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Male reproductive success and population control in the Mediterranean Fruit Fly, *Ceratitis capitata*

Thesis submitted for PhD

by

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“Time flies like an arrow; fruit flies like a banana”

- Anon

Abstract

The traits that determine male reproductive success in *Ceratitis capitata* (medfly) are largely unknown. This comes despite decades of research into the reproductive behaviour of this agricultural pest. An understanding of what makes a successful male is of great importance in this lekking species, as one male has the ability to dominate access to females. In addition species-specific pest control techniques such as the Sterile Insect Technique (SIT) and Release of Insects carrying a Dominant Lethal (RIDL) rely on the mating competitiveness of mass-reared 'sterile' males to disrupt natural mating systems and reduce population numbers. However, it is well known that the mass-rearing process required to generate these individuals produces less competitive males, despite this an understanding of the sexual selection processes that are involved is severely lacking. In this thesis I used the medfly as a model organism to investigate the context-dependent nature of male reproductive success. In Chapters 2 and 3 I investigate the effect of manipulating the adult sex ratio on pre- and post-mating reproductive traits. Chapter 2 uses proximate manipulation of the adult sex ratio to show how the relationship between pre- and post-mating success is affected by the levels of male competition. Chapter 3 shows that selection lines are unable to select for the traits that predict male reproductive success under altered levels of male competition. Chapter 4 describes the attempts to produce RIDL genetic constructs with embryonic lethality. Chapter 5 demonstrates that RIDL lines of medfly display characteristics that make them suitable for wild population control and may exceed the performance of existing SIT lines. Chapter 6 shows that manipulation of the larval diet can alter the gut microbiota of adults; however these manipulations have no effect on reproductive behaviour. Instead there was a significant effect of high-sugar larval rearing on mating success and body mass. This effect persisted for a single generation in offspring of these flies, even when they were reared on an inferior starch-based larval diet. Finally Chapter 7 summarises the thesis and discusses the implications of this work and future directions for both research in the reproductive behaviour of the medfly, and the future of RIDL technology in medfly population control.

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Chapter 1: General Introduction

This thesis is a summary of my research into male reproductive success and population control in the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). The medfly is a lekking insect, males aggregate in order to display and compete for the chance to mate with females. In lekking species, males provide no parental investment (PI), so that the benefit gained by the females expressing mate choice is primarily indirect via genetic input into their offspring. The continued maintenance of sexual selection in the face of strong directional female choice for the 'best' male genotypes has been recognised as a conundrum that needs explanation.

In this introductory chapter I discuss the basis of sexual selection theory and the attempts to resolve the above 'lek paradox' (section 1.1.1). I also discuss the role of the mating environment and of sex ratio on the opportunity for sexual conflict (section 1.1.2), another factor of considerable importance in a lekking species, where male-male competition is often high. I introduce the medfly in section 1.2, outlining its status as a pest insect and here I also discuss the mating behaviour of males in greater detail.

Research into male reproductive success is important in general to understand the identity and mechanisms underlying fundamental components of fitness. However, such research has additional importance in pest species such as the medfly. This species has been targeted by pest control methods such as the Sterile Insect Technique (SIT) (sections 1.3 and 1.4) and more recently by the Release of Insects carrying a Dominant Lethal (RIDL) (section 1.5). Both techniques rely on the release of large numbers of mass-reared males to disrupt reproduction of the wild type. Studies into the basis of sexual selection and sex ratio are key to the success of these techniques; the success of released males in these programs is dependent on their ability to elicit female responses to their courtship attempts under extremely male biased conditions. There are potentially large benefits for incorporating the latest sexual selection theory into attempts to improve mass-reared male mating performance. Despite decades of research into the sexual behaviour of the medfly, what determines the success or failure of male courtship is unknown. These issues

are described in section 1.5. I conclude this chapter with an introduction to the outline of my thesis (section 1.6).

1.1 Sexual selection and sexual conflict

The concept of sexual selection was introduced by Darwin (1859, 1871) to address the apparently paradoxical observation of why, in so many species, there are sex-specific traits that can impede the ability of individuals to survive. Sexual selection explains the presence of these so-called 'secondary' sexual characteristics, e.g. ornate peacock feathers and the sexually dimorphic size and strength of male elephant seals (Darwin, 1871, Andersson, 1994). Darwin realised that the fitness of an individual is not only dependent on its ability to survive but also upon their ability to gain mates and reproduce. "...this leads me to say a few words on what I call Sexual Selection. This depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring" (Darwin, 1859). Sexual selection is a form of intraspecific competition that can occur before, during and often after mating. Before copulation, intrasexual competition – usually between males - may take place to determine which males have priority positions in mating aggregations (e.g. the well-known 'lek', described in section 1.1.1) and hence the best access to females. It may therefore significantly influence mate choice and intersexual selection. Intersexual selection occurs when one sex – usually females - chooses between potential mates on the basis of secondary sexual characteristics. After copulation, selection may continue in the form of male-male competition via sperm competition, or through cryptic female choice in which females with internal fertilisation may bias paternity by preferentially choosing which sperm fertilise their eggs. Again it was Darwin who first pointed out that the "sexual struggle is of two kinds, between individuals of the same sex, generally the males, in order to drive away or kill their rivals, the females remaining passive; whilst in the other... the females, which no longer remain passive, but select the more agreeable partners" (Darwin, 1871).

