Order	*rare wt*	*rare* spa
AAB	spa spa wt	wt wt *spa*
ABA	spa wt spa	wt *spa* wt
BAA	wt spa spa	*spa* wt wt

Table 3.1: Combination of male triplet orders and B male genotypes used. Males were either wild-type (wt) or *sparkling poliert* (*spa*). In addition to these six combinations, the A males were either full siblings (related) or randomly selected from the population (unrelated). The B male was always unrelated, and all three males were unrelated to the female.

between genotypes, half of the triplets had *spa* as the 'B genotype', the other half, wt (Table 3.1). Since comparisons between orders would not be made, families contributed up to six males to the overall experiment, but within any one order, families only contributed either one or two males.

Behavioural observations

I aspirated each of 294 females, without anaesthesia, into a vial containing the first male within 30 minutes of lights on, and observed pairs every five minutes to record the start and end time of mating. I assigned females a random identification number that was visible during the experiment so that the observations were performed blind.

On the first day when all flies were virgins, observations stopped after 3h when all but one pair (which was discarded) had mated. In the subsequent days, I observed pairs every five minutes for 10h before separating any unmated pairs overnight. Once a mating had ended, I immediately discarded the male and left the female in the vial to lay eggs until the following morning.

For females that didn't mate, I aspirated the male into a fresh vial in the evening then aspirated the female into the vial containing the same male the following morning. Females were given up to three days to mate with the second male, and up to four days to mate with the third male, with all experiments ending after six days. I excluded from the experiment any females that did not successfully mate with all three males in this period (N=108). I discarded females that died during the seven days of experiments or escaped while being transferred between vials and recorded them as censored on the day of death (N=5). I immediately transferred females after they had mated with the third male to a fresh vial to lay eggs for 24h. These offspring were allowed to develop into adults for 13-14

days before they were frozen and I counted the number of offspring in each vial, using eye phenotype to detect paternity.

I excluded females that produced a very small total number of offspring (fewer than 20, given mean total number of offspring = 69.7, SE=2.31) from the analysis. I treated third matings with males that sired an unexpectedly low number of offspring given an expected 80% last-male sperm precedence (proportion of offspring sired by B less than 0.5 for AAB given mean=0.937, SE=0.01, greater than 0.5 for ABA given mean=0.129, SE=0.02, greater than 0.2 for BAA given mean=0.006, SE=0.002) as unsuccessful matings and discarded. This produced a final sample size of 100 females/male triplets: 32 AAB (15 related, 17 unrelated), 32 ABA (15 related, 17 unrelated), 36 BAA (15 related, 21 unrelated); with 47 rare wt B genotype, 53 rare spa B genotype.

Analysis

I calculated mating duration as the end time of the mating - start time of the mating in minutes. I calculated latency to mating as start time of the mating - end time of the previous mating in minutes. It is important to note that unlike other definitions (e.g. Tan *et al*. 2012), this time includes the overnight periods when the flies were separated and unable to mate. This overnight period will affect sperm competition because the female will continue to fertilise her eggs, depleting the sperm stored in the spermatheca. I analysed each order of male triplets separately, performing linear models in R v. 3.5.1 (R Core Team, 2014) and survival models in JMP 14.0.0 (Cary, 2012). Alpha was set as P = 0.05.

I analysed each trait in a separate model with B genotype, triplet relatedness and their interaction as the explanatory variables. Where the interaction was not significant, I

removed it from the model. For mating duration, I used a linear model to analyse the duration of the second and third matings for the AAB order, whereas for the ABA and BAA orders, I only analysed the duration of the third mating as any effect of triplet relatedness would only occur after the female has been presented with both A males. For paternity share, I used a quasibinomial general linear model, and for male fitness and female fecundity, I used a quasipoisson general linear model, to account for the overdispersion present in a model with binomial and poisson distributions respectively. To test for differences in latency to remating, I ran a proportional hazards survival analysis on latency to the second mating for the AAB order, and latency to the third mating for all orders.

Results

Mating duration

There was no significant difference in the mating durations analysed between triplets with two full siblings and an unrelated B male (related triplet) and two unrelated males and a third unrelated B male (unrelated triplet), nor a significant effect of the B male's genotype or the interaction between B genotype and relatedness (Table 3.2, Figures 3.1 and 3.2).

Latency to remating

In the AAB order, there was no significant effect of triplet relatedness on the latency to second mating (Table 3.2, Figure 3.3). Similarly, females did not significantly discriminate between triplet relatedness in the latency to third mating for any order (Table 3.2, Figure 3.4). The genotype of the B male did not affect latency to the second or third mating for the AAB and BAA orders but had a significant effect on the latency to third mating for the ABA (χ^2_1=6.93, p=0.0085), with females mating faster with the third male if the male genotypes were wt, *spa*, wt rather than *spa*, wt, *spa*.

Paternity share

There was no significant effect of the relatedness of the A males on the paternity share of the B male after the third mating for any order, nor B genotype, nor their interaction (Table 3.2, Figure 3.5).

Male fitness

For all orders, there was no significant difference in the number of offspring sired by the B male after the third mating between related triplets and unrelated triplets, nor between B genotypes, nor their interaction (Table 3.2, Figure 3.6).

Female fecundity

Female fecundity after the third mating was not affected in any order by triplet relatedness or B genotype (Table 3.2, Figure 3.7).

Analysis	Order	Variable	Test statistic	P value
Second mating duration	AAB	Relatedness	$F_{1, 26}$=0.175	0.680
		B genotype	$F_{1, 26}$=0.880	0.357
		Interaction	$F_{1, 26}$=0.153	0.700
Third mating duration	AAB	Relatedness	$F_{1, 28}$=2.89	0.100
		B genotype	$F_{1, 28}$=2.13	0.156
		Interaction	$F_{1, 28}$=0.0946	0.761
	ABA	Relatedness	$F_{1,28}$=0.546	0.466
		B genotype	$F_{1,28}$=0.0195	0.890
		Interaction	$F_{1,28}$=0.234	0.633
	BAA	Relatedness	$F_{1,31}$=0.655	0.424
		B genotype	$F_{1,31}$=0.118	0.733
		Interaction	$F_{1,31}$=2.93	0.0967
Latency to second mating	AAB	Relatedness	χ^2_1 =0.930	0.335
		B genotype	χ^2_1 =2.42	0.120
Latency to third mating	AAB	Relatedness	χ^2_1=0.0640	0.800
		B genotype	χ^2_1 =3.27	0.0689
	ABA	Relatedness	χ^2_1=0.664	0.415
		B genotype	χ^2_1=6.93	0.0085*
	BAA	Relatedness	χ^2_1 = 3.19	0.0739
		B genotype	χ^2_1 = 2.15	0.143
Paternity share of B male after the third mating	AAB	Relatedness	$Dev_{1,28}$=153	0.0965
		B genotype	$Dev_{1,29}$=169	0.0691
		Interaction	$Dev_{1,27}$=0.388	0.799
	ABA	Relatedness	$Dev_{1,28}$=20.3	0.197
		B genotype	$Dev_{1,29}$=20.3	0.214
		Interaction	$Dev_{1,27}$=36.1	0.0853
	BAA	Relatedness	$Dev_{1,33}$=2.20	0.282
		B genotype	$Dev_{1,32}$=1.51	0.373
		Interaction	$Dev_{1,31}$=1.52	0.371
Male fitness after the third mating	AAB	Relatedness	$Dev_{1,28}$=3.43	0.515
		B genotype	$Dev_{1,29}$=8.68	0.301
		Interaction	$Dev_{1,27}$=14.6	0.179
	ABA	Relatedness	$Dev_{1,28}$=15.2	0.297
		B genotype	$Dev_{1,29}$=24.5	0.185
		Interaction	$Dev_{1,27}$=38.1	0.0981
	BAA	Relatedness	$Dev_{1,32}$=1.44	0.420
		B genotype	$Dev_{1,33}$=2.79	0.261
		Interaction	$Dev_{1,31}$=1.32	0.440
Female fecundity after the third mating	AAB	Relatedness	$Dev_{1,28}$=0.671	0.762
		B genotype	$Dev_{1,29}$=16.6	0.13
	ABA	Relatedness	$Dev_{1,28}$=1.50	0.660
		B genotype	$Dev_{1,29}$=4.30	0.458
	BAA	Relatedness	$Dev_{1,32}$=0.904	0.737
		B genotype	$Dev_{1,33}$=6.054	0.385

Table 3.2: Full list of model results. Neither the relatedness of the A males nor the genotype of the rare B male had an effect on any of the traits measured, except for the latency to the third mating, where the female was faster to remate with the third male if the male genotypes were wt, *spa*, wt rather than *spa*, wt, *spa*.

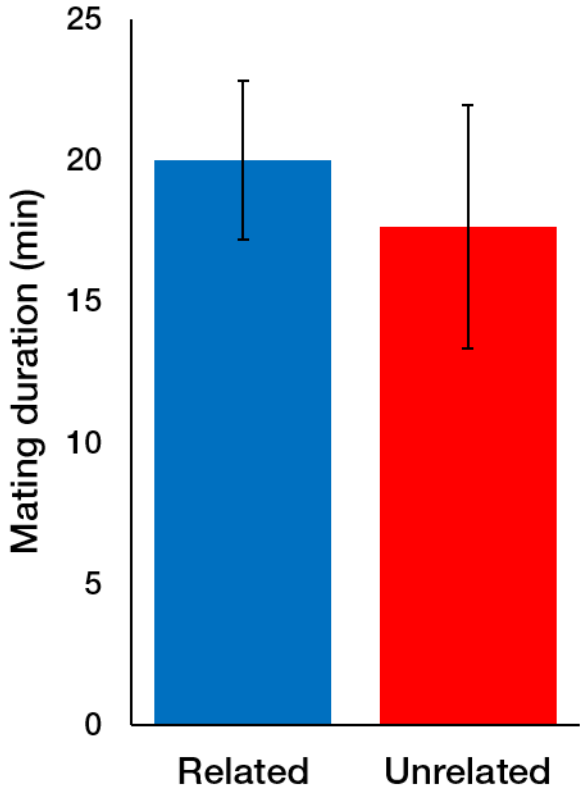


Figure 5.1: Relatedness had no effect on the duration of the second mating in the AAB order. A female presented with the brother of her previous mate mated for just as long as a female presented with an unrelated male of the same genotype as her previous mate. Sample size: 15 related, 17 unrelated, bars represent mean ± one standard error.

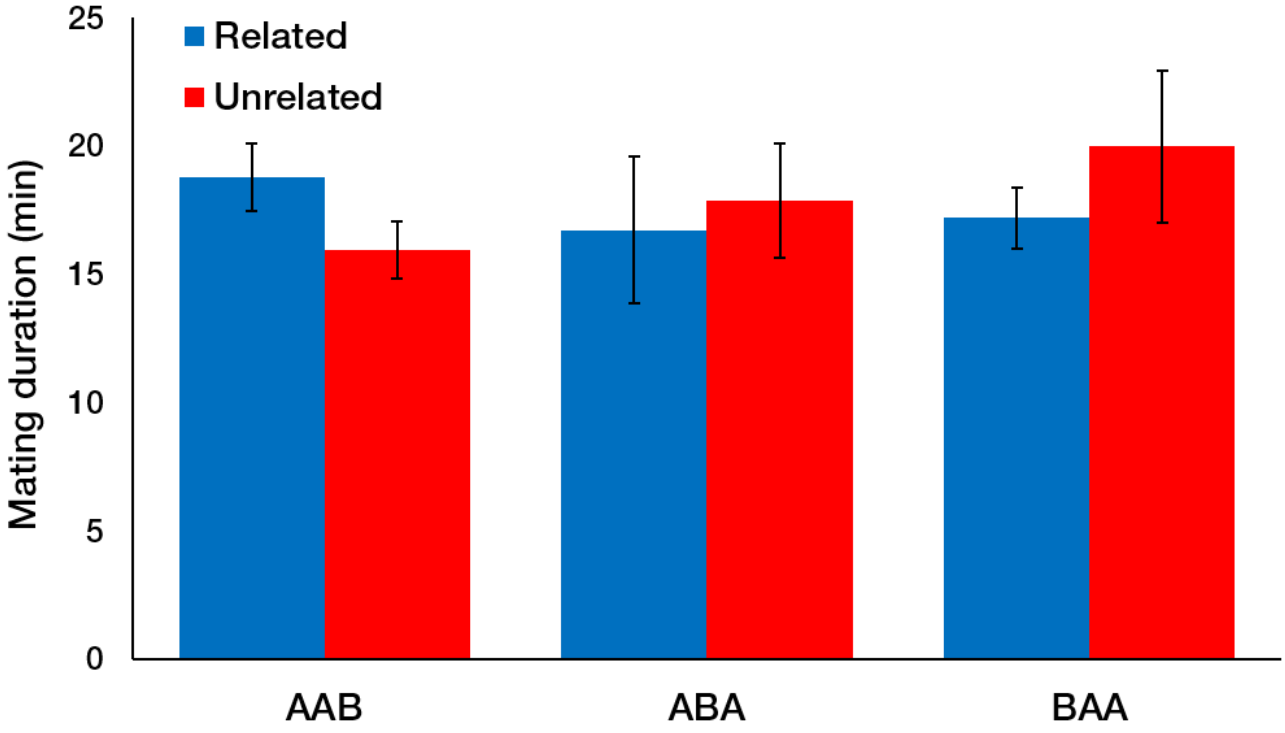


Figure 3.2: Relatedness had no effect on the duration of the third mating in any order. When presented with either an unrelated male and two males that were related to each other but not the female (related) or three unrelated males (unrelated), there was no difference in the duration of the mating with the third male, regardless of the order in which these males were presented. Sample size: AAB - 15 related, 17 unrelated; ABA - 15 related, 17 unrelated; BAA - 15 related, 21 unrelated, bars represent mean ± one standard error.

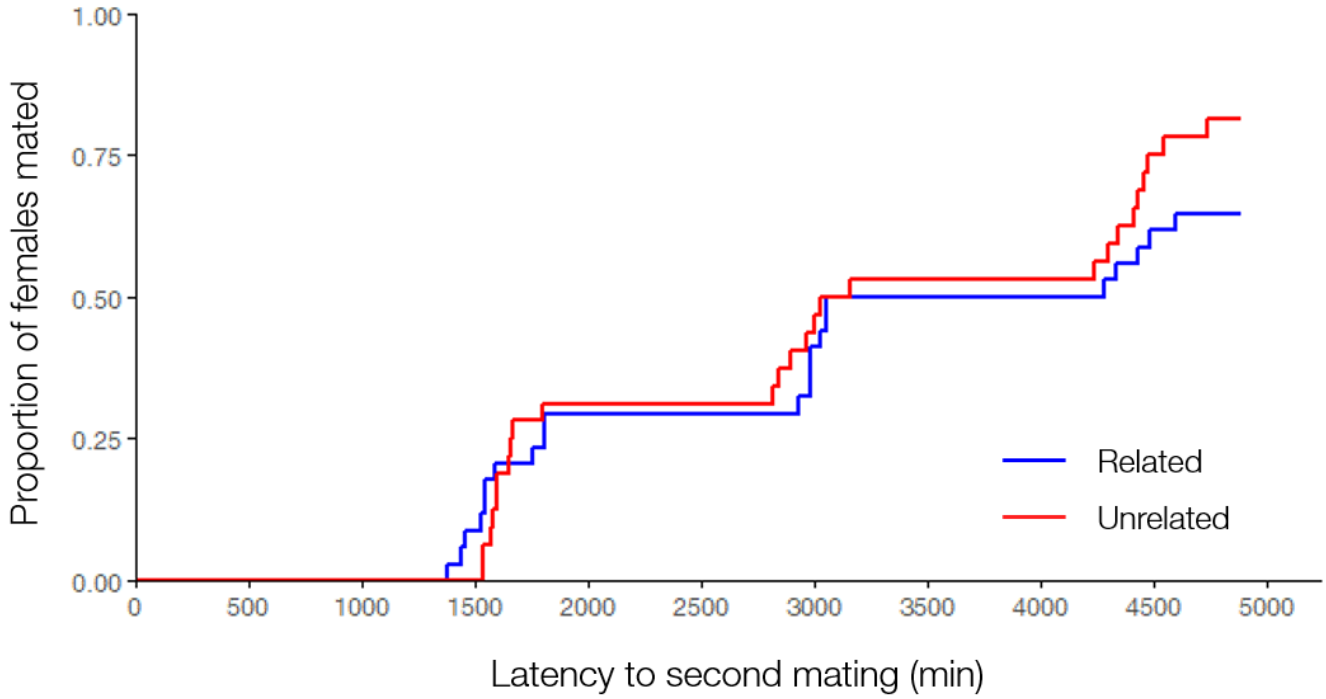
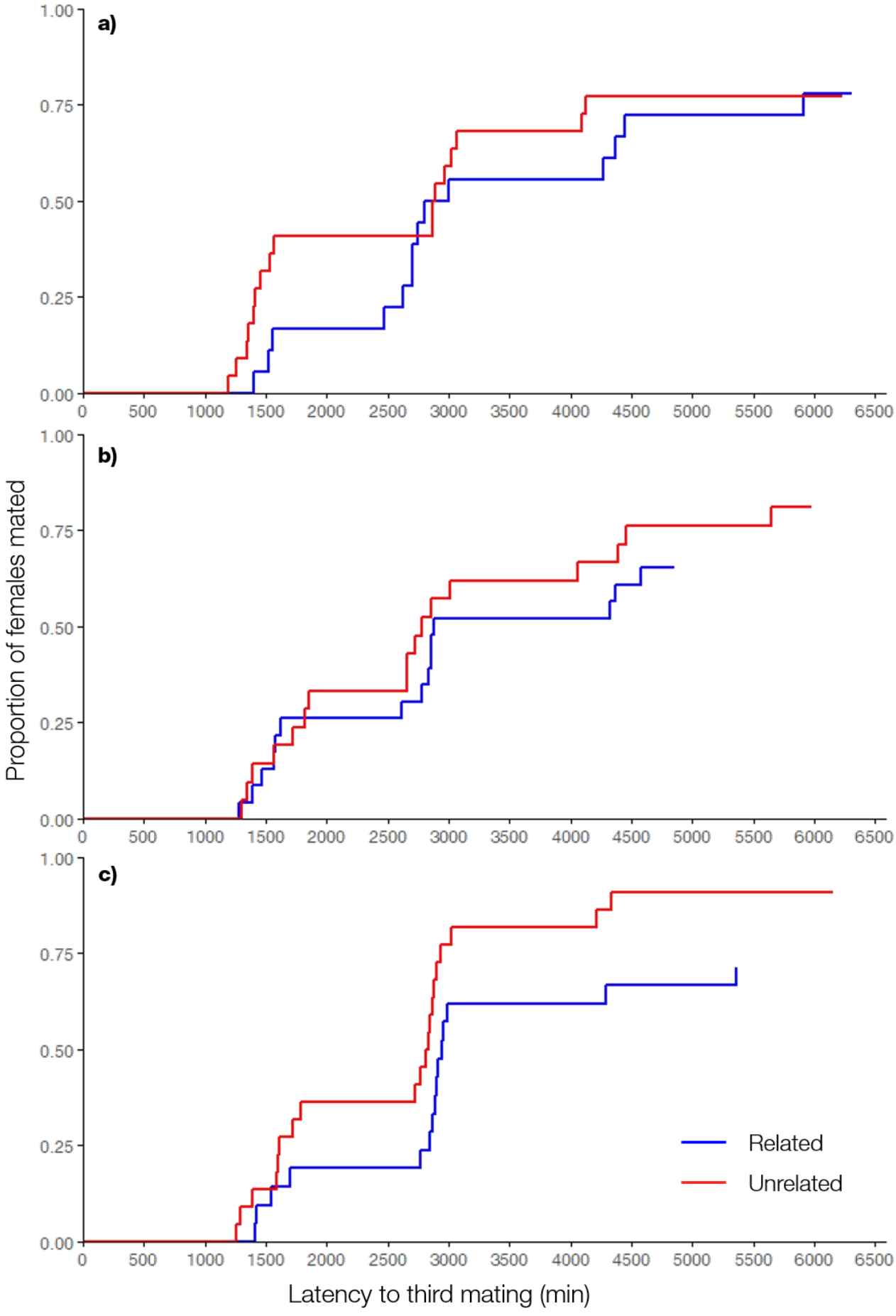


Figure 3.3: Relatedness had no effect on the time between the first and second mating in the AAB order. Females were equally fast to mate with the brother of her previous mate (blue) as with an unrelated male of the same genotype (red). Lines represent the cumulative proportion of females mated over time. Females that did not mate with the second male during the period of the experiment were included as right-censored data. For the related triplets (blue), n=22,12, for the unrelated triplets (red) n=26,6 (mated, censored).

Figure 3.4 (overleaf): Relatedness had no effect on the time between the second and third matings in any order. Females were just as fast to mate with the third male in a triplet containing two brothers (blue) as in a triplet containing all unrelated males (red). Females that did not mate with the third male during the period of the experiment were included as right-censored data. Lines represent the cumulative proportion of females mated over time in the **a)** AAB order, related n=14,4, unrelated n=16,5 **b)** ABA order, related n=15,8, unrelated n=17,4 **c)** BAA order, related n=15,6, unrelated 20,2 (sample sizes presented as number mated, number censored).


a)
b)
c)
Proportion of females mated
Latency to third mating (min)
Related
Unrelated
1.00
0.75
0.50
0.25
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500
6000
6500

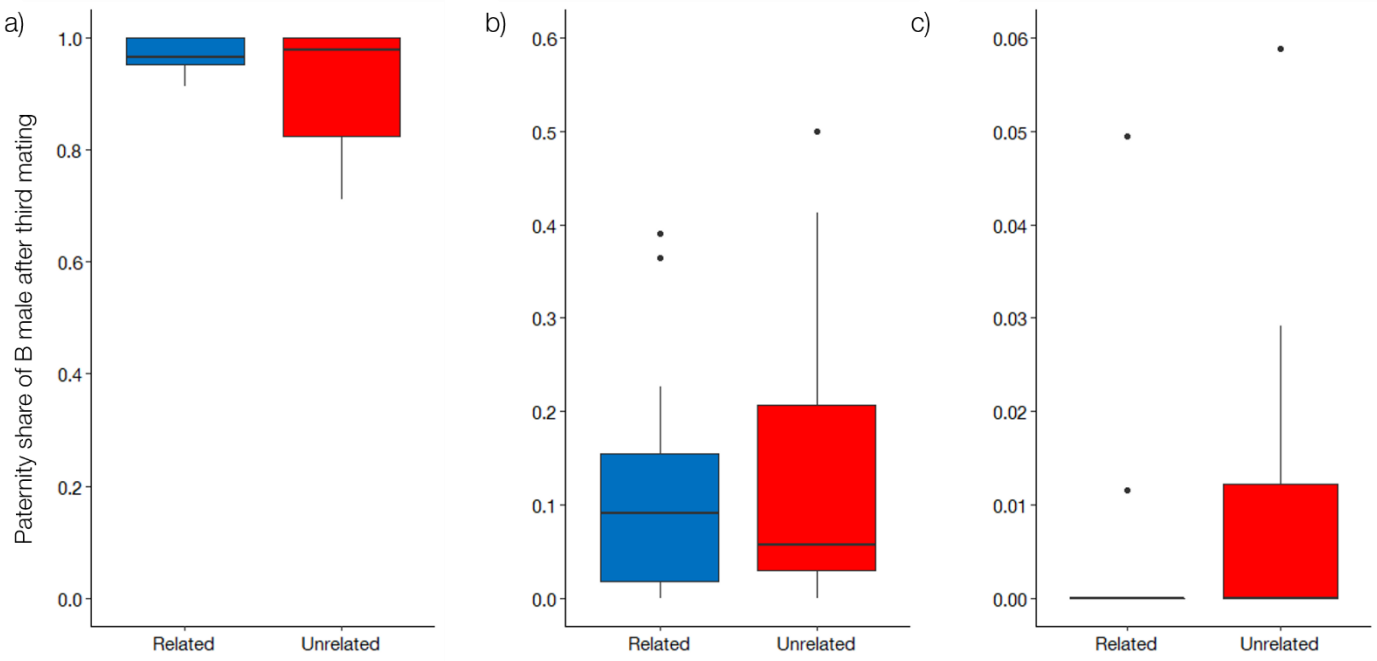


Figure 3.5: Relatedness had no effect on the paternity share of the B male after the third mating in any order. In triplets containing two brothers and an unrelated male (related) or triplets containing all unrelated males (unrelated), the unrelated B male sired the same proportion of adult offspring after the third mating in the **a)** AAB order, **b)** ABA order, **c)** BAA order. Sample size: AAB - 15 related, 17 unrelated; ABA - 15 related, 17 unrelated; BAA - 15 related, 21 unrelated.

