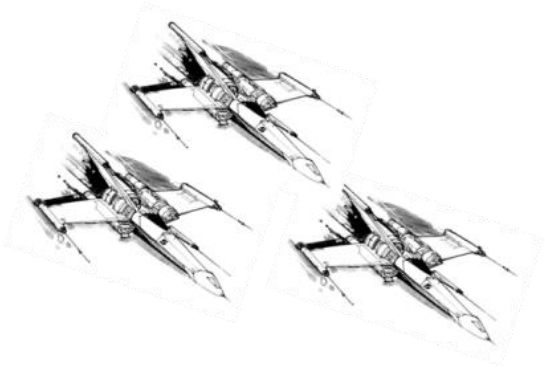
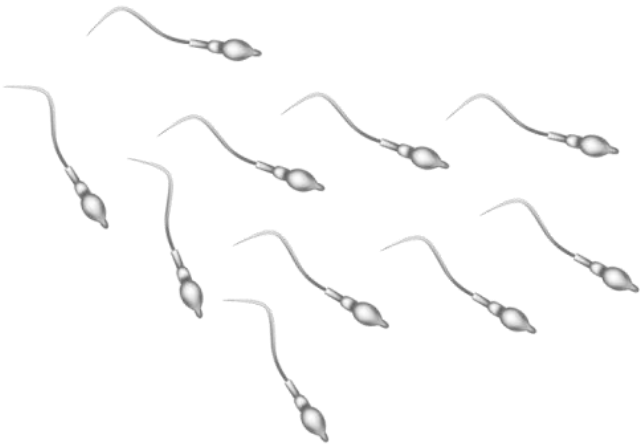
A study of X-chromosome meiotic drive in the Palearctic fly *Drosophila subobscura.*





Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy (or other degree as appropriate) by Rudi Verspoor.

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**fighter picture credit Tom Simpson A-NC-ND 2.0, www.flickr.com*

Abstract

This thesis examines a particular selfish genetic element (SGE), X-chromosome meiotic drive (XCMD), in the species *Drosophila subobscura*. XCMD is a system where the X-chromosome kills or disables Y-chromosome sperm to enhance their own transmission to the next generation. This also results in those males producing female biased broods. This selfish enhancement of their own transmission results in conflict with the rest of the genome that can be a potent force in evolution.

The first chapters deal with sex and mating behaviour and how XCMD and other SGEs are linked to it. Chapter three focusses on the marking techniques and mating behaviour in three species of *Drosophila*. This work was completed while establishing the XCMD system from wild populations Chapter four presents case studies of how SGEs are intrinsically linked to sex. Chapter five examines XCMD in *D. subobscura* and reveals that this species is completely monandrous. This shows that polyandry does not play a role in preventing the spread of XCMD in this species, unlike in a number of other taxa which have XCMD. I also demonstrate weak female choice against XCMD in this chapter. In chapter six and seven I examine the XCMD phenotype when it is expressed in different population genetic backgrounds. I test for evidence of suppression and incompatibilities, when XCMD is exposed in four different populations (Tunisia, Morocco, Spain, and UK). I find evidence of suppression in North Africa, but no suppression in Europe. I also find evidence for severe incompatibilities specific to XCMD on European genetic backgrounds, which are absent in North African backgrounds. These results are consistent with genetic conflict causing rapid evolution in North Africa between XCMD and suppressors, which results in XCMD specific hybrid incompatibilities in naïve European populations.

My final chapter evaluates how the testes proteomes of two species, *D. subobscura* and *D. pseudoobscura*, differ between XCMD and non-XCMD individuals. This ongoing work identifies some putative candidate genes that could be involved in the network that results in XCDM in these species. Interestingly, very few strong candidate genes overlapped in the two species, supporting the idea that separate genes and mechanisms are responsible for the two XCMD systems.

Contents

List of abbreviations

XCMD= X-chromosome meiotic drive

SR*s* = The Sex-ratio distorting X-chromosome meiotic drive system in *Drosophila subobscura*

PCR = Polymerase Chain Reaction

SNP = Single Nucleotide Polymorphism

ANOVA – Analysis of Variance

1 - Acknowledgements

I count myself as lucky to have maintained a great interest in the system that I work on, and the subject area more broadly. Much of this is because my department has been a supportive place to work in.

A special thanks my supervisors Tom and Greg. Tom has been outstanding both in calling out my terrible plans and helping me run with some of the better ones. Developing ideas, sharing some top whiskey and hiking the Swiss Alps while “working on meiotic drive’ were particular highlights. Greg, your dedication to all your students, their interests, and your own work has been an inspiration. Thanks for taking the time to run with a crazy edible termite plan with me. Your experience and persona made you an exceptional teacher.

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I’d like to thank all the staff and co-students in IIB for their various help,particularly Rob and Lynn for their time and patience. I would also like to thank Kelly Dyer, Catherine Montchamp-Moreau, Bojan Kenig, and Ana Llopart for all their help with collaborations and assistance with visits and fieldwork abroad.

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Finally I would like to thank my flies. The vast majority of them were great, and this thesis would be truly terrible without them.

2- General introduction

During my PhD, I was a part of a meiotic drive conference that resulted in a general review of meiotic drive being written by all attending members. The details of the full review are attached as an appendix and represent a collaborative project undertaken during my PhD.

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2.1 - What are selfish genetic elements and why should we study them?

One could argue that it is not possible to understand how an organism functions and behaves without recognising the potential for conflict between components of their genome, and incorporating these conflicts into our study of these organisms. An organism is a complex collection of elements at different scales, from individual nucleotides and genes through chromosomes and organelles within cells that make up specialised organs within the body. Many of these elements can experience diverging evolutionary interests that can result in elements behaving in their own interests, and thus they are termed selfish genetic elements (SGEs).

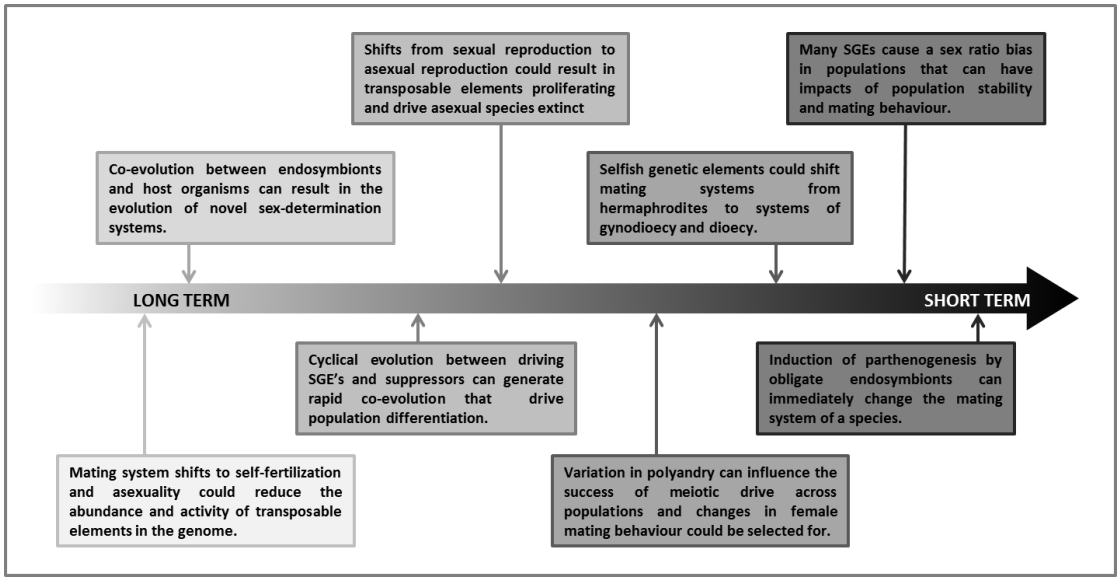

Figure 2.1 A timeline showing examples of the different interactions and impacts that selfish genetic elements and aspects of sex can have on each other (From Verspoor and Price, 2016)

SGEs are constituent parts of an organism that increase their own frequency in subsequent generations to a degree greater than that expected by Mendelian inheritance, without increasing the fitness of the organism that carries them (Burt and Trivers, 2006). They are ubiquitous across the tree of life and are highly diverse, varying widely in the methods they use to increase in frequency (Burt and Trivers, 2006). This diversity means that we are far from fully understanding how SGEs impact on processes of evolution and

ecology. It is also likely that we are underestimating the total number of SGEs and that many new SGEs remain to be discovered.

By selfishly distorting transmission to future generations SGEs create conflicts of interest with other regions of the genome, which lose out in transmission as a result. This intragenomic conflict generated by SGEs can have major consequences (Hurst and Werren, 2001, Presgraves, 2010, Hancks and Kazazian, 2012). The simple conflict between genes within an individual can cause rapid co-evolution (Bastide et al., 2011), huge skews in population sex-ratio (Charlat et al., 2007), or massive phenotype changes (McClintock, 1951). These impacts, alongside the ubiquity of selfish genetic elements, means it is important to investigate their role in shaping ecology and evolution across the tree of life. Rice, adopting the famous narrative of Dobzhansky, writes that 'Nothing makes sense in genetics, except in the light of genomic conflict' (Rice, 2013). Increasingly, it is recognised that SGEs have profound implications for the ecology and evolution of species, affecting mating behaviour, extinction (Dyer, 2012), speciation, and influencing genome size (Agren and Wright, 2011) (Figure 2.1). However, much of our understanding of SGEs currently derives from study in model organisms, and as a result it is likely that the extent of their impact on evolution and ecology is being underestimated.

2.2 - Selfish genetic elements in the age of genetic modification

The coming century will see a number of serious challenges to our biological systems, including the spread and risk from disease, and the resilience of our food systems to a changing climate. Genetic engineering and modification of organisms could play an increasing role in tackling these challenges.

Many natural SGEs, for example homing endonucleases or chromosome drive systems, are interesting because they have the capacity to increase in frequency rapidly, and so spread through populations naturally (Lindholm et al., 2016). Synthetic drive systems could be modelled on these to spread traits that make pest populations vulnerable or innocuous, or reduce their population sizes. One could imagine releasing a driving chromosome into a vector population to create male biased sex-ratios and population collapse. Recent advances in genetic engineering, for example CRISPR-Cas, are making genetic modification in a range of non-model organisms an increasingly achievable prospect.

We have already seen artificial drive systems being engineered in target organisms (Hammond et al., 2016), making the era of modified wild populations of organisms a reality in the next decade. The transplanting of genetic systems between organisms, like moving endosymbionts from one species to another, is another area of current interest. For example, the introduction of a foreign *Wolbachia* into the yellow fever vectoring mosquito *Aedes aegypti*, to reduce disease spread, is currently ongoing (Hoffmann et al., 2011, Jeffery et al., 2009).

Understanding core questions of how drive works is especially pertinent in the light of these advances. For example, many sperm-killing drive systems in nature remain restricted to particular populations (Jaenike, 2001, Lindholm et al., 2016). What forces are stopping these systems from spreading? SGE systems can drive the rapid evolution of resistance (Bastide et al., 2011). However, how commonly suppression evolves, how much of the genome it affects, and whether artificial drive systems will be equally vulnerable to suppression remain open questions. There is also evidence that SGEs are linked to population traits like mating behaviour, and may be able to influence

population structure, and even speciation. Understanding these processes is important both for predicting the success of engineered genetic systems and for recognising potential impacts on target populations. In this thesis I approach some of these questions using the X-chromosome meiotic drive systems in *Drosophila subobscura.*

2.3 - X-chromosome meiotic drive and the *Drosophila subobscura* system

This thesis focuses on a particular type of selfish genetic element known as sperm-killing X-chromosome meiotic drive (XCMD). In this system, X chromosomes act during gametogenesis in males to kill or disable Y-chromosome bearing sperm, thus gaining a transmission advantage by being passed up to 100% of a male's offspring (Figure 2.2A; Jaenike, 2001). As XCMD is sex-linked, this bias in transmission has the added effect of creating female biased sex-ratios (Jaenike, 2001). These systems are found in a number of insects, and autosome drive systems have been found in fungi and mammals. However, despite being known for almost 100 years (Gershenson, 1928), the number of systems that have been found to date are relatively few and are predominantly described in model organisms or their close relatives (See Lindholm et al., 2016 for recent review), although there are likely to be many more currently unknown to science. Nonetheless, sperm-killing XCMD can have a considerable impact of the evolution and ecology of a species, potentially impacting on mating system evolution (Price and Wedell, 2008), rapid evolution of suppression (Bastide et al., 2013, Bastide et al., 2011), population extinctions (Hamilton, 1967, Price et al., 2010, Dyer, 2012) and speciation (Phadnis and Orr, 2009).

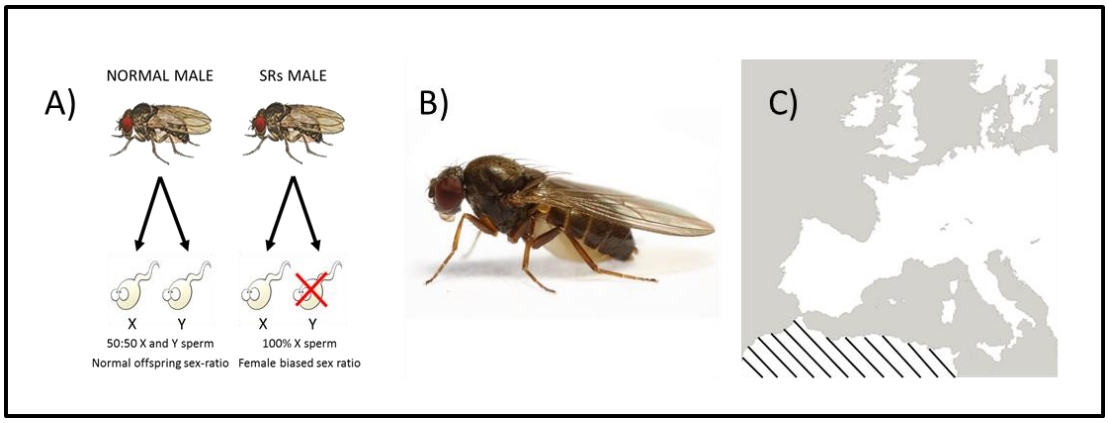

Figure 2.2 A) A schematic showing how males that carry SR*s* kill the Y-chromosome sperm that is carried by a male. B) A photograph of a *D. subobscura* adult female (photo credit D. Obbard). C) A map showing the widespread distribution of *D. subobscura* in white, and the restricted range of SR*s* shown with black diagonal lines.

In this thesis, I examine sperm-killing XCMD in *Drosophila subobscura,* a fruit fly native to forests in North Africa, Europe and Asia, that has colonised much of the world over the past 40 years (Figure 2.2B and 2.2C; Krimbas, 1993, Prevosti et al., 1989). The sperm killing XCMD in *D. subobscura* was first discovered by Jungen (1967) in Tabarka, Tunisia. It is associated with a complex inversion arrangement on the X-chromosome, as is the case with many drive systems (Jungen, 1967, Jungen, 1968, Jaenike, 2001). SR*s* is a strong driver, producing 85-100% female offspring (Hauschteckjungen, 1990). However, the sex-ratio distorting phenotype was only confirmed from Tunisia where it was first recorded (Jungen, 1968), despite the associated chromosomal arrangement being reported from Morocco in the 1970s, and more recently in Spain (Sole et al., 2002, Prevosti et al., 1984; data chapter 2).

To clarify nomenclature, I will refer to this specific driver throughout as SR*s* (Sex-Ratio because of the female biased broods it creates, and *s* to refer to the species *subobscura*). The chromosomal inversion set associated with it is named $A_{2+3+5+7}$, which has also been used as a name for the driver (Jungen, 1967), but this name is unwieldy, I choose to use SR*s* instead. While this system was discovered over 50 years ago, it has received relatively little research attention in comparison to the well-studied sperm-killing meiotic systems in *D. melanogaster*, *D. pseudoobscura*, *D. simulans*, and *Mus musculus*. My thesis, therefore, is the first study of this system for over 20 years. I aim to re-establish basic understanding of the meiotic drive system and further our scientific knowledge of the system and other systems like it. I first examine mating behaviour in relations to SR*s*. I then test for suppression and incompatibilities caused by SR*s* across parts of the species range. I also aim to further our understanding of the distribution and history of the system. Finally I highlight aspects of this thesis that I think are promising future research questions in the conclusions section.

2.4 - Overview of the thesis chapters

This thesis is focussed predominantly on the SR*s* system in *D. subobscura,* however, each chapter addresses self-contained questions. Each chapter is presented in manuscript format. Details of any published manuscripts, book chapters, or those currently in review are presented at the beginning of each chapter. Pdf's of published manuscripts or additional material I have collaborated on during my PhD has been appended in pdf format at the end of the thesis. The following are short summaries each chapter and why these questions were deemed important areas to examine at the time. In addition, I have tried to summarise the useful and unique aspects of their outcomes.

Chapter 3 - Dyeing insects for behavioural assays

This is a standalone chapter in the respect that it is not related to SR*s* in *D. subobscura.* This work was conducted as a productive project I could complete while I was still working to isolate the SR*s* system in the laboratory. This short methods paper examines how different methods of marking three species of *Drosophila* might affect their mating behaviour. This was an important precursor to the work carried out in later chapters. Previously, almost all methods papers focussed on *Drosophila* examine *D. melanogaster*. This paper provides the first study to examine the effects of CO2 anaesthesia on mating behaviour in three different species of *Drosophila*.

Chapter 4 – Sex and selfish genetic elements.

This chapter is an encyclopaedia chapter targeted at a general academic audience, as well as providing a baseline article that could be used for undergraduate studies. The two themes examined, *sex* and *selfish genetic elements*, are central to later chapters in this thesis. It also aims to be an interesting and accessible article about the intrinsic link between selfish genetic elements and sex for undergraduates or non-specialists.

Chapter 5 – The ability to gain matings, not sperm competition, reduces the success of males carrying a selfish genetic element in a fly.

This chapter addresses a subject of much recent interest in meiotic drive systems: namely the relationship between female mating behaviour (female choice and polyandry) and sperm-killing meiotic drive. A few systems have found female preference against traits linked to XCMD, for example in stalk-eyed flies (Johns et al., 2005). In other systems however, females seem to have no preference (Price et al., 2012). I found that in *D. subobscura,* females exhibit strong preference in all comparisons between male genotypes, but the direction of preference is heavily dependent on the genetic background that SR*s* is found on and the non-driving X-chromosome it is competed against. This observation suggests that pre-copulatory female preference is important in this species, as would be expected in a species where females only mate once. However, the result suggests that females have not evolved specific avoidance of mating with SR*s* males, despite the costs that mating with an SR*s* male imposes on them. Female remating, which results in sperm competition, has been shown to be particularly costly to XCMD males in a number of systems (Wilkinson and Fry, 2001, Price et al., 2014, Pinzone and Dyer, 2013, Sutter and Lindholm, 2015). I show that in contrast to these systems, female *D. subobscura* mate only once, which means there can be no sperm competition. This makes the *D. subobscura* a unique system to study XCMD under monandry.

Chapter 6 - Incipient reproductive isolation prevents the spread of a meiotic drive element

The focus of this chapter is on the role that XCMD plays in speciation. The idea that XCMD could cause incompatibilities was first suggested in the early 90s (Frank, 1991, Hurst and Pomiankowski, 1991). More recently there is increasing evidence linking meiotic drive and hybrid incompatibilities from a number of systems (See Presgraves, 2010, Johnson, 2010 for review). Two reasons make meiotic drive a good candidate for causing incompatibilities. First, it is expected that strong XCMD will cause other areas of the genome to rapidly evolve mechanisms to suppress the driver (Carvahlo and Vas, 1999, Bastide et al., 2011). Second, the genes involved in the process of XCMD are by definition involved in the killing or disabling of sperm. In this chapter I find strong incompatibilities between SR*s* from Tunisia, and

different genetic backgrounds in Spain, which become more severe with increased introgression onto Spanish backgrounds, despite non-SR*s* flies from Tunisia and Spain being fully reproductively compatible. I also find no evidence for suppression in Spain suggesting the population is naïve to SR*s* and may have only been exposed very recently. This is the first time a driver has been shown to create reproductive incompatibilities between two adjacent populations within the same species, providing compelling evidence that meiotic drive might can cause hybrid incompatibilities in natural populations.

Chapter 7 – The history of SRs in D. subobscura

This chapter uses all three field collections from throughout my PhD to ask questions about the phylogeny of the SR*s* XCDM, and investigates the genetic variation found in SR*s* and non-drive males. It is important to know if all the drive chromosomes collected from across different populations derive from a single common ancestor, or whether drive has evolved multiple times in this system. Investigating the genetic variation of the SR*s* chromosomes could also give an indication of how much they differ from each other, and whether they exchange genetic material with non-drive chromosomes. SR*s* chromosomes from all 3 populations were observed to be very closely related to each other, suggesting a recent evolution or sweep of drive (or this drive genotype) across North Africa and into southern Spain. This chapter also investigates whether SR*s* from Tunisia shows incompatibility with genetic backgrounds from Morocco, which would suggest local adaptation of drive and drive suppression. There was no evidence for incompatibilities between SR*s* from Tunisia and genetic backgrounds from Morocco, suggesting North Africa acts as a continuous population, and that both SR*s* and its suppressors have spread throughout the area.

Chapter 8 – A comparison of the testes proteomes of sperm-killing and non-sperm killing males in two species of fly.

The final chapter is a direct comparison of testes from XCMD males and non-drive males from two species, *D. subobscura* and D. *pseudoobscura* using a broad-spectrum proteomics approach. Identifying genes involved in natural

drive systems is increasingly of relevance for developing artificial drive systems. Recognising patterns between different wild systems may also allow patterns to emerge in terms of the genes most likely to develop the ability to drive. To our knowledge this will be the first time this approach has been used on testes of XCMD males to identify candidate genes of interest that may be involved in drive. A number of genes emerged as being highly differentiated between drive and non-drive males. These genes were, however, not the same for the two species. Notably, some of the genes have previously been implicated in segregation distortion systems in *D. melanogaster*. This works forms an exciting first step towards identifying the genes that cause drive in these two system. From this basis, future targeted knock-out work targeting specific genes and identifying their role in XCMD in these two species.

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3 - Dyeing insects for behavioural assays: the mating behaviour of anaesthetised *Drosophila*

This chapter has been published in the Journal of Visualized Experiments:

Verspoor, Rudi L., Chloe Heys, and Thomas AR Price (2015) Dyeing insects for behavioral assays: the mating behavior of anesthetized *Drosophila*. *Journal of visualized experiments: JoVE* 98.

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3.1 - Abstract

Mating experiments using *Drosophila* have contributed greatly to the understanding of sexual selection and behaviour. Experiments often require simple, easy and cheap methods to distinguish between individuals in a trial. A standard technique for this is CO_2 anaesthesia and then labelling or wing clipping each fly. However, this is invasive and has been shown to affect behaviour. Other techniques have used coloration to identify flies. This article presents a simple and non-invasive method for labelling *Drosophila* that allows them to be individually identified within experiments, using food colouring. This method is demonstrated by using it in trials where two males compete to mate with a female. Dyeing allows quick and easy identification. There was however, some difference in the strength of the colouration across the three species tested. Data is presented showing the dye has a lower impact on mating behaviour than in *Drosophila melanogaster*. Data is presented showing that the impact of CO_2 anaesthesia and intestinal dyeing depends on the species of *Drosophila*, with *D. pseudoobscura* and *D. subobscura* showing no impact, whereas *D. melanogaster* males had reduced mating success. The dye method presented is applicable to a wide range of experimental designs.

Short Abstract:

This protocol describes a simple, cost effective way to individually identify *Drosophila* or other insects. Demonstration data investigating mating success across three species of *Drosophila* show this method is comparable to the use of CO_2 anaesthesia.

3.2 - Introduction

Over the last few decades there has been increasing interest in how sexual selection and competition between males impact on evolution (Parker, 1970, Hosken et al., 2009). Experiments on mating behaviour have played an important role in developing and testing theories of sexual selection (Chapman et al., 1995, Avent et al., 2008). In particular, research using species of the genus *Drosophila*, has contributed greatly to our understanding of sexual selection and behaviour. However, it is important to investigate whether commonly used techniques might artificially bias the results of standard mating experiments (Barron, 2000, Mooers et al., 1999).

Anaesthesia is often used for handling and identification in experiments (Ashburner and Thompson, 1978). For example, flies are commonly collected before mating, or sorted into genotypes or experimental treatments using carbon dioxide (CO_2) anaesthetic. In experiments where two or more individuals need to be distinguished, it is common practice to anaesthetise the flies and clip part of the wing off to identify each individual or treatment group (Barron, 2000, Powell, 1997). It is vital, however, to understand how CO_2 treatment will affect behaviour. The effect of CO_2 anaesthesia has been examined in *Drosophila melanogaster* in which males exposed to CO_2 took significantly longer to mate and overall had lower mating success than non-anaesthetised males or males anaesthetised using exposure to cold (Barron, 2000). This effect was observed both when anaesthesia was applied on the day of the experiment and when flies were given one day to recover. However, this study was limited in only examining trials where a single male was presented to each female. A more realistic scenario is for a female to encounter multiple males (Moore and Moore, 1999, Hollocher et al., 1997), allowing competition between males, which might allow the detection of more subtle losses of male fitness due to anaesthesia. The use of CO_2 anaesthesia has also been found to reduce fecundity and longevity of adult *D. melanogaster* when they are exposed shortly after eclosion, as is common when collecting virgin flies (Perron et al., 1972).

An alternative to CO_2 anaesthesia is to mark flies by feeding them food coloured with dye (Avent et al., 2008, Mooers et al., 1999, Ashburner and Thompson, 1978, Hollocher et al., 1997, Wu et al., 1995, Melcher and Pankratz, 2005). This dye enters the intestines of the fly and is visible through the abdomen, allowing coloured flies to be distinguished from uncoloured flies, or from flies labelled with a different colour. Methods differ in how this can be applied; being added directly to the food (Wu et al., 1995), via dyed supplementary yeast paste (Mooers et al., 1999) or via exposure to a novel dyed food substrate (Melcher and Pankratz, 2005). These marking techniques appear to show no effect on mating performance (Avent et al., 2008, Mooers et al., 1999). However, a paper directly examining the effects of the same food colouring on adult *D. melanogaster* found a strong reduction in lifespan(Kalaw et al., 2002). Previous work has also focussed almost entirely on *D. melanogaster,* both with regard to the effects of CO2 anaesthesia (Barron, 2000, Perron et al., 1972) and food colouring methods (Kalaw et al., 2002). Currently, there is little information on how CO_2 anaesthesia or the use of intestinal colouring affects the mating behaviour of other *Drosophila*.

The following study evaluates the effect of CO_2 anaesthesia on the mating behaviour of three species of *Drosophila (D. melanogaster, D. pseudoobscura and D. subobscura)*. The effect of collecting flies on CO_2 was examined in both single and two male mating trials. The effect of CO_2 has also been found to vary in *D. melanogaster* (Barron, 2000) and so different latency periods between exposure to CO_2 and mating were tested. An alternative marking method to anaesthesia and wing clipping: the use of food dyes to stain the flies is also evaluated.

3.3 - Protocol

1. Protocol 1 – Preparation of fly food with food colouring

1.1 Take a standard *Drosophila* vial with approx 20ml of food in the bottom (Figure 3.1). Use the following recipe for food mix using 1L of boiling water: 10g Agar, 85g dextrose, 60g maize flour, 40g yeast, stir for 5 minutes of simmering, then add 25ml 10% nipagen once the mixture has cooled to 75°C.

1.2 After the food has cooled and solidified add two drops (approx. 0.5-1ml) of blue food colouring (Cost: £0.85) to the top of the food and spread over the whole surface of the vial (Figure 3.1). Use a different colour dye if preferred.

1.3 Leave the food for two days in the fridge so that the dye is absorbed by the top layer of food; this avoids excessive moisture damaging the flies during the maturation period. Add a small piece of tissue paper if excessive moisture is still a problem to blot up extra moisture and then subsequently remove it.

1.4 Transfer flies onto the food either individually or in groups.

Note: Flies will gain intestinal staining within 1 day of being placed on the food. Alternatively, fully mature the flies on the dyed food prior to the experiment (increased mortality during the maturation period was not observed from exposure to food dye).

1.5 Check that the dyed flies can be easily distinguished from the non-dyed flies. If they cannot be distinguished, repeat steps 1.1-1.5 using either a higher concentration of dye, or a different dye.

2 *Protocol 2 - Two male mating trials using food colouring*

2.1 For producing progeny, set up multiple vials containing pairs of female and male flies (small groups of males and females are also suitable, although care should be taken to avoid crowding of larvae). Allow the females to lay eggs and move the flies to new vials every 5-7 days. Store vials at a suitable temperature for the species of interest (22°C for *D. pseudoobscura* and *D. subobscura* and 25°C for *D. melanogaster*).

2.2 Before collecting experimental flies remove all flies from the collecting vials at a set time before collecting males and females to ensure they will be virgins (*D. melanogaster* – 6 hrs at 25°C, *D. pseudoobscura* – 18 hrs at 22°C, and *D. subobscura* – 24 hrs at 22°C). Note: If flies are not virgin this will bias their behaviour in mating trials (Friberg, 2006).

2.3.1 In this experiment, store male individually in standard 75x20mm plastic vials (containing ~ 20ml of food). This avoids the negative impacts on male mating behaviour and fitness seen in some species when males are kept in groups (Lize et al., 2014).

2.3.2 Expose half of the males to the desired treatment (CO_2 anaesthesia in this case). Use a CO_2 mat or tap to expose the flies for the required time. Half of the males in each treatment should then be stored on coloured food until the mating takes place. This will make them visually distinguishable during the mating trials.

2.3.3 For transferring flies use an aspirator (Yeh et al., 2013). It is important to label each vial to identify both the treatment and the colour status of the male. In the case of our example data there were four treatments (Anaesthesia, non-coloured = G-NC, anaesthesia, coloured = G-C, no anaesthesia, non-coloured, NG-NC, and no anaesthesia, coloured, NG-C)

2.4 Transfer newly emerged females into fresh food vials to mature as groups of 10.

2.5 Allow flies to mature to the specified age at which the mating is going to take place (*D. melanogaster* – 3 days, *D. pseudoobscura* – 5 days, *D. subobscura* – 7days (Holman et al., 2008). Store flies at a suitable temperature for the species being studied (e.g. 22°C for *D. pseudoobscura* and *D subobscura* and 25°C for *D. melanogaster*).

2.6 Move females to individual vials (containing ~ 20ml of food) 1 day before the mating trial. This allows them to acclimatise to the mating vial. Label these vials so that vials can be differentiated. Be careful to blind the experiment by using neutral labelling (i.e. 1-150) so it is not possible to guess the identity of the flies in any vial.

Note: The person who places the flies into each vial will have to know the identity of the flies placed in each vial as they will note which treatment was stained. However, the observer who watches the matings should not know their identity. To do this at least two experimenters will be needed, one to set up and one to observe.

2.7 Begin the mating trials between 10-12am, or at a time that coincides with the light coming on in the light/dark cycle the flies are exposed to ("dawn" for the flies). Add two male flies to each mating vial (containing a single female fly) using an aspirator. Ensure that the two males are from different treatments (anaesthesia or control) and that one has intestinal staining to make it possible to differentiate them from each other, and note which male is stained.

2.8 If copulation occurs, record the status of the male that mates (either coloured or non-coloured). If trials last for two hours, assume the female will not mate Note: Two hours is suitable for these species, but other *Drosophila* may need more or less time.

3 *Protocol 3 - Single male mating trials*

3.1 For single male trials, repeat Protocol 2 with two changes:

3.1.1 In step 2.3 do not keep males on coloured food.

3.1.2 In step 2.7, add only a single male to each vial.

3.1.3 In step 2.8 Record the time the fly is added to the vial, the time the mating starts and the time the mating finished should be recorded. From these values mating success, latency, and duration can be calculated.

4. Protocol 4 - Data analysis

4.1 Use suitable statistics package for analysis. If the data are normal and only have two treatments, t-tests or equivalent GLM's could be used. For two male experiments binomial tests or a binomial GLM should be used. These would be available in any basic statistics package. Note: For the example data, all analyses were carried out in R version 3.0.3 (R development team, 2011).

4.2 Check the mating latency and mating duration data for normality, by plotting frequency histograms of latency and duration for each treatment (Crawley, 2005), and using a test for normality such as Shapiro-Wilk. If it is not normal, transform it, or use non-parametric equivalent statistics (Crawley, 2005).

Note: For the example data from the single male experiments log transformation met the requirements of normality and equal variances.

4.3 If the data can be normalised, use t-tests to examine differences between mating latency and duration in the single male mating trials when using two treatments (Dytham, 2010). If multiple treatments are used, try an Analysis Of Variance (ANOVA) (Crawley, 2005). If the data cannot be normalised, try a Mann-Whitney non-parametric test (Dytham, 2010).

4.4 Use binomial tests to test for an effect of either food colouring or CO_2 anaesthesia on the mating success of competing males (Crawley, 2005). If multiple treatments are used, as is the case with the example data a General Linear Model (GLM) with binomial error structure can be used (Dytham, 2010).

4.5 For the two male trials in the example data GLMs with binomial error structures were used. One GLM examined colour as a response variable (coloured = 0 and non-coloured =1) with species, gas status, and gas treatment fitted. One GLM examined CO_2 as a response variable (gassed = 0 and not-gassed =1) with species, colour status, and gas treatment fitted. Model simplification based upon AIC was then performed (Crawley, 2005).

3.4 - Representative Results

Two male mating trials – The effect of CO_2 anaesthesia on mating behaviour

The superior model found to explain the variation in the effect of CO2 anaesthesia contained species as a factor (with *D. pseudoobscura* and *D. subobscura* fused as they showed no differences between each other). In *D. pseudoobscura* and *D. subobscura* there was no significant effect of CO_2 anaesthesia on mating success in two male trials ($Z_{1,589}$ = 0.087, p = 0.931; Table 3.1). For *D. melanogaster*, males exposed to CO_2 anaesthesia had significantly lower mating success ($Z_{1,589}$ = 2.467, p = 0.014). There was also a significant interaction between species and treatment using a chi-squared model difference test ($X^2_{1,589}$=6.83, p=0.009) with a greater effect being found when D. melanogaster were exposed to gas at collection or 1 day before mating trials (table 3.1). This effect was not found when *D. melanogaster* males were exposed to CO_2 two days before the experimental trial.

Table 3.1 Results from Two Male Choice Experiments Across All Species and Treatments Examined.

Treatment	Species	No. trials	Prop. of coloured flies successful	Post-hoc Binomial p-value	Proportion of gassed flies successful	Post-hoc Binomial p-value
Collection on CO_2	*D. mel*	73	0.49	1	0.36	0.034
	D. pse	79	0.52	0.822	0.56	0.368
	D. sub	71	0.56	0.342	0.46	0.635
Exposed to CO_2 18 hrs prior	*D. mel*	57	0.49	1	0.33	0.016
	D. pse	65	0.49	1	0.48	0.804
	D. sub	68	0.56	0.396	0.52	0.904
Exposed to CO_2 2days.	*D. mel*	56	0.34	0.022	0.57	0.350
	D. pse	70	0.46	0.550	0.47	0.720
	D. sub	56	0.52	0.8939	0.46	0.689

Two male mating trials – The effect of intestinal colouring on mating behaviour

Model simplification showed no significant effect of food colouring being found for any of the three species (p > 0.1). The proportion of successful mating for colored flies across treatments is shown in table 3.1 along with post-hoc binomial tests. The difference in colouration between flies kept on

coloured and uncoloured food can be seen in Figure 3.1. The intensity of the intestinal food colouring was greater in *D. pseudoobscura* and *D. subobscura* than in *D. melanogaster*.

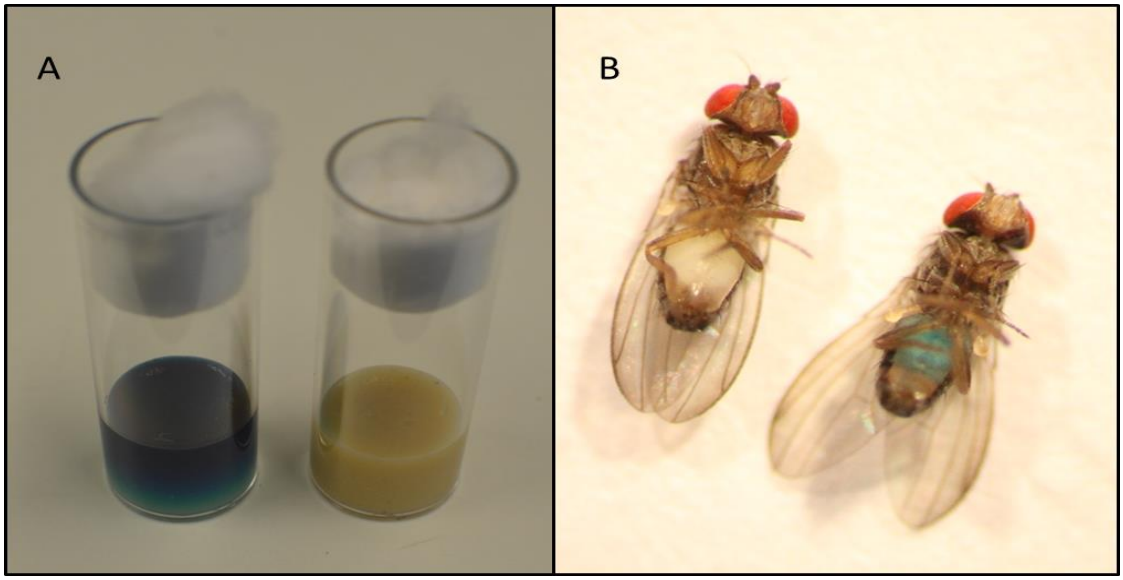

Figure 3.1 Photograph showing vials of coloured and non coloured fly food (A) and the strength of intestinal colouration in male *D. subobscura* (B).

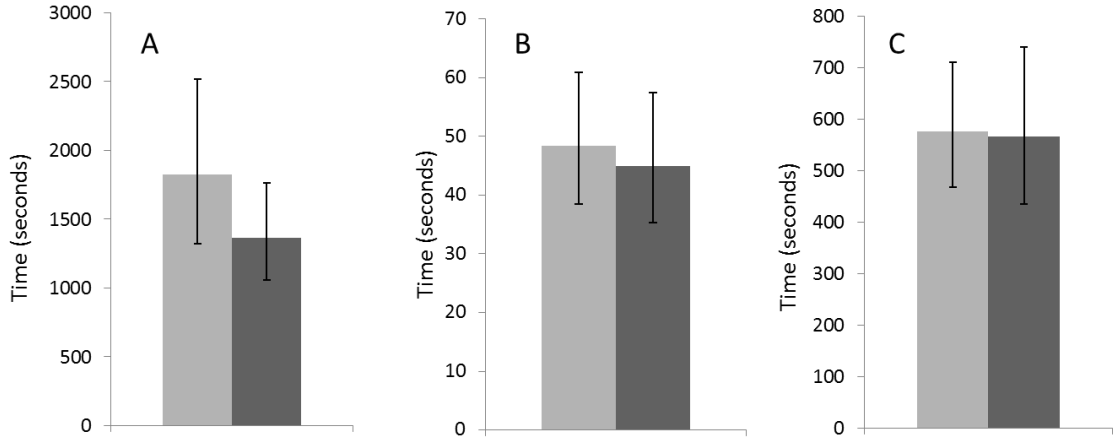

Figure 3.2 The mean and 95% confidence intervals for copulation latency for the three speciesinvestigated in single male trials (A = D. melanogaster, B = D. pseudoobscura, C = D. subobscura), when males were anaesthetised (light bars) or not anaesthetised (dark bars) when collected as virgins before sexual maturity.