However it was not until 80 years after Darwin's key tenets of sexual selection were laid down that evidence started to emerge of the different benefits gained by the sexes in reproductive activities (Bateman, 1948). This evidence started to explain why the sexes might adopt distinct roles in reproduction. For example, it is common for males to compete strongly and indiscriminately for females, whereas females may often express strong mate choice for particular males. It was realised that an important basis of sex differences in the strength of selection acting on various reproductive activities arose from variation in reproductive success and also in how the sexes benefit from additional matings (Bateman, 1948). In Bateman's now classic fruit fly study in *Drosophila melanogaster* groups of five adult males and five adult females, each bearing different chromosome markers, were combined allowing the attribution of offspring to their parents. From this, Bateman generated a striking result, from the mating experiments only 4% of females failed to produce any progeny, by contrast 21% of males contributed no offspring to the next generation. In other words males were five times more likely to fail to reproduce than females. Importantly he also discovered that in males there was direct proportionality between number of mates and fertility, so that while some males produced no offspring, the most successful males produced nearly three times as many offspring as the most successful female. By contrast a female's reproductive success did not increase significantly beyond the first mating.

Sex differences in potential reproductive rates are rooted in anisogamy, i.e. in relative differences in energetic investment in the production of gametes. It is generally assumed that the investment per gamete is small for male gametes (sperm) and large for those of the female (eggs). This difference is proposed to be sufficient for a male's reproductive success to be limited not by his ability to produce sperm but by his ability to fertilise eggs with those sperm. Females are generally assumed to face the opposite problem, with their reproductive success being limited by the ability to produce eggs rather than having them fertilised (Bateman, 1948, Parker, 1970, Parker *et al*., 1972, Parker, 1979, Johnstone *et al*., 1996, Chapman, 2006).

1.1.1 Lek mating systems and the ‘lek paradox’

In many species female reproductive capacity is further constrained by the often greater relative level of PI made by females in comparison to males. PI includes metabolic investment in sex cells and any other actions that benefit the young (Trivers, 1972). An extreme case of sex differences in PI is found in the so-called ‘lekking’ species (Emlen & Oring, 1977). A lek is a non-resource based aggregation of males, where each male may occupy a small area or territory, and engage in aggressive interactions with other males in order to perform mating displays to attract females. Lekking species are common across a wide range of mammals, birds, fish and insects (including the medfly) (Schuster, 1976, Steiner, 1978, Avery & Sherwood, 1982, Rossiter, 1994, Eberhard, 2000, Birkhead *et al*., 2006, Widemo & Johansson, 2006). The successful males are generally those with the most extreme plumage, vocalisations or sexual displays. Importantly, in a lek females receive no material benefits or parental care from males. Understanding the evolutionary forces that drive preferences for males with exaggerated traits including those expressed in lekking species has been the subject of much theoretical debate. Of particular interest is the phenomenon known as the ‘lek paradox’ (Kirkpatrick & Ryan, 1991). Simply put, this refers to the idea that strong directional selection on the secondary sexual characteristics of males arising from female choice should quickly deplete genetic variation underlying those traits. The finding that this does not occur is therefore deemed ‘paradoxical’ and in need of explanation (Kirkpatrick & Ryan, 1991). For instance, why should females continue to express strong preferences for males when there are ever-smaller benefits of doing so?

There have been several attempts to resolve the ‘lek paradox. For example, ‘Runaway selection’ proposed by R.A. Fisher (1915), is a mechanism by which a reproductive trait, which can be arbitrary with regards to male survival/condition, can become exaggerated as a result of a genetic correlation with the female preference. It requires the existence of a genetic correlation between preference and trait. This can be facilitated by a pre-existing ‘sensory bias’ in females for traits in males, which would then result in males possessing a particular trait or trait value leaving more offspring. If trait and preference are co-inherited then evolution of the male trait and

evolution of the female preference for the trait can become exaggerated together. 'Runaway' occurs as the existence of the female preference confers an advantage on the male trait, which in turn selects for exaggeration of the female preference, so that equilibrium cannot be reached (Fisher, 1915, Fisher, 1958, Lande, 1981). Ultimately runaway is halted when either genetic variability is exhausted or the trait becomes so exaggerated that male viability is compromised (Kirkpatrick & Ryan, 1991); hence it is predicted that traits will eventually cease to be subject to sexual selection once natural selection acts counter to any benefits of sexual selection.

An alternative mechanism for the maintenance of female choice is the 'good genes' hypothesis (Kirkpatrick & Ryan, 1991). This is based on the intuitive idea that female preference for male traits is rooted in the ability of females to detect and express a preference for high male genetic quality, which will therefore result in increased viability in the female's offspring (Kirkpatrick & Ryan, 1991, Andersson, 1994, Rowe & Houle, 1996). The 'Sexy sons' mechanism is a similar concept, but one which focuses on the indirect benefit to a female in terms of the increased reproductive potential of sons resulting from mating with 'sexy males' (Fisher, 1958). Under this scenario, if the male traits chosen by females are heritable, then a female may gain an increase in fitness through the number of grandchildren produced by mating with a sexy male. The sons, being sexy, have the potential to produce large numbers of offspring by mating with multiple females (Bateman, 1948, Wedell & Tregenza, 1998). However as with 'runaway selection', generations of selection for preferred traits in males that allow females to gain 'good genes' or 'sexy sons' is expected to erode genetic variance, as females select only those males which display the 'best' genotype, placing traits under strong directional selection (Falconer, 1981). This should invariably erode the benefits of female choice, as all males end up carrying the same 'good genes'. Why then would strong female preference persist if there is no benefit of choice? This can only be resolved if genetic variation in the traits that underpin sexually selected traits in males is maintained.