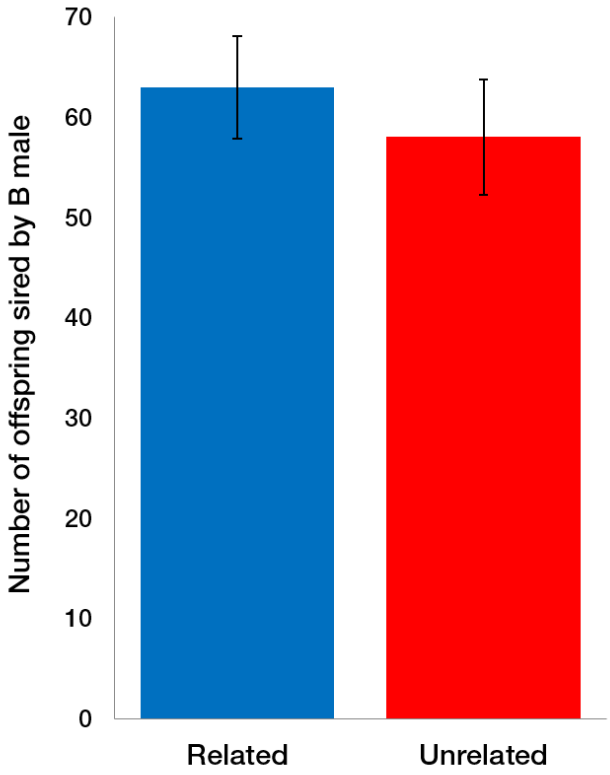


Figure 3.6: Relatedness had no effect on the number of offspring sired by the B male after the third mating in the AAB order. A B male mated to a female who had previously mated two brothers (related) sired the same number of adult offspring as a B male mated to a female who had previously mated two unrelated males (unrelated). There was similarly no effect of relatedness in either the ABA or BAA orders, where the number of offspring sired by the B male after the third mating were much lower. Sample size: 15 related, 17 unrelated, bars represent mean ± one standard error.

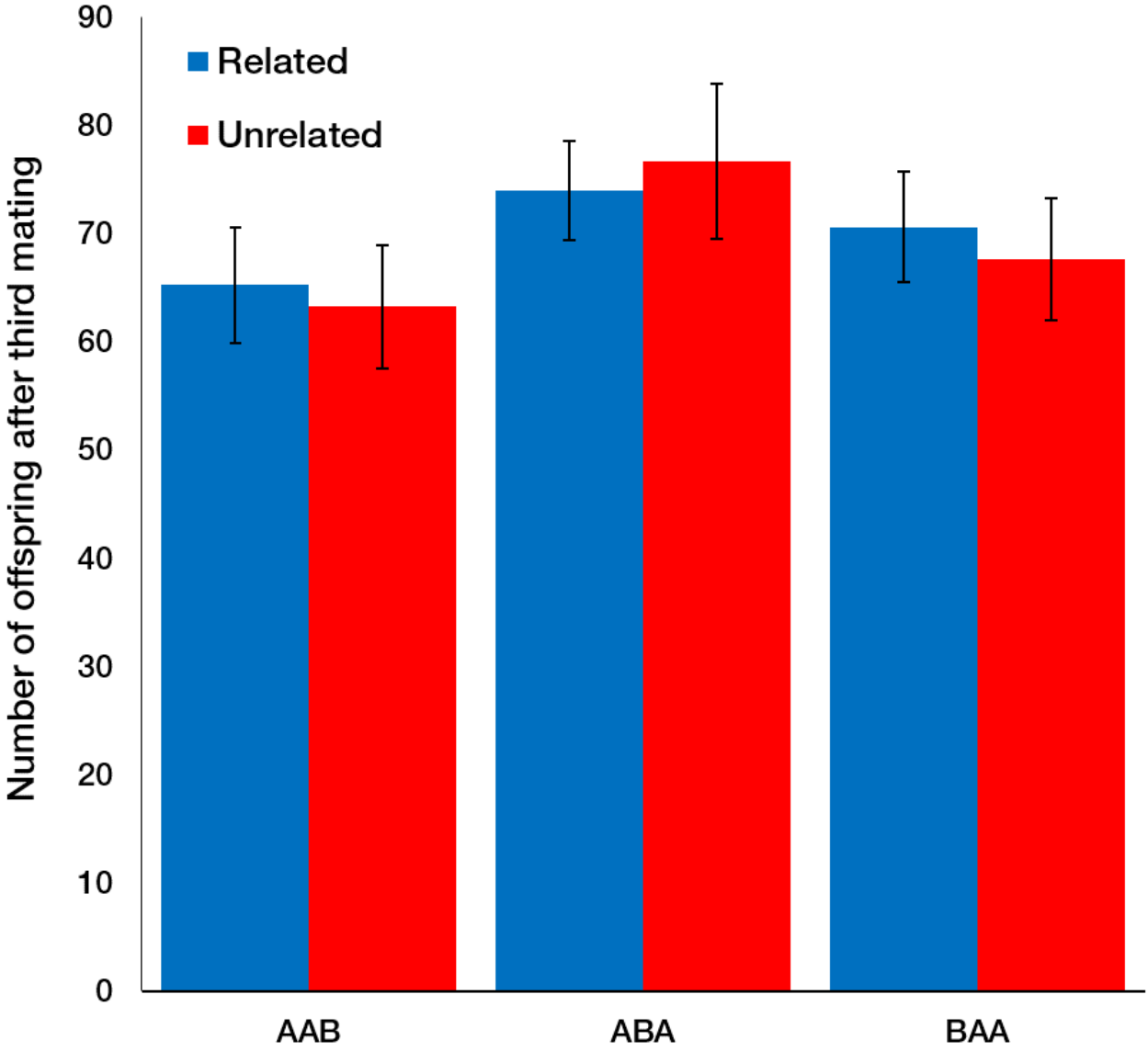


Figure 3.7: Females fecundity after the third mating was not affected by the relatedness of her mates in any order. After mating with either triplets containing two brothers and an unrelated male (related) or triplets containing all unrelated males (unrelated), females produced the same number of adult offspring, regardless of the order in which the three males were presented to her. Sample size: AAB - 15 related, 17 unrelated; ABA - 15 related, 17 unrelated; BAA - 15 related, 21 unrelated, bars represent mean ± one standard error.

Discussion

Previous work had found that an unrelated male competing against two brothers over an unrelated female had a strong reproductive advantage (Carazo *et al.*, 2014). To test whether this advantage holds when the males are presented to the female sequentially rather than simultaneously, I presented females with triplets of males in the orders AAB, ABA and BAA, where the A males were either two brothers or two unrelated males of the same genotype, and the B male was of a different genotype. In this sequential system, the relatedness of the A males did not affect the duration of matings, the latency to rematings, the B male's share of offspring sired after the third mating, the number of offspring sired by the B male after the third mating, nor the total number of offspring produced by the female after the third mating.

Are females capable of discriminating in a sequential system?

Tan *et al*. (2013)demonstrated that when presented with two individuals of the opposite sex, male *D. melanogaster* prefer to court females that are novel both in terms of the male's sexual experience with that female and his sexual experience with similar phenotypes, which is in line with the Coolidge effect. In contrast, females favoured males that were related to their previous mate. This female preference for familiarity is in part surprising because it contradicts the 'rare male effect', where males of a rare genotype experience greater mating success. One explanation for neophobia in females is that by mating with phenotypically similar males, females may be indirectly increasing the relatedness between her mates and thus reducing the harm caused to herself.

In contrast, there was no evidence from the present study that females were faster to mate with related males. For example, when presented with only the first two males in the AAB order, females were no faster to mate with the brother of her previous mate than with an unrelated male of the same genotype (Figure 3.3). Nor did the relatedness of previous partners affect the speed of remating to a third male (Figure 3.4). To alter her mating behaviour based on relatedness, the female must have some mechanism of kin discrimination, whether this be 'true' kin discrimination based on genetic qualities, or 'indirect' kin discrimination based on other signals such as familiarity cues. The absence of an effect of relatedness suggests that the females may either 'choose' not to discriminate based on relatedness or may be unable to, e.g. females may need to directly compare the males at the same time to be able to discriminate between them or may not be able to 'remember' previous mates.

Female *D. melanogaster* have been shown to alter their mate preferences based on previous mates, with the size of mates being remembered for a day after exposure (Dukas, 2005). Females also display mate copying, where they are more willing to mate with males they have seen with other females. Mate copying based on visual cues has been detected for three days after exposure to good and poor condition males (Mery *et al.*, 2009), although such memory has also been associated with a reduction in fitness (Mery and Kawecki, 2003; Burns, Foucaud and Mery, 2011). The related and unrelated A males in the AAB order were presented to the female only a day apart, which based on previous evidence, should not prove prohibitively demanding.

It is important to note that the daily remating rates of females were lower than in previous studies for unknown reasons, generating large variation in latency to mating. This could potentially mask any effect of relatedness on latency to mating and increase the

level of sperm depletion between matings as the female continues to use old sperm, potentially altering her behaviour.

Is the unrelated male advantage mediated by male-male effects?

Whilst there was clearly last-male sperm precedence (Figure 3.5), the unrelated B male sired neither more offspring nor a greater proportion of the female's offspring when competing against two unrelated males versus two brothers. This would indicate that the female is not biasing fertilisations based on relatedness in this sequential system, nor is the B male stimulating her fecundity differentially between treatments. It could be the case that the female needs all males present simultaneously to discriminate, but it could also be that either the unrelated male effect is explained by pre-copulatory dynamics or that it is not under female control. The experimental design removed direct competition between males, as they are both unable to fight and unable to assess their competitors, therefore if the effect is under male, not female, control then we would not expect to see an effect when the males are unaware of their reproductive environment.

Whilst Carazo *et al*. (2014) found that in a simultaneous AAB mating system, A males were equally likely to fight with their brother as with the B male, and the courtship and mating rates did not differ between the A and B males, these results were based on observations from only the first three hours of each day for the first three days of the experiment. Furthermore, they were not compared against a group with two unrelated A males and a B male of a second genotype. It is possible, therefore, that male-male interactions do differ in a simultaneous system; for example, the A males may have tired more rapidly over the full course of the experiment than the B males.

One limitation of this study is that only offspring after the third mating were counted, and it would be important to consider paternity over the lifetime of the female before coming to conclusions about the relative fitnesses of the males. Male ejaculate compounds can stimulate short term fecundity in females and alter her latency to remating (Fricke *et al.*, 2009; Perry, Sirot and Wigby, 2013). When examined alone, each may not show a strong response to relatedness, but when summed over the course of a female's lifetime, may make a significant difference in the male's lifetime reproductive success. However, given the magnitude of the reproductive advantage seen by Carazo *et al*. (2014), it is unlikely that we would not have seen a difference in paternity share and latency to remating in our sequential system were the reproductive advantage to hold.

Implications

The reproductive advantage previously seen for an unrelated male simultaneously competing against two brothers could have been mediated by male-male interactions, or male-female interactions, or a combination of the two. In our sequential system, we removed male-male interactions, and subsequently saw no reproductive advantage for the unrelated male. This would suggest that it is male-male interactions driving the effect, or that any differential female behaviour requires all males to be present simultaneously.

This raises the question as to the evolutionary stability of sexual altruism. Both males and females benefit from males competing within a triplet of three siblings versus a triplet of three unrelated males, with indirect inclusive fitness benefits mitigating the tragedy of the commons. However, males benefit from being an unrelated male in a triplet with two other brothers. As the present study suggests that any advantage is likely due to

male effects, this may undermine the evolutionary stability of a male strategy to compete against relatives, as brothers are vulnerable to competition from unrelated males.

It is important to further elucidate the mechanisms underpinning the unrelated male advantage to identify the nuances of this aspect of sexual conflict. Since individual recognition in *D. melanogaster* is largely an olfactory cue, it may be possible to expose a male to the scent of a group of competitors of different relatednesses. This would potentially allow the male to perceive his level of competition and adjust his behaviour towards the female and ejaculate composition accordingly, while removing any effects of male-male aggression. Whilst using a simple laboratory setup for these experiments provides a useful tractable system, the simplicity of the environment reduces the costs of courtship and mating for males and in doing so can increase sexual conflict (MacPherson *et al.*, 2018). Repeating the simultaneous mating experiment in a slightly more complex environment might illuminate other important variables mediating the unrelated male advantage.

CHAPTER 4:

LARVAE DEVELOP FASTER AND HAVE HIGHER SURVIVAL RATES IN UNRELATED GROUPS

Abstract

Intraspecific competition for food has been well studied in *Drosophila melanogaster*. As larval crowding and competition for food increases, development time increases, survival rates decrease and adult body mass decreases. Kin selection theory suggests the possibility of reduced competition among related groups of larvae, whereas niche partitioning suggests the possibility of reduced competition among diverse groups of larvae. I compared the development of groups of 15 full siblings or 15 non-siblings from egg to adult in vials with three different quantities of food. I found that the pupal and adult development time was shorter in unrelated groups than in related groups, survival rates were higher in unrelated groups, particularly at the lowest level of competition, but there was no difference in male or female adult body mass between the groups. These observations lend support to the 'elbow-room' hypothesis, where more genetically diverse groups have more diverse foraging strategies so are better able to exploit the food resource, thus lowering competition in the group.

Introduction

Food is essential for survival. In the absence of food, animals die. Intraspecific competition for food has been well studied in a myriad diverse taxa, in particular focussing on strategies that can increase an individual's competitiveness (Davies, Krebs and West, 2012). This may be through a more efficient searching technique (e.g. Pyke *et al*. 1977), or better adapted feeding anatomy (e.g. Christiansen and Wroe 2007), or increased aggression towards rivals for the same food patch (e.g. Isbell 1991). As the density of individuals on a patch of food increases and the resource becomes more crowded, we typically see increased competition and subsequently reduced fitness, borne out through lower survival rates, reduced growth and reduced resistance to physiological stress or disease.

Much of the research into crowding and competition for food has assumed that individuals are competing against unrelated rivals. In this scenario, the fitness of the individual depends solely on its ability to exploit the resource. However, this changes when the individual is competing against relatives. When competition occurs among relatives, an individual can gain fitness indirectly through the offspring of its competitors, which may lead to more complex strategies such as restraint from competition, or cooperation (Hamilton, 1964). When crowding on a food patch occurs between relatives, we might therefore expect to see less competition than with an unrelated group, if the costs to the individual of reduced competitiveness are outweighed by the increase in fitness to the relatives.

A contrasting hypothesis is niche partitioning, or the 'elbow-room' hypothesis (Young, 1981). This proposes that more functionally diverse groups will be able better to

exploit a resource as each individual has a slightly different niche, thereby reducing the direct competition between individuals. If the increased genetic diversity in unrelated groups leads to increased functional diversity in foraging strategies, we might expect to see increased fitness in unrelated groups, rather than in related groups.

The responses to crowding and larval competition are extremely well characterised in *Drosophila melanogaster* (Lewontin 1955; Miller and Thomas 1958; see Ashburner *et al*. 2005 for further references). Larvae at very high densities take longer to develop, have a more variable development time, have lower larval and pupal survival, grow to a smaller adult body size, have a more variable body size and have lower reproductive success. These crowding effects are primarily caused by the reduced concentration of yeast rather than the lack of space per se (Klepsatel, Procházka and Gáliková, 2018), but are also mediated by the build up of toxic metabolites in the substrate, in particular urea (Joshi, Knight and Mueller, 1996) and ammonia (Roy, Aditya and Ghosh, 2018), and by increased cannibalism (Vijendravarma, Narasimha and Kawecki, 2013; Ahmad *et al.*, 2015).

However, at lower densities, larvae can benefit from increased crowding (Wertheim *et al.*, 2002; Lihoreau *et al.*, 2016). Larvae use aggregation pheromones (Mast *et al.*, 2014) that allow clusters of larvae to more efficiently tunnel down below the surface of a liquid substrate . The churning activity of many larvae feeding in a substrate not only alters the abiotic state of the food (Wertheim *et al.*, 2005) but the biotic too, increasing the number of beneficial micro-organisms and suppressing the growth of competitive fungi and bacteria that also compete for the same substrate (Wertheim *et al.*, 2002; Golden and Dukas, 2014).

Crowding is thought to be common in wild populations of *D. melanogaster* (Mueller, 1985; Prasad and Joshi, 2003), and is near ubiquitous among laboratory

populations. Experimental evolution experiments have shown that there are multiple, heritable adaptations to crowding, such as increased development time traded off against reduced feeding rate and smaller body size (Rajamani *et al.*, 2006; Vijendravarma, Narasimha and Kawecki, 2012). However, we know less about plastic responses to crowding, in particular how competition for food changes with the relatedness of the group. Here I test whether the well known developmental responses to larval competition for food are affected by the composition of the group, using equal sized groups of either full siblings or unrelated larvae at three levels of competition. This will shed light on whether larvae are fitter in related groups, as predicted by kin selection, or in unrelated groups, as predicted by the elbow-room hypothesis.

Predictions

- Kin selection theory predicts that related groups will compete less because competition between siblings will harm the individual's indirect fitness. Therefore, at a given quantity of food, I predict larvae in related groups will have higher survival rates, shorter development times and higher adult body mass than larvae in unrelated groups, as a result of lower competition.
- A less competitive strategy between relatives will be most influential at the highest level of competition. I predict that the difference between related and unrelated groups will therefore be most apparent at the smallest quantity of food.

Methods

Fly population

I used a laboratory adapted, wild-type Dahomey stock of *Drosophila melanogaster*, maintained in large, outbred populations since 1970 at 25°C and a 12:12 light:dark schedule. All flies were maintained in cages containing bottles of Lewis medium with overlapping generations.

Experimental flies

To generate the experimental flies, I created families using parents that were two days post-eclosion, and had been collected as eggs from the stock population and reared at a standard larval density of approximately 150 eggs per bottle at 25°C.

To produce full siblings, I paired a single virgin male and female at lights-on for 12h in individual larval collection chambers containing a Petri dish filled with hard grape agar (550 ml water, 25 g agar, 300 ml grape juice concentrate and 21.25 ml 10%w/v Nipagin) spread with a thin film of live yeast paste (150µl per dish of 20g dried yeast, 40ml water), before discarding both parents. I kept the plates with eggs at 20°C overnight.

The next morning (Day 1, 24h after pairing), I picked eggs with a mounted needle into the treatment vials, collecting 30 eggs in total per family. Any families that failed to produce 30 eggs were excluded.

Competition treatments

To create the three different competition treatments, I added 8ml of firm plain agar (5g agar, 20ml Nipagin, 800ml water) to each 36ml vial to provide moisture, and then syringed either 1000µl, 100 µl or 50 µl of live yeast paste (20g dried yeast, 30ml water) in a

layer on top of the agar to form the 'low', 'high' and 'very high' competition vials respectively. To each vial, I then added 15 eggs, either all from the same family (related) or one from each of 15 different families (unrelated).

I performed each competition treatment in a separate block so that I had sufficient eggs laid in a short time period to use the same families for the related and unrelated treatments within each competition treatment. The families were randomised so that each family contributed to the same number of related vials (1 vial per family) and unrelated vials (15 vials per family), and no single family contributed more than one egg to the same unrelated vial. Therefore, each family was equally represented in the related and unrelated vials within a single treatment, but different families were used for each treatment. The sample sizes were 99, 100 and 84 for the low, high and very high competition treatments respectively.

Measuring development

All experimental vials were kept at 25°C and 70% humidity. In preliminary trials at 35% humidity, young larvae commonly desiccated whilst crawling up the walls of the vials, particularly in the very high competition vials, presumably in search of an alternative food source. Increasing the humidity reduced the number of deaths from desiccation.

I counted the number of flies that had pupated and eclosed daily, aspirating adults from their experimental vials to fresh vials for storage before freezing at -18C. For the low competition vials on day 9 (after all of the larvae had pupated but before they had eclosed) I additionally added approximately 1g of agar powder to form a thin layer on the surface of the yeast in each vial, to prevent adults from drowning in the remaining yeast paste. This

was not necessary for the higher competition vials as the smaller volume of yeast paste added meant the surface was more solid.

I froze the adults on day 17, day 27 and day 80 for the low, high and very high competition vials respectively, and censored any adults that emerged after these days. After freezing, I counted the number of adult males and females from each vial, and weighed the wet mass for each sex, providing a mean wet mass for males and females for each vial. For vials with large variation in development times, it was difficult to determine when the experimental generation ended and the offspring of the experimental generation began. I examined the daily number of pupations and eclosions, and used days 11, 15 and 18 as the cut-off point for pupations, and days 14, 17 and 20 as the cut-off point for eclosions for the low, high and very high competition treatments respectively.

Statistical analysis

I performed all models in R (R Core Team, 2014) with an alpha value of P=0.05. I removed any vials with 0 adults weighed (1 vial removed from the very high competition treatment) or more than 15 adults weighed (1 vial removed from the low competition treatment) from all analyses. I removed any vials with more total adults recorded than total pupae from the development time and survival analyses (9 vials removed, 3 from each treatment). As each competition treatment was performed several weeks apart using different families, it is impossible to extricate the effects of competition for food and any differences between experimental blocks. However, as the effects of competition for food on fly development are well characterised, I am only interested in whether these known effects remain constant when we change the group composition from unrelated to related. Therefore I analysed all treatments together, and when the interaction between

relatedness and the level of competition was significant, I performed the same analysis on each competition treatment separately to highlight which treatment was driving the effect. The results of the separate analyses for each variable are provided in the supplementary results.