Single mating trials – The effect of using CO_2 anaesthesia on mating behaviour

There was no significant difference in mating latency for any of the three species when CO_2 anaesthesia was used to collect recently emerged adults (Figures 3.2 and 3.3; Table 3.2).

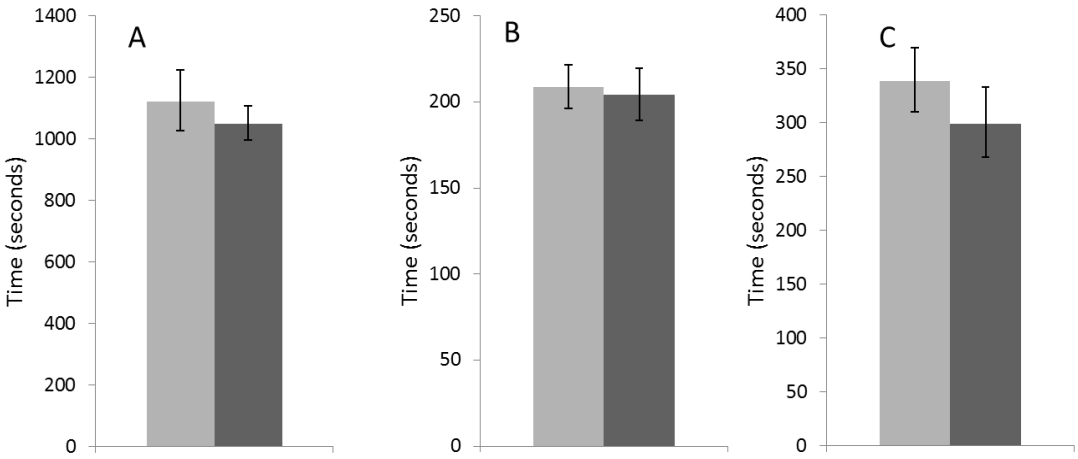

Figure 3.3 The mean and 95% confidence intervals for copulation duration for the three species in single male trials (A = D. melanogaster, B = D. pseudoobscura, C = D. subobscura), when males were anaesthetised (light bars) or not anaesthetised (dark bars) when collected as virgins before sexual maturity.

Table 3.2 Results from single mating experiments examining the effect of collection on CO_2 anaesthesia on the mating latency and duration. Tests were carried out on three *Drosophila* species (*D. melanogaster, D. pseudoobscura and D. subobscura*)

Species	Trait	d.f.	t-value	p-value
D. melanogaster	Latency	58	1.379	0.174
	Duration	58	1.243	0.221
D. pseudoobscura	Latency	109	0.419	0.676
	Duration	109	0.436	0.664
D. subobscura	Latency	83	0.098	0.922
	Duration	83	1.767	0.081

3.5 - Discussion

These data show that the impact of CO_2 anaesthesia is inconsistent between species, with two of three species showing little impact. However, our results suggest labelling with food dye had a lower impact on male mating success than CO_2 anaesthesia for *D. melanogaster*. These experiments demonstrate that food dyes can easily and cheaply be used to label flies for mating assays involving multiple males.

Of the three model *Drosophila* species examined, only *D. melanogaster* showed an effect of CO_2 anaesthesia on mating performance in a competitive situation. In contrast, none of the species showed an effect of collection on gas in single mating trials in terms of mating latency, contrary to previous results for *D. melanogaster* (Barron, 2000). The effect of competition could therefore be highlighting more subtle fitness effects of CO_2 anaesthesia, which are only detectable under situations where there is male-male competition. Exposure at early collection and one day prior to the trial have a negative effect on the ability of males of *D. melanogaster* to gain a mating. Exposure two days prior to the trial however did not show any effect. Both *D. pseudoobscura* and *D. subobscura* did not show any effect of exposure to gas in any of the trials. One explanation is that *D. melanogaster* was vulnerable to early exposure to CO_2 because it must be collected earlier in life (0-6hrs old) than the other species to ensure males are virgin. Hence male *D. melanogaster* of this age may be more sensitive as the cuticle of the fly is still hardening, compared to the other species which have had longer for their cuticle to harden. In general, this supports the idea that the effects of CO_2 anaesthesia are species specific and investigators should appropriately test the effect in their target species. Currently, the majority of work on the effect of CO_2 anaesthesia has been carried out on *Drosophila melanogaster (*Barron, 2000, Perron et al., 1972, Kaiser, 1995*)* and therefore may not be appropriate to apply to other related species.

The alternative non-invasive method presented to differentiate flies is food dye. Results suggest this treatment had no effect in *D. pseudoobscura* and

D. subobscura. However, these species also showed no effect of CO_2 anaesthesia. There was a negative effect of the food dye detected in *D. melanogaster* for one of the three trials. However, overall the results suggest the effect of CO_2 anaesthesia is greater than the effect of dye in *D. melanogaster*. However, while its use was successful in providing a cheap and easily visible marker for distinguishing between individuals it should be noted that the dye was easier to distinguish in *D. pseudoobscura* and *D. subobscura* than in *D. melanogaster.* Previous authors have used several colours (red, green and blue) (Avent et al., 2008, Mooers et al., 1999). We found blue colouring to be the easiest to distinguish in all species, particularly *D. pseudoobscura* and *D. subobscura*. Using several colours would potentially allow more complex experiments with many individually marked flies. However, preliminary tests of different dyes are essential, as some food dyes fail to colour the flies, possibly being digested when consumed. Other dyes can have toxic effects and reduce survival of the flies, and should be avoided (Kalaw et al., 2002). Alternative food colouring methods using more expensive stains have also been used for examining intestinal integrity for *D. melanogaster* (Rera et al., 2011). These may provide an alternative, although more expensive, dying method (Rera et al., 2011).

The dye method is as quick as CO_2 and subsequent marking by wing clipping as flies can be stored on food from collection. Alternately, uptake of the food was rapid (~3hrs) so storage overnight on coloured food would be sufficient to mark flies, as used in other studies[6]. However, the duration of the colouring is relatively short (~5-6 hrs) compared to wing clipping (permanent) or fluorescent dust marking (10-12 days) (Crumpacker, 1974). As *Drosophila* species vary in appearance, different dyes will be more or less effective for different species, and as some strains (e.g. knock-out mutants) can be vulnerable to changes in diet, any use of dye requires a preliminary test of its effectiveness particularly if longer term exposure to dyes can be toxic[14]. In contrast, we found no significant mortality after storage for multiple days on coloured foods for *D. melanogaster* (3 days), *D. pseudoobscura* (5 days), or *D. subobscura* (7 days). This is likely due to the difference in dye used. The critical step for successful use of the dye technique is therefore step 1.5,

validating that the chosen dye works well with the species and strain being used. An alternative used by some studies involves applying coloured dust to the outside of the fly prior to use in field experiments[24]. This method has been used for tracking individuals in the field due to the duration of marking and the ease of mass marking flies (Crumpacker, 1974). Although we have not explicitly tested this method in mating trials, it would be important to examine any effects that dust could have on the senses important in mating, particularly in *Drosophila* (Jallon, 1984, Liimatainen and Jallon, 2007). In species, however, where intestinal dying is not possible, these methods could be suitable.

In conclusion we found that in two of the three species tested (*D. pseudoobscura and D. subobscura*) there was no effect found of either CO_2 anaesthesia or food colouring on mating ability of males. For *D. melanogaster* a negative effect of CO_2 anaesthesia and food colouring was detected. Overall, the dye method provides a simple and cheap non-invasive method for identifying individual *Drosophila* that is equivalent to using methods that require CO_2 anaesthesia. It is likely this method would work across a wide range of species.

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4 – Sex and Selfish Genetic Elements

The following is a peer-reviewed book chapter written for the encyclopaedia of evolutionary biology, produced by Elsevier, written by myself and my supervisor Dr Thomas Price.

The chapter was published within the Encyclopaedia of Evolutionary Biology in May 2016

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4.1 - Abstract

Selfish genes that distort the rules of ‘fair’ Mendelian inheritance are intrinsically linked with sex. How sex occurs in a species determines the arena in which selfish genes compete to bias transmission in their favour. In turn, selfish genetic elements can drive rapid and fundamental changes in how sex occurs. These bidirectional impacts on evolution and ecology can affect both the immediate and long term biology of a species. The continuing expansion of genomic data is certain to reveal many more selfish genetic elements, and discover new ways in which they manipulate reproduction.

4.2 – Introduction

Sexual reproduction is extremely widespread across eukaryotes. This process, by which genetic material is inherited from generation to generation, involves the production of haploid gametes that subsequently fuse into (predominantly) diploid offspring (Figure 4.1). This fusion of genes from two parents, alongside recombination that allows genetic exchange between chromosomes, provides the offspring with a novel and diverse genetic assemblage. Within this broad definition of sex, a huge degree of complexity and variety exists, with important implications for biology. For example, many organisms are split into male and female sexes that invest in either many low-cost gametes or fewer expensive gametes respectively (anisogamy), as is the case with sperm and eggs in mammals. Mating systems, which describe how individuals interact with one another sexually, also show a remarkable variety in nature (Emlen and Oring, 1977, Goodwillie et al.,

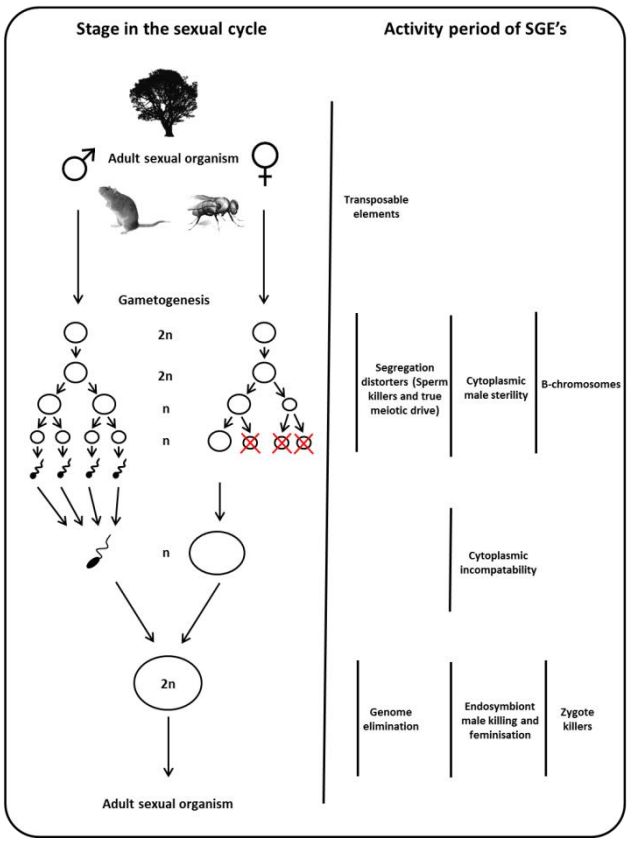

Figure 4.1 A schematic of the stages of sexual reproduction and the different points at which selfish genetics elements can be active.

2005). For example, two closely related plant species can have completely different mating systems, where one can self-fertilise while the other requires gametes from two different parents. Mating system diversity ranges far beyond this, including individuals that change sex depending on age or the presence of rivals, parthenogenetic species that require sperm from males of other species to reproduce, and unusual genetic systems such as haplodiploidy (Figure 4.2). How this diversity has come about, and the impacts it has, is a major focus of biological research. Increasingly, the causes and consequences of mating systems are being found to be intimately related to the existence and proliferation of selfish genetic elements (SGEs) - rogue genes that disobey Mendel's laws of inheritance.

From the process of sex, Mendel established our early understanding of genetic inheritance. He suggested that gametes are generated and recombined in a random manner, resulting in an equal chance for any part of the parental genome to reach the next generation. This `fair' Mendelian ratio of segregation during the production of gametes ensures that the interests of genes are aligned- and all will benefit when the collective (diploid organism) is more successful. However, there has been a growing realisation that this cooperative view of the genome is not the whole story. Increasing numbers of cases have been found where genes act selfishly within the genome and it is now recognised that the living world contains a wide range of SGEs, ubiquitous across the tree of life (Burt and Trivers, 2006). The character that unifies these diverse elements is that they all increase their own frequency in subsequent generations to a degree greater than that expected by Mendelian inheritance, without increasing the fitness of the organism that carries them. The methods for achieving this, however, vary widely between different SGEs: from transposable elements that can replicate and proliferate within the genome of a cell to killer chromosomes that sabotage the production of gametes that do not carry a copy of themselves (Burt and Trivers, 2006; Figure 4.1). In their most extreme form SGEs can result in 50% of the genome being lost. One of the best examples of this is the Paternal Sex Ratio (PSR) system in the wasp genus *Nasonia*. PSR is an extremely small extra chromosome that is not essential for the survival of males and does not

appear to increase the fitness of males in any way. Such supernumerary chromosomes are widespread in nature, and referred to as “B chromosomes”. However, what is (so far) unique about PSR is that it is transmitted only through males, making daughters a dead end for PSR. It has therefore evolved an extraordinarily damaging method of ensuring it is passed on only to sons. In *Nasonia*, males are haploid, carrying a single copy of each chromosome, while females are diploid, inheriting one copy of each chromosome from each parent. PSR eliminates all paternally derived chromosomes following the fusion of gametes, which means that individuals carrying the B-chromosome always develop into males. This ensures the continued transmission of the PSR B-chromosome, but reduces the fitness of all the other genes carried by the male to zero.

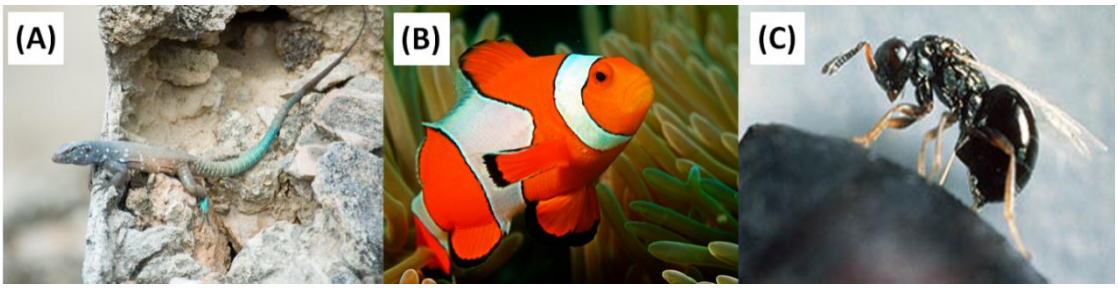

Figure 4.2 Examples of different mechanisms of having sex. (A) Whiptail lizards can have species with two sexes that reproduce sexually, or have a single sex and reproduce parthenogenetically. (B) Clown fish have males that can turn female when the dominant female dies. (C) *Nasonia,* an example of a wasp species that has haplodiploid sex determination. It is this wasp genus that harbours the PSR system where the paternal genome is eliminated.

Across these diverse examples, it is the transmission advantage gained to the next generation that is central in defining SGEs. Sex, and all the diversity and variation associated with it, is responsible for how genetic material reaches the next generation. Therefore it follows that SGEs and sex are inextricably linked. This chapter explores four case studies of how sex and SGEs interact. Using these, we hope to highlight the diversity of interactions that occur across a range of organisms. The timeframe in which these processes occur can be extremely wide and the interactions between SGEs and sex can be bidirectional (Figure 4.3). These emerging fields of research offer many unresolved questions and there remains exciting scope for future

discoveries. More broadly, the interactions of SGEs and sex have the potential to inform a wide range of subject areas in evolution and ecology: from behaviour and population ecology to genome architecture, speciation and extinction.

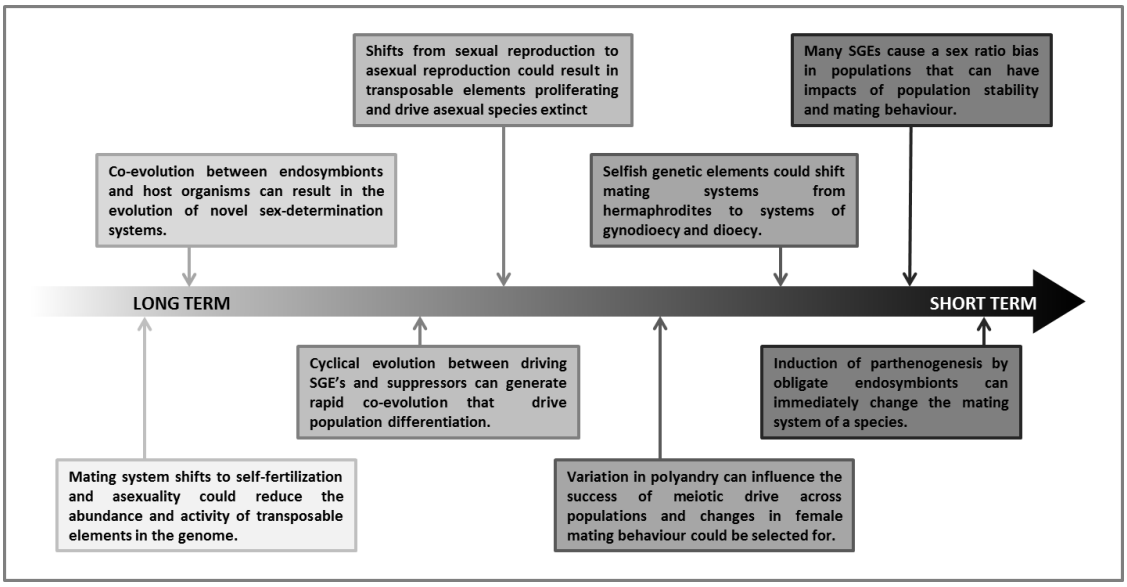

Figure 4.3 A timeline showing examples of the different interactions and impacts that selfish genetic elements and aspects of sex can have on each other.

4.3 - Short term impacts of mating behaviour on SGEs : Sperm-killing meiotic drive and polyandry

The interaction between female remating behaviour (known as “polyandry”) and sperm-killing meiotic drive provides a compelling example of how mating system can impact on an SGE. Sperm-killing meiotic drive occurs when one chromosome selfishly increases its own transmission by eliminating sperm that carry the rival chromosome during spermatogenesis. These drivers can be located on sex-chromosomes, which has the added consequence of producing sex-ratio biases in broods (Jaenike, 2001). These systems have evolved repeatedly in a broad range of organisms, with classic examples in mice, mosquitos, *Drosophila* and stalk-eyed flies (Jaenike, 2001, Lindholm et al., 2016), with pollen drive being a parallel system in plants (Taylor and Ingvarsson, 2003). Sperm-killing meiotic drive typically results in the carrier producing only half as many sperm as a standard male. Males, however, typically produce vastly more sperm than females produce eggs. As males generally produce such huge numbers of sperm, losing half to the action of a selfish meiotic-drive element may not result in a significant fertility cost when females mate once. This is because a male will typically still be able to transfer sufficient sperm to fertilise all the females’ eggs. However, in an estimated 90% of internally fertilising animal species, females mate with more than one male, allowing sperm from multiple males to compete within females to fertilise her ova (Taylor et al., 2014). In general, males that transfer more sperm to females are more successful in this competition between sperm, and father more offspring (Simmons, 2001). Therefore, if a male loses half of his sperm due to carrying a meiotic drive element, his fitness could be severely impaired if females remate frequently (Haig and Bergstrom, 1995).

Wu (1983) was the first to examine sperm competition in the *sex-ratio* (“*SR*”) drive system in the fruit fly *Drosophila pseudoobscura.* It was shown that males carrying *SR* transfer half the number of sperm to female than standard males (Price et al., 2014), and as a result father far fewer offspring than standard males when females mate with multiple males (Wu, 1983b, Wu, 1983a). Subsequently, this pattern of meiotic drive males being poor sperm

competitors has been found in other *Drosophila* (Angelard et al., 2008), other fly genera (Wilkinson and Fry, 2001) (Wilkinson & Fry 2001), and mice (Sutter and Lindholm, 2015). Experimental work has also shown that when female remating is artificially suppressed then meiotic drive elements can spread rapidly through laboratory populations (Price et al., 2010). A meiotic drive element at high frequency can also result in biased population sex ratios in nature (Bryant et al., 1982). These changes in population sex ratio could alter per capita birth rate, affecting a population's ability to compete with neighbouring populations or other species within the community (Unckless and Clark, 2014). Alternately, extreme sex-ratio biases could destabilize populations, potentially causing population extinctions(Price et al., 2010).

Polyandry is extremely variable in nature, with differences in the level of polyandry between species (Taylor et al., 2014), populations (Pinzone and Dyer, 2013, Price et al., 2014), seasons (Torres-Vila, 2004) and even individual females within a population (Price et al., 2011). Recently, studies in two distantly related species of *Drosophila* have shown that patterns of polyandry and the frequency of meiotic drive are linked in populations across North America. Populations *D. pseudoobscura* and *D. neotestacea* across North America carry sperm killing X-chromosome drivers, but the driver in each species has evolved independently. Recent work has shown that in both species the frequency of meiotic drive in a population could be predicted by the prevalence of polyandry in that population (Pinzone and Dyer, 2013, Price et al., 2014; Figure 4.4). This strongly suggests that high levels of polyandry results in sperm competition that eliminates drive from natural populations. A study of a semi-natural population of house mice in a barn in Switzerland also suggested that polyandry could explain the observed population extinction of the t-haplotype meiotic drive element (Manser et al., 2011). Overall, there is compelling evidence that sperm competition plays a major role in determining the frequency of meiotic drive males in wild populations.

The influence of mating systems on meiotic drive, however, does not necessarily have to be in a single direction. Currently, the reasons polyandry

varies within and between species is poorly understood (Taylor et al., 2014). Are drivers playing a role in the evolution of mating behaviour? A number of SGEs which are costly to females (including meiotic drive), also reduce the sperm competitive ability of males (Price and Wedell, 2008). As a result, increased polyandry may allow females to reduce the costs of mating with SGE-carrying males. In populations of *D. pseudoobscura* kept in the laboratory, females rapidly evolved increased polyandry in populations where they were at risk of mating with meiotic drive bearing males (Price et al., 2008). The question remains, however, can polyandry evolve as a response to the presence of an SGE in nature?

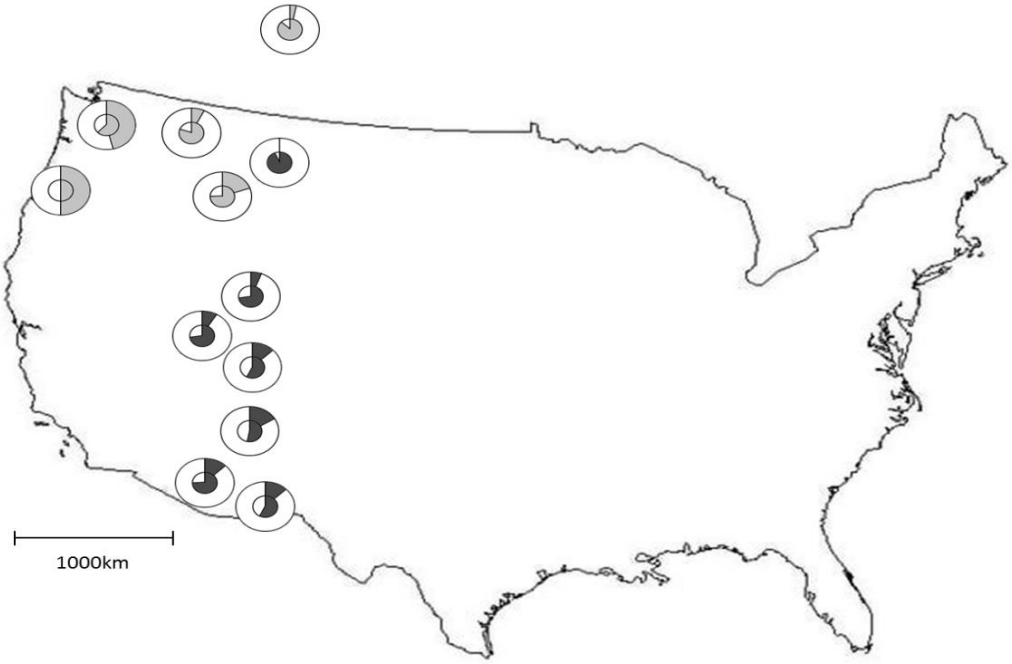

Figure 4.4 The distribution of X chromosome meiotic drive (outer circles) and polyandry (inner circles) in populations of two species of fruit fly, *Drosophila neotestacea* (gray circles) and *Drosophila pseudoobscura* (black circles), across North America. In both species, the frequency of meiotic drive decreases to the north, and the frequency of polyandry increases in parallel, suggesting that higher polyandry may reduce the success of the driving X (Pinzone and Dyer, 2013, Price et al., 2014).

More broadly, the interaction between polyandry and meiotic drive could play a role in population stability and extinction. In the laboratory, sex-linked meiotic drive is observed to rapidly drive populations extinct through the extreme sex-ratio bias it creates (Price et al., 2010), if females are forced to

mate only once. The likelihood of observing localized population extinction events caused by sex-linked meiotic drive in the wild is probably low. Despite this, there is some evidence of a sex-linked meiotic driver causing a population collapse in *D. neotestacea*, in a population with little polyandry (Pinzone and Dyer, 2013). If novel sex chromosome meiotic drivers regularly evolve then there is a risk that these could spread to a high enough frequency to drive the species extinct (Carvahlo and Vas, 1999). Recently, an X-chromosome drive system in *D. simulans* originating in south east Africa has spread across the continent and into Europe and Asia, rapidly creating female biased population sex ratios (Bastide et al., 2011). In this case, within a few years a genetic resistance allele that prevents the killing of Y-bearing sperm also spread from the same origin, returning sex ratios to approximately equal numbers of males and females throughout Africa (Bastide et al., 2013). In polyandrous species, pre-existing genetic variation in predisposition to polyandry (Price et al., 2011), might allow species to rapidly evolve higher levels of polyandry in the presence of costly sex ratio distorting drive. However, in species where females remate extremely rarely or not at all (monandry), there may be little or no ability to evolve increased polyandry, potentially increasing their vulnerability to extinction by sex ratio drive. Hence the prevalence of polyandry as a mating system might not simply be due to increased fitness of polyandrous females, but might also be influenced by selection at the population or species level (Price et al., 2010, Unckless and Clark, 2014).Are monandrous species more likely to go extinct than polyandrous ones, if novel sex chromosome drivers regularly evolve and spread through the species unchecked by polyandry?

Summary:

Males carrying sperm killing meiotic drivers typically have low success when females remate, because they produce fewer sperm than standard males. As a result, in populations where females mate with many males, meiotic drive is rare or absent, showing that mating system can determine the prevalence of an SGE. Monandrous species might be particularly vulnerable to extinction caused by the spread of a sex chromosome meiotic driver, potentially creating species level selection for polyandry. However, the SGE can also

impact on the evolution of mating systems- meiotic drive can cause the evolution of increased polyandry, because polyandrous females avoid many of the costs of mating with drive-bearing males.

4.4 - Long term impacts of mating systems on SGEs: Transposable elements, sex and mating systems

Transposable elements (TEs) have been described as the most abundant genes in nature (Aziz et al., 2010). They are self-replicating units that can copy themselves into other locations in the genome. As a result, they can proliferate within the genome of an individual, with TE-derived material comprising 10-90% of the genome in various species. The maize genome for example contains approximately 85% TE derived material (Schnable et al., 2009; Figure 4.5), while our own genome is around 50% TEs (Lander et al., 2001). Meanwhile, in bdelloid rotifers many retrotransposon TEs appear to be completely absent from their genomes (Arkhipova and Meselson, 2000; Figure 4.5). Despite this abundance, harboring TEs is usually harmful (Pasyukova et al., 2004), and they are implicated in human diseases, including cancer and hemophilia (Burt and Trivers, 2006, Callinan and Batzer, 2006, Hancks and Kazazian, 2012). Nevertheless, variation generated by TEs can sometimes be adaptive (Kidwell and Lisch, 1997). For example, some TEs have been implicated in telomere repair in *Drosophila* (Biessmann et al., 1992). Given the huge range of TEs that exist, and the importance of their activity, understanding the general forces that influence their abundance and distributions remains a central goal in evolutionary genetics. One factor that can have a large impact on the TE dynamics in a population is the mating system of an organism (Wright and Finnegan, 2001, Charlesworth and Wright, 2001, Arkhipova and Meselson, 2005).

Both the presence of sexual versus asexual reproduction, as well as the mating systems of sexual organisms, vary enormously across eukaryotes. A species may be an obligate sexual (e.g. Humans and *Drosophila*), cyclically sexual and asexual (e.g. yeast and daphnia), or purely asexual (e.g. bdelloid rotifers). These differences in the presence and frequency of sex will impact on TE dynamics (Wright and Finnegan, 2001, Arkhipova and Meselson, 2005, Dolgin and Charlesworth, 2006). In one respect, sex allows the movement of TEs between genetic lineages. In disease, increased transmission rates tend to select for higher virulence, whereas diseases that tend to persist long term

in a single host are selected for lower harm and lower virulence. Similarly, higher transmission between lineages via sex is likely to select for higher rates of transposition by TEs (Charlesworth and Langley, 1986). This suggests that asexual lineages might harbor lower numbers of TEs with lower transposition rates (Charlesworth and Langley, 1986). However, sexual lineages are also predicted to have a greater capacity for removing harmful TEs from the population via purifying selection, while the absence of sex in obligate asexual species could allow the rapid proliferation of TEs (Arkhipova and Meselson, 2005, Dolgin and Charlesworth, 2006). This potential proliferation of harmful TEs in asexual lineages, and inability to remove them via recombination, could lead to such high costs that it results in population extinction.

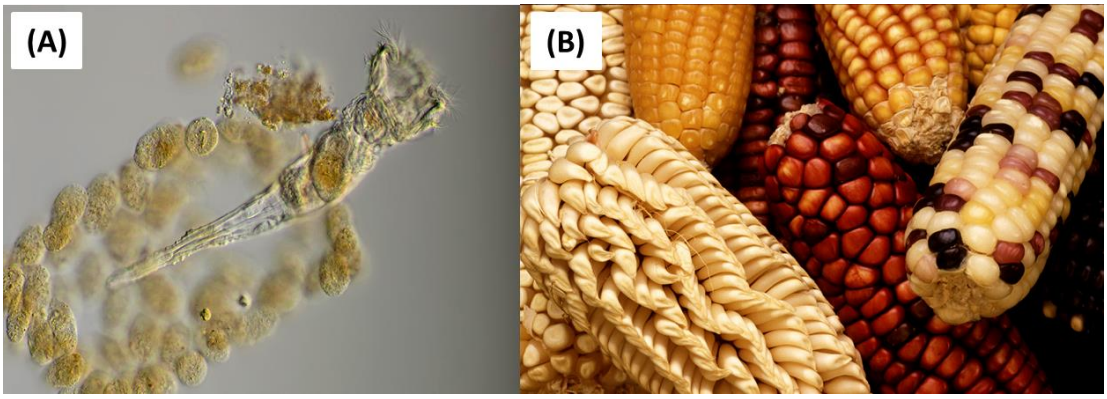

Figure 4.5 (A) A bdelloid rotifer feeding through algae. These anciently asexual organisms harbour extremely low numbers of retrotransposons in their genome. (B) The maize genome is composed of 85% TE derived genetic material. Maize is also the organism where TEs were first characterised by Prof. Barbara McClintock in the 1950s.

In sexual species, how sex occurs is also likely to impact on the success of TEs in the genome. Sexual mating systems vary in how gametes are mixed, with some species requiring gametes of two different parents (obligate out-crossing), while others can combine gametes from the same parent (self-fertilization) (Charlesworth, 2006). Shifts between these systems have evolved repeatedly, and are expected to have important effects on the dynamics of TEs within a species (Charlesworth and Wright, 2001). For example, recessive costs of TE insertions might be more frequently exposed in self-fertilizing species due to increased homozygosity. Hence, these TEs may be purged by selection more effectively from a self-fertilizing population

(Byers and Waller, 1999). The spread of TEs may also be inhibited by a lack of outcrossing, and may rapidly be lost in species with high levels of self-fertilization (Morgan, 2001, Le Rouzic and Capy, 2005, Boutin et al., 2012). On the other hand, increased self-fertilization will have the effect of reducing the effective population size of a group, increasing the effect of genetic drift (the stochastic fluctuation in allelic frequency due to random sampling across generations) (Dolgin et al., 2008, Wright et al., 2008). This reduced effective population size of self-fertilizing populations could also result in selection being less effective at removing TEs (Tenaillon et al., 2010). Following this argument, TE numbers might be more stochastic immediately following the evolution of self-fertilizing, while over longer time periods outbreeding populations will harbor lower numbers of TEs. Therefore, although differences between sexual and asexual species and variation in mating systems within sexual species will influence TE dynamics, there are competing theories about the direction of these effects.

The effects of asexuality on the dynamics of transposable elements have been examined in a number of model organisms. A study that introduced a TE into sexual and asexual lineages of yeast found the spread was faster and more consistent in sexual lineages, supporting the theory that sex facilitates the spread of TEs (Zeyl et al., 1996). Asexual strains of the water flea *Daphnia pulex* also carried lower numbers of TEs than cyclically sexual types (Schaack et al., 2010). In contrast, an exciting study in the wasp *Leptopilina clavipes*, where infection by an endosymbiont *Wolbachia* induces parthenogenesis, found evidence of TE proliferation in the asexual types consistent with the initial spread of TEs following a shift to asexuality (Kraaijeveld et al., 2012). Interestingly, this proliferation was specific to certain families of TEs. Meanwhile, a recent comparative study of the evening primrose *Oenothera* failed to find evidence for a reduction in TE abundance linked to functional asexuality (Agren et al., 2014). Studies focussing on how differences in outbreeding or self-fertilizing affect TE dynamics have also shown mixed results. While some studies show increased TE copy number in outbreeding species (Morgan, 2001, Hu et al., 2011), others have shown either little effect or the opposite results (Dolgin et al., 2008, Tam et al.,

2007). Across three genomes in the genus *Capsella*, the self-fertilizing *C. orientalis* lineage (self-compatible), that developed selfing earlier, did show lower numbers of TEs. Meanwhile the two more recent sister species *C. rubella* (self-compatible) and *C. grandiflora* (outcrossing) showed little difference in TE abundance. This could be a result of *C. orientalis* having been self-compatible being longer (Agren et al., 2014). Equally, this study looks only at copy number, and not transposition rate, making it difficult to differentiate between more efficient selection and differences in transposition rate that have been selected for. Overall, the relative importance of outcrossing facilitating the spread of TEs and reduced efficacy of selection allowing them to accumulate in self-fertilizing lineages remains unclear.

Sex and mating systems clearly play a role in facilitating the spread of TEs in a number of instances, but there remain exceptions. Some of these differences may be explained by the fact that these studies observe only a snapshot of the genome in time, which may be at different stages following a mating system shift. Short term dynamics of TEs may be more stochastic, and the forces governing TE dynamics could change over time (Dolgin and Charlesworth, 2006, Boutin et al., 2012, Agren et al., 2014). Refining the phylogenies, and better determining the evolutionary timings of mating system shifts, as well as increasing the number of study systems, will be vital to gain a broader understanding. Population parameters are also likely to be important in influencing the level of variation and the importance of genetic drift (Dolgin and Charlesworth, 2006, Tenaillon et al., 2010). A number of studies reported differences between classes of transposable elements, making it likely the genomic ecology and behavior of different TEs is a crucial factor. Nonetheless, how TE dynamics interact with mating systems is important to consider when approaching broader questions. What role does TE proliferation and the distribution of mating systems across species play in explaining variation in genome size (Whitney et al., 2010, Agren, 2014)? How do TE dynamics and mating system shifts contribute to differences in gene expression between related species (Hollister et al., 2011)? If mating system shifts consistently change the short or long term genomic burden of TEs in a species, how could this impact on speciation and extinction rates (Oliver and

Greene, 2009, Agren, 2013, Arkhipova and Meselson, 2005)?

Summary:

Despite their deleterious fitness effects, TEs constitute a huge proportion of the genome for many species and their numbers are highly variable between species. Models suggest that mating systems shifts have major impacts on TE dynamics, with a number of models supporting the loss of TEs in asexual or highly selfing species. However, a number of empirical studies have found that self-fertilizing or asexual species harbor lower number of TEs. This interaction between mating system and TE dynamics may impact on genome size, gene expression, mutation rate, and speciation.

4.5 - Long term impacts of SGEs on mating systems: The case of cytoplasmic male sterility

Conflict within the genome fundamentally arises when a genetic element can increase its own transmission without benefitting the rest of the genome. When genes carried by an individual have different patterns of inheritance, this can create conflicts of interest, potentially resulting in selfish behaviour. A classic example of this is uniparental inheritance of organelles. Eukaryote genomes include nuclear genes, arranged on one or more chromosomes contained within the nucleus, and genes contained within other organelles (additional membrane bound cell structures other than the nucleus). Although not all organelles contain DNA, some vitally important ones such as mitochondria and plastids do. These DNA carrying organelles are typically inherited through the maternal line, and almost never from the father, which means the DNA in these organelles are not passed on through male gametes such as sperm or pollen. These organelles therefore gain no fitness by being carried by a male. Instead, these organelles increase their representation in the next generation by maximising the number of daughters they produce. As nuclear genes are passed on through both male and female gametes, nuclear genes have a clear evolutionary interest in producing sons. This imbalance, with sons having high value for nuclear genes, but no value for organelle genes, creates a perfect scenario for intragenomic conflict and the evolution of selfish genes.

Mitochondria are found in all eukaryotes, and are essential for a range of key metabolic processes. In particular, the synthesis of adenosine triphosphate (ATP), the main molecule responsible for intracellular energy transport, is dependent on genes found only in the mitochondria. By interfering with these vital pathways, mitochondrial genes might be able to increase the proportion of offspring produced that are female, or increase the success of daughters, at a cost to the individual's ability to produce sons. In cytoplasmic male sterility (CMS), this is exactly what happens. Cytoplasmic male sterility occurs when a normally hermaphroditic plant has its ability to produce pollen drastically curtailed by the selfish action of the mitochondria it carries. In many cases, CMS eliminates all pollen production in individuals carrying

CMS causing mitochondria. In these species, populations consist of hermaphrodites with non-selfish mitochondria, and females carrying CMS mitochondria, fundamentally altering the mating system in that population.

CMS is found in a very wide variety of plant species. However, the molecular mechanisms by which CMS occurs is poorly understood for most species, with only a few model systems having been well characterised (McCauley and Olson, 2008). The elimination of pollen production occurs in very distinct ways in different species, including CMS strains that convert pollen producing stamens directly into seed producing carpels, thereby clearly increasing seed production and increasing the transmission of the CMS mitochondria (Chase, 2007). However, in some CMS strains stamens are converted to petals, or pollen is produced but degrades as it matures. In these cases it is not really clear how this damage to pollen actually increases seed production or benefits the CMS mitochondria.