The handicap principle, first suggested by Amotz Zahavi (1975), attempted to explain the persistence of female choice in the face of directional selection by invoking the idea of 'costly signals'. If a trait is to communicate fitness, then it must be reliable, or it will cease to be selected by females as an honest signal. In order for

a trait to be reliable then it must be costly to produce, as through imparting a survival cost to the bearer, the cost makes producing a false signal of fitness impossible. Two main objections to this idea have been proposed. First, that constant directional selection should lead to insufficient genetic variation to allow the handicap to act as a signal of fitness. Second, that the male handicap impedes survival and will also be transmitted to male offspring, lessening any advantage of choice (Maynard Smith, 1976). There is no evidence to support the first objection. It is reported that there is significantly higher additive genetic variation in sexually selected traits than non-sexually selected traits (Pomiankowski & Moller, 1995). The second objection to the handicap principle may be less easily overcome. Theory assessing the relative strength of these processes has often concluded that the burden of a handicap may outweigh the advantages of choice, and hence the process may come to resemble runaway selection (Maynard Smith, 1976).

An alternative mechanism for honest signalling is the parasite hypothesis (Hamilton & Zuk, 1982). This idea stems from the observation that parasites are a ubiquitous feature of metazoan life, and that the continuous co-evolution between parasites and their hosts leads to a large class of genes in the host with effects on resistance that are under cyclic co-adaptation. The fundamental prediction of the parasite hypothesis assumes that the full expression of secondary sex characteristics is condition-dependent, and can be limited by parasitic infection. Females then choose males with exaggerated sexual characters in order to avoid parasite infection in their offspring, through the inheritance of immunity genes. However, a key feature is that the trait itself while 'costly' to produce, need not be a survival handicap. Variation in male secondary sexual characters is maintained due to constant co-evolution with parasites. Unequivocal evidence for the parasite hypothesis has proven difficult as healthy males are always likely to mate more, and there are many reasons why females may prefer to mate with them, including direct selection (females attempting to avoid infection) (Moller 1990).

A more recent resolution to the lek paradox has been suggested by Rowe and Houle (1996), with the 'genic capture' hypothesis. This idea proposes that all genetic variance within an organism can theoretically be 'captured' by condition dependent traits (Rowe & Houle, 1996, Tomkins *et al*., 2004). The difference between this idea and the handicap principle is subtle but important. Rather than having a trait which is

so costly to produce that low-quality individuals cannot bear it, such as large antlers or a long tail, genic capture proposes that signals are kept honest simply because they are intimately tied to an individual's physical condition. As such it encompasses and extends the range of honest signalling proposed by the parasite hypothesis. Genic capture theory makes several clear predictions: 1) that an individual's expression of sexually selected traits should be condition dependent; 2) the condition of an organism is affected by a combination of general health, nutrition, parasitic load and many other factors; 3) thus condition is highly correlated with an organism's genotype and the result of the epistatic interactions of multiple loci (Tomkins *et al*., 2004). Essentially, all the genetic variation in a male responsible for condition may theoretically be 'captured' in the expression of these honest sexual signals.

Genic capture represents the most complete hypothesis regarding the maintenance of strong sexual selection to date and supporting evidence is growing. There are now many examples of condition dependent variation in sexually selected characters (Cotton *et al*., 2004, Birkhead *et al*., 2006, Bonduriansky, 2007), although empirical evidence for the 'cost' of traits is still lacking. Excitingly, mechanisms are beginning to emerge, by which condition may be 'captured' for signalling. There is evidence for example that the insulin/insulin-like growth factor (IGF) pathway may be key to the integration of physiological condition with sexually selected trait expression (Emlen *et al*., 2012). The insulin/IGF pathway is an essential regulator of tissue growth and overall body size, and is conserved across multicellular taxa (Teleman, 2009). On the surface this would indicate that the biggest males will always have the biggest sexually selected characters, as traits scale proportionately with body size (Shingleton *et al*., 2005). However, it turns out that traits differ in their responsiveness to insulin/IGF signalling; in *Drosophila*, genital size is relatively invariant with respect to insulin/IGF signalling, while wing size is highly sensitive to it. As a result wing size correlates with body size through larval nutrition, while genital size does not (Shingleton *et al*., 2005). Disruption of this same pathway with RNAi in the rhinoceros beetle (*Trypoxylus dichotomus*) demonstrated that the sexually selected trait of horn size was eight times more sensitive to this pathway than other morphometric traits such as eyes, legs and wings (Emlen *et al*., 2012). Genic capture now represents the most plausible mechanism for the development of exaggerated sexually selected traits in males. This new understanding of how natural

selection and sexual selection can work together through condition dependent traits puts classic studies of reproductive behaviour in a new light, and is of increasing importance in studies of male reproductive success.

1.1.2 Sexual conflict

Mating systems have a profound effect on the probability that sexual conflict will occur (Parker 1979). Sexual conflict occurs because of the potentially different reproductive rates in males and females. It is predicted for example, that conflict will occur over the mode and frequency of mating, as the optimum mating frequency for males is generally higher than it is for females. This can lead to sexually antagonistic coevolution, in which selection for adaptations that benefit males is balanced by counter-selection in females. Males are generally predicted to invest in courtship attempts that maximise mating frequency, and seminal fluid products that increase paternity, even to the detriment of the health of females. These favourable traits in males are predicted to directly or indirectly decrease fitness in females, and females are predicted to counter-adapt by evolving resistance to mating and male-induced harm. The levels of sexual conflict in a population are not fixed, and changes to the availability of mates and the levels of competition can alter the 'load' of sexual conflict that exists in a population. This can have profound impacts on sexual selection and mating behaviours, and is of particular relevance to mass-rearing effects on medfly for SIT (section 1.4), where studying alterations to the natural mating system can identify those male traits which are under subject to selection arising from sexual conflict.

Holland and Rice (1999) were among the first to investigate the effect of altering mating systems on the intensity of sexual conflict. They set up two populations where sexual selection was removed through enforced monogamous matings, or retained in promiscuously mating controls. They found that monogamous regimes, in which the ability of males and females to make mate choices and mate multiply was removed, rapidly reduced intersexual conflict as males evolved to invest less in courtship and induce less harm to females in mating. Simultaneously

female resistance to harm was reduced, such that mating costs with 'control' males were higher. These results have since been confirmed in several other experimental evolution studies (Hosken *et al*., 2001, Hosken & Ward, 2001, Martin & Hosken, 2003).