I used a linear model to test whether the mean wet mass of adult males and females was affected by the relatedness of the vial. To analyse egg-to-pupae, egg-to-adult and pupae-to-adult survival, I ran generalised linear models, measuring the effect of relatedness on the number of flies from the later stage as a proportion of the number of flies from the earlier stage. I used a quasibinomial error structure in this model, as using a binomial error structure produced a high dispersion parameter, whereas a quasibinomial distribution can better account for overdispersion. I used an F-test of equality of variances to test for differences in the variance of survival and adult body mass. To test for differences in the development time, I ran a Cox proportional hazards mixed-effects model on the number of new pupae or adults developing each day, with relatedness as a fixed effect and the vial as a random effect.

Results

Survival

Egg to Pupae

Across all treatments, there was an interaction between the level of competition and relatedness on how many individuals survived to pupation ($F_{2, 268}$ = 8.40, P=0.00029), but there was no significant main effect of relatedness ($F_{1, 270}$ = 2.049, P = 0.154).

In the low competition (1000µl food per vial) treatment, more individuals survived to pupation in the unrelated vials than related vials (Odds ratio = 0.851, $F_{1,94}$ = 12.8, P=0.000547, Figure 4.1a), but there was no difference in the variance in survival between vials ($F_{42/53}$ = 1.58, P=0.117). In the high competition (100µl food per vial) treatment, relatedness had no effect on survival to pupation ($F_{1,95}$ = 1.18, P=0.280) and related and unrelated vials had equal variance ($F_{42/53}$ = 1.58, P=0.117). In the very high competition (50µl food per vial) treatment, relatedness had no effect on the proportion of individuals surviving to pupation ($F_{1,79}$=0.102, P=0.750), but the variance in survival was significantly greater in the related vials ($F_{41/38}$ =2.63, P=0.00329).

Egg to Adult

There was both a significant main effect of relatedness (Odds ratio = 0.114, $F_{1, 270}$= 6.534, P = 0.0111) and interaction between relatedness and competition ($F_{2, 268}$ = 5.01, P = 0.00728) on the survival from egg to eclosion, with higher survival in unrelated groups.

In the low competition treatment, more individuals survived to eclosion in the unrelated vials than related vials (Odds ratio = 0.851, $F_{1,94}$ = 11.2, P = 0.00119, Figure 4.1b),

and the variance in survival was higher in the related vials ($F_{46/48}$ = 5.80, P<0.001). In the high competition treatment, there was no difference in survival between the related and unrelated vials ($F_{1,95}$ = 0.0338, P=0.855) nor any difference in the variance ($F_{42/53}$ = 1.29, P=0.373). In the very high competition treatment, relatedness had no effect on survival to eclosion ($F_{1,79}$ = 0.712, P=0.400) but the variance in survival was higher in the related vials ($F_{41/38}$ = 2.03, P=0.0300).

Pupae to Adult

More individuals survived from pupation to eclosion in the unrelated vials than related vials when all treatments were combined (Odds ratio = 0.544, $F_{1,270}$ = 6.11, P = 0.0141, Figure 4.1c), but the interaction between relatedness and level of competition was not significant ($F_{2, 268}$ = 1.18, P=0.310).

Development Time

Pupation

Eggs developed into pupae more quickly in unrelated vials than in related vials (Hazard ratio = 1.33, z = 2.88, P=0.004, Figure 4.2) and there was no interaction between relatedness and competition (X^2=2.00, P=0.366).

Eclosion

Eggs developed to eclosion more quickly in unrelated vials (Hazard ratio = 1.125, z=2.05, P=0.040, Figure 4.3) and there was no interaction between relatedness and competition (X^2=1.30, P=0.523).

Adult body mass

Relatedness had no significant effect on the wet body mass of adult males ($F_{1,274}$ = 2.34, P = 0.127, Figure 4.4a) nor adult females ($F_{1, 272}$=1.82, P=0.178, Figure 4.4b). There was no interaction between relatedness and competition for males ($F_{2,274}$=2.22, P=0.110) or females ($F_{2, 272}$= 0.652, P=0.521).

Figures

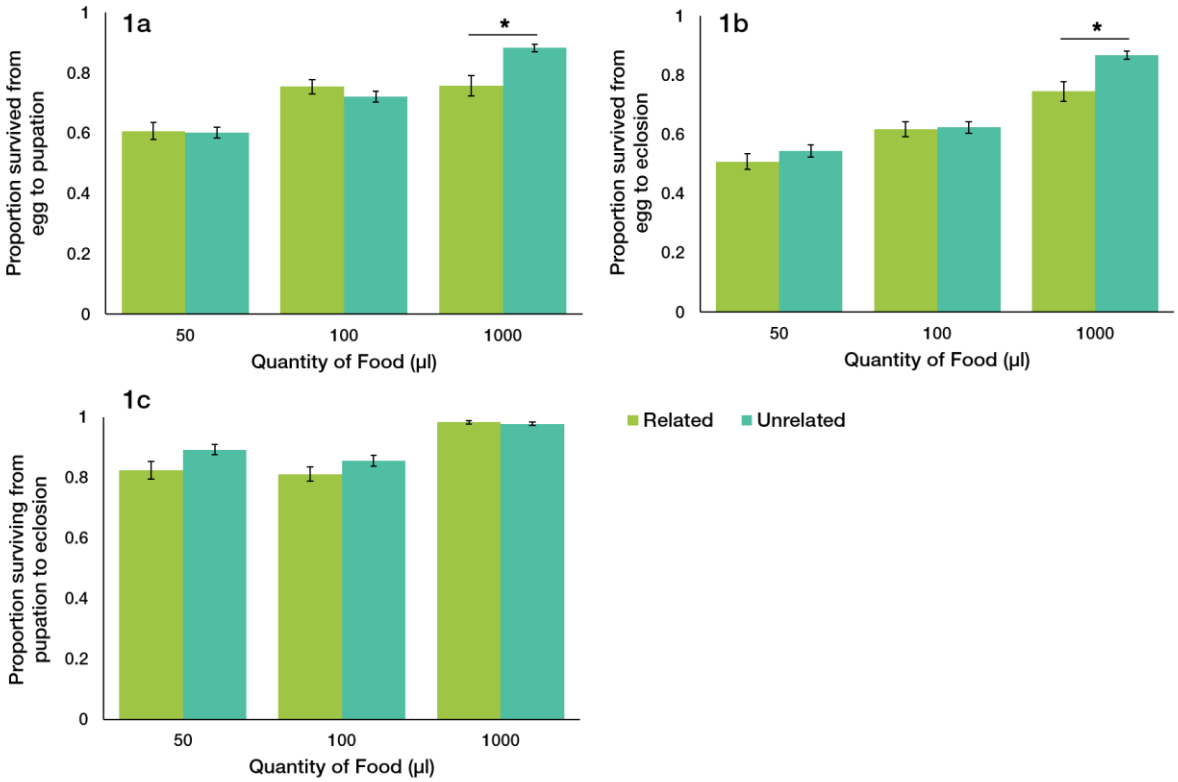


Figure 4.1: Survival of flies between stages of development in vials of full siblings (related) and unrelated individuals at three different levels of competition; very high competition (50µl food), high competition (100µl food) and low competition (1000µl food). At the lowest level of competition, egg-to-pupae and egg-to-adult survival was higher in unrelated vials ($F_{1,94}$ = 12.8, P=0.000547; $F_{1,94}$ = 11.2, P = 0.00119 respectively). Pupae-to-adult survival was higher in unrelated vials across all competition treatments ($F_{1,270}$ = 6.11, P = 0.0141). a) Mean proportion of eggs per vial (15) that reached pupation ± one standard error. b) Mean proportion of eggs per vial (15) that reached eclosion ± one standard error. c) Mean proportion of pupae that reached eclosion ± one standard error.

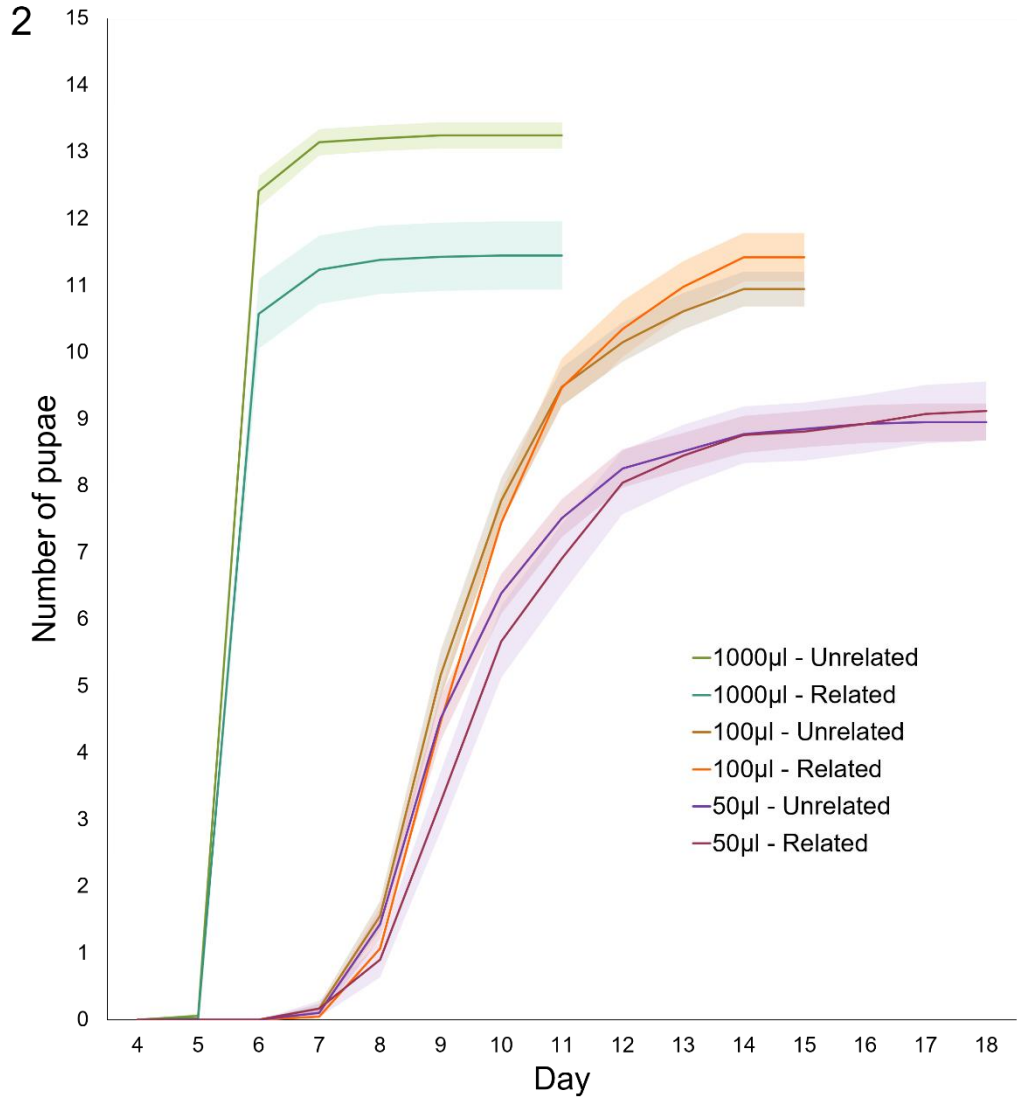


Figure 4.2: Development of flies over time from egg to pupae in vials of 15 full siblings (related) and 15 unrelated individuals (unrelated) at low, high and very high levels of competition for food. Flies in unrelated vials developed to pupation significantly faster regardless of competition treatment ($z = 2.88$, $P=0.004$). The total number of pupae in unrelated vials is higher than in related vials at the lowest level of competition (see Figure 4.1a) Lines show the cumulative mean number of pupae per vial each day with the shaded area representing one standard error above and below the mean.

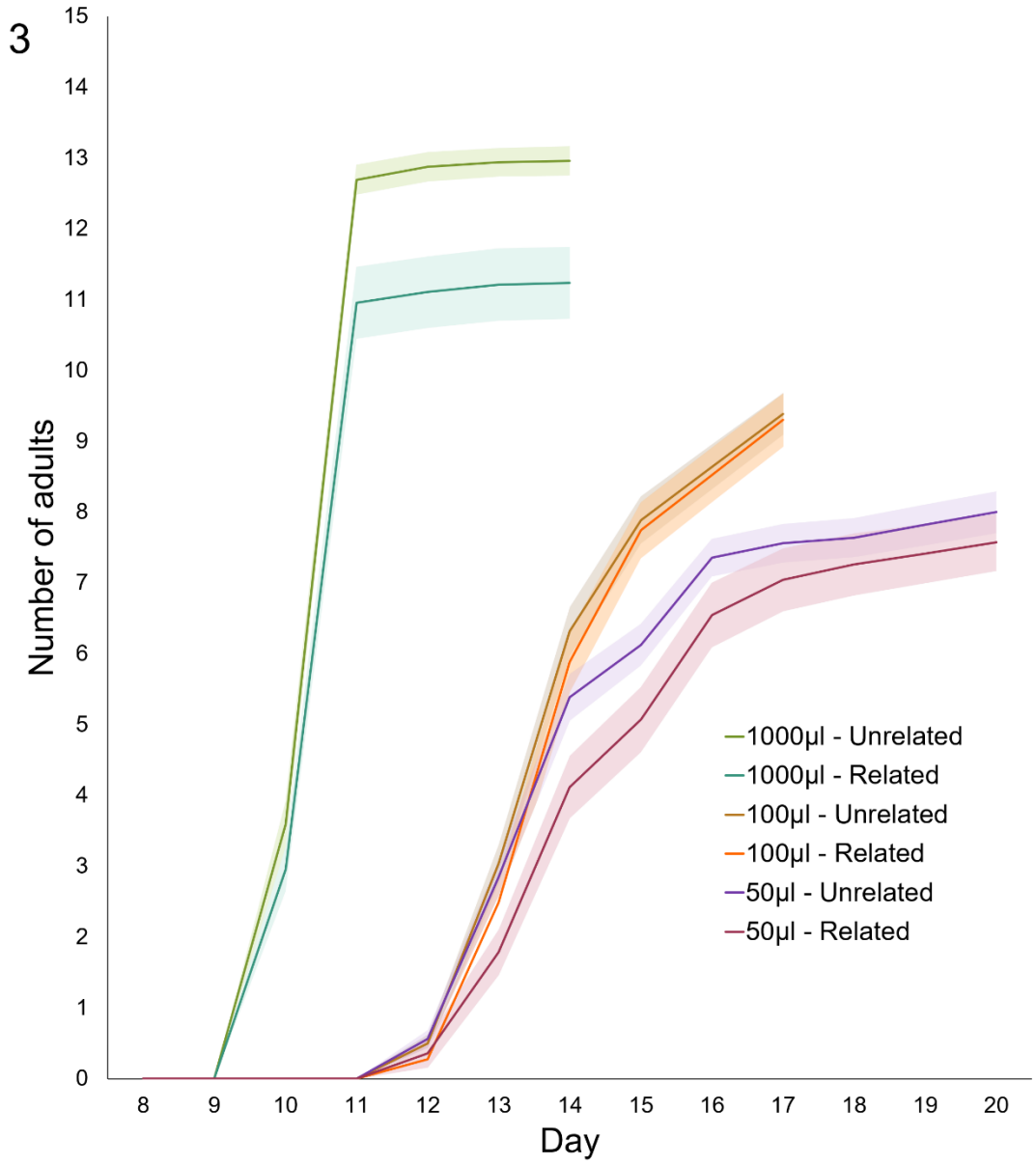


Figure 4.3: Development of flies over time from egg to adult in vials of 15 full siblings (related) and 15 unrelated individuals (unrelated) at low, high and very high levels of competition for food. Flies developed to eclosion faster in unrelated vials than related vials ($z=2.05$, $P=0.040$). The total number of adults emerging in unrelated vials is higher than in related vials at the lowest level of competition (see Figure 4.1b) Lines show the cumulative mean number of adults per vial each day with the shaded area representing one standard error above and below the mean.

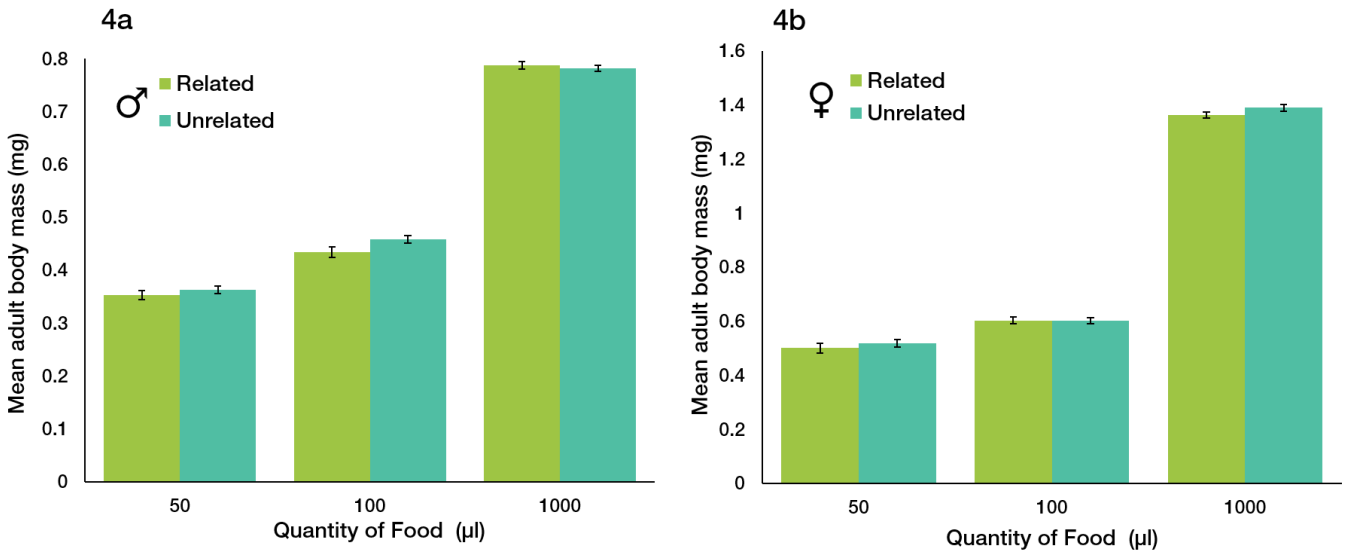


Figure 4.4: Wet body mass of adult males and females in vials of full siblings (related) and unrelated individuals at three different levels of competition; very high competition (50µl food), high competition (100µl food) and low competition (1000µl food). Relatedness had no effect on the body mass of males or females (males: $F_{1,274} = 2.34$, $P = 0.127$; females: $F_{1,272}=1.82$, $P=0.178$). a) Mean wet body mass of adult males ± one standard error of the vial means. b) Mean wet body mass of adult females ± one standard error of the vial means.

Discussion

I reared equal sized groups of related (full siblings) and unrelated individuals from egg to adult with three different quantities of food, and tested whether relatedness affected development. Groups of unrelated individuals developed faster both to pupation and eclosion and there were no differences in the subsequent adult body mass of males or females. Survival from pupation to eclosion was higher in unrelated groups at all levels of competition, but larval survival was only higher in unrelated groups at the lowest level of competition.

Relatedness had no effect on adult body mass

Although, as expected, males and females had lower adult body masses when provided with less food, the relatedness composition of the group had no effect (Figure 4.4). In *D. melanogaster*, adults do not continue to grow after eclosion (Ashburner *et al.*, 2005), and body mass is a very strong predictor of fecundity, fertility and lifespan (Bonduriansky, 2001; Morimoto, Pizzari and Wigby, 2016). The absence of a difference in body mass between related and unrelated groups fails to support the hypothesis that there are adult fitness consequences resulting from variation in the relatedness of larval groups. This suggests that either any changes in larval behaviour and physiology did not occur in a trade off with adult body mass or that there were multiple contributing factors to body mass with equal and opposite effects. Experimental evolution studies in this species have found different developmental strategies; larvae either develop quickly but have low adult body mass, or develop slowly but have higher adult body mass (Joshi and Mueller, 1996; Prasad and Joshi, 2003; Rajamani *et al.*, 2006; Vijendravarma, Narasimha and Kawecki, 2012; Rodrigues *et al.*, 2015). We do not see these facultative developmental

strategies in our data. However, as we did not measure adult fitness directly, we cannot be sure whether there are hidden trade-offs, such as investing in somatic body mass rather than the reproductive organs, that might make equally sized adults less successful.

Unrelated groups develop faster without sacrificing body mass

Larvae in unrelated groups developed to both pupation and eclosion faster than larvae in related groups, regardless of the quantity of food provided, which contrasts with predictions from kin selection. This may be an example of larvae choosing the least bad strategy for survival. If kin are less competitive towards each other, we would expect the level of competition to be higher in unrelated vials. Larvae in these vials may therefore increase their rate of development in order to escape larval competition sooner. If this were the case, we would expect to see this rapid development come at the cost of either survival or adult body mass, yet unrelated vials had higher survival rates and equal body mass.

A potential benefit of developing in an unrelated group would be if the emergent effects of the more diverse larvae on their environment promote more suitable growing conditions (as opposed to reducing direct competition, which I will discuss next). One study found adult females prefer to oviposit on food with more larval activity, even though larvae develop slower and smaller on this 'social food' (Golden and Dukas, 2014). Previously this has been explained by the texture of the food rather than the presence of larvae per se (Atkinson, 1983), but this mechanistic explanation does not explain the evolutionary benefits perceived by the mother.

Larvae face threats from parasitoids and from microbes that may either infect them directly or outcompete the yeasts on which the larvae feed (Sokolowski, 1985; Wertheim *et al.*, 2005; Reaume and Sokolowski, 2006). There is increasing evidence to suggest that the higher number and diversity of fly microbiota found in patches with more larvae act to suppress competitive bacteria and fungi that compete with the larvae for the food source (Wertheim *et al.*, 2002, 2005). Furthermore, younger larvae are less well adapted for tunnelling into the substrate than older larvae, and instead typically feed on the surface, where they are vulnerable to parasitoids. By growing alongside older larvae, younger larvae may be able to hide from parasitoids in the tunnels excavated by the older larvae (Golden and Dukas, 2014). Although parasitoids are not present in the laboratory environment, airborne moulds and bacteria are, and larvae may benefit from developing in an unrelated vial with a more diverse, beneficial microbiome.

The elbow-room hypothesis: findings from the field of the evolution of sex

Developing among unrelated larvae may be a good strategy because of niche partitioning or the 'elbow-room' hypothesis (Young, 1981). With the increased genetic diversity in an unrelated group, we might expect to see a greater diversity in feeding strategies, foraging propensity and food preferences. This might allow larvae to stratify into multiple 'micro-niches', with some specialising in feeding in the oxygen-rich surface layer and others specialising in tunnelling to the deeper, less disturbed food. If unrelated individuals have more dissimilar micro-niches, the level of direct competition in the group would be reduced, which may lead to faster development at no cost to adult fitness, which

is consistent with my data. This would be consistent with my findings that larvae benefit from developing in unrelated groups.

