Reproductive Interference and Satyrisation; An examination of theoretical components and potential practical application.

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Image courtesy of Professor Tracey Chapman, University of East Anglia

Abstract:

The aim of this thesis was to investigate evolutionary forces and other factors that promote the occurrence of Reproductive Interference. Reproductive Interference, also known as satyrisation, is defined as reproductive activity between individuals of different species, creating fitness costs for one or both participants. The associated fitness costs can often be asymmetrical between sex and species, impacting niche partitioning outcomes and producing a potential avenue for pest population control. I conducted a comprehensive literature review, to determine elements that could affect the presence and severity of satyrisation. This covered the mechanisms underpinning satyrisation, the relative fitness costs of these mechanisms and identifying other factors which could influence satyrisation. This review concluded with a theoretical approach to implementing satyrisation as part of a pest management scheme. To complement this, a series of experiments was then conducted on female age, a factor that could influence heterospecific mating interactions. In many insect species, male age is known to influence female mate choice, but less research examines how female age may affect mate choosiness. I conducted behavioural assays on female *Drosophila melanogaster* and *D. mauritiana* placed in heterospecific and conspecific crosses, over time, to determine whether the frequency of heterospecific reproductive interactions changed as the females aged. These experiments showed that differences in the frequency of mating behaviours were more strongly related to whether females were paired with conspecifically or heterospecifically rather than age itself. However, there was a large discrepancy in mortality rates between con- and heterospecific crosses, with females of both species surviving longer when paired heterospecifically. These findings were placed into a wider context and ideas for further study discussed, in a final concluding chapter. Overall, this thesis explored various determinants of Reproductive Interference, highlighting the diverse factors and outcomes of heterospecific interactions, and suggesting considerations of these processes in further research.

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Chapter 1 – Existing knowledge and thesis outline

Thesis Summary

The aim of this thesis is to investigate evolutionary forces and other factors that promote the occurrence of Reproductive Interference. Reproductive Interference, also known as satyrisation, is defined as reproductive activity between individuals of different species, creating fitness costs for one or both participants. This can occur at any stage of reproductive behaviour, from initial disruption of mate detection, up to unsuccessful hybridisation. As this process is largely an issue of mistaken identity, it primarily affects closely related species that are undergoing incipient speciation or experiencing secondary contact. Interestingly, the fitness costs of these interactions can often be asymmetrical between sex and species, impacting outcomes of niche partitioning and producing a potential avenue for control of pest populations.

As satyrisation can be impacted by variable facets of species interactions, I conducted a comprehensive literature review, examining both theoretical and empirical work, to determine underpinning elements that could affect the presence and severity of satyrisation. This covered the mechanisms by which Reproductive Interference could occur, the relative fitness costs of the underpinning mechanisms, identifying other biotic and abiotic factors which could alter heterospecific reproductive interactions. This review concluded with a theoretical approach to implementing satyrisation as part of a pest management scheme, with attention paid to testing the efficacy of potential methods before implementation.

To complement this, a series of experiments was then conducted on one such factor that could influence the frequency of heterospecific mating interactions, namely female age. In many insect species, male age is known to influence female mate choice, but less attention is paid to how female age may affect mate choosiness. Previous research by others has shown that both female and male age appeared to impact female mate choosiness between *Aedes aegypti* and *A. albopictus* mosquitoes, with older females being less discerning than younger females, and older males and females engaging in interspecific mating significantly more than their younger counterparts. Building from this rationale, I conducted a series of behavioural assays on a cohort of female *Drosophila melanogaster* and *D. mauritiana* placed in heterospecific and conspecific treatment crosses, over time, to determine whether the frequency of heterospecific reproductive interactions changed as the females aged. These experiments showed that the differences in the frequency of mating behaviours was more strongly related to whether females were paired with conspecific or heterospecific males rather than age itself. However, there was a large discrepancy in mortality rates between con- and heterospecific crosses, with females of both species surviving longer when paired heterospecifically, in comparison to those paired conspecifically. These findings were placed into a wider context and ideas for further study discussed, in a final concluding chapter.

Overall, the research completed for this thesis explored various determinants of Reproductive Interference, highlighting the diverse factors and outcomes of conspecific and heterospecific interactions, and speculating on how researchers should include consideration for these processes in further research, with its potential impacts on evolutionary history, species migration and pest control.

Chapter 1 - attribution statement: The literature review of this chapter has been published as review in the *Journal of Pest Science* (Published: 08 February 2022, 95:1023-1036; Appendix 3). I designed the approach to the review in discussion with my supervisor, Professor Tracey

Chapman. I conducted all the literature searching and synthesis and wrote the first draft. I incorporated feedback from my supervisory team and colleagues (Stewart Leigh, Luke Alphey, Wilfried Haerty), revised the MS in response to external reviewer comments and produced the final proofed version.

Introduction

Reproductive Interference occurs when individuals from two different species/diverging population have their fitness negatively impacted by incorrectly engaging in reproductive behaviours with each other. These errors in behaviour are intriguing because they help inform our understanding of reproductive character displacement, and how mating systems are maintained or changed during speciation. Furthermore, these interactions can have asymmetric fitness costs between the interacting parties – often called satyrisation. This has implications for niche partitioning and species displacement and could also be exploited to enact control on pest populations. The applied potential of satyrisation is significant but has so far been relatively under-researched. This was the core topic discussed in this thesis, in order to expand our current understanding of this phenomenon, and its potential for application in pest control protocols.

Due to the varied nature of preexisting research on Reproductive Interference it was decided that a comprehensive literature review should first be conducted to assess the extent of current knowledge. The aim was to assist in determining a suitable direction for further research and identify gaps in existing knowledge to pursue with an experimental approach. The review considered both theoretical and empirical work on this phenomenon, with a focus on considering the ramifications of fitness costs on affected individuals/populations/species, and how satyrisation could be utilised to assist with control of pest populations. This literature review was published in the Journal of Pest Science (*Journal of Pest Science*, *95*(3), 1023-1036, https://doi.org/10.1007/s10340-022-01476-6) and is included below, as the core part of this introductory chapter.

Introduction to Reproductive Interference

The study of the rapid evolution of reproductive traits and their divergence between closely related species is of fundamental interest to researchers in the context of speciation. It also gives insights into introgression and biodiversity conservation (Pfennig and Pfennig 2010; Rice and Pfennig 2010; Shuker and Burdfield-Steel 2017). There is empirical evidence that the divergence of different reproductive traits between closely related species, whether morphological or behavioural, can occur at variable rates. This can result in the phenomenon whereby individuals from the diverging species cannot form fertile hybrids but can suffer negative fitness costs due to interspecific sexual interactions. These reproductive interactions can take various forms and are collectively referred to as 'Reproductive Interference'. In vertebrates and invertebrates this process is often termed Satyrisation (after the sexually promiscuous half-goat man of Greco-Roman myth; Ribeiro and Spielman 1986; Bargielowski et al. 2013). The effects and fitness costs of reciprocal matings between species are often asymmetric, and it is this aspect that has implications for species coexistence (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017; Kyogoku 2020) as well as pest management. Some authors originally used the term Satyrisation to refer exclusively to *asymmetric* Reproductive Interference. However, the usage of this term has since broadened and in this review, we define Satyrisation as symmetric or asymmetric Reproductive Interference that occurs in vertebrate and invertebrate mating systems (Glossary).

Reproductive Interference sits at the interface between evolutionary biology and ecology. For instance, there is a growing realisation that it can help to resolve unexplained features of competitive relationships between species, such as when species exclusion cannot be explained by resource competition (Park et al. 1948; Birch et al. 1951; Kishi et al. 2009). There is also a growing awareness that Reproductive Interference can be a driver of reproductive character displacement, in addition to reinforcement and the Templeton effect (Templeton 1981; Butlin and Ritchie 2009; Hollander et al. 2018).

Reproductive Interference is also relevant for conservationists, as it could influence the invasion success of non-native species, and result in impacts upon other species with which the invasives could potentially interbreed (Liu et al. 2007; Gröning and Hochkirch 2008; D'Amore et al. 2009). Reproductive Interference is of significant applied interest in terms of its potential utility in controlling harmful species including disease vectors such as *Aedes* mosquitoes (Gröning and Hochkirch 2008; Bargielowski and Lounibos 2016). Satyrisation is being considered as a potential pest control method, both independently and in conjunction with other current pest-suppression strategies (Leftwich et al. 2015; Honma et al. 2018).

The first aim of this review is to summarise the ways in which Satyrisation is expressed within vertebrate and invertebrate systems and to determine the factors that result in asymmetric fitness costs, using illustrative test cases. The second aim is to consider how the principles underlying Satyrisation could be deployed for the control and management of dangerous insect pests. To do this, we reviewed the current literature on Satyrisation, defining the factors that cause its effects to vary and used this to inform how it can be deployed directly or indirectly as a method of pest control.

We restrict this review to the consideration of situations in which any hybrid progeny that are produced from matings between species have zero fitness (i.e., they are inviable or sterile). The topics of hybrid matings leading to introgression and hybrid vigour are covered in detail elsewhere (Huxel 1998; Hill et al. 2020) and are not considered within the scope of this review.

Glossary:

Reproductive Interference – *reproductive interactions across plants and animals, usually between individuals of closely related species, which do not lead to the production of fertile hybrids and instead result in negative fitness costs for the interactants.*

Satyrization – *a term referring to Reproductive Interference in vertebrate and invertebrate animal systems.*

Conspecific – *Belonging to the same species*

Heterospecific – *Belonging to a different species*

Sexual exclusion – *Exclusion of a species as a result of negative fitness costs arising from Reproductive Interference*

Reproductive Interference

Reproductive Interference is a broad term that is used to define the situation when there are sexual/reproductive interactions, usually between individuals of closely related species, which do not lead to the production of fertile hybrids and instead result in negative fitness costs for the interacting individual males and / or females (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017; Kyogoku 2020). This can include interactions between even reasonably diverged species, such as when a territorial male seeks to exclude individuals of other species, as well as its own, during mating competitions. In this way Reproductive Interference can represent a potential intersection between resource competition and heterospecific rivalry (Drury et al. 2015). However, Reproductive Interference more often occurs between species which are closely related/ recently diverged, due to the existence of incomplete mating barriers.

Therefore, Reproductive Interference is fundamentally linked to reproductive character displacement, reinforcement and speciation. It can occur, in principle, over a broad range of plant and animal taxa (Levin 1970). The study of Reproductive Interference, to date, has been focussed mostly within the study of plant science (Weber and Strauss 2016). In particular, the emphasis has been on determining the mechanisms and origin of Reproductive Interference in the formation of postzygotic barriers leading to speciation. Asymmetries in Reproductive Interference in plants have also been reported in terms of unilateral incompatibility (Bedinger et al. 2011; Lewis and Crowe 1958; Marta et al. 2004) and vestigial viable pollen (Whitton, et al. 2017). These factors are known to limit species co-occurrence.

In vertebrate and invertebrate mating systems, reproducing individuals are usually mobile and may exhibit a complex range of reproductive behaviours. This has the potential to offer a greater number of scenarios in which Reproductive Interference might occur, in comparison to plants, and to lead to stronger selection to avoid costly interspecific coupling (Levin 1970). Reproductive Interference that occurs within animal mating systems is usually referred to an Satyrisation and is divided into 7 categories (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017) each related to distinct types of mating barrier. These mechanisms can be pre- or post-copulatory, often work in conjunction, and can have potentially different ecological impacts. The mechanisms are: signal jamming, heterospecific rivalry, misdirected courtship, heterospecific mating attempts, erroneous female choice, heterospecific mating, and hybridization (summarised, with examples, in Table 1).

Reproductive Interference shares some features of resource competition, and is density dependent (Gröning and Hochkirch 2008). For example, it can result in population or species exclusion, or coexistence through divergence (Kuno 1992). This has been modelled using a

Lotka-Volterra competition framework (Ribeiro and Spielman 1986; Kuno, 1992). As with competition, Reproductive Interference can result in either exclusion of the ‘weaker’ species, divergence (parapatry), or coexistence through niche partitioning, reproductive character displacement, eventual evolution of complete mating barriers (Kyogoku 2020). However, unlike resource competition, Reproductive Interference lacks a true ‘shared resource’, and instead occurs due to errors in, or incomplete, mate recognition, resulting in fitness reductions in individuals of the interacting species (Gröning and Hochkirch 2008).

Due to the shared features of Reproductive Interference and resource competition, it can often be difficult to disentangle the relative importance of these different forms of interspecific interactions on reproductive behaviours, particularly within field settings. However, there is a growing realisation that Reproductive Interference may play a larger role in species competition and speciation than previously considered (Hochkirch et al. 2007). For example, it may help to explain the results of experiments initially attributed to competitive exclusion in which the seemingly weaker resource competitor excluded the ‘more efficient’ species (Park et al. 1948; Birch et al. 1951; Kishi et al. 2009). Reproductive Interference may even be maintained in some cases due to what Drury et al. (2019) refer to as an ‘Evolutionary Catch-22’, wherein the cost to males of mating with heterospecific females is lower than that of missing conspecific mating opportunities, thereby limiting divergence in male mate recognition and female reproductive characteristics. Whilst this is unlikely to be a feature of all species that can experience Reproductive Interference due to differences in male fitness costs, it is nevertheless interesting to consider in the context of factors that may limit the evolution of reproductive character displacement (Drury et al. 2015; Drury et al. 2019).

Overall, our knowledge of Reproductive Interference is important in the context of how we consider species interactions and their possible ecological outcomes. This is particularly relevant to increased invasion events, in which consideration must be given to the effects of Reproductive Interference on invasion success and how it impacts upon introgression into at-risk species (Liu et al, 2007; Gröning and Hochkirch 2008; D'amore et al. 2009).

Table 1: Summary of different Satyrisation categories (Gröning and Hochkirch 2008) together with illustrative examples.

Type of reproductive interference	**Form of incomplete mating barrier**	**Description**	**Examples**
Signal jamming/Signal Interference	Pre-mating	Disruption of reproductive signals due to presence of heterospecific signals such as pheromones, mating calls, visual displays.	Responses to heterospecific pheromones in Lepidopterans (Landolt and Heath 1987).
Heterospecific rivalry	Pre-mating	Heterospecifics are mistaken for conspecific rivals and become subject to aggression.	Interspecies fighting and territoriality in *Hetaerina* damselflies (Drury et al. 2015).
Misdirected courtship	Pre-mating	Courtship of heterospecifics due to mistaken identity / similarity of courtship behaviours and responses.	Courtship of heterospecific females by males of *Leptidea sinapis* and *Leptidea juvernica* (Friberg et al. 2013).
Heterospecific mating attempts	Pre-mating	Incomplete heterospecific copulations, which can have fitness costs arising from bodily harm, or harassment effects.	Attempted forced copulation between male *Poecilia reticulata* with heterospecific female *Skiffia bilineata* (Valero et al. 2008).
Erroneous Female Choice	Pre-mating	Females actively choosing heterospecific males due to mistaken identity or pre-existing sensory bias.	Erroneous female choice in *Paratrechalea ornata* spiders, in which females accept nuptial gifts and engage in misdirected courtship with male *Paratrechalea azul* (Costa-Schmidt, and Machado 2012).
Heterospecific mating	Post-mating, pre-zygotic	Successful heterospecific coupling, where fitness costs can arise from bodily harm, gamete wastage, and the induction of refractoriness to further matings.	Heterospecific mating and insemination between male *Aedes albopictus* and female *Aedes aegypti* (Nasci et al. 1989).
Hybridization	Post-mating, post-zygotic	Production of zero fitness hybrid offspring, from heterospecific mating. Fitness costs depend on extent of energetic costs expended on the production of hybrid offspring.	Production of sterile hybrids in *Drosophila arawakhana* × *Drosophila dunni* crosses. (Hill et al. 2020).

Asymmetric Reproductive Interference/Satyrisation

An intriguing aspect of Reproductive Interference is the high degree of asymmetry in fitness costs often observed in reciprocal interactions between species (Gröning and Hochkirch 2008). This can rapidly increase the probability or rate of competitive exclusion or niche partitioning. Within invertebrates, Satyrisation is beginning to garner attention as a potential mechanism for intentional exclusion to achieve pest control (Leftwich et al. 2015; Honma et al. 2018). The term 'Satyr' was first utilised in this context by Ribeiro and Spielman (1986) and was originally defined as *asymmetric* Reproductive Interference by reference to a mathematical model that explored the fitness costs of reciprocal interspecific interactions. However, since then, Satyrisation has generally been used to describe the symmetric *and* asymmetric Reproductive Interference that occurs in vertebrates and invertebrates, and this is the definition we adopt here. An example of asymmetric Satyrisation can be found in cryptic butterfly species, where the less competitive and less reproductively efficient species are observed to exhibit rapid niche partitioning with respect to their more competitive counterparts. This is thought to arise at least partly to avoid costly misdirected courtships from heterospecific males (Dincă et al. 2013; Friberg et al. 2013). Satyrisation was first described several decades ago (e.g. Ribeiro and Spielman 1986; Miller et al. 1994) and interest in it is growing partly as it provides an explanation for the observed reduction of *Aedes aegypti* populations in North America (particularly in the panhandle of Florida) following the invasion of *A. albopictus* (Parker et al. 2019). Satyrisation has been thoroughly studied in the *Aedes* system, in both laboratory and field experiments (Nasci et al. 1989; Tripet et al. 2011; Carrasquilla and Lounibos, 2015; Bargielowski and Lounibos, 2016; Honório et al. 2018; Bargielowski et al. 2019). This has led researchers to evaluate how prevalent it might be in nature, and to explore methods to utilise its principles to reduce or exclude pest species in favour of more benign ones (Honma et al. 2018). The main applied focus on Satyrisation stems from the finding that it often has asymmetric effects on fitness. The fitness costs to females engaging in courtship with heterospecifics is typically higher than the costs for males of heterospecific interactions, due to the generally higher levels of reproductive investment made by females. This scenario sets up the risk of energetic costs due to gamete wastage, potential harm from mating with males with incompatible morphology or physiology, or opportunity costs of lost mating opportunities due to the induction of post-mating refractoriness (Bath et al. 2012; Bargielowski and Lounibos, 2016; Yassin and David, 2016; Leigh et al. 2020). An example is described by Tripet et al. (2011) in which low (0.01-12.3%) mating rates to conspecifics were observed in female *A. aegypti* following injection with *A. albopictus* male accessory gland extracts, which induce refractoriness to remating in both species. Failure to mate with a conspecific will incur significant fitness costs. Asymmetry in costs in reciprocal interactions between species pairs is also common, with, for instance, females of one species suffering much higher costs heterospecific interactions than the other. Tripet et al. (2011) provide evidence, by observing that *A. aegypti* females are rendered refractory to mating by the heterospecific male accessory gland proteins of *A. albopictus,* whereas the insemination of *A. albopictus* females by *A. aegypti* male accessory gland proteins has no such effect.

Differential rates of character divergence and the underlying drivers are key candidates for producing asymmetric effects of Satyrisation. Studying the mechanisms of these asymmetries could also yield important insights into the relative plasticity or conservation of genes that regulate sexual behaviour and physiology and the rate at which they evolve, as well as strengthening our overall understanding of reproductive isolation. Asymmetric Satyrisation could also potentially inform new methods of control by the repression or replacement of pest species, in a manner that could bypass restrictions and concerns associated with genetic modification (Alphey et al. 2013; Leftwich et al. 2015; Honma et al. 2018). The effects of Satyrisation within existing control programmes is also of potential significance. For example, Satyrisation between modified males released to effect control with heterospecifics resident in the target control area (e.g. release males courting heterospecific non-target females, or heterospecific males bocking matings for release males) might reduce the efficacy of control, by lowering the frequency of conspecific matings between released males and wild females. Research into Satyrisation, as a direct method of pest control, is still in its infancy. However, its potential to interfere with key reproductive processes means that knowledge of the fundamental mechanisms involved could indicate new routes for manipulating pest populations into increased vulnerability (Honma et al, 2018). A key aspect is to understand which factors most influence asymmetric fitness costs between species. In addition, it will be important to determine if control could be rendered more successful by simultaneously manipulating multiple factors that increase Satyrisation asymmetry, or by tailoring the approach to target asymmetries to which any specific target population is particularly vulnerable. The factors of greatest importance in determining overall levels of Satyrisation are likely to vary with control scenario and are discussed in more detail below.

Factors impacting the degree of asymmetry in Satyrisation

The efficacy of Satyrisation at driving species exclusion (whether via sexual exclusion or a combination of sexual and competitive exclusion) or niche-partitioning, is highly dependent on the degree of asymmetry in fitness costs between the interacting species (Ribeiro and Spielman

1986). The asymmetry is strongly influenced by a variety of factors including density dependence, evolutionary history and life-history trade-offs. These factors and their effects are illustrated in Table 2.

There is an inherent difficulty in disentangling the relationships between Satyrisation and species character traits in order to ascertain whether an existing character trait simply exacerbates Satyrisation, or if Satyrisation itself was, or is, a driver of trait evolution. For example, we need to understand whether resource competition simply intensifies the effects of Satyrisation or if individuals of the less competitive species will be selected to specialise to avoid Satyrisation, as is suggested to occur in conflicts between the ladybirds *Harmonia axyridis* and *H. yedoensis* (Noriyuki et al. 2012).

It should also be noted that the extent of Satyrisation is also highly likely to be influenced by changes to abiotic factors and habitat structure. Examples include habitat loss or climate change potentially pushing related species together or preventing niche partitioning. This could increase the frequency at which Satyrisation occurs, by either creating sympatry where species were once allopatric (i.e. creating new habitat overlaps between species) or by increasing population densities in hybrid zones (Liu et al. 2007). Such factors may also cause changes to preferred ecological niches, which may act in conjunction with Satyrisation. The following sections discuss in more detail the various factors proposed to affect the efficacy/frequency of Satyrisation (Table 2).

Population density/species ratio

As with resource competition, the relative abundance of each competing species will play a role in whether Satyrisation is strong enough to result in species exclusion. Under resource competition, increased number of competitors results in resource limitation, whereas under Satyrisation, an uneven species ratio or a high density can result in a high frequency of heterospecific encounters, increasing the likelihood that costly heterospecific courtship will occur (Kyogoku and Sota, 2017; Kyogoku 2020). This phenomenon was observed in simulations by Takafuji et al. (1997) based on interactions between two closely related spider mites, in which the initial density ratios heavily affected the extent of competitive exclusion that occurred. This has significant implications for the success of invasion by non-native species which can reproductively interfere with native species.

Pre-existing resource competition asymmetry

As Satyrisation often occurs between closely related species, resource competition may be strong as there may not yet have been sufficient divergence to avoid niche overlap. Theory by Kishi and Nakazawa (2013) predicts some of the ways in which Satyrisation and resource competition can interact. In situations where the more resource-efficient species also suffers fewer fitness costs from Satyrisation, this should result in exclusion of the weaker species being more likely or more rapid. In contrast, when fitness cost asymmetries in resource competition and heterospecific reproductive interactions occur in opposite directions, i.e. the more resource-efficient species is more negatively affected by heterospecific reproductive interactions and *vice versa*, Satyrisation could theoretically lead to species coexistence, or even favour the weaker competitor. Another example of how resource competition and Satyrisation can have a combined effect on local species exclusion is found in Pied and Collared Flycatchers on the Swedish Island of Öland (Vallin et al. 2012). Resource competition between these two species over mating territories led to young Pied Flycatcher males being unable to establish territories. This in turn reduced the abundance of conspecific Pied Flycatchers males available, leading to an increase in heterospecific matings, the costly production of low-fitness hybrids and eventual local exclusion. The excluded species was found to have partitioned across separate islands, potentially to avoid the combined effects of resource competition and Satyrisation (Vallin et al. 2012).

Number of generations in sympatry/allopatry

Researchers investigating Satyrisation in *Aedes* have shown that mild forms of resistance to Satyrisation can evolve within just a few generations (Bargielowski et al. 2013; Bargielowski and Lounibos, 2016; Bargielowski et al. 2019). However, this means that allopatric populations may often be more susceptible to Satyrisation. Bargielowski and colleagues have observed that in *A. aegypti,* resistance to Satyrisation was associated with an increased female choosiness in sympatric populations, with allopatric females showing lower levels of discrimination against heterospecifics (Bargielowski et al. 2013; Bargielowski and Lounibos, 2016; Bargielowski et al. 2019). Similarly, Kyogoku (2020) observed that Satyrisation is more likely to occur during secondary contact (e.g., previously allopatric species coming into contact) than within coexisting (e.g., sympatric or parapatric) species.

Table 2. Factors that affect the efficacy/degree of asymmetry in Satyrisation.

Factors influencing the extent of asymmetry in Satyrization	**Consequences of factors**
Relative abundance, population density and sex ratio of target species and satyr species upon introduction	Affects the frequency of heterospecific interactions and matings
Pre-existing asymmetry in resource competition	Can exacerbate population dynamics that influence reproductive interference and increase likelihood of exclusion
Number of generations spent in sympatry or allopatry	Influences degree of selection pressure to prevent interspecific reproductive interactions
Presence/degree of pre-mating barriers	Mate recognition, choosiness, phenology of mating, courtship differences can alter asymmetry of fecundity costs of hybrid mating between species
Presence/degree of post-mating barriers	Effectiveness of responses to heterospecific seminal fluid proteins, extent of con or heterospecific sperm precedence, refractory period and capacity to hybridize can all alter asymmetry of fecundity costs of hybrid mating between species
Degree of intraspecific sexual conflict within the target species and satyr species	Can influence asymmetry of heterospecific mating fitness costs
Fitness costs of Satyrization resistance genes	Influences likelihood of resistance evolution/how long it takes for resistance to evolve/how long resistance genes will stay in the population if the species become allopatric
Life History trade-offs: parasite load, predation, changes in fecundity over time, life history, etc.	General fitness effects that can influence relative abundance and fecundity
Mating system	Differences in mating system will result in species differing in pre-mating and post-mating investment.
Presence of multiple interbreeding species	Could alter relative fitness costs between species and change selection pressures

Presence/degree of pre-mating barriers

The presence, and effectiveness, of pre-mating barriers between closely related species will necessarily affect the extent and frequency with which negative fitness costs are experienced. Hence, these barriers are key to the existence and extent of Satyrisation. For example, in diverging species that retain the capacity to interbreed, one direction of cross may often be more common than the reciprocal, due to one species having developed stronger pre-mating barriers than the other. This is likely to be dependent on the evolutionary history of divergence between species. Hence consideration of the time since divergence and / or phylogenetic relatedness may allow researchers to estimate the accumulation of changes in reproductive characteristics (Coyne and Orr 1989) and thus the likely strength of pre-mating barriers. An example of the evolution of pre-mating barriers that lead to fitness cost asymmetries is observed between *Drosophila occidentalis* and *D. suboccidentalis,* with *D. suboccidentalis* females being less receptive to heterospecific mating than *D. occidentalis* females, when measured in a series of no-choice tests (Arthur and Dyer 2015).

Presence/degree of post-mating barriers

The completeness of post-mating, pre-zygotic mating barriers between closely related species can affect the fitness costs of Satyrisation. The magnitude of post-mating barriers will, as for pre-mating ones, depend upon the evolutionary history of divergence between the species involved. An example is found in the phenomenon of conspecific sperm-precedence, in which same-species sperm are used preferentially over that of any other species sperm present in the female reproductive tract. Hence even if heterospecific mating can be costly, the fitness costs of gamete-wastage could potentially be mitigated via conspecific sperm-precedence, provided that females can or have previously mated with a conspecific male. Price (1997) and Rugman-Jones and Eady (2007) observed conspecific sperm precedence in *Drosophila simulans* and *Callosobruchus subinnotatus,* respectively, and noted that conspecific sperm was not only used preferentially for fertilisation but was also stored preferentially in spermathecae. However, it was not evident to what extent these phenomena were controlled by preferential female use, or by physiological effects of male seminal fluid proteins. A recent model by Iritani and Noriyuki (2021) of the reproductive interactions between the ladybird beetles *Harmonia axyridis* and *H. yedoensis* suggested that conspecific sperm precedence would not be sufficient to the counteract the negative effects of Satyrisation. This was due to the high costs of increased refractoriness to conspecific mating following a heterospecific mating. Overall, the efficacy of post-mating barriers in reducing the costs of Satyrisation will vary between species according to the relative costs of pre- vs post-mating effects on reproductive success.

Degree of intraspecific sexual conflict within the target species and Satyr species

Some research into Satyrisation has suggested that intraspecific sexual conflict between the evolutionary interests of each sex may play a role explaining asymmetry in the fitness costs of hybrid matings between species (Shuker et al. 2015; Leigh et al. 2020). In species that experience high levels of sexual conflict, females may be better adapted to tolerating the aggressive actions of seminal fluid proteins or persistent courtships. Similarly, females from species subject to lower levels of sexual conflict might be ill equipped to mitigate the coercive and harmful effects of mating with ‘harmful’ heterospecific males. Yassin and David (2016) found evidence to support this hypothesis as they observed differences in female mortality between hybrid crosses in the *Drosophila melanogaster* species subgroup. In crosses with higher mortality, females were often found to have higher levels of melanisation in their abdominal regions, suggesting wounds from heterospecific mating were more severe in some crosses than others. Similarly Kyogoku and Sota (2015) found that exaggerated genital spines in the sexually competitive males of the seed beetle *Callosobruchus chinensis* mediated the costs of Satyrisation in *C. maculatus* females. This suggested a direct link between male-male intraspecific competition adaptations, and fitness cost asymmetries in Satyrisation.

Fitness costs of 'Satyrisation resistance' genes

If Satyrisation carries high asymmetric fitness costs, it is likely to select for the evolution of resistance within the species which suffers highest costs (Bargielowski et al. 2013; Bargielowski et al. 2019). However, if selection is relaxed, e.g., if exposure to the Satyr species is reduced, Satyrisation resistance genes may be rapidly eliminated. This has been observed by Bargielowski et al. (2019) who described a reduction in Satyrisation resistance traits in *A. aegypti* when they were no longer found in sympatry with *A. albopictus*. The fitness costs were unknown but were suggested to be related to increased female choosiness, which can act to prevent hybrid matings when both species are in sympatry but which may restrict mating opportunities with conspecifics in allopatry. The impact of resistance gene costs is therefore important to consider, as it can influence the maintenance of resistance to Satyrisation and determine which populations will be or become more susceptible to it.

Life History Trade-offs

Factors such as predation, parasite load and nutritional resources that influence selection pressures and life history will likely have impacts on the existence of Satyrisation, its level of asymmetry and effect on sexual exclusion. For example, Drury et al. (2015) considered that Satyrisation was being maintained in sympatric populations of *Hetaerina* damselflies due to weak selection pressure on male mate choice and limitations in female character displacement, as a result of the requirement to maintain crypsis and avoid predation. In addition, Bargielowski et al. (2019) observed an increase in receptivity to heterospecific mating (in *A. aegypti* ♀ x *A. albopictus* ♂ crosses) as individuals aged, likely due to a willingness in females to accept lower quality mates as age-specific fecundity decreased. This could itself have density-dependent effects, since the time to find a mate (or at least a male) is likely to increase as density decreases. We conclude that accurate determination of the occurrence and effects of Satyrisation requires consideration of demography and many different biotic interactions.

Mating system

Mating systems are expected to have major effects on fitness costs associated with hybrid matings. For example, for the mating systems in which each reproductive episode involves significant investment (e.g., by the giving of nuptial gifts) or in species in which there are limited reproductive opportunities, then even small differences in reproductive characteristics between species could alter the level of Satyrisation asymmetry and result in divergent fitness costs. This phenomenon is evident in interactions between different biotypes of the haplodiploid whitefly *Bemisia tabaci.* Being haplodiploid (i.e. haploid males produced from unfertilised eggs and diploid females from fertilised) the frequency and success of mating is an important determinant of sex ratio, and thus can greatly affect population growth. It was found that between the B and Q biotypes of *B.tabaci,* the B biotype was more behaviourally plastic. When exposed to Satyrisation effects from exposure to the Q biotype, B biotype females more readily accepted copulations from B males, allowing for maintenance of sex ratio. In contrast, Q biotype appeared invariant in their mating acceptances, and did not upregulate their acceptance of con-biotype mates (Crowder et al. 2010).