Criticisms were initially levelled at experimentally evolved monogamous lines, because of the potential for differing effective population sizes from the polygamous control lines they were tested against. The argument was that in some of these studies, the differences reported could be due to inbreeding differences between treatments, and that many of these experiments used only two replicate lines per treatment (Snook, 2001, Wigby & Chapman, 2004). An alternative suggestion for manipulation of mating systems was to generate experimentally evolved lines with altered adult sex ratios, in which male biased lines would increase the intensity of sexual selection, while female biased lines decreased the intensity of sexual selection (Wigby & Chapman, 2004, Reuter *et al*., 2008). A second alternative was to leave sex ratio balanced but increase the population density in order to increase the potential for promiscuity and sexual conflict (Martin & Hosken, 2004). Comparisons between these studies and the earlier work were largely consistent (Wigby & Chapman, 2004, Reuter *et al*., 2008). Initial concerns about potentially confounding differences in effective population sizes proved to be largely groundless (Rice and Holland 2005, Snook *et al*. 2009). A follow up experiment by Reuter *et al*. (2008), experimentally altered the effective population sizes to levels much smaller than any estimates from previous studies (Holland & Rice, 1999), and still found no evidence for confounding effects. In the light of these findings, it must be concluded that experimental manipulation of mating systems is an extremely useful tool for studying traits that are undergoing sexual selection in males and females. This is of critical importance for studies in the medfly, where populations are exposed to radically altered sex ratios and densities when undergoing mass-rearing for SIT (section 1.3). In SIT strains, males are expected to perform competitively against wild males for matings with wild females, but in fact consistently underperform (see problems with SIT section 1.4).

1.2 The model species, *Ceratitis capitata*

Ceratitis capitata (medfly) (Wiedemann) is a member of the Tephritidae family of 'true' fruit flies, distinct from the family Drosophilidae from which it is separated by c.150 million years of evolution (Hedges *et al*., 2006). The Tephritid family comprises over 500 genera and nearly 5000 species of fruit flies. Studies into basic physiology, ecology, evolution, animal-plant interactions and sexual selection have been carried out in this family for over a century, and this group has been extensively studied both in the lab and the field (Dìaz-Fleischer & Aluja, 2000). In addition, many tephritids are considered to be of major economic importance, mostly as pest insects of various fruits and plant crops (Dìaz-Fleischer & Aluja, 2000).

The medfly is the most polyphagous of all the tephritids and is distributed across almost every tropical and temperate region of the world. It is a major pest due to the constant threat of invasion and reinvasion (Christenson & Foote, 1960, Bateman, 1972, Carey, 1996). Its polyphagous nature and global spread makes the medfly the most economically significant of the tephritids, and potentially the most economically damaging crop pest in the world (USDA & NASS, 2006). Adult females lay their eggs in batches of one to ten eggs, depending on the size of the fruit (McDonald & McInnis, 1985), just beneath the peel, particularly in pre-existing cracks or wounds (Silvestri, 1914, Back & Pemberton, 1915). Females display incredibly plastic oviposition behaviour and will accept many fruits as hosts even if they do not support larval development (Carey, 1984, Prokopy *et al*., 1984, Katsoyannos, 1989, Dìaz-Fleischer *et al*., 2000).

The sexual behaviour of the medfly has been extensively studied due to its status as a pest insect and because of the knowledge needed to successfully implement the sterile insect technique (SIT) to control it. SIT employs the mass-release of sterile males to disrupt the normal mating of wild females (Knipling, 1955) (see section 1.3). Initial concerns for the use of SIT centred on whether mass-reared sterile males would exhibit sufficiently wild type like characteristics and as a consequence of addressing this, the normal sequence of lekking, courtship and copulation has been well studied (Hendrichs *et al*., 2002, Robinson *et al*., 2002). Male medflies typically gather together in small leks of between 3 to 4 males, but

under SIT conditions this can rise to as many as 16 males in a single lek (Shelly *et al.* 1994). These aggregations represent classical leks in that they provide no resources for females, but offer the opportunity to choose a mate (Shelly *et al*., 1994). Male mating success is non-randomly distributed, it is assumed due to female choice, hence a few males have the capacity to monopolise access to female ova (Whittier & Kaneshiro, 1995). While participating in leks, males release pheromones that attract males and females usually over a distance of less than 1km (Shelly *et al*., 1993, Eberhard, 2000). Courtship, as first described by Féron (1962), begins when a female lands on a leaf supporting a male that then initiates courtship by 'pheromone calling'. This is a readily observable behaviour in which the abdomen curls dorsally and a rectal epithelial sac is everted to release pheromones (Arita & Kaneshiro, 1989). Pheromone calling is followed quickly by 'wing buzzing'. If the female orients towards the male he will vibrate his wings for a period of 15 seconds or more with the pheromone sac still everted, presumably to fan pheromones directly towards the female. In the final stage of courtship, the rectal sac is often retracted and wing movement shifts to an 'intermittent wing buzzing', where the wings twitch back and forwards while continuing to buzz. At this point the male stands directly opposite the female and the edges of the female's wings can be blown backwards by male wing fanning (Kaneshiro *et al*., 1995). Accompanying 'intermittent wing buzzing' is 'head rocking', this is performed at intervals, possibly to demonstrate the sexually dimorphic anterior orbital bristles. However, removal of one of these bristles does not appear to reduce courtship success (Mendez *et al*., 1999, Robinson *et al*., 2002), which suggests that this trait is not sexually selected. After the male has finished courting, he will attempt copulation, leaping over the female, before quickly turning around and clasping the female abdomen with his rear legs (Briceno *et al*., 1996). It is at this point when most copulations fail, as females often fall or fly from the surface to which they are attached, dislodging the male in the process. Successful copulations usually last between 90min to 3 hours, copulations lasting less than 15 minutes do not result in any sperm transfer (Seo *et al*., 1990, Taylor *et al*., 2000).