The elbow-room hypothesis was developed in the field of evolution and maintenance of sexual reproduction in light of the two-fold cost of sex, and whether the increased genetic diversity of offspring produced through sexual (rather than asexual) reproduction could provide an explanation (Maynard Smith, 1978; Young, 1981) . It was originally described as "genetic diversity leads to ecological diversity, so that a sexually-produced sibship can exploit the environment more fully than a genetically uniform one" (Young, 1981). It has since been tested theoretically (Maynard Smith, 1978) and empirically (Pérez-Tomé and Toro, 1982; Garcia and Toro, 1992), largely in plant species (Schmitt and Ehrhardt, 1987; Argyres and Schmitt, 1992; Cheplick and Kane, 2004; Masclaux *et al.*, 2010), where the ability to 'self' and reproduce asexually makes the conundrum of sex more apparent. The balance between related groups behaving more altruistically and unrelated groups being more efficient at exploiting resources seems to be highly species dependent (Cheplick and Kane, 2004).

My experimental design shares some similarities to that of Martin *et al.* (1988) who set out to test the elbow-room hypothesis in *D. melanogaster*. Inadvertently, their design also tested the role of kin selection and competition among groups of kin. In their setup, they compared groups of larvae that were either an uncontrolled mix of siblings and half-siblings from 10 mothers and one father ("homogeneous"/related) or an uncontrolled mix of half-siblings and unrelated larvae from 10 mothers and ten fathers ("heterogeneous"/unrelated). They measured egg-to-adult survival ("adult productivity") and egg-to-adult development time, once with a fixed number of larvae at a single quantity of food, and once with an unspecified number of larvae with food at either 100% or 50%

concentration. At a standardised density, they found no difference in survival to eclosion, but egg-to-adult development time was faster in unrelated groups, and when provided with food at 50% concentration, egg-to-adult survival was higher in unrelated groups. Both their results and mine suggest that the elbow-room hypothesis is a more appropriate explanation of the effects of relatedness on larval crowding than reduced competition from kin selection.

Early-stage survival benefits are only seen at the lowest level of competition

Unlike early-stage survival (egg to pupae), late-stage survival (pupae to adult) was higher in unrelated vials regardless of the quantity of food provided, yet the body mass after eclosion was the same between related and unrelated vials. Flies do not feed between the wandering, late third instar stage and eclosion (Ashburner *et al.*, 2005), so this result would suggest that there is an aspect of pupal fitness responding to relatedness that is not linked to the quantity of food consumed as a larva. Physical injury from cannibalism could cause such an effect, as wandering instar larvae and pre-pupae are particularly vulnerable to cannibalism from younger larvae, which results in lower pupal survival (Vijendravarma, Narasimha and Kawecki, 2013). However, in Chapter 5 I found that larvae prefer to cannibalise unrelated individuals, and preliminary work suggests that cannibalism rates are higher in vials with unrelated groups than related groups (Williams and Price, pers. comm). This would reduce pupal survival in unrelated vials, which is the opposite of what I observed.

I only measured the body mass of adults, thus excluding any flies that did not survive from pupation to eclosion. As pupal survival rates were lower in related vials but

there was no ultimate effect on adult body mass, it is possible that pupal death was non-randomly associated with pupal mass, and smaller pupae were more likely to die. This would increase the mean body mass of adults compared to pupae. If this were the case, larvae in related vials may have been feeding less and gaining less body mass, but these differences in distribution were masked at adulthood by survivorship bias. Measuring the pupal mass of all pupae before eclosion would provide evidence to confirm or reject this hypothesis.

Egg-to-pupal survival was the only phenotype that showed a competition-dependent response to relatedness, with more larvae surviving in the unrelated vials at the lowest level of competition and provided with the most food, whereas relatedness had no effect at the two high competition levels. By changing the quantity of food in each vial, I simultaneously changed both quantity of food (and subsequently the level of competition) and the volume of food, which will have affected the spatial diversity. In vials with 50 and 100µl of yeast paste, the paste did not completely cover the surface of the plain agar whereas 1000µl of paste fully covered the surface in a 2mm deep layer. If this larger volume of food provided more micro-niches, this would lend further credence to the elbow-room hypothesis, as the more diverse (unrelated) group was only able to better exploit the food when there was a larger variety of micro-niches available.

Future work

Taken together, the data suggest that larvae do better developing in an unrelated group rather than in a group of relatives, which can potentially be explained by the elbow-room hypothesis, as more diverse groups are better able to exploit an environment. The composition of a group of larvae in nature is largely due to the ovipositioning choices of

the mothers. Although we still know comparatively little about oviposition behaviours, particularly in wild *Drosophila melanogaster* (Reaume and Sokolowski, 2006), it is clear that females colocate and are attracted to sites with other larvae present (del Solar and Palomino, 1966; Mainardi, 1968; Golden and Dukas, 2014). This behaviour would decrease the relatedness of larval groups compared to a behaviour where females were repelled by the presence of other larvae, and would in turn increase the fitness of her offspring.

We use *D. melanogaster* in laboratory conditions to provide a tractable system in which to test ideas about behaviour and evolution. However, such experiments always leave the question of whether what we observe is adaptive or a laboratory artefact, and whether such behaviours are present in wild systems. For example, the dispersal behaviour that would allow a larva to seek out new food patches when the present patch has been exhausted would be adaptive both in wild conditions and in the laboratory population cages where other food sources are available. In the experimental vials, however, there was only a single food patch, and dispersing larvae risked dessication in the cotton wool, despite the high ambient humidity. Increasingly, *Drosophila* are being tested in habitats of intermediate complexity, ranging from patchy distributions of food on a Petri dish, to clusters of vials of food in a larger container (Yun *et al.*, 2017; MacPherson *et al.*, 2018), to outdoor cages with fruits and living plants (Debelle, in prep). Since foraging strategies are intrinsically linked to environmental complexity and resource heterogeneity, it would be fruitful to test if unrelated groups outperform related groups in these intermediate-complexity environments.

Supplementary results

Survival

Egg to Pupae

In the low competition (1000µl food per vial) treatment, more individuals survived to pupation in the unrelated vials than related vials (Odds ratio = 0.851, $F_{1,94}$ = 12.8, P=0.000547, Figure 4.1a), but there was no difference in the variance in survival ($F_{42/53}$ = 1.58, P=0.117).

In the high competition (100µl food per vial) treatment, relatedness had no effect on survival to pupation ($F_{1,95}$ = 1.18, P=0.280) and related and unrelated vials had equal variance ($F_{42/53}$ = 1.58, P=0.117).

In the very high competition (50µl food per vial) treatment, relatedness had no effect on the proportion of individuals surviving to pupation ($F_{1,79}$=0.102, P=0.750), but the variance in survival was significantly greater in the related vials ($F_{41/38}$ =2.63, P=0.00329).

Egg to Adult

In the low competition treatment, more individuals survived to eclosion in the unrelated vials than related vials (Odds ratio = 0.851, $F_{1,94}$ = 11.2, P = 0.00119, Figure 4.1b), and the variance in survival was higher in the related vials ($F_{46/48}$ = 5.80, P<0.001).

In the high competition treatment, there was no difference in survival between the related and unrelated vials ($F_{1,95}$ = 0.0338, P=0.855) nor any difference in the variance ($F_{42/53}$ = 1.29, P=0.373).

In the very high competition treatment, relatedness had no effect on survival to eclosion ($F_{1,79}$ = 0.712, P=0.400) but the variance in survival was higher in the related vials ($F_{41/38}$ = 2.03, P=0.0300).

Pupae to Adult

In the low competition treatment, relatedness had no effect on the survival rate from pupation to eclosion ($F_{1,94}$ = 0.348, P=0.557).

In the high competition treatment, there was no difference in survival between related and unrelated vials ($F_{1,95}$ = 2.69, P=0.105).

In the very high competition treatment, pupal to adult survival was higher in the unrelated vials than in related vials (Odds ratio = 0.544, $F_{1,79}$ = 5.10, P=0.0267, Figure 4.1c).

Development Time

Pupation

Relatedness had no effect on the development of eggs to pupation in the low competition treatment (z = 0.970, P =0.330) or the high competition treatment (z = 1.66, P = 0.096). However, flies developed faster to pupation in unrelated vials in the very high competition treatment (Hazard ratio = 1.33, z = 2.80, P = 0.005, Figure 4.2).

Eclosion

There was no effect of relatedness on the development of eggs to eclosion in the low competition treatment (z = 0.750, P = 0.450) or the high competition treatment (z = 0.83, P = 0.410). In the very high competition vials, flies in unrelated vials developed faster to eclosion than in related vials (Hazard ratio = 1.25, z = 2.04, P = 0.042, Figure 4.3).

Adult body mass

Relatedness had no significant effect on the wet body mass of adult males in the low competition treatment ($F_{1,96}$ = 0.489, P = 0.486) or the very high competition

treatment ($F_{1,80}$ = 0.728, P = 0.396). In the high competition treatment, males weighed more from unrelated vials (Estimate = 0.0250, $F_{1,98}$ = 4.59, P = 0.0346, Figure 4.4a).

Relatedness had no significant effect on the wet body mass of adult females at any level of competition (low: $F_{1,96}$ = 2.55, P = 0.1133; high: $F_{1,98}$ = 0.0045, P = 0.946; very high: $F_{1,78}$ = 0.611, P = 0.437, Figure 4.4b).

CHAPTER 5:

LARVAE PREFER TO CANNIBALISE UNRELATED AND UNFAMILIAR VICTIMS

Abstract

Cannibalism is a social behaviour with extreme fitness consequences for both the cannibal and the victim. As such, we might expect to see the relatedness between the cannibal and victim play a role in mediating the behaviour, with kin selection theory suggesting that cannibals might favour unrelated victims. Cannibalism has recently been documented in *Drosophila melanogaster*, with second instar larvae targeting 'wandering' third instar larvae that have ceased feeding before pupation. I designed choice tests using GFP tagged larvae to observe whether second instar larvae were more likely to cannibalise related or unrelated victims, socially familiar or socially unfamiliar victims, and whether they were influenced by the relatedness of the other cannibals on a victim. Larvae were more likely to cannibalise an unrelated unfamiliar victim than a related unfamiliar victim, and also more likely to cannibalise an unfamiliar related victim than a familiar related victim. The relatedness of the other cannibals on a victim did not affect cannibalism preferences but larvae were consistently attracted to the more cannibalised victim across experiments. These data support the kin selection view that larvae benefit from avoiding cannibalising kin, which may have evolutionary consequences for oviposition strategies and larval foraging strategies.

Introduction

Few behaviours can have as extreme fitness consequences as saving yourself from starvation by killing and feeding on a conspecific, particularly if there is the possibility that the conspecific is a relative. Cannibalism can come in many different forms: parents eating offspring (filial cannibalism (Bonsall and Klug, 2011): *e.g. fishes* (FitzGerald, 1992), *assassin bug* (Thomas and Manica, 2003), *wolf spider* (Anthony, 2003), *rainbow boa* (Lourdais *et al.*, 2005)); females eating male mates (sexual cannibalism (Buskirk, Frohlich and Ross, 1984): *fishing spider* (Arnqvist and Henriksson, 1997), *praying mantis* (Birkhead *et al.*, 1988)); offspring eating their mother (matriphagy: *earwig* (Suzuki, Kitamura and Matsubayashi, 2005), *pseudoscorpion* (Tizo-Pedroso and Del, 2005)); nestlings eating their weakest sibling (*birds of prey* (Collingwood, 1959)); migrating groups eating each other (*locusts* (Guttal *et al.*, 2012)); each with different adaptive causes and consequences.

Cannibalism in fruit flies

Frequently, we focus on the cannibalistic behaviours of carnivorous animals as their typical diet predisposes them to be adapted to feeding on conspecifics. However, a review of non-carnivorous insects (Richardson *et al.*, 2010) found reports of cannibalism in 130 species, both in the wild and in laboratory conditions. In 75% of these cases, cannibalism was seen in larvae, often consuming eggs or smaller larvae. The most well represented taxa were unsurprisingly the most studied; Coleoptera and Lepidoptera, with only seven Dipteran species represented in the list.

The pattern of evidence in documenting cannibalism in a non-carnivorous species seems to be that, at first, we do not think that the species ever cannibalises, then we observe that it can cannibalise but assume this to be a rare occurrence (Richardson *et al.*,

2010), then the more we observe the species, the more commonplace cannibalism in this species becomes. Despite the enormity of research on the model organism *Drosophila melanogaster*, cannibalism was first documented in this species as recently as 2013 (Vijendravarma, Narasimha and Kawecki, 2013) and has been studied very little since (Ahmad *et al.*, 2015).

Unusually for insect cannibalism, reports thus far indicate that it is primarily the younger larvae cannibalising older larvae in *D. melanogaster*, particularly first, second and early third instar larvae seeking and attacking late stage third instar or 'wandering' larvae (Vijendravarma, Narasimha and Kawecki, 2013). Although this size relationship is the reverse of that more commonly seen among cannibals (Bilde and Lubin, 2001), wandering larvae are within a few hours of pupation, have stopped feeding and become progressively less mobile until the prepupation stage (Ashburner *et al.*, 2005). They are therefore easy targets for cannibalism, especially as it takes younger larvae hours of rasping before potentially cutting through the cuticle. Larvae will also cannibalise eggs and adult carcasses, and adults will cannibalise damaged larval carcasses, but lack the mouth anatomy to cannibalise intact larvae (Ahmad *et al.*, 2015). Individuals can develop from egg to adult on an entirely cannibalistic diet, and show no morphological differences as adults beyond body size (Ahmad *et al.*, 2015) although female fecundity is approximately halved compared to females fed a normal diet (Vijendravarma, Narasimha and Kawecki, 2013). One fascinating response to cannibalism is that larvae raised on a cannibalistic diet facultatively develop 20% more teeth on their mouth hooks as third instars than larvae raised on a poor quality, yeast-based diet, and larvae experimentally evolved under low nutrition have cannibalistic tendencies twice as high as normal (Vijendravarma, Narasimha

and Kawecki, 2013). This demonstrates that cannibalism is both an adaptive and plastic response to low nutrition in *D. melanogaster*.

Cannibalism and inclusive fitness

As cannibalism usually results in the death of the victim and can often prevent death from starvation in the cannibal, this behaviour has both very high costs and benefits from a Hamiltonian perspective (Hamilton, 1964). Being able to discriminate between cannibalising a relative or non-relative could have major inclusive fitness benefits, particularly as wandering instar larvae are much closer to reaching sexual maturity and therefore are likely to provide indirect benefits to the cannibal if related.

The role of relatedness in mediating cannibalism is highly species specific. The spider *Stegodyphus lineatus* cannibalised more in groups of non-kin than kin, although only when in low nutrition environment, not when well fed (Bilde and Lubin, 2001). Some species show alternate strategies as larvae, with herbivorous/insectivorous morphs and cannibal morphs. Omnivorous larval spadefoot tadpoles *Scaphiopus bombifrons* prefer to associate with groups of siblings, whereas cannibal larvae prefer to associate with non-siblings, meaning they are less likely to attack kin (Pfennig, Reeve and Sherman, 1993). The cannibals nip at conspecifics and either release them if they are siblings or eat them if they are non-siblings, though they become less selective when under starvation. Virgin adult flour beetles *Tribolium confusum* cannibalise more unrelated eggs than related eggs, but dramatically reduce their rate of egg cannibalism after mating which reduces the likelihood of them eating their own offspring (Parsons, Zhong and Rudolf, 2013), showing another behavioural change which can result in kin discrimination without necessarily requiring kin recognition. Both insectivore and cannibal morphs of larval *Ambystoma tigrinum*

nebulosum salamanders cannibalised fewer related individuals, but families with more cannibal morphs showed better kin discrimination (Pfennig, Sherman and Collins, 1994). Pfenning *et al*. (1994) also showed that kin discrimination in this species was based on olfaction, as larvae were unable to discriminate between kin once their nares had been covered. In another species of salamander, *Ambystoma opacum*, larvae showed fewer cannibalistic behaviours towards kin than non-kin and this response was independent of environmental familiarity (Walls and Roudebush, 1991). In contrast, neither green poison frog larvae *Dendrobates auratus* nor willow leaf beetle larvae *Plagiodera versicolora* showed any effect of relatedness on cannibalistic behaviours (Goff and Stevens, 1995; Gray, Summers and Ibáñez, 2009).

Adult female *D. melanogaster* frequently oviposit together (del Solar and Palomino 1966; though see Atkinson 1983) and two pheromones and a chemosensory neurone have been identified that mediate larval attraction to other larvae (Mast *et al.*, 2014), so larvae can expect to encounter both related and unrelated larvae during their development. In the wild, larvae develop on rotting fruits (Reaume and Sokolowski, 2006), which are an ephemeral resource, so it is reasonable to assume that larvae will face conditions of starvation if their food patch becomes depleted. Both conditions set up a situation where discriminating between cannibalising kin and non-kin might be both frequently encountered over evolutionary time and have strong fitness consequences.

Previous research into larval cannibalism in *D. melanogaster* (Vijendravarma, Narasimha and Kawecki, 2013; Ahmad *et al.*, 2015) has not been able to follow the behaviour of multiple families due to the lack of common phenotypic larval markers in contrast to those available for adult studies such as eye colour. Ongoing unpublished work is testing rates of cannibalism in a single family of cannibals presented with either related

unfamiliar victims or unrelated unfamiliar victims (Williams and Price, pers. comm.). No one has studied whether larvae prefer to cannibalise one victim over another when presented with the choice of related/unrelated or familiar/unfamiliar victims, nor have multiple families of cannibals been studied at the same time, so we do not know anything about how families interact in this behaviour.

By using a transgenic GFP strain to identify families, I am able to test the behaviours of multiple families of victims and cannibals at the same time and measure their interactions. In this system I independently manipulate relatedness (full siblings or non-siblings) and familiarity (reared in the same vial of food or in a different vial), thereby beginning to unpick the mechanistic cues underpinning the cannibalistic preferences. I test the cannibalistic preferences of second instar larvae between related familiar and related unfamiliar third instar victims and between related unfamiliar and unrelated unfamiliar victims. I also examine whether the relatedness of the other cannibals on a larval victim affects cannibalism.

Predictions

- Kin selection theory would predict that cannibals may benefit from avoiding cannibalising kin, as killing a relative would reduce the cannibals' indirect fitness. Therefore, I predict cannibals will prefer to cannibalise unrelated unfamiliar victims over related unfamiliar victims
- Larvae may similarly avoid cannibalising familiar victims if they are using familiarity as a partial proxy for relatedness. If this is the case, I predict larvae will prefer to cannibalise related unfamiliar victims over related familiar victims

- Cannibals may choose to join a group of related cannibals over a group of unrelated cannibals so that their personal expenditure in rasping through the victim's cuticle benefits their relatives, thereby increasing their own indirect fitness. This would manifest as the two families of cannibals being unevenly distributed on each victim.

Methods

Stocks

I backcrossed a GFP-moesin transgene (Edwards *et al.*, 1997) into a Dahomey background for five generations. This transgene is on the second chromosome and acts on actin in cytoskeletons, thereby creating a whole-body GFP fly at all developmental stages to enable identification (Figure 5.1). Larvae shed their skin between moults so painting the cuticle is not an option, and dyed food visible in the gut is an ephemeral marker that is excreted as the larva eats non-dyed food. I crossed this GFP strain with a curly (*CyO*) balancer strain, also in the Dahomey background, for stock maintenance. A wild-type (WT) strain was also produced as a result of this cross and was kept as the control for the GFP strain. Both strains were maintained in 177ml polyethylene bottles with 60ml Lewis medium (Lewis, 1960) with overlapping generations at 20˚C for 19 months until required, with a minimum effective population size of 50.

General methods

To create the singly mated parents of the experimental, full sibling larvae, I reared the strains in bottles at 25˚C and collected virgins directly from the bottles within eight hours of eclosion under ice anaesthesia. Virgin males and females were then immediately paired in polystyrene 36ml vials which contained 8ml Lewis medium and excess live yeast on day 1 and housed at 25˚C to mate and lay eggs. On day 5, I transferred the adult pairs from their yeasted vials ("early vials") to vials with Lewis medium but no live yeast ("late vials"). The fresh medium stimulated egg production (pers. obs.) and the absence of live yeast made it easier to collect second instar larvae.

The cannibalism observations occurred on day 7 after pairing. Observations took place on a plate of charcoal agar (1000ml water, 40g agar, 20g wood charcoal) in 55mm diameter, non-vented Petri dishes. Charcoal absorbs stray incident fluorescent light and increases contrast between the larvae and the background. The agar was firm enough to prevent larvae from burrowing.

I picked second instar larvae from the late vials using a fine paintbrush, determining their developmental stage by their size and the shape of their mouthparts. I transferred 20 second instar larvae to the centre of each plate. I picked wandering third instar larvae from the early vials, selecting larvae displaying locomotion to reduce the number of victims pupating during the period of observations. I pinned the victims with entomological pins of 0.1mm diameter, securing them to the agar 15mm from the centre of the plate along the same diameter (Figure 5.1). For the familiarity experiment, I was able to blind myself to the familiarity of the victims, but this was impossible to achieve for the other two experiments due to the visible phenotypes. Each family was used on only one plate; any families that did not have enough larvae of the required age were not included.

The experimental design in this study differs from previous work as I used entomological pins to secure the victims to the agar rather than staples (Vijendravarma, Narasimha and Kawecki, 2013), as the latter introduced a higher incidence of victim escapism. Whilst piercing the victims' body with a pin will increase rates of cannibalism, this method was the same for all treatments and thus unbiased.

I made observations at 1, 2, 3 and 6 hours after establishing the plate, counting how many second instar larvae were cannibalising each victim. I recorded a second instar larva as cannibalising a victim if it was within a 5mm radius of the victim (Figure 5.1). I transferred any larvae that had crawled onto the lid of the Petri dish with a paintbrush

back to the central starting position to reduce mortality from desiccation. The plates were kept at 25˚C and 70-85% humidity.

Larvae within the 5mm radius may not have been cannibalising; a small number were passing through without interacting with the victim, whilst others may have been interacting with the victim but not for cannibalistic reasons. Larvae may have been attracted to the increased humidity or micro-variations in light intensity produced by the presence of the victim on an otherwise flat plain of agar. Furthermore, although I frequently observed rasping behaviours of the second instar larvae on the cuticle of the victims, it is possible that they were feeding off small quantities of Lewis medium transferred with the victim from its previous vial. Whilst I avoided transferring Lewis medium with the victims, I did not want to fully wash the victims as this may have interfered with the relatedness and familiarity cues I was trying to detect. However, any variation in this respect would not be biased towards one treatment.

Familiarity experimental methods

To test whether larvae differentially cannibalised victims based on familiarity, I presented cannibals with related familiar and related unfamiliar victims. On each plate, all larvae were from the same family from the WT control strain, with both victims and cannibals full siblings. To generate the related familiar victim, on day 5 after pairing the parents, I picked three third instar larvae from the early vial and transferred them to the late vial before adding the parents. On the day of the experiment, I then collected one third instar larva from the early vial (related unfamiliar) and one from the late vial which had been in the same vial as the cannibals (related familiar), taking care to select victims of approximately equal size. All 20 second instar cannibals were from the late vial.