Presence of multiple interbreeding species

The dynamics of interspecies breeding can be complex if more than one reproductively-interfering species is present in sympatry. This can affect relative fitness costs depending on the frequency at which each species courts/interbreeds with others. Females could mate heterospecifically with different species, potentially on multiple occasions. Shuker et al. (2015) considered heterospecific mating and harassment between 4 species from the bug family Lygaeidae (*Lygaeus equestris, Spilostethus pandurus, Lygaeus creticus* and *Oncopeltus fasciatus*) and found rare but consistent patterns of heterospecific matings between all species. In mass-breeding experiments, the presence and / or identity of the companion bug sex and species had significant effects on nymph production. In no-choice mating assays, heterospecific pairings between female *L. equestris* and male *S. pandurus* resulted in a particularly large reduction in *L. equestris* female longevity and fecundity. Some of these species have overlapping distributions in nature, thus Satyrisation has the potential to occur between these species in the wild. It would be interesting to investigate such instances of Satyrisation between multiple interacting species because of the wide variety of ecological outcomes to which they could lead.

Satyrisation as a control method

Following the observations that Satyrisation effects arising from *Aedes albopictus* were likely to have been a primary driver behind the population decrease of *Aedes aegypti* in North America (Tripet et al. 2011; Bargielowski et al. 2013; Bargielowski et al. 2015) researchers have become interested in exploring the principles of Satyrisation for intentional population exclusion (Leftwich et al. 2015; Honma et al. 2018). The fact that Satyrisation had occurred within *Aedes* species has been key to the increasing interest in its use for control, as decades of research have sought to discover effective methods to limit these important arbovirus vectors that spread globally significant pathogens such as dengue, chikungunya and Zika viruses (Alphey et al. 2013; World Health Organisation 2014; Parker et al. 2019).

However, despite being observed in North American *Aedes* populations, it is challenging to determine how frequently Satyrisation occurs in the field (Crowder et al. 2010; Bargielowski et al. 2015). If Satyrisation were to be used for control, the release of both sexes of the interfering species would operate via population replacement (replacing the pest with a more benign species). In contrast, the release of just one sex would function via population suppression (reducing or eliminating the pest; Alphey et al. 2013; Alphey 2014). However, it is possible that any replacement species could cause additional and potentially unanticipated problems. For example, *A. albopictus* is itself an arbovirus vector of medical significance, though it may be a less efficient vector for the transmission of relevant arboviruses than is *A. aegypti* (Alphey et al. 2013; Hugo et al. 2019). The relative vector competence of *Aedes* species is highly dependent on which disease and disease strain they carry (Vega-Rúa et al. 2014). Even if *A. albopictus* was confirmed as a less competent vector, it is not yet clear whether the release of more vectors could offset any benefit created by the reduction of original pest species.

Additional traits may also deserve consideration. For example, *A. albopictus* is reported to exhibit more aggressive biting behaviour than *A. aegypti*. Hence the additional nuisance of releasing more biting insects into a target are for control should be assessed. For this reason, in scenarios involving disease vectors such as *Aedes* species, it is generally beneficial to release only males, as it is females that bite, require bloodmeals and result in further disease transmission (Alphey et al. 2013; Gilles et al. 2014; Zhang et al. 2015). Provided that the females of the target pest show sufficient susceptibility to heterospecific courtship/mating, and that this incurs sufficient fitness costs, male-only releases could be compatible with the aim of population control via Satyrisation. As a consequence, there is interest in understanding the molecular mechanisms of Satyrisation in order to engineer Satyr strains for control that could target both inter and intra-specific reproductive interactions. For example, there is evidence to suggest that Satyrisation can occur between isolated populations within species, which are undergoing incipient speciation (Wu et al. 1995; Ting et al. 2001). Therefore, it may be possible to identify or engineer strains to confer control through within species Satyrisation effects, which, when combined with recognition of the factors described above that increase population susceptibility to Satyrisation, could be fruitful. In effect, this could resemble control via sterile-males or SIT (Release of sterile males to interrupt reproduction of pest populations) and would also resemble an interference control strategy originally developed in *Culex pipiens fatigans* (Krishnamurthy and Laven 1976) in which strains of the same species were available that were incompatible (though not initially known, the basis of this incompatibility was infection with different types of *Wolbachia*).

As with all control methods, Satyrisation will be susceptible to evolution of resistance (Bargielowski et al. 2013) and being rendered less effective by the expression of sexual traits such as conspecific sperm-precedence. However, resistance genes often carry fitness costs (Crowder et al. 2010; Bargielowski et al. 2019) meaning that in the absence of any selective pressure due to the presence of 'Satyr' individuals, resistance should decay. This creates an opportunity to determine which pest populations are more likely to be susceptible to Satyrisation by analysing how long they have been in sympatry or allopatry.

The dependence of Satyrisation on incorrect mate choice could also create opportunities for synergies with other control methods, with the aim of inducing additive or even multiplicative effects (Leftwich et al. 2015). Analysis and alteration of the genetic qualities of a target population and release strain, such as non-target loci, could be used to complement primary control strategies (Leftwich et al. 2020). For example, one could ensure that any release strain intended to confer one primary mechanism of control, such as via *Wolbachia* infection, was also sensitive to Satyrisation. Release of such insects for control could then introgress Satyrisation sensitivity alleles into the target population simultaneous with any primary targeting genes (Alphey et al. 2009). This would create an opportunity to subsequently exploit the sensitivity to Satyrisation introgressed into the target population, to enhance the efficacy of future management.

Similarly, Honma et al. (2018) examined "Sterile Interference", i.e., a combined application of sterile insect technique (mass release of sterile males) and Satyrisation. In this, they explored how control programs could be made cost-effective, using the initial reduction of the conspecific population to increase the ratio of heterospecific males to conspecific males, and therefore increase the likelihood of interspecific mating. Any control programmes in which engineered or manipulated individuals are released into a target population (e.g. such as Sterile Insect, or Incompatible Insect Techniques) should consider the possible effects of Satyrisation. Any appreciable frequency of courtships or matings between released individuals and heterospecifics in the area will decrease the efficacy of control by increasing mating interference and reducing the probability of the conspecific pairing upon which control is predicated.

The idea that Satyrisation may be affecting pre-existing control methods underlines that Satyrisation shares characteristics with these successful management schemes, namely the utilisation of signal jamming and mating disruption to exert control over pest populations. The potential difference between these methods may be that Satyrisation could have a greater role in effecting pre-mating fitness costs, which could be used to bolster the reproductive losses experienced by the pest population due to unsuccessful copulations. In addition, Satyrisation control programmes could reap the benefits of single sex release, but without the fitness losses from treatments that induces sterility in Sterile Insect Technique release individuals.

Our understanding of the potential of Satyrisation as a direct method of control is as yet undeveloped. However, while this means the Satyrisation is not likely to be applied in the near future, its understanding is vital both to understand its potential impacts upon control via other mechanisms and to inform potential new routes for control. By considering Satyrisation when designing control initiatives, we can determine and anticipate its likely positive or negative impacts.

A framework for control via Satyrisation

A potential framework for considering Satyrisation for control would require several key steps and these are outlined below and in Table 3. Of primary importance would be to identify the target population requiring control and from this to determine (i) whether it has any closely related species with which is it shows Satyrisation, and (ii) if these species occur in sympatry. If no such examples exist, additional research would be required to determine if Satyrisation has been observed between any related species in laboratory experiments. Subsequent steps would be to consider whether it is ethical, straightforward and beneficial to potentially release the 'controlling' Satyr species into the area containing the target species, through a series of standard risk assessments (Touré et al. 2003; Bale et al. 2008). Analyses from previous biological control and genetic pest management schemes could be used as a foundation (FAO/IAEA 2006; Oye et al. 2014). There are clear parallels between the potential use of Satyrisation and biological control, either in its standard or augmented form (i.e. if ongoing releases are required). Whether releasing the Satyr species/population complies with this current and well-established legislation for biological control would need to be carefully assessed (Turner et al. 2018) as well as considering biosafety frameworks advised by global authorities on biosecurity and public health (WHO and UNICEF 2010; United Nations 2013; Engineering Biology Research Consortium 2020).

It would be important in this assessment to focus on elements of the process that are potentially Satyrisation-specific. These might centre around the relationship between field and laboratory studies and the potential for resistance. Satyrisation in field populations with a long history of sympatry might represent 'resistant' genotypes, and effective control strategies via Satyrisation in this context would be encouraging. Isolated populations of the target species may be much more susceptible to Satyrisation, and this could be revealed by laboratory studies. However, such populations could rapidly acquire resistance. From a regulatory perspective, there may be quite a difference between introducing a new species (to create Satyrisation) vs supplemental releases of one of two species already in sympatry.

If it is determined that release of a Satyr population for control is ethical, safe, and beneficial, it will be necessary to examine how each factor functions between the target and Satyr population (Table 2). This may include:

1. Conducting field cage and then open field observations of interspecific interactions, both sexual and competitive.
2. Population and demographic surveys and modelling of populations.
3. Laboratory and field cage recreations of mating assays to determine mating frequency and to observe pre- and post-mating barriers.
4. Crossing species over multiple generations, first in the laboratory and then in semi natural conditions, to ascertain how resistance genes arise and persist.
5. Examining the genetic and geographical history of the target population, to determine their susceptibility to Reproductive Interference.
6. Examining the degree of intraspecific sexual conflict in each species/population.

If after examining these factors, Satyrisation remains a viable prospect, it should be considered whether it can synergise with other control methods, such as SIT (Honma et al. 2019). Following this, the development of practical control elements would be followed (Table 3) and under guidance from the various regulatory authorities (Vanderplank 1944; FAO/IAEA, 2006; Bale et al. 2008; Turner et al. 2018).

Overall, considering the ever-growing problems of resistance to standard chemical pesticides, and with such pesticides often being non-specific and harming non-target species, it is important to consider all potential alternative methods for control (Alphey et al. 2013; Shelton et al. 2020). Satyrisation could easily be added to this list, as it is a naturally existing phenomenon that could be harnessed in a number of different ways. Our growing understanding of Satyrisation invasion dynamics and the potential ecological complications of species release, will aid in the future development of principles of Satyrisation as a pest control method.

Table 3: Overview of planning elements for potential Satyrization control protocol development and associated steps.

Required plan components	Reasonable steps
Target identification and rationale	(i) Identify target species
	(ii) Identify potential "Satyr" species
	(iii) Determine frequency of RI through observations in sympatry or laboratory experiments
Risk assessment and regulation compliance	Research local regulations on species release and control protocols. Consider ethical and ecological ramifications of control.
Examine efficacy of potential satyrization control procedure	(i) Consider factors discussed in Table 2, and how these may affect the frequency and success of Satyrization.
	(ii)Examine potential synergies with other control methods
Consider practical applicatory elements	(i) Cost-effectiveness
	(ii) Duration, location, and frequency of application
	(iii) Communication with stakeholders and public

Conclusions

Satyrisation operates at the interface between evolutionary genetics and ecology and there is a growing body of literature to demonstrate its importance in the natural world via effects on species exclusion, speciation, and partitioning (Ribeiro and Spielman 1986; Kuno 1992). There are various factors that can influence the presence and degree of Satyrisation, including density-dependent factors such as species abundance, through to rates of species divergence and variation in sexual conflict. From an ecological point of view, we need to consider how Satyrisation may shape species distributions, and how it may alter invasion success and dynamics. From an evolutionary perspective, we must also consider the extent to which Satyrisation has influenced speciation and reinforcement. From an applied perspective, appropriate use of Satyrisation may aid in suppressing disease vector populations, or to increase crop yield by limiting crop pest populations.

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Thesis direction

Following the publication of my introductory review, I used the insights gained from the literature review to inform the direction of research for the rest of the thesis. I conducted several experiments that I felt would test some important factors considered in my literature review, and act as a proof-of-concept for experiments testing the efficacy of satyrisation for pest population control.

I decided to use the model fruit fly, *Drosophila melanogaster*, as a focal species. Alongside having relatively simple husbandry needs, a short lifespan and relatively high fecundity, the evolutionary history and reproductive isolation of the *Drosophilidae* in general is well
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established, making it a strong candidate for examination of the nature of reproductive barriers. The secondary species used was *D. mauritiana* and was selected because it has complete postzygotic isolation from *D. melanogaster,* while retaining the ability to engage in reciprocal interspecific matings (Turissini et al, 2018).

After I had identified these suitable species that would allow me to observe heterospecific reproductive interactions, I planned experiments to help illuminate how key factors could influence the efficacy of satyrisation and thus to gain knowledge of how it might be implemented for control (Table 3). The key factor I tested was the effect of female age on the probability of Reproductive Isolation. The context to this choice is outlined below. The experiments I conducted are reported in Chapter 2. I then concluded the thesis research by synthesising all the results and discussing the potential wider relevance of the work and future directions in a concluding Chapter 3.

Rationale to test female age as a potential factor impacting satyrisation

In order to assess the potential efficiency of Reproductive Interference for control, it is necessary to understand both the mechanisms of Reproductive Interference and the factors that cause it to vary. One key factor of particular interest is the effect of age on the probability and effects of Reproductive Interference. Age has known effects on mortality rate, sexual receptivity, and fecundity in both sexes. However, the precise effects of age on the probability of hybrid matings and the magnitude of the effects of hybrid matings that occur at different ages is not yet known. Thus, the implications of age effects on the effectiveness of control methods based on Reproductive Interference could be significant. That age effects could be important is supported by findings of the effects of age on matings within species. In a 2007 study a difference in mating propensity and mating competitiveness was reported between even 2-3 day versus 4-day old male Mediterranean Fruit Flies (*Ceratitis capitata*) that were destined to be used in Sterile Insect Technique control programmes (Shelly et al. 2007). The importance of such age effects is further supported by findings that 4–12-day-old females of this same species preferred younger males, but 20-day-old females displayed a loss of ability to discriminate between males of different ages (Anjos-Duarte et al. 2011).

It is not yet known whether the mechanisms that govern age-related effects on matings within species are shared with those that occur between species. However, assuming that they are at least somewhat related, then the many reports of variation in mating selectivity with age of both sexes, summarised below, support the hypothesis that age will be an important factor governing the probability of interspecific matings.

Effects of female age on male mate choice have been found in the polygamous moth species *Ephestia kuehniella*, in which males displayed pre-copulatory mate choice, with a preference for younger females and females who weighed more. Female fecundity and fertility also significantly decreased with age in this species (Xu and Wang, 2009). A wide-ranging review of female mate choice in insects concluded that female choosiness is generally expected to decrease with age, due to reproductive senescence (Kelly, 2018). For example, Moore and Moore (2001) found a significant decrease in the duration of courtship needed to elicit female mating responses in the cockroach *Nauphoeta cinerea*, in comprisons between young and old females. It was suggested that this may be due to the increasing cost of retaining viable oocytes as females age. Female mate choice can also be significantly influenced by an interaction between male density and female age, e.g., in the field cricket, *Gryllus lineaticeps*, with female choosiness decreasing with age, and with low density (Atwell and Wagner, 2014). Age in *Drosophila* fruit flies is known to have effects on multiple reproductive characteristics. This includes an effect of male age and prior mating experience on mating success (Long et al. 1980, Saleem et al. 2014) and on female choice (Avent et al. 2008). Effects of maternal and grandmaternal age on offspring fitness are also reported (Hercus & Hoffmann, 2000). Differences in the acoustic qualities of wing song also occur with age, and may be an important factor governing the probability of interspecific matings between *D. melanogaster* and *D.simulans* (Moulin et al. 2001). Koref-Santibáñez (2001) note that in assays conducted with *Drosophila pavani* and *Drosophila gaucha*, age impacted the frequency of heterospecific matings, with old male *D. guacha* more frequently mating heterospecific females of any age, when compared with younger *D. guacha* males. Conversely, *D. pavani* males were more likely to mate heterospecifically than their older counterparts, with a particular preference for older heterospecific females.

This body of research suggests that we would expect significant effects of age on the incidence and severity of satyrisation. This is supported by the results of a study by Bargielowski et al. 2019, in which it was determined that *Aedes aegypti* and male *A. albopictus* mosquitoes displayed higher frequencies of interspecific mating as individuals of either sex in both species aged. This effect may be due to older individuals more readily accepting interspecific matings, due to a decrease in selectivity in mate choice as a result of diminishing reproductive value towards the end of life. Another potential cause is the breakdown in mating barriers as various mating signals e.g., cuticular hydrocarbons, auditory cues, degrade with age. Whilst the underlying mechanism determining this age effect remains elusive, the existence of an interspecific mating age effect between two important vector species emphasises the need to investigate the effects of age on Reproductive Interference more broadly. Many insect systems may be subject to these same mechanisms of age-related acceptance of heterospecific mates, due to their similarly short lifespans, and mixed mating cues.

In the work described in Chapter 2, I investigated the effect of female age on the incidence and severity of Reproductive Interference in two closely related species of *Drosophila* (*D. melanogaster* and *D. mauritiana*). I examined the effects of exposure to con and heterospecific individuals of the opposite sex on female lifespan and reproductive rate. In addition, analyses of multiple reproductive behaviours were undertaken to determine whether certain behaviours underpinned fitness cost asymmetries between crosses, and to test if the frequency of these behaviours changed as females aged. This spanned from courtship up to mating behaviour, to cover both pre- and post-mating fitness costs. Included in this was also conducting homospecific mating assays to determine baseline data on mating habits, in order to see if any asymmetries in the cost of RI existed between the different heterospecific crosses.

The aim was to examine survival effects and fecundity data - hence I focussed on the effect of the con and heterospecific pairings on females. This allowed me to track the survival, fecundity, and reproductive success of individual females. Previous research also suggests that there are likely to be high survival costs to females arising from interspecific matings (Yassin and David, 2016). For example, there was a reported 5-fold increase in mortality for *D. santomea* females which mated with *D. mauritiana* males, due to incompatible genitalia causing internal wounding to the *D. santomea* female reproductive tracts.

The focal species *D. melanogaster* and *D. mauritiana* are considered to exhibit post-zygotic isolation. Hybrid matings occur in both directions. However, no fertile offspring are produced from either cross (Turissini et al. 2018). Female *D. melanogaster* x male *D. mauritiana* produce sterile females but no males, whereas female *D. mauritiana* x male *D. melanogaster* produce sterile males and no females.

I followed focal females throughout their lifetime, which allowed us to track both individual and population changes in receptivity, fecundity, and survival between crosses, and gave resolution into the effects of age on con- vs heterospecific matings.

References

Alphey L, McKemey A, Nimmo D, Neira-Oviedo M, Lacroix R, Matzen K, Beech, C (2013) Genetic control of *Aedes* mosquitoes. *Pathogens and Global Health* 107:170-179.

Alphey L (2014) Genetic control of mosquitoes. Annual Review of Entomology 59.

Alphey N, Bonsall M, Alphey L (2009) Combining pest control and resistance management: synergy of engineered insects with Bt crops. *J Econ Entomol* 102:717.

Arthur NJ, Dyer KA (2015) Asymmetrical sexual isolation but no postmating isolation between the closely related species *Drosophila suboccidentalis* and *Drosophila occidentalis*. *BMC Evolutionary Biology* 15:1-9.

Bale JS, Van Lenteren JC, Bigler F, (2008) Biological control and sustainable food production. *Phil Trans Roy Soc* B 363:761-776.

Bargielowski IE, Lounibos LP, Carrasquilla MC (2013) Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. *Proc Natl Acad Sci USA* 110:2888-2892.

Bargielowski IE, Lounibos LP, Shin D, Smartt CT, Carrasquilla MC, Henry A, Navarro JC, Paupy C, Dennett JA (2015) Widespread evidence for interspecific mating between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in nature. *Infection, Genetics and Evolution* 36:456-461.

Bargielowski IE, Lounibos LP (2016) Satyrization and satyrization-resistance in competitive displacements of invasive mosquito species. *Insect Science* 23:162-174.

Bargielowski IE, Honório NA, Blosser EM, Lounibos LP (2019) Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency. *J Medical Entomology* 56:329-333.

Bath E, Tatarnic N, Bonduriansky R (2012) Asymmetric reproductive isolation and interference in neriid flies: the roles of genital morphology and behaviour. *Animal Behaviour* 84:1331-1339.

Bedinger PA, Chetelat RT, McClure B, Moyle LC, Rose JK, Stack SM, van der Knaap E, Baek YS, Lopez-Casado G, Covey PA, Kumar A (2011) Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. *Sexual Plant Reproduction* 24:171-187.

Birch LC, Park T, Frank MB (1951) The effect of intraspecies and interspecies competition on the fecundity of two species of flour beetles. *Evolution* 1:116-132.

Butlin RK, Ritchie MG (2009) Genetics of speciation. *Heredity* 102*:*1–3.

Carrasquilla MC, Lounibos LP (2015) Satyrization without evidence of successful insemination from interspecific mating between invasive mosquitoes. *Biology Letters* 11:20150527.

Costa-Schmidt LE, Machado G (2012) Reproductive interference between two sibling species of gift-giving spiders. *Animal Behaviour* 84:1201-1211.

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. *Evolution* 43:362-381.

Crowder DW, Sitvarin MI, Carrière Y (2010) Plasticity in mating behaviour drives asymmetric reproductive interference in whiteflies. *Animal Behaviour* 79:579-587.

D'Amore A, Kirby E, Hemingway V (2009) Reproductive interference by an invasive species: an evolutionary trap. *Herpetological Conservation and Biology* 4:325-330.

Dincă V, Wiklund C, Lukhtanov VA, Kodandaramaiah U, Norén K, Dapporto L, Wahlberg N, Vila R, Friberg M (2013) Reproductive isolation and patterns of genetic differentiation in a cryptic butterfly species complex. *J Evolutionary Biology* 26:2095-2106.

Drury JP, Okamoto KW, Anderson CN, Grether GF (2015) Reproductive interference explains persistence of aggression between species. *Proc Roy Soc B* 282:20142256.

Drury JP, Anderson CN, Cabezas Castillo MB, Fisher J, McEachin S, Grether GF (2019) A general explanation for the persistence of reproductive interference. *Am Nat* 194:268-275.

Engineering Biology Research Consortium (2020) Security & engineering biology. https://ebrc.org/focus-areas/security/

Friberg M, Leimar O, Wiklund, C. (2013) Heterospecific courtship, minority effects and niche separation between cryptic butterfly species. *J Evolutionary Biology* 26:971-979.

FAO/IAEA (2006) Status and risk assessment of the use of transgenic arthropods in plant protection. Vienna, Austria.

Gilles JR, Schetelig MF, Scolari F, Marec F, Capurro ML, Franz G, Bourtzis K (2014) Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. *Acta Tropica,* 132:S178-S187.

Gröning J, Hochkirch A (2008) Reproductive interference between animal species. The *Quarterly Review of Biology* 83:257-282.

Hill T, Rosales-Stephens HL, Unckless R (2020) Rapid divergence of the copulation proteins in the *Drosophila dunni* group is associated with hybrid post-mating-prezygotic incompatibilities. *bioRxiv*. https://doi.org/10.1101/2020.05.20.106724

Hochkirch A, Gröning J, Bücker A (2007) Sympatry with the devil: reproductive interference could hamper species coexistence. *J Animal Ecology* 76:633-642.

Hollander J, Montaño-Rendón M, Bianco G, Yang X, Westram AM, Duvaux L, Reid DG,Butlin RK (2018) Are assortative mating and genital divergence driven by reinforcement? *Evolution letters* 2:557-566.

Honma A, Kumano N, Noriyuki S (2019) Killing two bugs with one stone: a perspective for targeting multiple pest species by incorporating reproductive interference into sterile insect technique. *Pest Management Science* 75:571-577.

Honório NA, Carrasquilla MC, Bargielowski IE, Nishimura N, Swan T, Lounibos LP (2018) Male origin determines satyrization potential of *Aedes aegypti* by invasive *Aedes albopictus*. *Biological Invasions* 20:653-664.

Hugo LE, Stassen L, La J, Gosden E, Ekwudu OM, Winterford C, Viennet E, Faddy HM, Devine GJ, Frentiu FD (2019) Vector competence of Australian *Aedes aegypti* and *Aedes albopictus* for an epidemic strain of Zika virus. *PLoS Neglected Tropical Diseases* 13:e0007281.

Huxel GR (1999) Rapid displacement of native species by invasive species: effects of hybridization. *Biological Conservation* 89:143-152.

Iritani R, Noriyuki S (2021) Reproductive interference hampers species coexistence despite conspecific sperm precedence. *Ecol Evol* 00:1– 13.

Kishi S, Takayoshi N, Yoshitaka T (2009) Reproductive interference determines persistence and exclusion in species interactions. *J Animal Ecology* 78:1043-1049.

Kishi S, Nakazawa T (2013) Analysis of species coexistence co-mediated by resource competition and reproductive interference. *Pop Ecol* 55:305–313.

Krishnamurthy BS, Laven H. (1976) Development of cytoplasmically incompatible and integrated (translocated incompatible) strains of *Culex pipiens fatigans* for use in genetic control. *Journal of Genetics* 62:117-129.

Kronforst MR, Young LG, Gilbert LE (2007) Reinforcement of mate preference among hybridizing *Heliconius* butterflies. *J Evolutionary Biology* 20:278-285.

Kuno E (1992) Competitive exclusion through reproductive interference. *Res Popul Ecol* 34:275–284.

Kyogoku D (2020) When does reproductive interference occur? Predictions and data. *Pop Ecol* 62:196-206.

Kyogoku D, Sota T (2017) A generalized population dynamics model for reproductive interference with absolute density dependence. *Scientific Reports* 7:1-8.

Landolt PJ, Heath RR (1987) Role of female-produced sex pheromone in behavioral reproductive isolation between *Trichoplusia ni* (Hübner) and *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae, Plusiinae). *Journal of Chemical Ecology* 13:1005-1018.

Leigh S, Rostant WG, Taylor MI, Alphey L, Chapman T (2020) Satyrization in *Drosophila* fruitflies. *J Evolutionary Biology*.

Leftwich PT, Bolton M, Chapman T (2016) Evolutionary biology and genetic techniques for insect control. *Evolutionary Applications* 9:212-230.

Leftwich PT, Spurgin LG, Harvey-Samuel T, Thomas CJ, Paladino LC, Edgington MP, Alphey L 2021. Genetic pest management and the background genetics of release strains. *Phil Trans Roy Soc B* 376:20190805.

Levin DA (1970) Reinforcement of reproductive isolation: plants versus animals. *Am Nat* 104:571-581.

Lewis D, Crowe LK (1958) Unilateral interspecific incompatibility in flowering plants. *Heredity* 12:233-256.

Liu SS, De Barro PJ, Xu J, Luan JB, Zang LS, Ruan YM, Wan FH (2007) Asymmetric mating interactions drive widespread invasion and displacement in a whitefly. *Science* 318:1769-1772.

Marta AE, Camadro EL, Díaz-Ricci JC, Castagnaro AP (2004) Breeding barriers between the cultivated strawberry, *Fragaria* × *ananassa*, and related wild germplasm. *Euphytica* 136-139.

Matute DR (2014) The magnitude of behavioral isolation is affected by characteristics of the mating community. *Ecology and Evolution* 4:2945-2956.

Nasci RS, Hare SG, Willis FS (1989) Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory. *J American Mosquito Control Association* 5:416-421.

Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, Lightfoot SBY, McNamara J, Smidler A, Collins JP (2014) Regulating gene drives. *Science* 345:626-628.

Park T (1948) Experimental studies of interspecies competition. 1. Competition between populations of the flour beetles, *Tribolium confusum* Duval and *Tribolium castaneum Herbst.Ecol Monogr* 18:265-307.

Parker C, Ramirez D, Connelly CR (2019) State-wide survey of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Florida. *J Vector Ecology* 44:210-215.

Pfennig DW, Pfennig KS (2010) Character displacement and the origins of diversity. *Am Nat* 176:S26-S44.

Price CS (1997) Conspecific sperm precedence in *Drosophila*. *Nature* 388:663-666.

Ribeiro JMC, Spielman A (1986) The satyr effect: a model predicting parapatry and species extinction. *Am Nat* 128:513–528.

Rice AM, Pfennig DW (2010) Does character displacement initiate speciation? Evidence of reduced gene flow between populations experiencing divergent selection. *J Evolutionary Biology* 23:854-865.

Rugman-Jones PF, Eady PE (2007) Conspecific sperm precedence in *Callosobruchus subinnotatus* (Coleoptera: Bruchidae): mechanisms and consequences. *Proc Roy Soc B* 274:983-988.

Shelton AM, Long SJ, Walker AS, Bolton M, Collins HL, Revuelta L, Johnson LM, Morrison NI (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. *Frontiers in Bioengineering and Biotechnology* 7:482.

Shuker DM, Currie N, Hoole T, Burdfield-Steel ER (2015) The extent and costs of reproductive interference among four species of true bug. *Population Ecology* 57:321-331.

Shuker DM, Burdfield-Steel ER (2017) Reproductive interference in insects. *Ecol Entomol* 42: 65-75.

Smadja C, Ganem G (2005) Asymmetrical reproductive character displacement in the house mouse. *J Evolutionary Biology* 18:1485-1493.

Takafuji A, Kuno E, Fujimoto H (1997) Reproductive interference and its consequences for the competitive interactions between two closely related *Panonychus* spider mites. *Experimental and Applied Acarology* 21:379-391.

Templeton AR (1981) Mechanisms of speciation-a population genetic approach. Annual *Review of Ecology and Systematics* 12:23-48.

Ting CT, Takahashi A, Wu CI. (2001) Incipient speciation by sexual isolation in *Drosophila*: concurrent evolution at multiple loci. *Proc Natl Acad Sci USA* 98:6709-6713.

Touré YT, Oduola AM, Sommerfeld J, Morel CM (2003) Bio-safety and risk assessment in the use of genetically modified mosquitoes for disease control. Ecological aspects for application of genetically modified mosquitoes. Wageningen: *Springer/Frontis* pp.217-222.

Tripet F, Lounibos LP, Robbins D, Moran J, Nishimura N, Blosser EM (2011) Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. *American Journal of Tropical Medicine and Hygiene* 85:265-270.

Turner G. Beech C, Roda L (2018) Means and ends of effective global risk assessments for genetic pest management. *BMC Proceedings* 12:13.

United Nations Cartagena Protocol on Biosafety to the Convention on Biological Diversity (2003). https://treaties.un.org/doc/Treaties/2000/01/20000129%2008-44%20PM/Ch_XXVII_08_ap.pdf

Valero A, Macías Garcia C, Magurran AE (2008) Heterospecific harassment of native endangered fishes by invasive guppies in Mexico. *Biology Letters* 4:149-152.

Vallin N, Rice AM, Arntsen H, Kulma K, Qvarnström A (2012) Combined effects of interspecific competition and hybridization impede local coexistence of *Ficedula* flycatchers. *Evolutionary Ecology* 26:927-942.

Vanderplank FL (1944) Hybridization between *Glossina* species and suggested new method for control of certain species of tsetse. *Nature* 154:607-608.

Vega-Rúa A, Zouache K, Girod R, Failloux AB, Lourenço-de-Oliveira R (2014) High level of vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor in the spread of Chikungunya virus. *Journal of Virology* 88:6294-6306.

Weber MG, Strauss SY, 2(016). Coexistence in close relatives: beyond competition and reproductive isolation in sister taxa. *Ann RevEcol, Evol Systemat* 47:359-381.