Termination of copulation is believed to be initiated by males, as males must tug repeatedly to remove their intromittent organ from within the long female ovipositor. However the precise proportion of control over copulation duration attributable to each sex is unknown, as female resistance to intromission may

influence copulation duration, as larger and older females tend to mate for longer (Field *et al*., 1999).

Particular males have been shown to dominate leks, which would suggest high variance in male reproductive success. However, female rematings sometimes occur off the lek and therefore have the potential to drastically reduce the reproductive success of the dominant lek males (Whittier & Kaneshiro, 1995, Hendrichs *et al*., 2002). The extent to which female medflies will remate in the wild has been a source of considerable research, both from the perspective of lek mating systems, but also because female remating can drastically reduce the efficiency of SIT. The frequency of female remating as experimentally tested in wild derived females is relatively high during the first four days after a first mating, with 3.3-13.3% of females observed re-mating over this time period, falling sharply after the 5th day (Kraaijeveld & Chapman, 2004). Female remating as assessed from paternity of offspring of wild flies was determined by use of microsatellites at between 4-28% (Kraaijeveld & Chapman, 2004). Refractoriness to remating is reported to be induced by male accessory gland products and the presence of sperm in the female reproductive tract (Jang, 1995, Miyatake *et al*., 1999, Jang, 2002). In *D.melanogaster* a combination of accessory gland products and sperm transfer is required to induce the highest levels of female refractoriness to remating (Wolfner, 1997, Swanson, 2003, Chapman, 2008, Fricke *et al*., 2009). However, such detailed investigations have yet to be done in the medfly. Initial studies identified thirteen genes with male-specific accessory gland expression in the medfly. Of these, nine have homology with known accessory gland genes in *D.melanogaster* (four of these also share homology with three seminal fluid protein gene sequences in *Aedes aegypti*, (Sirot *et al*., 2011)). Another four genes identified represent novel sequences, the function of which remains to be empirically tested (Davies & Chapman, 2006).

Despite the extensive descriptive detail of courtship and copulation in medfly little is known about the key determinants of male reproductive success. In addition, observations of secondary sexual characteristic traits in medfly have rarely been placed into a sexual selection framework (Boake *et al*., 1996). Also little attention has been paid to the impact of condition dependent variance in these traits. Studies into male mating behaviour and the competitiveness of mass-reared males for SIT almost exclusively use adult densities and sex ratios that are not comparable to those

found in the wild or under SIT suppression conditions. This is a significant omission given the extensive research effort applied to this organism in order to achieve population control.

1.2.1 Gut microbe-host interactions of the medfly

Hitherto overlooked is the influence of the gut microbiome on reproductive success in the medfly. Until recently associations between the Mediterranean fruit fly and bacteria received little scrutiny (Marchini *et al.*, 2002). However, it is now known that medfly guts contain a relatively simple, discrete community of bacteria, concentrated in the esophegeal bulb. This community is dominated by bacteria from the *Enterobacteriaceae* (*Klebsiella*, *Enterobacter*, *Pectobacterium*, *Citrobacter* and *Pantoaea* spp) (Behar *et al*., 2008b, Ben-Yosef *et al*., 2008a, Ben-Yosef *et al*., 2008b). However mass-reared medflies also have increased levels of the potentially pathogenic *Pseudomonas* spp (Ben Ami *et al.*, 2010b). It has been previously demonstrated that gut bacteria are capable of altering host behaviour and fitness in medfly and are at least partially capable of vertical transmission (Behar *et al*., 2005, Behar *et al*., 2008a, Behar *et al*., 2008b, Ben-Yosef *et al*., 2008b, Ben Ami *et al*., 2010a). This is also of interest because of the potential for the gut microbiome to induce reproductive isolation. In a recent experiment in *Drosophila* melanogaster, it was reported that the mechanism underlying the evolution of reproductive isolation through ecological adaptation in a classic study (Dodd, 1989); was due instead to differentiation of the gut microbiome (Sharon *et al*., 2010). There may therefore be the potential for tighter evolutionary relationships between gut bacteria and medflies than previously appreciated, and the effect of the domestication process on the gut microbiota is of particular importance for the study of mating behaviour and fitness in mass-reared flies. The gut bacterial communities of mass-reared flies are proposed to change significantly under domestication of strains for SIT, the principles of which are described in the next section.

1.3 SIT – The Sterile Insect Technique

The Sterile Insect Technique (SIT) is a species-specific and non-polluting method of insect population control that relies on the mass-rearing, sterilisation and release of large numbers of the target insects (Knipling, 1955, Knipling, 1979, Krafsur, 1998). The principle behind this system is that by releasing large numbers of sterile individuals over a wide area it is possible to overwhelm the natural population to the extent that fertile individuals almost invariably end up mating with their sterile counterparts and hence no offspring will be produced (Knipling, 1955).