To blind myself from the familiarity status of the victims during observations, I randomly assigned which side (left or right) the familiar victim would be placed. The victims were picked by an assistant who then assigned an ID to each plate. I observed a total of 53 plates.

Co-cannibalism experimental methods

This experiment was to determine whether the choice of which victim to cannibalise is affected by the relatedness (and familiarity) of the other cannibals surrounding that victim. On each plate, I transferred 10 second instar larvae from one GFP family and 10 from one WT control family. Cannibals were therefore related and familiar within their own family, and unrelated and unfamiliar to the other family. To ensure that the victims were equally unrelated and unfamiliar to both families, I took victims from the large outbred Dahomey population. I allowed flies from this population to lay eggs in bottles from which I then directly collected third instar wandering larvae.

The number of cannibals of each family was counted for the left and right victim, using colour to determine cannibal family status. I observed a total of 49 plates.

Relatedness experimental methods

In this setup, I tested whether relatedness affected the cannibals' choice of who to cannibalise, while also accounting for the influence of other cannibals. On each plate, 10 cannibals and one victim were from a single GFP family, and 10 cannibals and one victim were from a single WT control family. The victims and cannibals were from their respective family's early and late vials so were not familiar.

I observed how many cannibals of each family were on the GFP and WT victims, using colour to determine cannibal family status. I observed a total of 64 plates.

General statistical methods

It is unlikely that cannibalistic larvae act independently of each other (though see Niewalda *et al*. 2014), but we do not yet know the dependence structure of how the activity of one larva affects the others. Hence these data violate the assumption of independence required for linear modelling. Therefore, I calculated a single measure per plate and analysed each time point in a separate model to mitigate pseudoreplication and non-independence. Because the sample size for each experiment was large (~50), each plate is independent, the distribution of measurements on each plate comes from the same distribution on all plates and the dependence structure is likely to be the same on all plates, the central limit theorem leads to the distribution of measurements being Gaussian. I performed an approximate test of this distribution against a standard normal distribution ("z test"), using a two tailed test with an alpha value of P=0.025 for each tail. All analyses were performed in R (R Core Team, 2014).

Familiarity statistical analysis

For each plate and time point, the number of cannibals on the unfamiliar victim was subtracted from the number on the familiar victim, creating a single difference measure per plate for each time point which was analysed as described above.

Co-cannibalism statistical analysis

To determine if the GFP and WT populations had different intrinsic rates of cannibalism, I analysed the difference between the number of WT cannibals on both victims and the number of GFP cannibals on both victims.

For each plate, I identified which victim had on average the most cannibals and assigned this as the "more cannibalised victim", and the other as the "less cannibalised victim". For each of these two classes of victim, I then analysed the difference between the number of WT and GFP cannibals.

Relatedness statistical analysis

As before, I analysed the intrinsic rates of cannibalism between the two genotypes. I then analysed the difference between the total number of cannibals on their respective unrelated victim (WT cannibals on GFP victims, GFP cannibals on WT victims) and the total number of cannibals on their respective related victim (WT cannibals on WT victims, GFP cannibals on GFP victims).

Post-hoc analysis of victim skew

After finding that the preference for unrelated victims decreased over time, but the preference for unfamiliar victims increased over time, I wanted to further examine how the difference in the number of cannibals on each victim changed over time with each experiment (see **Discussion** for further explanation). For each plate, I calculated the difference between the number of cannibals on each victim (skew) at each time point, then found the linear regression coefficients (slope and intercept) for skew with time. I

compared the distribution of coefficients for the relatedness and co-cocannibalism experiments using a Welch's t-test.

Methods figures

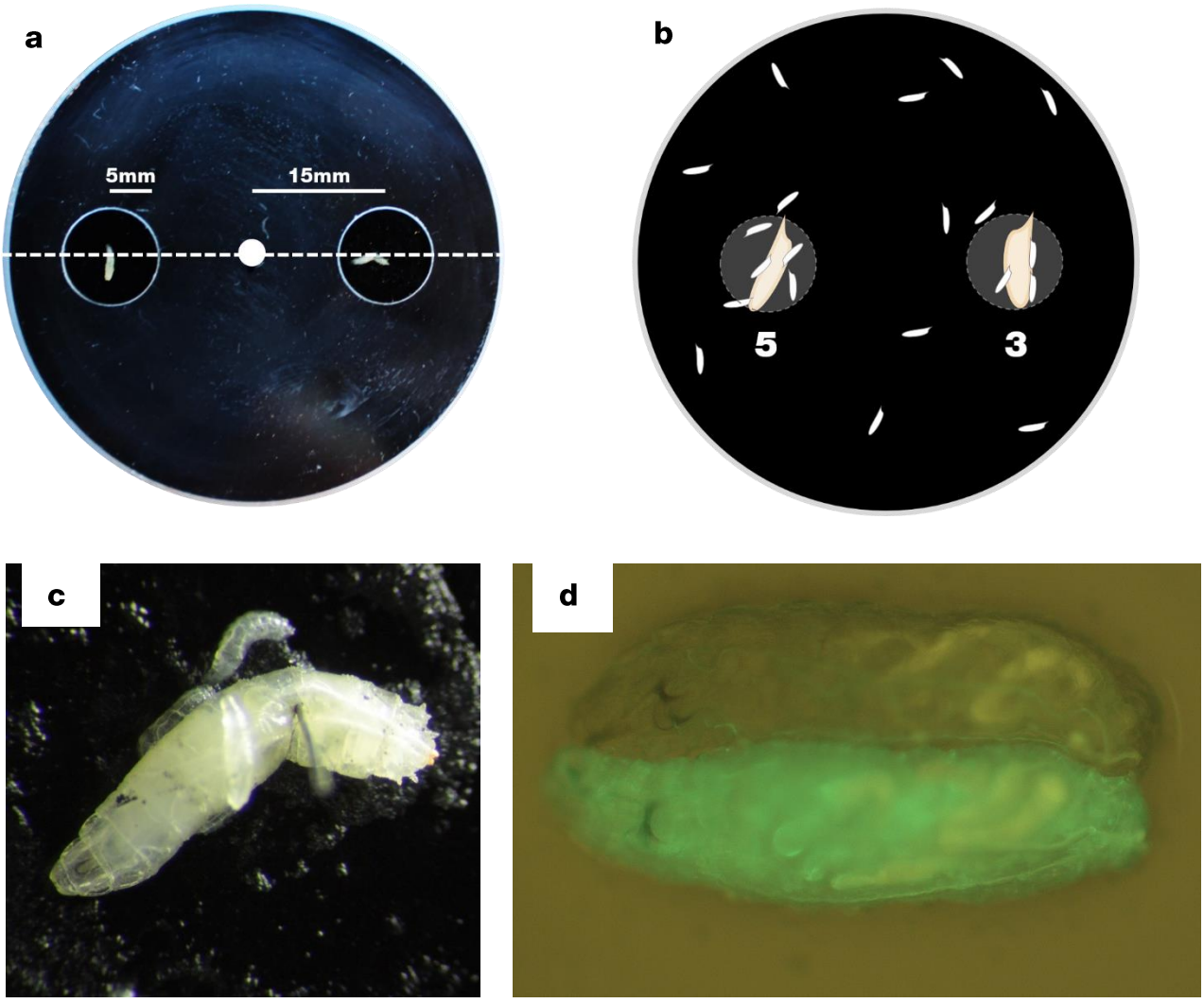


Figure 5.1 – Experimental design for cannibalism assays. 1a: Photograph of 55mm diameter Petri dish filled with charcoal agar onto which two third instar wandering larvae have been pinned 15mm from the centre. Twenty second instar larvae are placed in the centre at the start of the experiment and were recorded as cannibalising when within a 5mm radius of the victim, visualised by drilling 10mm diameter holes in a Petri dish lid. **1b:** Diagrammatic representation of the larvae during cannibalism observations (not to scale). In this scenario, the left victim would be recorded as having 5 cannibals, and the right victim, 3 cannibals. **1c:** Micrograph of four second instar larvae (one obscured at the lower edge of the victim) on a pinned third instar victim. **1d**: Micrograph showing the contrast between the WT (upper) and GFP (lower) phenotypes under a fluorescence microscope.

Results

Familiarity experiment

After 6h of being introduced, more second instar larvae cannibalised the related unfamiliar victim rather than the related familiar victim (Mean difference= -1.17, z=-2.24, P=0.025, Figure 5.2a). Although there was no significant effect of familiarity at the earlier time points (1h: z=-1.59, P=0.112; 2h: z=-1.63, P=0.103; 3h: z=-1.58, P=0.115), the difference was consistently skewed towards cannibalising the unfamiliar victim, with the magnitude of this difference increasing over time (Figure 5.2b).

Co-cannibalism experiment

At each time point, there was no difference in the total number of cannibals from each genotype (1h: z=-0.339, P=0.735; 2h: z=1.66, P=0.0965; 3h: z=0.348, P=0.345; 6h: z=0.534, P=0.593, Figure 5.3). On both the more and less cannibalised victim, the cannibals on each victim were equally represented by the two cannibal families, suggesting there is neither a strong preference nor a strong aversion to cannibalising within a group of related/unrelated cannibals (More cannibalised victim 1h: z=-0.468, P=0.640; 2h: z=1.15, P=0.247; 3h: z=0.335, P=0.738; 6h: z=0.864, P=0.387; Less cannibalised victim 1h: z=0.227, P=0.820; 2h: z=1.64, P=0.100; 3h: z=1.67, P=0.0950; 6h: z=-0.200, P=0.841, Figure 5.4).

Relatedness experiment

At each time point, there was no difference in the total number of cannibals from each genotype (1h: z=1.55, P=0.121; 2h: z=0.870, P=0.385; 3h: z=0.214, P=0.831; 6h: z=0.791, P=0.429, Figure 5.5). For all cannibals and all victims, second instar larvae were more likely to cannibalise the victim unrelated to them, but only for the first two hours (1h:

mean difference= 0.246, z=2.32, P=0.0204; 2h: mean difference=0.294, z=2.279, P=0.0294). After two hours, there was no preference for either the related or unrelated victim (3h: mean difference=0.0952, z=0.642, P=0.521; 6h: mean difference=-0.143, z=-0.890, P=0.373, Figure 5.6).

Victim skew across experiments

I found no statistical difference between the familiarity and relatedness experiments for either the slope or intercept of the skew between victims over time (Slope: t_{94}=0.0816, P=0.935; Intercept: t_{108}=0.844, P=0.401, Figure 5.7a). Both experiments show that the skew increases over time (relatedness slope = 0.695±0.0621, co-cannibalism slope = 0.687±0.0825, Figure 5.7b), which implies that as one victim becomes more cannibalised, this has a positive feedback loop encouraging further cannibalism, and that the strength of this feedback loop is the same across experiments.

Results figures

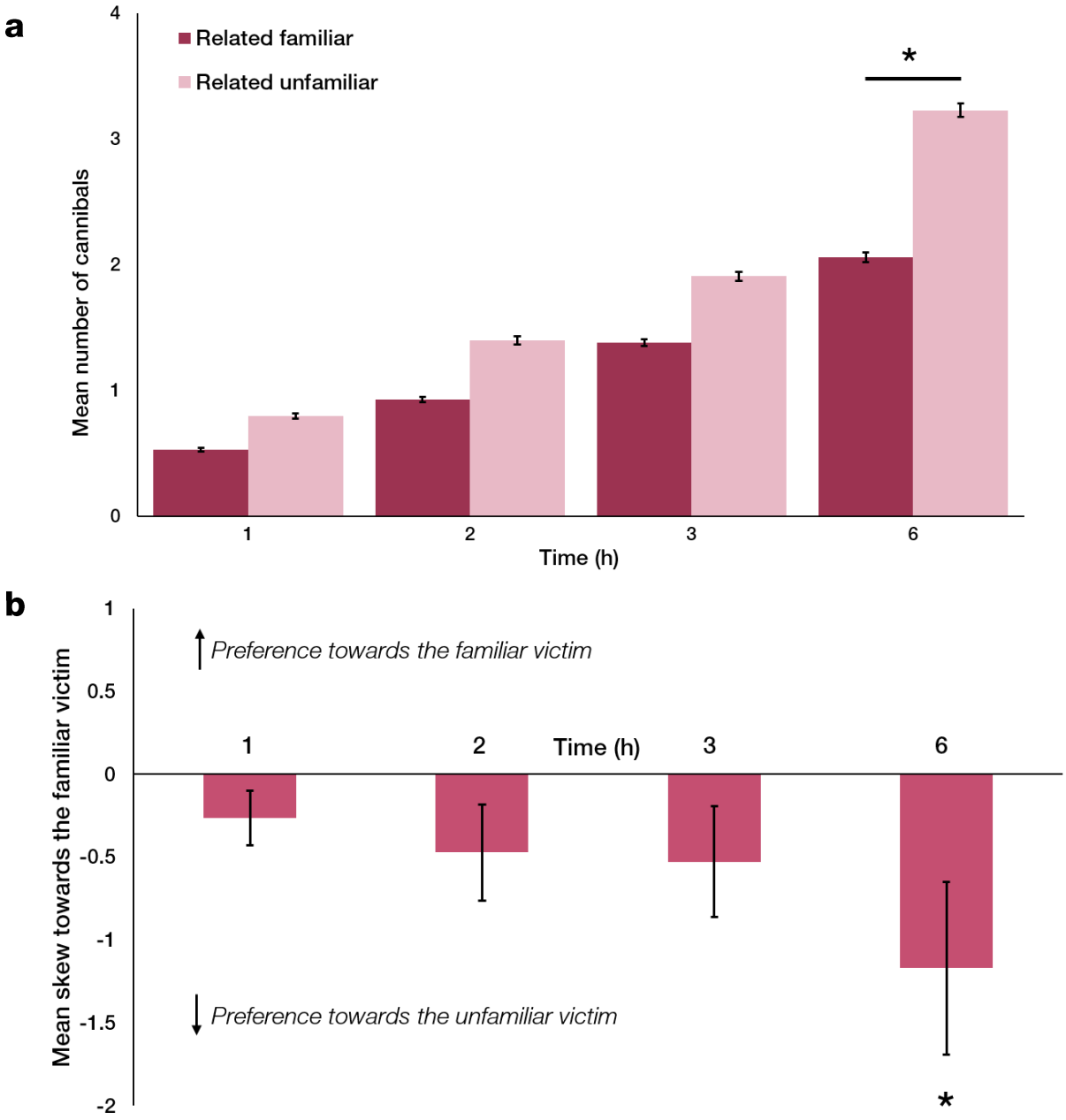


Figure 5.2 – Familiarity experiment: Cannibals preferentially target a sibling they are unfamiliar with over a sibling they are familiar with after six hours (z=-2.24, P=0.025). **a)** Bars show the mean number of second instar cannibals out of twenty on each wandering instar victim. **b)** The skew towards the familiar victim is calculated as the number of second instar larvae on the related familiar victim minus the number on the related unfamiliar victim on each plate. N=53, error bars ±SE

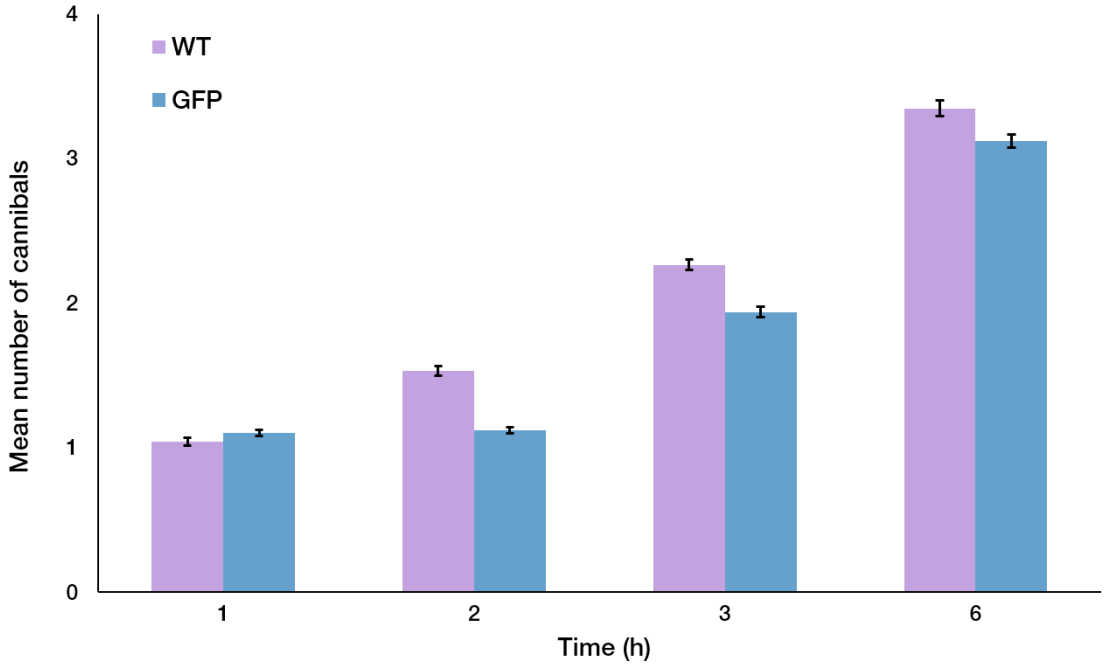


Figure 5.3 – Co-cannibalism experiment: WT and GFP genotypes did not cannibalise at statistically different rates as measured by the number of second instar larvae out of ten per family on both unrelated unfamiliar victims combined (P>0.34). N=49, error bars ±SE

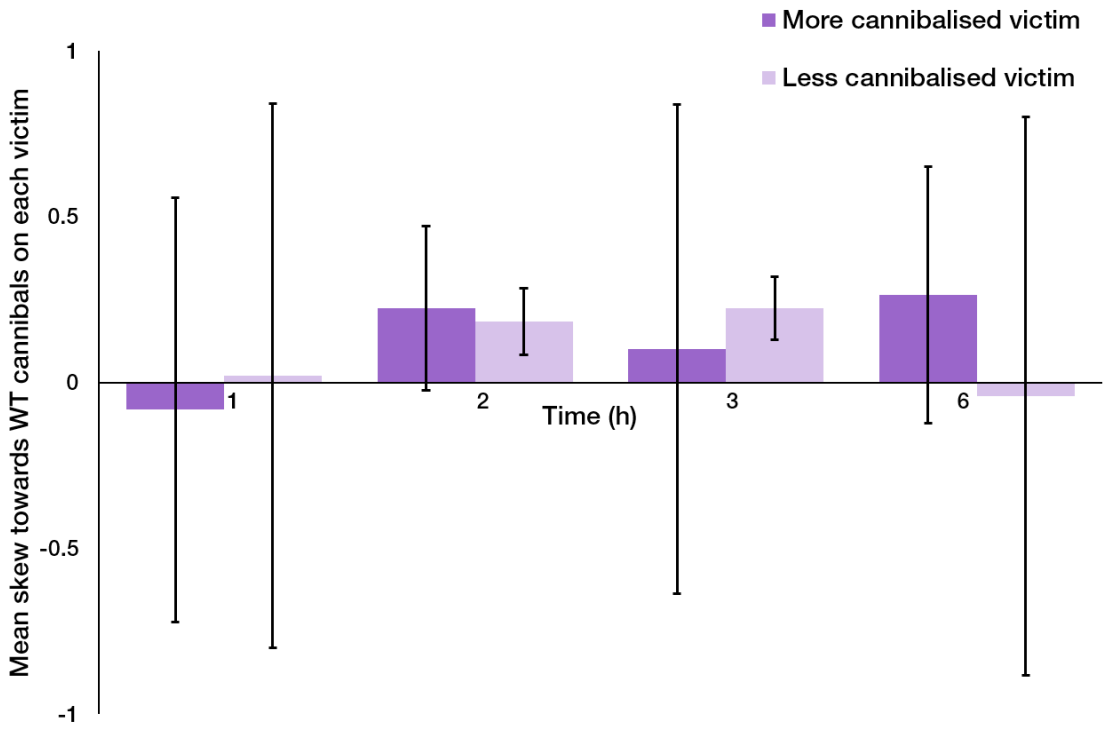


Figure 5.4 – Co-cannibalism experiment: Both families of cannibals were equally represented among cannibals on both the more and less cannibalised victim (P>0.1). Skew is calculated as the number of WT second instar larvae out of a possible ten minus the number of GFP second instar larvae cannibalising the victim. N=49, error bars ±SE

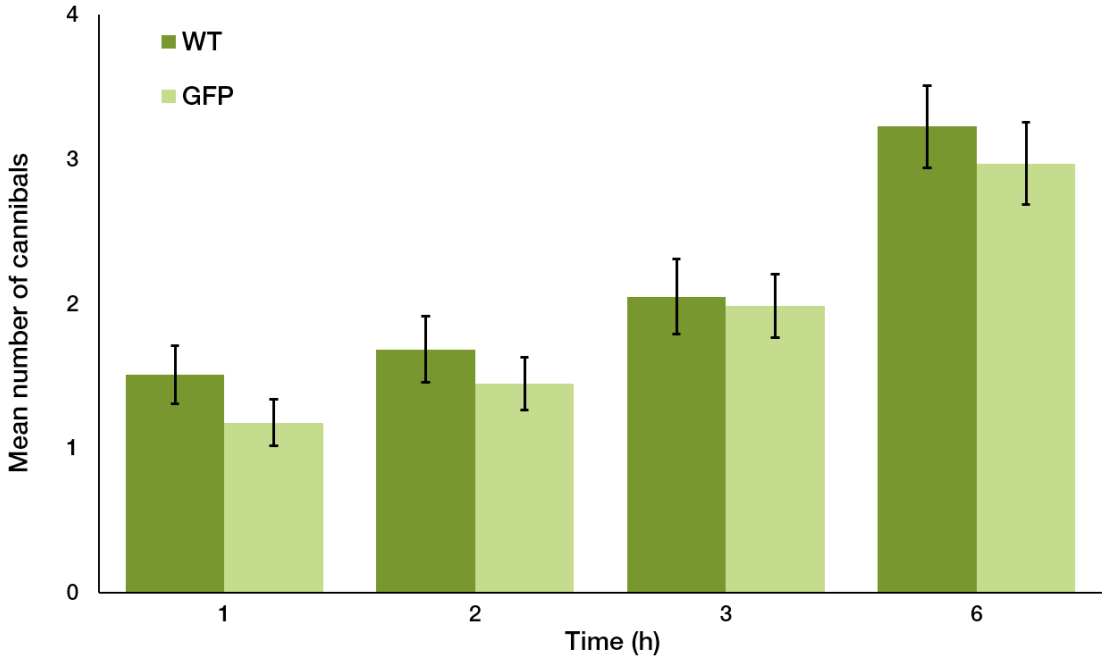


Figure 5.5 – Relatedness experiment: WT and GFP genotypes did not cannibalise at statistically different rates as measured by the number of second instar larvae out of ten per family on both victims combined (P>0.34). N=64, error bars ±SE