Whitton J, Sears CJ, Maddison WP (2017) Co-occurrence of related asexual, but not sexual, lineages suggests that reproductive interference limits coexistence. *Proc Roy Soc B* 284:20171579.

World Health Organization and UNICEF (2010) Progress and prospects for the use of genetically modified mosquitoes to inhibit disease transmission. World Health Organization, Geneva.

World Health Organization (2014) Global Brief on Vector-Borne Diseases. World Health Organization, Geneva.

Wu CI, Hollocher H, Begun DJ, Aquadro CF, Xu Y, Wu ML (1995) Sexual isolation in *Drosophila melanogaster*: a possible case of incipient speciation. *Proc Natl Acad Sci USA* 92:2519-2523.

Yassin A, David JR (2016) Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila*. *J Evolutionary Biology* 29:455-460.

Zhang D, Lees RS, Xi Z, Gilles JR, Bourtzis K (2015) Combining the sterile insect technique with Wolbachia-based approaches: II-a safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. *PloS One* 10:e0135194.

Chapter 2: Effects of Female Age on Behavioural and Fitness Responses to Conspecific and Heterospecific Male Exposure

Chapter 2 - attribution statement: I designed and conducted all insect husbandry and experimental work with additional input from my supervisor, Professor Tracey Chapman. Statistical analysis was conducted by me, with assistance from Lauren Harrison. Experimental reporting was written by me, with feedback from Tracey Chapman and Lauren Harrison.

Abstract:

Disruptions to reproductive processes that arise due to matings or interactions between species are of significant interest to identify fundamental mechanisms of reproductive isolation and speciation, and because they can highlight potential mechanisms to control pest populations. A wealth of studies have investigated the mechanisms of mate selection, including identifications of the reproductive traits and characteristics that individuals find attractive in prospective mates or that lead to success in mating. Linked to this, many researchers have necessarily documented instances in which mechanisms to ensure mate attraction or mating success have become misdirected towards individuals of other species or even inanimate objects. This can lead to matings or attempted matings between species, with the potential to result in significant fitness costs either through loss of conspecific mating opportunities, or energy invested in the production of sterile hybrids. In this context, changes in the accuracy of mate recognition and attraction with age, could be a vital component in the occurrence of reproductive interference. Many reproductive traits, such as fecundity, are observed to senesce with age. Here I tested the

prediction that the accuracy of mating choice and selectivity also shows this pattern. Thus, I investigated whether there was an increase in the frequency of heterospecific matings between between two closely related *Drosophila* species (*Drosophila melanogaster* and *D. mauritiana*) with advancing female age. In doing this I also investigated the effects of female age on the behavioural responses to con- and heterospecific mating stimuli. The results showed that the frequency of mating behaviours was most strongly related to whether focal females were paired with conspecific or heterospecific males, rather than age, with conspecifically paired females exhibiting higher frequencies of behaviour. However, there was a large discrepancy in mortality rates between con- and heterospecific crosses, with females of both species surviving longer when paired heterospecifically, in comparison to those paired conspecifically. The results show that there are distinct con- and heterospecific female fitness costs of mating, which should be considered in tests of the viability of satyrisation applications in pest population control.

Introduction

Reproductive Interference occurs when reproductive behaviours occur between members of different species (heterospecific individuals), resulting in a fitness cost to one or either party (Gröning & Hochkirch (2008), Burdfield-Steel & Shuker, 2011). Studying Reproductive Interference is of fundamental interest, particularly in evolutionary biology, as it can help elucidate the mechanisms underlying speciation (Cothran, 2015). Interest in Reproductive Interference and satyrisation (Reproductive Interference with asymmetric fitness costs between species) is also growing because of the realisation that it could be deployed to develop novel methods for control of pest populations (Mitchell et al. 2022). However, in order to assess the potential efficiency of Reproductive Interference for control, we need to understand both the mechanisms of Reproductive Interference and the factors that cause it to vary.

Key to the extent of Reproductive Interference is the probability of matings between species within the context of the evolution of species isolating barriers. Whether mechanisms selected to refine mate choice and preferences within species are those that are also deployed to protect against heterospecific matings is not yet clear. However, given that mate recognition and selectivity are likely to play key roles in determining the extent of Reproductive Interference it is important to understand the underlying drivers of variation in these traits. One overlooked area of study is how the magnitude of reproductive interference is affected by major life history traits such as ageing, and this is the main topic of the research I tackle in this chapter.

The focus of interest is the effect of female age on the probability and effects of Reproductive Interference. Age has known effects on mortality rate, sexual receptivity, and fecundity in both sexes. However, the precise effects of age on the probability of hybrid matings and the magnitude of the effects of hybrid matings that occur at different ages is not yet known. Thus, the implications of age effects for efficacy of control methods based on Reproductive Interference could be significant. That age effects could be important is supported by findings of a 2007 study that reported a difference in mating propensity and mating competitiveness between even 2-3 day versus 4-day old male Mediterranean Fruit Flies (*Ceratitis capitata*) that were destined to be used in Sterile Insect Technique control programmes (Shelly et al., 2007). The importance of such age effects is further supported by findings that 4–12-day-old females of this same species preferred younger males, but 20-day-old females displayed a loss of ability to discriminate between males of different ages (Anjos-Duarte et al. (2011). It is unclear as yet the extent to which the mechanisms that might govern age-related effects on matings within species are likely to be shared with those that occur in matings between species. However, it is generally assumed that recognition and selectivity mechanisms are at least somewhat related within and across species. Therefore, the many reports of variation in mating selectivity with age observed in both sexes, summarised below, support the hypothesis that age will be an important factor governing the probability of interspecific matings.

Effects of female age on male mate choice have been found in the polygamous moth species *Ephestia kuehniella*, in which males display pre-copulatory mate choice, with a preference for younger females and heavier females. Female fecundity and fertility also significantly decreased with age (Xu and Wang, 2009). A wide-ranging review of female mate choice in insects concluded that female choosiness is generally expected to decrease with age, due to reproductive senescence (Kelly, 2018). For example, Moore and Moore (2001) found a significant decrease in the duration of courtship needed to elicit female mating responses in the cockroach *Nauphoeta cinerea*, in comparisons between young and old females. It was suggested that this may be due to the increasing cost of retaining viable oocytes as females age. Female mate choice can also be significantly influenced by an interaction between male density and female age, e.g., in the field cricket, *Gryllus lineaticeps*, with female choosiness decreasing with age, and with low density (Atwell and Wagner, 2014).

Age in *Drosophila* fruit flies is known to have effects on multiple reproductive characteristics. This includes an effect of male age and prior mating experience on mating success (Long et al. 1980, Saleem et al. 2014) and on female choice (Avent et al. 2008). Effects of maternal and grandmaternal age on offspring fitness are also reported (Hercus & Hoffmann, 2000). Differences in the acoustic qualities of wing song also occur with age and may be an important factor influencing the probability of interspecific matings between *D. melanogaster* and *D. simulans* (Moulin et al. 2001). Koref-Santibáñez (2001) note that in assays conducted with *Drosophila pavani* and *Drosophila gaucha*, age impacted the frequency of heterospecific matings, with old male *D. guacha* more frequently mating heterospecific females of any age, when compared with younger *D. guacha* males. Conversely, *D. pavani* males were more likely to mate heterospecifically than their older counterparts, with a particular preference for older heterospecific females.

This body of research suggests that we should expect significant effects of age on the incidence and severity of satyrisation. This is supported by the results of a study by Bargielowski et al. (2019), in which it was determined that *Aedes aegypti* and male *A. albopictus* mosquitoes displayed higher frequencies of interspecific mating as individuals of either sex in both species aged. This effect may be due to older individuals more readily accepting interspecific matings, due to a decrease in selectivity in mate choice as a result of diminishing reproductive value towards the end of life. Another potential cause is the breakdown in mating barriers as various mating signals e.g., cuticular hydrocarbons, auditory cues, degrade with age. Whilst the underlying mechanism determining this age effect remains elusive, the existence of an interspecific mating age effect between two important vector species emphasises the need to investigate the effects of age on Reproductive Interference more broadly. Many insect systems may be subject to these same mechanisms of age-related acceptance of heterospecific mates, due to the deployment of mixed mating cues. Hence in the experiments described here, I also recorded courting behaviour, to determine if age affects could be attributed to specific frequency changes in certain pre-copulatory behaviour, and whether this was the basis for asymmetries in fitness costs between heterospecific crosses.

I investigated the effect of female age on the incidence and severity of Reproductive Interference in two closely related species of *Drosophila* (*D. melanogaster* and *D. mauritiana*). I examined the effects of exposure to con and heterospecific individuals of the opposite sex on female lifespan and reproductive rate. In addition, analyses of multiple reproductive behaviours were undertaken to determine whether specific behaviours were associated with any fitness cost asymmetries observed between crosses, and to test if the frequency of these behaviours changed as females aged. The behaviours recorded were courtship through to mating behaviour, to allow for estimates of both pre- and post-mating fitness costs. Baseline mating rate assays within species were conducted to determine baseline data on mating habits, in order to detect any asymmetries in the costs of Reproductive Interference between the reciprocal heterospecific pairings. I reasoned that if heterospecific mating events were sufficiently frequent or damaging, the fitness costs of these matings could inflate the mortality rates of focal females in those treatments. Alternatively, if these events were too infrequent, or the fitness costs to females were negligible, heterospecific cross females may even have a decreased mortality risk, due to fewer energetic drains from male mating attempts.

The aim was to examine effects on survival and reproductive output (fecundity). Hence, I focussed on measuring the effect of con and heterospecific pairings on focal females as they aged. This allowed me to track the survival, fecundity, and reproductive success of individual females. Previous research suggests that there are likely to be high survival costs to females arising from interspecific matings (Yassin and David, 2016). For example, there was a reported 5-fold increase in mortality for *D. santomea* females which mated with *D. mauritiana* males, due to incompatible genitalia causing internal wounding to *D. santomea* female reproductive tracts.

The focal species used were *D. melanogaster* and *D. mauritiana*. Reciprocal hybrid matings are reported to occur in both directions. However, there is post-zygotic reproductive isolation, and no fertile offspring are produced from either cross (Turissini et al. 2018). Female *D. melanogaster* x male *D. mauritiana* are reported to produce sterile females but no males, whereas female *D. mauritiana* x male *D. melanogaster* produce sterile males and no females. I exposed individual focal females of each species with either con or heterospecific males, which were renewed 7 days, until all focal females were dead. I followed focal females throughout their lifetime, which allowed me to track both individual and population changes in receptivity, fecundity, and survival between crosses, and gave resolution into the effects of age on con- vs heterospecific matings. Specifically, I collected data on movement, courtship and mating behaviour from all treatments twice weekly with an interval of one day between these assays.

Methods and Materials:

Pilot experiments to test the effects of con and heterospecific pairings on fitness in *D. melanogaster* and *D. mauritiana* females of two discrete ages and over time

Prior to the conduct and completion of the main experiment, two pilot studies were conducted to check methodology and to refine husbandry techniques of the species used. These initial studies followed similar protocols to the main experiment reported in this chapter, with minor exceptions. The first pilot study tested the effects of discrete female age cohorts rather than following focal females continuously over time. This was done to test whether reproductive behaviours were observed sufficiently frequent to warrant additional experimental study. The second pilot study followed females over time and allowed me to determine relative fecundity differences between the focal species and adjust the main experimental design to ensure sufficient sample sizes.

Laboratory and rearing conditions:

All experiments were carried out using Dahomey Wild-Type *D. melanogaster* and *D. mauritiana* (NDSSC:14021-0241.150) laboratory strains. These originated from Benin, West Africa (1970 collection) and Le Reduit, Mauritius (2006 collection) respectively. All cultures were held at 25 °C, under 12:12 h light:dark cycle, at 50% relative humidity. Stocks used to generate individuals for the experiment were raised under density-controlled conditions in milk bottles containing Sugar-Yeast-Agar (SYA) solid media [15 g Agar, 50 g granulated sugar, 100 g Brewer's yeast, 3 ml Propionic acid, 30 ml Nipagin solution (10% w/v solution in ethanol), 970 ml Water]. Due to differences in fecundity between the two species (as determined in the pilot studies) the *D. melanogaster* and *D. mauritiana* cultures were set up under different density conditions, to standardise the rearing conditions across both species, while generating sufficient individuals for analysis. To achieve this, 60:60 of *D. melanogaster* and 100:100 of *D. mauritiana* were placed in 4 and 6 standard SYA medium bottles respectively (1/3 pint glass bottles each containing 70 ml SYA medium) and moved to new food every day, in order to generate a timeseries of cohorts of offspring that emerged simultaneously, for use in assays. Experimental individuals were collected from the bottle cultures seeded at the same time; hence parents were standardized for age and mating experience.

Following eclosion, individuals were sexed and collected under ice anaesthesia, to ensure initial virginity and stored in groups of the same sex and species in SYA vials (25 x 75 mm flat bottomed soda glass vials) in controlled density conditions of 10 flies per vial, until they had reached sexual maturity. This was a duration of 3 and 5 days for females and males, respectively. New sets of replacement non focal male individuals were similarly generated every 7 days. During storage prior to use in the experiment, flies were moved onto new food vials every 2 days.

The experimental treatments were all 4 combinations of conspecific (♀ *D. melanogaster* x ♂ *D. melanogaster* and ♀ *D. mauritiana* x ♂ *D. mauritiana*) and heterospecific (♀ *D. melanogaster* x ♂ *D. mauritiana* and ♀*D. mauritiana* x ♂ *D. melanogaster*) crosses, each with a starting sample size of N = 50 at the beginning of the experiment. Virgin females were randomly assigned to treatment groups and then assigned an ID number. Females were then transferred into individual vials containing 2 conspecific or heterospecific males and kept in those treatment groups with 2 males each throughout their lives. Flies were transferred to new food every 2-3 days using CO_2 anaesthesia. To keep reproductive activity high, 2 new virgin 5-day old non focal males were introduced to each female every 7 days. Female identity was used to track treatment, survivorship, reproductive behaviour, and fecundity at an individual level, over the duration of the experiment.

Behavioural assays of courtship and mating in conspecific and heterospecific environments:

The behaviours recorded during this experiment were chosen to capture a proxy of the fitness costs throughout the mating process. These included pre-mating behaviours such as courtship, which we designated as focal females being chased or having wing song directed at them by experimental males, and movement, which was designated as any movement by the focal female not mediated by male chasing. These behaviours were chosen as they may by proxies for energetic fitness costs to females as they could indicate actions taken to avoid male harassment or searching behaviour to find suitable mates. I also chose to observe instances of attempted and successful mating. Successful mating can incur fitness costs to females associated with physiological changes from seminal fluid proteins, bodily harm, time expenditure, refractory periods, and gamete use. These costs are of course mitigated in females mating conspecifically that successfully produce offspring. Such costs can be significant in heterospecific pairings or in matings with conspecific but low-quality males. Attempted mating behaviour can also be costly due to time / injury costs in the absence of a mating that might potentially produce offspring. Successful mating was scored if males mounted the focal females for an extended period, without being rejected. Attempted mating was determined by a male mounting a focal female for a short duration before being forced to dismount due to a female rejection behaviour (decamping, kicking and other repelling behaviours as in Aranha and Vasconcelos, 2018).

To assess the frequency and intensity of reproductive behaviour in each of the treatments, I made behavioural spot checks twice weekly, within an hour of lights being turned on, and consisted of a 2h period in which all vials were inspected for courtship and mating behaviours every 20 minutes. The recording of behaviour was anonymised with respect to treatment identity. The behavioural spot checks recorded the presence or absence of behaviour, as follows:

1 = Courting (chasing and wing song directed at focal females)

2= Not courting

3 = Moving (movement of focal females that was not mediated by male chasing)

4 = Not moving

5 = Attempted mating (attempted mounting events, without successful copulation)

6 = Successful mating (mounting events resulting in successful copulation and conjoining of mating individuals)

Female fecundity and progeny production in con and heterospecific environments:

Fecundity data were generated by counting the eggs laid in each set of recently vacated vials after the flies were transferred to new food. Fecundity was sampled 3 times during the experiment, at day 13, 20 and 27. The progeny emerging from these eggs (conspecific crosses only) were also counted by retaining these vials for 11 days, allowing the offspring to emerge, or freezing them at -4˚C for later counting.

Focal female survival in con- and heterospecific mating environments.

Age at death was recorded for each focal female throughout the experiment. Following the conclusion of behavioural assays after 4 weeks, at day 32 of the experiment, males were discarded and the surviving females were subsequently kept isolated and transferred using CO_2 anaesthesia onto fresh food, twice weekly as before, until all females had died.

Statistical analysis:

(i) General approach

All statistical analyses were conducted in R studio (R version 4.2.3) (R Core Team, 2023). To determine whether the treatment cross (conspecific or heterospecific) or age had an impact on frequency of each mating behaviour, I collated counts of behavioural data from each behavioural assay, to calculate an average percentage frequency of a given behaviour for each treatment, for the age focal females were on the day of the assay. Sample sizes at each sampling time point were noted, as were the dates on which new non focal males were introduced (up until day 32). It should be noted that most of the behavioural and fecundity data collected were zero-inflated as they were measures of frequency of relatively rare events. Measures were taken to accommodate this, with each model being assessed for normality and adherence to model assumptions via visual assessment of the data, and with use of simulated residuals generated by the R Package *DHARMa*. The individual R packages used in the analyses were *ggplot2* (Wickham, 2016), *Tidyverse* (Wickham et al. 2019), *ggrepel* (Slowikowski, 2021), *ggfortify* (Horikoshi, 2018) , *car* (Fox, Weisberg, 2019), *boot* (Canty and Ripley, 2022), *dplyr* (v1.1.2; Wickham et al, 2023), *glmmTMB* (Brooks et al. 2017), *DHARMa* (Hartig, 2022), *gapminder* (Bryan, 2023), *tidyr* (Wickham, Vaughan and Girlich, 2023), *hablar* (Sjoberg, 2023), *bbmle* (Bolker and R Development Core Team, 2022), *emmeans* (Lenth, 2023). All data were analysed except for ‘attempted mating’. For that trait, the lack of observations of heterospecific mating attempts meant that analysis by GLMM was not possible because of severe zero-inflation and model non convergence.

(ii) Analysis of movement frequency of each treatment cross over time.

Analysis of the frequency of movement behaviour was performed using a generalized linear mixed model (GLMM) as follows:

*Movement GLMM output (Formula: Movement ~ Cross*Female Age + (1|Female Identity) +(1|observation), Family = Compois)*

The model included the fixed effect of the interaction of Treatment Cross and Female age. I included female identity as a random effect to account for multiple measures of the same individual with age and included observation number as a random effect to account for overdispersion in the data. After exploring model assumptions, this GLMM was run with Compois as the family. The model was tested for adherence to assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on focal female movement.

(iii) Analysis of courtship frequency of each treatment cross over time.

Courtship frequency was analysed using the following model:

*Courtship GLMM output (Formula: Courtship ~ Cross*Female Age + (1|Female Identity) +(1|observation), Family = Poisson).*

Analysis of the frequency of courtship behaviour was performed using a GLMM and included the interaction of Treatment Cross and Female age as a fixed effect. I included female identity as a random effect to account for multiple measures of the same individual with age and included observation number as a random effect to account for overdispersion in the data. As the statistical models were run on count data, the GLMM model was run with the Poisson family error structure. This statistical model was tested for adherence to model assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on courtship directed at focal females.

(iv) Analysis of Attempted Mating frequency of each treatment cross over time.

There were too few attempted mating data recorded to run a meaningful analysis for this trait.

(v) Analysis of Mating frequency of each treatment cross over time.

Analysis of the frequency of mating behaviour was performed using a generalized linear mixed model (GLMM) as follows:

*Analysis of mating frequency of each treatment cross over time. Mating GLMM output (Formula: Mating ~ Cross*Female Age + (1|Female ID), Family = nbinom2).*

The included the interaction of Treatment Cross and Female age as a fixed effect. I included female identity as a random effect to account for multiple measures of the same individual with age. A negative binomial model distribution had the best fit following tests of model assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on successful mating of focal females.

(vi) Female survival data analysis:

Focal female survival was tracked throughout the experiment. The survival data were recorded as days upon which deaths or censors (e.g. loss during transfer, etc.) occurred. A Cox proportional hazards analysis was run, with treatment cross as the fixed effect to determine the impact of treatment cross of focal female survival. A pairwise survival test with treatment cross as the fixed effect and a Bonferroni p-value correction was run to assess the differences in female survival between treatment crosses. In addition to the packages listed in prior analyses, survival analyses also used the following packages: *survival* (v3.5-5; Therneau, 2023), *survminer* (v0.4.2; Kassambara et al. 2021).

(vii) Fecundity and progeny data analysis:

To determine whether the treatment cross (conspecific or heterospecific) or age had an impact on focal female fecundity or offspring production, both egg number and offspring counts were conducted. Analysis of the egg number was performed using a GLMM and included the interaction of Treatment Cross and Female age the fixed effect. I included female identity as a random effect to account for multiple measures of the same individual with age. Due to zero-inflation, this GLMM was run with a negative binomial family and included a zero-inflation parameter applied to all observations. To analyse the number of offspring, a GLMM that included the interaction of Treatment Cross and Female age as the primary factor, as well as Female Identity as a random effect and a random observation factor was used. Similarly, to the egg number (fecundity) scores, these data were heavily zero-inflated, which we accommodated by using a truncated negative binomial distribution and by including a zero-inflation parameter applied to the interaction of treatment cross and female age. This generated a conditional model and a zero-inflated model.

Results:

Frequency of mating behaviours between conspecific and heterospecific mating treatments.

(i) Courtship frequency in con and heterospecific treatments over time

The frequency of courtship varied significantly between treatment crosses over time (Figure 1).

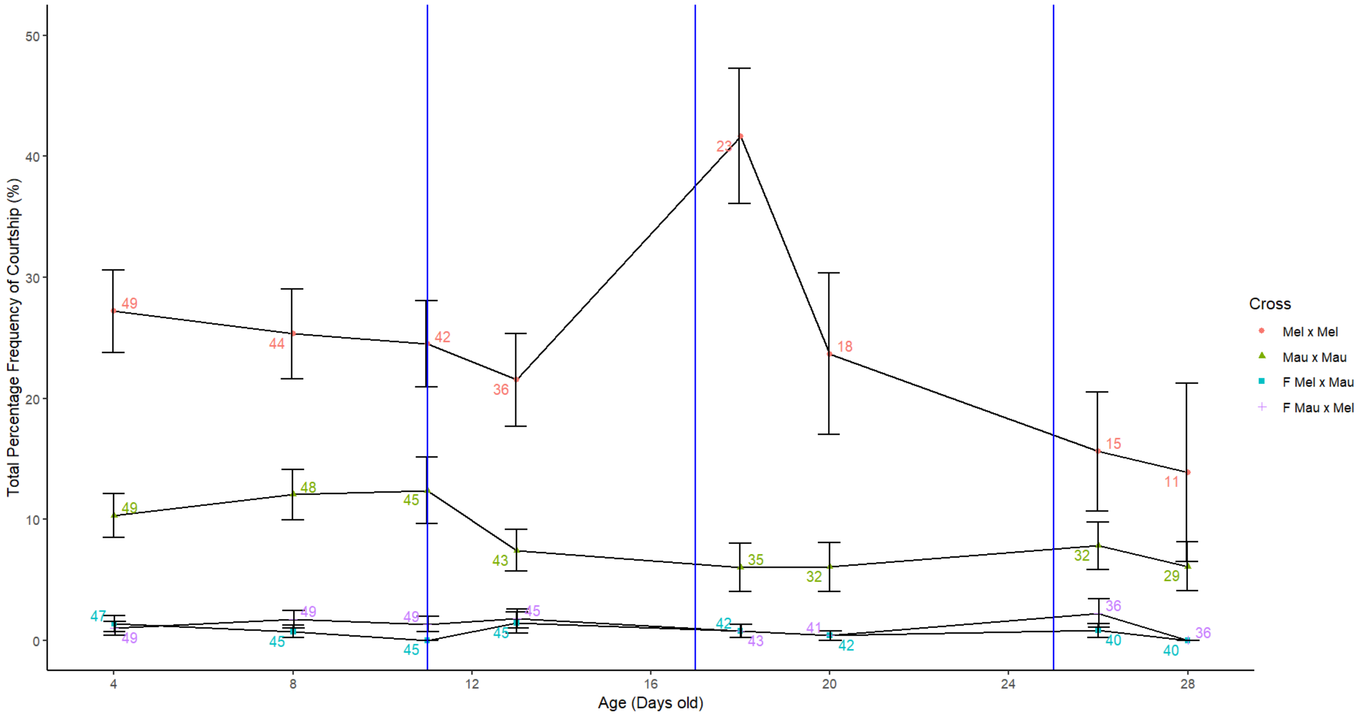

Figure 1. Average percentage frequency of observations (± 1 SE) in which courtship was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific. *Vertical blue lines depict the points at which new, young non focal males were introduced. Numbers associated with each point indicate sample size (number of focal females) at the given age. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

Females held with conspecific males received a higher frequency of courtship than did females held in heterospecific crosses, with *D. melanogaster* x *D. melanogaster* showing a significantly higher observed courtship than *D. mauritiana* x *D. mauritiana*. The frequency of courtship seen in the heterospecific crosses was very low (Table 1).

Table 1. Analysis of courtship frequency of each treatment cross over time. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of courtship observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	0.31	0.10	3.14	0.002**
Mau x Mau	-0.95	0.15	-6.22	4.94e – 10 ***
Mel (f) x Mau (m)	-3.22	0.36	-8.85	<2e – 16 ***
Mau (f) x Mel (m)	-2.92	0.31	-9.25	<2e – 16 ***
Mel x Mel*Age	-5e-4	3e-4	-1.68	0.092
Mau x Mau*age	-2.3e-4	4e-4	-0.48	0.629
Mel (f) x Mau (m)* age	-1e-3	1.1e-3	-0.86	0.385
Mau (f) x Mel (m)* age	-8e-4	1.e-3	-0.84	0.399

The results showed that conspecific *D. melanogaster* pairs showed a significantly higher courtship frequency than all other treatment crosses (Table 1). Age did not seem to significantly affect the frequency of courtship. To test for differences in courtship between all treatments, a series of pairwise analyses was conducted. This confirmed that each species pair had significantly different frequencies of observed courtship behaviour within the assays (p = <0.0001) with *D. melanogaster* conspecific treatments having a much higher frequency of courtship (Figure 1). The two heterospecific crosses showed a low frequency of courtship and did not significantly differ from one another (z = -1.005, p = 0.7464). These results show that the absence of observed courtship in heterospecific crosses was bidirectional and was not driven by the focal female species identity.

(ii) Movement frequency in con and heterospecific treatments over time

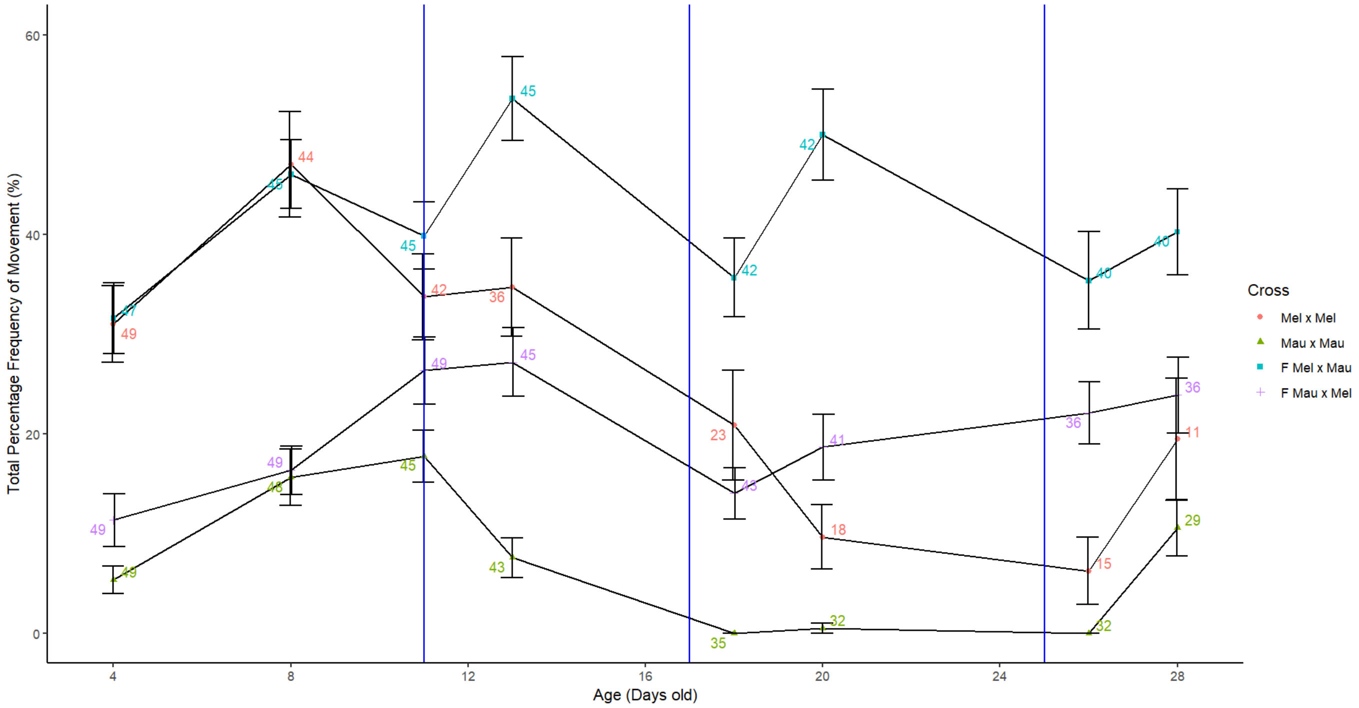

Figure 2. Average percentage frequency of observations (± 1 SE) in which movement was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific. *Vertical blue lines depict the points at which new, young non focal males were introduced. Numbers associated with each point indicate sample size (number of focal females) at the given age. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

Movement rate was observed to differ between the treatment crosses and to vary across female age (Figure 2; Table 2).

Table 2. Analysis of movement frequency of each treatment cross over time. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of movement observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	0.83	0.88	9.45	<2e – 16 ***
Mau x Mau	-1.27	0.15	-8.30	<2e – 16 ***
Mel (f) x Mau (m)	0.03	0.12	0.29	0.77
Mau (f) x Mel (m)	-0.79	0.13	-6.14	8.08e – 10 ***
Mel x Mel*Age	-2e-3	3e-4	-6.16	7.27e – 10 ***
Mau x Mau*age	3e-4	5e-4	0.75	0.455
Mel (f) x Mau (m)* age	1.9e-3	3e-4	5.43	5.64e – 08 ***
Mau (f) x Mel (m)* age	2.3e-3	3e-4	5.95	2.68e – 09 ***

The two conspecific crosses (Mel x Mel and Mau x Mau, respectively) appeared to exhibit differing behaviours (Table 2), with *D. melanogaster* females (z = 9.456, p <2e – 16) being significantly more mobile than their *D. mauritiana* counterparts (z = -8.305, p = <2e – 16). More interestingly, female age had no consistent effect on movement behaviour across species pair treatments. For conspecific *mauritiana* crosses, age had no impact on their already limited movement frequency (z =0.746, p = 0.455), but *melanogaster* conspecific crosses showed a marked decline in female movement with age (z = -6.16, p = 7.27e – 10). *D. melanogaster* (♀) x *D. mauritana* (♂) pairings appear to have a minor uptick in movement with age (z = 5.43, p

=5.64e – 08). This was also observed in the other heterospecific pairing, with *D. mauritiana* females paired with male *D. melanogaster* displaying an increase in the movement frequency with age. The output from pairwise comparisons showed that the different treatments resulted in different observed movement frequency (p = <.0001). The one exception to this was *D. melanogaster* x *D. melanogaster* and *D. mauritiana* (f) x *D. melanogaster* (m) which had comparable movement frequency (z = 1.450, p = 0.4681).