The first pest to be targeted with SIT was the New World screw-worm fly (*Cochliomyia hominvorax*, Coquerel). The screw-worm is an obligate parasite of mammals that causes primary myiasis in cuts and abrasions. Myiasis begins when a female screw-worm is attracted to an open wound (though occasionally this extends to the mouth, nasal cavity or the navel of newborns) and lays up to 450 eggs (Baumhover, 1966). The resulting larvae from a single oviposition bout can kill small animals as they feed on the host's living tissue. Larger animals such as cattle are often killed as a result of infections that result from the large open sores. In some cases humans have also died from the direct or indirect consequences of screw worm infection (Dove, 1937, Baumhover, 1966, Krafsur *et al*., 1987). Prior to the implementation of SIT, screw-worm was managed by animal husbandry techniques such as checking and cleaning wounds, and by chemical spraying. However, these techniques were often expensive, laborious and relatively ineffective, making screw-worm a significant agricultural pest (Baumhover, 1966). A concerted program of SIT designed to eliminate screw-worm from the southern United States began in 1958 and was widely regarded as a great success, with no reported cases of screw-worm since 1982 (Krafsur *et al*., 1987, Krafsur, 1998). On the back of this success SIT programs were rolled out across the Americas, with successful follow up programs in El Salvador, Honduras, Nicaragua, Costa Rica and Panama (Wyss, 2000). When an outbreak of New World screw-worm was reported in Libya in 1990, the Food and Agriculture Organization (FAO) of the United Nations launched a massive SIT eradication program. Due to the techniques and infrastructure built up and perfected over decades of SIT in the United States, the eradication process took less than 6 months (Lindquist *et al*., 1992).

The successful example set by SIT eradication of the screwworm, led to the first large SIT program being set up against medfly in Mexico in 1977 - the Moscamed mass-rearing program. This succeeded in eradicating medfly infested areas of southern Mexico, and an SIT barrier was established to prevent reinvasion and maintain the fly-free status of Mexico and the USA (Hendrichs *et al*., 1983, Hendrichs *et al*., 2002). SIT has also been implemented prophylactically in some medfly free areas such as California, where it is estimated that an infestation of medfly, if it occurred, would cost the region around $2 billion a year (Siebert, 1999). In areas where the medfly is a persistent problem there has been a gradual shift towards suppression over eradication and the economic feasibility of using SIT for medfly has been supported by cost-benefit analyses (Siebert, 1999). Even without including the environmental benefits, costs per hectare per year of protecting orchards for an integrated area-wide approach using SIT is roughly equivalent to the costs for area-wide application of bait-sprays and lower than for non area-wide conventional cover sprays (Enkerlin & Mumford, 2001, Mumford, 2005). These savings to the fruit industry, and the general environmental benefits, reveal the potential gains for the establishment of commercial SIT mass-rearing facilities.

The first attempts at SIT released both sexes, as large-scale sex separation was not feasible. However it was readily apparent that male-only releases were preferable, as in many species it is only the adult females that are potentially damaging. For example, in mosquitoes it is the females that bite and act as potential disease vectors, while in Tephritids it is females that are responsible for crop damage (Knipling, 1959, Knipling, 1979). More generally, released sterile females may ‘distract’ sterile males from finding wild females with which to mate; leading to sterile-sterile matings, each of which represents a failure to disrupt mating between wild individuals (Shelly & Whittier, 1996, Rendon *et al*., 2004). In one particularly disastrous attempt at SIT control in *Bactrocera oleae* Rossi (olive fly), mixed-sex releases produced no reduction in population numbers at all. This ultimately proved to be due to a slight asynchrony in the time of day at which mass-reared and wild flies would attempt to mate, so that released sterile males only attempted to court and mate with sterile females (Economopoulos & Zervas, 1982, Estes *et al*., 2012). When a second attempt at this program was attempted with male-only releases, the asynchrony in mating times was less of an issue, as males dispersed in search of wild

females (Estes *et al*., 2012). Large scale sets of field trials into mixed-sex vs. male-only medfly SIT revealed a 3-5 fold increase in efficacy with male-only releases (Shelly & Whittier, 1996, Rendon *et al*., 2000, Rendon *et al*., 2004).

The idea of improving the efficiency of SIT through male-only releases was suggested early on in the development of SIT (Knipling, 1959). However, in most species it was considered either too laborious or was simply not possible to effectively separate males and females. Whitten (1969) was the first to propose that genetic sexing strains (GSS) could be developed, by inducing the translocation of a visible genetic marker to the male sex chromosome. This would then allow the sexes to be distinguished relatively easily. GSS are derived from single individuals in whom a unique irradiation-induced translocation has occurred. Genetic diversity is usually then reintroduced into the resulting strains through backcrossing with wild type and screening in order to prevent inbreeding (Franz *et al*., 1997). The first GSS developed in the medfly used inducible-female lethality coupled to the alcohol dehydrogenase locus (ADH) to generate male-only (97%) cohorts under the restrictive conditions (Robinson *et al*., 1986). This strain contained males that had an *ADH* null mutation translocated to the male determining chromosome; this meant that *ADH* activity was higher in females than males. When larvae were exposed to allyl alcohol - *ADH* converted this to a toxic ketone, as female enzymatic activity is higher in this strain, they are able to tolerate lower levels of allyl alcohol than males; and by using the correct concentrations it was therefore possible to produce male-only cohorts (Robinson *et al*., 1986). The success of GSS has been their ability to produce male-only cohorts for mass release under SIT factory conditions. This has led to their almost universal adoption in SIT programs – a GSS currently used widely in medfly SIT programs is VIENNA-8 (Robinson *et al*., 1999, Robinson, 2002, Franz, 2005, Pereira *et al*., 2007). VIENNA strains have two Y-linked translocation markers, which create male-specific expression of the wild type alleles for *white pupae* (*wp*) (Rossler, 1979) and *temperature sensitive lethal* (*tsl*) (Franz *et al*., 1994). These strains contain females that are homozygous for *wp* and *tsl*, which means they can be killed with a heat shock during development, and the lines easily screened for effective female lethality by scoring the resulting pupae for the absence of the *wp* marker (Fisher, 1998).