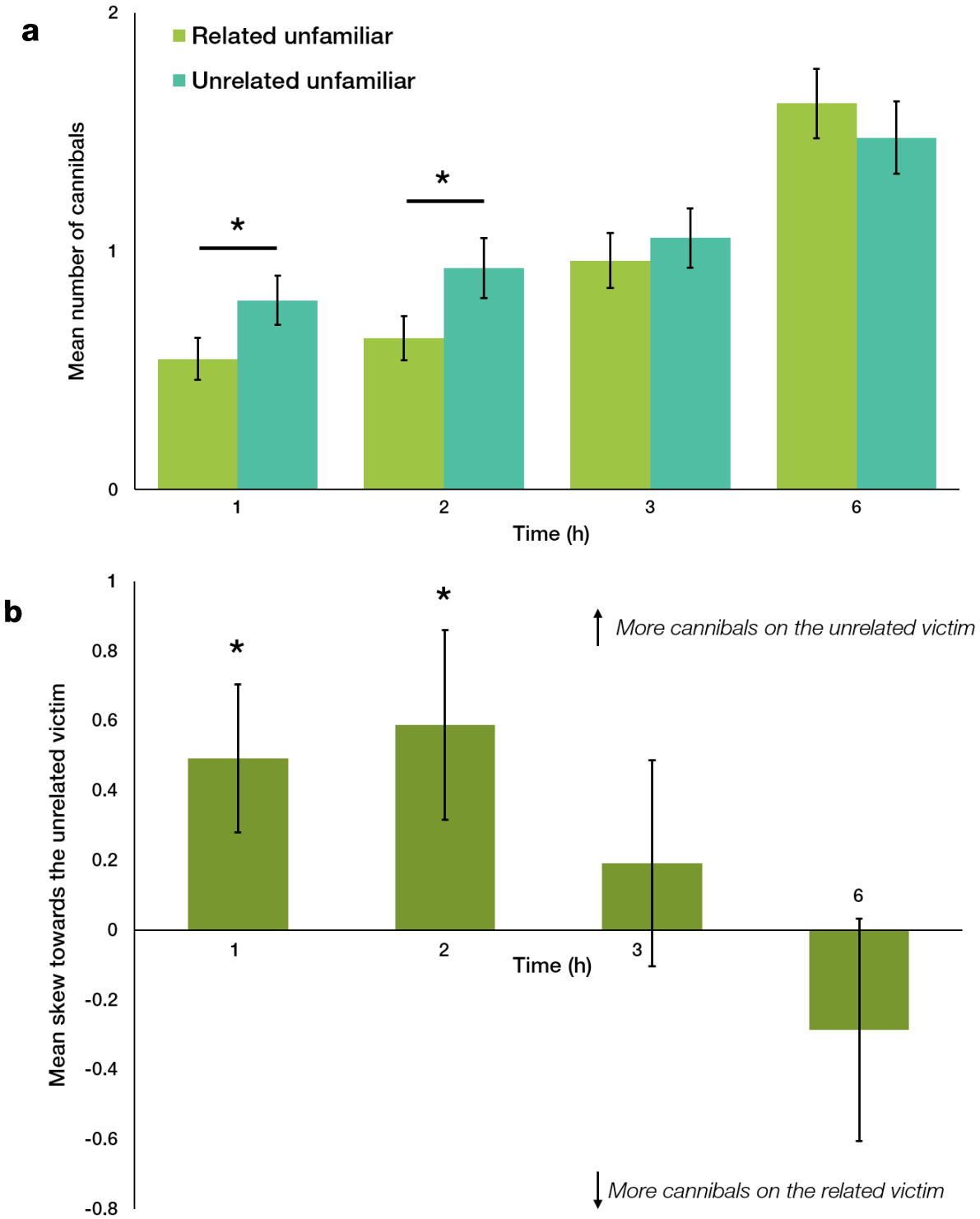
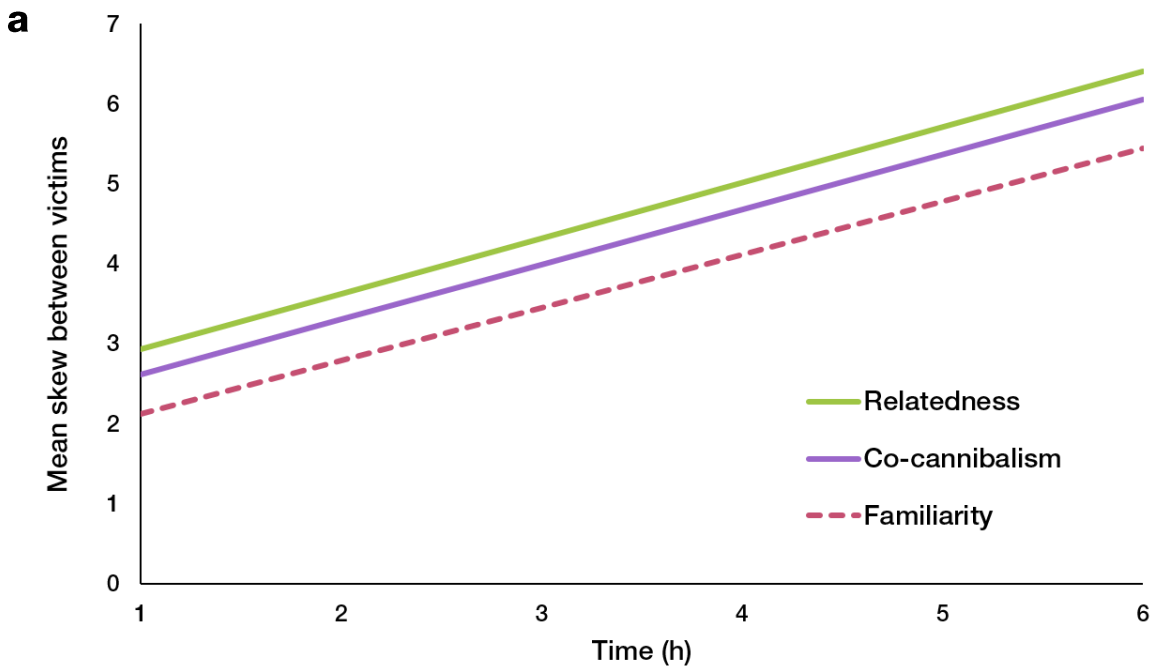


Figure 5.6 – Relatedness experiment: Second instar larvae from one family were more likely to cannibalise an unrelated victim than a related victim for the first two hours (1h: z=2.32, P=0.0204, 2h: z=2.279, P=0.0294) but not after three hours (P>0.37). **a)** Bars show the mean number of second instar cannibals out of twenty on each wandering instar victim. **b)** Skew is calculated as the number of larvae out of a possible twenty on the unrelated unfamiliar victim minus the number on the related unfamiliar victim. N=64, error bars ±SE



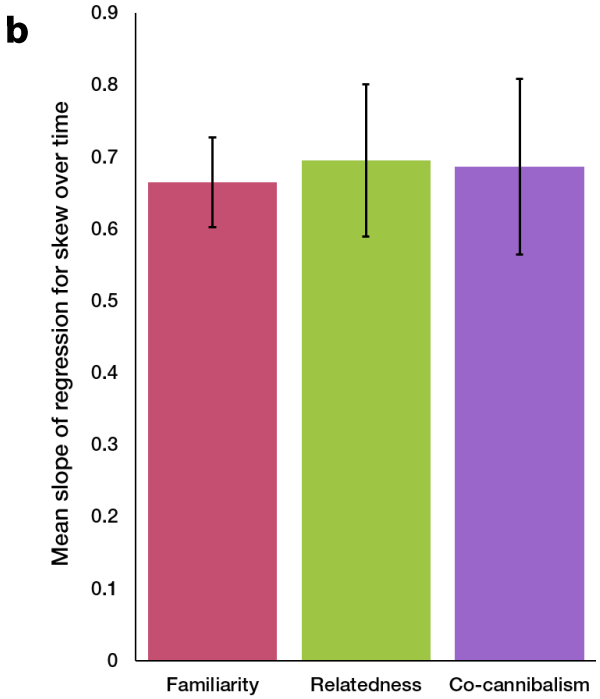


Figure 5.7 – Victim skew: For each plate I took a linear regression of the difference in the number of cannibals between victims (skew) over time and calculate the mean slope and intercept of these regressions. The degree of skew and the increase of skew over time was the same between the relatedness and co-cannibalism experiment (Intercept: P=0.401, Gradient: P=0.935). i.e. in all three experiments, second instar larvae increasingly favoured one victim over time, and the rate at which they favoured one victim over the other was the same in all experiments. Unlike the other two experiments, the familiarity experiment used only one family of cannibals so were not included in the statistical analysis.
a) Lines plot the mean slope and mean intercept in each experiment. **b)** Bars show the mean gradient of regression of skew over time from each plate, error bars ±SE. N=53, 64, 49 for familiarity, relatedness and co-cannibalism experiments respectively.

Discussion

In light of the recent observation of cannibalism in the otherwise non-carnivorous *Drosophila melanogaster* (Vijendravarma, Narasimha and Kawecki, 2013; Ahmad *et al.*, 2015), I tested whether larval cannibalistic behaviours are affected by the relatedness and familiarity of their victims and co-cannibals. I found that second instar larvae prefer to cannibalise unrelated pinned third instar wandering larvae when presented with a choice of an unrelated and related unfamiliar victim (Figure 5.6b). Similarly, cannibalism was higher on unfamiliar victims when presented with a choice of a related familiar and related unfamiliar victim (Figure 5.2b). However, second instar larvae showed no preference or avoidance towards cannibalising alongside other second instar larvae from either their own family or an unfamiliar family (Figure 5.4).

These results support an inclusive fitness view, where larvae avoid reducing their indirect fitness by preferentially cannibalising unrelated or unfamiliar victims over related or familiar victims. This preference is tempered by the preference for the more cannibalised victim. Where these two preferences align, we see an increasing preference for one victim over the other (Figure 5.2b), whereas where these two preferences oppose, we see a decreasing strength of preference over time (Figure 5.6b). Although larvae showed a preference for unrelated and/or unfamiliar victims, as would be expected from inclusive fitness theory, there was still a not insignificant minority of larvae cannibalising the related and/or familiar victims, even only a few hours after removing the cannibals from their food source. However, just because a behaviour appears to be adaptive kin discrimination does not mean that is has evolved for indirect benefits. Perhaps, for instance, under starvation conditions, larvae are attracted to less familiar chemical cues as

seeking novelty may increase their chance of finding an alternative food source. Whilst both victims in this setup were unfamiliar to the cannibals, any genetic component of odour would be less familiar among unrelated individuals.

Larvae prefer to cannibalise unfamiliar victims

The preference we observed towards avoiding cannibalising related familiar victims in favour of related unfamiliar victims is in contrast to the absence of discrimination between related familiar and unfamiliar groups in adults (Carazo *et al.*, 2015; Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015; Martin and Long, 2015; Le Page *et al.*, 2017). The degree of sociality in larvae and adults is genetically decoupled in this species; gregarious behaviours in larvae do not correlate to gregarious behaviours in adults across genetic lines (Anderson, Scott and Dukas, 2016).

The difference we see in discrimination in this study may be explained by fitness consequences – there may be no net benefit to discriminating between familiar and unfamiliar adults whereas there may be a net benefit to this behaviour as larvae – or it may result from different recognition mechanisms at the different life stages. Studies on adult familiarity preferences frequently use larval familiarity, not adult familiarity; and it is quite possible that any larval preferences are subject to change as the individual undergoes metamorphosis.

Relatedness does not affect who cannibalises with whom

We observed that, when presented with two equally unfamiliar and unrelated victims, second instar larvae preferred to cannibalise one larva over the other, however, this preference was not affected by the relatedness of the cannibals to each other (Figure

5.4). Larvae were related and familiar within each cannibal family but were unrelated and unfamiliar to larvae from the other family, therefore it is unlikely that this lack of preference is due to an inability to discriminate between siblings and non-siblings and instead is more likely to suggest the absence of a fitness benefit to discriminating.

Cannibalism in *D. melanogaster* is a costly exercise. Second instar larvae preferentially seek out wandering instar larvae to attack as they are no longer actively feeding and it is hypothesised that the cost of retaliation is therefore lower (Vijendravarma, Narasimha and Kawecki, 2013). However, young larvae, which typically feed on yeast and fruit sugars, are not particularly well adapted to biting through the intact cuticle of another living larva. Cannibalism in this species is therefore a prolonged process, which will occur at the expense of dispersing to find an alternative food source. Though expensive, cannibalism may be still the best option, particularly if the alternative is starvation and death.

With sufficient cannibals, it is possible for the cuticle of the wandering larva to 'burst', leaving the inner contents of the victim more accessible (pers. obs.). One might therefore consider cannibalism to be a 'public good', with each individual benefiting from a damaged cuticle, but not wanting to pay the personal costs of cannibalising the victim. One way to avoid this dilemma is to increase the relatedness of the group, as now each individual also benefits indirectly from the gains of the rest of the group. However, in the present study, the groups of individuals cannibalising a victim were no more related than the local population; both of the two cannibal families were equally represented on each victim. We could test whether the relatedness of the group affects the efficacy of rupturing the cuticle by comparing the time taken for an unrelated, unfamiliar victim to burst with a group of either full siblings or unrelated cannibals.

Cannibalism occurs more frequently under periods of stress when food is less available. It may be that the total loss of direct fitness from starvation makes any cost of cannibalism the least worst option. However, one might imagine a cheating strategy whereby an individual is attracted to a group of cannibals on a victim and reduces their own cannibalistic effort but still benefits from the group effort. The absence of a behaviour in a laboratory adapted population does not preclude the existence of that behaviour in a wild population, and greater temporal and spatial variation in food resources and oviposition may greatly affect the relatedness of groups of co-cannibals. Micro-satellite analysis has shown copulating pairs of wild *D. melanogaster* are more related than by chance (Robinson, Kennington and Simmons, 2012a), and a similar technique on groups of wild cannibals may prove enlightening.

Larvae prefer to cannibalise unrelated victims, but this preference reduces over time as they favour the more cannibalised victim

When we presented two families of cannibals with a victim from each family, the second instar larvae initially preferred to cannibalise the unrelated victims, but this preference disappeared after two hours (Figure 5.6b).

The loss of preference towards unrelated victims may reflect a 'tug-of-war' for a potential cannibal; whether to move towards an unrelated victim with fewer co-cannibals or whether to move towards a related victim with more co-cannibals. As a victim becomes more injured, be this through needle pricks, parasitism or cannibalism, it becomes more attractive to potential cannibals (Vijendravarma, Narasimha and Kawecki, 2013), and this can clearly be seen in the increasing difference or skew between the number of cannibals on each victim in all three experiments (Figure 5.7a). As the skew increases over time, the

potential cannibal's preference for injured victims may outweigh its preference for unrelated victims (Figure 5.8). It may also be that once a relative is sufficiently cannibalised that its chances of survival are bleak, the cannibal no longer avoids cannibalising it, or that as the cannibal becomes increasingly starving, it becomes less exacting in its choice of victim, as seen in other species (Pfennig, Reeve and Sherman, 1993; Taylor and Schmidt, 1994).

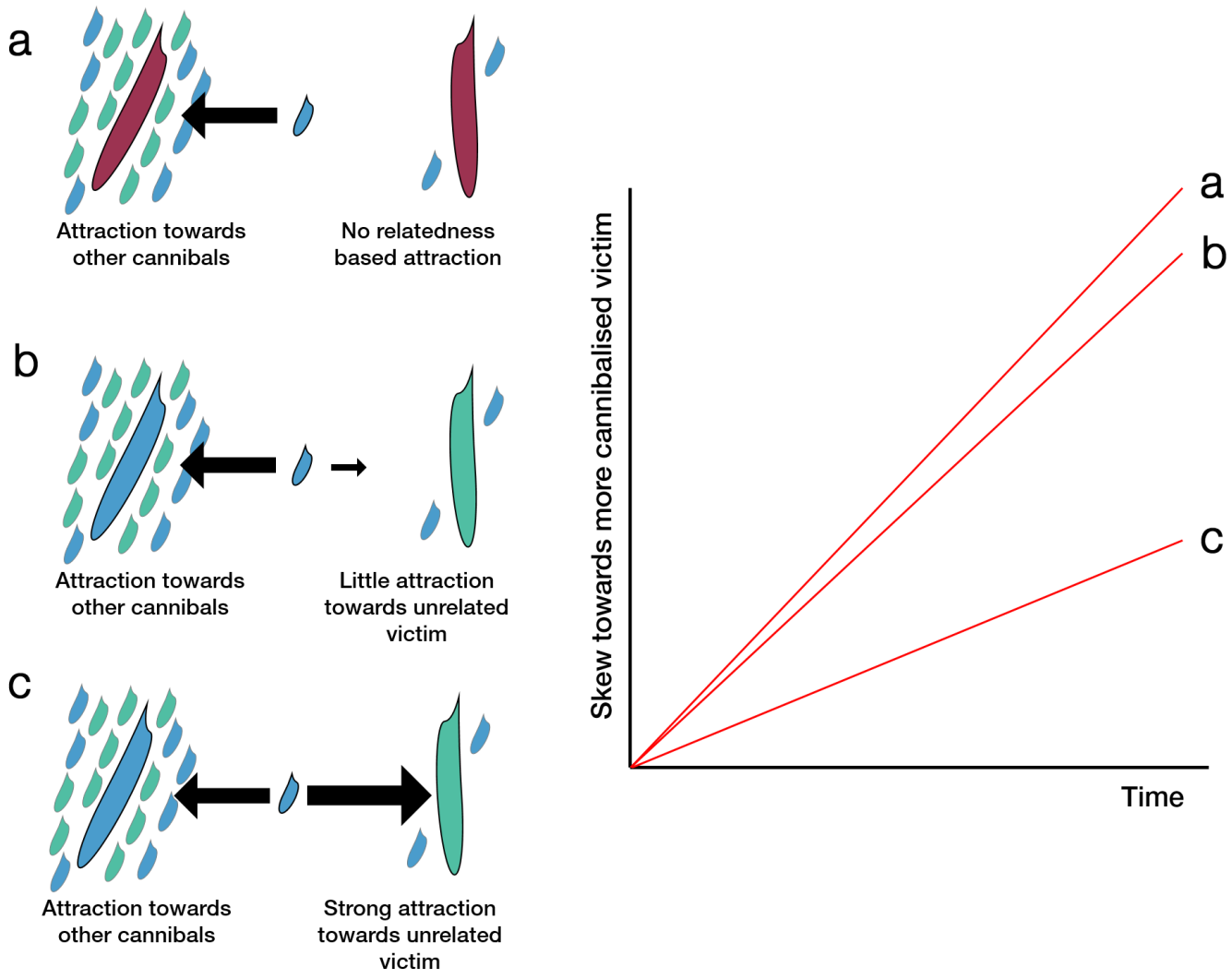


Figure 5.8: Hypothetical effect of tug-of-war between contrasting preferences for unrelated victims and more cannibalised victims on victim skew. In the co-cannibalism experiment **a**, both families are equally unrelated to each victim and there cannot be an effect of relatedness. Therefore, any increase in skew over time represents the strength of larval preference for the more cannibalised victim. The relatedness experiment could either resemble **b** or **c** depending on the strength of attraction for unrelated victims. In **b** the strength of attraction for unrelated victims is low, so there is little opposition to the preference for the more cannibalised victim, thus the increase in skew over time resembles that of **a**. In **c**, there is stronger attraction towards the unrelated victim, providing an opposing preference for those larvae related to the more cannibalised victim. Therefore, the positive feedback loop of larvae joining the more cannibalised victim is less extreme, and the increase in skew over time is less steep.

To test this 'tug-of-war'hypothesis post-hoc, I compared the change in skew over time between the relatedness experiment and co-cannibalism experiment. The two experiments both had 10 second instar larvae from each of two cannibal families and two unfamiliar victims, so differ only in the different relatednesses of the victims. In the co-cannibalism experiment, both victims were equally unrelated to the cannibals and so there should be no relatedness preference in opposition to a preference for more cannibalised victims. In the relatedness experiment, one victim was related and the other unrelated, allowing the two preferences to compete. The skew increased across time at the same rate in both experiments (Figure 5.7a), meaning that cannibals were always attracted to the more cannibalised victim.

In the relatedness experiment, there were two families of cannibals, so each victim was the related victim to half of the cannibals, and the unrelated victim to the other half of the cannibals. Early in the experiment, before the identity of the more-cannibalised victim had time to be established, more larvae were cannibalising the unrelated victim (Figure 5.6b). Later in the experiment, when skew is higher, this preference is no longer seen. As there were no differences in the cannibalism rates between families (Figure 5.5), each victim was equally likely to become the more-cannibalised victim, and for the cannibals related to that victim, preferring the unrelated victim lost the tug-of-war to preferring the more-cannibalised victim (scenario **b** in Figure 5.8). This suggests that whilst larvae prefer to cannibalise unrelated victims, the strength of this preference is weak in comparison to the strength of their preference for more-cannibalised victims.

This may also explain why we see a significant preference for unfamiliar victims only at the 6h timepoint. In the familiarity experiment, all second instar larvae were from the same vial and they would all share the same preference for the same victim. Any initial

preference for the unfamiliar would have been exacerbated by a preference for more-cannibalised victims with a positive feedback effect, with no opposing preference as seen in the relatedness experiment.

The gradient of skew is identical for all three experiments, that is to say that, regardless of the choice of victims, the attraction towards the more cannibalised victim over time remained the same (Figure 5.7b). With this knowledge, it would be interesting to directly manipulate the skew in the number of cannibals on each victim and observe how that affects a focal cannibal's choice between victims of different familiarities and relatednesses.

Experimental design

One limitation with the experimental design of the familiarity experiment is that only the familiar victims were transferred into new vials prior to the observations. Therefore, for the two days before the observations, the familiar victims were competing with predominantly younger larvae in fresh food that had not been liquefied by dense larval feeding. In the other vial, unfamiliar victims were competing with a much wider age range of larvae, which were on average several days older than those in the late vial, and more numerous as the female had been laying in this vial for longer. Having older and more numerous larvae in the vial will have altered the composition and viscosity of the food.

Whilst we might expect the quality of the food to decrease with more larvae feeding on it, there is also an Allee effect in the larval response to density, with survival decreasing at the lowest densities (Wertheim *et al.*, 2002). This reduced survival at very low densities may be both because larvae aggregate more on firmer food and can

collectively feed more efficiently (Durisko *et al.*, 2014) and because higher densities of larvae reduce the proliferation of fungae that would otherwise compete for the same food resource (Rohlfs, 2005). It is possible that whilst I tried to size-match the victims, the larvae from the early vial (unfamiliar larvae) were imperceivably more attractive as a target of cannibalism because of more efficient feeding.

It would be possible to mitigate any handling effects by also transferring the unfamiliar larvae into a new vial with a similarly fresh substrate as the familiar vial. However, it would not be possible for the unfamiliar larvae to be in contact with first and second instar larvae of the same family as the familiar larvae are, as dividing the newly laid eggs between two vials would not leave enough larvae of the appropriate developmental stage for the experiment.