The costs of CMS to nuclear genes often drives the evolution of suppressors of CMS in the nuclear genome. As a result, CMS is often cryptic, and only revealed when distantly related individuals crossbreed (Budar et al., 2003). This coevolution of CMS and suppressors can occur rapidly and independently, even in nearby subpopulations. Hence CMS and suppressors can create enormous variation in mating system in different populations and over time (Bailey and Delph, 2007). There is substantial evidence that this conflict may also help produce reproductive incompatibilities between populations, and hence drive speciation (Agren, 2013). Moreover, as suppressors seem to be effective only against one CMS type and populations can harbour several different strains of CMS, there is the potential for cycles of different CMS strains to become locally abundant, and then be suppressed by the increase in frequency of a specific nuclear suppression allele (Taylor et al., 2001). Hence, it is possible that mating systems in many species may be determined by stochastic factors involving which CMS strains and suppressors happen to have been present in the founders of the population (Nilsson & Ågren 2006).

However, CMS is also implicated in longer term changes in mating system. Gynodioecy, a mating system in which females and hermaphrodites coexist, is found in 5-10% of angiosperm plants (Renner and Ricklefs, 1995). In some cases this is likely to be driven by a balance between CMS and suppressors. However, in other cases, gynodioecy appears to be controlled by nuclear genes, with no mitochondrial involvement (Dufay and Billard, 2012). It is likely that CMS drives the initial evolution of gynodioecy in most cases, but then details of the ecology of the population can either select strongly for suppressors and a return to pure hermaphroditism, or can stabilise gynodioecy. The benefits of gynodioecy, accrued by nuclear genes as well as mitochondria, are likely to involve removing the risk of self-fertilisation. CMS has not only driven a change in mating system in many species from all hermaphroditic to gynodioecy, it is also thought to be a major step in the evolutionary transition from hermaphroditic to dioecious (Touzet, 2012). However, it is still not clear how a gynodioecious species would then transition to full dioecy (Spigler and Ashman, 2012). Nevertheless, transitions from hermaphroditism to gynodioecy, and then to dioecy, are tentatively supported by the available phylogenetic data (Spigler and Ashman, 2012).

An enduring mystery of CMS is why mitochondrial elimination of male gametes in hermaphrodites seems so rare in animals. A reduction in sperm should benefit mitochondria in hermaphroditic animals if this results in a reallocation of resources and the production of increased numbers of eggs. Around 5% of animal species are hermaphroditic, so there is ample opportunity for CMS to have evolved in animals (Jarne and Auld, 2006). In contrast to this expectation, hermaphroditic animals are actually far more likely to evolve androdioecy, a mating system in which individuals are either hermaphrodite or male. The transition from hermaphroditism to androdioecy is ten times as common as the transition to gynodioecy (Weeks, 2012), with only nine gynodioecious animal species recorded, including corals, sponges, two worms and a hagfish. The reasons why animal mitochondria seem to be unable to create gynodioecy are unclear. Most reviews suggest that the genomes of animal mitochondria are too small to evolve CMS. However, although plant mitochondrial genomes can be several megabases, compared

to 16 kilobases in most animals (Touzet and Delph, 2009), and have more complex genomes with potential for recombination (McCauley and Olson, 2008), the number of proteins, rRNAs and RNAs produced by the mitochondria are similar between plants and animals (Chase, 2007). Moreover, transmission manipulation by selfish mitochondria has been found to be widespread in natural populations of a roundworm (Clark et al., 2012). The reasons why gynodioecy is so uncommon in animals therefore remain mysterious.

Summary:

The transmission of organelle DNA only through females creates a conflict of interest between organelle and nuclear DNA, resulting in mitochondria that eliminate male gametes. These are very common in plants, and can create a mating system where individuals are either female or hermaphroditic. Rapid evolution, chance, and local ecology can cause this conflict to create differences in mating systems between subpopulations, and changes in the mating system of a single population over time. In the long term, this conflict can play a major role in shifting a species mating system from one where all individuals are hermaphrodites, to one where there are males and females.

4.6 - Short term impacts of SGEs on mating systems: Endosymbiont manipulation

Many organisms carry intracellular endosymbionts, such as *Wolbachia*, *Cardinium*, *Rickettsia* and *Spiroplasma* (Figure 4.6). These are microorganisms that infest cells of the host organism, and are extremely widespread and in some clades can be extremely common. They are typically inherited in the same manner as mitochondria, and have the same conflict of interest with nuclear genes, benefiting from transmission through female gametes, but gaining nothing from sons. As a result, they have evolved a wide variety of mechanisms for increasing their transmission through female gametes (Werren et al., 2008). However, endosymbionts can also be beneficial to the host, with some endosymbionts providing protection for their hosts from attack by parasitoids (Jaenike et al., 2010) or viruses (Hedges et al., 2008). Despite the benefits of endosymbiont infection in some cases, in many or most species endosymbionts reduce the fitness of the rest of the genome. In many cases this occurs because the endosymbiont manipulates the host to ensure it is transmitted through more female gametes. The form of this reproductive manipulation is heavily dependent on the details of the mating system of the species. However, endosymbiont infection can also transform the mating system of the host.

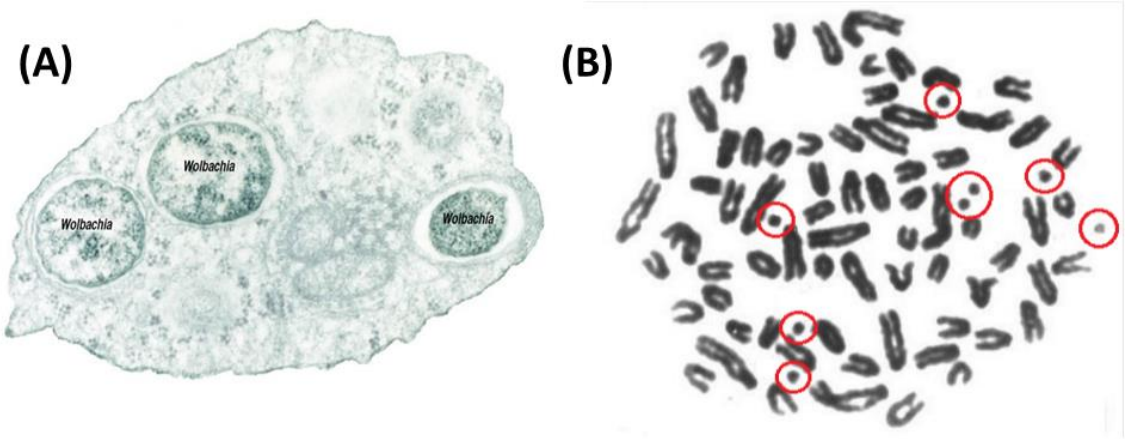

Figure 4.6 (A) An example of a moth cell that contains a number of bacterial cells within it from the obligate endosymbioint Wolbachia. (B) An image showing the karyotype (chromosome compliment) of a Siberian roe deer. Multiple supernumerary B-chromosomes carried by this individual are circled in red.

In many organisms, infection by endosymbionts can feminise the host individual, converting genetic males into functional females. As a result, the endosymbiont is passed on through female gametes in an individual that would usually produce endosymbiont-free sperm. However, similar to the X chromosome meiotic drive case, feminisation is likely to cause a heavily female biased population sex ratio, and nuclear genes will lose fitness by not being expressed in males. Endosymbionts may also kill males in which they occur, if this results in the concentration of resources on their sisters and improves their fitness. In the butterfly *Hypolimnas bolina* in Polynesia, male killing endosymbionts created populations where females outnumbered males 100:1, for decades. Recently a nuclear suppressor of the male killing mechanism spread through Polynesia, rapidly returning sex ratios to a 1:1 ratio (Charlat et al., 2007a, Hornett et al., 2014). Although in continental Asia, males of this species compete with one another for access to females, and females mate with only their preferred males, the male killer completely altered the mating system in Polynesia, with males unable to mate with all the females they encountered and evolving to be extremely choosy, while females evolved to compete with one another for access to males (Charlat et al., 2007b). Hence endosymbionts can rapidly distort sex ratios and modify mating systems, but the rapid evolution of suppression or removal of the endosymbionts may make this transitory, at least in some species.

Summary:

Endosymbionts, parasitic bacteria living in the cells of their hosts, are typically passed on only through female gametes, creating a potential conflict of interest with the nuclear genome. As a result, endosymbionts have evolved a wide range of mechanisms for manipulating reproduction that reduce investment in sons and male gametes, and increase it in daughters and female gametes, and evidence that this can rapidly drive changes in sex ratio and mating system.

4.7 - Conclusions

These four case studies demonstrate that the mating system of the host organism plays a key role in determining the arena in which SGEs operate, and so greatly influences their evolution. Moreover, SGEs can themselves alter the mating system of their host, through mechanisms such as changing sex ratios and sex determinations systems, altering the costs and benefits of multiple mating, and eliminating populations, species or lineages that display mating systems that make them vulnerable to the spread of SGEs.

Beyond those already mentioned in this chapter, there exists an enormous range of known SGEs that manipulate reproduction (Burt and Trivers, 2006). SGEs have the potential to evolve whenever there is a conflict of interest over transmission of the DNA carried by an organism, making it likely that SGEs occur in all sexual organisms, and potentially all life. The vast majority of organisms have never been examined for the presence of SGEs, and many types of SGE are hard to detect, requiring multigenerational examinations of transmission and fitness. Hence it is likely that an enormous array of novel SGEs await discovery. Recent discoveries of selfish mitochondria (Clark et al., 2012), sperm based zygote killers (Seidel et al., 2008) in model nematodes, and a novel form of meiotic drive in human oogenesis (Ottolini et al., 2015) also support the idea that many novel types of SGE have not even been thought of, let alone searched for. As a result, it is likely that the interrelationship of mating system and SGE evolution, action and prevalence, may be even more closely linked than currently known.

4.9 - References

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5 - The ability to gain matings, not sperm competition, reduces the success of males carrying a selfish genetic element in a fly.

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5.1 - Abstract

Females are expected to avoid low quality males fathering their offspring. X-chromosome meiotic drive (XCMD) makes males very low quality mates. XCMDs are X-chromosomes that, in males, cause the failure of all Y chromosome sperm, so all functional sperm carry the driving X and produce daughters. This transmission advantage can allow the XCMD to spread through populations. However, XCMD males typically have low fertility, are very poor at sperm competition, only produce daughters, and bear low fitness alleles associated with XCMD. This imposes significant costs on females that mate with these males. Recently, several studies have shown that females can reduce the risk of their offspring inheriting XCMDs by mating with multiple males (polyandry), as XCMD males typically lose out to normal males in sperm competition. Hence it has been suggested that increased polyandry may be likely to evolve whenever a costly XCMD is common in a population, and that polyandry may be a key factor in preventing XCMDs spreading through populations. We test this by examining the fruit fly *Drosophila subobscura*, in which females are known to mate only once in European populations where XCMDs are absent. However, in North African populations the XCMD, referred to as "SR*s*", occurs. If the association between XCMDs and polyandry is true, then these North African populations should have evolved polyandry. However, we find no evidence of polyandry in North African *D. subobscura* populations. Instead, we find some evidence that males that carry SR*s* are slightly less successful at gaining matings in non-competitive situations. These results show that polyandry does not necessarily evolve in response to the presence of harmful X-chromosome drive. With both sperm competition and female choice both being unlikely to substantially reduce the success of XCMD in *D. subobscura*, the factors that prevent SR*s* spreading through these populations remain mysterious.

Keywords

Female preference, mate-choice, meiotic-drive, monandry, multiple mating, sperm competition

5.2 - Introduction

For females, the fitness benefits of choosing a high quality male mate can directly increase the number of offspring she produces, or can enhance them via genetic benefits (Andersson and Simmons, 2006, Trivers, 1972). In many species, however, males carry traits or genes that make them very poor mates, e.g. sexually transmitted diseases (Hurst et al., 1995) or low fitness genes (Lesna and Sabelis, 1999). Mating with these males may be costly, and there can be selection for females to avoid mating with these males. In many species, females can choose to avoid mating with low quality males (Jennions and Petrie, 1997). Alternatively, females may avoid these costs after mating through selecting to use sperm only from high quality males or dumping ejaculates from low quality males (Birkhead and Pizzari, 2002, Parker, 1970, Simmons, 2001). If high quality males produce ejaculates that outcompete other ejaculates inside the female, then females can increase the average quality of their offspring's sire by simply mating with multiple males (Parker, 1970).

Mating with the wrong male can be costly to females when some males carry harmful selfish genetic elements. Selfish genetic elements (SGEs) are genes that increase their own frequency in subsequent generations beyond fair Mendelian inheritance, without increasing the fitness of the organism that carries them (Burt and Trivers, 2006). These systems are near ubiquitous across the living world, ranging from transposable elements which replicate within an organisms genome, to extreme systems such as the supernumerary chromosome PSR that in male wasps eliminates all other chromosomes carried by the male. As SGEs are often costly to the individual that carries them, it has been proposed that females, across a broad range of taxa, will benefit by reducing the risk that SGE-bearing males will father their offspring (Lande and Wilkinson, 1999, Tregenza and Wedell, 2000).

One particular SGE, X chromosome meiotic drive ("XCMD"), has been found to be intimately related to female mating decisions (Wilkinson et al., 1998, Pinzone and Dyer, 2013, Price et al., 2008b, Taylor et al., 2014, Price et al., 2014). XCMD occurs when a particular X-chromosome carries the ability to

kill Y-chromosome bearing sperm produced by the host male (Jaenike, 2001). This benefits the XCMD chromosome, which is passed on to most or all offspring, resulting in strongly female biased broods. The transmission advantage gained by the driving X can allow XCMD to spread rapidly through populations (Bastide et al., 2011a, Carvahlo and Vas, 1999, Price et al., 2010), and reach substantial frequencies in nature (Jaenike, 2001, Pinzone and Dyer, 2013, Price et al., 2014). However, for a female, mating with an XCMD male typically imposes significant costs. Firstly, XCMD males typically transfer small ejaculates, due to the loss of half their sperm, which may reduce a female's ability to produce offspring (Price and Wedell, 2008). Secondly, drive loci are often associated with large areas of low recombination, such as inversions, which can result in an accumulation of linked deleterious mutations that reduce the carriers fitness (Jaenike, 2001). Thirdly, XCMDs typically cause female biased population sex ratios. As offspring fathered by an XCMD male are all female, their value is lower in female biased populations in almost all circumstances (Bryant et al., 1982, Jaenike, 2001, Fisher, 1930). These costs suggest that females could evolve measures to reduce the likelihood that an XCMD male will father their offspring (Haig and Bergstrom, 1995, Jaenike, 2001). Polyandry and female choice are two mechanisms by which females can avoid the costs of mating to XCMD males (Manser et al., 2015, Cotton et al., 2014, Wilkinson et al., 1998, Wilkinson and Fry, 2001, Wu, 1983, Pinzone and Dyer, 2013, Price et al., 2014, Sutter and Lindholm, 2015)

Polyandry, females mating with multiple males within a single reproductive episode, is a general method by which females can reduce the chance of XCMD males fathering their offspring. Carrier males are expected to be disproportionately poor sperm competitors compared to standard males because drive kills half of their sperm (Haig and Bergstrom, 1995), and laboratory studies support this (Angelard et al., 2008, Price et al., 2008a, Sutter and Lindholm, 2015, Wilkinson and Fry, 2001). Moreover, increased polyandry can evolve in response to the presence of XCMD rapidly in the laboratory (Price et al., 2008b). Recent work in wild populations, in two species of *Drosophila*, has shown negative correlations of polyandry and the

frequency of XCMD (Pinzone and Dyer, 2013, Price et al., 2014). These results mean polyandry has been suggested as a general mechanism that suppresses meiotic drive (Holman et al., 2015, Manser et al., 2011, Pinzone and Dyer, 2013, Price et al., 2014, Price et al., 2010). This proposition, however, is challenged by an XCMD system at apparently stable frequencies in *Drosophila subobscura*, a species in which females are reported as monandrous in their European range (Fisher et al., 2013, Smith, 1956).

Alternatively, there could be differences in a male's ability to gain matings, either through female preference or male competitive ability. Female preference requires a detectable trait to be linked to the XCMD locus (Lande and Wilkinson, 1999), however, as XCMD systems often involve large areas of low recombination, traits important for female choice or for males to compete for females may be linked to drive loci. Female choice against males carrying XCMD has been found in stalk eyed flies in relation to eye stalk length, a trait linked to XCMD (Wilkinson et al., 1998, Cotton et al., 2014, Johns et al., 2005). However, in a well-studied XCMD system in *Drosophila pseudoobscura,* there is no evidence that females are able to identify and avoid XCMD males, despite the system being very old (Price et al., 2012) and being associated with large inversions (Beckenbach, 1996, Sturtevant and Dobzhansky, 1936). This is true in several other systems (Price and Wedell, 2008), although female preference in majority of XCMD systems remains to be investigated (Jaenike, 2001).

Drosophila subobscura, a Palearctic woodland fly that has recently spread worldwide (Krimbas, 1993), harbours an XCMD system in North African populations, henceforth referred to as "SR*s*" (Jungen, 1967a). SR*s* only exists in North Africa at up to 30% frequency (Hauschteckjungen, 1990, Jungen, 1967a). As outlined earlier, mating with an SR*s* male is expected to be costly for multiple reasons. Hence female *D. subobscura* are expected to have evolved mechanisms to reduce the likelihood of SR*s* males fathering their offspring.

However, while most *Drosophila* species are polyandrous (Holman et al., 2008, Simmons, 2001), *D. subobscura* from Europe are monandrous (Smith,

1956, Fisher et al., 2013, Loukas et al., 1981). Monandry is an unusual mating system (Taylor et al., 2014), and is expected to have major impacts on pre- and post-copulatory mate choice mechanisms (Hosken et al., 2009). Firstly, monandrous females cannot use sperm ejection or sperm competition to bias paternity away from XCMD males. Secondly, monandrous females are often expected to be highly choosy in their mates (Hosken et al., 2009). Firstly, because they cannot trade up by remating, but also because when females mate only once, the operational sex ratio is likely to be male biased, giving females an extensive choice of mates. Selection pressure to avoid mating with XCMD males may therefore be greater in a monandrous species. *Drosophila subobscura* is also selective of mates in other circumstances (Immonen et al., 2009, Lize et al., 2014, Verspoor et al., 2015b).

Polyandry cannot, however, be immediately discounted in *D. subobscura* as a mechanism to prevent SR*s* males fathering a females offspring. While *D. subobscura* is reported to be monandrous in Europe (Maynard-Smith 1956; Fisher et al. 2013; but see Loukas et al. 1981), North African populations may be polyandrous as female mating behaviour remains to be tested here. Significant latitudinal clines in polyandry have been observed across species ranges in several other species of *Drosophila* (Pinzone and Dyer, 2013, Price et al., 2014). Further, females in many insect species are more likely to remate when provided with a smaller ejaculate (Charlat et al., 2007b, Kaitala and Wiklund, 1995, Perry and Rowe, 2008). This may be reflected in females mating to SR*s* males being more likely to remate, as the males transfer fewer sperm (Hauschteckjungen et al., 1987).

Three central questions are examined. Firstly, are North African flies monandrous, like they are in European populations? We predict that North African flies will be polyandrous, because increased polyandry can evolve in response to the presence of meiotic drive (Price et al., 2008b), and because stable levels of meiotic drive in natural populations can indicate polyandry is present (Holman et al., 2015, Price et al., 2010). Secondly, we predict that females that mate with SR*s* males will be more likely to remate, because SR*s* males transfer small ejaculates, and because remating will allow the female to "trade-up" (Watson, 1991). Thirdly, we predict that SR*s* males will be less

able to gain matings than standard males, because the SR*s* inversion could have accumulated deleterious alleles (Dyer et al., 2007). These provide the first examination of pre- and post-copulatory mechanisms used by females in relation to a selfish gene in *D. subobscura*.

5.3 - Methods

Fly stocks and maintenance

We collected populations of *D. subobscura* from Tabarka, Tunisia (36.57°N 8.45°E) in April of 2013 using baited traps (see Verspoor et al. 2015 for details). Fifteen isofemale lines were established from wild caught females that had been mated prior to their capture. Over time, sibling mating between the offspring from a single wild female creates a highly inbred homozygous lineage- an "isoline". While flies within an isoline are virtually identical, collectively multiple isolines successfully preserve standing genetic diversity of the population when it was sampled in the field (David et al., 2005b). XCMD lines were maintained indefinitely as self-replicating inbred crosses as outlined in Supplementary figure 5.1. In this way, females can carry the SR*s* chromosome as either homozygous or heterozygous carriers, despite not exhibiting any sex-ratio distorting phenotype. An outbred population was generated from these isolines before the experiments (numbers of isolines: 15) to recreate some of the genetic diversity representative of the wild populations. Outbred populations were maintained as discrete generations every five weeks to produce all the females used in experiments below. Flies were kept as populations for a maximum of three generations to minimize the possibility of adaptation to the laboratory environment. All populations and isolines were stored in standard *Drosophila* vials on a medium of agar, sugar, maize and yeast, and kept at 18˚C on a 12 hourly day/night day cycle (lights on at 10 a.m. GMT). All flies used were 7-day-old virgins, as sexual maturity has been reported by this age for female *D. subobscura* (Holman et al., 2008). After collection, all adult flies were stored at 22˚C with an identical day/night cycle to that of the stock populations. All experiments were carried out at 22˚C (reported to be close to the optimum temperature for this species) (Krimbas, 1993). For all experiments mating vials were allocated random numbers to avoid any recorder bias. There were always two people present at any given mating, one adding the flies to the vials, with the second blind to the flies' identities who recorded the timings of the copulations.

Experiment 1 – Remating suppression of Tunisian females

The aim of experiment 1 was to measure the general remating rates of females from North Africa, to compare them with rates reported for European *D. subobscura* (Fisher et al., 2013). In order to make the remating rate measure comparable to that used by Fisher et al., (2013), their methods were replicated. In the general treatment females (at age 7-days) were mated to an equally aged male and the mating was observed to ensure it was successful. These males were then discarded and females were allowed to lay eggs for a period of 7days. Larval action was examined to check whether the first mating was fertile. This is important as females have been observed to remate if the first mating is not fertile (Fisher et al., 2013). After 7 days the female was moved to a new food vial and offered a new 7-day old male to mate with in a window of 2 hours. All flies used originated from the outbred population outlined above (see *Fly stocks and maintenance)*. In addition to examining general remating rates of the North African population, a second treatment looked at the willingness of a female to remate after mating first to an SR*s* male. This was carried out exactly as above, the only difference being that the first male a female mated to was an SR*s* male. SR*s* males were generated from the crosses outlines in Supplementary figure 5.1a.

Experiment 2 – Remating suppression with continuous remating opportunities

The aim of experiment 2 is to examine whether SR*s* males differ from non-driving males (from now on referred to as "ST*s")* in their ability to suppress female remating. In addition, compared to experiment 1 that examined remating in a fixed window experiment, the following experiment permitted remating opportunities with a 2nd male throughout a female's lifespan, and thus is a less restrictive in when the female can remate. For this experiment all females used were from the outbred Tunisian population outlined in *fly stocks and maintenance*. SR*s* and ST*s* males came from separate parental crosses outlined in Supplementary figure 5.1a. Each female was first mated to either an SR*s* male or an ST*s* male, when all flies were 7 days old. Half the females were then kept isolated to oviposit for four weeks. The other females had the males removed from their vials after mating, and were given 7 days

to lay eggs. This was used to check that the first mating had been successful and fertile, and to check the expected offspring sex-ratio that results from the genotype of the male. After this 7 day period, the female was moved to fresh vial with a male of the opposite genotype for the rest of the duration of the experiment (3 weeks). The sex ratio of the offspring produced each week was checked, with any remating detected by a deviation from the first week's sex ratio. Hence this experiment gave females constant exposure to a second male for three weeks.

Experiment 3 – Single male mating performance

Experiment 3 aimed to evaluate the mating performance of XCMD males compared to males that do not carry the driver. Two experiments were carried out. Firstly, the mating performance of SR*s* males was compared to that of a pool of ST*s* male genotypes. This mix of genotypes was produced by crossing general population males (see Supplementary figure 5.1a) to either homozygote SR*s* females or to random population females which would only carry ST*s* X-chromosomes. These generated F1 males that carried either the SR*s* chromosome or a range of ST*s* X-chromosomes with a range of population backgrounds.

However, in this experiment we could not be certain that any differences in mating performance were due entirely to the SR*s* chromosome, as males may have differed in many chromosomes, and were also reared in different vials. So we ran a second experiment in which the SR*s* chromosome was introgressed onto a single ST*s* isoline. The SR*s* line was crossed into the isoline, which were then backcrossed to that isoline for a minimum of 5 generations, resulting in a line that carried the SR*s* X-chromosome but whose autosomes and Y chromosome originated from the ST*s* isoline. Three randomly chosen ST*s* isolines were used for these introgressions of SR*s*. We then crossed these introgressed lines back to the ST*s* isoline to produce females heterozygous for SR*s*/ST*s* (Supplementary figure 5.1a). These females, when mated to a random ST*s* male, produce broods where half their sons carry ST*s* and half carry SR*s*, but whose autosomes and Y

chromosomes are completely shared (Supplementary figure 5.1c). The sons from this cross are then effectively genetically identical and full siblings, differing only in their X-chromosome. An additional benefit of this setup also means is that they are reared in the same vial, preventing bias due to rearing in different vials. SR*s* and ST*s* siblings were then compared for their mating performance, with the genotype of the male (SR*s* or ST*s*) was assigned post experiment by measuring the sex ratio of their offspring. Observer bias during mating is impossible because male genotype was only determined after the experiment has been run.

For both experiments, the measures of mating performance used were copulation latency and duration. Mating latency, which is the time to achieve copulation, is a standard measure of pre-copulatory choice in female *Drosophila* (Avent et al., 2008, Prathibha et al., 2011, Somashekar and Krishna, 2011). Preferred males are expected to require a shorter time to gain a mating, and low mating latencies tend to correlate with other measures of attractiveness in *Drosophila*. Copulation duration, which is the amount of time a male spends copulating, can be considered an estimate of a male *Drosophila*'s investment in the mating (Bretman et al., 2011, Price et al., 2012). For example, in *D. pseudoobscura*, copulation duration does directly correlate with the number of sperm transferred for some male genotypes (Price et al., 2008a). However, in *D. melanogaster*, copulation duration does not directly correlate with the number of sperm transferred, which occurs in the first minutes of copulation, but does correlate with the transfer of other ejaculate components that suppress female remating. For several other *Drosophila* species, copulation duration also correlates with female offspring production (Avent et al., 2008, Singh and Singh, 2014) , or reduces female willingness to remate (Mazzi et al., 2009), suggesting that the transfer of ejaculate proteins is higher during longer copulations across a broad range of *Drosophila*. Following the 7-day maturation period, a single male was added to a vial containing a virgin female and given a 2 hour window to mate. Males were then removed, and the female were allowed to lay eggs for a period of 7 days. Females were then removed and the larvae were left to develop into

adults. These offspring, when they emerged as adults, were then counted and sexed.

Experiment 4 –two-male competitive mating trial

Experiment 4 aimed to independently retest the mate preference patterns found in *Experiment 3.* A single male mating trial and a two-male choice trial were repeated for a single genetic background. Two-male trials have been suggested to closer resemble nature, where males can actively compete against each other for a female (Moore & Moore, 1999). For these experiments SR*s* and ST*s* males were generated from separate parental crosses (SR*s* males generated from homozygote SR*s* female crossed to ST*s* males while ST*s* males came from homozygote ST*s* females crossed to ST*s* males; Supplementary figure 5.1a). SR*s* had been introgressed into the standard background for nine generations to homogenise their genetic background. For the single mating experiment the same methods were use as described in Experiment 1, to independently test the pattern found for genetic background A. For the two male mating trials, in order to differentiate males from each other one male from each trial had their wing clipped, under CO_2 anaesthesia, at 2 days old. CO_2 exposure was carried out at this period to minimize the impacts on mating behaviour (Barron, 2000, Verspoor et al., 2015c). This was carried out in a balanced design to avoid bias, with 50% of each genotype of male being clipped. Two males were then added to each vial containing a single female and given a 2 hour window to achieve a mating. The genotype of the successful male was recorded to test whether one genotype was consistently more successful in gaining the mating.

Data Analysis

For Experiment 1 a Generalised Linear Model with a binomial error structure was used to compare the different treatments with the response variable being the number of remating events. Both the total number of remating events, as well as only rematings events where the 1st mating was fertile, are tested. For Experiment 2, in order to test for individual rematings, binomial tests were used on individual flies, comparing the sex-ratio of the offspring produced in week 2 and week 4. In order to correct for multiple testing the

Holm-Bonferroni correction was applied. For Experiment 3 *t*-tests were used to examine differences in mating duration, mating latency, offspring production. For Experiment 4 where males carrying three specific non-driving chromosomes were compared to SR*s* males, two way ANOVAs were used to examine the effects of background and male X-chromosome type on mating duration and latency and *t*-tests were used to examine differences between mating duration and latency for the single male mating data. A binomial GLMM was used with an expected proportion of 0.5 if there was no evidence for female preference; wing clipping was incorporated as a random factor. A binomial test was used to test for an effect of wing-clipping. All analyses were carried out in R, version 3.0.3 (R development team, 2011).

5.4 - Results

Experiment 1 – Remating suppression of Tunisian females by SRs and STs males

North African mated females showed extremely low (0-1%) rates of true polyandry (remating after a fertile first mating), irrespective of whether they were first mated to a random population male without drive or if they were first mated to a SR*s* male, with no significant difference between the treatments ($P > 0.2$, Table 5.1). The rate of pseudomatings (mating after an infertile first mating), and the total rate of all rematings (5-8%) also did not differ between treatments (differences $P > 0.2$, Table 5.1).

Table 5.1 Results from observed remating trials for ST*s* and SR*s* males, using Tunisian females. The first two columns show details of the samples sizes and the number of remating events when females were exposed to two different conditions. The total number of remating events are subsequently split into true remating and pseudoremating. A second mating was considered a pseudoremating if a female did remate but failed to produce any offspring in the 7 days after her first mating, which typically indicates that the first mating failed.

Treatment	Sample (*N*)	Total Remating	True Remating	Pseudo Rematings
ST*s* first	92	8	1	7
SR*s* first	51	3	0	3

Experiment 2 – Remating suppression with continuous remating opportunities

There was very little difference between the sex-ratio on week 1 and week 4 for all the treatments (Table 5.2), which suggests remating was extremely rare. Examining individual pairs between week 1 and week 4 showed that there was strong evidence for remating in three out of a total of 58 (*N*=30 SR-ST, *N*=28 ST-SR) individuals across the four weeks. All of these individuals were females that had first mated to a SR*s* male and were subsequently

mated to a standard male. These three females explained the slight trend towards a lower proportion of female offspring produced by week 4 (92.3%) compared to week 1 (98.6% female) in the SR*s* first mating ST*s* remating treatment. It should be noted that during Week 3, there was a reduction in the sample size of some of the treatments because 17 vials were discarded across the treatments due to an outbreak of fungus killing the offspring in these vials.

Table 5.2 Results from a relaxed window mating experiment comparing SR*s* males and ST*s* males. This uses changes in the sex-ratio over time to infer potential remating events. This table shows the mean proportion of female offspring and confidence intervals for the four female remating treatments across four weeks.

	Percentage of female offspring			
Treatment	Week 1	Week 2	Week 3	Week 4
SR – ST	98.6±0.9 (*N*=35)	97.0±2.5 (*N*=35)	94.8±3.5 (*N*=29)	92.3±4.5 (*N*=30)
SR	98.0±2.5 (*N*=18)	97.7±2.2 (*N*=18)	97.8±2.6 (*N*=12)	98.7±1.77 (*N*=14)
ST – SR	49.8±1.6 (*N*=35)	49.8±1.7 (*N*=35)	52.6±2.1 (*N*=26)	51.7±2.3 (*N*=28)
ST	50.1±3.1 (*N*=17)	53.2±6.4 (*N*=17)	49.3±6.0(*N*=8)	54.0±3.0 (*N*=15)

Experiment 3 – Single male mating performance

Males carrying ST*s*-chromosomes were significantly quicker than drive males to obtain a mating ($t_{1,38}$ = 2.401 P = 0.022; 18SR*s* males and 21 ST*s* males), obtaining a mating on average twenty three minutes earlier (Figure 5.1a). SR*s* males copulated for significantly longer than ST*s* males ($t_{1,38}$ = 2.141 P = 0.039), copulating for, on average, three minutes longer (Figure 5.1b). However, there was no significant difference in the mean number of offspring produced by the two different classes of males ($t_{1,37}$ = 1.181 P = 0.249; Figure 5.1c).

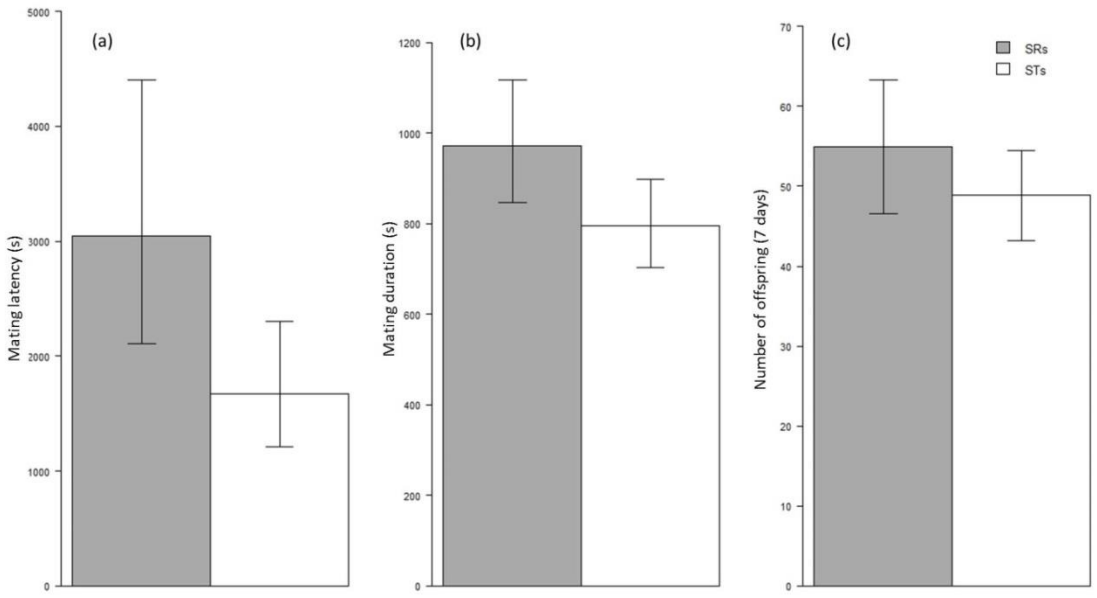

Figure 5.1 Shows the differences in differences in (a) copulation latency (b) copulation duration and (c) offspring production over a 7 day period, between SR*s* and ST*s* males on a range of population backgrounds in single mating trials. Bar plots show the mean and 95% confidence intervals.

For the experiment that introgressed SR*s* into three ST*s* lines, there was a significant interaction between background and X chromosome ($F_{2,216}$ = 5.921, P = 0.003; 222 trials in total : SR(A) :63, ST(A) : 51 SR(B) : 28, ST (B) : 24, SR (C) : 22, ST (C) :34), with SR*s* males being faster to obtain matings than their ST*s* siblings in one genotype, but slower to mate in two others (Figure 5.2a). The three backgrounds also differed significantly in mating latency, when SR*s* and ST*s* are pooled ($F_{2,216}$ = 5.133, P < 0.007; Figure 5.2a). However, beyond the significant interaction between background and X chromosome, there was no evidence of a significant difference in the main effect comparing SR*s* and ST*s* for mating latency ($F_{1,216}$ = 0.001, P = 0.981 Figure 5.2a). For mating duration there was no significant interaction between background and X chromosome (P > 0.2; Figure 5.2b). Both the background ($F_{2,218}$ = 21.03, P < 0.001; Figure 5.2b) and X-chromosome ($F_{1,218}$ = 15.01, P < 0.001; Figure 5.2b) explained a highly significant amount of the variation in mating duration, with SR*s* male mating for longer than non-driving males, and

males from Genotype A mating for longer than those from Genotypes B and C.

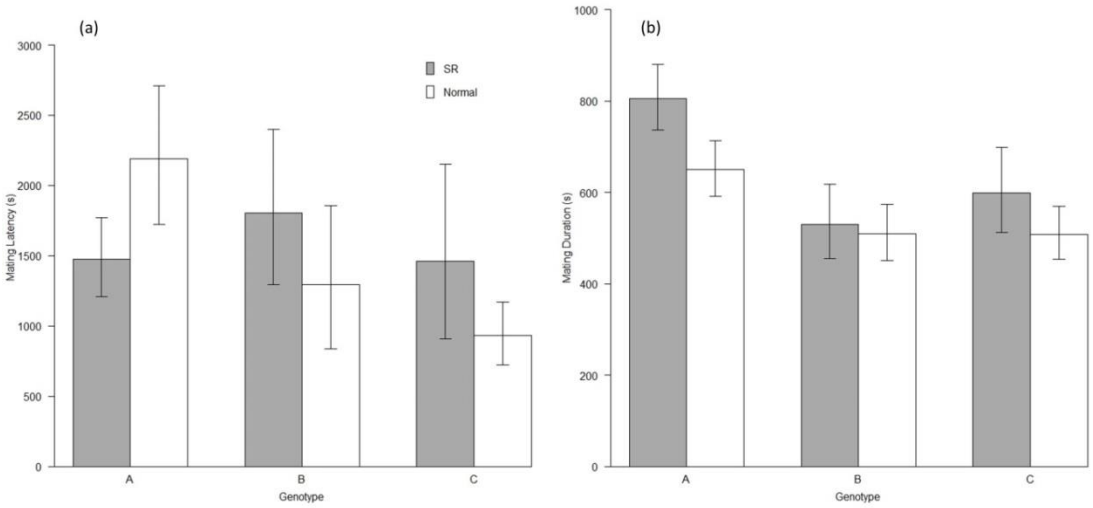

Figure 5.2 Shows the differences between SR*s* and ST*s* males in (a) copulation latency and (b) copulation duration, for three different genetic backgrounds. Different genotypes represent the SR*s* chromosome introgressed onto a different isofemale line collected from Tabarka, Tunisia. Bar plots show the mean and 95% confidence intervals.

Experiment 4 – Two-male competitive mating trials

SR*s* males were significantly more successful at gaining a mating in a competitive environment (*N SRs wins* = 49, *N* trials = 77, $Z_{1,76}$ = 2.362 : *P* = 0.018), gaining 64 percent of the matings. This is consistent with the shorter latency time found for SR*s* males compared to ST*s* males, on background A (Figure 5.2a). There was no overall effect detected of wing clipping (*N* clipped = 46, *N* trials=77 binomial test: *P* = 0.110). The single male trials of SR*s* and ST*s* background A males, first performed in Experiment 1, were also repeated alongside the two male trials, to check the consistency of the single male results. SR*s* males of genetic background A again achieved matings faster than their non-SRs brothers ($t_{1,119}$ = 7.691 , *P* < 0.001). There was, however, no significant difference in mating duration ($t_{1,119}$ = 1.619 , *P* < 0.108), but there was a trend for longer matings by SR*s* males.