(iii) Frequency of attempted matings observed in con and heterospecific treatments over time

Attempted mating was infrequently observed (Figure 3). Unfortunately, because of the low frequency of observations, the data on attempted mating was unable to be statistically analysed, due to issues with model convergence, which could not be rectified with hurdle or zero-inflated models. Attempted matings were only observed in conspecific treatment crosses, primarily in *D. melanogaster*. Overall, the frequency of conspecific attempted matings did appear to decline with age. This is with exception of the final two timepoints for *D. melanogaster*.

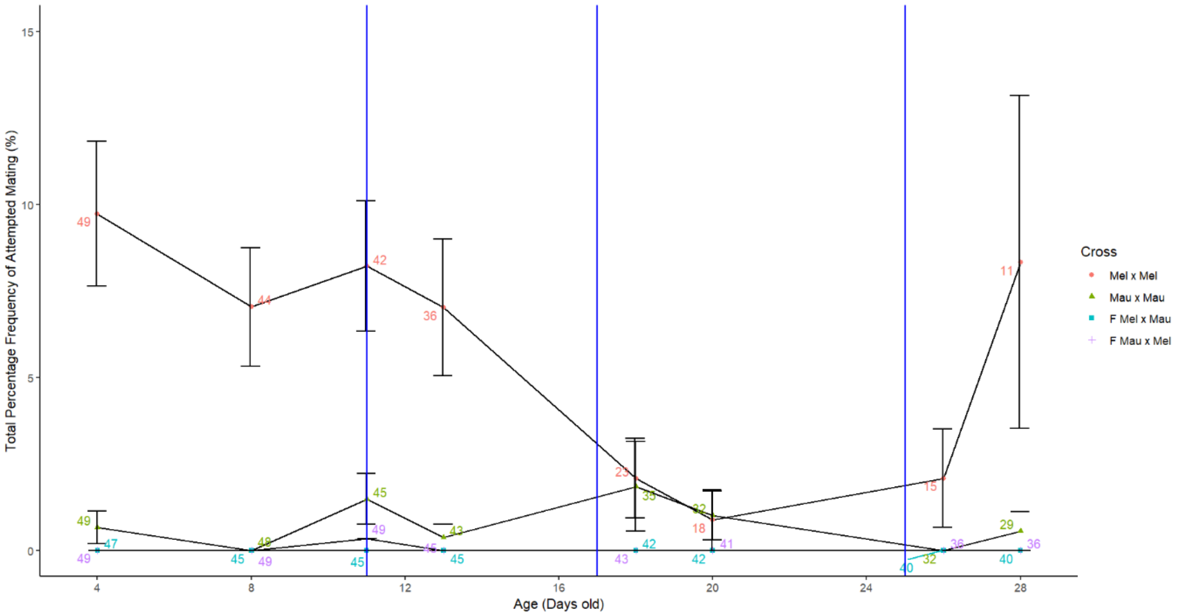

Figure 3. Average percentage frequency (± 1 SE) in which attempted mating was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific. *Vertical blue lines depict the points at which new, young non focal males were introduced. Numbers associated with each point indicate sample size (number of focal females) at the given age. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

(iv) Frequency of matings observed in con and heterospecific treatments over time

Successful mating attempts were also relatively rare (Figure 4). To incorporate this within the analysis, random observation effects were removed from the GLMM model for mating frequency.

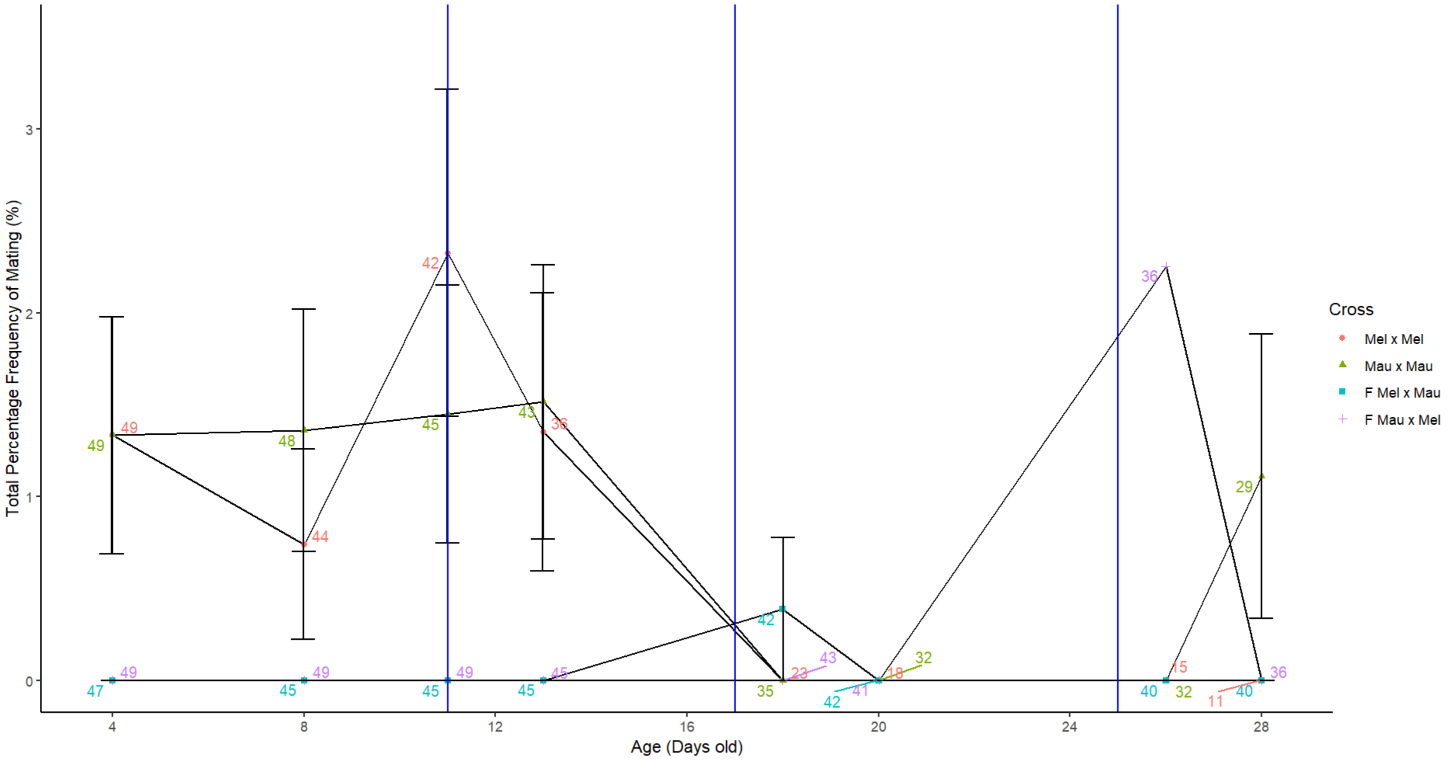

__Figure 4. Average percentage frequency (± 1 SE) in which mating was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific.__ Vertical blue lines depict the points at which new, young non focal males were introduced. Numbers associated with each point indicate sample size (number of focal females) at the given age. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.

Table 3. Analysis of mating frequency of each treatment cross over time. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of mating observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**p.value**
Mel x Mel	-2.68	0.50	-5.32	1.01e – 07 ***
Mau x Mau	-0.24	0.56	-0.44	0.660
Mel (f) x Mau (m)	-3.80	1.62	-2.34	0.018 *
Mau (f) x Mel (m)	-5.75	2.21	-2.61	0.009**
Mel x Mel*Age	-4e-4	2.6e-3	-1.78	0.073 ‘
Mau x Mau*age	2.6e-3	2.9e-3	0.89	0.369
Mel (f) x Mau (m)* age	4.9e-3	4.4e-3	1.12	0.264
Mau (f) x Mel (m)* age	0.01	3.9e-3	2.75	0.005 **

My analysis showed that age was not a significant predictor of the frequency of matings between species pairs, with the one exception of female *D. mauritiana* paired with male *D. melanogaster* (z = 2.752, p = 0.00591). Figure 4 showed that for this cross, there was an uptick in matings as females aged. There was no significant interaction of mating frequency with age, and the differences in mating frequency were due to treatment cross (Table 3). Pairwise comparisons showed that there was no significant difference in mating frequency in comparisons between individual treatments (p = >0.05). The one exception was for *D. mauritiana* conspecific crosses and *D. melanogaster* females paired heterospecifically (z = 2.669, p = 0.0382).

(v) Female survivorship in con and heterospecific treatments

Survival of focal females differed between the different treatment crosses, with an overall trend for higher survivorship over time for females paired heterospecifically (Figure 5).

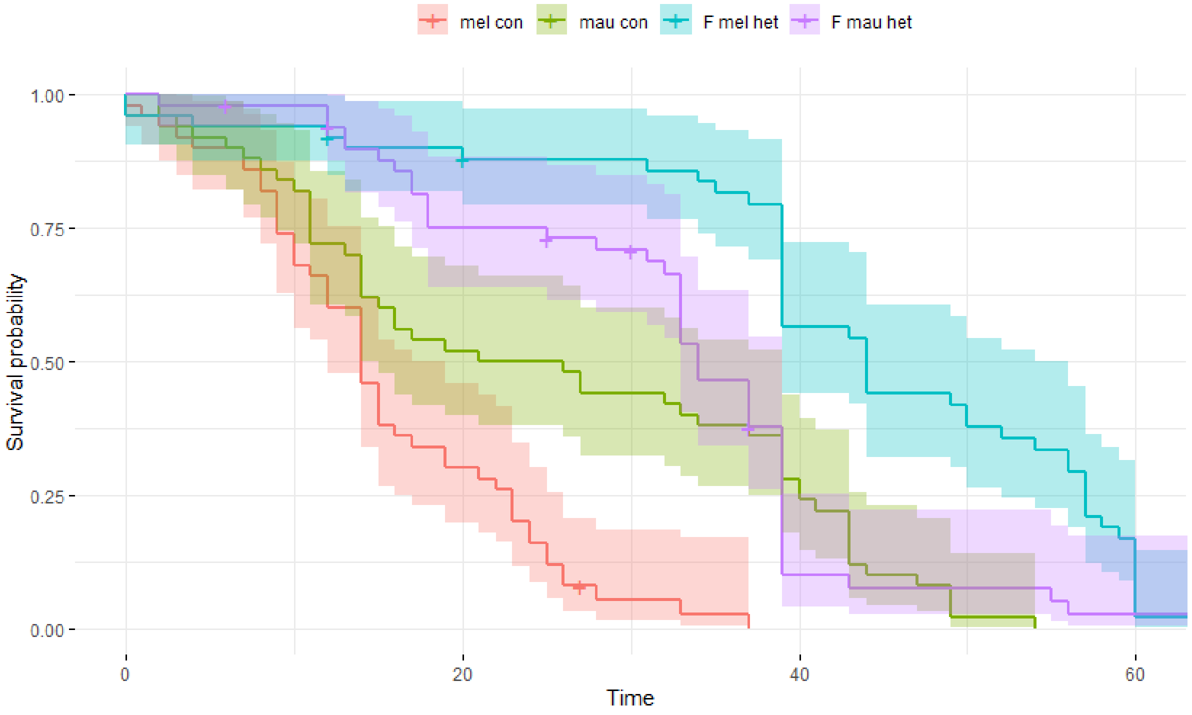

Figure 5. Focal Female survival probability against time in days. *Lines denote survival curves of each treatment cross over time, with shaded areas displaying 95% confidence intervals. D. melanogaster (mel) and D. mauritiana (mau); F = female; con = conspecific cross, het = heterospecific cross.*

Table 4. Pairwise comparisons of the survival outcomes of conspecific and heterospecific crosses *using Log Rank Test with Bonferroni p-value correction. D. melanogaster (mel) and D. mauritiana (mau); f = female, m= male*

	mel x mel	mau x mau	mel (f) x mau (m)
mau x mau	0.00016	-	-
mel (f) x mau (m)	<2e-16	1.00E-07	-
mau (f) x mel (m)	5.40E-12	1	0.00014

Drosophila melanogaster females paired with conspecific males had the lowest survival (Table 5. Median (days)= 14 ± 13.5). A Cox proportional hazards analysis suggested that all crosses had significantly higher survival compared to the *D. melanogaster* conspecific cross. In comparison to conspecific *D. melanogaster* crosses, *D. mauritiana* focal females in conspecific crosses survived significantly longer (Median (days) = 23.5 ± 29) than did *D. melanogaster* focal females in the conspecific treatment (Hazard Ratio, HR = 0.29255, p <0.05). *D. mauritiana* females crossed with *D. melanogaster* males had significantly higher survival than did conspecific *D. melanogaster* females (HR = 0.22307, p = <0.05). *D. melanogaster* females crossed with heterospecific males survived significantly longer than did their conspecific mating counterparts (HR = 0.09196, p = <0.05) with an approximately 3-fold increase in median survival (Table 5). It is interesting to note that *D. mauritiana* females had significantly higher survival than *D. melanogaster* females, regardless of their mating partners, with heterospecific crosses only showing marginally better survival than their conspecific counterparts in *D. maurtiana.*

Table 5. Median survival of focal females from all treatment crosses. *D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Treatment Cross	**Median survival (Days)**	**Interquartile Range (days)**
Mel x Mel	14	13.5
Mau x Mau	23.5	29
Mel (f) x Mau (m)	44	18
Mau (f) x Mel (m)	33	21

(vi) Female fecundity in con and heterospecific treatments

The two focal species were unable to produce fertile/viable offspring in cross species matings. I recorded both fecundity and progeny counts so that measures of offspring output and offspring survival/viability could be determined. This was to determine whether treatment cross had significant impacts on focal female fecundity and reproductive output. Figure 6 shows the number of eggs laid by each cross at 3 different ages, with the time points chosen to generate a snapshot of female egg production over time. There was a decline in egg laying with age, across all crosses, suggesting that regardless of species, females were more fecund when younger. In fact, age had a significant effect on egg number only for crosses involving *D. melanogaster* females, with conspecific crosses seeing a significant decrease with age ($z = -5.278$, $p=1.31e-07$) and heterospecific crosses showing a decrease followed by a slight uptick ($z = 2.115$, $p = 0.0345$) (Table 7). It appears that *D. melanogaster* crosses had a higher propensity to egg laying than *D. mauritiana* regardless of whether paired with con- or heterospecific males. This may suggest a difference in mating strategy between the two species.

Pairwise comparisons of the treatments revealed that treatment crosses were significantly different each other in their egg number with the exception of conspecific *D. melanogaster* crosses being comparable to both heterospecific crosses (z = -0.919, p = 0.7946) when compared with *D. melanogaster* (f) x *D. mauritiana* (m), and z = 2.198, p = 0.1238 when compared with *D. mauritiana* (f) x *D. melanogaster* (m)).

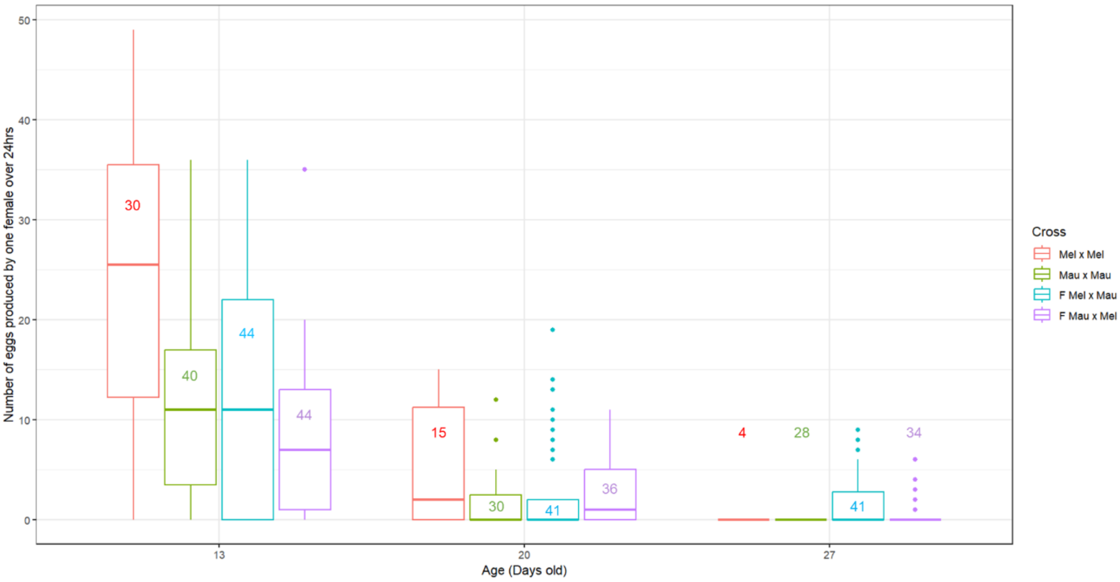

Figure 6. Number of eggs produced by focal female Drosophila depending on treatment cross i.e., con- or heterospecific over 3 age points. *Numbers denote sample sizes of egg collections for each cross, at each timepoint. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

Table 6. Analysis of fecundity of each treatment cross over 3 time points. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in fecundity of in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**p.value**
Mel x Mel	5.85	0.56	10.34	< 2e – 16 ***
Mau x Mau	0.31	0.71	0.45	0.656
Mel (f) x Mau (m)	-1.46	0.64	-2.28	0.026 *
Mau (f) x Mel (m)	-1.59	0.67	-2.37	0.018 *
Mel x Mel*Age	-0.20	0.04	-5.28	1.31e – 07 ***
Mau x Mau*age	-0.07	0.05	-1.51	0.130
Mel (f) x Mau (m)* age	0.87	0.41	2.12	0.035 *
Mau (f) x Mel (m)* age	0.06	0.44	1.30	0.193

Number of offspring of both sexes over time can be seen below (Figure 7).

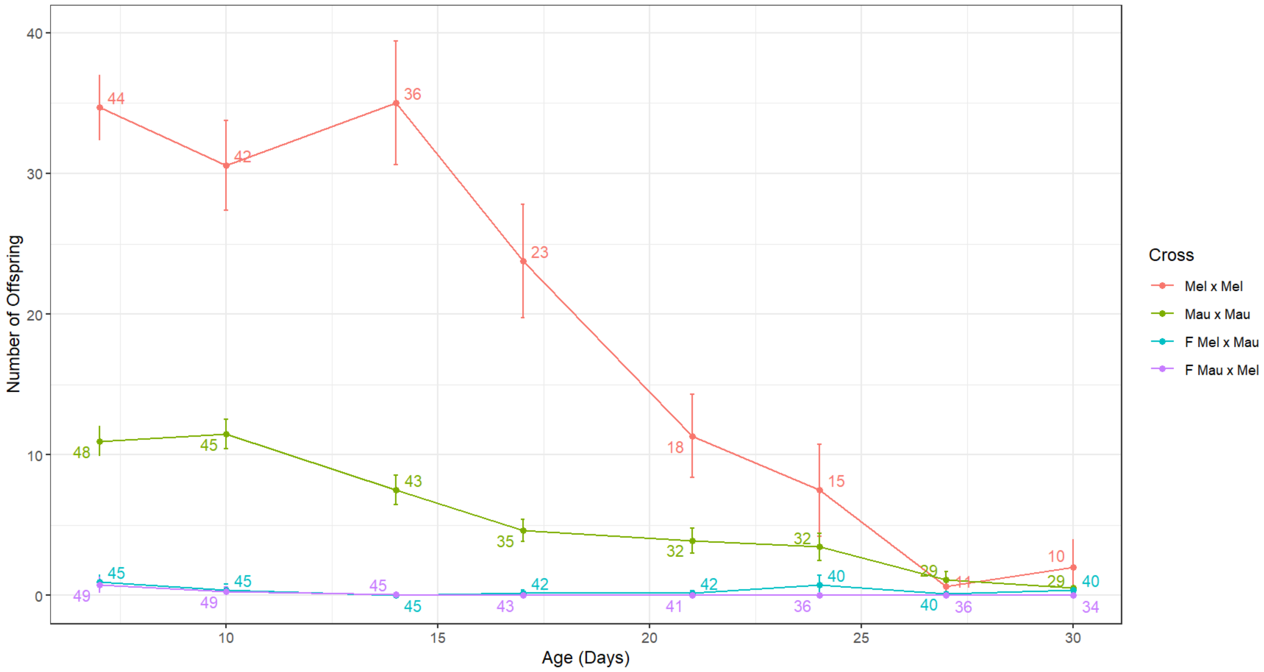

Figure 7. Number of offspring produced by focal female Drosophila depending on treatment cross i.e., con- or heterospecific over time. *Numbers denote sample sizes for each cross, at each timepoint. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

There was a clear reduction in offspring production with increasing age in the conspecific crosses. Statistical analysis (Tables 7 and 8) showed that age only significantly impacted offspring production for conspecific *D. melanogaster* crosses (z = -5.52, p = 3.32e-08). The model suggested that age impacted offspring production in *D. mauritiana* (f) x *D. melanogaster* (m) crosses (z = -2.42, p = 0.015578). Overall, mating treatment appeared to be more predictive than did female age for offspring production.

Table 7. Analysis of offspring of each treatment cross over time. *Shown is the output of the GLMM conditional model specified in the statistical methods section, to test for differences in the number of offspring produced by con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	4.09	0.12	33.23	<2e – 16 ***
Mau x Mau	-1.01	0.17	-5.89	3.80e – 09 ***
Mel (f) x Mau (m)	-1.52	0.43	-3.55	3.82e-4***
Mau (f) x Mel (m)	1.02	0.99	1.03	0.303
Mel x Mel*Age	-0.05	0.01	-5.52	3.32e – 08 ***
Mau x Mau*age	-3.8e-3	0.01	-0.32	0.075
Mel (f) x Mau (m)* age	0.04	0.02	1.78	0.074 ‘
Mau (f) x Mel (m)* age	-0.24	0.09	-2.42	0.015 *

Table 8. Analysis of total offspring of each treatment cross. *Shown is the output of the GLMM zero-inflated model specified in the statistical methods section, to test for differences in the total number of offspring produced by con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**p value**
Mel x Mel	-3.77	0.54	-6.92	4.48e – 12 ***
Mau x Mau	1.17	0.65	1.79	0.072
Mel (f) x Mau (m)	6.89	0.919242	7.49	6.87e – 14 ***
Mau (f) x Mel (m)	5.75	1.22	4.72	2.37e – 06 ***
Mel x Mel*Age	0.16	0.03	5.54	3.09e – 08 ***
Mau x Mau*age	-0.04	0.04	-1.05	0.296
Mel (f) x Mau (m)* age	-0.15	0.05	-3.14	0.001 **
Mau (f) x Mel (m)* age	-8e-3	0.09	-0.09	0.932

As offspring production was very low in heterospecific crosses, a zero-inflation model was also run (Table 8). As well as many zeros observed for the heterospecific crosses, the model also suggested that there were many zeroes (no offspring produced) in the *D. melanogaster* conspecific cross (z=-6.921, p=4.48e-12) and that this is increased as females aged (z = 5.536, p = 3.09e – 08). Pairwise analysis showed that all crosses were significantly different from each other, with the exceptions of conspecific *D. mauritiana* crosses being comparable to both heterospecific crosses (z = -1.325, p = 0.5472 for *D. melanogaster* (f) heterospecific crosses and z = 2.413, p=0.0747 for *D. mauritiana* (f) heterospecific crosses).

Discussion:

The hypothesis tested in this experiment was that the frequency of heterospecific matings would increase with focal female age, in both *Drosophila melanogaster* and *D. mauritiana*. As seen in the results, this hypothesis was not supported. Instead, it was found that the frequency of reproductive behaviours observed in females was largely impacted by treatment cross rather than female age, in most instances. This did not support the overall prediction that heterospecific matings would increase in frequency with female age. The findings from the main and pilot experiments were consistent (Appendix 1 and 2) and are discussed in more detail below.

Effects of treatment cross and female age on reproductive behaviour frequency

My analysis showed that courtship was largely unaffected by female age, and that species cross was a stronger predictor for the observed frequency of courtship toward focal females. Notably, there were differences between the two conspecific crosses, and not just between conspecific and heterospecific groups. Whilst the two heterospecific crosses had minimal courtship and did not significantly differ in their frequency of courtship, there was a distinct difference in the frequency of courtship for conspecific *D. melanogaster* crosses and *D. maurtiana* crosses, with *D. melanogaster* being much more active in courtship. This trend was also consistent with the results of my pilot studies (Appendix 1 and 2). It is important to note that as I measured courtship by the presence of singing and chasing behaviour, my data should reflect the attractiveness of focal females to experimental males. In the future, assays could be conducted to also account for female courtship receptivity behaviours, such as decreased locomotion and increased abdominal grooming (Aranha and Vasconcelos, 2018). A point of note is that across behavioural assays, homosexual courtship behaviour was observed between males on multiple occasions, notably chasing and attempted mounting behaviours. Due to the anonymised coding, the species in which this happened was not identified. However, it is relevant to the relative attractiveness of males in con- and heterospecific crosses, with some males attempting courtship towards males rather than females. This could suggest that some markers of attractiveness are more closely tied to species than just sex. Homosexual courtship between males has been observed between *Drosophila* males in other studies, and it has been suggested that younger males produce pheromones that stimulate courtship behaviour in mature males (Vaias et al.1993). It is proposed that these behaviours could be adaptive in allowing for males to improve their courtship skills when they do encounter females. A study on *D. melanogaster* and *D. affinis* shows that homosexual courtship can even be expressed interspecifically (McRobert & Tompkins 1988) which is relevant to estimating costs of satyrisation.

D. melanogaster females were more mobile than their *D. mauritiana* counterparts. When paired conspecifically, *D. mauritiana* females only displayed limited amounts of movement, in comparison to conspecific crosses *of D. melanogaster*. Interestingly, when crossed heterospecifically, females of both species showed increased movement. When *D. mauritiana* females were paired heterospecifically, their movement was comparable to that of conspecific *D. melanogaster* crosses. When *D. melanogaster* females were paired with heterospecific males, they were significantly more mobile than the other crosses. In data from pilot studies (Appendix 2) this was even more pronounced, with both heterospecific crosses having a higher movement frequency than their conspecific counterparts.

One reason for an increase in movement frequency in heterospecifically paired females could be that they are increasing their searching behaviour, in the absence of any conspecific males. The observations also suggest that *D. melanogaster* females have a higher relative level of activity than *D. mauritiana*. Whether this is a result of a more energetically intense mating strategy, as suggested by Yassin and David (2016), or perhaps more female involvement in mate discovery is unknown, but as this movement was not mediated by male chasing, it may not have been influenced by an increase in male attention. Finally, all but one cross (*D. mauritiana* conspecific) displayed a reduction in movement frequency with age. This may be another component of senescence, with females limiting their energy expenditure as they begin aging. It was not captured in *D. mauritiana* conspecific crosses, due to their already low movement activity.

Matings were infrequently observed, but those that were observed were consistent with published data (Turissini et al. 2018). The conspecific crosses displayed higher frequencies of mating than their heterospecific counterparts, and mating frequency appeared to decline with age. This was largely consistent with my findings from the pilot experiments (Appendix 1 and 2). As heterospecific mating was so rarely observed, the likelihood of significant fitness costs arising from this source on a population level seems unlikely. That being said, the fitness costs to individuals making erroneous mate choices is still likely to be high due to issues of off target responses to seminal fluid proteins, gamete wastage, bodily harm and a sterile refractory period (Yassin and David, 2016). The low frequency of heterospecific matings between these species could reflect a strong reproductive barrier as a result of potentially high individual fitness costs. To examine if this is the case, it would be useful to perform a similar experiment with larger sample size to gain a larger sample size of these infrequent behaviours. This would allow for determination of the severity of individual fitness costs to heterospecific mating individuals, and to test whether this would be sufficient to induce reproductive character displacement and the creation/maintenance of a reproductive barrier.

Effects of treatment cross and female age on focal female survival

Analysis of focal female survival showed that females paired with conspecific males suffered a higher mortality than their heterospecfically paired peers. This seemed to match best with the frequency of courtship observed during the experiment, suggesting that the energetic costs of fielding male courtships were severe enough to potentially impact female mortality risk. What is most interesting is that *D. melanogaster* females had far higher survival when paired with males of a different species. These results suggest that female survival is mediated more by intraspecific mating costs. Yassin and David (2016) found that there were asymmetries in satyrization between *D. santomea* and *D. mauritiana*, depending on within-species reproductive costs/intraspecific sexual conflict. It could be that *D. melanogaster* exhibits a higher level of sexual conflict within species. This would lead to earlier mortality rates when placed with conspecific males, which induce high reproductive costs, but an increased longevity when paired with heterospecific males from species with less sexual conflict, due to an increased resilience to reproductive costs. Due to the apparent mortality differences found in this study, it is clear that in further experiments on reproductive interference, the fitness costs of heterospecific reproductive behaviours should also be contrasted to the fitness costs associated with intraspecific sexual conflict. An interesting avenue for this could be conducting similar behavioural assays experiments as in my research, but selecting a species pair whose intensity of intraspecific sexual conflict is more comparable. Not only would this serve to help test if these results are consistent with Yassin and David’s (2016) speculations but could also help elucidate differences in sexual conflict between species that may have previously been less apparent.

Effects of treatment cross and female age on focal female fecundity

As in many studies looking at reproductive interference, data analysis was impeded by the relatively rare occurrence of heterospecific sexual interactions. In my study, behavioural assays looking at attempted matings and successful matings were particularly affected by these issues. One way to potentially improve this, and to more realistically look at mating behaviours, would be to move to cage matings with multiple mating pairs. Whilst this would make tracking of individuals more difficult, the increase in data collection could elucidate more general trends between crosses, when placed in more natural setting that allow for enhanced expression of mate choice. This could also prove more useful for research aiming to determine the viability of satyrisation as a control method, as experiments would be set within closer to natural settings.

In my experiment, egg laying was observed by focal females in all treatment crosses, with conspecifically paired *D. melanogaster* females having the largest observed egg production. Fecundity seemed to decline with age for all crosses, but my analysis showed this was only significant in *D. melanogaster* crosses. Analysis of offspring production showed that viable offspring were only observed in the two conspecific crosses, as expected based on previous work on reproductive isolation between these two species (Turissini et al. 2018). Whilst a decline in offspring number with age was found, my analysis showed that offspring number was more strongly predicted by treatment cross than female age. Whether these results reflect a lack of sexual senescence on offspring production or low sample sizes, is unclear. Heterospecific crosses did occasionally produce progeny - these hybrid offspring were non-viable and displayed intersexual morphology. The production of non-viable offspring represents a high fitness cost to females so, despite being infrequent, it would be important to account for this when considering satyrisation applied for control of pest populations.

Conclusions

This experiment was conceived with the notion that sexual senescence with age may also have implications for the frequency of reproductive interference. As females age, it is presumed that, due to their increasing limited opportunities to mate, they would be less selective with their mate choice, opening an opportunity for reduced mate discrimination to result in heterospecific matings through erroneous mate choice. This reasoning was supported by evidence that hydrocarbon cuticular profile, a common means of determining mate fitness, may change or degrade with age (Braga et al. 2016), which could result in further misdirected reproductive behaviours. The results of this experiment did not find female age to be of significant impact on the frequency of reproductive behaviours and fecundity, but nevertheless revealed interesting additional findings. Consistent with the literature, heterospecific interactions were infrequent, meaning that conspecific treatment cross tended to be a better predictor of behaviours of interest. However, the results generally showed a trend of *D. melanogaster* females being more active than their *D. mauritiana* peers, when in similar treatment crosses. This could be reflective of overall difference in mating strategy between these species, with *D. melanogaster* being more active/aggressive with reproductive behaviours and output. This could also be related to the differences in female survivorship depending on treatment cross, with heterospecific paired *D. melanogaster* females having significantly higher survivorship than their conspecific counterparts. My results did not match the prediction as regards to female age and sexual senescence, as I found that age was not of significant importance for reproductive behaviours overall. Further research is required to test whether these findings are consistent across multiple experiments.