Evolutionary implications

Cannibalism between siblings affects not only the fitness of the cannibal and the victim but also the fitness of the mother. Adults produce an aggregation pheromone that induces aggregated oviposition in *D. melanogaster* (del Solar and Palomino, 1966), thought to potentially inhibit the growth of fungal competitors (Wertheim *et al.*, 2002; Rohlfs, 2005). In the house fly, *Musca domestica*, older larvae cannibalise younger larvae and so whilst adult females similarly co-oviposit, there is a strong selection to avoid ovipositing next to older eggs. Females are able to signal the age of their eggs via a bacterial cue that attracts oviposition at low densities, but transforms into an oviposition repellent as the bacteria proliferate (Lam *et al.*, 2007).

Drosophila melanogaster contrast with *M. domestica* in that it is primarily the younger larvae that cannibalise the older larvae, and as a result there may be selection for

female fruit flies to prevent other females from ovipositing when her own eggs have already started to develop. This is particularly the case if the food patch is likely to decline in quality before her offspring can pupate, and if the rival female's larvae are more likely to target unrelated victims. By the exact same logic, however, it is also in the female's interest to lay her eggs near older eggs if the resource is likely to deplete so that her own offspring can cannibalise those of another family, potentially generating a race to lay the last eggs on a patch. We currently do not know if adult females manipulate their oviposition preferences in response to potential larval cannibalism, nor even whether larval cannibalism substantially affects their own fitness. A crude cost-benefit analysis in Harlequin ladybirds, *Harmonia axyridis* found that offspring cannibalism only reduces the mother's fitness when food was plentiful (Osawa, 1992).

Whilst theoretical models of cannibalism certainly predict cannibalism to increase during food shortages (O'Connor, 1978; Parker, Mock and Lamey, 1989) , cannibalism does not only occur in the absence of food. Sibling cannibalism (in the form of larvae eating eggs) may not only increase larval survival but may also reduce competition or provide a more favourable diet for alternative morphology (Schultner *et al.*, 2014; Bolívar-Silva, Guedes and Guedes, 2017) . Although cannibalism in early instar *D. melanogaster* larvae does increase the number of teeth that develop in later instars (Vijendravarma, Narasimha and Kawecki, 2013), there appears to be no resulting difference in adult morphology beyond body size (Ahmad *et al.*, 2015). However, the role of cannibalism in eliminating competitors and thereby manipulating local population demographics cannot be so easily ruled out in this species and warrants further research.

Future research

Cannibalism is such an intrinsically social behaviour with such extreme fitness consequences that it provides a fertile breeding ground for evolutionary conflict and adaptive trade-offs. The recent establishment in the literature of cannibalism in the model organism *D. melanogaster*, and the findings of this study that larvae can and do discriminate between kin can hopefully provide a model system in which to empirically test these ideas.

A fully factorial choice design of relatedness and familiarity, additionally with olfactory mutants and antibiotics would help isolate the mechanism underpinning these behaviours. Comparing rates of cannibalism between wild populations would help clarify the extent and subtleties of the competing cues that may have been lost in a homogeneous laboratory environment. Finally an experimental evolution approach, evolving larvae in high-cannibalism conditions and competing against low-cannibalism lines might reveal both larval and adult adaptations to this complex behaviour.

CHAPTER 6:

GENERAL DISCUSSION

Each of the previous chapters has its own discussion highlighting the research implications and future directions relevant to each study. Here I will discuss the general themes common to all the research I have presented. The aim of this thesis has been to identify both adult and larval social behaviours in which *Drosophila melanogaster* show kin discrimination, and to begin to elucidate the underlying requirements for this response.

Summary of main results

Male relatedness and familiarity are required to modulate male-induced harm to females

In Chapter 2, I showed that females mated to triplets of males that were related and familiar to each other had higher lifetime reproductive success, slower reproductive ageing, longer reproductive lifespan and longer lifespan. Whilst females suffered less harm when mated to related triplets than unrelated triplets, I only saw this effect of relatedness between males that had been reared together in a familiar larval environment. There was no difference between related and unrelated males that had been reared apart in separate larval environments. I could not detect any differences in male aggression, courtship or mating rates. These data show that relatedness is necessary to mediate sexual conflict, and familiarity alone is not sufficient.

Unrelated males do not retain their reproductive advantage in a sequential mating system

In Chapter 3, I showed that the reproductive advantage experienced by an unrelated male competing for mates against brothers did not exist when males were presented to the female sequentially, rather than simultaneously. There was no difference in mating duration, latency to remating, paternity share, number of offspring sired by the focal male or female fecundity between a triplet of unrelated males or a triplet of two brothers and one unrelated male. This suggests that male-male interactions are required to mediate the unrelated male advantage.

Larvae develop faster and have higher survival rates in unrelated groups

In Chapter 4, I showed that larvae develop faster to pupation and eclosion in unrelated groups than in related groups and have higher egg-to-pupal and egg-adult survival in unrelated groups with the greatest quantity of food. There were no differences in male or female adult body mass between related and unrelated groups. This suggests that larvae in unrelated groups are benefiting from the increased niche partitioning and reduced competition predicted by the 'elbow-room hypothesis'.

Larvae prefer to cannibalise unrelated and unfamiliar victims

In Chapter 5, I showed that second instar larvae prefer to cannibalise wandering instar larvae that are unrelated to them over related victims when both victims are unfamiliar. When both victims are related, larvae preferred to cannibalise unfamiliar victims over victims with whom they had grown up in the same vial. However, the relatedness of the other second instar cannibalising a victim did not influence their preference of victim.

Future directions

Proximate mechanisms in *Drosophila* kin discrimination

Now that my research has provided evidence for kin discrimination in both adult and larval *Drosophila melanogaster*, the next important step will be to identify the proximate mechanisms by which flies can detect the relatedness of other individuals. The

primary candidate for a recognition system is cuticular hydrocarbons (CHCs); a diverse suite of chemicals on the insect's surface that protect against desiccation but have also been implicated in olfactory individual recognition across a range of insect species (Stanley-Samuelson and Nelson, 1993; Ferveur, 2005; Blomquist and Bagnères, 2010).

In *D. melanogaster*, the CHC profile is affected by genetics (Ferveur and Jallon, 1996), the social environment such as mating (Everaerts *et al.*, 2010), and gut microbiota (Lizé, McKay and Lewis, 2013; Lewis and Lizé, 2015). Gut microbes are transmitted from mother to offspring via the chorion (Bakula, 1969; Markow and O'Grady, 2008), which larvae eat as soon as they hatch. This makes gut microbes a partially heritable component of CHC profile, along with genetic factors.

The first step to determining whether flies use CHCs to discriminate between kin would be to use flies lacking the ability to detect olfactory cues and determine whether they exhibit the same response to relatedness. One of the benefits of using the model organism *Drosophila melanogaster* is the ready availability of mutant strains, such as the olfactory mutant lacking *odorant receptor co-receptor*, *Orco* (Larsson *et al.*, 2004). Previous work has used *Orco* mutants to demonstrate the role of the olfactory sense in mediating adult responses to mates (Tan *et al.*, 2013). If *Orco* mutants do not display the same responses as the wild type flies in the experimental designs from Chapters 2 and 5, this would strongly suggest that olfaction is a critical sense in mediating kin discrimination. If *Orco* mutants do display the same response, this could either be because flies are using a set of redundant cues to determine relatedness, of which olfaction is only one (Bretman *et al.*, 2011), or that olfaction is not used to detect relatedness.

If olfaction does mediate flies' response to relatedness, we can isolate the CHC profiles of related unfamiliar individuals and unrelated familiar individuals using a solvent

such as hexane and performing gas chromatography mass spectrometry on the solutes (Ferveur, 2005). I showed in Chapter 2 that adults discriminate between related familiar and related unfamiliar individuals. Thus, the CHC profiles of these two groups must be distinct if CHCs are to be the proximate mechanism, and similarly with related and unrelated familiar individuals. If there are no differences in the spectrographs, it is unlikely that CHCs are the method of kin discrimination in adult *D. melanogaster*.

Another advantage of using *Drosophila melanogaster* as a model organism for studying kin selection is the ability to artificially manipulate both the CHC profile and microbiome of flies. By adding low doses of antibiotics such as streptomycin to the food we can remove the microbiome (Heys *et al.*, 2018). Previous studies have reintroduced individual bacterial species to axenic flies (Venu *et al.*, 2014), and it should thus be possible to inoculate axenic eggs or larvae with the bacterial colony of another related or unrelated individual. If flies show the same response to unrelated individuals with the microbiome of related individuals as they do to related individuals, this would strongly implicate the microbiome in the kin recognition response.

From the lab to the field

Many of the experiments in this thesis and those suggested above are only possible because *Drosophila melanogaster* has been so well studied for the over a century (Bellen and Yamamoto, 2015). As such, we have characterised many of its behaviours and underlying genetic mechanisms, have a library of mutant strains readily available, and have optimised rearing conditions in laboratory conditions. The single greatest benefit of using a model laboratory organism is the tractability of the system, and the relative ease with which one can manipulate a single variable, *ceteris paribus*.

However, the single greatest limitation of my findings is that they were produced in a laboratory system and as such we do not know the extent to which they are applicable to wild *D. melanogaster*. The Dahomey strain used in this thesis was first collected in present-day Benin in 1970 (Partridge and Farquhar, 1983). Assuming a 12 day egg-to-egg lifecycle, this population has been adapting to laboratory conditions for over 1400 generations. Many of the behaviours we observe will be the product of selection under artificially dense populations with little to no environmental stochasticity, no natural predators, limited dispersal and highly simplified spatial environments. This therefore limits our ability to make inferences from laboratory *D. melanogaster* and apply them to wild animals with such different ecologies.

One compromise that is being developed across a number of laboratories is to study *Drosophila* in spatially complex yet captive environments to more closely mimic the habitat of wild *Drosophila*. This allows us to study the role of environmental complexity, resource patchiness, and abiotic fluctuations while retaining the practical benefits of working with laboratory organisms, such as the ease of tracking focal individuals over their lifespan. Such work has produced novel findings. Previously the benefit to a female of being highly fecund appeared to trade off with the increased harassment and male-induced harm from being the most attractive female. In slightly more complex, but still unrealistic, arenas, the frequency of sexual encounters was reduced, reducing sexual conflict and the cost of attractiveness to females (Yun *et al.*, 2017; MacPherson *et al.*, 2018).

Many of my findings have implications for the evolution of dispersal and fine-scale population structure. For example, larvae develop better in unrelated groups and females might therefore choose to oviposit gregariously, however, cannibalism is more frequent

between an unrelated cannibal and victim, so the temporal longevity of the oviposition site may be as important as the social status of the site. We cannot simply take flies from the wild that are adapted to these more spatially and temporal habitats and test them in an artificial environment, as these naïve populations will display non-adaptive behaviours. However, we could experimentally evolve populations under different habitat regimes and test whether the kin discrimination behaviours still hold across populations.

Finally, it is possible to study wild *Drosophila melanogaster* directly, although such studies are largely observational rather than manipulative. One crucial piece of knowledge we are lacking is the genetic structure of wild populations. Microsatellite analysis has been used to show that mating pairs of wild *D. melanogaster* are more related to each other than to the population (Robinson, Kennington and Simmons, 2012a) and that wild females mate multiply with 4-6 males (Imhof *et al.*, 1998). With the ever-decreasing cost of sequencing, similar techniques could be used to measure the relatedness of larvae within and between patches, such as apples in an orchard. This would provide information on the relative likelihood of an adult encountering a related/unrelated and familiar/unfamiliar individual, and therefore whether it would be beneficial for discriminatory behaviours based on relatedness and familiarity to evolve in wild populations.

Drosophila as a model organism for testing kin selection

Kin selection has been a fruitful field of research for over half a century. During this time, a large proportion of empirical research has focussed on Hymenoptera, whether to test eusociality (Queller and Strassmann, 1998) or local mate competition (Herre, 1985), or bacteria, to test cooperation (Griffin, West and Buckling, 2004) and spite (Inglis *et al.*,

2009). However, our ability to manipulate the Hymenopteran genome is limited, and bacteria cannot be used to test many complex behaviours of multicellular organisms. There have been numerous studies on social behaviour in wild vertebrates (Clutton-Brock, 2002), but these are largely observational and there is a limit to how much we can ethically manipulate these species.

In contrast, *Drosophila melanogaster* has been used as a model organism across large swathes of biological research, from genetics to proteomics to neuroscience to behaviour. We not only understand many aspects of its biology, but have also already developed the tools and techniques to artificially manipulate them (Gramates *et al.*, 2017). In this thesis, I have shown that *D. melanogaster* demonstrate not only the ability to distinguish between kin and non kin, but also that they behave differently towards individuals of different relatednesses. We can now develop theoretical models in synergy with empirical designs, making full use of the advantages of *Drosophila melanogaster* as a model organism.

Concluding remarks

Drosophila melanogaster was once thought to have evolved in populations too crowded and too unstructured to evolve kin discriminatory behaviours (Chippindale *et al.*, 2015; Hollis, Kawecki and Keller, 2015). Very little was known about social behaviours at the larval stage, and even less about whether relatedness mediated these larval behaviours. In this thesis, I have provided new evidence that relatedness, over and above just environmental familiarity, plays a role in both adult and larval social behaviours. This opens up the species to further study into social behaviour and provides a new model organism for testing ideas about kin selection.

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APPENDIX 1

Chapter 2 as published in Proceedings of the Royal Society, B.

PROCEEDINGS B

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Research

Cite this article: Le Page S, Sepil I, Flintham E, Pizzari T, Carazo P, Wigby S. 2017 Male relatedness and familiarity are required to modulate male-induced harm to females in *Drosophila*. *Proc. R. Soc. B* **284**: 20170441. http://dx.doi.org/10.1098/rspb.2017.0441

Received: 2 March 2017
Accepted: 10 July 2017

Subject Category:
Evolution

Subject Areas:
behaviour, evolution, ecology

Keywords:
sexual conflict, sexual selection, kin selection, inclusive fitness, social behaviour, *Drosophila*

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3832528.

THE ROYAL SOCIETY PUBLISHING

Male relatedness and familiarity are required to modulate male-induced harm to females in *Drosophila*

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Males compete over mating and fertilization, and often harm females in the process. Inclusive fitness theory predicts that increasing relatedness within groups of males may relax competition and discourage male harm of females as males gain indirect benefits. Recent studies in *Drosophila melanogaster* are consistent with these predictions, and have found that within-group male relatedness increases female fitness, though others have found no effects. Importantly, these studies did not fully disentangle male genetic relatedness from larval familiarity, so the extent to which modulation of harm to females is explained by male familiarity remains unclear. Here we performed a fully factorial design, isolating the effects of male relatedness and larval familiarity on female harm. While we found no differences in male courtship or aggression, there was a significant interaction between male genetic relatedness and familiarity on female reproduction and survival. Relatedness among males increased female lifespan, reproductive lifespan and overall reproductive success, but only when males were familiar. By showing that both male relatedness and larval familiarity are required to modulate female harm, these findings reconcile previous studies, shedding light on the potential role of indirect fitness effects on sexual conflict and the mechanisms underpinning kin recognition in fly populations.

1. Introduction

The evolutionary strategies that maximize female fitness may simultaneously hamper male fitness and *vice versa*, generating sexual conflict over reproductive decisions [1–3]. This conflict often arises because intense competition among males over access to mating and fertilization opportunities can harm females (i.e. reduce their fitness). Such harm has been likened to a tragedy of the commons [4–7], in which selfish exploitation results in the depletion, or even destruction, of a shared resource. Male harm of females may occur through a number of pathways including sexual harassment, sexual coercion, traumatic insemination, male accessory gland products, pathological polyspermy and infanticide [2]. In all these cases, sexual selection promotes male strategies even if they happen to harm females in the process (i.e. collateral female harm), or precisely because they harm females (e.g. [8]). Male harm of females is emerging as an important factor in population ecology and evolution, as increasing evidence indicates its role in a number of fundamental processes, such as dispersal [9], population extinction [10] and intersexual coevolution [2]. However, the mechanisms underpinning the variation in the severity of female harm observed across species and populations remain little understood.

Recent theoretical work has suggested that indirect fitness effects might play a key role in modulating male harm to females [6,11–14]. This happens

whenever males tend to compete with males to whom they are more genetically related than the population average, for example when population viscosity limits dispersal and competition is not exclusively local [14,15]. In this context, a male may indirectly reduce his own inclusive fitness by harming females that could also reproduce with his male relatives, and this is expected to relax male–male competition and selection for male traits that harm females [6,11–14].

While the expectation that, under some circumstances, within-group male relatedness reduces the intensity of intra sexual competition has received empirical support (e.g. [16–19]), the notion that within-group male relatedness might also reduce female harm is only beginning to be investigated. Consistent with this idea, female least killifish, *Heterandria formosa*, died younger and produced progressively smaller offspring when experimentally mated to males that are unrelated to each other, compared with females mated to males highly related to each other (but always unrelated to the female; [20]). Similarly, female bulb mites, *Rhizoglyphus robini*, laid more eggs over a 2-day period when paired for 5 days with males that had experimentally evolved in populations comprising their full siblings than when paired with stock males [21].

The influence of male relatedness on female harm has also been explored in the fruit fly, *Drosophila melanogaster*. Carazo *et al.* [22] found that females had higher lifetime reproductive success and slower reproductive ageing (a more gradual decline in fecundity and fertility with age) when exposed to a triplet of brothers that were unrelated to the female but had been raised together as larvae than when exposed to a triplet of males that were unrelated to each other and had been raised separately as larvae. These patterns have now been explored by different research groups and in different *D. melanogaster* populations [23–26] resulting in some studies reporting results consistent with Carazo *et al.*'s findings and others reporting no effects (summarized in electronic supplementary material, table S1), suggesting that these effects are not entirely consistent and that they might be modulated by other mechanisms.

One such mechanism might be familiarity. Hollis *et al.* [24] identified larval familiarity among males as a requirement for reduced harm to females. By introducing a new treatment in which females were exposed to males that were related to each other but raised apart as larvae, this study showed that males were only benign to females when they were related and raised together as larvae. These results are consistent with larval familiarity acting as a kin recognition mechanism, as demonstrated in other taxa [27–32]. In principle, these results may also indicate that male flies might have evolved to reduce female harm strategically in response to male familiarity *per se*, independently of relatedness, through direct (rather than indirect) fitness effects [24]. For example, mechanisms such as reciprocity might reduce competition among familiar males, and this may in turn reduce female harm.

A scenario in which variation in male harm is entirely predicted by relatedness, not familiarity, would suggest that flies use genetic cues to recognize kin and reduce harm in the presence of relatives to gain indirect fitness benefits. A scenario in which variation in male harm is entirely predicted by male familiarity, not relatedness, would be consistent both with the idea that direct benefits associated with familiarity drive changes in female harm, and the idea that female harm is driven by indirect effects, whereby flies may rely entirely on familiarity cues to recognize kin. Finally, variation in male harm may be predicted by the interaction between relatedness and familiarity cues. For example, indirect fitness effects may reduce male harm to females when males are related, but male flies may only be able to recognize relatives under familiarity [33]. However, because no study has tested the fully factorial combination of relatedness and familiarity [22–26], the relative roles of these factors remain unresolved.

In this study, we conducted an experiment using a novel, fully factorial design to isolate the separate effects of relatedness, familiarity (shared larval environment) and their interaction on male sexual behaviour (as measured through assays of male–male aggression, courtship and mating rates) and female harm (as measured through female lifetime reproductive success, reproductive ageing, lifespan and reproductive lifespan) in *D. melanogaster*. We used four different social environments in which males were: (i) related and familiar, (ii) related and unfamiliar, (iii) unrelated and familiar and (iv) unrelated and unfamiliar. While we found no effect on male behaviours, we did observe an interaction between male relatedness and larval familiarity, thereby showing that larval familiarity alone is insufficient to reduce harm to females. Male relatedness increased female reproductive success, lifespan and reproductive lifespan, and slowed reproductive ageing, but only when males were familiar.

2. Methods

(a) Stock cultures

We used a laboratory-adapted, wild-type Dahomey stock of *Drosophila melanogaster*, maintained in large, outbred populations since 1970 [34,35] at 25°C in a non-humidified room and a 12 : 12 h light : dark cycle. This is the same stock used by Carazo *et al.* [22,23]. All flies were maintained in cages containing bottles of Lewis medium [36] with overlapping generations.

(b) Male treatments

We produced triplets of males belonging to one of four treatments generated from a fully factorial cross of relatedness and familiarity in the larval environment: related and familiar, related and unfamiliar, unrelated and familiar, and unrelated and unfamiliar (figure 1).

To generate each experimental male triplet, we created families using parents that were 2 days post-eclosion, and had been collected as eggs from the stock population and reared at standard larval density at 25°C [34]. We paired a single virgin male and female for 12 h in individual larval collection chambers containing a Petri-dish filled with hard grape agar (550 ml water, 25 g agar, 300 ml grape juice concentrate and 21.25 ml 10%w/v Nipagin) with a smear of live yeast paste, before discarding the males. Twenty-four to thirty-six hours after egg laying, we picked larvae with a mounted needle into 36 ml vials containing 8 ml of Lewis medium, collecting 60 larvae in total per family over a period of 3 days. Any families that failed to produce 60 larvae were excluded.

From each of 135 families, 45 larvae were divided equally among three 'single family' vials and 15 larvae were distributed individually among each of 15 'mixed family' vials. Thus, each 'single family' vial contained 15 larvae from a single family, and each 'mixed family' vial contained 15 larvae from 15 randomly allocated families (figure 1). These vials were kept at 18°C and adult virgin males were collected within 16 h of eclosion.

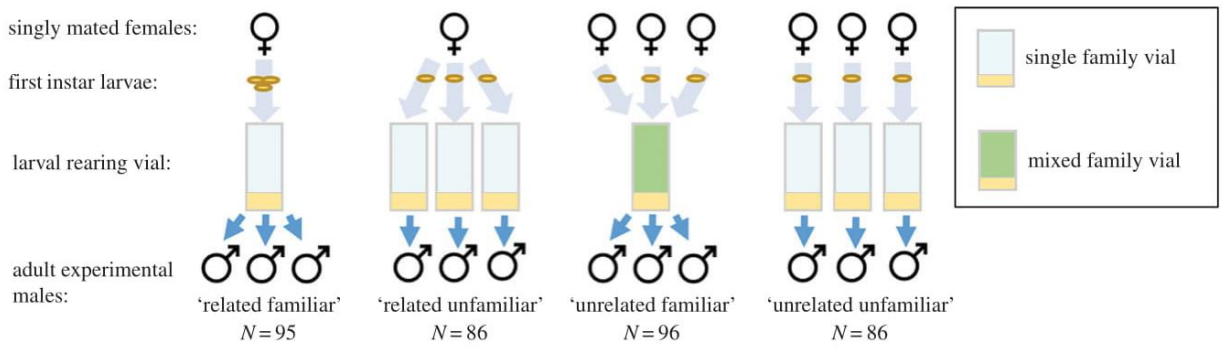

Figure 1. Scheme of how we generated the four male treatments. Each rearing vial contained 15 larvae, either all 15 from one singly mated female (single family vial) or one larva from each of 15 singly mated females (mixed family vial). We collected adult virgin males from these rearing vials, which were immediately housed in their experimental triplets: 'related familiar', 'related unfamiliar', 'unrelated familiar' or 'unrelated unfamiliar'.