5.5 - Discussion

Contrary to our expectations, and in contrast to previous work on XCMD systems in *Drosophila*, stalk-eyed flies and mice, we found no evidence that polyandry reduces the risk of a females offspring being fathered by XCMD carrying males. We found no evidence of significant polyandry in North African *D. subobscura*, whether mating with XCMD or non-XCMD males. However, we did find evidence for weak female choice against XCMD carrying males. This pattern was complicated, as the evidence suggested that males carrying different non-XCMD chromosomes differed significantly in their ability to gain matings.

Polyandry

Previous studies of female remating in *D. subobscura* found extremely low rates of polyandry (Maynard-Smith 1956, Fisher et al 2013), and where females do remate it is usually because the first mating failed. Considering our remating experiments covered three weeks after the female's first mating, and field estimates suggest less than 35% of females will survive a week after mating (Rosewell and Shorrocks, 1987), wild remating may be even rarer than our results suggest. Overall, we found no evidence that rates of polyandry are higher in North Africa than in European populations (Fisher et al 2013), in contrast to species of *Drosophila* that show clines in polyandry across their range (Pinzone & Dyer, 2013; Price et al., 2014). It would be ideal to examine remating in the wild to be certain of this monandry, however, laboratory estimates of polyandry are reflective of wild populations in *D. pseudoobscura* (Price et al., 2011).

Polyandry has been suggested as a general mechanism for reducing the transmission advantage of meiotic drivers, and suppressing their spread (Wu 1983; Wilkinson and Fry 2001; Angelard et al 2008; Price et al., 2008; Pinzone & Dyer 2013; Price et al 2014). Indeed, it has been shown in *D. pseudoobscura* that monandrous laboratory populations harbouring XCMD are rapidly driven extinct (Price et al., 2010). However, the persistence of the SR*s* system in the wild for a minimum of 50 years (and probably far longer) suggests otherwise for *D. subobscura* (Hauschteck-Jungen, 1990; Jungen,

1967). Hence *D. subobscura* provides a counter-example of an XCMD system that persists where females are monandrous. As SR*s* frequencies appear to be stable in North African populations, other factors must be counteracting the transmission advantage of SR*s*. Currently, for *D. subobscura* these factors are unknown. Evidence from other systems have suggested other factors which could impact the success of SR*s* in the wild, for example homozygous costs in females (Wallace, 1948), differences in survival and success in multiple life stages (Beckenbach, 1996), or effects of abiotic factors (Dyer, 2012). Monitoring fluctuations in SR*s* frequency within and between years would prove invaluable to understanding how natural ecology affects drive dynamics.

Transitions are from monandry to polyandry remain poorly understood. A rare case of rapid evolution of increased polyandry occurred in laboratory populations of *D. pseudoobscura* where females were exposed to XCMD males (Price et al., 2008b), suggesting that polyandry might evolve in direct response to XCMD. However, our results show no evidence of increased polyandry in the presence of meiotic drive, either generally in North Africa, or specifically when a female first mates with an XCMD male. Hence polyandry has not evolved in response to XCMD in this case. It is possible that there is simply not enough genetic variation in mating behaviour in *D. subobscura* for polyandry to evolve. Understanding the basis for monandry in *D. subobscura* is important to understand how possible a shift from monandry to polyandry is, however the factors that underlie monandry are unknown. For example, if monandry is due to male suppression of remating, the evolution of polyandry might be highly constrained (Hosken et al., 2009). In highly promiscuous species, sperm completion is expected to hinder the establishment and spread of XCMD (Holman et al., 2015). Therefore the establishment of XCMD in species might be facilitated by monandry.

Male mating performance

We found some evidence that SR*s* males are slower to achieve matings than ST*s* males. However, this occurred only in two ST*s* genotypes, with a third ST*s* genotype consistently showing longer latencies to achieve matings. This

is puzzling, as theory suggests SR*s* males should have worse mating success than STs males, either because SR*s* is associated with deleterious alleles that reduce male fitness and reduce their courtship success, or because females prefer to mate with STs males over SR*s* males. To confirm that SR*s* males were more successful at mating in this third genotype, we competed SR*s* against ST*s* males of this genotype in two-male trials, in which SR*s* males again outcompeted ST*s* males.

Overall, our results on male mating performance are inconclusive. However, in all three genotypes an SR*s* and ST*s* males differed in their mating performance, although the direction of this difference depended on genotype. These results are unusual for *Drosophila*, where a number of species show no difference in the ability of XCMD-bearing males to gain a mating (Price & Wedell 2008; Price et al. 2012). Previously female choice against meiotic drive carrying males has been reported in Diopsid stalk-eyed flies and mice (Cotton et al., 2014, Wilkinson et al., 1998). In one genetic background, SR*s* males were faster than males carrying ST*s* to secure matings in single-male trials two-male competition trials, which shows consistency between one and two male scenarios. Although the mating situation of *D. subobscura* in North Africa is not characterized, they can reach densities in the wild in the UK which make both one and two male scenarios possible (Begon, 1976). Hence SR*s* males cannot simply always be inferior competitors; instead there appears to be strong variation in male attractiveness or competitive ability based on their ST*s* X chromosomes, with our SR*s* genotype falling within the range of performance that exists between different ST*s* genotypes. This weak and variable difference between SR*s* and ST*s* mating performance is unlikely to play a major role in restricting the spread of SR*s* through populations. Nonetheless, all tests yielded strong differences in male performance even if the competitor males were full siblings, suggesting that the X chromosome is very important in determining differences in male performance in *D. subobscura*.

Mating duration and offspring production

We also found that SR*s* males mate for longer than ST*s* males. However, it is difficult to know what mating duration signifies in a monandrous fly. Theory suggests that the operational sex ratio should be highly male biased in *D. subobscura*, as males can mate many times during their life, but females only mate once. If on average males are unlikely to mate more than once, why not invest everything in a given mating? If copulation duration is a measure of mating effort as in other *Drosophila* (Price et al. 2012), why should drive males put in more effort? One possible explanation is that SR*s* males are more exhausted from spending longer courting females and so spend longer in a subsequent mating (Verspoor et al., 2015). However, our result from experiment 2 where SR*s* was competed against multiple backgrounds does not support this hypothesis. Instead, SR*s* males mate for longer on average even when they achieve matings more quickly (Figure 5.2). Another potential reason is that if an SR*s* male loses half his sperm to drive, then by mating for longer it provides supplementary sperm or seminal fluid to enter the female to compensate. However, there was no evidence that SR*s* males produced fewer offspring, which is consistent with a previous study on this system (Hauschteck-Jungen et al., 1987), suggesting that both male genotypes provide enough sperm for females to remain fertile for a substantial period.

5.6 - Conclusions

In contrast to many studies, we find no evidence that polyandry plays any role in suppressing the fitness of an XCMD system in *D. subobscura.* This suggests polyandry has not evolved as a response to XCMD and supports *D. subobscura* being monandrous across its species range. We present evidence of a weak female preference against XCMD carrying males. However, results were highly dependent on the competitor males' genotypes. It is unlikely that this preference evolved specifically in response to XCMD, but rather monandrous females are generally choosy and XCMD males on average are less attractive. Consistent with previous studies on a number of species we find no evidence that males carrying XCMD produce less offspring in a single mating situation. Our results contradict recent findings that polyandry (Pinzone & Dyer, 2013; Price et al., 2014; Price, et al., 2008) and female preference (Cotton et al., 2014; Johns et al., 2005; Wilkinson et al., 1998) are important for preventing the spread of XCMD systems through populations. However, this means that the factors that have caused SR*s* to remain at 20% frequency in Tunisia for the past 50 years remain unknown.

5.7 - Acknowledgements

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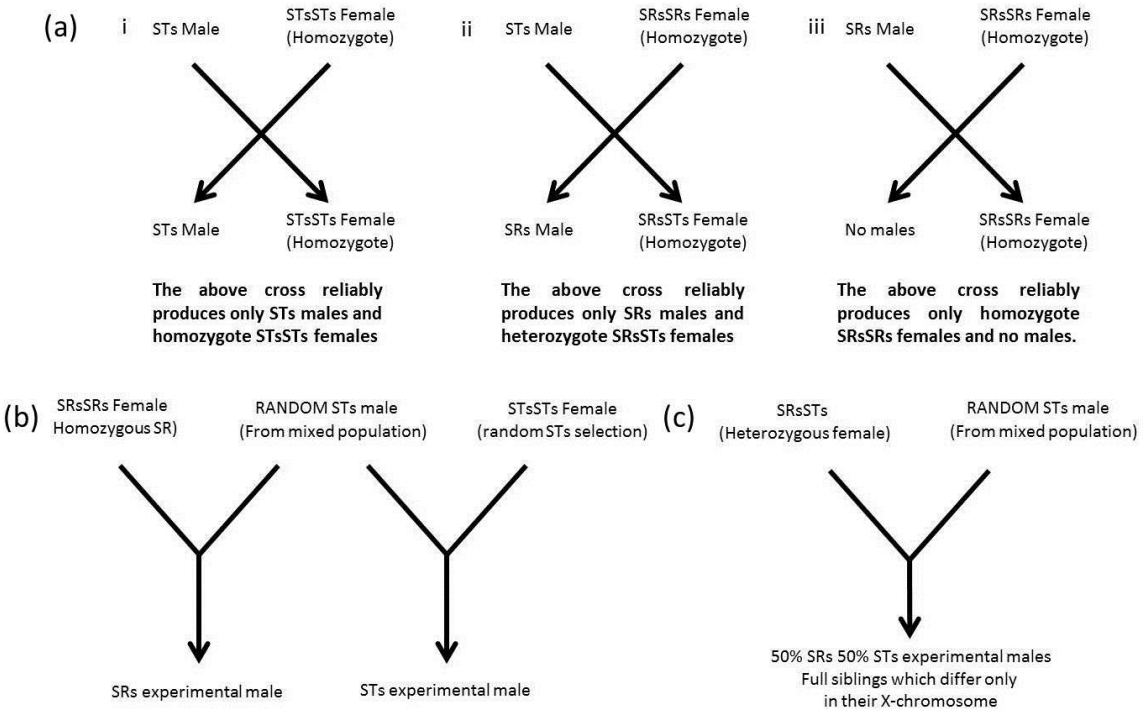
5.9 - Supplementary material



Supplementary figure 5.1. Crossing schematic showing how the SRs X-chromosome can be kept to produce all the different mixes of genotypes required for the experiment.

6 - Incipient reproductive isolation prevents the spread of a meiotic drive element

This chapter is currently in review at *Nature: Ecology and Evolution*.

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6.1 - Abstract

Divergent trajectories of rapid antagonistic co-evolution associated with selfish genetic elements have been suggested to be a major force driving the evolution of reproductive isolation between populations (Crespi and Nosil, 2013, Johnson, 2010a, Presgraves, 2010a). Meiotic drivers, selfish chromosomes that increase their own transmission at a cost to the rest of the genome, are potentially major contributors to this 'Conflictual Speciation'. Indeed, drive elements are implicated in hybrid incompatibilities between several sister-species (Phadnis and Orr, 2009a, Tao et al., 2001b, Zanders et al., 2014, Zhang et al., 2015). However, it remains unclear whether in these examples meiotic drive systems created the population incompatibilities, or whether isolation occurred through other means and meiotic drive evolved subsequently (Johnson, 2010a). We present evidence of incipient reproductive isolation caused by an X-chromosome meiotic driver in natural populations of the fly *Drosophila subobscura*. This driver, when introgressed into neighbouring and more distant populations, caused greater than two-fold costs to F1 males and complete loss of fertility in F2 hybrid males. These costs were specific to the driving chromosome, with the populations otherwise fully compatible. Consistent with the CS process, we also observed weak suppression of drive within the native range of the driver. These results provide critical evidence of early reproductive isolation caused by a meiotic driver in otherwise compatible natural populations that exchange migrants (Balanya et al., 2006). Witnessing drive-specific reproductive isolation, without other incompatibilities between adjacent populations, strongly suggests that genomic conflict between drivers and suppressors can initiate strong reproductive isolation, and hence that conflictual speciation is an underestimated force in the generation of biodiversity.

6.2 - Main text

Two main mechanisms have been proposed as key sources of reproductive isolation between previously compatible populations (Crespi and Nosil, 2013). In 'Ecological Speciation', adaptation to specific ecological factors drives divergence between populations resulting in reproductive isolation. This process is well documented, both in terms of incipient and complete isolation, for a number of species pairs (Nosil, 2012). In 'Conflictual Speciation', selfish genetic elements such as endosymbionts, transposable elements, or meiotic drivers manipulate reproduction to increase their own transmission relative to rival parts of the genome. Such biased transmission imposes costs on the fitness of the rest of the genome (intragenomic conflict) (Burt and Trivers, 2006), leading to cycles of adaptation to suppress the selfish element and counter adaptation by the element to re-establish its transmission advantage. Population specific co-evolution between genes which distort gametogenesis and their suppressors could lead to an arms race of increasingly potent drivers and suppressors. These strong driving elements, when exposed to naïve populations may then be so potent that they cause a breakdown of gametogenesis by overdriving, killing all gametes including those carrying the meiotic drive element, resulting in hybrid breakdown (Hurst and Pomiankowski, 1991b, Frank, 1991a).

To date, there is good evidence for the process of rapid coevolution between selfish genetic elements and their host genome (Obbard et al., 2009, Juchault et al., 1992, Bastide et al., 2011b), and also evidence that the action of these genes contributes to hybrid sterility (Phadnis and Orr, 2009a, Tao et al., 2001b, Zhang et al., 2015, Zanders et al., 2014). However, these sterility phenotypes may have evolved after the evolution of reproductive isolation. Intriguingly, there are a few studies where crosses between particular meiotic drive carrying isolines are incompatible with isolines derived from elsewhere in the species range (Hauschteck-Jungen, 1990, Simon et al., 2016), compatible with incipient reproductive isolation associated with meiotic drive. However, compelling evidence that meiotic drive creates the reproductive isolation that initially isolates species is lacking, because there are no examples where a driving chromosome creates reproductive incompatibility

when crossed between neighbouring natural populations that are otherwise fully fertile, indicating both that the driver itself is responsible for the incompatibility and that the incompatibility is population-wide and thus establishes barriers to gene flow.

The fly *Drosophila subobscura* carries a sperm-killing X-chromosome meiotic drive system (henceforth referred to as "SR*s*" – Sex-Ratio *subobscura*) that has close to 100% transmission advantage making almost all offspring female. Historically this element has been restricted to North African populations where it persists at intermediate frequencies (Figure 6.1; 1967 frequency: 16% n=140 (Jungen, 1967b); 1990 frequency: 15% n=320 (Hauschteck-Jungen, 1990); 2013 frequency: 9% n=156 Supplementary table 6.1). Recent collections showed low frequency of the SR*s* chromosome in Southern Spain (1970 frequency: 0% n=71 (De Frutos, 1972): 1998: frequency: 5% n=63 (Solé et al., 2002); 2013: frequency: 1% n=330 Supplementary table 6.1). *D. subobscura* is a highly mobile fly, having invaded and spread across the whole of the Americas in ten years (Pegueroles et al., 2013a). This high capacity for migration (Serra et al., 1987), and the low frequency of SR*s* in Spain is compatible with a model of recurrent migration from North Africa and local selection against the element, implying flies carrying the driving chromosome have substantial fitness deficits in Spain. A study examining the fitness of SR*s* when crossed to a Swiss strain is consistent with this theory (Hauschteck-Jungen, 1990). Hauschteck-Jungen et al (1990) crossed SR*s* into a laboratory strain of *D. subobscura,* demonstrating that an active driving element can cause incompatibility. However, this study used only a single strain, from a distant population unlikely to have ever encountered SR*s*. For meiotic drive to be a strong driver of hybrid incompatibilities, potentially resulting in reproductive isolation, the incompatibility must be maintained even in adjacent populations experiencing gene flow. Here we examine whether this drive system creates incompatibilities in hybrids between adjacent natural populations that could exert a selective force stopping the spread of SR*s* through Spain.

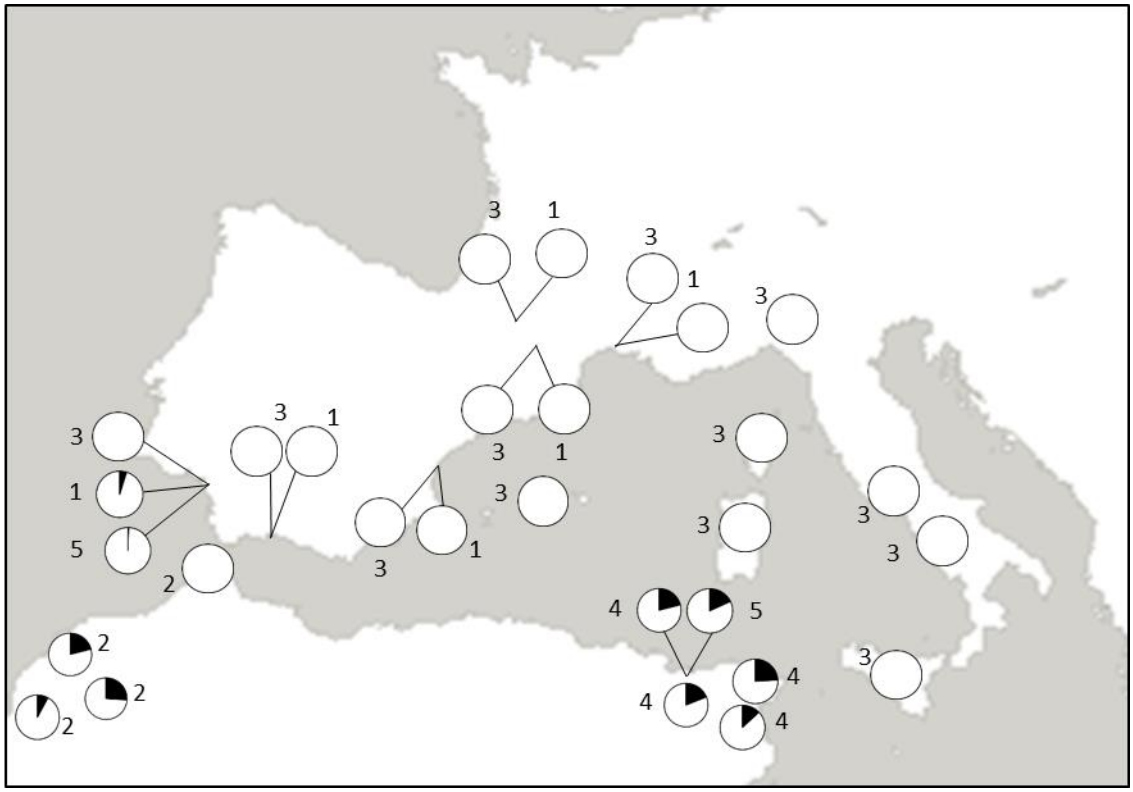

Figure 6.1 Map of driving and non-driving X-chromosomes across Southern Europe and North Africa. Pie charts show the proportion of SR*s* (in black) and non-driving (white) X chromosomes. The numbers represent the years and sources of the collections (1 - 2002 (Solé et al., 2002), 2 – 1974 (Prevosti, 1974), 3 – 1984 (Prevosti et al., 1984), 4 – 1968 (Jungen, 1968), 5 – 2013 Supplementary table 6.1).

We established the source of selection against the SR*s* element by examining the fitness of males carrying SR*s*, non-driving North African, or non-driving Spanish X-chromosomes on their native and hybrid backgrounds (Figure 6.2a). We found a highly significant interaction between X chromosome type and genetic background (Figure 6.2a; ANOVA: $F_{2,419}$ = 30.64 p <0.001). Males that carried a non-driving X chromosome showed equally high levels of fitness, irrespective of whether they were on a Spanish, Tunisian, or mixed genetic background (Tukey's post hoc test: P>0.861 in all comparisons, Supplementary table 6.2). The fitness of SR*s* males that had a Tunisian genetic background equalled the high fitness of non-driving males (Tukey's post hoc test: P>0.875 in all comparisons, Supplementary table 6.2). However, males that carried SR*s* on a mixed Spanish/Tunisian genetic background produced fewer than half the number of offspring than all other male types (Tukey's post hoc test: P<0.001 in all comparisons,

Supplementary table 6.2). This is consistent with a previous study that found incompatibilities specific to crosses between the X-chromosome meiotic drive from North Africa and a laboratory strain from Switzerland (Hauschteck-Jungen, 1990). We confirm that the hybrid incompatibility is specific to only the SR*s* chromosome and that the adjacent populations are otherwise fully compatible with each other (Figure 6.2).

We then examined whether the costs of SR*s* would be higher in a population that has never been exposed to SR*s*. We crossed SR*s* into a number of isofemale lines from the native Tunisian population, the neighbouring Spanish population, and a distant UK population. There was equally strong evidence of incompatibility when the SR*s* chromosome is expressed on F1 hybrid backgrounds from both neighbouring (Spain) and distant (UK) populations (Figure 6.2B; ANOVA: $F_{2,36} = 43.91$ $p < 0.001$; see Supplementary table 6.2). Expressing the SR*s* chromosome on an increasingly Spanish genetic background in an F2 backcross resulted in almost complete infertility of SR*s* males. Over ninety percent of these males produced fewer than five offspring, compared to Spanish X-chromosomes which show normal offspring production (Wilcoxon rank: n=176 W = 6958 $p < 0.001$; Figure 6.3). Incompatibility caused by the SR*s* chromosomes therefore increases when expressed on an increasingly Spanish genetic background. This supports previous studies using single laboratory strains [17], and suggests these incompatibilities form real barriers to the invasion of a selfish genetic element into Europe. To further demonstrate that these incompatibilities were caused by an interaction between SR*s* and Spanish autosomes/Y chromosomes, we attempted to rescue the fertility of the SR*s* chromosome by backcrossing the few female F3 offspring that were produced to Tunisian males to reintroduce a Tunisian genetic background. The resulting F4 SR*s* sons had restored fertility (Supplementary table 6.3).

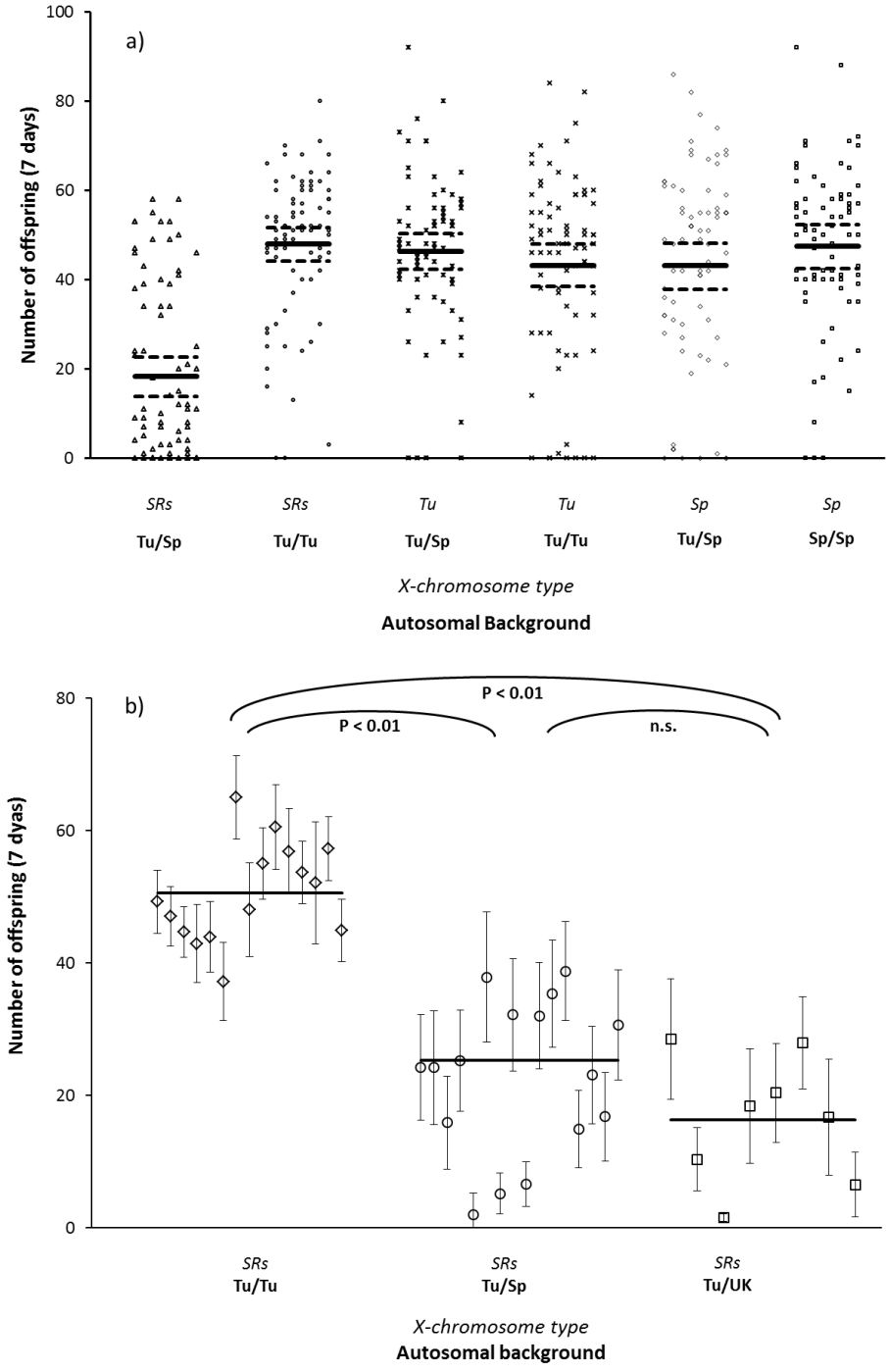

Figure 6.2 a) The number of offspring produced by three different categories of X-chromosome (*SRs*, non-driving Tunisian – *Tu*, non-driving Spanish – *Sp*) on different autosomal backgrounds (100% native autosomes or 50% foreign autosomes). Reduced fitness only occurs when SR*s* chromosomes occur in a hybrid background. Solid lines and dashed lined show the means and two SEM respectively. b) Mean number of offspring produced by SR*s* males with different genetic backgrounds, showing low fitness on hybrid backgrounds. Each point indicates the mean and 95% confidence intervals for a single isoline. Main lines show population means.

This observation thus extends the pioneering work by Hauschteck-Jungen et al 1990 by showing that meiotic drive specific incompatibilities cause extreme costs in the F1 and F2 males between adjacent natural populations. The incompatibility occurs across a broad set of Spanish genetic backgrounds, and the degree of incompatibility increases with increased genetic introgression. Hence the driving SR*s* chromosome from Tunisia are entering the neighbouring Spanish population (Solé et al., 2002), but create strong hybrid sterility on this background and these costs are sufficient to prevent spread of the driver through the Spanish population and into Europe. Thus, SR*s* causes a form of incipient reproductive isolation between North Africa and Spain that is currently specific only to the selfish genetic element.

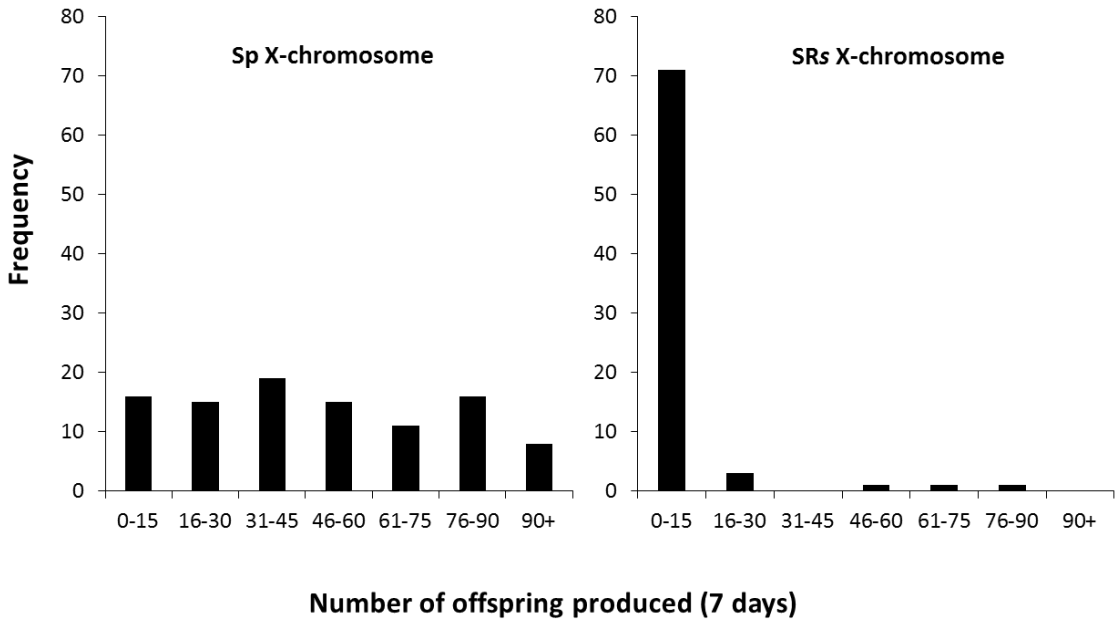

Figure 6.3 Histogram of offspring production for males introgressed into the Spanish population for two generations, carrying the Spanish (Sp) X chromosome or SR*s*.

The conflictual speciation model postulates that hybrid sterility is associated with a history of evolutionary conflict over suppression of drive in the native population (Crespi and Nosil, 2013). Here, we formally test this by comparing the strength of drive in three different populations, with the CS model predicting drive is weakest in the native population where there is a history of

coevolution between driver and genome. We observed that drive is stronger in hybrids with partial Spanish and UK genetic backgrounds than in the Tunisian background ($F_{2,36}$=17.71 p < 0.001; figure 6.4). We also demonstrate differences in the strength of drive between different genetic backgrounds from Tunisia are consistent and repeatable, as noted previously (Hauschteck-Jungen, 1990). The most parsimonious explanation for these data is that weak suppression of SR*s* has evolved in Tunisia, suggesting active genetic conflict between SR*s* and suppressors. We also conclusively demonstrate that this is true suppression, as 17 of the 18 sons produced by SR*s* fathers were fully fertile males carrying a Y chromosome (Supplementary figure 6.1) with only one pseudomale (Cobbs, 1992), meaning the SR*s* X chromosome was failing to transmit to these offspring.

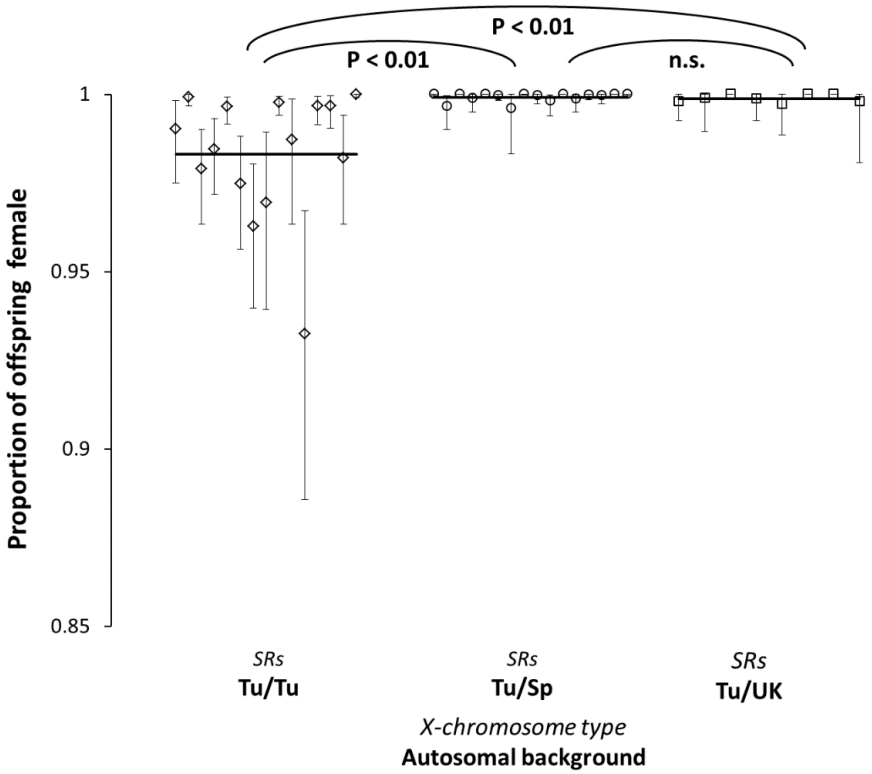

Figure 6.4 Offspring sex ratio of SR*s* males on native and Spanish/Tunisian and UK/Tunisian hybrid backgrounds. Each point indicates the mean and 95% confidence intervals for a single isoline. Main lines show population means.

The varying level of suppression is variable between lines from North Africa (Figure 6.4), which also suggests ongoing co-evolutionary conflict between

suppressors and X-chromosome meiotic drive. The increased strength of drive in Spanish and UK hybrids suggests that the low fitness seen in these hybrids is likely to be caused by an overdrive phenotype (McDermott and Noor, 2010a) in which mechanisms that kill the Y chromosome sperm in Tunisia act so strongly in hybrids that almost all sperm that carry the SR*s* X are also killed.

This demonstration of hybrid incompatibility that is specific only to a meiotically driving sex chromosome and associated with ongoing genomic conflict provides strong evidence in support of conflictual speciation occurring in natural populations (Johnson, 2010a). The study establishes the isoline specific hybrid incompatibilities associated with drive in previous work (Hauschteck-Jungen, 1990) exist at a population level before the establishment of complete reproductive isolation. This observation of incipient isolation indicates that other examples of drive genes which have been implicated in causing reproductive isolation are also likely to have been the root cause of divergence between populations, rather than phenotypes that arose post speciation (Phadnis and Orr, 2009a, Tao et al., 2001b, Zanders et al., 2014, Zhang et al., 2015).

What processes might lead to full isolation? Conflictual speciation via meiotic drive requires three processes to occur. First, there must be the spread of a meiotic driver that occurs locally, but not globally, within a species. Second, there must then be a build-up of incompatibilities between adjacent populations that reduce the fitness of interpopulation hybrids. Third, there are three likely routes to complete reproductive isolation: a) a build-up of extremely high drive frequencies, making incompatibility universal, b) fixation within the drive bearing population of reproductive changes that reduce drive harm but which create interpopulation incompatibilities, or c) the low fitness of hybrids creates reinforcement selection for avoidance of interpopulation matings, as shown for *Wolbachia* induced cytoplasmic incompatibility (Jaenike et al., 2006a). This paper provides evidence for the first two processes, the local spread of a meiotic driver and the build-up of meiotic drive specific incompatibilities between two neighbouring populations.

The theory that meiotic drive could cause speciation was first proposed in 1991 (Hurst and Pomiankowski, 1991b, Frank, 1991a) Recent genomic evidence has provided considerable evidence across taxa that meiotic drive and hybrid incompatibilities are strongly linked. Our evidence that meiotic drive can be part of the initial phase of the generation of reproductive isolation, combined with the ubiquity of meiotic drive and other elements that cause intragenomic conflict (Burt and Trivers, 2006, Lindholm et al., 2016a), suggests that conflictual speciation may be a major contributor to the generation of biodiversity.

6.3 - Methods

Hybrid incompatibility of SRs and non-driving males on native and foreign population genetic backgrounds.

We searched for costs associated with the selfish SR*s* X chromosome by testing the fitness of three types of X-chromosomes (one driving SR*s* chromosome and a selection of non-driving X-chromosomes from Tunisia and Spain) on different genetic backgrounds (either 100% from the population of origin (Native), or a 50:50 mix of native and foreign backgrounds). For details of collections see (Verspoor et al., 2015a). SR*s* males from Tunisia, and non-driving males from both Tunisia and Spain were crossed to create experimental males with either their full native or 50% non-native genetic background (Supplementary figure 6.2a). For each treatment, 80 replicates were set up. To make sure that the population of origin of the female was not important, males were mated to either a female from Spain or from Tunisia. Female origin had no impact on offspring production or sex ratio (ANOVA $F_{1,418}$=0.474 p=0.492), so this factor was removed from the ANOVA. All flies used were seven days old, to ensure sexual maturity (Holman et al., 2008). Males were paired individually to females for seven days to lay eggs. The number of offspring was counted and analysed using ANOVA and Tukey's post hoc tests. Offspring sex ratio was used to confirm male X chromosome genotype (>85% female = SR*s*). All analyses were carried out in R (R Core Team, 2011).

Hybrid incompatibility of SRs males across multiple populations.

We further tested the strength of the hybrid incompatibility of SR*s* by comparing its fitness across different isofemale lines from three populations. Each isofemale line (or "isoline") comprises the highly inbred descendents of a single wild caught female, and members of an isoline are effectively genetically identical (David et al., 2005a). Experimental males were produced by crossing SR*s* homozygote females to males from an isoline (Tunisia – 15 isolines; Spain – 16 isolines; UK; 8 isolines; supplementary figure 6.2b). These produced experimental SR*s* hybrid males with a 50% Tunisian and 50% either UK or Spanish genetic background. From each of the 39 isolines,

40 SR*s* males were paired with a virgin female from an outcrossed Tunisian population. Pairs were mated and offspring recorded as above.

The fitness of the SRs chromosome in an F2 hybrid genetic background.

We then tested the fitness of SR*s* when it had been introgressed for 2 generations into a foreign Spanish background. To produce the experimental males we crossed F1 heterozygote females carrying one SR*s* X chromosome to a male from an outbred Spanish population. The resulting male offspring now carried either an SR*s* or a Spanish non-driving X-chromosome with a ~25% Tunisia/75% Spain genetic background. These focal males were mated as above to a Spanish female, and their offspring recorded. Focal males that produced fewer than 5 offspring could not be reliably assigned by offspring sex ratio, and so their X-chromosome type was confirmed by sequencing the G6P gene region (see SOM for details). The median number of offspring produced was analysed using a Wilcoxon rank test.

Testing for rescue of SRs phenotype by backcrossing to the Tunisian genetic backround

Although the F2 SR*s* hybrid males were almost entirely infertile, a few female F3 offspring were produced. To examine whether SR*s* fertility could be rescued by increasing the proportion of the background that was Tunisian, we crossed half of these females to a male from an outbred Tunisian population, and half to an outbred Spanish population. As the focal females were heterozygotes, carrying one SR*s* chromosome, half of their sons would be expected to carry SR*s*. Male offspring of each focal female were mated as above. Males were subsequently assigned to three phenotype categories: SR*s* if the sex ratio of their offspring was >85% female, non-driving if the sex ratio was 50:50, and unknown if they produced 5 or fewer offspring. If Tunisian autosomes do rescue SR*s*, then the SR*s* phenotype would only appear in the backcross to Tunisian males, as SR*s* would be sterile in the Spanish background.

Sex-ratio distortion of SRs males across multiple populations

Here we tested whether the strength of the SR*s* sex-ratio distorting phenotype differed when it was in hybrid genetic backgrounds between different populations. For details of the F1 Experimental males see supplementary figure 6.2b. Pairs were mated as above, and offspring were sexed and counted. Proportion of female offspring was transformed using an arcsine transformation before analysis. Mean sex ratio for each line was calculated and populations were compared using ANOVA and Tukey's post hoc tests.