From the conclusions of the experiment and the relative consistency of the findings across the main and pilot experiments, it follows that additional work to distinguish between pre- and post-copulatory effects should be conducted. Due to the obvious differences in mating frequency between the crosses, this would allow for determination of the relative costs of pre-copulatory effects on heterospecific pairs and illuminate whether this represents a viable route for controlling fitness. It could also help to determine the severity of post-copulatory fitness costs, which could help elucidate mechanisms of reproductive character displacement. To achieve this, one could conduct experiment which aims to separate out pre-copulatory and post-copulatory behaviours. An example would be to conduct an experiment where females are subject to pre-copulatory behaviours without the risk of mating, and *vice versa*. This could potentially be done by allowing females to interact with sterilised males and/or interact with unsterilised males with some barrier to contact, to isolate pre-copulatory effects and to simultaneously conduct artificial inseminations on other females, to isolate post-copulatory effects. Whilst this would not capture the potential costs of bodily harm from mating, it would allow for some observation of the differences between pre-copulatory fitness costs and post-copulatory fitness costs. Further research could also be conducted on other potentially interesting cofactors that could influence the frequency of heterospecific mating, such as species density, sex-ratio, etc. Conducting similar research on other species pairs could also prove fruitful in trying to understand underlying mechanisms of reproductive interference.

Overall, while the findings of these experiments were not consistent with my hypothesis, the results show the requirement for additional factors to be considered in studies of reproductive interference. This is highly relevant for research that aims to determine if satyrisation could be utilised for pest population control, as each species pair is likely to be somewhat unique in its precise satyrisation fitness costs, which could influence the efficacy of control. Whilst research of this type is hindered by the relative infrequency of heterospecific reproductive interactions, limiting the capacity for statistical analysis, it none-the-less is an important avenue of research, which could help elucidate mechanisms of reproductive character displacement, niche partitioning outcomes, and the maintenance of certain reproductive characteristics.

References:

Aranha, M. M., & Vasconcelos, M. L. (2018). Deciphering ^female innate behaviors. *Current Opinion in Neurobiology*, 52:139-148.

Anjos-Duarte, C. S., Costa, A. M., & Joachim-Bravo, I. S. (2011). Influence of female age on variation of mate choice behavior in Mediterranean fruit fly (Diptera: Tephritidae). *Journal of insect behavior*, 24(1):11-21.

Atwell, A., & Wagner Jr, W. E. (2014). Female mate choice plasticity is affected by the interaction between male density and female age in a field cricket. *Animal behaviour*, 98:177-183.

Avent, T. D., Price, T. A. R., & Wedell, N. (2008). Age-based female preference in the fruit fly *Drosophila pseudoobscura*. *Animal Behaviour*, *75*(4), 1413-1421.

Bargielowski, I., Honório, N. A., Blosser, E. M., & Lounibos, L. P. (2019). Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency. *Journal of Medical Entomology*, 56(2):329-333.

Bolker B, R Development Core Team (2022). _bbmle: Tools for General Maximum Likelihood Estimation_. R package version 1.0.25, <https://CRAN.R-project.org/package=bbmle>.

Braga, M. V., Pinto, Z. T., de Carvalho Queiroz, M. M., & Blomquist, G. J. (2016). Effect of age on cuticular hydrocarbon profiles in adult Chrysomya putoria (Diptera: Calliphoridae). *Forensic science international,* 259:e37-e47.

Brooks M.E., Kristensen K., van Benthem K.J., Magnusson A., Berg C.W., Nielsen A., Skaug H.J., Maechler M. and Bolker B.M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. The R Journal, 9(2), 378-400. doi: 10.32614/RJ-2017-066.

Bryan J (2023). _gapminder: Data from Gapminder_. R package version 1.0.0, <https://CRAN.R-project.org/package=gapminder>.

Burdfield-Steel, E. R., & Shuker, D. M. (2011). Reproductive interference. *Current Biology,* 21(12):R450-R451.

Canty A, Ripley BD (2022). boot: Bootstrap R (S-Plus) Functions. R package version 1.3-28.1.

Cothran, R. D. (2015). The importance of reproductive interference in ecology and evolution: from organisms to communities. *Population Ecology*, 57(2):339-341.

Fox J, Weisberg S (2019). An R Companion to Applied Regression, Third edition. Sage, Thousand Oaks CA. https://socialsciences.mcmaster.ca/jfox/Books/Companion/.

Gröning, J., & Hochkirch, A. (2008). Reproductive interference between animal species. *The Quarterly review of biology*, 83(3):257-282.

Hartig F (2022). _DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models_. R package version 0.4.6, <https://CRAN.R-project.org/package=DHARMa>.

Hercus, M. J., & Hoffmann, A. A. (2000). Maternal and grandmaternal age influence offspring fitness in *Drosophila*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1457):2105-2110.

Horikoshi M, Tang Y (2018). ggfortify: Data Visualization Tools for Statistical Analysis Results. https://CRAN.R-project.org/package=ggfortify.

Kassambara A, Kosinski M, and Biecek P (2021). survminer: Drawing Survival Curves using 'ggplot2', version 0.4.9, https://cran.r-project.org/package=survminer

Kelly, C. D. (2018). The causes and evolutionary consequences of variation in female mate choice in insects: the effects of individual state, genotypes and environments. *Current Opinion in Insect Science*, 27:1-8.

Koref-Santibáñez, S. (2001). Effects of age and experience on mating activity in the sibling species *Drosophila pavani* and *Drosophila gaucha*. *Behavior Genetics*, 31(3):287-297.

Lenth R (2023). _emmeans: Estimated Marginal Means, aka Least-Squares Means_. R package version 1.8.5, <https://CRAN.R-project.org/package=emmeans>.

Long, C. E., Markow, T. A., & Yaeger, P. (1980). Relative male age, fertility, and competitive mating success in *Drosophila melanogaster*. *Behavior genetics*, *10*(2):163-170.

McRobert, S. P., & Tompkins, L. (1988). Two consequences of homosexual courtship performed by *Drosophila melanogaster* and *Drosophila affinis* males. *Evolution*, 42(5):1093-1097.

Mitchell, C., Leigh, S., Alphey, L., Haerty, W., & Chapman, T. (2022). Reproductive interference and Satyrisation: mechanisms, outcomes and potential use for insect control. *Journal of Pest Science*, 1-14.

Moulin, B., Rybak, F., Aubin, T., & Jallon, J. M. (2001). Compared ontogenesis of courtship song components of males from the sibling species, D. melanogaster and D. simulans. *Behavior Genetics*, 31(3):299-308.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Saleem, S., Ruggles, P. H., Abbott, W. K., & Carney, G. E. (2014). Sexual experience enhances *Drosophila melanogaster* male mating behavior and success. *PloS one*, 9(5):e96639.

Shelly, T. E., Edu, J., & Pahio, E. (2007). Age-dependent variation in mating success of sterile male Mediterranean fruit flies (Diptera: Tephritidae): implications for sterile insect technique. *Journal of economic entomology*, 100(4):1180-1187.

Sjoberg D (2023). _hablar: Non-Astonishing Results in R_. R package version 0.3.2, <https://CRAN.R-project.org/package=hablar>.

Slowikowski, K(2021). ggrepel: Automatically Position Non-Overlapping Text Labels with 'ggplot2'. R package version 0.9.1. https://CRAN.R-project.org/package=ggrepel

Therneau T (2023). A Package for Survival Analysis in R. R package version 3.5-5, https://CRAN.R-project.org/package=survival.

Turissini, D. A., McGirr, J. A., Patel, S. S., David, J. R., & Matute, D. R. (2018). The rate of evolution of postmating-prezygotic reproductive isolation in *Drosophila*. *Molecular Biology and Evolution*, 35(2):312-334.

Vaias, L. J., Napolitano, L. M., & Tompkins, L. (1993). Identification of stimuli that mediate experience-dependent modification of homosexual courtship in *Drosophila melanogaster*. *Behavior genetics*, 23:91-97.

Wickham H (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H (2019). “Welcome to the tidyverse.” Journal of Open Source Software, 4(43), 1686. doi: 10.21105/joss.01686.

Wickham H, François R, Henry L, Müller K, Vaughan D (2023). dplyr: A Grammar of Data Manipulation. https://dplyr.tidyverse.org, https://github.com/tidyverse/dplyr.

Wickham H, Vaughan D, Girlich M (2023). _tidyr: Tidy MessyData_. R package version 1.3.0, <https://CRAN.R-project.org/package=tidyr>.

Xu, J., & Wang, Q. (2009). Male moths undertake both pre-and in-copulation mate choice based on female age and weight. *Behavioral Ecology and Sociobiology*, 63(6):801-808.

Yassin, A., & David, J. R. (2016). Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila*. *Journal of Evolutionary Biology*, 29(2):455-460.

Chapter 3: Concluding Chapter

Chapter 3 - attribution statement: The concluding chapter was researched and written by me with incorporated feedback from my supervisor, Professor Tracey Chapman.

The existence of Reproductive Interference is of significant interest to a wide variety of researchers. The major reductions in fitness that can arise due to heterospecific reproductive interactions is relevant in several distinct contexts. It prompts study of mechanisms that govern reproductive character displacement, the formation of reproductive barriers leading to incipient speciation, as well the potential for using Reproductive Interference for pest control.

The goal of this thesis was to examine the practical and theoretical implications of Reproductive Interference and satyrisation. To achieve this end, I first conducted an in-depth literature review (Chapter 1 and Appendix 3). This review included a consideration of theoretical studies of outcomes in species subject to Reproductive Interference (Ribeiro and Spielman, 1986; Kuno 1992; Kishi and Nakazawa, 2013; Kyogoku, 2020) as well as empirical studies that observed Reproductive Interference in nature and described the interactions involved (Landolt and Heath, 1987; Friberg et al. 2013; Bargielowski et al. 2015). The findings of my review demonstrated that Reproductive Interference can be shaped by multiple factors, both biotic and abiotic. These factors can alter the costs of interspecific interactions, meaning that whilst Reproductive Interference can be observed across many taxa, the specific impacts of these interactions and their subsequent fitness costs can vary greatly between interacting species or populations. The review also emphasised research into using satyrisation as a pest control method in its own right, or in conjunction with other strategies such as the Sterile Insect Technique (SIT) (Gröning and Hochkirch 2008; Bargielowski and Lounibos 2016; Leftwich et al. 2015; Honma et al. 2018).

By collating the studies in this review, I was able to clearly pull into focus some current gaps of knowledge in the field. A recurring challenge to the study of Reproductive Interference and satyrisation is that infrequent heterospecific reproductive interactions can, by their nature, be hard to study empirically due to the difficulty of gaining large sample size datasets. Potential inflation of the frequency of Reproductive Interference as a result of studies within laboratory settings is also possible (Gröning and Hochkirch 2008). For this reason, theory papers that could model the effects of ecological variables such as density were useful to include as they can predict potential outcomes of satyrisation for scenarios that can be difficult to investigate empirically (Ribeiro and Spielman, 1986; Kuno 1992; Kishi and Nakazawa, 2013).

My synthesis of the literature led to the conclusion that it is likely that the fitness costs associated with reproductive interference may vary significantly between species pairs and even between reciprocal interactions between the same species pairs. This variation suggests that extracting quantitative overarching conclusions about satyrisation could require a meta-analysis. Such an analysis was originally planned for my thesis research, though ultimately, time constraints prevented me from completing it. A valuable systematic review of Reproductive Interference has been conducted by Gröning and Hochkirch (2008). However, at present, there is a lack of sufficient quantitative data on the magnitude of fitness costs resulting from Reproductive Interferences, especially for reciprocal interactions between species, which would be needed to test for asymmetries. However, there have been some meta-analyses that have investigated factors that affect the degree of satyrisation, such as species recognition mechanisms, conspecific sperm precedence etc (Mendelson and Shaw, 2012; Howard, 1999). This suggests that additional meta-analyses on adjacent topics could still be very useful to broaden our knowledge of Reproductive Interference. My published literature review has since been cited by Ceratsi et al (2023) in their study researching the application of satyrisation in the control of agricultural pest *Drosophila suzukii.* In doing so, Ceratsi et al (2023) emphasised the value of creating a structured framework within which to approach the study and application of Reproductive Interference in a methodical way.

In addition to its translational control potential, the study of Reproductive Interference could also be very useful in determining impacts on species displacement outcomes when resident populations are subject to the effects of invasive species with which they may interact (Gröning and Hochkirch 2008; Kishi and Nakazawa, 2013). With increasing concerns about the spread of invasive species as a result of climate change and human introductions, considering the impact of satyrisation on the success of species invasion could prove useful in determining at-risk species and populations, and in identifying where to focus management strategies.

I used the findings of my review to influence my decisions on my subsequent research study direction. After consideration of the various factors that could impact the frequency or intensity of fitness costs of satyrisation experienced between interacting species, I decided to test for the impact of female age on the prevalence and costs of reproductive interference in *Drosophila melanogaster* and *Drosophila mauritiana*. These species both belong to the same species subgroup, are known to occur sympatrically and to exhibit interspecific reproductive interactions, albeit at low frequency. Matings between *Drosophila melanogaster* and *Drosophila mauritiana* do not produce fertile offspring (hybrid matings result in either inviable or sterile individuals, depending upon the direction of the hybrid mating). Collectively, these characteristics make them a suitable candidate for testing of satyrisation (Turissini et al. 2018). Female rather than male age was chosen, as effects of male age have been better researched to date, with male age often reported as influencing female mate choice in many insect species (Shelly et al. 2007). Of greater relevance is that studies of reproductive interference have found that female age was associated with female mate selectivity (Anjos-Duarte et al. 2011). Generally, it was found that older females were less selective as they were reached the stage of sexual senescence (Kelly, 2018). This was central to the hypothesis I tested, that as females aged, their ability to express mate selectivity would decrease, leading to an increase in the frequency of hybrid matings under a no choice scenario.

Following a series of pilot studies to optimise rearing, methodology and successful husbandry techniques (Chapter 2, Appendix 1 and 2), I tested the reproductive behaviour and fitness of focal females of *D. melanogaster* and *D. mauritiana* exposed to either con or heterospecific males. This then allowed for direct comparisons between treatment crosses in females of the same but advancing age. The results showed that differences in the observed frequency of reproductive behaviours was largely dictated by treatment cross, with *D. melanogaster* generally being more active than *D. mauritiana* in most reproductive behaviours in either conspecific or heterospecific crosses. These findings were generally consistent between both pilot experiments and the main final experiment. The findings did not support the hypothesis tested, that there would be a significant age effect leading to increased frequency of heterospecific matings, as suggested by prior literature on sexual senescence. However, the results did illuminate a potential difference in life history strategies between the two focal species, with *Drosophila melanogaster* engaging more frequently in reproductive behaviours than *D. mauritiana*. The behaviours selected for the observations were courtship, movement, attempted mating and successful mating, and the experiment focused on the fitness costs for focal females. Whilst these behaviours were chosen due to their relative ease of observation, and ease as proxy measures for fitness costs (energy expenditure, harassment, bodily harm, etc.), they may not capture the full scope of fitness costs of satyrisation. The experimental design also did not include some interactions that could show these additional aspects, e.g., my design did not include measurements of potential fitness costs of interspecific competition between males.

One of the most interesting findings was differences in female survivorship between treatment crosses. Females in conspecific crosses suffered higher mortality than their heterospecific treatment counterparts. Interestingly, *D. melanogaster* females suffered the highest mortality in conspecific mating conditions, but the least mortality when paired with a heterospecific *D. mauritiana* male. *D. melanogaster* females engaged more frequently in reproductive behaviours than did *D. mauritiana* in both conspecific and heterospecific crosses. This could suggest that the asymmetries in behaviour found are due to a difference in mating strategy between the two species, with *D. melanogaster* being much more active, and incurring higher energy expenditure in the expression of reproductive behaviour.

Fecundity analysis showed that egg production occurred across all treatment crosses, but that, as expected, viable offspring resulted only when females paired with conspecific males. This aligns with existing literature (Turssini et al. 2018) which suggests that our two focal species have complete post-zygotic isolation. The literature suggests that while these species produce inviable hybrid offspring, the sexual identity of each hybrid varies between the reciprocal cross (e.g., ♀ *D. melanogaster* x ♂ *D. mauritiana* produce sterile hybrid male offspring and no female offspring, whereas ♀ *D. mauritiana* x ♂ *D. melanogaster* produce sterile hybrid female offspring and no male offspring – Hybrid offspring from either cross also have decreased viability). However, my findings found that hybrid offspring often displayed intersexual morphology (e.g., abdominal colouring and shape.). As dissections were not performed, we are unable to ascertain the full extent of this phenotype. It is interesting to consider whether the reproductive barrier between these species may be maintained in part by the degradation of functional sexual dimorphism in hybrid offspring.

Following the completion of these experiments, I considered what further empirical work could be done. The study of reproductive interference can often challenged by the infrequency of heterospecific reproductive interactions. My pilot studies (Chapter 2, Appendix 1, 2) showed evidence of this difficulty. The design I eventually chose, of following individual focal females exposed continually to con or heterospecific males in the absence of competition allowed me to consider individual female survival and fitness effects over age and increased the number of reproductive interactions seen for all treatments. This being said, an obvious beginning for future research would be upscaling. Gröning and Hochkirch (2008) suggest that assays performed in laboratory settings with limited numbers of individuals interacting may artificially inflate the frequency of reproductive interference. Hence in the future, larger-scale cage experiments could be used to better replicate natural conditions, though this would make tracking individuals and the collection of individual level fitness data more difficult.

Another reasonable progression of these experiments could be to determine fitness cost asymmetries more granularly at both the pre-copulatory and post-copulatory stages. Due to the lack of heterospecific matings, post-copulatory fitness costs may generally be lower and have minimal effects at the population level. It could be very interesting to untangle the relative effects of both pre- and post-copulatory effects. This could be done by testing the effects of injections of heterospecific ejaculates, to simulate cross species artificial insemination experiments. As the semen proteins injected often have strong stimulatory effects on a female's egg production, such experiments could reveal the potential costs of post-copulatory fitness costs of hybrid mating. Determining the precise elements contributing to pre-copulatory costs could be more complex as it could involve multiple behaviours. However, the use of sterilised males or physical barriers between individuals could be utilised to pick apart the different contributing elements. Another point of interest would be to conduct similar assays as in my experiment but considering the impacts on male fitness costs, such as interspecific competition, gamete wastage, etc.

As noted in my literature review, experiments conducted to determine the impacts of the ecological factors listed in Table 2 could be of interest. Theory has already been used to evaluate the importance of species densities (Ribeiro and Spielman, 1986; Kuno 1992; Kishi and Nakazawa, 2013). I selected age as the biotic factor to test further due to its ease of study and for implications of varying age under more natural settings within potential satyrisation control programmes. Some research has begun to consider the effects of allopatry/sympatry and the evolution of satyrisation resistance (Bargielowski et al. 2013; Bargielowski and Lounibos, 2016; Bargielowski et al. 2019). All these factors could be a potential avenue for research but, as noted in the review, the relative impact of certain factors on the frequency and severity of Reproductive Interference, is likely to be highly dependent on the species pair(s) being studied. Because of this, I suggest that future research should be tailored around the species involved and their traits and life history, as well as determining whether there are general methods that could be applied across any prospective systems.

It may also be practical to explore other ecological interactions, outside of reproduction, that could influence the incidence of satyrisation or the fitness costs arising from it. My research was conducted with a focus on the frequency/severity of reproductive interference and the resulting fitness costs on involved individuals. This was because I was testing whether this strategy could be exploited for pest control, and specifically whether the magnitude of fitness costs resulting from satyrisation could be sufficient to achieve this. However, there are still many other aspects of satyrisation that could be elucidated. One example is research to investigate the relationship between reproductive interference and species exclusion/niche partitioning. Existing research has already aimed to determine the relative effects of resource competition and reproductive interference on the outcomes of species exclusion events (Park et al. 1948; Birch et al. 1951; Kishi et al. 2009). Some data show that sexual exclusion is the determinant of species exclusion, more so than resource competition. Such studies show that our initial understanding of interspecific competition may be incomplete without factoring in the possibility of Reproductive Interference. They also underscore that satyrisation’s potential capacity for species exclusion should be considered when determining the risks of species invasions. Similarly, another potentially fruitful avenue of study is research on the evolutionary mechanisms underlying satyrisation, including how resistance to it evolves (Bargielowski et al. 2013; Bargielowski and Lounibos, 2016; Bargielowski et al. 2019). Drury et al. (2019) referred to an “Evolutionary Catch-22” i.e., the maintenance of Reproductive Interference between two species/populations as a result of additional factors impeding reproductive character displacement. Investigating the mechanisms involved could help clarify the precise traits that mediate the occurrence of Reproductive Interference.

Another avenue for future work could be to investigate the underlying genetic mechanisms of reproductive interference and specifically, to identify the genomic signatures of fitness costs. One example could be found in the effects of Seminal Fluid Proteins, which can alter female physiology following a mating (Chapman, 2001; Poiani, 2006). If the effects of these SFPs differed between species, or if one species is more resistant to heterospecific SFP effects than the other, this could easily create asymmetries in fitness cost following heterospecific matings. With this in mind, a study utilising transcriptomics techniques to reveal the profiles of gene expression could prove quite fruitful. My research focussed primarily on pre-mating factors, as my rationale was that if heterospecific mating was infrequent, the relative costs of post-mating effects would be negligible. However, this may not be the case across all systems, meaning that post-mating effects could have stronger effects on fitness cost asymmetries in certain species pairs in which heterospecific matings are more frequent. Studies of this nature could help elucidate differences in the relative rates of evolution of sexual characteristics and give insight into the maintenance of variation in certain sexual traits.

A significant topic for further study should be into the use of satyrisation as a potential control method. While in its relative infancy, there is promise in the application of Reproductive Interference for pest control. Reproductive Interference could be used as a complementary strategy, assisting in reducing pest numbers alongside more established control methods such as Sterile Insect Technique (Honma et al, 2018), or perhaps eventually as an independent control method. Regardless, the perturbation of pest species reproductive activity is of keen interest in pest management, and satyrisation's variability makes it an attractive potential tool (Leftwich et al. 2015; Honma et al. 2018; Leftwich et al. 2020).

Overall, my thesis stresses how Reproductive Interference and satyrisation are compelling phenomena in need of ongoing research. My literature review compiled existing knowledge, while highlighting gaps. After designing my experiments with this in mind, I determined that one factor of import to the frequency of satyrisation, female age, had very little influence on the extent or effects of Reproductive Interference between *Drosophila melanogaster* and *D. maurtiana*. However, findings of differences in survival between the respective conspecific and heterospecific crosses might be evidence of a difference in mating strategy which may have impacts on reproductive interference elsewhere. These experiments also begin to suggest a means of considering reproductive interference and important cofactors experimentally, which could be of use to further research, especially that which considers use of satyrisation for population control. My research emphasizes the broad scope of research that could be conducted on these phenomena and suggests what directions could be taken in the future.

References

Anjos-Duarte, C. S., Costa, A. M., & Joachim-Bravo, I. S. (2011). Influence of female age on variation of mate choice behavior in Mediterranean fruit fly (Diptera: Tephritidae). *Journal of insect behavior*, 24(1), 11-21.

Bargielowski IE, Lounibos LP, Carrasquilla MC (2013) Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. *Proc Natl Acad Sci USA* 110:2888-2892.

Bargielowski IE, Lounibos LP, Shin D, Smartt CT, Carrasquilla MC, Henry A, Navarro JC, Paupy C, Dennett JA (2015) Widespread evidence for interspecific mating between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in nature. *Infection, Genetics and Evolution* 36:456-461.

Bargielowski IE, Honório NA, Blosser EM, Lounibos LP (2019) Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency. *J Medical Entomology* 56:329-333.

Birch LC, Park T, Frank MB (1951) The effect of intraspecies and interspecies competition on the fecundity of two species of flour beetles. *Evolution* 1:116-132.

Cerasti, F., Mastrantonio, V., Dallai, R., Cristofaro, M., & Porretta, D. (2023). Applying Satyrization to Insect Pest Control: The Case of the Spotted Wing Drosophila, *Drosophila suzukii* Matsumura. *Insects*, 14(6), 569.

Chapman, T. (2001). Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity*, 87(5), 511-521.

Drury JP, Anderson CN, Cabezas Castillo MB, Fisher J, McEachin S, Grether GF (2019) A general explanation for the persistence of reproductive interference. *Am Nat* 194:268-275.

Friberg M, Leimar O, Wiklund, C. (2013) Heterospecific courtship, minority effects and niche separation between cryptic butterfly species. *J Evolutionary Biology* 26:971-979.

Gröning J, Hochkirch A (2008) Reproductive interference between animal species. The *Quarterly Review of Biology* 83:257-282.

Honma A, Kumano N, Noriyuki S (2019) Killing two bugs with one stone: a perspective for targeting multiple pest species by incorporating reproductive interference into sterile insect technique. *Pest Management Science* 75:571-577.

Howard, D.J., 1999. Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics*, 30(1):109-132.

Kelly, C. D. (2018). The causes and evolutionary consequences of variation in female mate choice in insects: the effects of individual state, genotypes and environments. *Current Opinion in Insect Science*, 27:1-8.

Kishi S, Takayoshi N, Yoshitaka T (2009) Reproductive interference determines persistence and exclusion in species interactions. *J Animal Ecology* 78:1043-1049.

Kishi S, Nakazawa T (2013) Analysis of species coexistence co-mediated by resource competition and reproductive interference. *Pop Ecol* 55:305–313.

Kuno E (1992) Competitive exclusion through reproductive interference. *Res Popul Ecol* 34:275–284.

Kyogoku D (2020) When does reproductive interference occur? Predictions and data. *Pop Ecol* 62:196-206.

Landolt PJ, Heath RR (1987) Role of female-produced sex pheromone in behavioral reproductive isolation between *Trichoplusia ni* (Hübner) and *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae, Plusiinae). *Journal of Chemical Ecology* 13:1005-1018.

Leftwich PT, Bolton M, Chapman T (2016) Evolutionary biology and genetic techniques for insect control. *Evolutionary Applications* 9:212-230.

Leftwich PT, Spurgin LG, Harvey-Samuel T, Thomas CJ, Paladino LC, Edgington MP, Alphey L 2021. Genetic pest management and the background genetics of release strains. *Phil Trans Roy Soc B* 376:20190805.

Mendelson, T. C., & Shaw, K. L. (2012). The (mis) concept of species recognition. *Trends in ecology & evolution*, 27(8):421-427.

Park T (1948) Experimental studies of interspecies competition. 1. Competition between populations of the flour beetles, *Tribolium confusum* Duval and *Tribolium castaneum Herbst.Ecol Monogr* 18:265-307.

Poiani, A. (2006). Complexity of seminal fluid: a review. *Behavioral Ecology and Sociobiology*, 60:289-310.

Shelly, T. E., Edu, J., & Pahio, E. (2007). Age-dependent variation in mating success of sterile male Mediterranean fruit flies (Diptera: Tephritidae): implications for sterile insect technique. *Journal of economic entomology*, 100(4):1180-1187.

Turissini, D. A., McGirr, J. A., Patel, S. S., David, J. R., & Matute, D. R. (2018). The rate of evolution of postmating-prezygotic reproductive isolation in *Drosophila*. *Molecular Biology and Evolution*, 35(2):312-334.

Ribeiro JMC, Spielman A (1986) The satyr effect: a model predicting parapatry and species extinction. *Am Nat* 128:513–528.

Yassin A, David JR (2016) Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila*. *J Evolutionary Biology* 29:455-460.

Appendix 1: Pilot study summary

The relationship between female age and female choosiness and willingness to hybrid mate in crosses between D. melanogaster and D. mauritiana

Methods and Materials

Laboratory and rearing conditions:

All experiments were carried out using Dahomey Wild-Type *D. melanogaster* and *D. mauritiana* (NDSSC:14021-0241.150) laboratory strains. These originated from Benin, West Africa (1970 collection), and Le Reduit, Mauritius (2006 collection) respectively. All cultures were held at 25 °C, under 12:12 h light:dark cycle, at 50% Relative Humidity. To generate individuals for the experiment, adult flies of each species were put in density-controlled Sugar-Yeast-Agar vials. Due to differences in fecundity between the species, the number of vials and density per vial varied between the species, with *D. melanogaster* requiring 15 vials at a density of 3♀ x 3♂, and *D. mauritiana* requiring 25 vials at a density of 10♀ x 10♂. These vials were left for 3 days to allow time for mating and laying. After this, adult flies were transferred to fresh vials to continue stocks. The vacated vials were kept until the eclosion of any offspring. After a further 5 days, the newly emerged flies were sexed under ice anaesthesia within 8hrs of eclosion, to increase the reduce the likelihood of flies mating before being assigned treatments. Sexed flies were kept in sex-and-species-separated vials (10 per vial) until treatment age is met, being moved to new food every 2 days to prevent waste buildup. More eggs were collected from stock vials of each species to become the male mating cohort. The respective age treatments for the sexes were as follows: For mating ♂ = 5 days, for remating ♂ = 6 days, for Young ♀ = 3 days, for Old ♀ = 14 days. Due to shortages in the number of males, I later expanded age range for male flies from 3-5 days old. Before use in behavioural assays females were verified as virgins. Stocks were continued and egg collection maintained on a consistent schedule to allow for replacement males to be aged appropriately and replaced throughout the experiment in order to control for male age effects.

Mating assays

In this study, there were 8 treatment groups composed of the 4 different combinations of species crosses, and of 2 age groups. Exact sample sizes size per treatment are given below (Appendix 1, Table 1). Assays were conducted in 2 blocks, split between the old female crosses and the young female crosses, following the same methodology.

Appendix 1, Table 1. Summary of treatment group details from pilot study 1.

Treatment	Focal female species	Focal female age (days)	Con- or heterospecific pairing	Sample size (N)
Young Melanogaster ♀ x Melanogaster ♂	*D. melanogaster*	3	Conspecific	44
Old Melanogaster ♀ x Melanogaster ♂	*D. melanogaster*	14	Conspecific	48
Young Mauritiana ♀ x Mauritiana ♂	*D. mauritiana*	3	Conspecific	47
Old Mauritiana ♀ x Mauritiana ♂	*D. mauritiana*	14	Conspecific	46
Young Melanogaster ♀ x Mauritiana ♂	*D. melanogaster*	3	Heterospecific	41
Old Melanogaster ♀ x Mauritiana ♂	*D. melanogaster*	14	Heterospecific	49
Young Mauritiana ♀ x Melanogaster ♂	*D. mauritiana*	3	Heterospecific	45
Old Mauritiana ♀ x Melanogaster ♂	*D. mauritiana*	14	Heterospecific	56

One day prior to the mating assay, males were selected randomly from their treatment groups and placed into mating arenas. On the day of the assay, a single female was introduced (conspecific or heterospecific) via mechanical pooter and allowed to interact for a duration of 120 minutes. Every 20 minutes, spot behavioural assays were conducted to test for courtship and copulation. Courtship was defined as females being subject to male chasing and wing-song and was observed in a subset of 10 vials per treatment. Copulation was defined as successful mounting attempts followed prolonged coupling which we deemed to be sufficient evidence of copulation and observed in all samples. If mating was observed, the time of copulation initiation was noted, rounded to the nearest 5-minute mark, so that a measure of mating latency could be generated.

Once a pair were observed to copulate, they were removed from the assay pool. If heterospecific matings were infrequent/slow to occur (as suggested by the literature) these treatment mating arenas were be observed for a further 30 minutes, in order to help prevent an artificially small sample size leading to underpowered remating tests.