1.4 Shortcomings of SIT

Despite the many successes with SIT and the implementation of GSS, it is readily acknowledged that there are significant flaws with current SIT technology that drive up costs (Hendrichs *et al*., 2002). The investment in infrastructure, rearing and distribution of medfly are all firmly established, leading to efficient economies of scale for medfly suppression programs (Enkerlin & Mumford, 2001). However, further increases in cost-effectiveness for SIT are desirable (Hendrichs *et al*., 2002). The most promising scope for improvement of SIT medfly programs is recognised to lie with enhancements to the mating competitiveness of the released sterile males (Hendrichs *et al*., 2002, Robinson *et al*., 2002). It has been continuously reported that the sterile males used for release display impaired mating success rate with wild females. Even in the best SIT strains the mating success of released males is reported as less than half the mating rate of wild males (Shelly & Whittier, 1996, Cayol & Zarai, 1999, FAO/IAEA/USDA, 2003, Pereira *et al*., 2007, Pereira *et al*., 2011). The efficacy of an SIT strain can be reduced by the cumulative effects of the mass-rearing process and the sterilisation method, both of which can be subject to a large degree of variability (Briceno & Eberhard, 1998, Briceno *et al*., 2002, Lux *et al*., 2002a, Lux *et al*., 2002b, Parker & Mehta, 2007).

Initiation of a mass-rearing SIT strain from the wild is often difficult and wild-derived flies often fail to reproduce under mass-rearing conditions (Rossler, 1975). In overcoming this problem strong selection is placed upon females that, unlike their wild counterparts, will oviposit into fine nets or screens in the absence of colour, tactile or odorant cues (Schwarz *et al*., 1981). Because of these initial difficulties in establishing mass-rearing, the widely-used SIT strains are often relatively old. They have therefore usually been bottlenecked at the initiation stage, and isolated from their various originating wild populations for many generations. They often have very different rates and determinants of reproductive success than the wild populations from which they were derived (Cayol *et al*., 2002, Lux *et al*., 2002a, Pereira *et al*., 2007). Despite this, there is very little evidence of any strain or geographically based pre-mating reproductive isolation among SIT strains. Instead, a more important factor seems to be the strong selection placed on life history arising from adaptation to the crowded mass-rearing conditions. This leads to replicable

changes in a suite of similar behavioural traits across many different SIT strains in comparison to wild flies (Briceno & Eberhard, 1998, Cayol, 2000). Mass-reared males appear to join, call and remain in leks in the same way as do wild males, and also attempt to court at the same frequency. However, females are 3-4 times more likely to accept courtship and mating attempts from wild over released sterile males (Shelly *et al*., 1994, Briceno *et al*., 1996, Lance *et al*., 2000). While it has been observed that mass-reared males engage in each aspect of courting behaviour, overall courtship duration is significantly shorter in mass-reared males (Calkins, 1984). This is likely a result of selection for shorter courtship under mass-rearing driven by the crowded conditions needed to generate the large number of flies for SIT. Under the high densities of captivity, courtship sequences are often interrupted by other males (Briceno & Eberhard, 1998), hence there may be strong selection to truncate courtship and move earlier to mating attempts. This is expected to increase the rejection by wild females of courtship and mating attempts from released males.

The irradiation process used to sterilise males is highly effective, with >99% of males being completely infertile after treatment. However the negative effects on sexual competitiveness from irradiation are well documented (Hooper, 1972, Rossler, 1975, Wong *et al*., 1983, Kanmiya *et al*., 1987, Moreno *et al*., 1991, Lux *et al*., 2002b). Ionising radiation is used to sterilise males by causing lesions and breaks in the DNA of the actively dividing cells of the male germ line (i.e. the sperm). This results in the expression of lethal mutations, whilst leaving the more slowly dividing cells of the soma relatively unharmed (LaChance *et al*., 1967, Curtis, 1971). However, in reality calculating the radiation dose that ensures there is no residual male fertility in the released males, but minimal damage to the soma is extremely difficult. Invariably some somatic damage results from irradiation, leading to reduced lifespan and mating competitiveness in the released sterilised males (Hooper, 1972, Barry *et al*., 2003, Parker & Mehta, 2007, Lauzon & Potter, 2008).

The traditional GSS are based on radiation-induced translocations of wild type alleles to the Y chromosome, which compliment X-linked or autosomal recessive traits for pupal colour and temperature sensitive lethality (Robinson, 1989, Franz *et al*., 1997, Robinson *et al*., 1999). These chromosomal aberrations tend to be unstable, requiring stringent quality control every generation in order to maintain

GSS integrity (FAO/IAEA/USDA, 2003). They also reduce the fitness of the insects, making them less effective as agents for SIT (Robinson, 2002).

A significant cause for concern for SIT is the extent of female remating. SIT males produce no viable sperm; as a result, remating by females with wild males can completely negate the benefits of SIT. It has been consistently demonstrated that remating rates in females that have first mated an SIT male are considerably higher than when they first mated a wild male (Kraaijeveld & Chapman, 2004). This appears to be due to the fact that SIT males transfer few or no sperm (Miyatake *et al*., 1999, Jang, 2002). This inability appears to prevent the development of female refractoriness to remating. Supporting evidence for this view comes from the finding that a female's propensity to remate is directly correlated with the amount of sperm received during copulation (Mossinson & Yuval, 2003, Bertin *et al*., 2010).