Fertility and Y-chromosome status of sons of SRs males

To check the few males produced from SR*s* fathers were not pseudomales (Cobbs, 1992) we tested their fertility by pairing them to two random virgin females from an outcrossed Tunisian population. After mating as above, vials were checked for larval action to confirm the males were fertile. These same males were then assayed for the presence of a Y-chromosome using the *kl2* marker (Herrig et al., 2014). The presence of a Y-chromosome was confirmed using gel electrophoresis, with a positive and negative control.

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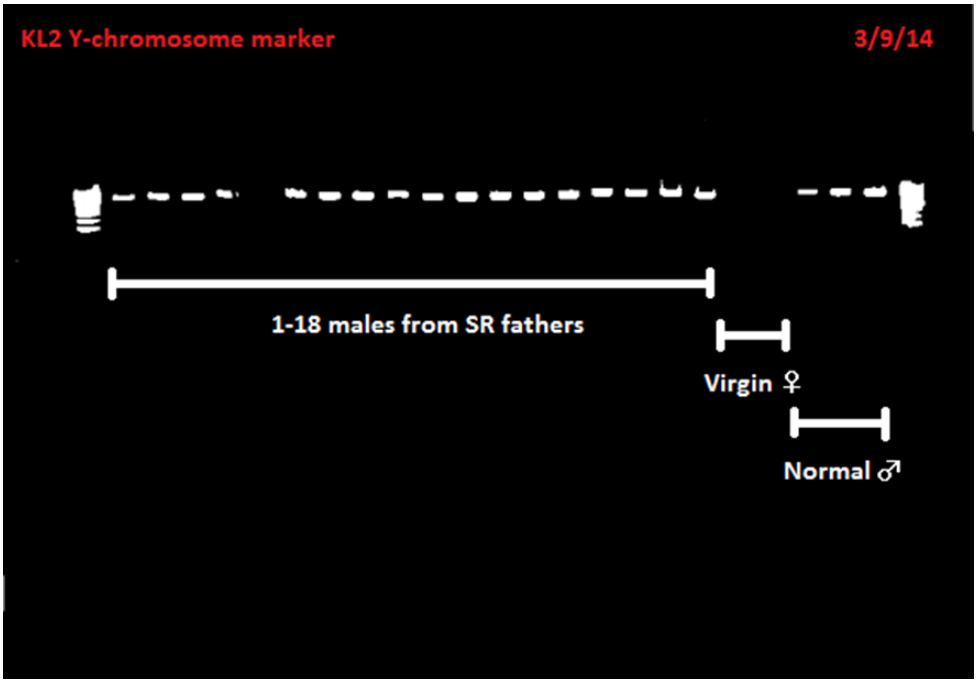
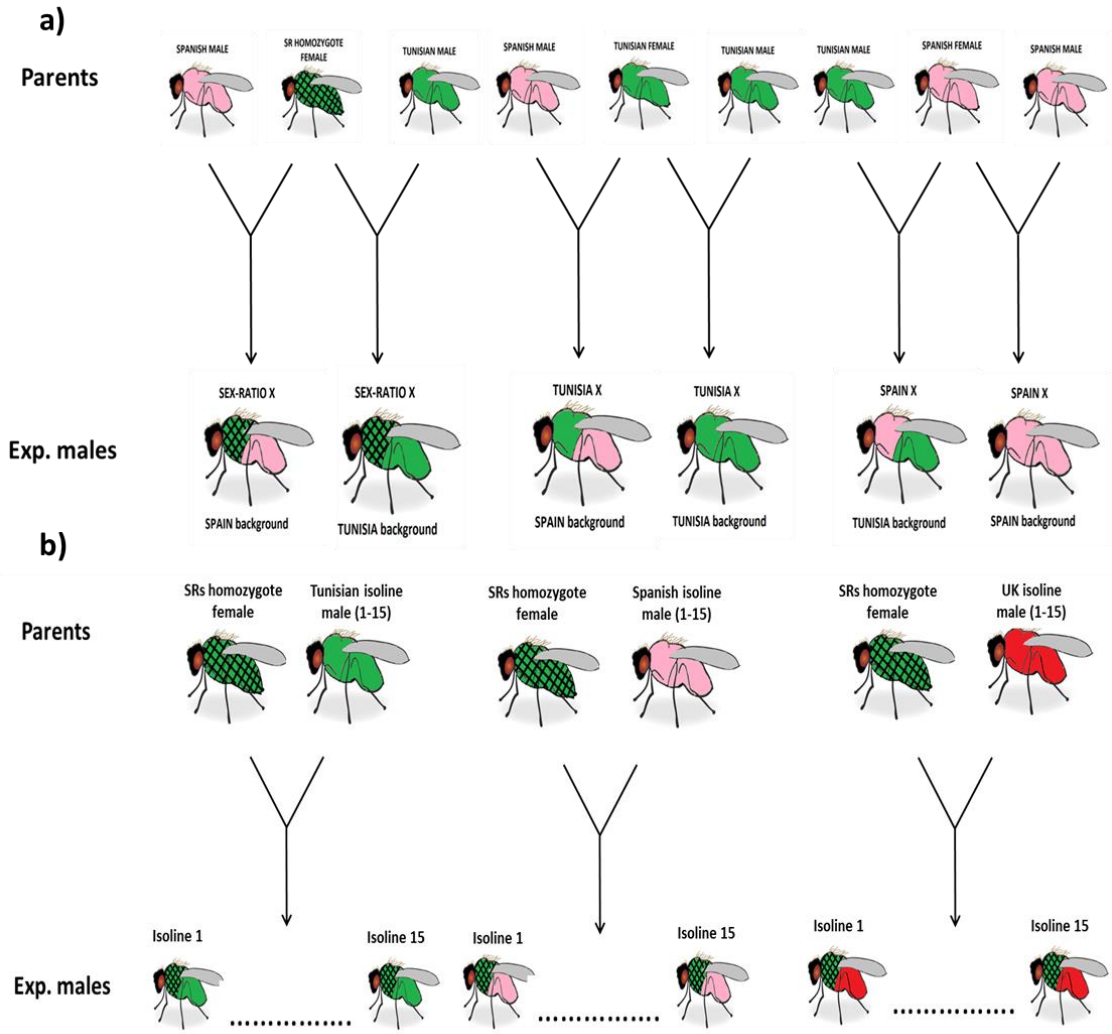
6.5 - Supplementary information



Supplementary figure 6.1 Gel electrophoresis image showing the amplification of the kl2 gene from the *Drosophila subobscura* Y-chromosome. PCR conditions were an initial 3min denaturing step, followed by 35 cycles of 94 for 30secs, 60 for 30 secs, 72 for 30secs, with a final elongation period of 10mins at 72. PCR products were determined using gel electrophoresis on a 1.5% agarose gel with 3µL Midori green per 100mL of TAE buffer. This image confirms that the few male offspring produced from SR*s* carrying males are carrying a Y-chromosome. All the males which carried a Y-chromosome were also found to be able to produce offspring when mated to a virgin female.



Supplementary figure 6.2. Showing the layout of the crossing schematics for a) Experiment 1 comparing the fitness of the SR*s* X chromosome and non-driving X-chromosomes from Tunisia and Spain on native and hybrid populations genetic backgrounds b) Experiment 2 and 4 comparing the fitness costs of SR*s* and the levels of suppression of SR*s* in multiple isofemale lines across three populations. Colours indicate different genetic backgrounds from different populations. Checked pattern indicates the SR*s* X-chromosome is present in the cross.

Supplementary information G6P locus

The G6P locus, located on the X-chromosome (A chromosome) was used to differentiate SR*s*, from Spanish X-chromosomes. The forward and reverse primers used can be seen below (Forward primer – ATCATACCGCTCTGGATCTCAT, Reverse primer – GTGGAGCTGAGGATCTTGTTG). The reaction profile was an initial 3min denaturing step at 95°C, followed by 35 cycles of 95°C for 30secs, 60°C for 30 secs, 72°C for 30secs, with a final elongation period of 10mins at 72°C. PCR products were determined using gel electrophoresis on a 2.5% agarose gel with 3µL Midori green per 100mL of TAE buffer. For one of the Spanish X-chromosomes, there was no amplification so SR*s* was scored based on the presence of a PCR product. For the two remaining X-chromosome types Sanger sequencing was used to identify the X-chromosome by SNP variation. PCR products were cleaned using antarctic phosphatase and exonuclease 1, with an incubation of 45mins at 37°C followed by 15mintes at 80°C. Sequencing products were amplified using BigDye3.1 protocol with a sequencing program of 35 cycles of 96°C for 10secs, 50°C for 5secs, 60°C for 4mins. Sequencing was precipitated using 3M sodium acetate and cleaned with 70% ethanol. 10ul of Hi-Di formamide was then added and sequencing was carried out on and ABI3500xL genetic analyser. SNPs in the region were called using the software Geneious version 7.1.3.

Supplementary table 6.1 shows the proportion of female offspring produced from wild males caught in Tunisia and Spain collected in 2013. Males were each crossed to a 7 day old virgin female from their same population of origin. Males were conservatively classed as the SR*s* phenotype if they produced >85% female broods.

Prop. of offspring female	Tunisia (n= 146)	Spain (n = 320)
0.3-0.4	1	9
0.4-0.5	32	86
0.5-0.6	83	175
0.6-0.7	22	41
0.7-0.8	3	7
0.8-0.9	3	0
0.9-1.0	12	2

Supplementary table 6.2 showing Tukey's post hoc tests on the differences in offspring produced by three types of X-chromosome (Driving SR*s* - "SR*s*", non-driving Tunisian – "Tun" and non-driving Spanish – "Spa") on two different population genetic backgrounds (100% their own native background – Nat or 50%/50% their own background and that of a different population – Hyb). The replicates for each category are as follows (SR*s*:Hyb n=61, SR*s*:Nat n=75, Spa:Hyb n=77, Spa:Nat n=70, Tun:Hyb n=73, Tun:Nat n=71)

Tukey's HSD tests SRs specific incompatibility (fig 2a)				
	Difference	lower CI	Upper CI	P adjusted
SRs:Hyb - Spa:Hyb	24.808	32.969	16.646	< 0.001
Tun:Hyb - Spa:Hyb	0.566	8.245	7.112	0.999
Spa:Nat - Spa:Hyb	3.122	10.912	4.667	0.861
SRs:Nat - Spa:Hyb	0.070	7.723	7.582	1.000
Tun:Nat - Spa:Hyb	1.7649	9.555	6.025	0.987
Tun:Hyb - SRs:Hyb	24.241	16.008	32.474	< 0.001
Pum:Nat - SRs:Hyb	21.686	13.349	30.022	< 0.001
SRs:Nat - SRs:Hyb	24.737	16.529	32.946	< 0.001
Tun:Nat – SRs:Hyb	23.043	14.707	31.380	< 0.001
Spa:Nat – Tun:Hyb	2.555	10.420	5.309	0.938
SRs:Nat – Tun:Hyb	0.496	7.232	8.225	0.999
Tun:Nat – Tun:Hyb	1.198	9.062	6.666	0.997
SRs:Nat – Spa:Nat	3.051	4.787	10.890	0.875
Tun:Nat – Spa:Nat	1.357	6.616	9.330	0.996
Tun:Nat – SRs:Nat	1.694	9.533	6.145	0.989

Supplementary table 6.3 showing differences in the offspring produced by SR*s* males when introgressed onto 39 isolines across three populations (Spain n=16, Tunisia n=15, UK n=8). Tukey's post-hoc tests were used to test how the populations differ from each other. The mean number of offspring produced for each isofemale line was calculated using 20-40 replicates.

TukeysHSD tests comparing population level off.produced (fig 3)				
	Difference	lower CI	Upper CI	P adjusted
Spain-UK	6.518	3.897	16.934	0.289
Tunisia-UK	34.293	23.762	44.824	< 0.001
Spain-Tunisia	27.775	19.130	36.420	< 0.001

Supplementary table 6.4 shows the X-chromosome status of males produced by backcrossing hybrid females carrying one SR*s* and one Spanish X-chromosome to either a Tunisian male, to test for the rescue of the SR*s* phenotype, or to a Spanish male. The backcross to a Tunisian male confirms rescue of the SR*s* phenotype in 7 males. Male types were classified based upon the sex-ratio of the offspring produced as follows: SR*s* if the sex ratio of their offspring was >85% female, non-driving if the sex ratio was 50:50, and unknown if they produced 5 or fewer offspring.

♂ **Parent**	**Sample (n)**	**SR*s***	**Spain**	**Unknown**
Tunisia	15	7	6	2
Spain	18	0	7	11

Supplementary table 6.5 Differences between three populations in the proportion of offspring that are female when an SR*s* males was introgressed onto an isoline from that population. Number of isofemale lines used differed across three populations (Spain n=16, Tunisia n=15, UK n=8). Tukey's post-hoc tests were used to test how the populations differ from each other. The mean proportion of female offspring was calculated for each isofemale line based on 20-40 replicate introgressed males from that isoline.

TukeysHSD tests comparing population level suppression for (fig 2b)				
	Difference	lower CI	Upper CI	P adjusted
Spain-UK	0.00682	0.042	0.0556	0.937
Tunisia-UK	0.08567	0.135	0.0363	< 0.001
Spain-Tunisia	0.09250	0.133	0.0519	< 0.001

7 - A single evolution of an X-chromosome meiotic drive system has spread across multiple populations of *Drosophila subobscura*

Authors

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This manuscript is currently in preparation for Ecology and Evolution

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7.1 - Abstract

Many selfish genetic elements (SGEs) manipulate reproduction to increase their own transmission, often at a cost to the rest of the genome. The intragenomic conflict this generates has profound implications for the evolution of species. One class of SGE, sperm killing X-chromosome meiotic drive (XCMD), involves X-chromosomes that kill or disable Y-chromosome sperm, allowing them to spread rapidly through populations. Theory suggests that in populations where XCMD is present, the rest of the genome should co-evolve to suppress XCMD and reduce the costs it imposes. This co-evolution between XCMD and the rest of the genome could cause a population carrying a driver to diverge rapidly in reproductive genes and traits, potentially creating incompatibilities between XCMD exposed populations and unexposed populations. Recently, an XCMD system in the fruit fly *Drosophila subobscura* thought to originate in North Africa, has reached European populations in southern Spain. Severe incompatibilities are found when the XCMD crosses into European genetic backgrounds. Here we investigate incompatibilities and suppression of Tunisian XCMD, when it crosses into genetic backgrounds from Morocco, an intermediate population between Tunisia and Spain. We find evidence for weak suppression of the XCMD but find no evidence for incompatibilities between the XCMD and genetic backgrounds from Morocco. We also examine the genetic relationship between XCMD males collected from Tunisia, Morocco and Spain. Six X-linked regions show that all XCMD carrying males are genetically very similar across Tunisia, Morocco and Spain. This supports the theory that a single evolution of XCMD has spread through North Africa co-evolving with populations, which experiences incompatibilities when it reaches populations in southern Spain.

7.2 - Introduction

Selfish genetic elements (SGEs) are diverse and ubiquitous across the tree of life (Burt and Trivers, 2006). They have been shown to have wide ranging impacts on the evolution of species (Hurst and Werren, 2001), including influencing genome size and structure (Kraaijeveld, 2010), causing rapid conflict driven evolution (Bastide et al., 2011) and affecting sexual selection (Price and Wedell, 2008). Some SGEs, such as meiotic driving sex chromosomes and endosymbionts, can also distort population sex-ratios, which may then change population growth rates (Unckless and Clark, 2014), influence mating behaviour (Jaenike et al., 2006), and affect population stability (Hamilton, 1967). Recently there has been a great deal of interest in whether SGEs play an important role in speciation (McDermott and Noor, 2010, Johnson, 2010, Presgraves, 2010). Rapid co-evolutionary cycles driven by conflict are particularly likely to create reproductive incompatibilities between populations because the interests between genetic actors does not reach a stable resolution, thus rapid evolutionary change is maintained indefinitely. SGEs, and the conflict they create with the rest of the genome, are likely to produce such co-evolutionary cycles.

X-chromosome meiotic drive (XCMD), a type of sperm killing SGE, has generated considerable interest as the cause hybrid incompatibilities (McDermott and Noor, 2010b, Frank, 1991, Hurst and Pomiankowski, 1991). XCMD is a selfish X-chromosome that in males either kills or disables Y-chromosome sperm. This ensures the majority of viable sperm carry the X-chromosome, thereby selfishly enhancing its own transmission to the next generation and also creating strong genetic conflict with the Y-chromosome (Jaenike, 2001, Lindholm et al., 2016). They can also spread rapidly through populations, distorting sex-ratios, and potentially driving local population extinctions (Pinzone and Dyer, 2013, Bastide et al., 2011). XCMD, by causing broods and populations to be female biased, imposes an additional cost on the rest of the genome. This is because producing sons not daughters is expected to give increasing fitness returns in increasingly female biased populations (Fisher, 1930). These costs can cause the rapid evolution of mechanisms to reduce the harm. Evolved responses to reduce

the harm caused by XCMD could work via direct suppression of the Y-sperm killing mechanism (Bastide et al., 2011), or through behavioural changes that reduce the risk of exposure to XCMD, such as mate avoidance (Johns et al., 2005) or increased polyandry (Price et al., 2014).

If suppressors of drive evolve, the XCMD is expected to evolve counter-adaptations to avoid being suppressed, leading to ongoing cycles of antagonistic co-evolution between the XCMD and the rest of the genome. This rapid evolution in the rest of the genome is expected to occur only in populations where XCMD is present. Moreover, separate populations that both contain the same XCMD might evolve independent forms of suppression, involving different loci. In these ways, the genomic conflict caused by XCMD is expected to drive rapid population-specific evolution, which could cause divergence in genes involved in sperm-killing and gametogenesis. This dynamo for divergence has been proposed as a cause of male-specific incompatibilities between populations, and thus a contributor to reproductive isolation (Hurst and Pomiankowski, 1991, Frank, 1991).

There is strong circumstantial evidence that XCMD play an important role in hybid incompatibilities and reproductive isolation. Of the limited number of genes known to be involved in speciation, more than half also show evidence of genomic conflict (Presgraves, 2010, Johnson, 2010, McDermott and Noor, 2010). There is also good evidence that suppression can rapidly evolve in response to XCMD (Bastide et al., 2011), and that suppression can be limited to local populations (Stalker, 1961). In addition, hybrid incompatibilities occur between populations and subspecies that are specifically associated with XCMD genes (Hauschteckjungen, 1990, Phadnis and Orr, 2009, Tao et al., 2001).

Existing evidence however, is not sufficient to conclude that XCMD does indeed play a major role in creating hybrid incompatibilities and speciation. Firstly, some systems combine evidence of past genomic conflict with current incompatibilities between separated populations or species (Phadnis and Orr, 2009). This makes it difficult to confirm if the genetic conflict originally created these incompatibilities, or whether this genetic conflict evolved after

separation. For example, if two populations or species are isolated, and an XCMD system subsequently evolves and spreads to fixation in one population but is entirely suppressed, then it may well become activated in hybrids that lack the suppressors, and generate hybrid incompatibilities, despite having evolved after the two populations separated. Stronger evidence for the role of XCMD in generating incompatibilities would come from finding currently active XCMD systems that create incompatibilities when crossed into otherwise compatible populations. A second weakness of the current evidence, is that evidence of incompatibilities from active XCMD systems have been restricted to crosses between distant laboratory strains, and may not reflect incompatibilities that occur in nature between neighbouring populations (Hauschteckjungen, 1990). Finding evidence that XCMD can create incompatibilities between neighbouring populations, between which immigration is ecologically reasonable, would provide stronger evidence for XCMD being important in contributing to reproductive isolation in nature.

Examining how locally co-evolution occurs between populations is important to understanding incompatibilities driven by genomic conflict. Testing where there is evidence for suppression, and where there is evidence for incompatibilities is a first step towards this. It is known XCMD can spread through populations. Would we expect suppression to evolve and track XCMD as it spreads, as was the case in *Drosophila simulans* (Bastide et al., 2011)? Alternately, suppression could evolve *de novo* in different populations exposed to either the same or different drivers, a pattern consistent with the two X-chromosome drive variants, one of which is locally suppressed, in *D. paramelanica* (Stalker 1961?). These independent evolutions of suppression could result in rapid divergence between populations and loci that are important for spermatogenesis and the suppression of drive, potentially contributing to incompatibilities between populations. These scenarios, one which sweeps a single suppressor across populations and the other that drives independent evolutions of suppression, could differ in their consequences.

The XCMD system in *D. subobscura* (henceforth referred to as 'SR*s*') presents an opportunity to examine these questions in natural populations. In contrast to systems where an XCMD is completely suppressed and only becomes active when crossed into distantly related populations, SR*s* shows active drive in the North African populations where it occurs (Jungen, 1967, Jungen, 1968). Co-evolution between SR*s* and suppressors is likely to be occurring in these populations, because different genotypes express different strengths of drive in Tunisia, ranging from 85-100% (Hauschteckjungen, 1990; Chapter 6). In Tunisia, SR*s* is always associated with an inversion complex covering the majority of the sex-chromosome ($A_{2+3+5+7}$) (Jungen, 1967). This arrangement has been found in other areas of North Africa and southern Spain (Prevosti, 1974, Sole et al., 2002). In addition, the XCMD sex ratio phenotype has recently been confirmed in Morocco and Southern Spain (Chapter 6). Currently, it is unknown if these drivers emerged from a single origin and subsequently spread, or represent separate evolutionary transitions. The observation of XCMD in Southern Spain could be an interesting case of a driver moving into previously unexposed European populations, possibly related to the ongoing spread of southern high temperature adapted *D. subobscura* genotypes towards northern Europe in response to climate change (Balanya et al., 2006). Alternately, drive in Spain and Morocco could represent distinct independent evolutions of XCMD. In light of the strong incompatibilities that are known to occur between the Tunisian SR*s* and Spanish populations (Chapter 6), further examining SR*s* in populations from North Africa and southern Spain is important to understand how they might persist and spread.

This paper aims to answer three questions. First, is there any evidence for incompatibilities of SR*s* from Tunisia in populations from Morocco, like those found when SR*s* is expressed on European genetic backgrounds (Chapter 6)? Second, is there evidence of suppression of SR*s* in Moroccan populations and if so what is the phenotype of this suppression like? If suppression to drive evolved independently in Morocco it might create incompatibilities with Tunisian SR*s*, whereas shared suppression across North Africa would be less likely to cause this. Third, does the X chromosome

of males that express drive in Morocco and Spain cluster with SR*s* X chromosomes from Tunisia, suggesting a single origin of drive in *D. subobscura,* and do they show signatures of recent selective sweeps indicative of a conflict?

7.3 Methods

Wild fly collections and screening SR*s* phenotype

Flies were collected from three populations; Tabarka, Tunisia (36.57_N, 8.45_E) and Punta Umbria, Spain (37.10_N, 6.57_W) in April 2013 (Verspoor et al., 2015) and Azmizmiz, Morocco (31.19_N, 8.25_W) in April 2016. Flies were caught from wild populations using banana, yeast and beer baits for collections at all three locations (Markow and O'Grady, 2005). Wild-caught females were brought into the laboratory and their offspring highly inbred to create isofemale lines (David et al., 2005b), which captures wild genotypes and minimizes adaptation to the laboratory. Wild caught males were mated to a laboratory female to measure the sex-ratio of the offspring they produce. A sex-ratio of >85% females was used to assign status of SR*s* to a male (Hauschteckjungen, 1990). One Tunisian male that was found to carry SR*s* was then mated to a daughter from a wild caught Tunisian female, to produce an isolate of SR*s* that could be kept as set of inbred lines in the laboratory (Verspoor et al., 2016), and used for the testing of the phenotype in the Moroccan population (outlined below).

Testing for incompatibilities and suppression of Tunisian SR*s* in Moroccan genetic backgrounds

The Tunisian SR*s*, outlined above, was crossed into 11 different isolines from Amizmiz to generate experimental males. These males are 50/50 Tunisia/Morocco hybrids, and carry a range of genetic backgrounds from the natural population in Morocco (Supplementary figure 7.1). SR*s* on a Tunisian background was used as a control for comparison of the strength of drive and offspring production. For each isoline and the Tunisian control 25 replicates were set up to measure incompatibilities and drive strength. Each of the Tunisian/Morocco SR*s* bearing male, and the pure Tunisian SR*s* males, was mated to a virgin Tunisian female collected from an outbred mass population originating from a mix of Tunisian isolines. All flies used were seven days old, to ensure sexual maturity (Holman et al., 2008). Males were paired with females for seven days to allow egg laying, then the parents were removed.

Pairs were stored on a 12:12 light dark cycle at 18°C. Seven days after the onset of eclosion, the number of offspring was counted and analysed using ANOVA and Tukey's post hoc tests. The sex-ratio was analysed using a binomial GLM, with the control line as the baseline sex-ratio for comparison. All analyses were carried out in R (R core R development team, 2011).

DNA sequencing and analysis

Six gene regions spanning the X-chromosome were sequenced. A previous study which used FISH confirmed that these all mapped to the X-chromosome in *D. subobscura* (Segarra and Aguade, 1992). Figure 7.3 shows where the expected regions fall in the chromosome based upon this mapping. Individuals had their DNA extracted using qiagen DNeasy blood and tissue kits. These loci were amplified for 25 SR*s* and 25 non-SR*s* males, collected from the three populations (Spain n=10, Tunisia n=18, Morocco n=21); All loci were amplified using standard PCR and Sanger sequencing methods (Supplementary table 7.1). SNPs in the region were called using the software Geneious version 7.1.3.

Analysis of sequence variation

There was evidence of potential recombination at the loci zeste, with three SR*s* individuals showing haplotypes shared with ST*s* (Figure 7.3). Therefore five loci were concatenated across the X-chromosome to produce the relationship between SR*s* and ST*s*. The optimal model of evolution was evaluated using the AIC score in MEGA (Tamura et al., 2011). A maximum-likelihood phylogenetic tree was then estimated in MEGA (SR*s* = 24 individuals, non-SR*s* = 25 individuals) (Tamura et al., 2011). Separate phylogenetic trees for each of the loci were also produced (Supplementary Figures 7.2-7). All trees were bootstrapped for 1000 replicates. For each locus, statistics of genetic diversity were examined for drive and non-drive males. Basic statistics for segregating polymorphic sites, genetic diversity, haplotype number and evidence of recombination were calculated in DNAsp (Librado and Rozas, 2009). Patterns of genetic differentiation were calculated using K_{ST} and Snn (Hudson, 2000, Hudson et al., 1992) in DnaSP (Librado and Rozas, 2009) using both geographic sampling location and SR*s* and

non SR*s* as groupings. 1000 random permutations were used to estimate the significance of individual loci (Librado and Rozas, 2009).

7.4 - Results

Testing for incompatibilities and suppression of XCMD in a Moroccan population

Hybrid crosses between Moroccan isolines and the Tunisian driver did not differ significantly in the number of offspring they produced when compared to a fully Tunisian background ($F_{11,245}$, p = 0.318; figure 7.1A). There were no significant differences between any of the isolines using Tukey's post-hoc tests (all p > 0.3). The strength of drive in the Moroccan hybrid flies was consistently strong, however there was a significant effect of background (chi.sq=30.07 df = 11 p = 0.002, figure 7.1B), which was entirely driven by one cross. There were some notable numbers of males produced in this cross, with a mean suppression of ~7% across all crosses from this line. This is consistent with the variability in drive suppression found in Tunisia.

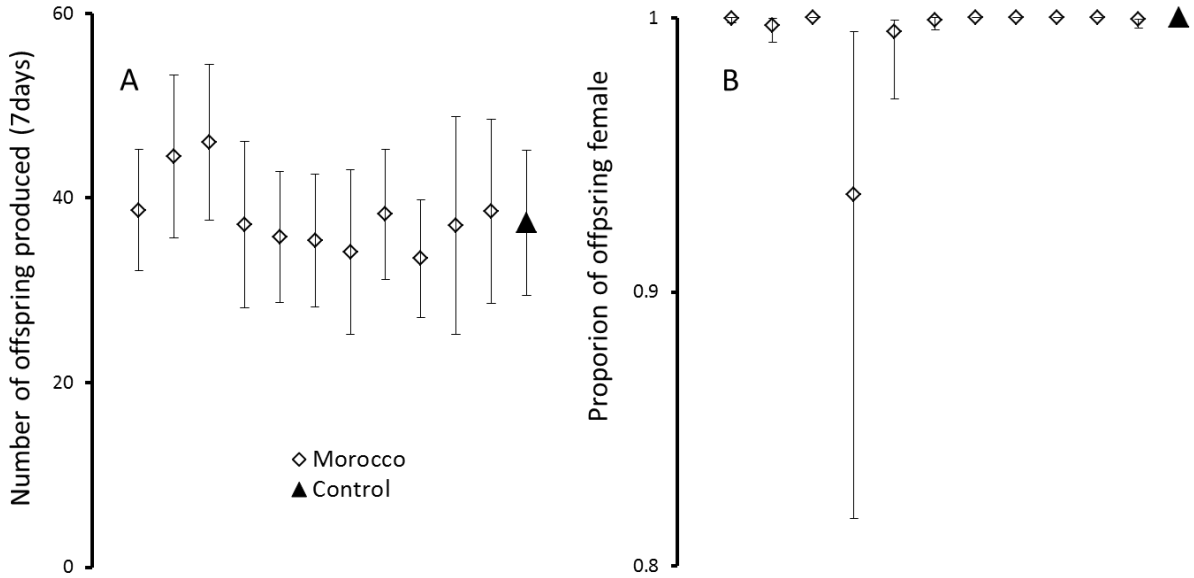

Figure 7.1 (A)Total offspring produced by hybrid Moroccan/Tunisian males carrying the Tunisian SR*s* (diamonds) and pure Tunisian males carrying SR*s* (triangle). (B) Proportion of offspring that are female from hybrid Moroccan/Tunisian males carrying the Tunisian SR*s* (diamonds) and pure Tunisian males carrying SR*s* (triangle). Points indicate the mean number of offspring produced, while error bars indicate 95% confidence intervals.

Sequence analysis of 6 X-linked regions across three populations containing SR*s*

Examining the phylogenetic tree estimated from the concatenated data of the five regions reveals all the SR*s* individuals, irrespective of geographic location, fall into a monophyletic group in which there is little sequence variation (Figure 7.2). This node is also one of the few nodes in the tree that is supported by a bootstrap value of greater than 50. In general, there is a lack of resolution, in terms of confidence scores, about the relationship of different non-SR*s* individuals to each other. The tree also shows that there is greatly reduced variation within SR*s* individuals. Indeed, a number of individuals that were from populations collected in Morocco and Tunisia, sites greater than 1000km apart, shared exactly the same genotype at all locations across the SR*s* X-chromosome. When examining trees individually no consistent pattern emerges between SR*s* and non-SR*s*, with some non-drive individuals being group together with the SR*s* grouping. However, there is no strong support for these branching structures.

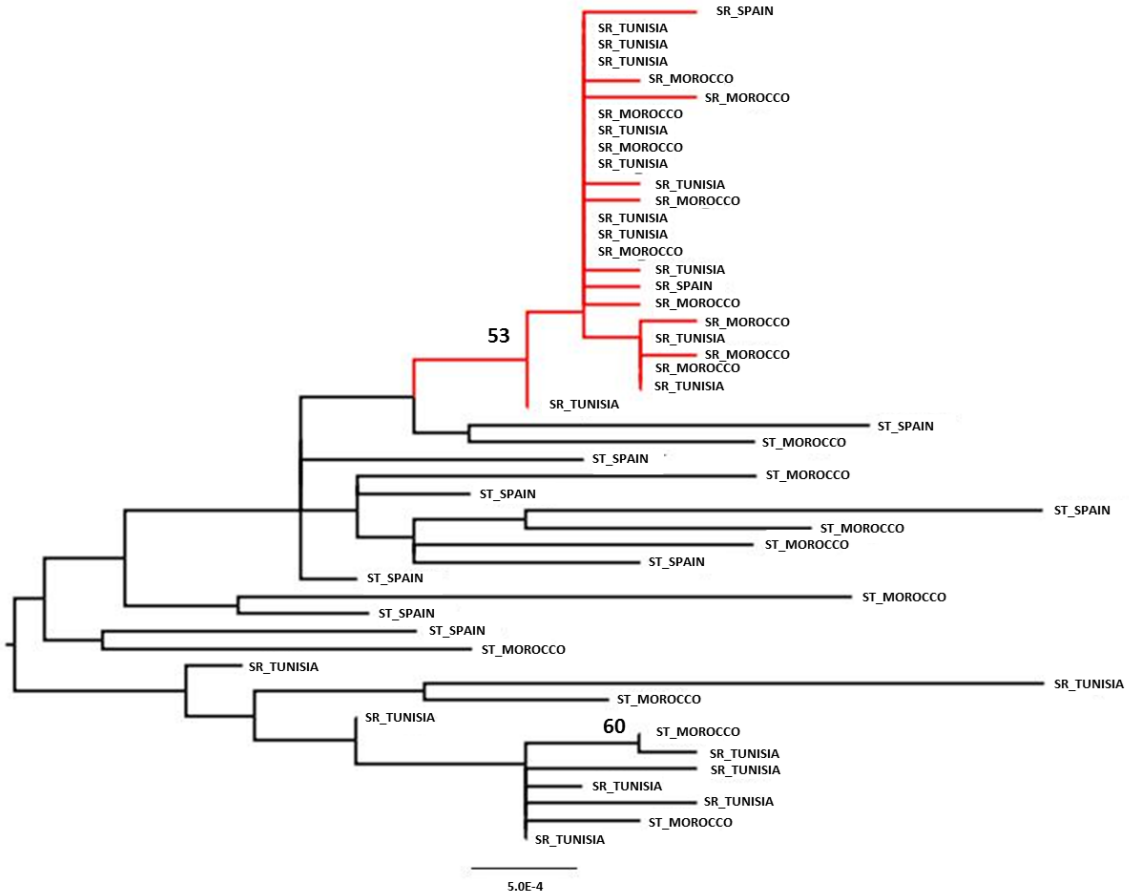

Figure 7.2 A maximum-likelihood phylogenetic tree produced from the concatenated sequence of five regions across the X-chromosome, estimated using the Tamura-Nei model (G+I). Bootstrap values of greater than 50 are included at branch nodes. Branches in red lead to individuals carrying the SR*s* phenotype.

SR*s* have low diversity compared to non-SR*s* individuals across all regions of the X-chromosome (Figure 7.3; supplementary table 7.2). This is also mirrored in there being lower haplotype diversity in the SRs group. There are very few shared polymorphisms (one or less) , with the exception of the Zst region (8 shared polymorphisms) (supplementary table 7.2) There are a large number of singletons within all of the X-chromosome regions except the *Zeste* region, which results in lower Tajima's D values than for non-SR*s*. However the only significant value is for the *white* region, which is likely due to the complete lack of variation at many of the other regions (Supplementary table 7.2).

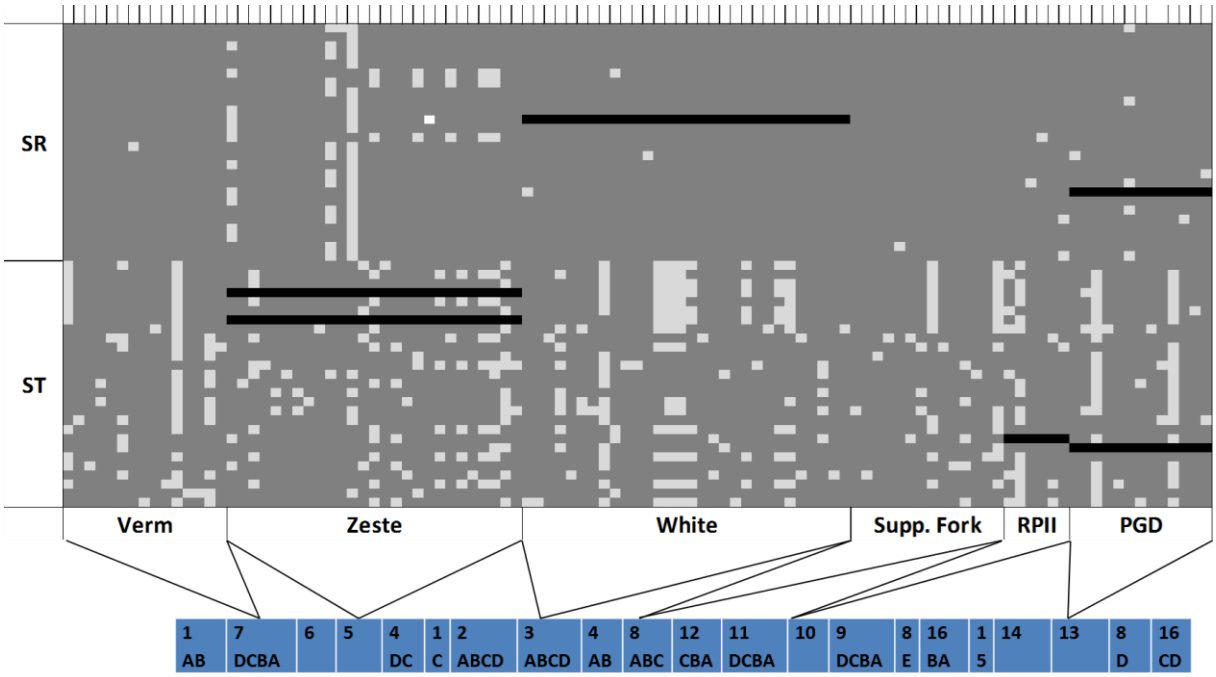

Figure 7.3 A summary of all the SNP variation across the six regions split by SR*s* and non-SR*s* phenotypes. Dark grey and light grey indicate different nucleotide states. Black areas indicate where the marker has failed to amplify for individuals. Below the plot is the structure of the inverted drive chromosome arrangement reported in (Jungen, 1967, Krimbas, 1993). Placement of the markers reflects FISH mapping (Segarra and Aguade, 1992)

There was highly significant differentiation found between SR*s* and non SR*s* grouping at each individual locus across the chromosome by both measures, Kst and Snn, when tested using 1000 replicates (Supplementary table 7.3). Between populations, including both SR*s* and non-SR*s* flies, significant

differentiation was only found at one out of six of the loci for Kst and Snn respectively, using the same test of 1000 replicates (Supplementary table 7.3). However, this test might not be a fair evaluation of divergence between the populations, because our inclusion of the large number of genetically similar SR*s* might artificially reduce divergence.

To remove this bias, differentiation between populations was then examined using only non-SR*s* individuals. The number of loci showing significant differentiation by population rose to three and one, for Kst and Snn respectively. However, all of these measures of differentiation were still lower than for those between SR*s* and non-SR*s.* When comparing all six regions differentiation grouped by SR*s* vs Non-SR*s* was significantly greater than by population both when all samples were included (Kst - t = 8.337, df = 7.949 p = 0.001; Snn - t=11.562, df= 9.568 p<0.001) and for only non SR*s* samples (Kst – t = 2.389, df = 9.998, p = 0.038; Snn – t = 9.105, d.f.=9.903, p<0.001) (Figure 7.4).