After the initial assays, male flies were removed from the mating arenas and females left in their fly vial to lay eggs. 24hrs after the initial mating assay, the number of eggs laid were counted and the female was removed from the vial and introduced to a single conspecific male in a new mating arena. Initially, the males to be used in these remating assays were virgin males, to ensure that they weren't sperm depleted. However, due to shortages in male numbers, some mated males were reused in remating assays, following a 24h period of recuperation. The same behavioural assays were then conducted, and the same mating data recorded, to examine remating behaviours across the treatments.

Statistical analysis:

All statistical analysis was conducted in R studio (R version 4.2.3) (R Core Team, 2023). Individual packages and package versions are listed alongside each analysis section.

(i) Analysis of courtship frequency of each treatment at two distinct female ages.

Due to the conflation of Female Age and experimental block, female age could not be included as a fixed effect in a singular model. To overcome this, two separate models were run for each Female age. Analysis of the frequency of courtship behaviour was performed using a generalized linear mixed models (GLMM) as follows:

Courtship GLMM output (Formula: Courtship ~ Cross, Family = Compois)

These models included the fixed effect of Treatment Cross and no random observations. After exploring model assumptions, these GLMMs were run with Compois as the family. The models were tested for adherence to assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed to determine if there were any differences between treatment crosses at each time point to ascertain the significance of the fixed factors on courtship directed toward focal females.

(ii) Analysis of mating frequency of each treatment cross over time.

Unlike the courtship model, as mating was only observed in conspecific pairings. As such, we were able to include female age within the model. Analysis of the frequency of mating behaviour was performed using a generalized linear mixed model (GLMM) as follows:

*Movement GLMM output (Formula: Mating ~ Cross*Age, Family = Compois)*

The model included the fixed effects of the interaction between treatment cross and female age. This GLMM was run with the Compois family error structure. The model was tested for adherence to assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on successful matings of focal females.

Results

(i) Frequency of Courtship between treatment pairs at two distinct female ages

In this first pilot experiment, it appeared that treatment cross had a significant impact on the frequency of observed courtship, with conspecifics being more likely to be observed courting then their heterospecific counterparts, at both age 3 and age 14 (Appendix 1, Figure 1). As the experiment was conducted in blocks, I did not include age as a variable in the models (Appendix 1, Tables 2 and 3) though a reduction in observed courtship in conspecific *melanogaster* crosses in the older female cohort was apparent (Figure 1). GLMMs run on each data set showed that for the 3-day-old cohort, all treatments were significantly different from each other (p = <0.05, Appendix 1, Table 2). A pairwise analysis showed that *D. mauritiana* treatments had similar frequencies of observed courtship. The two heterospecific treatments were also similar to each other. Most notably, there was a significant difference in frequency of observed courtship between conspecific and heterospecific *D. melanogaster* (p = <0.0001), Appendix 1, Figure 1, Plot A.) It appears that at 3 days old, the species of a *D. melanogaster* female's mating partner greatly impacted the frequency of courtship.

For the 14-day-old females, (Appendix 1, Table 3), while *D. melanogaster* conspecific cross was significantly different from zero, the two heterospecific crosses did not significantly differ in their average courtship frequency to the *D. melanogaster* treatment cross. The only treatment to differ significantly from the other was *D. mauritiana* conspecific cross. This was confirmed by a series of pairwise analyses with showed that all treatment crosses had similar frequencies of observed courtship, with the only cross that was significantly different from the others being conspecific *D. mauritiana* (p = <0.05).

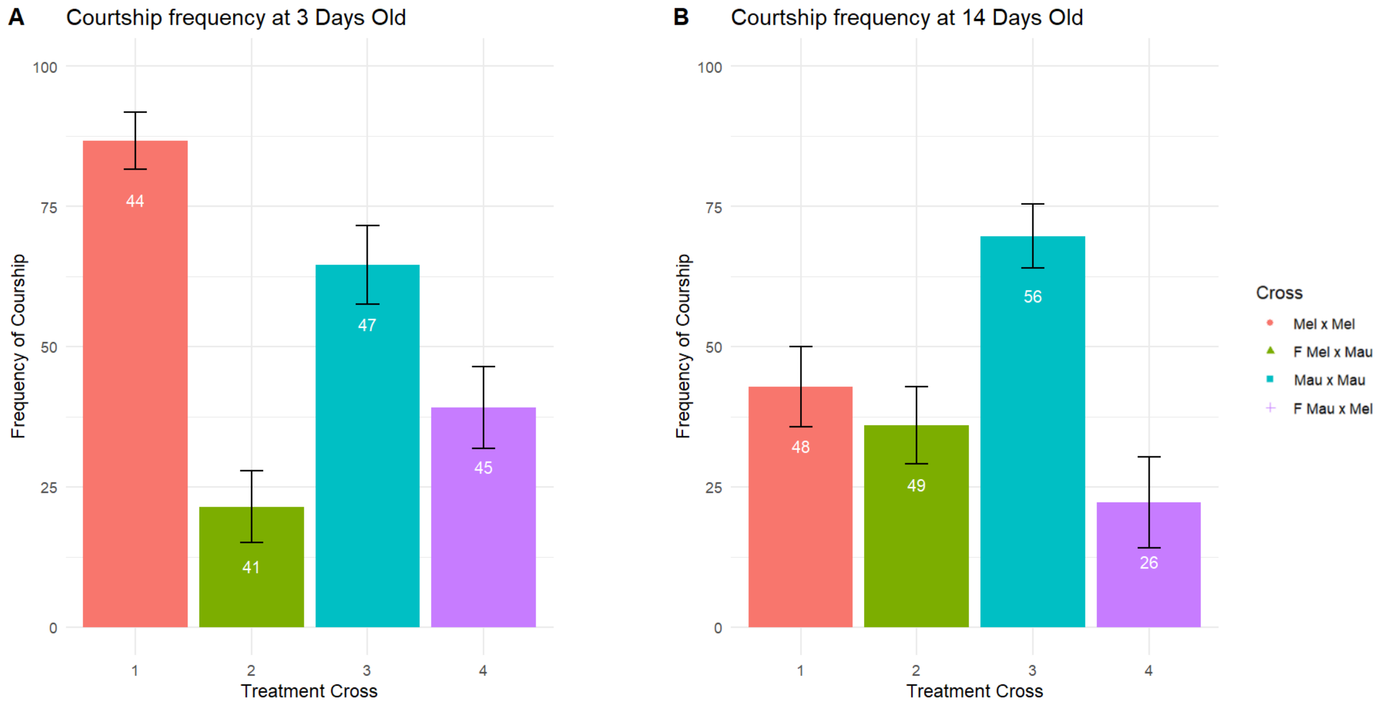

Figure 1. (A) -Average percentage frequency of observations (± 1 SE) in which courtship was observed in 3-day-old females, for the 4 treatment crosses i.e., conspecific or heterospecific*. D. melanogaster (Mel) and D. mauritiana (Mau); F = female. Colour coding consistent between both panels.*

(B) -Average percentage frequency of observations (± 1 SE) in which courtship was observed in 14-day-old females, for the 4 treatment crosses i.e., conspecific or heterospecific*. D. melanogaster (Mel) and D. mauritiana (Mau); F = female. Colour coding consistent between both panels.*

Table 2. Analysis of courtship frequency in 3-day-old females of each treatment cross. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of courtship observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	-0.14	0.05	-2.44	0.0144 *
Mel (f) x Mau (m)	-1.39	0.30	-4.63	3.50e-06 ***
Mau x Mau	-0.29	0.12	-2.41	0.015 *
Mau (f) x Mel (m)	-0.79	0.19	-4.12	3.78e-05 ***

Table 3. Analysis of courtship frequency in 14-day-old females of each treatment cross. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of courtship observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	-0.84	0.16	-5.13	2.8e-07 ***
Mel (f) x Mau (m)	-0.17	0.25	-0.69	0.486
Mau x Mau	0.48	0.18	2.64	8.17e-3 **
Mau (f) x Mel (m)	-0.65	0.39	-1.65	0.09

(ii) Frequency of mating between treatment pairs at two distinct female ages

As mating only occurred between conspecific crosses, the models were adjusted to remove heterospecific crosses, to prevent model nonconvergence due to zero-inflation. As such, Figure 2 includes these heterospecific groups for visualisation, but they were excluded from the analysis. Figure 2 shows that regardless of the two age groups, conspecific females had a high frequency of mating. Age appears to have some minor effects on mating frequency with a slight decline in mating frequency between 3-day-old and 14-day-old females, across both species crosses. A GLMM analysis (Appendix 1, Table 4) determined that at both ages, the two conspecific treatments were significantly different from 0 (p = 0.00012), but that there was no significant difference in average mating frequency between the conspecific crosses or the age groups (p = > 0.05). These findings were further solidified by a pairwise analysis that found the treatments were not significantly different to each other.

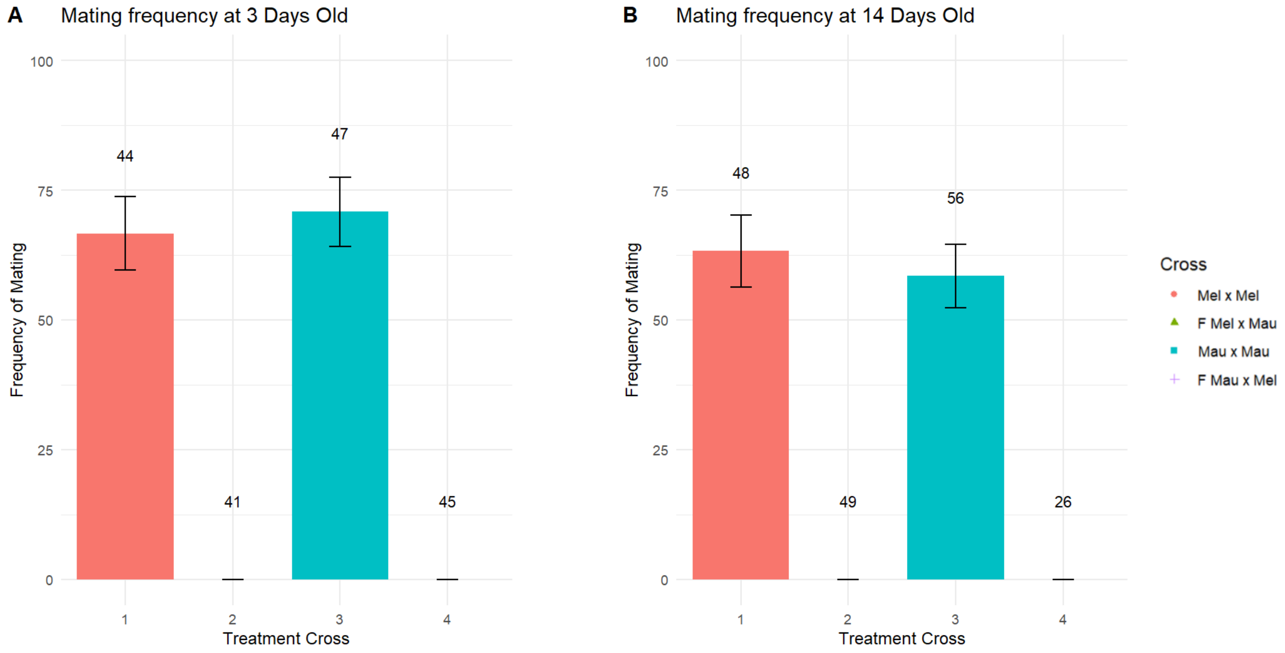

Figure 2. (A) -Average percentage frequency of observations (± 1 SE) in which mating was observed in 3-day-old females, for the 4 treatment crosses i.e., conspecific or heterospecific. *D. melanogaster (Mel) and D. mauritiana (Mau); F = female. Colour coding consistent between both panels.*

(B) -Average percentage frequency of observations (± 1 SE) in which mating was observed in 14-day-old females, for the 4 treatment crosses i.e., conspecific or heterospecific. *D. melanogaster (Mel) and D. mauritiana (Mau); F = female. Colour coding consistent between both panels.*

Table 4. Analysis of mating frequency of each conspecific treatment cross at two distinct female ages. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of mating observed in conspecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau), at two distinct female ages (3-days-old and 14-days-old, respectively).*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**p.value**
Mel x Mel * Age 3	-0.41	0.11	-3.84	1.2e-4***
Mau x Mau * Age 3	0.06	0.14	0.43	0.665
Mel x Mel * Age 14	-0.05	0.15	-0.34	0.729
Mau x Mau * Age 14	-0.15	0.21	-0.74	0.453

Discussion

These pilot data helped elucidate general trends in reproductive behaviour between con- and heterospecific crosses in our focal species. These findings aided in determining the direction and design of the main experiments and allowed me to improve husbandry methods.

Effects of treatment cross on Courtship frequency between 3-day-old and 14-day-old focal females

It was found that age had little significant effect on courtship frequency for the two heterospecific crosses. For conspecific *D. melanogaster* females, courtship frequency appeared to decline between the two age groups, in stark comparison to conspecifically paired *D. mauritiana*, in which courtship frequency remained consistent across the two age groups. This may be indicative of a difference in reproductive strategy between the two focal species, with *D. melanogaster* investing more in courtship displays toward younger females. This could either be due to a difference in female receptivity or male attraction toward females with age.

Effects of treatment cross on Mating frequency between 3-day-old and 14-day-old focal females

I observed matings only between females paired with conspecific males. Whilst this means I could not draw comparisons between con- and heterospecific treatments, I could infer that, if heterospecific mating occurs, that it is at a very low frequency. The findings did, however, show that regardless of the changes in courtship frequency between the two conspecific crosses with age, mating frequency remained consistent across the two age groups. This is interesting when placed in contrast to this experiment's findings on courtship frequency between the same cohort.

Appendix 2: Pilot study summary

Test of the relationship between female age and female choosiness and willingness to hybrid mate in crosses between D. melanogaster and D. mauritiana.

Following the initial pilot study, my protocol for an experiment looking into the effects of female age on the frequency of heterospecific reproductive behaviour was refined. This protocol was similar to the main experiment reported in this chapter.

Methods

Laboratory and rearing conditions:

All experiments were carried out using Dahomey Wild-Type *D. melanogaster* and *D. mauritiana* (NDSSC:14021-0241.150) laboratory strains. These originated from Benin, West Africa (1970 collection), and Le Reduit, Mauritius (2006 collection) respectively. All cultures were held at 25 °C, under 12:12 h light:dark cycle, at 50% Relative Humidity. Stocks used to generate individuals for the experiment were raised under density-controlled conditions in milk bottles containing Sugar-Yeast-Agar solid media. Parents of the experimental flies were collected from bottle cultures seeded at the same time. Hence parents were standardized for age and experience, to minimize the possibility of differential parental effects on the experimental individuals.

To standardize the rearing of the experimental flies, 3:3 females:males of *D. melanogaster* and 10:10 of *D. mauritiana* were placed in vials and moved to new food daily. This ratio was based upon data gathered in the initial pilot experiment, above, which found that this number of individuals resulted in approximately similar larval density in vials between the species. This limited the differences in larval size by aiming to standardize larval competition. Pilot experiments also showed that these adult densities generated a sufficient number of individuals. However, to account for the increased number of male individuals needed for mating experiments, and to account for losses from premature death or during transfer, the number of male collection vials was doubled (e.g., from 25 to 50 for *D. mauritiana*, and from 15 to 30 for *D. melanogaster*).

Once larvae eclosed into adult flies, these individuals were sexed and collected under ice anaesthesia. Collected flies were stored in conspecific and sex-specific vials of a density of 10 until use in experiments. For females, this was duration of 3 days, to ensure sexual maturity, and for males this will be a duration of 5 days. The same procedure was used to generate each of the new sets of males required for replacement every 7 days.

Experimental set up conditions

24h after collection, females were randomly allocated to one of the 4 treatment groups. The experimental treatments were all 4 combinations of conspecific (♀ *D. melanogaster* x ♂ *D. melanogaster* and ♀ *D. mauritiana* x ♂ *D. mauritiana*) and heterospecific (♀ *D. melanogaster* x ♂ *D. mauritiana* and ♀*D. mauritiana* x ♂ *D. melanogaster*) crosses. Each female was placed individually in a vial with 2 males of the appropriate species for the designated treatment group. To ensure that food quality was maintained, all flies were transferred, using CO_2 anaesthesia, to new food every 2-3 days (e.g. 3 transfers per week). On the 7th day, when being transferred onto new food, old males were discarded and replaced with new male individuals of 5 days of age, obtained from fresh cultures. This meant that males were kept the same relative ages across week-long durations (5 days old – 12 days old). Any female deaths were scored daily. Dead males were replaced by spares of the same age.

To generate fecundity data, eggs were counted from one set of newly vacated vials, from each treatment, per week. Eggs were counted directly after vials are vacated, to minimize the emergence of larvae, which could otherwise obscure the eggs to be counted. To generate courtship and mating data, behavioural spot checks were performed twice every week. Vials were placed randomly with respect to treatment on the observation board, with treatment labels obscured, the day before the assay to blind the samples on the day of observation. The duration of these checks was 2hrs long, with spot samples of behaviour conducted every 20 minutes. The following behaviours in focal females were scored: Courtship (defined as chasing), attempted copulation (defined as mounting attempts that are unsuccessful) and copulation (defined as successful mounting). At the end of each assay, vials were removed from the observation board, and the date and age of the individuals observed in each of these behavioural checks, noted. To record generated data, notation was be kept on the vials and on an associated sheet. To delineate the time of the observation, different pen colours were used for different time codes. To record the behaviour observed, a number system was used to determine the female's behaviour at that time:

1 = Courting (chasing and wing song directed at focal females)

2= Not courting

3 = Moving (movement of focal females that was not mediated by male chasing)

4 = Not moving

5 = Mating (mounting events resulting in successful copulation and conjoining of mating individuals for a prolonged duration)

Collected data were anonymised to treatment.

Statistical analysis:

The methodology of the second pilot study was similar to that of the final, main experiment reported in Chapter 2, but female identity wasn't tracked in this pilot. As such, female ID was not included as a random factor in this analysis.

(i) General approach

All statistical analyses were conducted in R studio (R version 4.2.3) (R Core Team, 2023). To determine whether the treatment cross (conspecific or heterospecific) or age had an impact on frequency of each mating behaviour, I collated counts of behavioural data from each behavioural assay, to calculate an average percentage frequency of a given behaviour for each treatment, for the age focal females were on the day of the assay. It should be noted that most of the behavioural data collected were zero-inflated as they were measures of frequency of relatively rare events. Measures were taken to accommodate this, with each model being assessed for normality and adherence to model assumptions via visual assessment of the data, and with use of simulated residuals generated by the R Package *DHARMa*. The individual R packages used in the analyses were *ggplot2* (Wickham, 2016), *Tidyverse* (Wickham et al. 2019), *ggrepel* (Slowikowski, 2021), *ggfortify* (Horikoshi, 2018) , *car* (Fox, Weisberg, 2019), *boot* (Canty and Ripley, 2022), *dplyr* (v1.1.2; Wickham et al, 2023), *glmmTMB* (Brooks et al. 2017), *DHARMa* (Hartig, 2022), *gapminder* (Bryan, 2023), *tidyr* (Wickham, Vaughan and Girlich, 2023), *hablar* (Sjoberg, 2023), *bbmle* (Bolker and R Development Core Team, 2022), *emmeans* (Lenth, 2023).

(ii) Analysis of courtship frequency of each treatment cross over time.

Analysis of the frequency of courtship behaviour was performed using a generalized linear mixed model (GLMM) as follows:

*Courtship GLMM output (Formula: Courtship ~ Cross*Age + (1|obs), Family = Compois)*

The model included the fixed effect of the interaction of Treatment Cross and Female age and observation as a random effect. After exploring model assumptions, this GLMM was run with Compois as the family. The model was tested for adherence to assumptions using simulated residuals generated by the R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on courtship directed at focal females.

(iii) Analysis of movement frequency of each treatment cross over time.

Analysis of the frequency of movement behaviour was performed using a generalized linear mixed model (GLMM) as follows:

*Movement GLMM output (Formula: Movement ~ Cross + Age + Cross*Age + (1|obs), Family = Poisson)*

The model included the fixed effects of the Treatment Cross, Age and their interaction and observation as a random effect. This GLMM was run with the Poisson family error structure. The model was tested for adherence to assumptions using simulated residuals generated by the

R package *DHARMa*. Following this, a series of pairwise analyses were performed on the interactions between treatment cross and female age to ascertain the significance of the fixed factors on focal female movement.

(iv) Analysis of Mating frequency of each treatment cross over time.

There were too few mating data recorded to run a meaningful analysis for this trait.

Results

(i) Courtship frequency in con and heterospecific treatments over time

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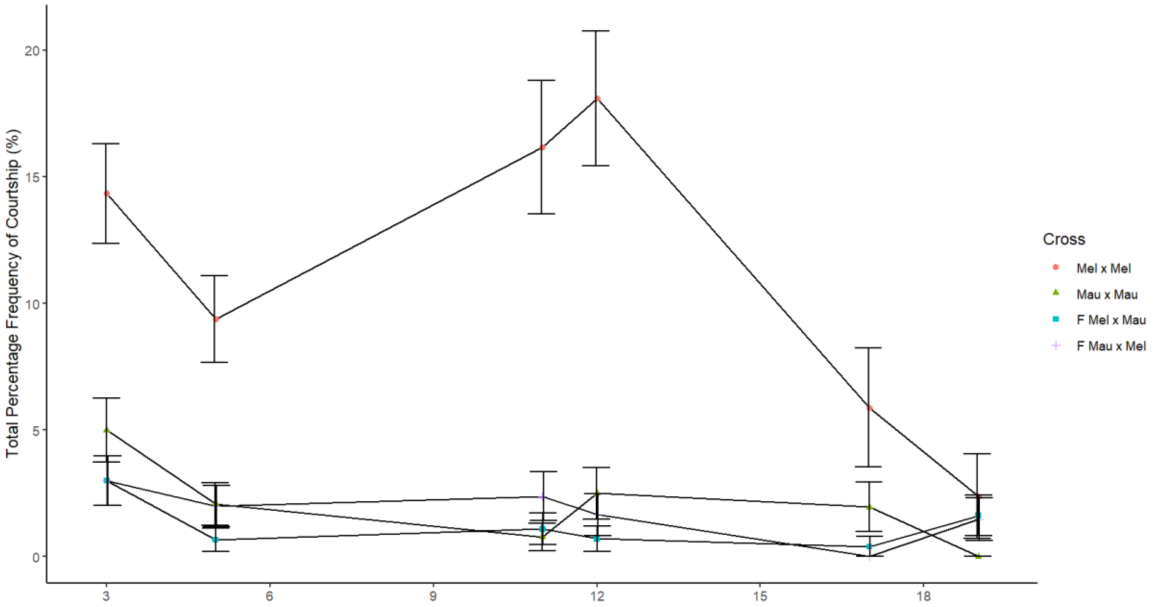

Figure 1. Average percentage frequency of observations (± 1 SE) in which courtship was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific*. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

In this experiment, the patterns of observed courtship between the crosses over time, were similar to those seen in the final experiment. Conspecifically paired *D. melanogaster* females experienced the most courtship, with a general decline in courtship frequency as female aged (Appendix 2, Figure 1, Table 1).

Table 1. Analysis of courtship frequency of each treatment cross over time. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of courtship observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	4.38	0.416681	10.514	< 2e – 16 ***
Mau x Mau	-1.55	0.637379	-2.441	0.014*
Mel (f) x Mau (m)	-2.58	0.69	-3.71	2.02e-4***
Mau (f) x Mel (m)	-1.93	0.64	-3.00	2.675e-3**
Mel x Mel*Age	-0.13	0.03	-3.38	7.26e-4***
Mau x Mau*age	2e-3	0.06	0.04	0.963
Mel (f) x Mau (m)* age	0.07	0.06	1.14	0.251
Mau (f) x Mel (m)* age	0.03	0.05	0.56	0.575

A pairwise analysis revealed that all treatment crosses had similar courtship frequencies, with the exception of conspecific *D. melanogaster* (p = <0.001).

(ii) Movement frequency in con and heterospecific treatments over time

The frequency of movement in focal females varied with treatment cross (Appendix 2, Figure 2, Table 2). Females paired heterospecifically were observed to have a higher frequency of movement than their conspecific counterparts. Interestingly, *D. melanogaster* females also moved more than *D. mauritiana* females in similar mating treatments. A pairwise analysis showed that all treatments were significantly different (p =<0.05). It is interesting to note that in both this pilot and the final, main experiment, heterospecifically *D. melanogaster* females had the highest observed movement. However, unlike the final experiment, the second most motile treatment cross here was heterospecifcally *paired D. mauritiana,* instead of conspecifically paired *D. melanogaster*.

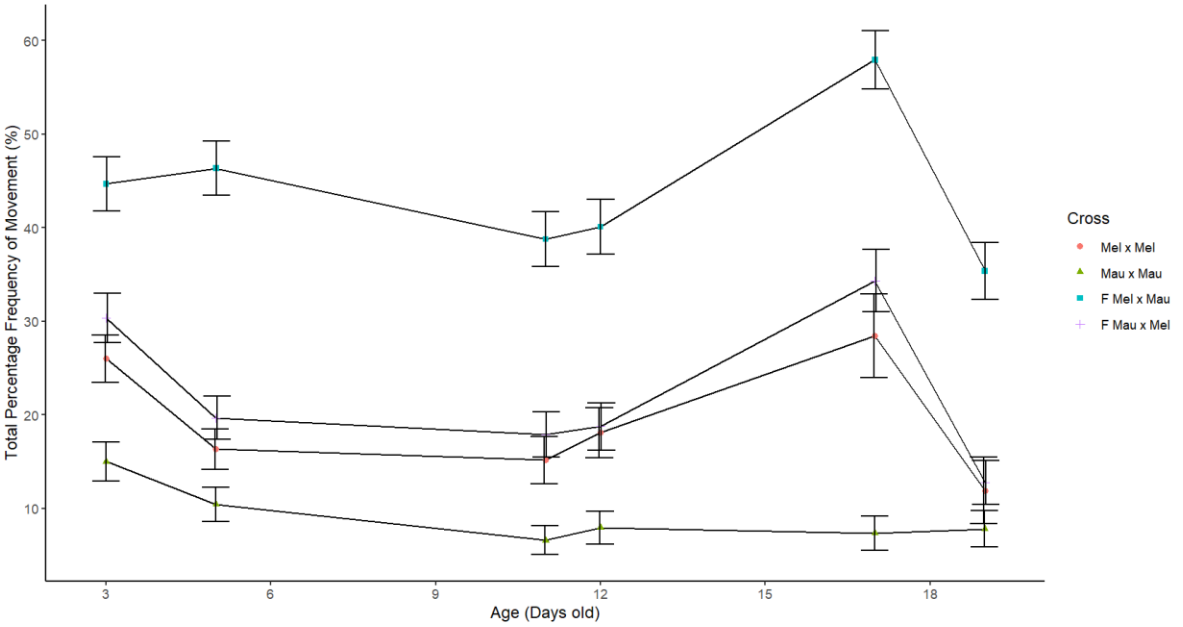

Figure 2. Average percentage frequency of observations (± 1 SE) in which movement was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific*. D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

Table 2. Analysis of movement frequency of each treatment cross over time. *Shown is the output of the GLMM model specified in the statistical methods section, to test for differences in the frequency of movement observed in con and heterospecific crosses between D. melanogaster (Mel) and D. mauritiana (Mau); f = female, m= male.*

Dependent variable/variable interactions	**Estimate**	**SE**	**z value**	**P value**
Mel x Mel	4.49	0.19	23.38	< 2e – 16 ***
Mau x Mau	-0.65	0.28	-2.26	0.023 *
Mel (f) x Mau (m)	0.45	0.25	1.79	0.073
Mau (f) x Mel (m)	-0.05	0.26	-0.20	0.838
Mel x Mel*Age	-0.08	0.02	-5.12	3.04e-07 ***
Mau x Mau*age	0.02	0.02	0.70	0.483
Mel (f) x Mau (m)* age	0.07	0.02	3.35	7.99e-4***
Mau (f) x Mel (m)* age	0.04	0.02	2.02	0.043 *

(iii) Mating frequency in con and heterospecific treatments over time

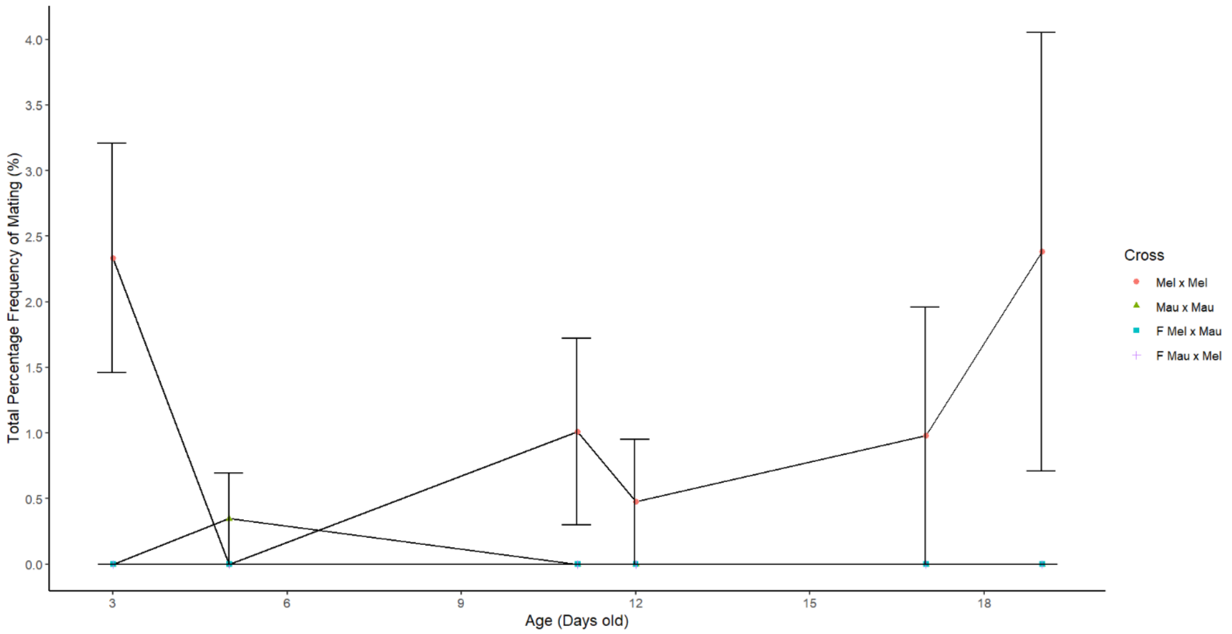

Figure 3. Average percentage frequency of observations (± 1 SE) in which mating was observed, against female age in days, for the 4 treatment crosses i.e., conspecific or heterospecific. *D. melanogaster (Mel) and D. mauritiana (Mau); F = female.*

In the second pilot study, mating was very infrequently observed (Appendix 2, Figure 2).

Unfortunately, because of the low frequency of observations, these mating data in Pilot 2 could not be statistically analysed, due to issues with model convergence, which could not be rectified with hurdle or zero-inflated models. I include a plot of the data to allow visual inspection (Appendix 2, Figure 2).

Matings were only observed in conspecific treatment crosses, primarily in *D. melanogaster*. While I predicted a decline in mating with age, conspecifically paired *D. melanogaster* did not follow this pattern, with mating frequency being rather variable.

Appendix 1 and 2 - attribution statement: I designed and conducted all insect husbandry and experimental work associated with both pilot experiments with additional input from my supervisor, Professor Tracey Chapman. Statistical analysis was conducted by me, with assistance from Lauren Harrison. Experimental reporting was written by me, with feedback from Tracey Chapman and Lauren Harrison.