Virgin males were immediately aspirated and housed in vials of Lewis medium and excess live yeast grains at 18°C in their experimental triplets: 'related familiar', 'related unfamiliar', 'unrelated familiar' and 'unrelated unfamiliar'. 'Related familiar' comprises three males collected from the same 'single family' vial. 'Related unfamiliar' comprises one male taken from each of the three 'single family' vials of the same family. 'Unrelated familiar' comprises three males taken from the same 'mixed family' vial. 'Unrelated unfamiliar' comprises one male taken from each of three 'single family' vials of three different families. No family contributed to more than one vial of each related familiar and related unfamiliar treatments, and families were randomly assigned so that each had an equal contribution to the unrelated familiar and unrelated unfamiliar treatments. Two days before the introduction of females, males were transferred to fresh vials and kept at 25°C. To produce virgin females, we reared eggs from the cage population at 18°C at standard density (approx. 250 flies per 75 ml bottle containing 45 ml of Lewis medium) in parallel with male collection, collected adult females under ice anaesthesia and aged them at 25°C in individual yeasted vials for 3 days before the start of the experiment.

We performed the experiment across two blocks, producing a combined total of 95 related familiar triplets (39 in block 1, 56 in block 2), 86 related unfamiliar triplets (37 in block 1, 49 in block 2), 96 unrelated familiar triplets (22 in block 1, 74 in block 2) and 86 unrelated unfamiliar triplets (33 in block 1, 53 in block 2). Differences in sample sizes across treatments are due to some flies escaping and stochastic variation in the number of adult males emerging in each family vial within the short collection period.

(c) Behavioural observations

On day 1, we added a single virgin female to each male triplet in a randomly numbered vial to blind the observer to the treatment throughout data collection. On days 2, 3, 4, 5, 8, 9, 10, 11 and 12, we observed the vials during eight scans in the morning (only seven scans on day 2, block 1), 10–20 min apart and recorded whether any males were displaying aggression [37], courtship [38] and mating behaviours. Note that in Carazo *et al.* [22], triplets of males were replaced at regular intervals to prevent males co-ageing with the female. The set-up we used to generate unrelated familiar males prevented us from replacing males during the experiment, therefore males were allowed to age with the female in this study, and as such, we did not expect a similarly strong level of female harm as reported in [22].

Flies were transferred to fresh vials under light CO_2 anaesthesia on days 3, 5, 8 and 11 in both blocks and additionally on day 15 in block 2, and the vials were retained to collect adult offspring (see Fitness measures). Vials were discontinued upon the female's death, and we recorded the day of death up to day 15 in block 1 and up to day 19 in block 2 after which time any remaining females (6% across both blocks) were censored. We also censored vials in the event of male death (four related familiar vials, one related unfamiliar vial, one unrelated familiar vial, one unrelated unfamiliar vial) or flies escaping during handling (one related familiar vial, one related unfamiliar vial).

(d) Fitness measures

Vials containing the offspring of experimental groups were reared at 25°C for 16 days, allowing sufficient time for offspring to develop to the pupal or adult stage, when they were then frozen. To account for different egg-to-adult development times between vials, we counted adult flies and pupae that had reached the P13 phanerocephalic pupal phase [39], identified by the black wing colour, and included both in offspring counts.

(e) Statistical analysis

Survival models were performed using JMP [40]. All other models were performed using the MASS package [41] in R [42] using type III sums of squares to calculate *p*-values. For all analyses, we included block—and all interaction terms that include block—as fixed effects. In all cases, the interaction terms that include block were not significant (electronic supplementary material, table S2), so we removed these terms from the models and kept block as a fixed main effect. While we know which families contributed to the related familiar, related unfamiliar and unrelated unfamiliar treatments, our experimental design makes it impossible to know the family identities of flies in the unrelated familiar treatment. As our knowledge of family identity is confounded with treatment, we were not able to include family identity in the full model analysis.

For aggression and courtship, we analysed the number of scans per day in which the behaviour was observed with a binomial penalized quasi-likelihood GLMM [43], with relatedness, familiarity and their interaction, and block as fixed effects, and day within vial ID as a nested random effect. For mating rate, we analysed whether or not a mating was observed for each day using a binomial penalized quasi-likelihood GLMM with relatedness, familiarity and their interaction, and block as fixed effects, and vial as a nested random effect.

For female reproductive success, we analysed the total number of offspring produced during the experiment. Only 27 of the 357 females were still reproducing at the end of the experiment, and short-term reproductive success is known to be a strong predictor of long-term reproductive success in this species [44]. Therefore, our measurements of reproductive success during the experiment can be considered a very close estimate of lifetime reproductive success. Vials in which a male died

before the death of the female were excluded from this analysis. We analysed lifetime reproductive success with a quasi-Poisson GLM with relatedness, familiarity and their interaction, and block as fixed effects. For female reproductive ageing, we divided the number of offspring laid in each time period by the number of days in that period to create an estimated daily offspring measure that accounts for the differing number of days in each time period. Vials in which a male died before the death of the female were right-censored in this analysis. We analysed daily offspring estimates with a Poisson penalized quasi-likelihood GLMM with relatedness, familiarity and day and their interactions, and block as fixed effects, and the vial ID as a random effect.

To estimate female reproductive lifespan, we used the last day of the last time period in which the female reproduced. We fitted proportional hazards models for female lifespan and female reproductive lifespan, with relatedness, familiarity and their interaction, and block as fixed effects. Vials were right-censored in the analysis when male death, male escape or the end of the experiment preceded female death or the end of reproduction.

3. Results

(a) Male behaviour

The frequency of male–male aggression, courtship and mating were not significantly affected by male relatedness, larval environmental familiarity or their interaction (electronic supplementary material, tables S3 and S4).

(b) Female harm

Female lifetime reproductive success was significantly increased by relatedness among male triplets ($t_{349} = -2.1$ $p = 0.034$), but there was no significant effect of familiarity ($t_{349} = -0.97$, $p = 0.33$) and no significant interaction ($t_{349} = 1.40$, $p = 0.16$; figure 2). To further investigate the possibility of an interaction, we ran the same model on the familiar and unfamiliar halves of the dataset separately. Females had a higher lifetime reproductive success when housed with related familiar males than unrelated familiar males, but this effect was marginally non-significant ($t_{184} = -1.9$, $p = 0.053$). However, there was no effect of relatedness when comparing the lifetime reproductive success of females exposed to related unfamiliar and unrelated unfamiliar males ($t_{168} = -0.123$, $p = 0.90$).

There was a significant effect of the interaction between relatedness and day on female reproductive ageing ($t_{895} = -3.14$, $p = 0.0017$), whereby the age-specific offspring production of females housed with unrelated male triplets declined faster than that of females housed with related male triplets. There was no significant interaction between familiarity and day ($t_{895} = -0.74$, $p = 0.46$), nor between relatedness, familiarity and day ($t_{895} = 1.04$, $p = 0.30$), on daily offspring production (electronic supplementary material, figure S2). Again, we ran the model on the familiar and unfamiliar datasets separately. When comparing familiar treatments, the interaction between relatedness and day remained significant ($t_{463} = -3.23$, $p = 0.0013$), with females ageing faster when housed with unrelated triplets. When comparing unfamiliar treatments, there was no significant interaction between relatedness and day ($t_{432} = -1.53$, $p = 0.13$).

There was a significant interaction between relatedness and familiarity on female reproductive lifespan (Wald $\chi^2 = 5.34$, $p = 0.021$; figure 3), whereby females housed with related familiar males reproduced for longer periods than females housed with unrelated familiar males (risk ratio = 1.37, $p = 0.041$; electronic supplementary material, table S5). The interaction between relatedness and familiarity also had a significant effect on female lifespan ($\chi^2_1 = 4.76$, $p = 0.029$; figure 3), with a marginally non-significant trend for females housed with related familiar males to live longer than those housed with unrelated familiar males (risk ratio = 1.322, $p = 0.069$; electronic supplementary material, table S5).

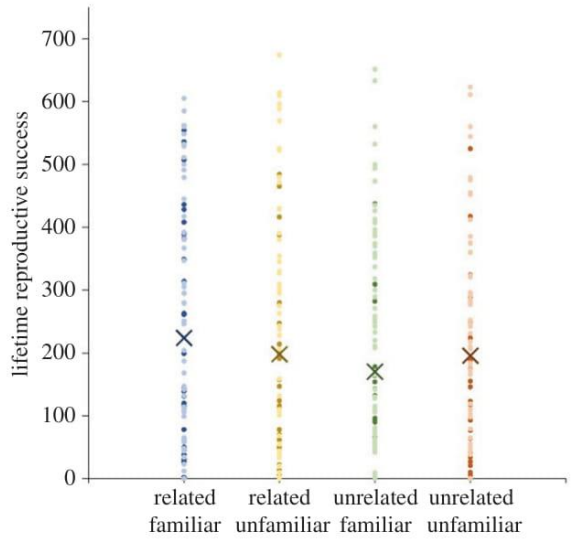

Figure 2. The effect of male relatedness and larval familiarity on female lifetime reproductive success. Points show the total number of offspring laid by each female during the experimental period that reached adult and P13 pupal stage from the first experimental block (dark points), the second experimental block (light points) and predictions from the generalized linear mixed model (crosses). Females mated to triplets of males that were related produced significantly more offspring than those mated to triplets of unrelated males ($p < 0.05$). There was no difference in lifetime reproductive success between females mated to triplets of familiar and unfamiliar males ($p > 0.05$).

Taken together, these results indicate that female harm is minimized when females are exposed to triplets of males that are both related and familiar to each other.

4. Discussion

The role of relatedness in sexual selection and sexual conflict has attracted increasing interest, given the potential for indirect fitness effects in structured populations [6,14,45]. An important challenge in this context is to disentangle the role of relatedness from that of social familiarity. Our results provide support to previous findings from some *D. melanogaster* populations, indicating that related familiar males are less harmful to females. Importantly, the use of a fully factorial design enables the present study to show that both genetic relatedness and familiarity during development are required for any modulation of male harm to females in our population of *D. melanogaster*.

The present study found that females housed with triplets of full brothers show a small but significant increase in reproductive success and slower reproductive ageing than females housed with triplets of unrelated males. While there was no significant interaction between male relatedness

and familiarity, this reduction in female harm due to relatedness was only apparent when comparing related and unrelated males in socially familiar treatments. Were familiarity not to play any role in mediating lifetime reproductive success, we would expect to see an effect of relatedness in both the familiar and unfamiliar halves of our data. Therefore, these results suggest that, despite the lack of a significant interaction, familiarity may play a role in mediating the effect of male relatedness on female lifetime reproductive success.

Consistent with this, male relatedness interacted with familiarity to affect both female reproductive lifespan and female lifespan: females both reproduced and survived for longer when housed with related familiar males than with unrelated familiar males, while there was no significant difference attributable to relatedness in the unfamiliar treatments. The role of relatedness can be seen clearly in figure 3: without an effect of an interaction between relatedness and familiarity, we would expect the lines to be flat (no effect of relatedness nor an interaction) or parallel (no interaction). The statistical significance of the effects above was generally weak, and thus some caution should be applied in their interpretation.

Similarly, neither male relatedness nor familiarity affected the rates of male–male aggression, courtship or mating, which seems to contradict previous findings [22,23,26]. The most likely reason for this is that the experimental design of the present study prevented us from replacing the males at regular intervals as in previous studies [22,24–26], and as such, we could not minimize the effects of co-ageing. Male co-ageing with the females is bound to underestimate differences in harm to females across treatments, because males in treatments where they are more harmful to females are also expected to age more quickly (and hence deteriorate faster). Elevated male ageing in high-harm treatments would tend to equalize the levels of female harm across treatments with time, and particularly so towards the end of their lifespan. Thus, our estimates of both overall female harm levels and treatment differences are conservative.

Collectively, these results indicate that, at least in the population we studied, within-group male relatedness plays a role in modulating male harm of females, in consort with familiarity. For all measures of female harm, females experienced the least harm when exposed to males that were both related and familiar. This is consistent with the hypothesis that indirect fitness effects contribute to explain reduced female harm when local male competitors are related to each other. For example, a focal male may be selected to invest less in competition with rival males, and be less harmful to females, if his rivals are more genetically related to him than to the population average and these females are likely to reproduce with his relatives. This is because of the indirect fitness the male would gain via the increased reproductive success of his male relatives, who would experience both less competition for fertilizations and have more fecund mates, and thus gain higher reproductive success. This would reduce both male–male competition and sexual conflict [12]. Males in our study appear capable of discriminating between individuals on the basis on kinship to adopt a less competitive and less harmful strategy with brothers and females, respectively. Crucially, however, we now show that male flies can only do this when raised together as larvae.

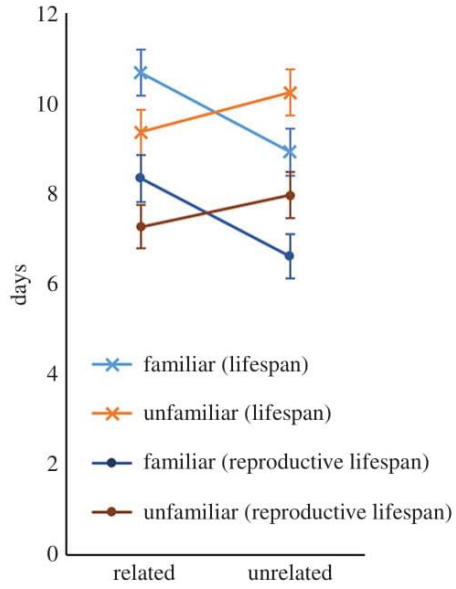

Figure 3. The effect of male relatedness and larval familiarity on female lifespan and reproductive lifespan. The mean number of days from the start of the experiment until the female died (lifespan; crosses) and the mean last day on which the female reproduced (reproductive lifespan; circles), with error bars representing $\pm$ one standard error. The last day of reproduction was estimated as the last day of the last time period in which the female reproduced. The interaction between relatedness and familiarity was significant for both lifespan and reproductive lifespan ($p < 0.05$).

These new results help reconcile those of previous studies. Specifically, Carazo *et al.* [22] compared related familiar and unrelated unfamiliar treatments and emphasized the role of male relatedness. Subsequently, Hollis *et al.* [24] added a related unfamiliar treatment, and by showing that related and unrelated males behave the same when unfamiliar to each other, they suggested that harm to females was driven by male–male familiarity. By using a fully factorial experiment, we show that both previous studies capture different aspects of a complex social behaviour: male flies do adjust female harm in response to the relatedness of their rivals, but only under conditions of larval familiarity. The results of the present study therefore also shed light on the proximate mechanisms of kin recognition in *D. melanogaster*. There is some evidence that female *D. melanogaster* preferentially mate with their own relatives [46–48]. In addition, there is evidence that both males and females recognize whether a new partner is related or unrelated to a previous partner (genetic familiarity) [49]. This latter result suggests that kin recognition has the potential to be at least partly based on genetic cues in this species.

Two possible, non-mutually exclusive mechanisms for kin recognition in *D. melanogaster* have been proposed: cuticular hydrocarbons (CHCs) [49,50] and gut microbiota [51]. CHCs have both a genetic and environmental component, and numerous insect species use CHCs to discriminate between kin [52]. Furthermore, CHCs can be modulated by gut microbiota [53], which are maternally transmitted to offspring via the egg and are also strongly influenced by diet [54]. In our study, we separated larvae after 24–36 h, so it is still possible that individuals are using familiarity cues in this very early period of life, which we would detect as an effect of relatedness in this experimental set-up. This would

be particularly true if flies were discriminating based on gut microbiota, as these are largely inherited from mothers via the egg casing [55,56]. Our experimental design differs in this respect from previous studies [22–26], which manipulated males at the egg, rather than larval, stage, thus reducing the possibility for maternal cues.

Kin recognition may be costly [57], and both its evolution and maintenance require adaptive explanations, albeit these need not be the same. Our study population has been adapted to laboratory conditions of large, dense, confined populations for over 45 years; over 1000 generations. It is hence possible that this population has not been especially structured beyond the microscale, an unlikely scenario for the evolution of kin recognition.

In this context, two mechanisms may contribute to explain mounting evidence for kin recognition in laboratory-adapted *D. melanogaster* populations: one adaptive and one non-adaptive.

First, these responses may reflect a relic of a plastic behaviour evolved in natural populations. While the initial evolution of this behavioural plasticity would have been presumably costly, the cost of maintaining a plastic response to kin under familiarity may be relatively low in laboratory populations where kin structure is expected to be limited. In this scenario, the evolution of kin recognition mechanisms would have been favoured by persistent population viscosity over multiple generations [25], and natural *D. melanogaster* populations in which recognition would have originally evolved must have been structured such that males could expect to grow up with related and unrelated individuals and compete with familiar individuals as adults.

In the wild, *D. melanogaster* live in orchards, feeding and laying eggs on rotting fruit [58]. Little is known about population viscosity and the family-level genetic structure of wild populations. Females co-locate at oviposition sites [59] (although this is likely due to substrate texture, not females actively seeking other larvae [60,61]), and larvae disperse [62], both of which would potentially reduce the likelihood of stable kin interactions. However, larval foraging behaviour, pupation site and adult choice of resting site all have a strong genetic component [58,63,64], and early adult habitat experiences shape later habitat preferences [65,66]. While there is little information on clutch sizes in wild populations, it is inferred from ovariole anatomy that females lay their eggs in small clutches [67], and indeed laboratory-reared females decide on the site quality between each egg [68]. Small clutch sizes, rather than laying eggs individually, could lead to genetic structure in wild populations by increasing the relatedness among neighbours, increasing the probability that adult males encounter related familiar competitors. There is also evidence of some genetic structure in a wild population, where mating males and females are more related to each other than to the average fly in the population [69].

A particularly important stage for the initial evolution of kin recognition and reduced female harm is likely to be at the colonization of a new patch. If a small number of females initially populate a new feeding site, the next generation will be small and will contain substantial variation in male relatedness. Any behaviours that increase female fecundity and male fitness at this stage of colonization may have large, long-lasting effects on the genetic distribution of the future population. While less relevant in established populations, which are larger and possibly less structured, kin recognition may be retained at relatively low costs as the relic of a highly successful strategy from the founding of the population. Another possibility is that fly populations may show some structure even in laboratory conditions. Flies are known to form non-random social networks even in small group sizes and small physical environments [70], therefore it is possible that some laboratory populations show some degree of relevant microstructure.

The second, non-adaptive mechanism that has recently been put forward to explain kin-biased sexual behaviour in flies is that, if individual levels of competitiveness (e.g. aggression and courtship) are at least partly heritable, triplets of related males are more likely to have similar levels of competitiveness than triplets of unrelated males. If males with similar levels of competitiveness competed less intensely than males with more variable levels of competitiveness, this would produce the effect of related males competing less [26]. This explanation, however, seems counterintuitive. As expected by contest theory and supported by a wealth of data across different taxa, males tend to invest more in competition with rivals of similar competitive value [71,72], a result also replicated by Martin and Long [26]. Also, if female behaviour changes in response to the variability among males, such as being more receptive in the presence of three unrelated (and hence more genetically dissimilar) males [73], this might in turn trigger a proximate increase in male–male fighting and sexual harassment of females, leading to greater female harm.

Another proximate explanation for groups of brothers harming females less than groups of unrelated males might represent a cognitive error. It is possible that if *D. melanogaster* males use variability of smell, be that CHC profiles or gut microbiota, as a measure of how many males they are competing with, they may underestimate the number of competitors when they are related, i.e. smell similar. Thus, if a male is surrounded by brothers, he may assume there is less competition and behave less competitively, harming the female less [13].

While our data show that groups of related familiar males are less harmful to females, we do not yet know whether this is mediated through pre- or post-copulatory effects. It is possible that there are post-copulatory differences between treatments for which we did not test. Male *D. melanogaster* are known to adjust the composition of their ejaculate according to the female's previous mating history and perceived competition [74–76]. In particular, we do not currently know if the levels of male accessory proteins transferred to the females differ between treatments.

The present study joins several others looking at the effect of relatedness on sexual behaviour in *D. melanogaster*, with some of the key findings of each study summarized in electronic supplementary material, table S1. Each used a very similar experimental design, but different laboratory populations of *D. melanogaster*. The Dahomey population used in Carazo *et al.* [22,23] and this study are the same. The three IV populations used in Hollis *et al.* [24], Chippindale *et al.* [25] and Martin & Long [26], while nominally the same, have been reared in separate laboratories for several decades. Apart from genetic differences, the Dahomey and IV populations differ substantially in rearing conditions. The Dahomey population, as used in this study, is maintained in cages with large, dense populations and overlapping

generations, which allows for selection to continue late in life. By contrast, the IV populations are maintained on a discrete 14-day generation culture cycle in vials at a controlled density of approximately 100 eggs per vial, which prevents selection from acting beyond that time point. This difference in culturing conditions could potentially alter sexual conflict-mediated selection on female ageing in Dahomey versus IV populations. However, there have been no direct tests of this hypothesis. It will be important for future studies to explore, via the fully factorial design applied here, whether relatedness and familiarity among males similarly interact to affect female harm in the IV and other *Drosophila* populations.

More generally, one implication of these studies is that local relatedness among male competitors may represent a possible modulator of the 'sexual tragedy of the commons' and population viability. An important avenue of future research, therefore, will be to explore whether the ecology of *D. melanogaster* across different laboratory and wild populations (e.g. fine-grained population structure) may be more or less conducive to kin-selected sexual cooperation.

Data accessibility. The datasets supporting this article can be accessed at the Oxford University Research Archive: https://doi.org/10.5287/bodleian:mq112gn2P.

Authors' contributions. S.W. and P.C. conceived of the study. S.L.P., P.C. and S.W. designed the study. S.L.P. and E.F. generated the experimental flies. S.L.P. and I.S. collected behavioural data. S.L.P. collected offspring data and carried out the statistical analyses. S.L.P., S.W., P.C. and T.P. drafted the manuscript. All authors gave their final approval for publication.

Competing interests. We have no competing interests.

Funding. S.L.P. is funded by a BBSRC studentship award (BB/J014427/1). S.W. and I.S. are funded by a BBSRC fellowship to S.W. (BB/K014544/1). P.C. is funded by a Ramón y Cajal research fellowship and by Spanish Research Grants (CGL2014-58722-P and CGL2016-81894-ERC) from the Spanish Ministry of Economy and Competitiveness (MINECO). T.P. is funded by a BBSRC-LINK grant with Aviagen Ltd (BB/L009587/1).

Acknowledgements. Thanks are expressed to Ju Morimoto and Eleanor Bath for their assistance in generating the experimental flies, Florian Klimm for assisting in writing a program to randomly assign larvae, George Nicholson, Dan Lunn and Nico Kist for their assistance in the statistical analyses, and to two anonymous reviewers for their constructive suggestions.

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