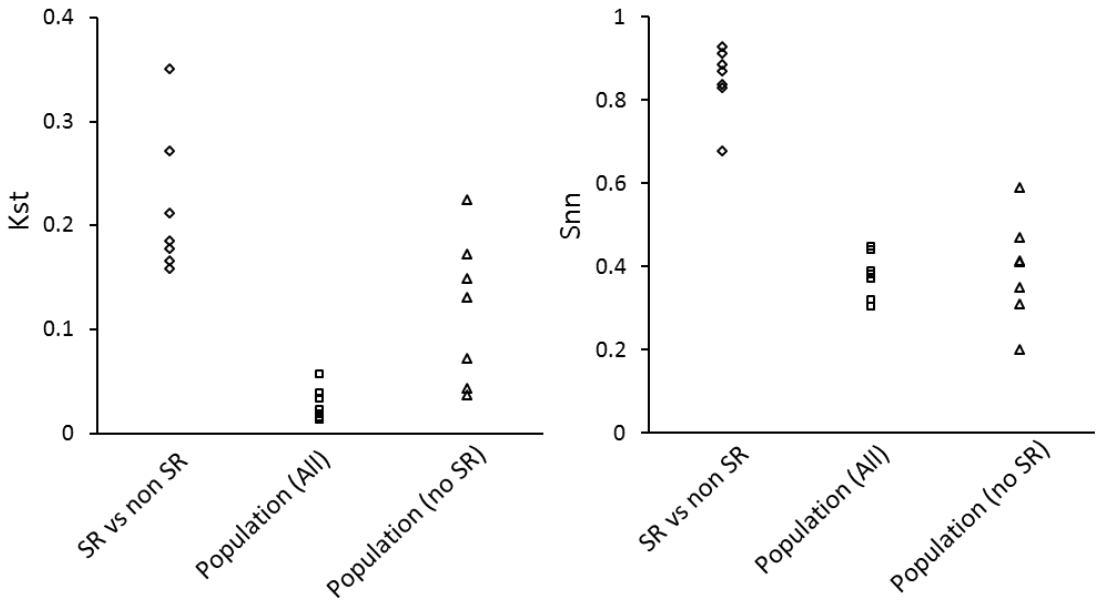

Figure 7.4 Plots showing two measures of differentiation, Kst and Snn, split by the X-chromosome type and pooling all populations (SR*s* vs Non SR*s* indicated by diamonds), or by the three populations, Spain, Tunisia and Morocco, while pooling SR*s* and non-SR*s* (indicated by squares) or by populations with only non-SR*s* individuals (indicated by triangles). Each point represents a different locus from the X-chromosome.

7.5 - Discussion

Incompatibilities and suppression of Tunisian XCMD in a Morocco

SR*s* from Tunisia was crossed into different Moroccan genetic backgrounds to test for incompatibilities, similar to those observed previously in crosses to European populations (Hauschteckjungen, 1990; chapter 6). We found no evidence of reduced offspring production when SR*s* occurred in hybrid Tunisian/Moroccan males suggesting complete compatibility of SR*s* with Moroccan genetic backgrounds. This is in contrast to the strong incompatibilities found when Tunisian SR*s* was crossed into genetic backgrounds from southern Spain consistent with the incompatibilities only occurring between SR*s* and European populations (Chapter 6).

We found evidence for very weak suppression of drive in Moroccan populations, comparable to those previously reported for Tunisian populations (Jungen, 1967; Chapter 6). These results are consistent with a single origin of SR*s* that spread through populations in North Africa, followed by either the spread or independent evolutions of weak suppressors in North Africa. However, if these suppressors are independently evolved, they do not result in obvious incompatibilities between Tunisia and Morocco. There are not thought to be barriers to gene flow between different European populations of *D. subobscura,* instead local variation is thought to be maintained predominantly by selection (Pegueroles et al., 2013). This could well be the case for the North African populations in Tunisia and Morocco, where the foothills of the Atlas Mountains may provide a continuous, if fragmented, zone of habitat. This is consistent with North Africa clustering together based upon karyotypes when compared to Europe (Krimbas, 1993). In this case there would be no barrier to prevent SR*s* and its suppressors spreading through North Africa, despite the large geographic distance. XCMD and concurrent suppression has been reported to have spread across large areas of the range in *D. simulans* (Atlan et al., 1997).

Currently we lack good estimates for genetic structure either within North African populations, or between North African and European populations. Large differences in karyotype frequencies and types have previously been reported (Prevosti, 1974b, Krimbas, 1993) in comparison to differences between European populations. It is possible that historically *D. subobscura* was separated into smaller populations in plant refugia during previous glaciations (Medail and Diadema, 2009). This combination of population structure imposed by glacial refugia and population specific evolution driven by genomic conflict could have aided the evolution of incompatibilities with SR*s* on secondary contact with Europe. Indeed, two large refuge areas are identified very close to, or encompassing where SR*s* occurs in North Africa. Generating a broader understanding of genetic relationships between North African and European populations, including understanding how different inversions maintain genetic differentiation and how differences in inversion types in North Africa are maintained will require further research.

Genetic analysis of XCMD and non-drive individuals

We found the most parsimonious explanation for sequence diversity on the X chromosome was monophyly for all SR*s* individuals, notwithstanding their collection from the three sites across Tunisia, Morocco, and Spain. Again, this observation supports a single evolution of SR*s* that subsequently spread in North Africa. Reduced genetic diversity and excess of singletons found within the drive individuals is consistent with a relatively recent evolution of SR*s* or a recent sweep has recently removed the majority of variation in the SR*s* chromosome, with sporadic low frequency variants arising since the last sweep. However, the reduced diversity in the SR*s* group could also partly a result of its reduced effective population size due to its low frequency (0-30%). This inference is also supported by us finding individuals that share their entire haplotype across all six regions across the X-chromosome, despite being sampled from populations that are greater than 1500km apart. However, there were no segregating variants that were fixed in to either SR*s* and non-SR*s* individuals in the regions examined. Greater resolution on the origin of SR*s* in North Africa might be provided by more variable markers such as microsatellites, or non-coding regions of the genome.

In the absence of any exchange and in light of their reduced population size, drive chromosomes are expected to build up a severe genetic load of deleterious mutations over time (Dyer et al., 2007). It has been suggested that even small amounts of genetic exchange may be important for allowing selection to remove deleterious mutations from the drive chromosome (Pieper and Dyer, 2016). We do find some evidence for recombination events contributing to genetic exchange between XCMD and non-XCMD individuals in one region. It could be that some segregating inversion types allow recombination over small parts of the X-chromosome. The restriction of SR*s* within North Africa to only moderate frequencies suggests something is preventing it from sweeping to fixation. In the XCMD system in *D. subobscura,* there is no evidence of lethal mutations as homozygote females are viable and fertile (Verspoor et al., 2016), however, more subtle fitness costs, either in XCMD males, or in drive carrying females remain to be examined. Other systems have reported abiotic variables correlated with XCMD frequency (Dyer, 2012). More generally, inversions clines in *D. subobscura* have been reported to be under environmental selection (Balanya et al., 2006). However, this remains to be examined in relation to SR*s* frequency in North Africa.

<u>An incipient system of suppression and incompatibility driven by genetic conflict</u>

Our results are consistent with the recent emergence of a driver that has spread in North Africa, resulting in weak suppression co-evolving in response within these populations. However, despite migration between North Africa and Spain (Sole et al., 2002), as well as extensive trade which has facilitated the spread of *D. subobscura* to the new world (Prevosti et al., 1989), SR*s* has failed to spread outside of North Africa. Rapid sweeping of meiotic drive chromosomes, followed by strong suppression has been documented on a continent wide scale before in the Paris system in *D. simulans* (Bastide et al., 2011). The P-element, a transposable element originating in *D. willistoni*, has also spread extremely rapidly in *D. melanogaster* achieving a global distribution (Engels, 1992, Engels, 1997). Here, we show the same meiotic drive element in *D. subobscura* is co-evolving in North Africa with

suppressors, while it is being prevented from spreading further into European populations by severe incompatibilities specific only to itself.

This case of hybrid incompatibility driven by intra-genomic conflict appears to be incipient, in which the conflict in North Africa between driving chromosome and suppression is ongoing. Meanwhile, migration is occurring from North Africa into southern Spain, and likely further afield through trade (Sole et al., 2002; chapter 6). Natural genetic exchange between populations could also increase, if North African populations behave like European ones, where locally adapted genetic assemblages are moving Northwards in response to climate change (Balanya et al., 2006). There are a number of routes through which full isolation between the North African and European populations could occur in this system in the future.

In one case, the SR*s* chromosome could increase in frequency, eventually reaching fixation in North Africa. This would mean every migrant from North Africa would carry an incompatible X-chromosome incompatible with Spanish backgrounds. Current evidence however suggests that SR*s* has occurred at similar frequencies in North Africa for at least the last 50 years and is not increasing in frequency (Jungen, 1967, Hauschteckjungen, 1990). However, the factors underlying this stability and the permanence or dynamics of SR*s* frequencies are not well understood (Verspoor et al., 2016). Factors suppressing drive in North Africa could also build up and contribute to incompatibilities if they directly contributed to incompatibilities when crossed into other populations. However, this is not consistent with incompatibilities being specific to the X-chromosome being expressed on foreign population backgrounds as we see in European populations (Chapter 6).

Severe hybrid incompatibility between XCMD and *D. subobscura* autosomes of Spanish origin could also fuel the evolution of reinforcing pre-zygotic isolation between populations. This has been observed in the *D. subquinaria/recens* system, where unidirectional cytoplasmic incompatibilities between hybrids are caused by Wolbachia (Shoemaker et al., 1999). In this case it was found that asymmetric mate choice evolved only in the populations where both species co-occurred and there was a risk of hybrid

incompatibilities (Jaenike et al., 2006b). *D. Subobscura* has a complex courtship, including the giving of nuptual gifts, suggesting female choice is important in this species (Immonen et al., 2009, Verspoor et al., 2015, Verspoor et al., 2016). If mate choice played a role in this case of hybrid incompatibilities, exposed populations in southern Spain would be expected to have evolved pre-zygotic isolation either from XCMD individuals or North African flies more generally. Meanwhile unexposed northerly European populations would not have a reason to have evolved these mechanisms of pre-zygotic isolation. SR*s* is also associated with a large inversion meaning a trait that is already important for mate selection could be differentiated and linked to SR*s*, facilitating female preference against SR*s* individuals. This has been seen in XCMD systems in stalk-eyed flies (Johns et al., 2005).

There is mounting evidence linking genetic conflict and reproductive isolation and hybrid incompatibility (Presgraves, 2010, Johnson, 2010, McDermott and Noor, 2010), suggesting it could be an underestimated driver of genetic differentiation and speciation. Developing our understanding of the genes involved in the processes underlying, SR*s*, its associated suppression in North Africa, and the hybrid incompatibilities it causes when crossed into Europe is an obvious route to further test conflictual speciation. Are the loci responsible for suppression in North Africa the same as those which are causing the incompatibilities in Europe? Will there be signatures of rapid selection at these regions suggesting recent and rapid changes in response to genetic conflict? Is there evidence of pre-zygotic isolation specific to SR*s* exposed population in Europe? These remain important questions for understanding the process of differentiation and speciation by genomic conflict.

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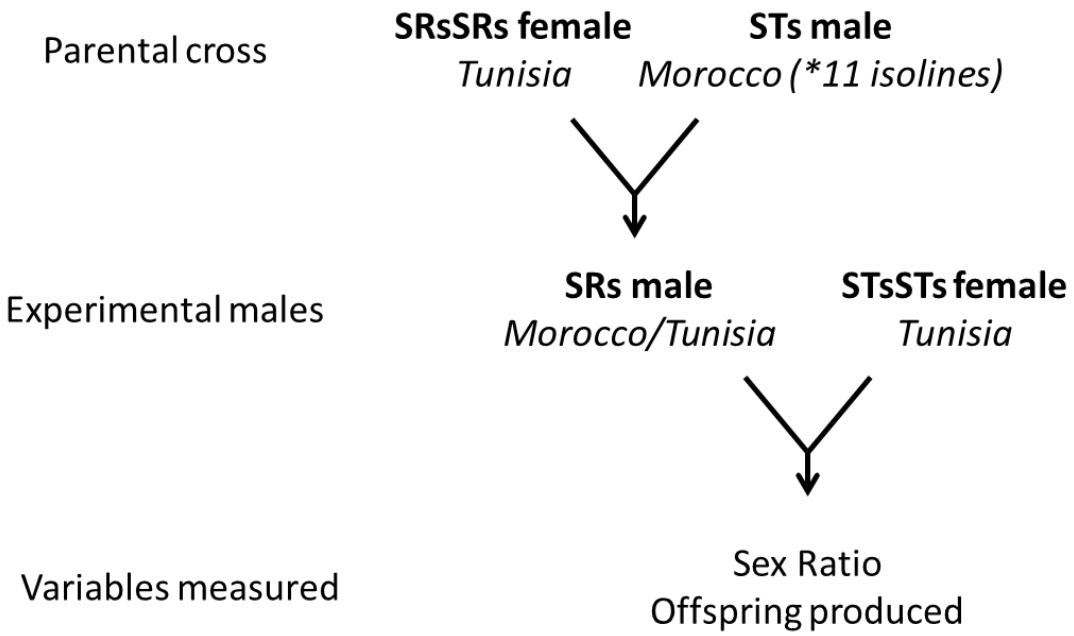
7.7 Supplementary material

Supplementary table 7.1 All loci were amplified using the same conditions. The reaction profile was an initial 3min denaturing step at 95°C, followed by 35 cycles of 95°C for 30secs, 60°C for 30 secs, 72°C for 30secs, with a final elongation period of 10mins at 72°C. PCR products were visualized using gel electrophoresis on a 2.5% agarose gel with 3µL Midori green per 100mL of TAE buffer. PCR products were cleaned using antarctic phosphatase and exonuclease 1, with an incubation of 45mins at 37°C followed by 15mintes at 80°C. Sequencing products were amplified using BigDye3.1 protocol with a sequencing program of 35 cycles of 96°C for 10secs, 50°C for 5secs, 60°C for 4mins. Sequencing was precipitated using 3M sodium acetate and cleaned with 70% ethanol. 10ul of Hi-Di formamide was then added and sequencing was carried out on and ABI3500xL genetic analyser.

Gene Name	Forward primer	Reverse Primer
Zeste	CGGTGGCTCGAATAAAACACATC	TGATCTGCAGTATGATCTCCTCG
White-1	CCAAGAACTACGGCACCCTG	CCATTAGCAGGATCTTGTCGAAG
Vermillion	GCCACTGGACTTTATGGACTTTC	CTCTTGTGGACGAGCTTGCT
Su(f)-1	TACAACATTGAGTCTTGGTCGGT	GGAACTGTGATGCCTGGTG
RPII-1	AAAGTTGGGTGGCCTCATGG	GGTCAAGTTCTGGGCTATCGAA
Pgd	GCCCCTGATCAAGCCCATC	CAGTTGGTGTGATGGAACTTGC



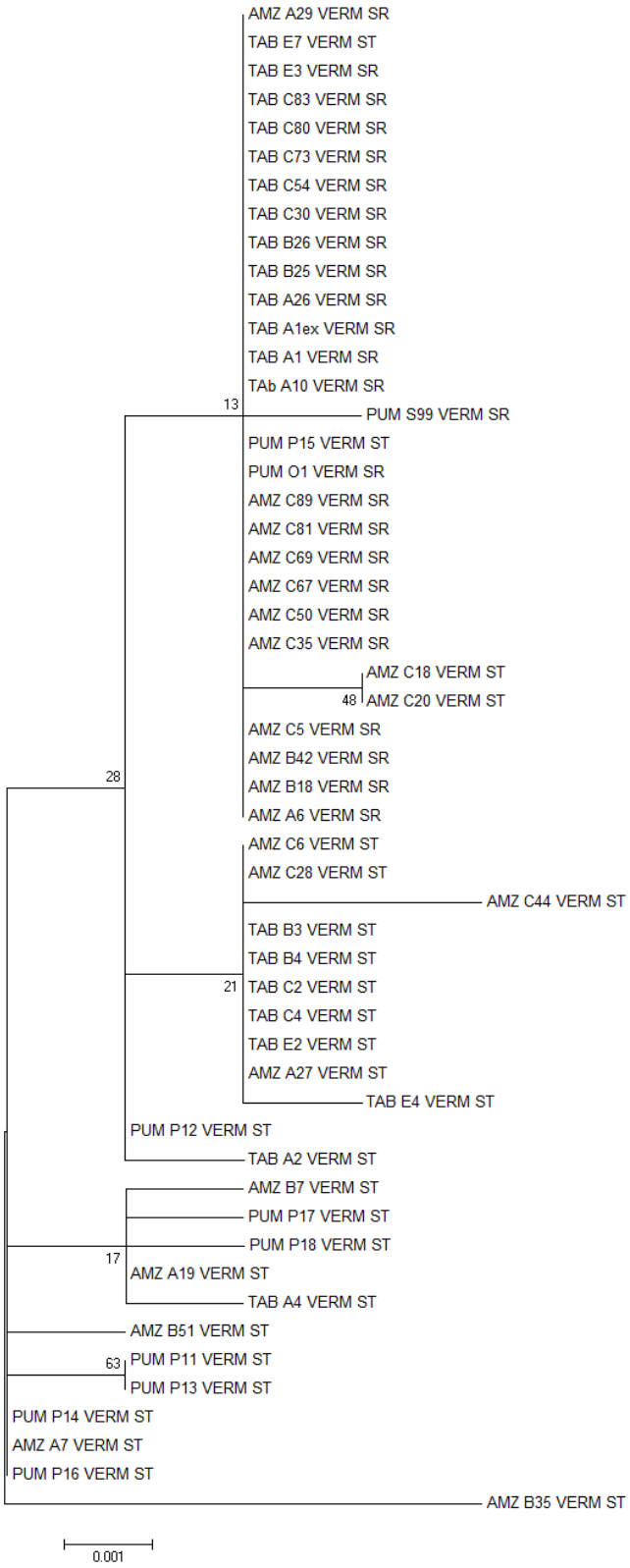
Supplementary figure 7.1 A crossing schematic for establishing 50:50 hybrids males, on Tunisian and Moroccan backgrounds.

Supplementary table 7.2 Summary of sample sizes, genetic diversity statistics, haplotype data, and tajima's D for the 6 regions across the X-chromosome.

Region	Type	n	Variable sites (singletons) (shared*)	Pi	Theta	Haplotype Number	Haplotype Diversity	Tajimas D
White	SR	26	10 (9) (1*)	0.0012	0.0036	5	0.289	-2.2236
	ST	26	28 (13) (1*)	0.0891	0.0102	17	0.917	-0.4672
PGD	SR	25	4 (3) (1*)	0.0008	0.0002	6	0.567	-1.4513
	ST	26	10 (5) (1*)	0.0021	0.0004	11	0.837	-1.213
Zeste	SR	26	10 (2) (8*)	0.0034	0.0037	6	0.671	-0.2409
	ST	26	25 (10) (8*)	0.0080	0.0092	21	0.967	-0.4907
Suppressor forked	SR	26	1 (1) (0*)	0.0001	0.0003	2	0.077	-1.1556
	ST	27	13 (6) (0*)	0.0030	0.0042	14	0.806	-0.9583
Vermillion	SR	25	1 (1) (0*)	0.0001	0.0003	2	0.077	-1.1556
	ST	25	14 (9) (0*)	0.0033	0.0047	16	0.906	-0.9981
RPII	SR	26	4 (3) (1*)	0.0005	0.0013	5	0.351	-1.7071
	ST	26	4 (1) (1*)	0.0014	0.0013	8	0.754	0.26114

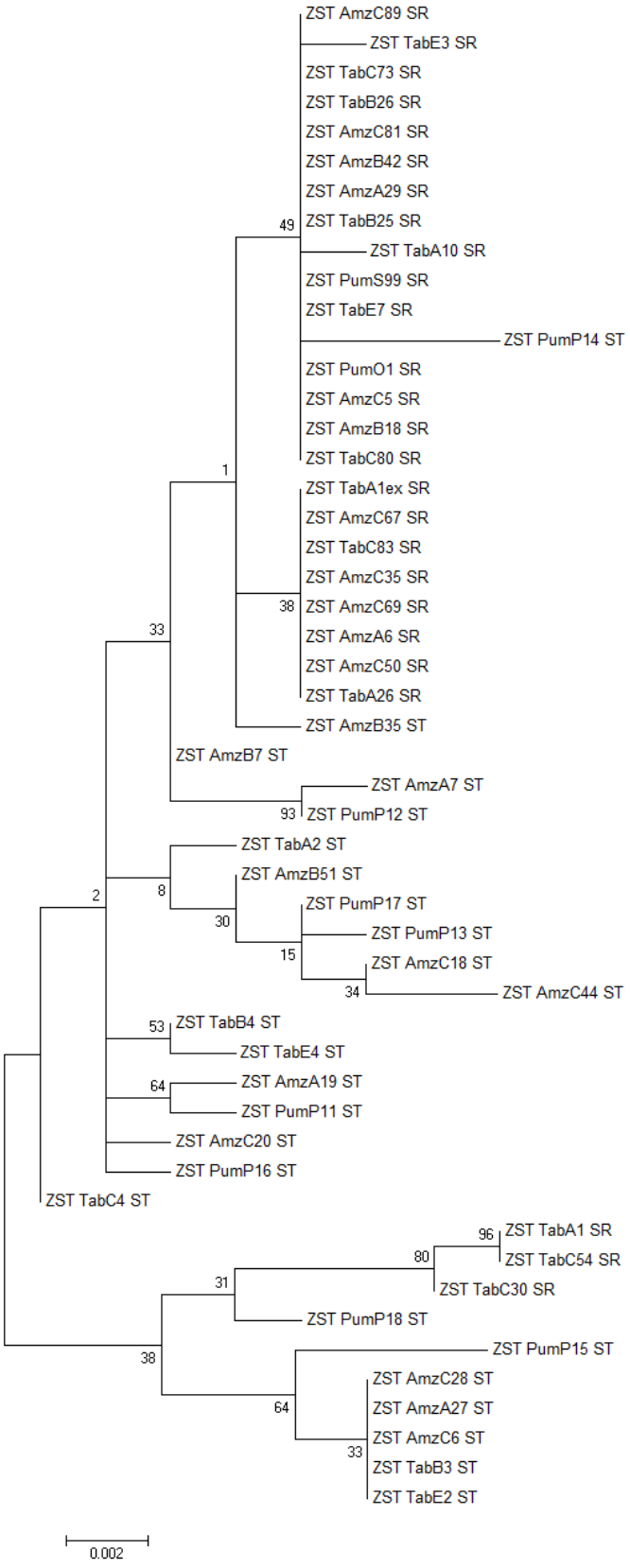
Supplementary table 7.3 Summary of genetic differentiation statistics Kst and Snn, as calculated in DNAsp (Librado and Rozas, 2009). Comparisons are for SR*s* vs non-SR*s* males, the three populations including all individuals and the three populations for only non-SR*s* individuals.

Region	Kst SRvs ST	Kst Pop.	Kst Pop. (No SR)	Snn SR vs ST	Snn Pop.	Snn Pop (No SR)
White	0.184*** (p<0.001)	0.015$^{n.s.}$ (p=0.179)	0.171** (p=0.003)	0.869*** (p<0.001)	0.368$^{n.s.}$ (p=0.325)	0.413n.s. (p=0.070)
PGD	0.349*** (p<0.001)	0.023$^{n.s.}$ (p=0.123)	0.042$^{n.s.}$ (p=0.948)	0.837*** (p<0.001)	0.302$^{n.s.}$ (p=0.876)	0.199$^{n.s.}$ (p=0.972)
Zeste	0.157*** (p<0.001)	0.013$^{n.s.}$ (p=0.808)	0.036$^{n.s.}$ (p=0.898)	0.928*** (p<0.001)	0.389$^{n.s.}$ (p=0.241)	0.307$^{n.s.}$ (p=0.598)
Supp Forked	0.176*** (p<0.001)	0.033$^{n.s.}$ (p=0.083)	0.223*** (p<0.001)	0.829*** (p<0.001)	0.381$^{n.s.}$ (p=0.178)	0.408$^{n.s.}$ (p=0.086)
Verm	0.270*** (p<0.001)	0.057** (p=0.009)	0.129*** (p=0.005)	0.885*** (p<0.001)	0.438* (p=0.012)	0.469* (p=0.027)
RPII	0.165*** (p<0.001)	0.018$^{n.s.}$ (p=0.818)	0.071$^{n.s.}$ (p=0.091)	0.676*** (p<0.001)	0.318$^{n.s.}$ (p=0.860)	0.347$^{n.s.}$ (p=0.275)
All	0.211*** (p<0.001)	0.039$^{n.s.}$ (p=0.056)	0.148*** (p<0.001)	0.910*** (p<0.001)	0.447$^{n.s.}$ (p<0.055)	0.587*** (p<0.001)

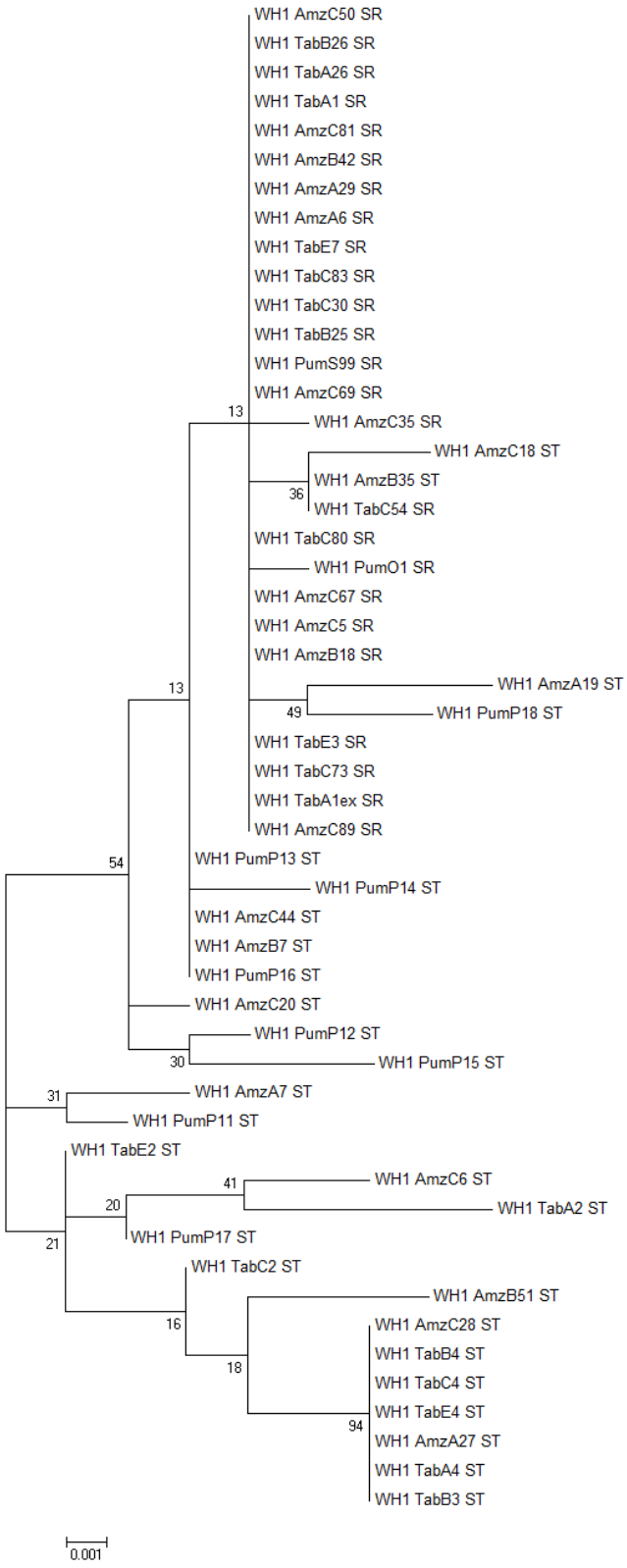


Supplementary figure 7.2 Maximum likelihood tree using the Tamura 92 (+G) model of evolution, with 1000 bootstrap resampling for the Vermilion

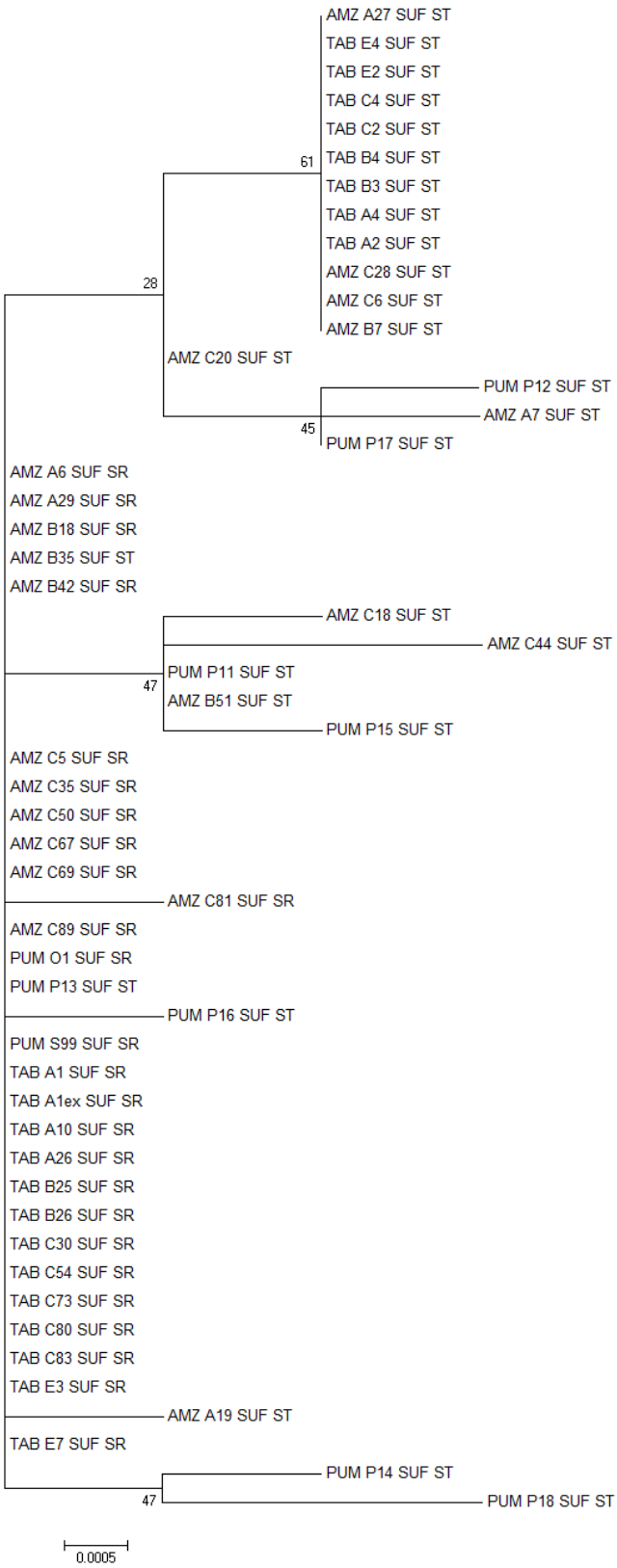
gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SRs and non-SRs individuals are labelled SR and ST respectively.



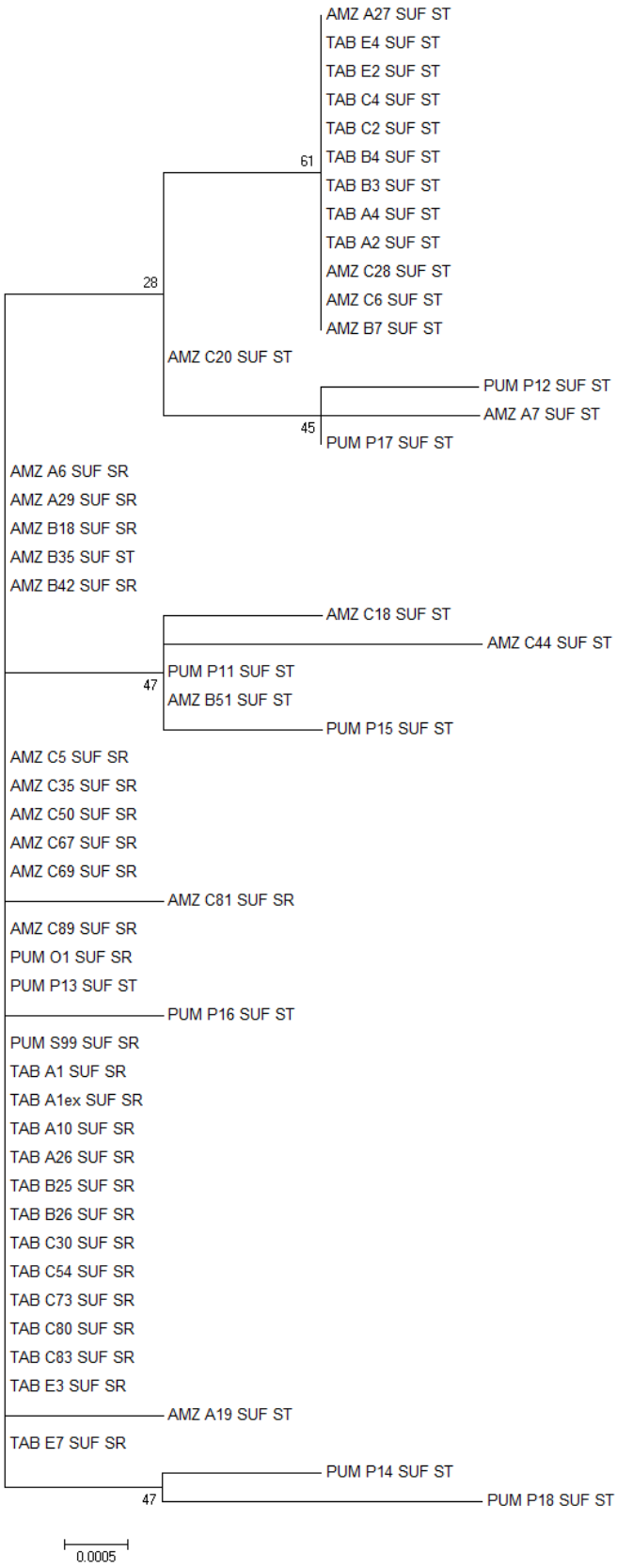
Supplementary figure 7.4 Maximum likelihood tree using the Jukes-Cantor (+G+I) model of evolution, with 1000 bootstrap resampling for the Zeste gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SRs and non-SRs individuals are labelled SR and ST respectively.



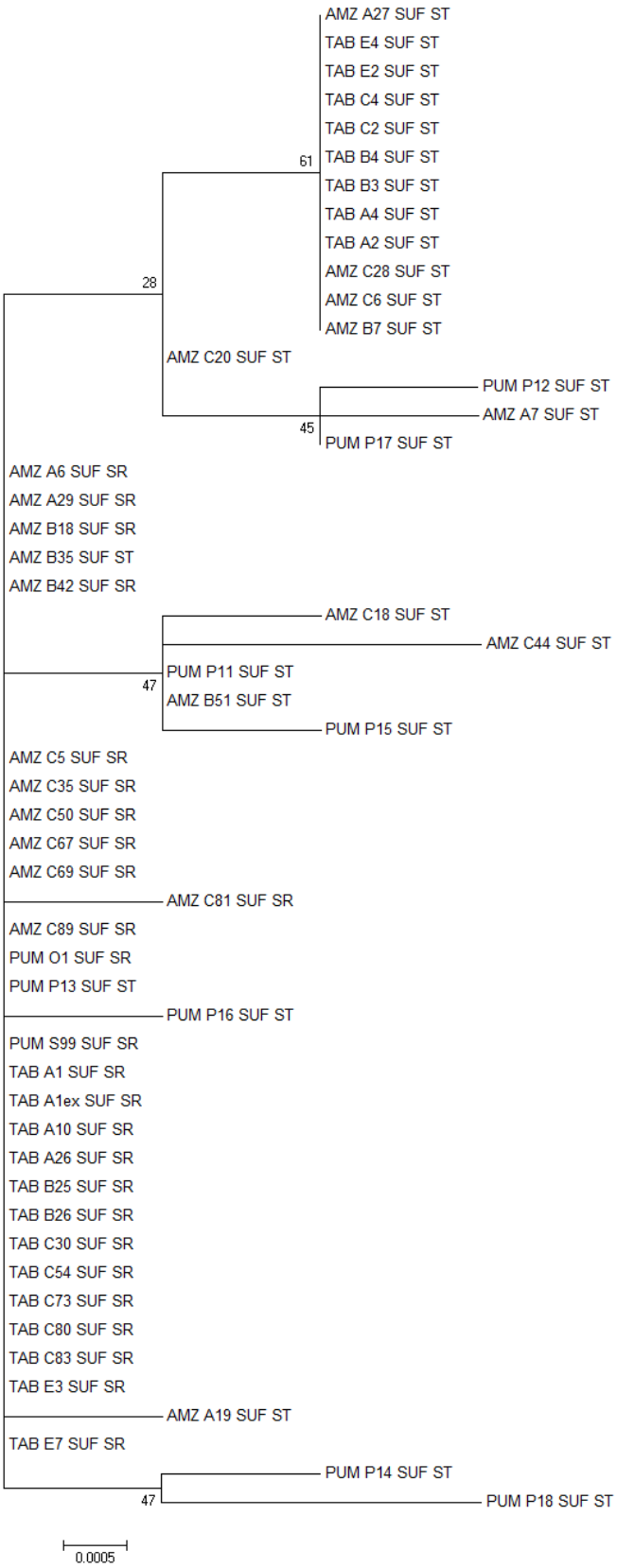
Supplementary figure 7.4 Maximum likelihood tree using the Tamura 92 (+G) model of evolution, with 1000 bootstrap resampling for the White gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SRs and non-SRs individuals are labelled SR and ST respectively.



Supplementary figure 7.5 Maximum likelihood tree using the Tamura 92 (+G) model of evolution, with 1000 bootstrap resampling for the Suppressor of forked gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SR*s* and non-SR*s* individuals are labelled SR and ST respectively.



Supplementary figure 7.6 Maximum likelihood tree using the Tamura-Nei (+G) model of evolution, with 1000 bootstrap resampling for the RPII gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SRs and non-SRs individuals are labelled SR and ST respectively.



Supplementary Figure 7.7 Maximum likelihood tree using the Tamura 92 (+G) model of evolution, with 1000 bootstrap resampling for the PGD gene. Three populations are labelled (Tunisia – Tab, Spain – Pum, Morocco – Amz). SRs and non-SRs individuals are labelled SR and ST respectively.

8 – Characterising the testes proteome of males that carry a sperm-killing selfish gene and males that do not, in two species of fly.

This part of my thesis has been collaboration with Prof. Rob Beynon and Dr Lynn McLean at the centre for proteomics in the University of Liverpool. I performed the dissection and removal of testes. Further processing, running of the proteomics raw analysis was carried out in the centre for proteomics. Further analysis of the list of proteins and discussion was performed by myself.