References:

Aranha, M. M., & Vasconcelos, M. L. (2018). Deciphering female innate behaviors. *Current Opinion in Neurobiology*, 52:139-148.

Anjos-Duarte, C. S., Costa, A. M., & Joachim-Bravo, I. S. (2011). Influence of female age on variation of mate choice behavior in Mediterranean fruit fly (Diptera: Tephritidae). *Journal of insect behavior*, 24(1):11-21.

Atwell, A., & Wagner Jr, W. E. (2014). Female mate choice plasticity is affected by the interaction between male density and female age in a field cricket. *Animal behaviour*, 98:177-183.

Avent, T. D., Price, T. A. R., & Wedell, N. (2008). Age-based female preference in the fruit fly *Drosophila pseudoobscura*. *Animal Behaviour*, *75*(4):1413-1421.

Bargielowski, I., Honório, N. A., Blosser, E. M., & Lounibos, L. P. (2019). Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency. *Journal of Medical Entomology*, 56(2):329-333.

Bolker B, R Development Core Team (2022). _bbmle: Tools for General Maximum Likelihood Estimation_. R package version 1.0.25, <https://CRAN.R-project.org/package=bbmle>.

Braga, M. V., Pinto, Z. T., de Carvalho Queiroz, M. M., & Blomquist, G. J. (2016). Effect of age on cuticular hydrocarbon profiles in adult Chrysomya putoria (Diptera: Calliphoridae). *Forensic science international*, 259:e37-e47.

Brooks M.E., Kristensen K., van Benthem K.J., Magnusson A., Berg C.W., Nielsen A., Skaug H.J., Maechler M. and Bolker B.M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. The R Journal, 9(2):378-400. doi: 10.32614/RJ-2017-066.

Bryan J (2023). _gapminder: Data from Gapminder_. R package version 1.0.0, <https://CRAN.R-project.org/package=gapminder>.

Burdfield-Steel, E. R., & Shuker, D. M. (2011). Reproductive interference. *Current Biology*, 21(12), R450-R451.

Canty A, Ripley BD (2022). boot: Bootstrap R (S-Plus) Functions. R package version 1.3-28.1.

Cothran, R. D. (2015). The importance of reproductive interference in ecology and evolution: from organisms to communities. *Population Ecology*, 57(2):339-341.

Fox J, Weisberg S (2019). An R Companion to Applied Regression, Third edition. Sage, Thousand Oaks CA. https://socialsciences.mcmaster.ca/jfox/Books/Companion/.

Gröning, J., & Hochkirch, A. (2008). Reproductive interference between animal species. *The Quarterly review of biology*, 83(3):257-282.

Hartig F (2022). _DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models_. R package version 0.4.6, <https://CRAN.R-project.org/package=DHARMa>.

Hercus, M. J., & Hoffmann, A. A. (2000). Maternal and grandmaternal age influence offspring fitness in *Drosophila*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1457):2105-2110.

Horikoshi M, Tang Y (2018). ggfortify: Data Visualization Tools for Statistical Analysis Results. https://CRAN.R-project.org/package=ggfortify.

Kassambara A, Kosinski M, and Biecek P (2021). survminer: Drawing Survival Curves using 'ggplot2', version 0.4.9, https://cran.r-project.org/package=survminer

Kelly, C. D. (2018). The causes and evolutionary consequences of variation in female mate choice in insects: the effects of individual state, genotypes and environments. *Current Opinion in Insect Science*, 27:1-8.

Koref-Santibáñez, S. (2001). Effects of age and experience on mating activity in the sibling species *Drosophila pavani* and *Drosophila gaucha*. *Behavior Genetics*, 31(3):287-297.

Lenth R (2023). _emmeans: Estimated Marginal Means, aka Least-Squares Means_. R package version 1.8.5, <https://CRAN.R-project.org/package=emmeans>.

Long, C. E., Markow, T. A., & Yaeger, P. (1980). Relative male age, fertility, and competitive mating success in *Drosophila melanogaster*. *Behavior genetics*, 10(2):163-170.

McRobert, S. P., & Tompkins, L. (1988). Two consequences of homosexual courtship performed by *Drosophila melanogaster* and *Drosophila affinis* males. *Evolution*, 42(5):1093-1097.

Mitchell, C., Leigh, S., Alphey, L., Haerty, W., & Chapman, T. (2022). Reproductive interference and Satyrisation: mechanisms, outcomes and potential use for insect control. *Journal of Pest Science*, 1-14.

Moulin, B., Rybak, F., Aubin, T., & Jallon, J. M. (2001). Compared ontogenesis of courtship song components of males from the sibling species, *D. melanogaster* and *D. simulans*. *Behavior Genetics*, 31(3):299-308.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Saleem, S., Ruggles, P. H., Abbott, W. K., & Carney, G. E. (2014). Sexual experience enhances *Drosophila melanogaster* male mating behavior and success. *PloS one*, 9(5):e96639.

Shelly, T. E., Edu, J., & Pahio, E. (2007). Age-dependent variation in mating success of sterile male Mediterranean fruit flies (Diptera: Tephritidae): implications for sterile insect technique. *Journal of economic entomology*, 100(4):1180-1187.

Sjoberg D (2023). _hablar: Non-Astonishing Results in R_. R package version 0.3.2, <https://CRAN.R-project.org/package=hablar>.

Slowikowski, K(2021). ggrepel: Automatically Position Non-Overlapping Text Labels with 'ggplot2'. R package version 0.9.1. https://CRAN.R-project.org/package=ggrepel

Therneau T (2023). A Package for Survival Analysis in R. R package version 3.5-5, https://CRAN.R-project.org/package=survival.

Turissini, D. A., McGirr, J. A., Patel, S. S., David, J. R., & Matute, D. R. (2018). The rate of evolution of postmating-prezygotic reproductive isolation in *Drosophila*. *Molecular Biology and Evolution*, 35(2):312-334.

Vaias, L. J., Napolitano, L. M., & Tompkins, L. (1993). Identification of stimuli that mediate experience-dependent modification of homosexual courtship in *Drosophila melanogaster*. *Behavior genetics*, 23:91-97.

Wickham H (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H (2019). "Welcome to the tidyverse." Journal of Open Source Software, 4(43), 1686. doi: 10.21105/joss.01686.

Wickham H, François R, Henry L, Müller K, Vaughan D (2023). dplyr: A Grammar of Data Manipulation. https://dplyr.tidyverse.org, https://github.com/tidyverse/dplyr.

Wickham H, Vaughan D, Girlich M (2023). _tidyr: Tidy MessyData_. R package version 1.3.0, <https://CRAN.R-project.org/package=tidyr>.

Xu, J., & Wang, Q. (2009). Male moths undertake both pre-and in-copulation mate choice based on female age and weight. *Behavioral Ecology and Sociobiology*, 63(6):801-808.

Yassin, A., & David, J. R. (2016). Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila*. *Journal of Evolutionary Biology*, 29(2):455-460.

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REVIEW

Reproductive interference and Satyrisation: mechanisms, outcomes and potential use for insect control

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Abstract

Reproductive Interference occurs when interactions between individuals from different species disrupt reproductive processes, resulting in a fitness cost to one or both parties involved. It is typically observed between individuals of closely related species, often upon secondary contact. In both vertebrates and invertebrates, Reproductive Interference is frequently referred to as 'Satyrisation'. It can manifest in various ways, ranging from blocking or reducing the efficacy of mating signals, through to negative effects of heterospecific copulations and the production of sterile or infertile hybrid offspring. The negative fitness effects of Satyrisation in reciprocal matings between species are often asymmetric and it is this aspect, which is most relevant to, and can offer utility in, pest management. In this review, we focus on Satyrisation and outline the mechanisms through which it can operate. We illustrate this by using test cases, and we consider the underlying reasons why the reproductive interactions that comprise Satyrisation occur. We synthesise the key factors affecting the expression of Satyrisation and explore how they have potential utility in developing new routes for the management and control of harmful insects. We consider how Satyrisation might interact with other control mechanisms, and conclude by outlining a framework for its use in control, highlighting some of the important next steps.

Keywords Reproductive interference · Satyr effect · Satyrisation · Interspecific interactions · Pest control · Pest

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Key Messages

- Reviews the current knowledge on interspecific mating interactions.
- Synthesises factors that impact frequency or asymmetry of Reproductive Interference.
- Analyses of implications/outcomes of interacting factors of Reproductive Interference with test cases.
- Generates framework for using fitness cost asymmetries for pest control.

Introduction

The study of the rapid evolution of reproductive traits and their divergence between closely related species is of fundamental interest to researchers in the context of speciation. It also gives insights into introgression and biodiversity conservation (Pfennig and Pfennig 2010; Rice and Pfennig

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2010; Shuker and Burdfield-Steel 2017). There is empirical evidence that the divergence of different reproductive traits between closely related species, whether morphological or behavioural, can occur at variable rates. This can result in the phenomenon whereby individuals from the diverging species cannot form fertile hybrids, but can suffer negative fitness costs due to interspecific sexual interactions. These reproductive interactions can take various forms and are collectively referred to as 'Reproductive Interference'. In vertebrates and invertebrates, this process is often termed Satyrisation (after the sexually promiscuous half-goat man of Greco-Roman myth; Ribeiro and Spielman 1986; Bargielowski et al. 2013). The effects and fitness costs of reciprocal matings between species are often asymmetric, and it is this aspect that has implications for species coexistence (Gröning and Hochkirch 2008; Shuker and BurdfieldSteel 2017; Kyogoku 2020), as well as pest management. Some authors originally used the term Satyrisation to refer exclusively to *asymmetric* Reproductive Interference. However, the usage of this term has since broadened, and in this review, we define Satyrisation as symmetric or asymmetric Reproductive Interference that occurs in vertebrate and invertebrate mating systems.

Reproductive Interference sits at the interface between evolutionary biology and ecology. For instance, there is a growing realisation that it can help to resolve unexplained features of competitive relationships between species, such as when species exclusion cannot be explained by resource competition (Park et al. 1948; Birch et al. 1951; Kishi et al. 2009). There is also a growing awareness that Reproductive Interference can be a driver of reproductive character displacement, in addition to reinforcement and the Templeton effect (Templeton 1981; Butlin and Ritchie 2009; Hollander et al. 2018).

Reproductive Interference is also relevant for conservationists, as it could influence the invasion success of nonnative species, and result in impacts upon other species with which the invasives could potentially interbreed (Liu et al. 2007; Gröning and Hochkirch 2008; D'Amore et al. 2009). Reproductive Interference is of significant applied interest in terms of its potential utility in controlling harmful species including disease vectors such as *Aedes* mosquitoes (Gröning and Hochkirch 2008; Bargielowski and Lounibos 2016). Satyrisation is being considered as a potential pest control method, both independently and in conjunction with other current pest-suppression strategies (Leftwich et al. 2016; Honma et al. 2019).

The first aim of this review is to summarise the ways in which Satyrisation is expressed within vertebrate and invertebrate systems and to determine the factors that result in asymmetric fitness costs, using illustrative test cases. The second aim is to consider how the principles underlying Satyrisation could be deployed for the control and management of dangerous insect pests. To do this, we reviewed the current literature on Satyrisation, defining the factors that cause its effects to vary, and used this to inform how it can be deployed directly or indirectly as a method of pest control.

We restrict this review to the consideration of situations in which any hybrid progeny that are produced from matings between species have zero fitness (i.e., they are inviable or sterile). The topics of hybrid matings leading to introgression and hybrid vigour are covered in detail elsewhere (Huxel 1999; Hill et al. 2020) and are not considered within the scope of this review.

Reproductive interference

Reproductive Interference is a broad term that is used to define the situation when there are sexual/reproductive interactions, usually between individuals of closely related species, which do not lead to the production of fertile hybrids and instead result in negative fitness costs for the interacting individual males and/or females (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017; Kyogoku 2020). This can include interactions between even reasonably diverged species, such as when a territorial male seeks to exclude individuals of other species, as well as its own, during mating competitions. In this way, Reproductive Interference can represent a potential intersection between resource competition and heterospecific (between different species) rivalry (Drury et al. 2015). However, Reproductive Interference more often occurs between species which are closely related/ recently diverged, due to the existence of incomplete mating barriers. Therefore, Reproductive Interference is fundamentally linked to reproductive character displacement, reinforcement and speciation (Smadja and Ganem 2005; Kronforst et al. 2007; Matute 2014). It can occur, in principle, over a broad range of plant and animal taxa (Levin 1970). The study of Reproductive Interference, to date, has been focussed mostly on the study of plant science (Weber and Strauss 2016). In particular, the emphasis has been on determining the mechanisms and origin of Reproductive Interference in the formation of post-zygotic barriers leading to speciation. Asymmetries in Reproductive Interference in plants have also been reported in terms of unilateral incompatibility (Bedinger et al. 2011; Lewis and Crowe 1958; Marta et al. 2004) and vestigial viable pollen (Whitton, et al. 2017). These factors are known to limit species co-occurrence.

In vertebrate and invertebrate mating systems, reproducing individuals are usually mobile and may exhibit a complex range of reproductive behaviours. This has the potential to offer a greater number of scenarios in which Reproductive Interference might occur, in comparison to plants, and to lead to stronger selection to avoid costly interspecific coupling (Levin 1970). Reproductive Interference that occurs within animal mating systems is usually referred to as Satyrisation, and is divided into seven categories (Gröning and Hochkirch 2008; Shuker and Burdfield-Steel 2017) each related to distinct types of mating barrier. These mechanisms can be pre- or post-copulatory, often work in conjunction, and can have potentially different ecological impacts. The mechanisms are: signal jamming, heterospecific rivalry, misdirected courtship, heterospecific mating attempts, erroneous female choice, heterospecific mating, and hybridization (summarised, with examples, in Table 1).

Reproductive Interference shares some features of resource competition and is density-dependent (Gröning and Hochkirch 2008). For example, it can result in population or species exclusion, or coexistence through divergence (Kuno 1992). This has been modelled using a Lotka-Volterra competition framework (Ribeiro and Spielman 1986; Kuno, 1992). As with competition, Reproductive Interference can result in either exclusion of the ‘weaker’ species, divergence (parapatry), or coexistence through niche partitioning/ reproductive character displacement/ eventual evolution of complete mating barriers (Kyogoku 2020). However, unlike resource competition, Reproductive Interference lacks a true ‘shared resource’, and instead occurs due to errors in, or incomplete, mate recognition, resulting in fitness reductions in individuals of the interacting species (Gröning and Hochkirch 2008).

Due to the shared features of Reproductive Interference and resource competition, it can often be difficult to disentangle the relative importance of these different forms of interspecific interactions on reproductive behaviours, particularly within field settings. However, there is a growing realisation that Reproductive Interference may play a larger role in species competition and speciation than previously considered (Hochkirch et al. 2007). For example, it may help to explain the results of experiments initially attributed to competitive exclusion in which the seemingly weaker resource competitor excluded the ‘more efficient’ species (Park et al. 1948; Birch et al. 1951; Kishi et al. 2009). Reproductive Interference may even be maintained in some cases due to what Drury et al. (2019) refer to as an ‘Evolutionary Catch-22’, wherein the cost to males of mating with heterospecific females is lower than that of missing conspecific (between same species) mating opportunities, thereby limiting divergence in male mate recognition and female reproductive characteristics (Shuker and Burdfield-Steel 2017). Whilst this is unlikely to be a feature of all species that can experience Reproductive Interference due to differences in male fitness costs, it is nevertheless interesting to consider in the context of factors that may limit the evolution of reproductive character displacement (Drury et al. 2015, 2019). Overall, our knowledge of Reproductive Interference is important in the context of how we consider species interactions and their possible ecological outcomes. This is particularly relevant to increased invasion events, in which consideration must be given to the effects of Reproductive Interference on invasion success and how it impacts upon introgression into at-risk species (Liu et al, 2007; Gröning and Hochkirch 2008; D’amore et al. 2009).

Asymmetric reproductive interference/ Satyrisation

An intriguing aspect of Reproductive Interference is the high degree of asymmetry in fitness costs often observed in reciprocal interactions between species (Gröning and Hochkirch 2008). This can rapidly increase the probability or rate of competitive exclusion or niche partitioning. Within invertebrates, Satyrisation is beginning to garner attention as a potential mechanism for intentional exclusion to achieve pest control (Leftwich et al. 2016; Honma et al. 2019). The term ‘Satyr’ was first utilised in this context by Ribeiro and Spielman (1986) and was originally defined as *asymmetric* Reproductive Interference by reference to a mathematical model that explored the fitness costs of reciprocal interspecific interactions. However, since then, Satyrisation has generally been used to describe the symmetric *and* asymmetric Reproductive Interference that occurs in vertebrates and invertebrates, and this is the definition we adopt here. An example of asymmetric Satyrisation can be found in cryptic butterfly species, where the less competitive and less reproductively efficient species are observed to exhibit rapid niche partitioning with respect to their more competitive counterparts. This is thought to arise at least partly to avoid costly misdirected courtships from heterospecific males (Dincă et al. 2013; Friberg et al. 2013). Satyrisation was first described several decades ago (e.g., Ribeiro and Spielman 1986; Miller et al.1994) and interest in it is growing partly as it provides an explanation for the observed reduction of *Aedes aegypti* populations in North America (particularly in the panhandle of Florida) following the invasion of *A. albopictus* (Parker et al. 2019).

Table 1 Summary of different Satyrisation categories (Gröning and Hochkirch 2008) together with illustrative examples

Type of reproductive interference	Form of incomplete mating barrier	Description	Examples
Signal jamming/Signal Interference	Pre-mating	Disruption of reproductive signals due to presence of heterospecific signals such as pheromones, mating calls, visual displays	Responses to heterospecific pheromones in Lepidopterans moths) (Landolt and Heath 1987)
Heterospecific rivalry	Pre-mating	Heterospecifics are mistaken for conspecific rivals and becomes subject to aggression	Interspecies fighting and territoriality in *Hetaerina* damsel-flies (Drury et al. 2015)
Misdirected courtship	Pre-mating	Courtship of heterospecifics due to mistaken identity/ similarity of courtship behaviours and responses	Courtship of heterospecific females by males of butterfly species *Leptidea sinapis* and *Leptidea juvernica* (Friberg et al. 2013)
Heterospecific mating attempts	Pre-mating	Incomplete heterospecific copulations, which can have fitness costs arising from bodily harm, or harassment effects	Attempted forced copulation between male guppies (*Poecilia reticulata* with heterospecific female Topmin-nows (*Skiffia bilineata* (Valero et al. 2008)
Erroneous Female Choice	Pre-mating	Females actively choose heterospecific males due to mistaken identity or pre-existing sensory bias	Erroneous female choice in *Paratrechalea ornata* spiders, in which females) accept nuptial gifts and engage in misdirected courtship with male *Paratrechalea azul* Costa-Schmidt, and Machado 2012)
Heterospecific mating	Post-mating, pre-zygotic	Successful heterospecific coupling, where fitness costs can arise from bodily harm, gamete wastage, and the induction of refractoriness to further matings	Heterospecific mating and insemination between male *Aedes albopictus* and female *Aedes aegypti* mosquitoes Nasci et al. 1989)
Hybridization	Post-mating, post-zygotic	Production of zero fitness hybrid offspring, from heterospecific mating. Fitness costs depend on extent of energetic costs expended on the production of hybrid offspring	Production of sterile hybrids in *Drosophila arawakhana* × *Drosophila dunni* crosses. (Hill et al. 2020)

made by females. This scenario sets up the risk of energetic costs due to gamete wastage, potential harm from mating with males with incompatible morphology or physiology, or opportunity costs of lost mating opportunities due to the induction of post-mating refractoriness (Bath et al. 2012; Bargielowski and Lounibos, 2016; Yassin and David, 2016; Leigh et al. 2020). An example is described by Tripet et al. (2011) in which low (0.01–12.3%) mating rates to conspecifics were observed in female *A. aegypti* following injection with *A. albopictus* male accessory gland extracts, which induce refractoriness to remating in both species. Failure to mate with a conspecific will incur significant fitness costs. Asymmetry in costs in reciprocal interactions between species pairs is also common, with, for instance, females of one species suffering much higher costs heterospecific interactions than the other. Tripet et al. (2011) provide evidence, by observing that *A. aegypti* females are rendered refractory to mating by the heterospecific male accessory gland proteins of *A. albopictus*, whereas the insemination of *A. albopictus* females by *A. aegypti* male accessory gland proteins has no such effect.

Differential rates of character divergence and the underlying drivers are key candidates for producing asymmetric effects of Satyrisation. Studying the mechanisms of these asymmetries could also yield important insights into the relative plasticity or conservation of genes that regulate sexual behaviour and physiology and the rate at which they evolve, as well as strengthening our overall understanding of reproductive isolation. Asymmetric Satyrisation could also potentially inform new methods of control by the repression or replacement of pest species, in a manner that could bypass restrictions and concerns associated with genetic modification (Alphey et al. 2013; Leftwich et al. 2016; Honma et al. 2019). The effects of Satyrisation within existing control programmes are also of potential significance. For example, Satyrisation between modified males released to effect control with heterospecifics resident in the target control area (e.g., release males courting heterospecific non-target females, or heterospecific males blocking matings for release males) might reduce the efficacy of control, by lowering the frequency of conspecific matings between released males and wild females.

Research into Satyrisation, as a direct method of pest control, is still in its infancy. However, its potential to interfere with key reproductive processes means that knowledge of the fundamental mechanisms involved could indicate new routes for manipulating pest populations into increased vulnerability. A key aspect is to understand which factors most influence asymmetric fitness costs between species. In addition, it will be important to determine if control could be rendered more successful by simultaneously manipulating multiple factors that increase Satyrisation asymmetry, or by tailoring the approach to target asymmetries to which any specific target population is particularly vulnerable. The factors of greatest importance in determining overall levels of Satyrisation are likely to vary with control scenarios and are discussed in more detail below.

Factors impacting the degree of asymmetry in Satyrisation

The efficacy of Satyrisation at driving species exclusion (whether via sexual exclusion or a combination of sexual and competitive exclusion) or niche partitioning, is highly dependent on the degree of asymmetry in fitness costs between the interacting species (Ribeiro and Spielman 1986). The asymmetry is strongly influenced by a variety of factors including density dependence, evolutionary history, and life history trade-offs. These factors and their effects are illustrated in Table 2.

There is an inherent difficulty in disentangling the relationships between Satyrisation and species character traits in order to ascertain whether an existing character trait simply exacerbates Satyrisation, or if Satyrisation itself was, or is, a driver of trait evolution. For example, we need to understand whether resource competition simply intensifies the effects of Satyrisation or if individuals of the less competitive species will be selected to specialise to avoid Satyrisation, as is suggested to occur in conflicts between the ladybirds *Harmonia axyridis* and *H. yedoensis* (Noriyuki et al. 2012).

It should also be noted that the extent of Satyrisation is also highly likely to be influenced by changes to abiotic factors and habitat structure. Examples include habitat loss or climate change potentially pushing related species together or preventing niche partitioning. This could increase the frequency at which Satyrisation occurs, by either creating sympatry where species were once allopatric (i.e., creating new habitat overlaps between species) or by increasing population densities in hybrid zones (Liu et al. 2007). Such factors may also cause changes to preferred ecological niches, which may act in conjunction with Satyrisation. The following sections discuss in more detail the various factors proposed to affect the efficacy/frequency of Satyrisation (Table 2).

Population density/species ratio

As with resource competition, the relative abundance of each competing species will play a role in whether Satyrisation is strong enough to result in species exclusion. Under resource competition, an increased number of competitors results in resource limitation, whereas under Satyrisation, an uneven species ratio or a high density can result in a high frequency of heterospecific encounters, increasing the likelihood that costly heterospecific courtship will occur (Kyogoku and

Table 2 Factors that affect the degree of asymmetry in Satyrisation

Factors influencing the extent of asymmetry in Satyrisation	Consequences of factors
Relative abundance, population density, and sex ratio of target species and satyr species upon introduction	Affects the frequency of heterospecific interactions and matings
Pre-existing asymmetry in resource competition	Can exacerbate population dynamics that influence reproductive interference and increase the likelihood of exclusion
Number of generations spent in sympatry or allopatry	Influences degree of selection pressure to prevent interspecific reproductive interactions
Presence/degree of pre-mating barriers	Mate recognition, choosiness, phenology of mating, courtship differences can alter asymmetry of fecundity costs of hybrid mating between species
Presence/degree of post-mating barriers	Effectiveness of responses to heterospecific seminal fluid proteins, the extent of con or heterospecific sperm precedence, refractory period, and capacity to hybridise can all alter asymmetry of fecundity costs of hybrid mating between species
Degree of intraspecific sexual conflict within the target species and satyr species	Can influence asymmetry of heterospecific mating fitness costs
Fitness costs of Satyrisation resistance genes	Influences likelihood of resistance evolution/how long it takes for resistance to evolve/how long resistance genes will stay in the population if the species become allopatric
Life History trade-offs: parasite load, predation, changes in fecundity over time, life history, etc	General fitness effects that can influence relative abundance and fecundity
Mating system	Differences in mating system will result in species differing in pre-mating and post-mating investment
Presence of multiple interbreeding species	Could alter relative fitness costs between species and change selection pressures

Sota, 2017; Kyogoku 2020). This phenomenon was observed in simulations by Takafuji et al. (1997) based on interactions between two closely related spider mites, in which the initial density ratios heavily affected the extent of competitive exclusion that occurred. This has significant implications for the success of invasion by non-native species which can reproductively interfere with native species.

Pre-existing resource competition asymmetry

As Satyrisation often occurs between closely related species, resource competition may be strong as there may not yet have been sufficient divergence to avoid niche overlap. Theory by Kishi and Nakazawa (2013) predicts some of the ways in which Satyrisation and resource competition can interact. In situations where the more resource-efficient species also suffer lower fitness costs from Satyrisation, this should result in the exclusion of the weaker species being more likely or more rapid. In contrast, when fitness cost asymmetries in resource competition and heterospecific reproductive interactions occur in opposite directions, i.e. the more resource-efficient species are more negatively affected by heterospecific reproductive interactions and *vice versa*, Satyrisation could theoretically lead to species coexistence, or even favour the weaker competitor. Another example of how resource competition and Satyrisation can have a combined effect on local species exclusion is found in pied and collared flycatchers on the Swedish Island of Öland (Vallin et al. 2012). Resource competition between these two species over mating territories led to young pied flycatcher males being unable to establish territories. This in turn reduced the abundance of conspecific Pied Flycatchers males available, leading to an increase in heterospecific matings, the costly production of low-fitness hybrids, and eventual local exclusion. The excluded species was found to have partitioned across separate islands, potentially to avoid the combined effects of resource competition and Satyrisation (Vallin et al. 2012).

Number of generations in sympatry/allopatry

Researchers investigating Satyrisation in *Aedes* have shown that mild forms of resistance to Satyrisation can evolve within just a few generations (Bargielowski et al. 2013, 2019; Bargielowski and Lounibos, 2016). However, this means that allopatric populations may often be more susceptible to Satyrisation. Bargielowski and colleagues have observed that in *A. aegypti*, resistance to Satyrisation was associated with an increased female choosiness in sympatric populations, with allopatric females showing lower levels of discrimination against heterospecifics (e.g., previously allopatric species coming into contact) than within coexisting (e.g., sympatric or parapatric) species.

Presence/degree of pre-mating barriers

The presence, and effectiveness, of pre-mating barriers between closely related species will necessarily affect the extent and frequency with which negative fitness costs are experienced. Hence, these barriers are key to the existence and extent of Satyrisation. For example, in diverging species that retain the capacity to interbreed, one direction of the cross may often be more common than the reciprocal, due to one species having developed stronger pre-mating barriers than the other. This is likely to be dependent on the evolutionary history of divergence between species. Hence, consideration of the time since divergence and/or phylogenetic relatedness may allow researchers to estimate the accumulation of changes in reproductive characteristics (Coyne and Orr 1989), and thus, the likely strength of pre-mating barriers. An example of the evolution of pre-mating barriers that lead to fitness cost asymmetries is observed between *Drosophila occidentalis* and *D. suboccidentalis*, with *D. suboccidentalis* females being less receptive to heterospecific mating than *D. occidentalis* females, when measured in a series of no-choice tests (Arthur and Dyer 2015).

Presence/degree of post-mating barriers

The completeness of post-mating, pre-zygotic mating barriers between closely related species can affect the fitness costs of Satyrisation. The magnitude of post-mating barriers will, as for pre-mating ones, depend upon the evolutionary history of divergence between the species involved. An example is found in the phenomenon of conspecific spermprecedence, in which same species sperm are used preferentially over that of any other species sperm present in the female reproductive tract. Hence, even if heterospecific mating can be costly, the fitness costs of gamete wastage could potentially be mitigated via conspecific sperm-precedence, provided that females can or have previously mated with a conspecific male (Burdfield-Steel et al. 2015). Price (1997) and Rugman-Jones and Eady (2007) observed conspecific sperm precedence in *Drosophila simulans* and *Callosobruchus subinnotatus*, respectively, and noted that conspecific sperm was not only used preferentially for fertilisation but was also stored preferentially in spermathecae. However, it was not evident to what extent these phenomena were controlled by preferential female use, or by physiological effects of male seminal fluid proteins. A recent model by Iritani and Noriyuki (2021) of the reproductive interactions between the ladybird beetles *Harmonia axyridis* and *H. yedoensis* suggested that conspecific sperm precedence would not be sufficient to counteract the negative effects of Satyrisation.

This was due to the high costs of increased refractoriness to conspecific mating following a heterospecific mating. Overall, the efficacy of post-mating barriers in reducing the costs of Satyrisation will vary between species according to the relative costs of pre- versus post-mating effects on reproductive success.

Degree of intraspecific sexual conflict within the target species and Satyr species

Some research into Satyrisation has suggested that intraspecific sexual conflict between the evolutionary interests of each sex may play a role in explaining asymmetry in the fitness costs of hybrid matings between species (Shuker et al. 2015; Leigh et al. 2020). In species that experience high levels of sexual conflict, females may be better adapted to tolerating the aggressive actions of seminal fluid proteins or persistent courtships. Similarly, females from species subject to lower levels of sexual conflict might be ill-equipped to mitigate the coercive and harmful effects of mating with ‘harmful’ heterospecific males. Yassin and David (2016) found evidence to support this hypothesis as they observed differences in female mortality between hybrid crosses in the *Drosophila melanogaster* species subgroup. In crosses with higher mortality, females were often found to have higher levels of melanisation in their abdominal regions, suggesting wounds from heterospecific mating were more severe in some crosses than others. Similarly, Kyogoku and Sota (2015) found that exaggerated genital spines in the sexually competitive males of the seed beetle *Callosobruchus chinensis* mediated the costs of Satyrisation in *C. maculatus* females. This suggested a direct link between male-male intraspecific competition adaptations, and fitness cost asymmetries in Satyrisation.

Fitness costs of ‘Satyrisation resistance’ genes

If Satyrisation carries high asymmetric fitness costs, it is likely to select for the evolution of resistance within the species which suffers the highest costs (Bargielowski et al. 2013, 2019). However, if the selection is relaxed, e.g., if exposure to the Satyr species is reduced, Satyrisation resistance genes may be rapidly eliminated. This has been observed by Bargielowski et al. (2019) who described a reduction in Satyrisation resistance traits in *A. aegypti* when they were no longer found in sympatry with *A. albopictus*. The fitness costs were unknown but were suggested to be prevent hybrid matings when both species are in sympatry but which may restrict mating opportunities with conspecifics in allopatry. The impact of costs of resistance genes is therefore important to consider, as it can influence the maintenance of resistance to Satyrisation and determine which populations will be or become more susceptible to it.