This chapter will form the main body of a manuscript currently in preparation for *scientific reports*

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8.1 - Abstract

Sperm-killing meiotic drive is a type of selfish genetic element (SGE) where a chromosome sabotages sperm that carry its sister chromosome during spermatogenesis, thus gaining a transmission advantage to the next generation. The conflict these chromosomes create in the genome have important impacts on the evolution and ecology of species. These selfish chromosomes can also spread rapidly through populations making them of interest for designing methods of genetically modifying wild species, particularly for pest and vector control. However, our current understanding of the molecular functioning of wild systems remains restricted to a few well studied examples. Expanding our knowledge beyond these model systems could help elucidate general patterns about how sperm-killing evolves, and identify gene networks and molecular pathways that are vulnerable to this selfish behaviour. Mass spectrometry based proteomics offers an exciting approach to explore the network of proteins involved in sperm-killing chromosome meiotic drive and identify novel genes of importance to this process. We characterised the proteomes of testes from *Drosophila pseudoobscura* and *D. subobscura* males, which either carried a selfish sperm-killing X-chromosome meiotic driver or did not. Overall proteomes of the testes of each species was characterised, giving a proteome of 1612 and 1170 proteins for *D. pseudoobscura* and *D. subobscura* respectively. The testes proteomes of the two species shared over 800 proteins. Within each species 73 and 67 proteins showed significant differential expression between drive and non-drive testes for *D. pseudoobscura* and *D. subobscura* respectively. However, there was little overlap between the species in the proteins that differed strongly between drive and non-drive individuals. We present subsets of genes of interest for both species and highlight candidate proteins for further investigation. These include some proteins already implicated in other segregation distortion and sperm-killing meiotic drive systems.

8.2 - Introduction

Selfish genetic elements (SGEs) are both diverse and widespread in the tree of life (Burt and Trivers, 2006). Sperm-killing meiotic drive is a particular type of SGE, where one chromosome kills or disables its sister chromosome during spermatogenesis, thereby ensuring it is passed to more offspring than expected by fair Mendelian inheritance (Lindholm et al., 2016). This 'unfair' pattern of inheritance creates genetic conflict between sister chromosomes. If the selfish chromosome is a sex chromosome, it will bias brood sex ratios away from the most adaptive value, creating conflict between the driver and the rest of the genome. The transmission advantage of meiotic drivers can allow them to rapidly spread through populations, and for sex chromosome meiotic drivers to potentially eliminate populations due to the lack of one sex (Dyer, 2012). The conflict may also create rapid and ongoing co-evolution between driving chromosomes and loci that act to suppress the drive mechanism. This rapid evolution caused by these sperm-killing drivers, and the genetic conflict they generate can influence both the ecology and evolution of species that harbour it; including speciation (Phadnis and Orr, 2009), extinction (Pinzone and Dyer, 2013), and changes in mating behaviour (Price and Wedell, 2008). Current knowledge the mechanisms underlying drive systems comes predominantly from model organisms and their close relatives (Lindholm et al., 2016, Jaenike, 2001). However, the conflict over which sister chromosome enters each gamete or offspring is almost universal when diploid organisms produce haploid gametes. Hence sperm-killing meiotic drive could be far more widespread in nature than currently acknowledged (Lindholm et al., 2016).

Currently, the majority of our understanding of the mechanisms of sperm-killing meiotic drive works comes from a few well studied cases such as the autosomal *SD* in *Drosophila melanogaster* (Presgraves, 2007) and t-haplotype in *Mus musculus* (Lyon, 2003), and the X-chromosome *Winters sex-ratio* system in *D. simulans* (Tao et al., 2007). In the *SD* system in *D. melanogaster*, a truncated nuclear transport gene (RanGAP) disrupts gradients across the nuclear membrane, causing sperm damage (Merrill et al., 1999). The t-haplotype in *M. musculus* carrying a mutated gene ($Smok^{tcr}$)

results in distortion, with Dynein genes being strong candidates as distorters, because in this system sperm are not killed but their motility is severely impaired (Lyon, 2003). However, other unidentified distorter candidates remain in this old and complex system. In *D. simulans* the *Winters* X-chromosome sex ratio distorting driver kills Y chromosome sperm via a novel mRNA gene dox, which has also given rise to a suppressor by retrotransposition to another part of the genome (Tao et al., 2007). This system, where a gene evolved a selfish phenotype and then retrotransposed to suppress this selfish behaviour, highlights how rapidly and simply these drivers can evolve and subsequently become suppressed. These well studied systems highlight the diverse means by which sperm-killing meiotic drive can occur, with Dynein genes, the nuclear transport gene RanGAP, and a novel mRNA being implicated. However the paucity of examples prevents any synthesis of which and what types of genes are likely to evolve drive.

Until recently, molecular and cellular study of these sperm-killing systems has been restricted to model organisms. However, novel non-targeted spectrometry based proteomics offers the potential to identify differences and candidate genes or gene networks in other systems. Increasing the number of systems where we can examine the molecular networks of sperm-killing meiotic drive could pave the way to broader questions about mechanism. Are there particular aspects of cellular mechanics that are vulnerable to being exploited? Do most of these systems evolve from *de novo* genes, as is the case in *D. simulans* with the *dox* gene (Helleu et al., 2016), or do most organisms share orthologous genes that can, in mutated forms, cause drive? To answer these questions, the mechanics of a wider range of sperm-killing systems must be investigated.

Here we use non-targeted spectrometry based proteomics to investigate two species, *D. pseudoobscura* and *D. subobscura,* that have independently evolved drive. These are both X-chromosome meiotic drive systems (XCMD), in which the X-chromosome kills or disables Y-chromosome sperm (for review see Jaenike, 2001). They both drive at close to 100%, resulting in the X-chromosome being inherited by all female offspring (Sturtevant and Dobzhansky, 1936, Jungen, 1967). They are also both associated with large

inversions covering 50-80% of their X-chromosomes. However, in other respects there are considerable differences between the two systems. The *D. pseudoobscura* system is thought to be hundreds of thousands of years old (Babcock and Anderson, 1996) while what little genetic evidence there is suggests *D. subobscura* XCMD is quite young and is not strongly differentiated from its non-driving chromosome types (Chapter 7). There is no evidence for suppression of drive in the *D. pseudoobscura* system, with all genotypes showing 100% drive across its range. Why suppression has not evolved against *D. pseudoobscura* drive remains a mystery, as drive imposes significant costs on the rest of the genome via distorted sex ratios (Price et al., 2014) and reduced fertility (Price et al., 2012), and it has enormous population sizes, providing many opportunities for the evolution of suppression. In *D. subobscura*, even relatively poor sampling within the range where XCMD is found suggests weak suppression exists (Chapter 6). Interestingly, in *D. subobscura* the XCMD system is also implicated in causing inter-population hybrid incompatibilities (Hauschteckjungen, 1990; Chapter 6), however there is no evidence of this in the *D. pseudoobscura* system. Identifying the candidate genes and molecules causing drive in these two species could help explain the differences between these systems.

As spermatogenesis is incomplete for Y-chromosome sperm in these XCMD systems, we assume that most spermatogenesis related proteins will be more highly expressed in the non-XCMD testes. Proteins overexpressed in XCMD testes are particularly likely to be involved in the drive mechanism. By looking at other systems and using factors that are known from XCMD in *D. pseudoobscura* and *D. subobscura* we can further develop some expectations of the proteins that might cause drive, and be overexpressed in XCMD testes (Table 8.1). For example, novel and unique genes have been shown to cause XCMD in *D. simulans* (Helleu et al., 2016). In this case, a novel gene that is unique to only one species might stand out. Alternatively, if patterns are shared with other drive systems, then proteins that have previously been implicated in meiotic drive, or associated processes might be highlighted (for example nuclear transport, as seen in *SD*). If this is the case, then we would expect key drive proteins to be overexpressed in the XCMD

testes of both species, and possibly will have been previously characterised in drive systems of other species. In the *D. subobscura* XCMD system, there is evidence that is also causes hybrid incompatibilities when crossed into neighbouring populations of the same species. Heterochromatin formation and binding have been found to be important in other systems where there is evidence of both XMCD and hybrid incompatibilities (McDermott and Noor, 2010). Heterochromatin and chromosome condensation processes might be expected to be highlighted in the *D. subobscura* XCMD testes.

Table 8.1 Three potential scenarios and the expectations of what they might show in the proteomics screen.

Scenario	**Proteins expected to be overexpressed in XCMD testes**
The system is unique and a novel mechanism is causing drive in the species. For example, the *dox* system in *D. simulans* (ref)	A protein which is unique to only that species and is not shared across multiple databases. It is unlikely that the gene has previously been described as having a role in XCMD and may not be characterised at all.
Drive systems across *Drosophila* share common functions, genes and processes.	Proteins that are implicated in other drive systems. Enrichment for biological processes known to other drive systems (for example nuclear transport in the RanGAP system in *SD* in *D. melanogaster*).
Incompatibilities in the *D. subobscura* system share features in common with other systems where XCMD and incompatibilities have been associated with each other.	Enrichment for heterochromatin formation and binding processes. This has been identified as important in a number of other drive/incompatibility systems (McDermott and Noor, 2010).

We used liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) to examine differences in the proteins in the testes of XCMD and non-XCMD males, for both *D. pseudoobscura* and *D. subobscura*. We provide the first proteomes of the testes for these species, which are compared to the well characterised sperm and testes proteome of *D. melanogaster*. We present candidate proteins, differentiated between XCMD and non-XCMD testes, which may be involved in the cellular networks of drive and provide a comparison of the two species. This work provides the

foundation for further targeted work specific genes and proteins that cause XCMD.

8.3 - Methods

Fly stocks and testes Dissections

Original collections of *D. pseudoobscura* were from Show Low, AZ, USA (110°00′W 34°15′ N), in September 2008, and *D. subobscura* flies were collected from Tunisia in May 2013 (Verspoor et al., 2015).Two types of male, those carrying XCMD and those without, were used from each of the two species, *D. pseudoobscura* and *D. subobscura*. Inbred lines of XCMD for each species have been maintained since collection (see supplementary figure 8.1). All flies were maintained on a standard food mixture (yeast, sugar, cornmeal and agar), between 18°C and 22°C on a 12:12 light dark cycle, since collection in the wild. Males either not carrying XCMD, or carrying XCMD were collected from separate crosses (supplementary figure 8.1 (i) and (ii)). Males were aged to 4 days to ensure they would be sexually mature, before their testes were dissected (Holman et al., 2008).

Male flies were anaesthetised on CO_2 mats in groups of 10 (5 XCMD and 5 non-XCMD) for no longer than 5 minutes prior to dissection. Immediately before dissection, the thorax of the anaesthetised male was crushed to kill the fly. The testes were then dissected into a drop of PBS on a sterile petri dish. Additional material removed with the testes was gently removed. Testes were removed from the PBS and placed into a 0.5ml siliconized test tube with 10ul of 50mM Ammonium Bicarbonate in the bottom of the tube. Ten pairs of each type of testes XCMD and non-XCMD, were dissected for each of the two species in preparation for proteomic analysis. All testes were frozen at -80°C within an hour of dissection.

Proteomic Analysis

Ten pairs of testes were suspended in 50µl of 25mM ammonium bicarbonate (AMBIC) then transferred to a micro tube containing stainless steel beads. The testes were homogenised at top speed, for 15 seconds, using a Precellys Minilys homogeniser (Peqlab, Southampton, UK). 20 µl of homogenate was removed, diluted 1:2 with 25mM AMBIC containing 0.05% Rapigest (Waters, Manchester) and shaken at 550 rpm for 10min at 80°C.

The sample was then reduced (addition of 2.5µl of 60 mM DTT and incubation at 60 °C for 10 minutes) and alkylated (addition of 2.5µl of 180 mM iodoacetamide and incubation at room temperature for 30 minutes in the dark). Trypsin (Sigma, Poole, UK, proteomics grade) was reconstituted in 50 mM acetic acid to a concentration of 0.2µg/µl and 2.5µL was added to the sample followed by overnight incubation at 37 °C. The digestion was terminated and RapiGest™ removed by acidification (1µl of TFA and incubation at 37 °C for 45 min) and centrifugation (15,000 x g for 15 min). To check for complete digestion each sample was analysed pre- and post-acidification by SDS-PAGE.

For LC-MS/MS analysis, a 1µl injection of each digest, corresponding to approximately 480 ng of sample, was analysed using an Ultimate 3000 RSLC™ nano system (Thermo Scientific, Hemel Hempstead) coupled to a QExactive™ mass spectrometer (Thermo Scientific). The sample was loaded onto the trapping column (Thermo Scientific, PepMap100, C18, 300 µm X 5 mm), using partial loop injection, for seven minutes at a flow rate of 4 µl/min with 0.1% (v/v) FA. The sample was resolved on the analytical column (Easy-Spray C18 75 µm x 500 mm 2µm column) using a gradient of 97% A (0.1% formic acid) 3% B (99.9% ACN 0.1% formic acid) to 60% A 40% B over 90 minutes at a flow rate of 300 nL min^{-1}. The data-dependent program used for data acquisition consisted of a 70,000 resolution full-scan MS scan (AGC set to $1e^6$ ions with a maximum fill time of 250ms) the 10 most abundant peaks were selected for MS/MS using a 17,000 resolution scan (AGC set to $5e^4$ ions with a maximum fill time of 250ms) with an ion selection window of 3 *m/z* and a normalised collision energy of 30. To avoid repeated selection of peptides for MSMS the program used a 30 second dynamic exclusion window.

We processed the data with Progenesis (version 4 Nonlinear Dynamics, Newcastle upon Tyne, UK). Samples were aligned according to retention time using a combination of manual and automatic alignment. Default peak picking parameters were applied and features with charges from 1^+ to 4^+ featuring three or more isotope peaks were retained. Database searching was performed using Mascot (Matrix Science, London, UK). A Mascot

Generic File, created by Progenesis, was searched against the reviewed entries of the reference proteome set of *Drosophila melanogaster* from Uniprot (20043 proteins) and *D. pseudoobscura* (UniProt: UP1819; 16756 proteins) added. A fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine were specified. A precursor mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.01 Da were applied. The results were then filtered to obtain a peptide false discovery rate of 1%.

Examination and comparison of proteomes

Full lists of the whole proteomes were generated for both species. Full list were checked in Flybase for gene identifiers (Gramates et al., 2017). Multiple hits that matched the same gene were combined. The proteomes were compared to two proteomes for *D. melanogaster* sperm (Wasbrough et al., 2010) and testes (Takemori and Yamamoto, 2009) to identify shared proteins. Differences between XCMD and non-XCMD for these proteins were scored using fold-change, number of unique peptides and an ANOVA score of the difference in quantity between the two types. To examine differences in gene ontology for genes that were particularly different between XCMD and non XCMD males, GOrilla was used to examine lists ranked by their ANOVA score as a measure of expression difference (Eden et al., 2009).

Proteins of particular interest were identified from the full proteome lists, with the criteria of having a significant ANOVA score based on difference in expression between XCMD testes and non-XCMD testes, a fold change of greater than x1.5, and more than 1 unique identifying peptide, to ensure the difference in expression level was real. The unique peptide measure details the number of peptides in the protein that are different between SR and ST. Details of these proteins were examined from Flybase to identify gene ontology and prior evidence of their role in important biological processes (Gramates et al., 2017). FlyAtlas was used to examine if these genes were previously shown to be differentially expressed in the testes tissue of *D. melanogaster* (Chintapalli et al., 2007). Flymine was used to identify novel information and other research relevant to sperm-killing meiotic drive (Lyne et

al., 2007). Lists were also compared between the two species for common proteins.

8.4 - Results

Overall testes proteomes of *D. pseudoobscura* and *D. subobscura*

For *D. pseudoobscura* we identified 1657 proteins in total from searching against the *D. melanogaster* and *D. pseudoobscura* protein databases, of which 1612 had gene hits in Flybase. Of these 1612 proteins, 88 were unique to *D. pseudoobscura,* with no *D. melanogaster* orthologs. Interestingly, almost half of these unique proteins are produced by genes located on the X-chromosome (43 of 88). For *D. subobscura* we identified 1196 proteins against the same two databases, of which 1170 had hits in Flybase. Of these 1170 proteins, 12 were unique to *D. suboobscura,* with no *D. melanogaster* orthologs found. The reduced number of unique hits in *D. subobscura* is likely a result of this species not having a dedicated gene database to search against, such that *D. subobscura* unique genes would be missed. *D. pseudoobscura* and *D. subobscura* shared 53% and 73% of their total proteomes of with each other, respectively.

Proteins shared with *D. melanogaster* sperm specific and testis specific proteomes

For both species, we compared the full proteomes to the sperm specific (Wasbrough et al., 2010) and testes specific (Takemori and Yamamoto, 2009) proteomes for *D. melanogaster*. For *D. pseudoobscura*, the proteins with hits in flybase shared 303 proteins with the sperm specific (total listed=1108) and 195 with testes specific (total listed=232) proteomes for *D. melanogaster.* For *D. subobscura*, the proteins with hits in flybase shared 221 with the sperm specific (total listed=1108) and 178 with testes specific (total listed=232) proteomes for *D. melanogaster.*

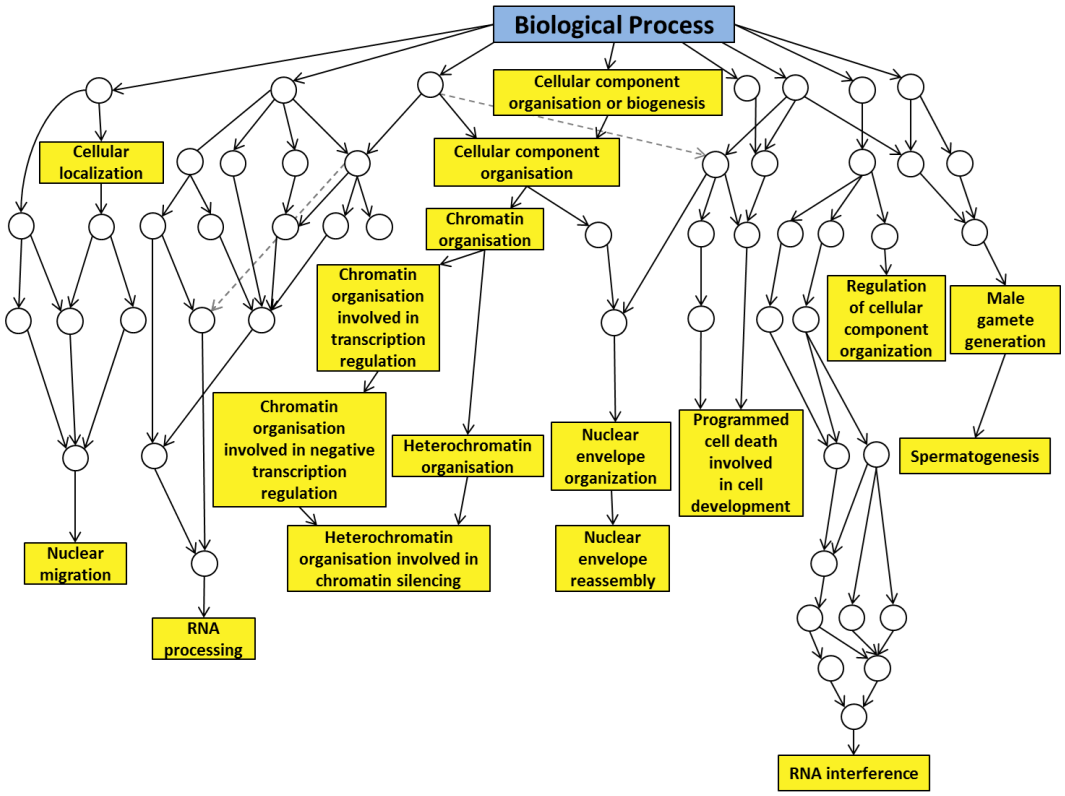

Figure 8.1 Output from GOrilla analysis of proteins that differed in expression between XCMD and non-XCMD testes in *D. subobscura*. Proteins were ranked by the ANOVA score of their difference in abundance between XCMD and non-XCMD testes. Highlighted boxes indicate gene ontologies where proteins that differ highly between XCMD/non-XMD testes are overrepresented.

GOrilla analysis of gene ontology for ranked lists of genes

We carried out a ranked list GOrilla analysis for each species based on biological function on lists ranked by ANOVA scores of differentiation between XCMD and non-XCMD batches of testes. For *D. subobscura* there were a number of biological processes where proteins that differed highly between XCMD and non-XCMD testes were significantly overrepresented (Figure 8.1). Of particular note is the gene ontology "chromatin organisation" and associated higher order GO terms (driven by 3 genes), male gamete generation and spermatogenesis (both driven by 15 genes) which are processes obviously associated with gamete production and the process of segregation. Another gene ontology term of interest is "nuclear migration" (driven by 4 genes), which is a process implicated in the *D. melanogaster* SD drive system), (Figure 8.1).

In *D. pseudoobscura* it is notable that a large number of the enriched processes centre on mRNA pathways. All of the nine processes were being driven by one highly differentiated protein *Aubergine*, which is a member of the *Argonaute* gene family.

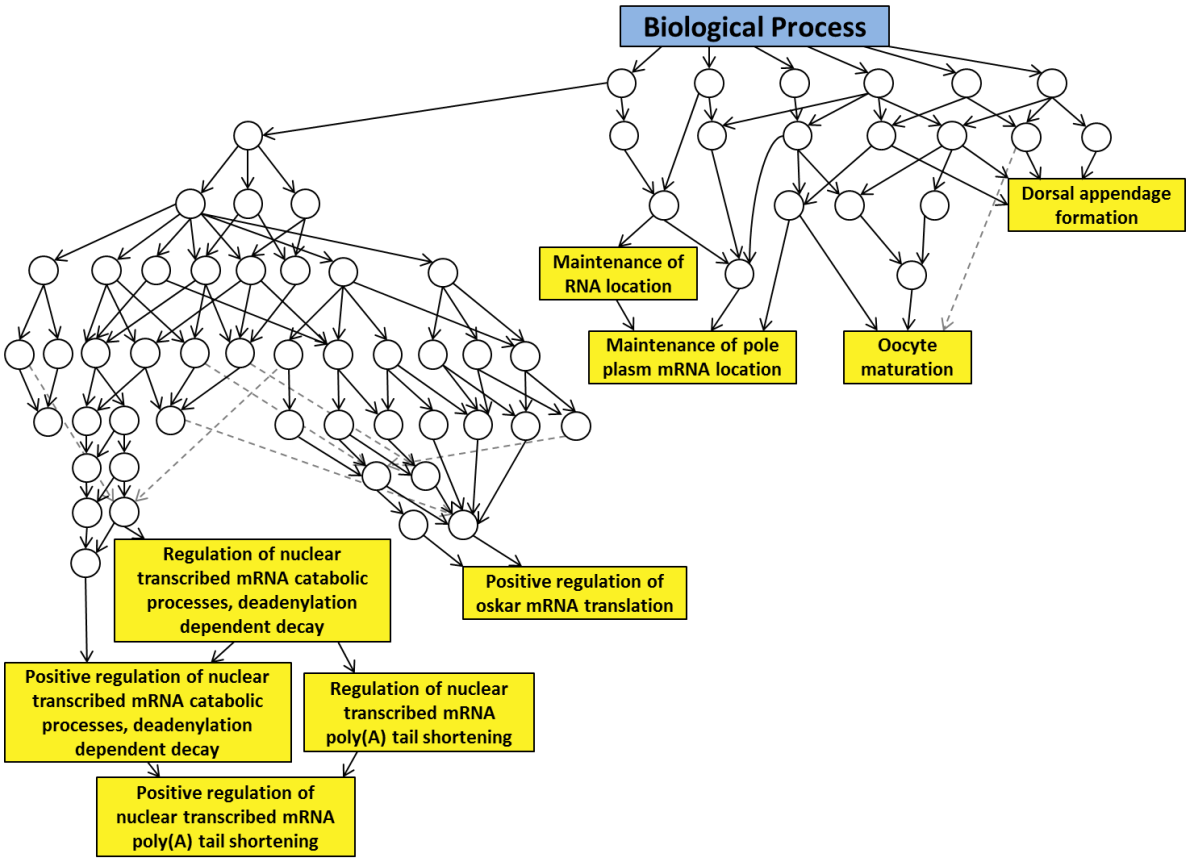

Figure 8.2 Output from GOrilla analysis of proteins that differed in expression between XCMD and non-XCMD testes in *D. pseudoobscura*. Proteins were ranked by the ANOVA score of their difference in abundance between XCMD and non-XCMD testes. Highlighted boxes indicate gene ontologies where proteins that differ highly between XCMD/non-XMD testes are overrepresented.

<u>Proteins with high differentiation between meiotic drive and non-meiotic drive testes</u>

For *D. pseudoobscura* a total of 73 proteins fulfilled the criteria of having an ANOVA score less than 0.05, and protein fold change greater than 1.5, with

at least 2 unique peptides (see supplementary table 8.1). Eleven of these were found to have no orthologs with *D. melanogaster* in flybase. For 54 of these proteins the highest abundance was in the non-meiotic drive male testes, with 19 having the highest state in meiotic drive male testes. 42% of the 73 proteins were found to be associated with X-linked genes. Several genes of particular note were found within this list, associated with genes known to be involved either directly in segregation distortion or associated biological processes (Table 8.2).

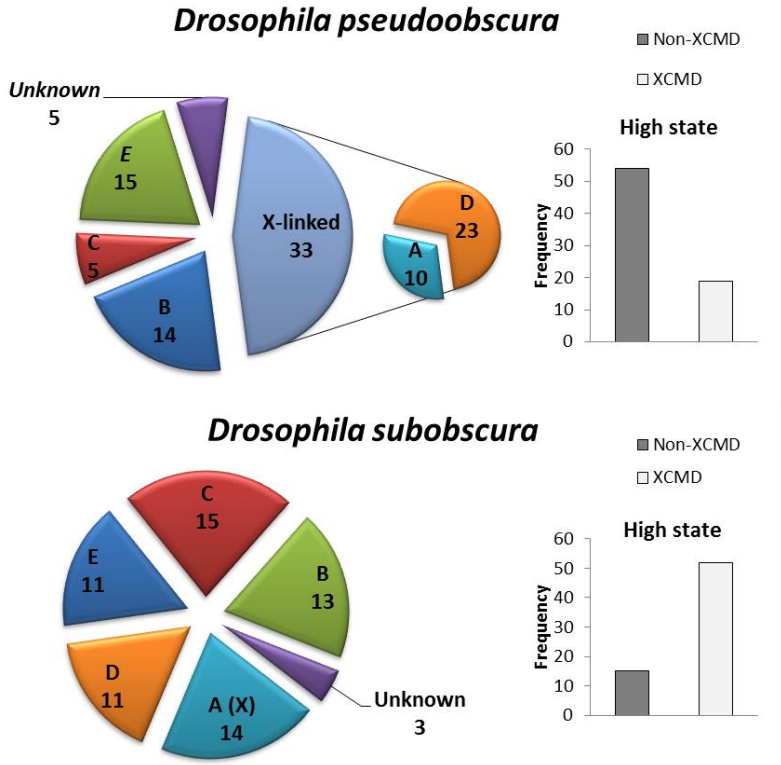

Figure 8.3 Charts showing how proteins with an anova score <0.05, fold change > 1.5 and more than one unique peptide are distributed across the chromosomes. Chromosome names are according to the original Muller elements in *Drosophila*. *D. subobscura* the X-chromosome is made of only element A, while in *D. pseudoobscura* the X-chromosome is a fusion of Muller elements A and D. The number of genes which had a high state for XCMD or non XCMD is also presented.

For *D.subobscura* a total of 67 proteins fulfilled the criteria of having an ANOVA score less than 0.05, and protein fold change greater than 1.5, and at least 2 unique peptides (see supplementary table 8.2). Three of these were found to have hits to *D. pseudoobscura* genes which shared no orthologs with *D. melanogaster*. For 15 of these proteins the highest

abundance was in the non-meiotic drive male testes, with 52 having higher expression in meiotic drive male testes. 22% of the 67 proteins were found to be associated with X-linked genes. Four of the genes could not be localised to a predicted genomic location. Several genes of particular note were found within this list, associated with genes known to be involved either directly in segregation distortion or associated biological processes (Table 8.2; Table 8.3).

Table 8.2 selected genes of high interest for *D. pseudoobscura* due to their differentiation between meiotic drive and non-meiotic drive individuals. *D. melanogaster* gene names, inferred genome location, ANOVA score, fold change, unique peptide count and if XCMD or non-XCMD was the high state are included. Biological processes of note and associations with segregation distortion in other systems are shown.

Gene name (*D. melanogaster*)	Notable associations
RanGEF (Chr. XR) Fold change: 2.258 Aov Score: <0.001 Unique pept: 3 High state: non-XCMD	Biological processes of note: regulation of mitotic cell cycle, mitotic chromosome condensation, chromosome condensation. Interacts directly with RanGAP, which is directly involved in segregation distortion of the SD system in *D. melanogaster*.
Sauron (Chr. 4) Fold change: 1.731 ANOVA: 0.024 Unique pept: 2 High state: non-XCMD	Biological processes of note: mitotic spindle assembly, meiosis I cytokinesis, male meiosis
Eukar. trans. init. factor 4G (Unknown) Fold change: 1.535 ANOVA: < 0.001 Unique pept: 16 High state: non-XCMD	Biological processes of note: spermatid differentiation, male germ-line cyst formation, spermatocyte division
Aubergine (Chr. 4) Fold change: 2.685 ANOVA: < 0.001 Unique pepti: 10 High state: non-XCMD	Biological processes of note: Chromosome condensation. Association of mutations in aubergine with the stellate segregation distortion system in *D. melanogaster* (Gell and Reenan, 2013).
Reg. part. non-ATPase 10 (Chr. XR) Fold change: 1.624 ANOVA: 0.010 Unique pept: 10 High state: non-XCMD	Biological processes of note: mitotic sister chromatid segregation
CDP diglyceride synthetase (Chr. XR) Fold change: 1.539 ANOVA: 0.029 Unique pept: 4 High state: XCMD	Biological processes of note: Sperm individualization, Reduced expression of CDP-DAG synthase changes lipid composition and leads to male sterility in *Drosophila*. High state in XCMD individuals.
Nessun dorma (Chr. 4) Fold change: 1.715 ANOVA: < 0.001 Unique pept: 3 High state: non-XCMD	Biological processes of note: Male meiosis cytokinesis, a novel central spindlin partner, is required for cytokinesis in *Drosophila* spermatocytes
γ-Tubulin at 23C (Chr. 4) Fold change: 2.146 ANOVA: 0.06 Unique pept: 2 High state: non-XCMD	Biological processes of note: centrosome organization, mitotic sister chromatid separation, mitotic nuclear division
GA26415 (Chr. XL) Fold change: 4.401 ANOVA: < 0.001 Unique pept: 2 High state: XCMD	Biological processes of note: Fourfold increase in expression. Specific to only *D. pseudoobscura*, with no *melanogaster* ortholog. High state in XCMD individuals.

Table 8.3 Selected genes of high interest for *D. subobscura* due to their differentiation between meiotic drive and non-meiotic drive individuals. *D. melanogaster* gene names, inferred genome location, ANOVA score, fold change, and unique peptide count are included. Biological processes of note and associations with segregation distortion in other systems are shown.

Gene name (*D. melanogaster*)	Notable associations
Decondensation factor 31(Chr. 2) Fold change : 1.501 ANOVA : 0.014 Unique peptides: 9 High state: XCMD	Biological processes of note: Chromatin organisation. A mutation in this gene was found to enhance chromosome meiotic drive in the SD system in *D. melanogaster* (McElroy et al., 2008).
Topoisomerase 2 (Chr. 2) Fold change : 1.850 ANOVA : 0.009 Unique peptides: 2 High state: XCMD	Biological processes of note: chromatin silencing, sister chromatid segregation, chromosome condensation, meiotic nuclear division. Associated with prevention of chromatid segregation in *D. melanogaster* mitosis via abnormal enrichment associated with satellite DNA (Ferree and Barbash, 2009)
Cut up (Chr. X) Fold change : 1.668 ANOVA : 0.009 Unique peptides: 2 High state: XCMD	Biological processes of note: spermatogenesis, sperm individualization, spermatid, nucleus elongation, microtubule anchoring at centrosome.
Wurstfest (Chr. 3) Fold change: 1.531 ANOVA: < 0.001 Unique peptides: 9 High state: XCMD	Biological processes of note: spindle assembly involved in male meiosis I, multicellular organism reproduction
Cullin 3 (Chr. 4) Fold change: 3.034 ANOVA: 0<0.01 Unique peptides: 3 High state: XCMD	Biological processes of note: Sperm individualisation, processes contributing to actin, tubulin and basic functions in spermatogenesis
Dicer-2 (Chr. 4) Fold change: 1.653 ANOVA: < 0.001 Unique peptides: 2 High state: XCMD	Biological processes of note: heterochromatin organization involved in chromatin silencing. Contributes to centrosome attachment during nuclear divisions.
Nessun dorma (Chr. 4) Fold change: 1.948 ANOVA: 0.004 Unique peptides: 2 High state: XCMD	Biological processes of note: Male meiosis cytokinesis, a novel central spindlin partner, is required for cytokinesis in *Drosophila* spermatocytes
Lamin (Chr. 4) Fold change: 1.586 ANOVA: < 0.001 Unique peptides: 4 High state: XCMD	Biological processes of note: spermatogenesis, centrosome organization, nuclear migration, heterochromatin maintenance, chromatin silencing
Imp (Chr. X) Fold change: 1.644 ANOVA: 0.030 Unique peptides: 2 High state: XCMD	Biological processes of note: Spermatogenesis, regulates ageing of the *Drosophila* testis stem-cell niche

8.5 - Discussion

The overall proteomes of *D. pseudoobscura* and *D. subobscura*

The proteomes of *D. pseudoobscura* and *D. subobscura* testes shared over half of their proteins with each other, suggesting a high degree of conservation across these species despite the ~14-21MYA since two species diverged (Gao et al., 2007). *D. pseudoobscura* had considerably more protein hits than *D. subobscura*, but it is highly likely the inflated number of protein hits for *D. pseudoobscura* is partly driven by the fact that we are using a database specific to this species. There were 88 hits specific only to the *D. pseudoobscura* database for the *D. pseudoobscura* analysis, compared to only 12 for the *D. subobscura* analysis. Developing a database of genes for *D. subobscura* would account for this bias. Nonetheless, there were a greater number of hits for *D. pseudoobscura* than *D. subobscura*, even after accounting for this. It is possible that using the same numbers of pairs of testes for both species resulted in there being greater biological material for *D. pseudoobscura,* because they have larger testes. This could have meant a greater number of proteins were identified for the *D. pseudoobscura* analysis than for *D. subobscura*. From these large lists, it remains to be examined which of these proteins are specific to the testes of these species. This question could be tackled by using tissue specific expression or protein analysis.

The proteomes of both species were compared to the specific sperm proteome of *D. melanogaster* (Wasbrough et al., 2010). For both *D. pseudoobscura* and *D. subobscura* approximately twenty percent of their proteins were also found in the sperm-specific proteome of *D. melanogaster.* It is likely there are a greater number of proteins shared, however many of these may be in quite low quantity, and so are masked or overshadowed by more abundant testes proteins in our analysis. Another potential source of discrepancy between *D. pseudoobscura* and *D. subobscura* compared to *D. melanogaster*, is that both of these species are sperm heteromorphic, producing a caste of small infertile sperm that, that have a role in increasing the survival of the larger fertile sperm (Holman et al., 2008). Our approach

was not designed to examine proteins that are important in this sperm heteromorphism (Holman et al., 2008). Nonetheless, many proteins important to sperm-heteromorphism will be within our overall proteomes, but would not be present in the sperm proteome of *D. melanogaster*. Sperm-heteromorphism is not an uncommon phenomenon, being found across a number of invertebrate species (Swallow and Wilkinson, 2002). However, our understanding of why sperm heteromorphism occurs or the genes, proteins and mechanisms underlying it remains incomplete.

Our proteomes were also compared to a list of testes specific proteins of *D. melanogaster* (Takemori and Yamamoto, 2009). For both species around 15% of proteins within the overall proteomes (*D. pseudoobscura*=12%, *D. subobscura*=15%), were found within the *D. melanogaster* list of testes specific proteins. It is likely that these proteins are also testes specific in *D. pseudoobscura* and *D. subobscura* and could represent a core testes specific proteomes for *Drosophila*. Examination of the specificity of these proteins to the testes of *D. pseudoobscura* and *D. subobscura* would confirm this. Overall this showed that out of the 232 testes specific proteins identified in *D. melanogaster*, a large percentage were found within the proteomes of the two species we analysed (*D. pseudoobscura* = 85%, *D. subobscura* = 76%)

GOrilla analysis of enriched biological processes

It is remarkable that so few of the biological processes that were found to be enriched in the GOrilla analysis of genes ranked by differentiation were shared between *D. pseudoobscura* and *D. subobscura* (Figure 8.2, Figure 8.3). In *D. subobscura* a range of genes were driving different biological processes. Notably, heterochromatin organisation and nuclear migration were enriched processes. It is known that the XCMD system in *D. subobscura* causes hybrid incompatibilities in inter-population hybrids, and heterochromatin formation has been implicated in hybrid male lethality in between *D. simulans* and *D. melanogaster* (Ferree and Barbash, 2009, Bayes and Malik, 2009). The link between hybrid incompatibilities and heterochromatin in the *D. subobscura* XCMD system definitely warrants closer examination. Nuclear migration also appeared as a significantly

enriched process for *D. subobscura*. Interestingly, the SD distorter system in *D. melanogaster* is known to work through disruption of nuclear migration pathways, specifically by the truncation of the transport protein RanGAP (Merrill et al., 1999).

In contrast, all of the enriched biological processes for the *D. pseudoobscura* analysis were driven by a single highly differentiated gene, *aubergine*. Interestingly, mutant alleles of this gene are known to play a role in the stellate segregation distortion system in *D. melanogaster* (Gell and Reenan, 2013). It has also been proposed that signatures of selection found in *D. pseudoobscura* linages could be a result of rapid evolution for testes specific function such as the suppression of transposable elements or the activity of meiotic drive (Lewis et al., 2016b). Finding this gene to be enriched could indicate *aubergine* is playing a role in the process of segregation and segregation disruption more generally. However, caution is warranted in the interpretation of these GOrilla analyses for two reasons due to their reliance on the *D. melanogaster* protein database for the analysis. Firstly, they will be completely excluding processes driven by proteins specific to *D. pseudoobscura* and *D. subobscura* that are not present in *D. melanogaster*. This would miss novel recently evolved XCMD proteins, like that of the Paris system in *D. simulans* (Helleu et al., 2016). Secondly, relying on the *D. melanogaster* database would not account for changes caused by recent losses or diversifications of particular gene families, as has been shown to be the case for the *Argonaute* gene family (Lewis et al., 2016a).