Life history trade-offs

Factors such as predation, parasite load, and nutritional resources that influence selection pressures and life history will likely have impacts on the existence of Satyrisation, its level of asymmetry, and its effect on sexual exclusion. For example, Drury et al. (2015) considered that Satyrisation was being maintained in sympatric populations of *Hetaerina* damselflies due to weak selection pressure on male mate choice and limitations in female character displacement, as a result of the requirement to maintain crypsis and avoid predation. In addition, Bargielowski et al. (2019) observed an increase in receptivity to heterospecific mating (in *A. aegypti* ♀ x *A. albopictus* ♂ crosses) as individuals aged, likely due to a willingness in females to accept lower quality mates as age-specific fecundity decreased. This could itself have density-dependent effects, since the time to find a mate (or at least a male) is likely to increase as density decreases. We conclude that accurate determination of the occurrence and effects of Satyrisation requires consideration of demography and many different biotic interactions.

Mating system

Mating systems are expected to have major effects on fitness costs associated with hybrid matings. For example, for the mating systems in which each reproductive episode involves a significant investment (e.g., by the giving of nuptial gifts) or in species in which there are limited reproductive opportunities, then even small differences in reproductive characteristics between species could alter the level of Satyrisation asymmetry and result in divergent fitness costs. This phenomenon is evident in interactions between different biotypes of the haplodiploid whitefly *Bemisia tabaci*. Haplodiploidy (i.e. haploid males produced from unfertilised eggs and diploid females from fertilised) renders the frequency and success of mating an important determinant of sex ratio, and thus can greatly affect population growth. It was found that between the B and Q biotypes of *B.tabaci*, the B biotype was more behaviourally plastic. When exposed to Satyrisation effects from exposure to the Q biotype, B biotype females more readily accepted copulations from B males, allowing for the maintenance of sex ratio. In contrast, Q biotype appeared invariant in their mating acceptances and did not upregulate their acceptance of con-biotype mates (Crowder et al. 2010).

Presence of multiple interbreeding species

The dynamics of interspecies breeding can be complex if more than one reproductively interfering species is present in sympatry. This can affect relative fitness costs depending on the frequency at which each species courts/interbreeds with others. Females could mate heterospecifically with different species, potentially on multiple occasions. Shuker et al. (2015) considered heterospecific mating and harassment between four species from the bug family Lygaeidae (*Lygaeus equestris, Spilostethus pandurus, Lygaeus creticus* and *Oncopeltus fasciatus*) and found rare but consistent patterns of heterospecific matings between all species. In massbreeding experiments, the presence and/or identity of the companion bug sex and species had significant effects on nymph production. In no-choice mating assays, heterospecific pairings between female *L. equestris* and male *S. pandurus* resulted in a particularly large reduction in *L. equestris* female longevity and fecundity. Some of these species have overlapping distributions in nature, thus Satyrisation has the potential to occur between these species in the wild. It would be interesting to investigate such instances of Satyrisation between multiple interacting species because of the wide variety of ecological outcomes to which they could lead.

Satyrisation as a control method

Following the observations that Satyrisation effects arising from *Aedes albopictus* were likely to have been a primary driver behind the population decrease of *Aedes aegypti* in North America (Tripet et al. 2011; Bargielowski et al. 2013, 2015) researchers have become interested in exploring the principles of Satyrisation for intentional population exclusion (Leftwich et al. 2016; Honma et al. 2019). The fact that Satyrisation occurred within *Aedes* species has been key to the increasing interest in its use for control, as decades of research have sought to discover effective methods to limit these important arbovirus vectors that spread globally significant pathogens such as dengue, chikungunya and Zika viruses (Alphey et al. 2013; World Health Organisation 2014; Parker et al. 2019).

However, despite being observed in North American *Aedes* populations, it is challenging to determine how frequently Satyrisation occurs in the field (Crowder et al. 2010; Bargielowski et al. 2015). If Satyrisation were to be used for control, the release of both sexes of the interfering species would operate via population replacement (replacing the pest with a more benign species). In contrast, the release of just one sex would function via population suppression (reducing or eliminating the pest; Alphey et al. 2013; Alphey 2014). However, it is possible that any replacement species could cause additional and potentially unanticipated problems. For example, *A. albopictus* is itself an arbovirus vector of medical significance, though it may be a less efficient vector for the transmission of relevant arboviruses than is *A. aegypti* (Alphey et al. 2013; Hugo et al. 2019). The relative vector competence of *Aedes* species is highly dependent on which disease and disease strain they carry (Vega-Rúa et al. 2014). Even if *A. albopictus* was confirmed as a less competent vector, it is not yet clear whether the release of more vectors could offset any benefit created by the reduction of original pest species.

Additional traits may also deserve consideration. For example, *A. albopictus* is reported to exhibit more aggressive biting behaviour than *A. aegypti*. Hence, the additional nuisance of releasing more biting insects into a target area for control should be assessed. For this reason, in scenarios involving disease vectors such as *Aedes* species, it is generally beneficial to release only males, as it is females that bite, require blood meals, and result in further disease transmission (Alphey et al. 2013; Gilles et al. 2014; Zhang et al. 2015). Provided that the females of the target pest show sufficient susceptibility to heterospecific courtship/mating and that this incurs sufficient fitness costs, male-only releases could be compatible with the aim of population control via Satyrisation. As a consequence, there is interest in understanding the molecular mechanisms of Satyrisation in order to engineer Satyr strains for control that could target both inter- and intraspecific reproductive interactions. For example, there is evidence to suggest that Satyrisation can occur between isolated populations within species, which are undergoing incipient speciation (Wu et al. 1995; Ting et al. 2001). Therefore, it may be possible to identify or engineer strains to confer control through within-species Satyrisation effects. This, when combined with recognition of the factors described above that increase population susceptibility to Satyrisation, could be fruitful. In effect, this could resemble control via sterile males or via the Sterile Insect Technique (SIT, or mass release of sterile males to effect population control) and would also resemble an interference control strategy originally developed in *Culex pipiens fatigans* (Krishnamurthy and Laven 1976) in which strains of the same species were available that were incompatible (though not initially known, the basis of this incompatibility was infection with different types of *Wolbachia*).

As with all control methods, Satyrisation will be susceptible to the evolution of resistance (Bargielowski et al. 2013) and being rendered less effective by the expression of sexual traits such as conspecific sperm-precedence. However, resistance genes often carry fitness costs (Crowder et al. 2010; Bargielowski et al. 2019) meaning that in the absence of any selective pressure due to the presence of 'Satyr' individuals, resistance should decay. This creates an opportunity to determine which pest populations are more likely to be susceptible to Satyrisation by analysing how long they have been in sympatry or allopatry.

The dependence of Satyrisation on incorrect mate choice could also create opportunities for synergies with other control methods, with the aim of inducing additive or even multiplicative effects (Leftwich et al. 2016). Analysis and alteration of the genetic qualities of a target population and release strain, such as non-target loci, could be used to complement primary control strategies (Leftwich et al. 2021). For example, one could ensure that any release strain intended to confer one primary mechanism of control, such as via *Wolbachia* infection, was also sensitive to Satyrisation. Release of such insects for control could then introgress Satyrisation sensitivity alleles into the target population simultaneously with any primary targeting genes (Alphey et al. 2009). This would create an opportunity to subsequently exploit the sensitivity to Satyrisation introgressed into the target population, to enhance the efficacy of future management.

Similarly, Honma et al. (2019) examined "Sterile Interference", i.e., a combined application of the sterile insect technique and Satyrisation. In this, they explored how control programmes could be made cost-effective, using the initial reduction of the conspecific population to increase the ratio of heterospecific males to conspecific males, and therefore increase the likelihood of interspecific mating. Any control programmes in which engineered or manipulated individuals are released into a target population (e.g., such as Sterile Insect, or Incompatible Insect Techniques) should consider the possible effects of Satyrisation. Any appreciable frequency of courtships or matings between released individuals and heterospecifics in the area will decrease the efficacy of control by increasing mating interference and reducing the probability of the conspecific pairing upon which control is predicated.

The idea that Satyrisation may be affecting pre-existing control methods underlines that Satyrisation shares characteristics with these successful management schemes, namely the utilisation of signal jamming and mating disruption to exert control over pest populations. The potential difference between these methods may be that Satyrisation could have a greater role in affecting pre-mating fitness costs, which could be used to bolster the reproductive losses experienced by the pest population due to unsuccessful copulations. In addition, Satyrisation control programmes could reap the benefits of single-sex release, but without the potential fitness losses from treatments that induce sterility in individuals released as part of Sterile Insect Technique programmes.

Our understanding of the potential of Satyrisation as a direct method of control is as yet undeveloped. However, while this means the Satyrisation is not likely to be applied in the near future, its understanding is vital both to understand its potential impacts upon control via other mechanisms and to inform potential new routes for control. By considering Satyrisation when designing control initiatives, we can determine and anticipate its likely positive or negative impacts.

A framework for control via Satyrisation

A potential framework for considering Satyrisation for control would require several key steps, and these are outlined below and in Table 3. Of primary importance would be to identify the target population requiring control and from this to determine (i) whether it has any closely related species with which is it shows Satyrisation, and (ii) if these species occur in sympatry. If no such examples exist, additional research would be required to determine if Satyrisation has been observed between any related species in laboratory experiments. Subsequent steps would be to consider whether it is ethical, straightforward and beneficial to potentially release the 'controlling' Satyr species into the area containing the target species, through a series of standard risk assessments (Touré et al. 2003; Bale et al. 2008). Analyses from previous biological control and genetic pest management schemes could be used as a foundation (FAO/IAEA 2006; Oye et al. 2014). There are clear parallels between the potential use of Satyrisation and biological control, either in its standard or augmented form (i.e. if ongoing releases are required). Whether releasing the Satyr species/population complies with this current and well-established legislation for biological control would need to be carefully assessed (Turner et al. 2018) as well as considering biosafety frameworks advised by global authorities on biosecurity and public health (WHO and UNICEF 2010; United Nations 2003; Engineering Biology Research Consortium 2020).

Table 3 Overview of planning elements for potential Satyrisation control protocol development and associated steps

Required plan components	Reasonable steps
Target identification and rationale	(1) Identify target species (2) Identify potential "Satyr" species (3) Determine the frequency of reproductive interference through observations in sympatry or laboratory experiments
Risk assessment and regulation compliance	Research local regulations on species release and control protocols. Consider ethical and ecological ramifications of control
Examine the efficacy of potential satyrisation control procedure	(i) Consider factors discussed in Table 2, and how these may affect the frequency and success of Satyrisation (ii) Examine potential synergies with other control methods
Consider practical applicatory elements	(i) Cost-effectiveness (ii) Duration, location, and frequency of application (iii) Communication with stakeholders and public

It would be important in this assessment to focus on elements of the process that are potentially Satyrisation-specific. These might centre around the relationship between field and laboratory studies and the potential for resistance. Satyrisation in field populations with a long history of sympatry might represent 'resistant' genotypes, and effective control strategies via Satyrisation in this context would be encouraging. Isolated populations of the target species may be much more susceptible to Satyrisation, and this could be revealed by laboratory studies. However, such populations could rapidly acquire resistance. From a regulatory perspective, there may be quite a difference between introducing a new species (to create Satyrisation) versus supplemental releases of one of two species already in sympatry.

If it is determined that the release of a Satyr population for control is ethical, safe, and beneficial, it will be necessary to examine how each factor function between the target and Satyr population (Table 2). This may include:

- Conducting field cage and then open field observations of interspecific interactions, both sexual and competitive.
- Population and demographic surveys and modelling of populations.
- Laboratory and field cage recreations of mating assays to determine the mating frequency and to observe pre- and post-mating barriers.
- Crossing species over multiple generations, first in the laboratory and then in semi-natural conditions, to ascertain how resistance genes arise and persist.
- Examining the genetic and geographical history of the target population, to determine their susceptibility to Reproductive Interference.
- Examining the degree of intraspecific sexual conflict in each species/population.

If, after examining these factors, Satyrisation remains a viable prospect, it should be considered whether it can synergise with other control methods, such as SIT (Honma et al. 2019). Following this, the development of practical control elements would be followed (Table 3) and under guidance from the various regulatory authorities (Vanderplank 1944; FAO/IAEA 2006; Bale et al. 2008; Turner et al. 2018).

Overall, considering the ever-growing problems of resistance to standard chemical pesticides, and with such pesticides often being non-specific and harming non-target species, it is important to assess all potential alternative methods for control (Alphey et al. 2013; Shelton et al. 2020). Satyrisation could easily be added to this list, as it is a naturally existing phenomenon that could be harnessed in a number of different ways. Our growing understanding of Satyrisation invasion dynamics and the potential ecological complications of species release, will aid in the future development of principles of Satyrisation as a pest control method.

Conclusions

Satyrisation operates at the interface between evolutionary genetics and ecology and there is a growing body of literature to demonstrate its importance in the natural world via effects on species exclusion, speciation, and partitioning (Ribeiro and Spielman 1986; Kuno 1992). There are various factors that can influence the presence and degree of Satyrisation, including density-dependent factors such as species abundance, through to rates of species divergence and variation in sexual conflict. From an ecological point of view, we need to consider how Satyrisation may shape species distributions, and how it may alter invasion success and dynamics. From an evolutionary perspective, we must also consider the extent to which Satyrisation has influenced speciation and reinforcement. From an applied perspective, appropriate use of Satyrisation may aid in suppressing pathogen vector populations or increasing crop yield by limiting crop pest populations.

Author Contributions

CM, TC and WH conceived and planned this article; CM wrote the first draft; all authors contributed to and approved the final manuscript.

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Data availability There are no raw data to deposit for this article.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

References

Alphey L (2014) Genetic control of mosquitoes. Annu Rev Entomol 59:205–224. https:// doi. org/ 10. 1146/ annur ev- ento- 011613-162002

Alphey N, Bonsall M, Alphey L (2009) Combining pest control and resistance management: synergy of engineered insects with Bt crops. J Econ Entomol 102:717–732. https://d oi.org/1 0 .1603/0 29. 102. 0233

Alphey L, McKemey A, Nimmo D, Neira-Oviedo M, Lacroix R, Matzen K, Beech C (2013) Genetic control of *Aedes* mosquitoes. Pathog Glob Health 107:170–179. https://d oi.org/1 0 .1179/2 047 7 73213Y. 00000 00095

Arthur NJ, Dyer KA (2015) Asymmetrical sexual isolation but no postmating isolation between the closely related species *Drosophila suboccidentalis* and *Drosophila occidentalis*. BMC Evol Biol 15:1–9. https:// doi. org/ 10. 1186/ s12862- 015- 0328-y

Bale JS, Van Lenteren JC, Bigler F (2008) Biological control and sustainable food production. Phil Trans Roy Soc B 363:761–776. https:// doi. org/ 10. 1098/ rstb. 2007. 2182

Bargielowski IE, Lounibos LP (2016) Satyrization and satyrizationresistance in competitive displacements of invasive mosquito species. Insect Sci 23:162–174. https:// doi. org/ 10. 1111/ 1744- 7917. 12291

Bargielowski IE, Lounibos LP, Carrasquilla MC (2013) Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. Proc Natl Acad Sci USA 110:2888–2892. https:// doi. org/ 10. 1073/ pnas. 12195 99110

Bargielowski IE, Lounibos LP, Shin D, Smartt CT, Carrasquilla MC, Henry A, Navarro JC, Paupy C, Dennett JA (2015) Widespread evidence for interspecific mating between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in nature. Infect Genet Evol 36:456–461. https:// doi. org/ 10. 1016/j. meegid. 2015. 08. 016

Bargielowski IE, Honório NA, Blosser EM, Lounibos LP (2019) Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency. J Med Entomol 56:329–333. https://doi.org/10.1093/jme/tjy153

Bath E, Tatarnic N, Bonduriansky R (2012) Asymmetric reproductive isolation and interference in neriid flies: the roles of genital morphology and behaviour. Anim Behav 84:1331–1339. https://doi.org/10.1016/j.anbehav.2012.08.025

Bedinger PA, Chetelat RT, McClure B, Moyle LC, Rose JK, Stack SM, van der Knaap E, Baek YS, Lopez-Casado G, Covey PA, Kumar A (2011) Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. Sex Plant Reprod 24:171–187. https://doi.org/10.1007/s00497-010-0155-7

Birch LC, Park T, Frank MB (1951) The effect of intraspecies and interspecies competition on the fecundity of two species of flour beetles. Evolution 1:116–132. https://doi.org/10.2307/2405763

Burdfield-Steel ER, Auty S, Shuker DM (2015) Do the benefits of polyandry scale with outbreeding? Behav Ecol 26:1423–1431. https://doi.org/10.1093/beheco/arv103

Butlin RK, Ritchie MG (2009) Genetics of speciation. Heredity 102:1–3. https://doi.org/10.1038/hdy.2008.97

Carrasquilla MC, Lounibos LP (2015) Satyrization without evidence of successful insemination from interspecific mating between invasive mosquitoes. Biol Let 11:20150527. https://doi.org/10.1098/rsbl.2015.0527

Costa-Schmidt LE, Machado G (2012) Reproductive interference between two sibling species of gift-giving spiders. Anim Behav 84:1201–1211. https://doi.org/10.1016/j.anbehav.2012.08.026

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43:362–381. https://doi.org/10.1111/j.1558-5646.1989.tb04233.x

Crowder DW, Sitvarin MI, Carrière Y (2010) Plasticity in mating behaviour drives asymmetric reproductive interference in whiteflies. Anim Behav 79:579–587. https://doi.org/10.1016/j.anbehav.2009.11.025

D'Amore A, Kirby E, Hemingway V (2009) Reproductive interference by an invasive species: an evolutionary trap. Herpetol Conserv Biol 4:325–330

Dincă V, Wiklund C, Lukhtanov VA, Kodandaramaiah U, Norén K, Dapporto L, Wahlberg N, Vila R, Friberg M (2013) Reproductive isolation and patterns of genetic differentiation in a cryptic butterfly species complex. J Evolut Biol 26:2095–2106. https://doi.org/10.1111/jeb.12211

Drury JP, Okamoto KW, Anderson CN, Grether GF (2015) Reproductive interference explains persistence of aggression between species. Proc Roy Soc B 282:20142256. https://doi.org/10.1098/rspb.2014.2256

Drury JP, Anderson CN, Cabezas Castillo MB, Fisher J, McEachin S, Grether GF (2019) A general explanation for the persistence of reproductive interference. Am Nat 194:268–275. https://doi.org/10.1086/704102

Engineering Biology Research Consortium (2020) Security & engineering biology. https://ebrc.org/focus-areas/security/

FAO, IAEA, (2006) Status and risk assessment of the use of transgenic arthropods in plant protection. Austria, Vienna

Friberg M, Leimar O, Wiklund C (2013) Heterospecific courtship, minority effects and niche separation between cryptic butterfly species. J Evolut Biol 26:971–979. https://doi.org/10.1111/jeb.12106

Gilles JR, Schetelig MF, Scolari F, Marec F, Capurro ML, Franz G, Bourtzis K (2014) Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. Acta Trop 132:S178–S187. https://doi.org/10.1016/j.actatropica.2013.08.015

Gröning J, Hochkirch A (2008) Reproductive interference between animal species. Q Rev Biol 83:257–282. https://doi.org/10.1086/590510

Hill T, Rosales-Stephens HL, Unckless R (2020) Rapid divergence of the copulation proteins in the *Drosophila dunni* group is associated with hybrid post-mating-prezygotic incompatibilities. biorxiv. https://doi.org/10.1101/2020.05.20.106724

Hochkirch A, Gröning J, Bücker A (2007) Sympatry with the devil: reproductive interference could hamper species coexistence. J Anim Ecol 76:633–642. https://doi.org/10.1111/j.1365-2656.2007.01241.x

Hollander J, Montaño-Rendón M, Bianco G, Yang X, Westram AM, Duvaux L, Reid DG, Butlin RK (2018) Are assortative mating and genital divergence driven by reinforcement? Evol Lett 2:557–566. https://doi.org/10.1002/evl3.85

Honma A, Kumano N, Noriyuki S (2019) Killing two bugs with one stone: a perspective for targeting multiple pest species by incorporating reproductive interference into sterile insect technique. Pest Manag Sci 75:571–577. https://doi.org/10.1002/ps.5202

Honório NA, Carrasquilla MC, Bargielowski IE, Nishimura N, Swan T, Lounibos LP (2018) Male origin determines satyrization potential of *Aedes aegypti* by invasive *Aedes albopictus*. Biol Invasions 20:653–664. https://doi.org/10.1007/s10530-017-1565-3

Hugo LE, Stassen L, La J, Gosden E, Ekwudu OM, Winterford C, Viennet E, Faddy HM, Devine GJ, Frentiu FD (2019) Vector competence of Australian *Aedes aegypti* and *Aedes albopictus* for an epidemic strain of Zika virus. PLoS Negl Trop Dis 13:e0007281. https://doi.org/10.1371/journal.pntd.0007281

Huxel GR (1999) Rapid displacement of native species by invasive species: effects of hybridization. Biol Cons 89:143–152. https://doi.org/10.1016/S0006-3207(98)00153-0

Iritani R, Noriyuki S (2021) Reproductive interference hampers species coexistence despite conspecific sperm precedence. Ecol Evol 00:1–13. https://doi.org/10.1002/ece3.7166

Kishi S, Nakazawa T (2013) Analysis of species coexistence comediated by resource competition and reproductive interference. Pop Ecol 55:305–313. https://doi.org/10.1007/s10144-013-0369-2

Kishi S, Takayoshi N, Yoshitaka T (2009) Reproductive interference determines persistence and exclusion in species interactions. J Anim Ecol 78:1043–1049. https://doi.org/10.1111/j.1365-2656.2009.01560.x

Krishnamurthy BS, Laven H (1976) Development of cytoplasmically incompatible and integrated (translocated incompatible) strains of *Culex pipiens fatigans* for use in genetic control. J Genet 62:117–129. https://doi.org/10.1007/BF02984218

Kronforst MR, Young LG, Gilbert LE (2007) Reinforcement of mate preference among hybridizing *Heliconius* butterflies. J Evolution Biol 20:278–285. https://doi.org/10.1111/j.1420-9101.2006.01198.x

Kuno E (1992) Competitive exclusion through reproductive interference. Res Popul Ecol 34:275–284. https://doi.org/10.1007/BF02514797

Kyogoku D, Sota T, (2015) Exaggerated male genitalia intensify interspecific reproductive interference by damaging heterospecific female genitalia. J Evol Biol 28(6):1283–1289

Kyogoku D (2020) When does reproductive interference occur? Predictions and data. Pop Ecol 62:196–206. https:// doi. org/ 10. 1002/ 1438- 390X. 12041

Kyogoku D, Sota T (2017) A generalized population dynamics model for reproductive interference with absolute density dependence. Sci Rep 7:1–8

Landolt PJ, Heath RR (1987) Role of female-produced sex pheromone in behavioral reproductive isolation between *Trichoplusia ni* (Hübner) and *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae, Plusiinae). J Chem Ecol 13:1005–1018. https:// doi. org/ 10. 1007/ BF010 20534

Leftwich PT, Bolton M, Chapman T (2016) Evolutionary biology and genetic techniques for insect control. Evol Appl 9:212–230. https:// doi. org/ 10. 1111/ eva. 12280

Leftwich PT, Spurgin LG, Harvey-Samuel T, Thomas CJ, Paladino LC, Edgington MP, Alphey L (2021) Genetic pest management and the background genetics of release strains. Phil Trans Roy Soc B 376:20190805. https:// doi. org/ 10. 1098/ rstb. 2019. 0805

Leigh S, Rostant WG, Taylor MI, Alphey L, Chapman T (2020) Satyrization in *Drosophila* fruitflies. J Evolution Biol 34:319–330. https:// doi. org/ 10. 1111/ jeb. 13733

Levin DA (1970) Reinforcement of reproductive isolation: plants versus animals. Am Nat 104:571–581. https:// doi. org/ 10. 1086/ 282691

Lewis D, Crowe LK (1958) Unilateral interspecific incompatibility in flowering plants. Heredity 12:233–256. https:// doi. org/ 10. 1038/ hdy. 1958. 26

Liu SS, De Barro PJ, Xu J, Luan JB, Zang LS, Ruan YM, Wan FH (2007) Asymmetric mating interactions drive widespread invasion and displacement in a whitefly. Science 318:1769–1772. https:// doi. org/ 10. 1126/ scien ce. 11498 87

Marta AE, Camadro EL, Díaz-Ricci JC, Castagnaro AP (2004) Breeding barriers between the cultivated strawberry, *Fragaria* × *ananassa*, and related wild germplasm. Euphytica 136(2):139–149. https:// doi. org/ 10. 1023/B: EUPH. 000003 0665. 95757. 76

Matute DR (2014) The magnitude of behavioral isolation is affected by characteristics of the mating community. Ecol Evol 4:2945–2956. https:// doi. org/ 10. 1002/ ece3. 1142

Miller JR, Spencer JL, Lentz AJ, Keller JE, Walker ED, Leykam JF (1994) Sex peptides: potentially important and useful regulators of insect reproduction. ACS Symposium Series: Natural and Engineered Pest Management Agents, Vol. 551 Chapter 14, pp 189–209

Nasci RS, Hare SG, Willis FS (1989) Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory. J Am Mosq Control Assoc 5:416–421

Noriyuki S, Osawa N, Nishida T (2012) Asymmetric reproductive interference between specialist and generalist predatory ladybirds. J Anim Ecol 81(5):1077–1085.

Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, Lightfoot SBY, McNamara J, Smidler A, Collins JP (2014) Regulating gene drives. Science 345:626–628. https://d oi.org/1 0 .1126 / scien ce. 12542 87

Park T (1948) Experimental studies of interspecies competition. 1. Competition between populations of the flour beetles, *Tribolium confusum* Duval and *Tribolium castaneum* Herbst. Ecol Monogr 18:265–307. https:// doi. org/ 10. 2307/ 19486 41

Parker C, Ramirez D, Connelly CR (2019) State-wide survey of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Florida. J Vector Ecol 44:210–215. https:// doi. org/ 10. 1111/ jvec. 12351

Pfennig DW, Pfennig KS (2010) Character displacement and the origins of diversity. Am Nat 176:S26–S44. https:// doi. org/ 10. 1086/ 657056

Price CS (1997) Conspecific sperm precedence in *Drosophila*. Nature 388:663–666. https:// doi. org/ 10. 1038/ 41753

Ribeiro JMC, Spielman A (1986) The satyr effect: a model predicting parapatry and species extinction. Am Nat 128:513–528. https:// doi. org/ 10. 1086/ 284584

Rice AM, Pfennig DW (2010) Does character displacement initiate speciation? Evidence of reduced gene flow between populations experiencing divergent selection. J Evolution Biol 23:854–865. https:// doi. org/ 10. 1111/j. 1420- 9101. 2010. 01955.x

Rugman-Jones PF, Eady PE (2007) Conspecific sperm precedence in *Callosobruchus subinnotatus* (Coleoptera: Bruchidae): mechanisms and consequences. Proc Roy Soc B 274:983–988. https:// doi. org/ 10. 1098/ rspb. 2006. 0343

Shelton AM, Long SJ, Walker AS, Bolton M, Collins HL, Revuelta L, Johnson LM, Morrison NI (2020) First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection. Front Bioeng Biotechnol 7:482. https:// doi. org/ 10. 3389/ fbioe. 2019. 00482

Shuker DM, Burdfield-Steel ER (2017) Reproductive interference in insects. Ecol Entomol 42:65–75. https:// doi. org/ 10. 1111/ een. 12450

Shuker DM, Currie N, Hoole T, Burdfield-Steel ER (2015) The extent and costs of reproductive interference among four species of true bug. Popul Ecol 57:321–331. https:// doi. org/ 10. 1007/ s10144- 014- 0470-1

Smadja C, Ganem G (2005) Asymmetrical reproductive character displacement in the house mouse. J Evolution Biol 18:1485–1493. https:// doi. org/ 10. 1111/j. 1420- 9101. 2005. 00944.x

Takafuji A, Kuno E, Fujimoto H (1997) Reproductive interference and its consequences for the competitive interactions between two closely related *Panonychus* spider mites. Exp Appl Acarol 21:379–391. https:// doi. org/ 10. 1023/A: 10184 23711 166

Templeton AR (1981) Mechanisms of speciation-a population genetic approach. Annu Rev Ecol Syst 12:23–48. https://d oi.org/1 0 .1146 / annur ev. es. 12. 110181. 000323

Ting CT, Takahashi A, Wu CI (2001) Incipient speciation by sexual isolation in *Drosophila*: concurrent evolution at multiple loci. Proc Natl Acad Sci USA 98:6709–6713. https:// doi. org/ 10. 1073/ pnas. 12141 8898

Touré YT, Oduola AM, Sommerfeld J, Morel CM (2003) Bio-safety and risk assessment in the use of genetically modified mosquitoes for disease control Ecological aspects for application of genetically modified mosquitoes. Springer/Frontis, Wageningen, pp 217–222

Tripet F, Lounibos LP, Robbins D, Moran J, Nishimura N, Blosser EM (2011) Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am J Trop Med Hyg 85:265–270. https:// doi. org/ 10. 4269/ ajtmh. 2011. 10- 0677

Turner G, Beech C, Roda L (2018) Means and ends of effective global risk assessments for genetic pest management. BMC Proc 12:13. https:// doi. org/ 10. 1186/ s12919- 018- 0112-2

United Nations Cartagena Protocol on Biosafety to the Convention on Biological Diversity (2003) https:// treat ies. un. org/ doc/ Treat ies/ 2000/ 01/ 20000 129% 2008- 44% 20PM/ Ch_ XXVII_ 08_ ap. pdf

Valero A, Macías Garcia C, Magurran AE (2008) Heterospecific harassment of native endangered fishes by invasive guppies in Mexico. Biol Let 4:149–152. https:// doi. org/ 10. 1098/ rsbl. 2007. 0604

Vallin N, Rice AM, Arntsen H, Kulma K, Qvarnström A (2012) Combined effects of interspecific competition and hybridization impede local coexistence of *Ficedula* flycatchers. Evol Ecol 26:927–942. https:// doi. org/ 10. 1007/ s10682- 011- 9536-0

Vanderplank FL (1944) Hybridization between *Glossina* species and suggested new method for control of certain species of tsetse. Nature 154:607–608. https://doi.org/10.1038/154607a0

Vega-Rúa A, Zouache K, Girod R, Failloux AB, Lourenço-de-Oliveira R (2014) High level of vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor in the spread of Chikungunya virus. J Virol 88:6294–6306. https://doi.org/10.1128/JVI.00370-14

Weber MG, Strauss SY (2016) Coexistence in close relatives: beyond competition and reproductive isolation in sister taxa. Ann RevEcol, Evol Systemat 47:359–381. https://doi.org/10.1146/annurev-ecolsys-112414-054048

Whitton J, Sears CJ, Maddison WP (2017) Co-occurrence of related asexual, but not sexual, lineages suggests that reproductive interference limits coexistence. Proc Roy Soc B 284:20171579. https://doi.org/10.1098/rspb.2017.1579

World Health Organization (2014) Global Brief on Vector-Borne Diseases. World Health Organization, Geneva

World Health Organization and UNICEF (2010) Progress and prospects for the use of genetically modified mosquitoes to inhibit disease transmission. World Health Organization, Geneva

Wu CI, Hollocher H, Begun DJ, Aquadro CF, Xu Y, Wu ML (1995) Sexual isolation in *Drosophila melanogaster*: a possible case of incipient speciation. Proc Natl Acad Sci USA 92:2519–2523. https://doi.org/10.1073/pnas.92.7.2519

Yassin A, David JR (2016) Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila*. J Evolution Biol 29:455–460. https://doi.org/10.1111/jeb.12784

Zhang D, Lees RS, Xi Z, Gilles JR, Bourtzis K (2015) Combining the sterile insect technique with Wolbachia-based approaches: II-a safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. PloS One 10:e0135194. https://doi.org/10.1371/journal.pone.0135194