Highly differentiated genes of interest for *D. pseudoobscura and D. subobscura*

When examining proteins that were identified as highly differentiated (ANOVA < 0.05, fold change difference > 1.5, at least 2 unique peptides) between the two species there were some obvious differences. There was only one protein, *Nessun dorma*, which was shared between the two species and this gene had its high expression state in opposite directions for each of the species. This suggests completely different genetic mechanisms underlying these two XCMD systems. More generally, the high expression state of this

gene was also different for the two species, being low in XCMD for *D. pseudoobscura* and high in XCMD for *D. subobscura*. Looking across the differentiated proteins for the two species, there is a general pattern of *D. pseudoobscura* being dominated by proteins with their low expression state in XCMD (54 of 73 proteins), whereas in *D. subobscura* the majority of proteins were more highly expressed in XCMD testes (52 of 67 proteins). This would be consistent with the conflict being more resolved in *D. pseudoobscura*, as might be expected in an ancient XCMD system (Babcock and Anderson, 1996). If this explanation is correct, it is possible that many of the genes that are differentiated are a result purely of the increased amount of mature sperm being produced in non-XCMD individuals. This would suggest that the XCMD system in *D. pseudoobscura* may have recruited genes to the X chromosome drive inversion that ameliorate any damage the drive mechanism causes. Alternatively, frequent and long term exposure to drive may have led the rest of the *D. pseudoobscura* genome to adapt to reduce the costs of drive, without directly suppressing the mechanism. These processes have parallels in the long term evolutionary trajectories of endosymbionts adapting to new hosts (Nakayama et al., 2015). In contrast, in the *D. subobscura* system, where the XCMD system is thought to be younger (Chapter 7), and where there is evidence of ongoing conflict between the driver and suppressors (Chapter 6), it could be that the greater conflict is resulting in more misregulation of proteins throughout spermatogenesis and that there has not been the time to mitigate these processes in XCMD testes.

There was a moderate number of candidate proteins where the genes localised to the X-chromosome for each species (*D. pseudoobscura* = 33/72 and *D. subobscura* -= 14/67). The greater number of X-linked genes in *D. pseudoobscura* is likely an effect of the fusion of Muller elements A and D into the sex-chromosome in this species. All of these X-linked genes could be considered potential candidate genes for XCMD in these species.

Within *D. pseudoobscura* there are a number of proteins that are worth discussing in further detail. Aubergine, mentioned earlier, is involved in chromosome condensation (Lyne et al., 2007) and has been implicated in the stellate segregation distortion system in *D. melanogaster.* In addition, hard

sweeps have been observed in *D. pseudoobscura* on genes from the argonaute family, resulting in them being linked to processes associated with genetic conflict. A number of genes (RanGEF, Sauron, Regulatory particle non-ATPase 10, Nessun Dorma) are also involved in mitotic and meiotic chromosome assembly, chromosome condensation, spindle assembly and cytokinesis. However, all of these genes have their high state in non-XCMD individuals, and therefore could be differentiated due to XCMD killing half of their sperm. RanGEF interacts directly with RanGAP, the protein responsible for drive in the SD system in *D. melanogaster* (Presgraves, 2007) and is also found on the right arm of the X-chromosome in *D. pseudoobscura* due to the fusion of Muller element A and D into a larger sex-chromosome for this species. Two genes also stand out due to their high state in XCMD individuals are CDP diglyceride synthetase and GA26415, both of which are located on the sex-chromosome. CDP diglyceride synthetase is involved in sperm individualisation, and has also been linked to male fertility in *D. melanogaster*. GA26415 is interesting because it has a more than four-fold change in expression and it has no ortholog in *D. melanogaster*, possibly indicating a novel or fast evolving gene on a sex-chromosome. More widely, it highlights the importance of not overlooking genes specific to *D. pseudoobscura* because they lack annotation of biological information.

Looking more closely at the genes with strong differentiation between XCMD and non-XCMD individuals in *D. subobscura* there are a number of proteins implicated in heterochromatin processes, chromatin silencing and chromosome condensation (*Dicer-2*, *Topoisomerase-2*, *Decondensation factor 31* and Lamin). However, none of these genes were inferred to be located on the X-chromosome of *D. subobscura*. However, they could be linked to a network of proteins involved in the killing process, which is triggered by another gene on the X-chromosome. Meanwhile, proteins involved in cytokinesis and spindle formation, two processes likely to affect chromosome migration during cell replication, also stand out (*Nessun dorma*, *Cullin-3*, *wurstfest*, and *Cut-up*). Notably, *Cut-up* is located on the X-chromosome and is also specifically involved in anchoring at the centrosome and nuclear migration processes, both of which could be important for

distorting segregation during mitosis or meiosis. Finally, *Imp* is located on the X-chromosome of *D. subobuscura*, is in a high state in XCMD individuals and has been reported to be important for spermatogenesis in *D. melanogaster*.

Summary

LC MS/MS proteomics were used to examine the testes of XCMD and non-XCMD males from two species of fly, *D. pseudoobscura* and *D. subobscura*. The majority of proteins identified across the two species were shared in the overall testes proteomes. Differentiated proteins contributed to enrichment biological processes such as nuclear migration, heterochromatin formation, and spermatogenesis in *D. subobobscura*. In *D. pseudoobscura*, mRNA specific processes were all driven by a single gene *aubergine*, which has previously been implicated in processes driven by genetic conflict and selfish genes. Examination of highly differentiated proteins between XCMD and non-XCMD individuals highlighted large differences in proteins involved in a nuclear migration, heterochromatin formation and cytokinesis in mitotic and meiotic cell division. We identify both candidate X-linked genes that could be involved in causing XCMD and also highlighted non-sex-linked genes that could be important to protein networks causing XCMD. Very few highly differentiated genes were shared between the two species suggesting XCMD is caused by different mechanisms.

8.6 - References

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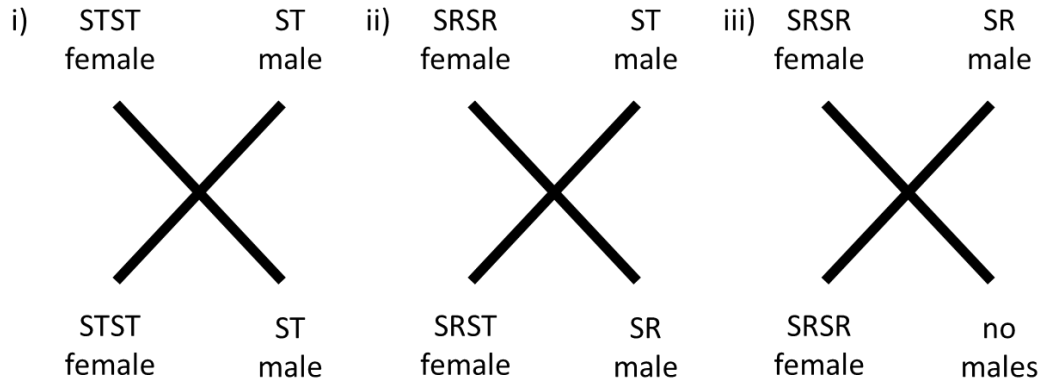
8.7 - Supplementary material



Supplementary figure 8.1 Outline of how XCMD can be kept as an inbred line in the laboratory. In the above example SR denotes the selfish XCMD. ST denotes a chromosome that is not selfish, non-XCMD. All X genotypes can be produced from the offspring generation that are required to re-establish the parental lines.

Supplementary table 8.1 List of proteins for *D. pseudoobscura* that have an anova score of less than 0.05, more than 2 unique peptides, and a fold change of greater than 1.5. Proteins are ranked by fold-change. The protein accession number and whether the protein was in a higher state in XCMD or non-XCMD is reported from the analysis. The gene name and gene location from Flybase are also reported. (*) indicates that the protein provided hits to more than one closely related ortholog in Flybase.

Acc. No.	High Exp.	Unique pept.	Anova (p)	Fold change	D.pse name	D.pse loc.
Q2M118	Non-XCMD	3	0.028	9.645	GA20539	X-linked
Q29L57	XCMD	2	0.002	8.617	GA18675	Autosome
B5DMZ2	XCMD	3	0.000	4.401	GA26415	X-linked
Q29F59	Non-XCMD	8	0.000	3.901	GA18688	X-linked
B5DHC1	Non-XCMD	2	0.000	3.879	GA25276	Autosome
Q29D12	Non-XCMD	2	0.000	3.689	GA17335	X-linked
Q29IB6	Non-XCMD	2	0.000	3.368	GA15297	X-linked
Q29I93	XCMD	2	0.000	3.119	GA20639	X-linked
Q29MX9	Non-XCMD	2	0.004	2.966	GA16409	Autosome
B5DUH6	XCMD	6	0.000	2.867	GA27866	Unknown*
Q29HN9	Non-XCMD	2	0.003	2.774	GA17789	X-linked
Q29P29	Non-XCMD	10	0.000	2.686	GA19382	Autosome
B5DRB2	XCMD	2	0.001	2.590	GA28518	X-linked
Q2LYQ1	Non-XCMD	2	0.004	2.520	GA21751	X-linked
B5DYF8	XCMD	2	0.003	2.426	GA26554	Autosome
Q29P53	Non-XCMD	2	0.006	2.334	GA18367	Autosome
Q29E69	Non-XCMD	3	0.001	2.258	GA10341	X-linked*
Q28Z07	XCMD	4	0.000	2.235	GA12807	Autosome
B5DMB2	Non-XCMD	8	0.000	2.193	GA27065	X-linked
Q29KG7	Non-XCMD	2	0.006	2.146	GA16328	Autosome
B5DVZ6	Non-XCMD	2	0.035	2.122	GA26730	Autosome
Q2M030	Non-XCMD	3	0.004	2.079	GA14613	X-linked
Q291T8	Non-XCMD	8	0.001	1.997	GA18184	Autosome
Q29LE6	Non-XCMD	3	0.013	1.991	GA14807	Autosome
Q299C3	XCMD	3	0.026	1.967	GA10159	Autosome
Q29LP5	Non-XCMD	2	0.002	1.909	GA16575	Autosome
B5DP49	Non-XCMD	3	0.000	1.885	GA24460	X-linked*
Q29GJ2	Non-XCMD	2	0.000	1.854	GA22125	X-linked
Q298N5	Non-XCMD	2	0.000	1.800	GA20078	Autosome
Q29FI4	XCMD	2	0.007	1.800	GA20324	X-linked
B5DKV3	Non-XCMD	2	0.002	1.785	GA22941	X-linked
Q29JW8	XCMD	6	0.000	1.784	GA18220	Autosome
Q295H4	Non-XCMD	2	0.004	1.773	GA16770	Autosome
Q9VFC4	XCMD	3	0.011	1.770	GA19834	Autosome
Q29E41	Non-XCMD	2	0.007	1.748	GA16941	X-linked
Q9VPQ7	Non-XCMD	2	0.027	1.745	GA11227	Autosome
Q9VQ93	Non-XCMD	2	0.025	1.731	GA25765	Autosome

Q2LZX1	Non-XCMD	2	0.024	1.725	GA19501	X-linked
Q29KL0	Non-XCMD	3	0.000	1.715	GA10522	Autosome
B5DIA7	Non-XCMD	5	0.001	1.708	GA25940	Autosome
Q2LZW3	Non-XCMD	5	0.000	1.707	GA15106	X-linked
B5DI57	Non-XCMD	2	0.037	1.693	GA25815	Autosome
Q28XX6	Non-XCMD	15	0.002	1.678	GA12219	Autosome
Q9W002	XCMD	2	0.025	1.677	GA24328	X-linked
Q295F4	XCMD	3	0.002	1.666	GA10588	Autosome
P22811	Non-XCMD	4	0.033	1.662	Ry	Autosome
Q2LZ85	XCMD	6	0.000	1.658	GA13433	X-linked
Q295N0	XCMD	5	0.000	1.653	GA19203	Autosome
B5DMZ5	Non-XCMD	2	0.039	1.636	GA26400	X-linked
Q29EX1	Non-XCMD	15	0.000	1.633	GA21956	X-linked
Q2M0C5	Non-XCMD	2	0.032	1.630	GA10541	X-linked
Q2M071	Non-XCMD	7	0.000	1.625	GA11342	X-linked
Q2LYK8	Non-XCMD	10	0.010	1.624	GA20484	X-linked
B5DWK7	Non-XCMD	2	0.004	1.622	GA27089	Autosome
B5DIT3	Non-XCMD	2	0.006	1.613	GA25658	Autosome
B5DV14	Non-XCMD	2	0.012	1.589	GA27986	Unknown*
Q28ZJ0	XCMD	3	0.018	1.583	GA17478	Autosome
Q297V0	Non-XCMD	15	0.001	1.579	GA16178	Autosome
B5DW01	Non-XCMD	3	0.024	1.562	GA26722	Autosome
I5APQ2	XCMD	5	0.000	1.562	GA15384	Autosome
Q28XN8	Non-XCMD	3	0.001	1.561	GA11023	Autosome
B5DLH2	XCMD	2	0.012	1.561	GA23060	X-linked
B5DHL1	Non-XCMD	10	0.000	1.544	GA22466	Unknown*
Q29FD7	XCMD	4	0.029	1.539	GA20725	X-linked
Q29A31	Non-XCMD	2	0.008	1.536	GA19377	Autosome
Q29CV3	Non-XCMD	16	0.000	1.535	GA10575	Unknown
B5DHY7	Non-XCMD	2	0.001	1.525	GA25872	Autosome
I5ANQ5	Non-XCMD	6	0.000	1.525	GA30141	Autosome
Q29EN8	Non-XCMD	7	0.015	1.518	GA20325	X-linked
Q29ET1	Non-XCMD	4	0.004	1.518	GA11051	X-linked
B5DSP9	Non-XCMD	6	0.003	1.513	GA23131	Unknown
Q2M0Z3	Non-XCMD	2	0.013	1.506	GA20215	X-linked
Q2LZ16	Non-XCMD	5	0.021	1.502	GA20345	X-linked

Supplementary table 8.2 List of proteins for *D. subobscura that* have an anova score of less than 0.05, more than 2 unique peptides, and a fold change of greater than 1.5. Proteins are ranked by fold change. The protein accession number and whether the protein was in a higher state in XCMD (SR) or non-XCMD (ST) is reported from the analysis. The gene name and gene location from Flybase are also reported.

Acc. No.	High exp.	Unique pept.	Anova (p)	Fold change	D. pse name	Location
Q9VCN3	XCMD	2	0.033	3.092	GA18181	Autosome
Q29KT9	XCMD	2	0.001	3.034	GA16511	Autosome
Q2LZL6	XCMD	2	0.008	2.601	GA18418	Autosome
B5DPE7	XCMD	2	0.000	2.537	GA23736	Autosome
Q293M1	Non-XCMD	2	0.018	2.457	GA19444	Autosome
Q28ZS9	Non-XCMD	3	0.002	2.325	GA19322	Autosome
B5DQM7	Non-XCMD	2	0.044	2.298	GA23591	Autosome
Q29ML2	Non-XCMD	2	0.021	2.239	GA21094	Autosome
P91638	XCMD	2	0.010	1.965	GA21172	Autosome
Q291A3	XCMD	3	0.000	1.948	GA19635	Autosome
Q294J8	XCMD	2	0.004	1.890	GA20909	Autosome
Q29GG1	XCMD	4	0.006	1.866	GA17546	X-linked
Q29K53	XCMD	2	0.009	1.850	GA10169	Autosome
A1Z707	XCMD	2	0.007	1.840	GA15262	Autosome
Q9VKW5	XCMD	5	0.007	1.832	GA25293	Autosome
Q2LZ83	XCMD	2	0.000	1.823	GA12600	Autosome
Q29PF6	XCMD	4	0.000	1.803	GA12794	Autosome
B5DJI7	Non-XCMD	2	0.016	1.790	GA28881	Autosome
B5DXU8	Non-XCMD	4	0.015	1.774	GA30021	Autosome
Q2M1C5	XCMD	2	0.024	1.764	GA20709	Autosome
B5DUL5	XCMD	5	0.000	1.763	GA23524	Unknown
Q29FS9	XCMD	7	0.005	1.763	GA17642	X-linked
Q2LZL7	XCMD	3	0.005	1.740	GA10461	Autosome
Q29CQ5	XCMD	2	0.000	1.738	GA21515	X-linked
I5AMN4	XCMD	2	0.017	1.729	GA15004	Autosome
Q29FI8	Non-XCMD	3	0.003	1.710	GA19890	Autosome
B5DN78	XCMD	2	0.009	1.668	GA25980	X-linked
Q29KL0	XCMD	2	0.004	1.653	GA10522	Autosome
Q29F59	XCMD	6	0.002	1.652	GA18688	Autosome
Q291W2	Non-XCMD	3	0.012	1.651	GA21819	Autosome
Q29GU3	XCMD	2	0.030	1.644	GA14212	X-linked
I7LPX5	Non-XCMD	2	0.011	1.629	GA18170	X-linked
Q28YH8	XCMD	4	0.000	1.629	GA10787	Autosome
Q29EZ4	XCMD	2	0.001	1.624	GA10597	Autosome
B5DU70	XCMD	12	0.035	1.623	GA22539	Autosome
Q29HB2	XCMD	9	0.000	1.620	GA14712	X-linked
Q28ZX4	XCMD	3	0.012	1.615	GA15343	Autosome
Q29HM1	Non-XCMD	3	0.002	1.607	GA14770	X-linked

Q29NU0	XCMD	4	0.001	1.586	GA19971	Autosome
Q292L3	XCMD	3	0.046	1.580	GA15449	Autosome
Q28X05	Non-XCMD	2	0.020	1.574	GA10842	Autosome
Q7K3W2	XCMD	2	0.001	1.571	GA21285	Autosome
Q29J14	Non-XCMD	2	0.019	1.568	GA13249	X-linked
B5DTM6	XCMD	2	0.026	1.564	GA22255	Unknown
Q9W3D8	XCMD	3	0.021	1.554	GA11405	X-linked
Q292G8	XCMD	3	0.000	1.554	GA18915	Autosome
Q297P9	XCMD	4	0.008	1.551	GA10599	Autosome
Q296L6	XCMD	2	0.043	1.546	GA11240	Autosome
E1JJL2	XCMD	2	0.043	1.545	GA23027	X-linked
Q290E7	XCMD	9	0.001	1.532	GA22160	Autosome
Q29L16	XCMD	2	0.000	1.531	GA17688	Autosome
P11147	XCMD	2	0.002	1.528	GA18066	Autosome
Q9VIE8	XCMD	2	0.026	1.523	GA21639	Autosome
Q28WX4	XCMD	5	0.002	1.521	GA16296	Autosome
B5DVT1	XCMD	4	0.001	1.520	GA27045	Autosome
I5APL5	XCMD	3	0.005	1.519	GA30215	Autosome
B5DXS9	XCMD	3	0.014	1.519	GA26951	Autosome
B5DYU6	Non-XCMD	8	0.013	1.514	GA26534	Autosome
Q28Z41	XCMD	5	0.004	1.510	GA18551	Autosome
B5DNY8	XCMD	2	0.029	1.510	GA22264	X-linked
E2QD63	XCMD	2	0.000	1.509	GA15538	X-linked
A1Z9N0	Non-XCMD	3	0.030	1.509	GA21105	Autosome
Q9W1I7	XCMD	2	0.000	1.506	GA18965	Autosome
Q9VKX2	Non-XCMD	4	0.004	1.506	CG5362	Autosome
Q29EI3	XCMD	2	0.006	1.504	GA18259	Autosome
Q29FX8	XCMD	3	0.007	1.502	GA12352	X-linked
Q29P07	XCMD	9	0.014	1.501	GA15302	Autosome

9 – General discussion.

Parts of this discussion are formed from collaborations with my supervisors for future work in the area of XCMD, genetic conflict, and hybrid incompatibilities.

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9.1 - Outline

This thesis addresses a diverse set of questions held together by the theme of X-chromosome meiotic drive, with a particular focus on *Drosophila subobscura*. Within this thesis, each chapter was presented in the format of a self-contained paper. In this chapter, I synthesize this information to explore the evolutionary dynamics of meiotic drive in natural populations. I then outline three areas that my research has directly or indirectly contributed to, and could be investigated further. First, the need for spatial and temporal sampling of meiotic drive in order to better understand the dynamics in natural systems. Second, the role of meiotic drive in speciation, using examples from a number of study systems as well as my own to lay out future research avenues in this exciting area of study. Finally, how the preliminary proteomics could be used as a step to directly manipulating genes of interest using gene editing tools.

9.2 – Progress to the study of SRs in *Drosophila subobscura*

I entered into this PhD in 2013 aiming to revive the study of the SR*s* system in *D. subobscura*, which was last studied in the early 1990s (Hauschteckjungen, 1990). A large part of the initial stages of my PhD was taken up with collecting and isolating the systems from the wild, during which time I also focussed on becoming acquainted with using *Drosophila* as a study organisms. As a result, chapter 3 is a standalone chapter that does not concern the SR*s* system or research questions that focus on selfish genetic elements (SGEs). I have subsequently focussed on two main themes in my thesis. Firstly, I have explored mechanisms that might prevent the spread of SR*s*, both within and between populations. Secondly I was interested in the history of the SR*s* system, where it can be found, and the genes that might be pivotal to the drive mechanism.

Understanding the frequency and spread of SRs

Understanding what forces hold in check the spread of selfish genetic elements is important for both fundamental understanding and application of SGEs. It is of fundamental interest because they are diverse and ubiquitous, and there is increasing evidence of their role in driving evolution in natural systems (Hurst and Werren, 2001, Burt and Trivers, 2006, Lindholm et al., 2016). However, currently our understanding of the factors that control drive frequencies in natural populations is incomplete (Lindholm et al., 2016). Applied interest comes from the increasing interest in using genetic engineering to produce artificial drive systems to tackle challenges of food security and global disease (Gantz et al., 2015, Hammond et al., 2016). While we have modified crop and livestock animals for thousands of years, we now stand on the cusp of being able to edit many of the pest insect species in the natural world.

There are a number of species in which X-chromosome meiotic drive (XCMD) are either restricted within certain populations, or present in a frequency cline across ranges (Lindholm et al., 2016; Chapter 4), *D. subobscura* included. However, the question of why they are restricted remains unsolved. Recently, there has been considerable interest in

differences in the levels of polyandry across populations, and how this could provide a mechanism that disadvantages XCMD males (Haig and Bergstrom, 1995, Price et al., 2014, Pinzone and Dyer, 2013). However, I have shown that there is no evidence that *D. subobscura* remate in North Africa (Chapter 5) making this system unique when compared to mice (Sutter and Lindholm, 2015), other *Drosophila* (Price et al., 2014, Pinzone and Dyer, 2013), and Stalk-eyed flies (Wilkinson and Fry, 2001), where remating has been shown to reduce the fitness of meiotic drive males.

Female choice has also been proposed as a mechanism to reduce the fitness of XCMD males. However, specific choice of a trait linked to drive, namely male eye-stalk length, has only been found in stalk-eyed flies (Wilkinson et al., 1998, Johns et al., 2005). We observed that whilst female mate choice is strong in *D. subobscura*; it is not necessarily discriminatory against males that carry SR*s (*Chapter 5; Appendix 1*)*. This does not exclude the possibility that fitness costs associated with SR*s* influence female choice in a more general manner, as we found that males carrying SR*s* were on average less successful at gaining matings than an average population male. Overall, however, these results suggest that female mating behaviour is not playing a crucial role in preventing the spread of SR*s* within North Africa. Currently, it remains unclear what prevents SR*s* from spreading in North Africa. I feel the next step to investigating what dictates the frequencies of SR*s* in natural populations in North Africa would require field collections across space and time to see how frequencies of SR*s* change. Unfortunately, this kind of established longitudinal data is lacking for many drive systems, including SR*s* (Lindholm et al., 2016; see section 9.3).

A second question I approached was how SR*s* might behave when entering new, previously unexposed populations of *D. subobscura* in Europe. There were three main reasons to examine this question in greater details. Firstly, understanding if SGEs are going to be able to move into populations as their ranges change in frequency is a novel question for which there is relatively little empirical study (but see Bastide et al., 2011). For example, there is already good evidence in *D. subobscura* for within population genetic changes in response to alterations in climate (Balanya et al., 2006).

Secondly, there was already evidence both from crosses with lab strains that incompatibilities with SR*s* exist between North Africa and Europe (Hauschteckjungen, 1990), and the karyotype arrangement associated with SR*s* was reported in Southern Spain for the first time (Sole et al., 2002). Thirdly, there has been considerable interest recently in the role of SGEs, by driving cycles of intragenomic conflict, in causing speciation (Johnson, 2010, Presgraves, 2010, McDermott and Noor, 2010).

My collections of *D. subobscura*, and the confirmation of the SR*s* phenotype in both Morocco and Spain (Chapter 6; Chapter 7) extend the known range of this system and confirm the recent supposition that driver is present in southern Spain (Sole et al., 2002). I have further shown that the drivers, across all three populations, are most closely related to each other, suggesting a single evolution of a driver that has subsequently spread across a large area (Chapter 7). Finally, and most excitingly, chapter 6 demonstrates that the incompatibilities that Hauschteck-Jungen (1990) showed between the driver and laboratory strains of *D. subobscura* from Switzerland also occur when the driver is presented on a range of natural genetic backgrounds from populations in south Spain. This is the same population where the driver has recently been found (Sole et al., 2002; Chapter 6), and demonstrates that these incompatibilities are a mechanism that can stop the spread of SR*s* into European populations. This greatly advances our understanding of the natural range of SR*s* and how we might expect it not to expand in spite of climatic shifts that might cause a northerly shift (Balanya et al., 2006). More broadly, this highlights that the SR*s* system in *D. subobscura* is an excellent candidate for studying how genetic conflict might drive the process of speciation (see section 9.4)

The history of the SRs system and potential candidate gene involved

Expanding the known range that the SR*s* drive phenotype is found, and beginning to examine the history of this drive system has also been a goal of my PhD. This question is pertinent for a number of reasons. Firstly, drive systems in other species vary from single very old ones like the *SR* system in *D. pseudoobscura*, to multiple different drive systems like in *D. simulans*

(Jaenike, 2001). Secondly, the SR*s* system has only been examined in detail from Tunisia, and there has not even been the confirmation of SR*s* phenotype in Morocco or Spain, despite suggestions that it could occur there (Prevosti, 1974, Sole et al., 2002). Thirdly, if this system is going to be used as a model for examining conflictual speciation, it is important to establish that there is a single drive system across the whole range, rather than independent drive systems producing vastly different phenotypes.

Collecting individuals from across three populations (Tunisia, Morocco, and Spain) I was able to use sequence data to confirm that all the SR*s* X-chromosomes were monophyletic, consistent with a single origin of drive. Sequence variation also suggested that there was very little variation among SR*s* chromosomes across individuals, consistent either with a small effective population size and restricted recombination, or a recent selective sweep event, or both in combination. However, much work remains to be done on the genetics of the SR*s* system. More broadly, there remains much work to be done on the population structure of *D. subobscura* in North Africa. Work that was carried out in the 70s suggests dramatic differences in karyotype frequencies (Prevosti, 1974), potentially indicating separate glacial refugia during previous peaks in glaciation across Europe. This history would be interesting to understand in the context of SR*s* if it played a role in allowing independent co-evolution of a drive system in North Africa that subsequently came into contact with a European population where it created significant incompatibilities.

A natural extension to understanding the population level effects of SR*s* is the exploration of the genetic basis of the SR*s* trait and its influences. Our untargeted proteomics approach represents the first steps towards this. Although exploratory, beginning to characterise the proteomic network of SR*s* establishes a good baseline for more focussed gene knockout work. A demonstration, that considerable differences can be found in genes that already have associations with segregation distortion in *D. melanogaster* also add weight to this method being an effective first sweep approach (Chapter 8). Eventually, characterising the suite of genes most suitable or susceptible to drive phenotypes, across a range of species, could be useful for designing

drive systems. The potential to apply CRISPR-Cas gene editing technology to disrupt specific genes in *D. subobscura* offers and exciting research avenue (Murakami et al., 2015, Tanaka et al., 2016).

9.3 - Why we need continuous field studies of meiotic drive?

In this thesis, I have revisited a system first documented in the 60s, again in the late 80s and now in 2013. I have reconfirmed the presence of the drive phenotype where it was first documented in Tunisia, and expanded the known range of the XCMD phenotype to include Morocco and Spain. However, if there are geographically or climatically driven ecological factors which are limiting the range of SR*s* in the wild, then this degree of sampling is completely insufficient to detect them. This is particularly relevant for the system in *D. subobscura* because I have found that polyandry is not playing a role in reducing the fitness of SR*s* males (Chapter 5). Many drive systems, even those that are well documented or have been known for upwards of 50 years, lack in depth spatial and temporal sampling (Lindholm et al., 2016). This glaring gap, in light of the variation in frequencies of drive in nature, and that these variations often occur across natural gradients or in predictable parts of species ranges, provides an obvious route for future research.

When collecting flies in for the work in this thesis, I realised how little is known about *D. subobscura* in North Africa compared to European populations, not only with respect to SR*s* but also more generally. If there was a suggestion I would make to someone in the future that wants to know why drive is found at the frequency it is in North Africa, I would recommend spatial and seasonal sampling of drive across multiple years. Indeed, the altitudinal gradients and patchwork of forest habitat make this an excellent site for such a study. It could also provide an excellent basis for collaboration between a Moroccan or Tunisian institution, and a European one. The ESEB Godfrey Hewitt Mobility award allowed me to perform sampling in Morocco, however an ongoing collaboration with an institute in Morocco would greatly facilitate more extensive spatial and temporal sampling.

Such work could be novel, relatively inexpensive and provide informative data from wild populations that could later be verified by experimental testing of particular hypotheses. Widening the scope of work on the system in the wild would also provide access to natural genetic diversity both in populations, and in the drive system itself, that is currently limited. Outside of

focussing on just SR*s* there are also interesting and pertinent questions to be asked about the origins of *D. subobscura* in North Africa, as populations found there harbour a diverse range of unique and potentially ancient karyotypes (one of which is SR*s*). In addition, there is a wealth of old karyotype data from the 60s and 70s (see Krimbas, 1993 for review), which would provide an excellent replicate of the interesting studies in Europe showing that karyotype frequencies across the continent are shifting in response to changing climate (Balanya et al., 2006).

9.4 - The role of meiotic drive in speciation

Speciation remains one of the most important, interesting, and productive areas of research in evolution. Understanding it as a process is fundamental to understanding the diversity of life. The idea that rapid co-evolution between a XCMD and suppressors could drive incompatibilities is extremely exciting (Hurst and Pomiankowski, 1991, Frank, 1991). However, until recently there was little experimental evidence to these theories and much scepticism (Charlesworth et al., 1993, Coyne et al., 1991). There have been a number of recent reviews linking genetic conflict to hybrid incompatibilities and speciation in the last decade (Presgraves, 2010, Johnson, 2010) and evidence of specific incompatibilities is mounting (Phadnis and Orr, 2009, Simon et al., 2016). However, there are two main difficulties with the evidence that comes from these systems. Some of them are quite far down the process of differentiation into new species, for example *D. pseudoobscura pesudoobscura* and *D. pseudoobscura bogotana,* which makes it difficult to ascertain if XCMD drive actually established the original differentiation, or the incompatibilities emerged as a by-product of rapid evolution after divergence (Johnson, 2010, Phadnis and Orr, 2009). The second criticism is of those studies which have demonstrated incompatibility occurring in very proximate strains, but have failed to demonstrate the process in a natural context (Hauschteckjungen, 1990, Simon et al., 2016).

In chapter 4 and 5 of my thesis, I focus on testing for incompatibilities between adjacent populations that are caused specifically by the active SR*s* chromosome from North Africa. This builds on a pioneering study by Hauschteck-Jungen (1990) by showing that these hybrid incompatibilities exist on a range of natural genetic backgrounds from populations that are exposed to the SR*s* chromosome in the wild (Chapter 5). This research shows that the SR*s* system in *D. subobscura* is perfect for studying the ongoing process of the evolution of hybrid incompatibilities that are specific to a selfish gene. This system could be used to test hypotheses about how these processes are occurring in the present day. One hypothesis which would be interesting to test using the SR*s* system is whether the genes involved in the cyclical evolution of drive and suppression in North Africa are

the same genes or loci that are involved in hybrid incompatibilities when crossed into Spanish populations.

The following approach, developed with my supervisors, outlines how to isolate the regions of the genome that are contributing to both the weak suppression, found within North Africa, and the strong incompatibilities, found when SR*s* is crossed into Spain. Firstly, extensive field sampling from both North Africa and Spain would be required, allowing the establishment of around 100 isolines from each. These would harbour the required variation in suppression and susceptibility to incompatibilities that would be required to map the genomic regions involved in these phenotypes. The first step would be screening these isolines for suppression and incompatibilities in the lab, to allow high and low lines to be identified for each of the two phenotypes (Figure 9.1). These could then be used to generate crosses, between high and low isolines that would recombine and create an admixture of different genetic variants that contribute to this phenotype. A large sample of the fifth generation offspring of these crosses would then be screened for how strongly they express either the suppression phenotype, or the hybrid incompatibility phenotype. Using the variability in the phenotypes of these offspring it should be possible to sequence a large number of the individuals and pull out regions that are associated with high or low levels of suppression and incompatibility.

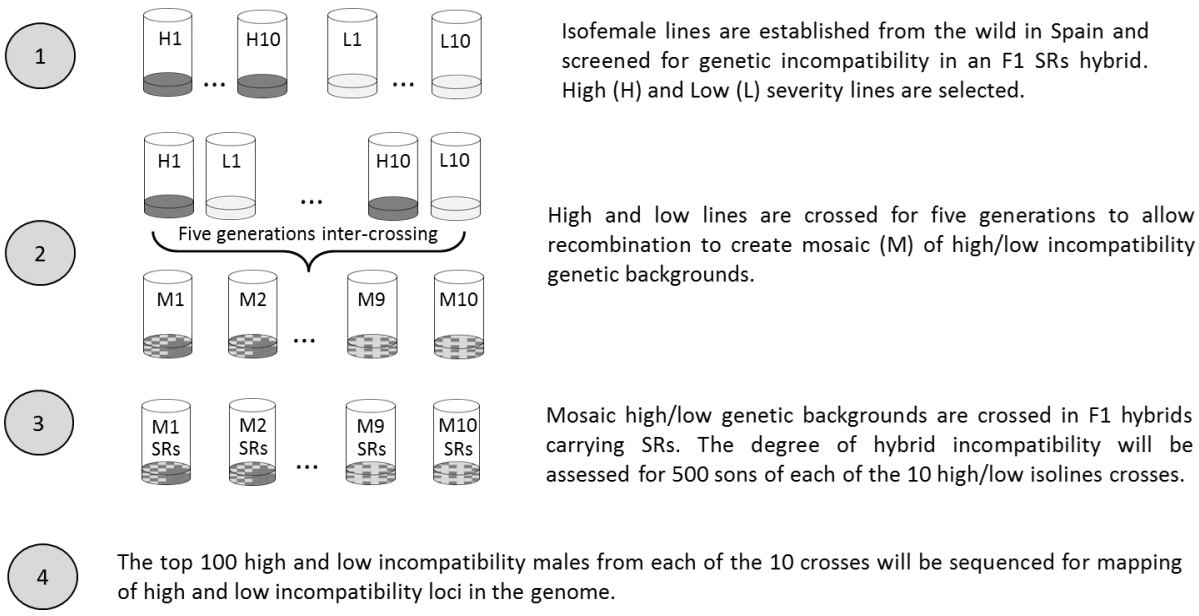

Figure 9.1 A crossing scheme outlining how to map regions associated with variation in incompatibilities caused by SRs.

Establishing if the regions associated with these phenotypes were similar would be informative to the theory of conflictual speciation. It would also allow a closer examination of the selection acting on these regions, using genomic signatures in genetic variation. Conflictual speciation predicts that the hybrid incompatibility is driven by rapid changes in the SGEs native population, driven by cyclical red-queen evolutionary processes. These rapid changes should cause genomic signatures of selection, as genes adapt to the rapid changes in meiosis, gametogenesis, and selective sweeps of hitchhiking genes. These effects could also have far reaching consequences within the organism. Examining whether the speed of evolution is higher in the SGEs native population than the naïve Spanish population and whether the same is true for the loci that suppress drive in North Africa and cause incompatibilities in Spain would provide an interesting test of the theory of conflictual speciation.

9.5 – Gene editing technology and SR*s*

Chapter 7 focusses on identifying candidate genes involved in causing drive, by examining differences in the proteomes of two species which have XCMD systems. Until recently, the underlying genetic mechanisms of only a few drive systems have been studied in detail, including SD in *D. melanogaster* (Presgraves, 2007), the t-haplotype in *Mus musculus,* and the drive systems in *D. simulans* (Helleu et al., 2016). However, advances in techniques to knock out genes in non-model organisms will revolutionize our understanding of the roles of individual genes play in the drive phenotype. This is extremely promising for narrowing down the list of genes for the XCMD systems in *D. subobscura* and *D. pseudoobscura* (Chapter 7). The use of CRISPR-Cas gene editing technology has developed in *D. melanogaster* (Gratz et al., 2013) and Mosquitos (Hammond et al., 2016, Gantz et al., 2015, Kistler et al., 2015), and has recently been demonstrated in some non-model sister species of *Drosophila*.

The next step for research in the SR*s* system would be to build on the recent demonstration of CRISPR-Cas mediated gene modification in *D. subobscura* (Murakami et al., 2015, Tanaka et al., 2016*).* This would allow the direct manipulation of those genes of interest identified in the proteomics scan, by designing a range of knock-out lines, screening phenotypes and establishing the role they play in generating the drive phenotype. This would provide direct access to the genes involved and how they work in concert with each other to create XCMD. More widely, understanding the genetic workings of natural drive systems will aid our understanding of how, where and from what gene systems these natural systems can evolve. It could also increase our ability to harness a multitude of genetic mechanisms to develop artificial drive systems.

9.6 - References

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Appendix 1 – The ecology and evolutionary dynamics of meiotic drive

This paper was the result of a collaborative workshop on meiotic drive, bringing experts from across the world to develop ideas and write a comprehensive review on meiotic drive.

The paper was accepted for publication in Trends in Ecology and Evolution and can be found using the following reference:

Lindholm, A., Dyer, K., Firman, R., Fishman, L., Forstmeier, W., Holman, L., Johannesson, H., Knief, U., Kokko, H., Larracuente, A., Manser, A., Montchamp-Moreua, C., Petrosyan, V., Pomianowski, A., Presgraves, D., Safronova, L., Sutter, A., Unckless, R., Verspoor, R., Wedell, N., Wilkinson, G. & Price, T. 2016. The ecology and evolutionary dynamics of meiotic drive. *Trends Ecol Evol,* 31**,** 315-326.

Appendix 2 - Age-based mate choice in the monandrous fruitfly *Drosophila subobscura*

This paper was the result of supervised project with an honours student, Michael Cuss, jointly supervised by my supervisor Tom Price and myself.

The paper was accepted for publication in animal behaviour and can be found using the following reference:

Verspoor R.L., Cuss M., Price T.A.R. (2015) Age-based mate choice in the monandrous fruitfly *Drosophila subobscura*, Animal Behaviour, 102, 199-207.

Appendix 3 – Observations of entomophagy across Benin – practices and potentials.

This paper was the result of an ongoing collaborative project I established during my masters. Although not explicitly on my PhD thesis subject, the project has been ongoing throughout the period of my PhD.

The paper was accepted for publication in Food Security and can be found using the following reference:

LG Riggi, M Veronesi, G Goergen, C MacFarlane, RL Verspoor (2016) Observations of entomophagy across Benin – practices and potentials. *Food Security* 8 (1), 139-149