1.5 Release of Insects carrying a Dominant Lethal (RIDL)

Genetic methods that enhance SIT have recently become available in the form of the 'Release of Insects Carrying a Dominant lethal' (RIDL) system developed by Oxitec Ltd (Thomas *et al*., 2000). RIDL functions in a similar way to traditional SIT, but the releases males are not 'sterile' in the traditional sense, rather they carry a conditionally expressed dominantly lethal gene which reduces the number of offspring they produce. Females that mate with these RIDL males produce no or only male offspring, substantially reducing the population size as occurs with SIT (Alphey, 2002). RIDL has been developed and applied by Oxitec Ltd. to several insect species, including medfly, olive fly, mosquitoes and cotton bollworm (Morrison *et al*., 2009, Harris *et al*., 2011, Simmons *et al*., 2011, Ant *et al*., 2012). Transgenic RIDL insects carry a dominant, repressible lethal gene system, which can be expressed in a female-specific pattern (Thomas *et al*., 2000). RIDL-like transgene systems potentially represent a more stable and targeted approach for inducing female lethality than GSS, by using a tetracycline-repressible transactivator fusion protein (tTA). This is introduced into the host genome by the transposable element *piggyBac* which can be subsequently removed, making the inserts as stable as any native section of the genome (Handler, 2002, Dafa'alla *et al*., 2006). In the absence

of tetracycline, tTA will drive the expression of any (e.g. lethal) genes that are under the control of the tetracycline responsive element (*tRe/tetO*) (Gossen & Bujard, 1992). Proof-of-principle tests for these methods were initially developed as a two-component driver and responder systems in *D. melanogaster* (Thomas *et al* (2000). Two versions were constructed i) using a female-specific promoter, the *yolk protein 3* (*Yp3*) fat body enhancer (Liddell & Bownes, 1991), to drive tTA expression in female larvae and adults but not males and ii) male and female tTA driven expression of a female-specific lethal gene, *msl-2*^{NOPU} (a gene used in males to regulate dosage compensation by hyper activating the X chromosome (Gebauer *et al*., 1998). It was realised that the female-specific lethal system could act as a combined sexing and pest control system without the requirement of sterilisation through ionising radiation (Thomas *et al*., 2000, Alphey, 2002, Alphey & Andreasen, 2002, Alphey *et al*., 2008). This is because removal of the tetracycline repressor of dominant female lethality in the release generation allows a male-only population to be produced, which can then be released to mate with wild females.

RIDL males, unlike their SIT counterparts, are therefore fully fertile and produce normal sperm. However matings with RIDL males will produce no viable female progeny, substantially reducing the effective population size of the insect. As all RIDL females die, the population will decrease and the RIDL construct itself will be rapidly lost from the population (Alphey *et al*., 2008). Subsequent refinement of RIDL has used simpler and more universally compatible genetic components, in order to make RIDL readily transferrable across species. For example, a simpler one-component tTA system has been constructed to replace the two-component system developed previously (Thomas *et al*., 2000, Gong *et al*., 2005, Alphey *et al*., 2008). This condensed RIDL construct places the *tTA* coding region under the control of the tTA responsive element (*tetO*), and can provide a fully repressible lethal system in medfly (Gong *et al*., 2005). This system works by generating a positive-feedback loop, in the absence of tetracycline, where tTA levels build up to lethal levels. Low levels of tTA are relatively harmless. However at high concentrations, tTA is thought to exert a toxic effect due to transcriptional ‘squelching’ or by interfering with ubiquitin-dependent protein degradation (Gill & Ptashne, 1988, Berger *et al*., 1990). One significant advantage of the ‘positive feedback’ system is that it does not need to use any species-specific gene promoters. Koukidou *et al* (2006) successfully used

this system to make a widely applicable genetic marker, by using a non-lethal tTA positive feedback system to drive expression of enhanced GFP (*EGFP*) in *D. melanogaster*, *C. capitata*, the nematode *Caenorhabditis elegans*, the tobacco plant *Nicotiana tabacum* and in a human *He-La* cell line. Female specific lethality in the one-component RIDL system in medfly came with the insertion of intron cassettes from the *C. capitata transformer* (*Cctra*) gene. Sex-specific alternative RNA splicing of these introns is such that the transcription of *tTA* in males is disrupted as the pre-messenger RNA (pre-mRNA) contains several stop codons, and only females produce mature mRNA and therefore the functional protein (Fu *et al*., 2007). Once again the key components of this system are not species-specific and so function across a broad phylogenetic range, making it relatively straightforward to use this system in multiple species (Fu *et al*., 2010).

RIDL is in various stages of development for different species, proof-of-principle caged experiments in the mosquito *Aedes aegypti* demonstrated that the steady release of RIDL males could be used to eliminate target wild populations (Valdez de *et al*., 2011). On the back of this success, the first open-field trial with a RIDL insect was carried out with *Aedes aegypti* on Grand Cayman in 2009 (Harris *et al*., 2011). The releases lasted for only 4 weeks, however over this period 9.6% of eggs captured from oviposition traps had RIDL parentage, while RIDL males were estimated at 16% of the total proportion of males in the field (Harris *et al*., 2011). While RIDL medfly lines have been developed and tested for mating compatibility with wild type females against GSS, no open field trials or mating compatibility tests with wild-derived females have yet been undertaken (Morrison *et al*., 2009).

Further development of RIDL in medfly requires a combined integration of studies into transgenic medfly fitness against wild type, GSS, and wild-derived flies. These tests need to be conducted both in the laboratory and the field to assess rearing characteristics and reproductive success in natural conditions in order to demonstrate RIDL as a viable alternative to SIT in the medfly. Though RIDL tackles the major issue of the deleterious effects of irradiation on fitness, it does not resolve the basic problem of strain adaptation to mass-rearing. This effect has been extensively characterised, but research has generally ignored the sexual selection framework in which this adaptation occurs. The extensive literature on the conditions which promote or retard male reproductive success under SIT are largely anecdotal, each

aimed at promoting a mechanism to improve SIT, without recourse to the underlying mechanisms of sexual selection. As a result, an integrated view of female choice and the selection on male secondary sexual characteristics is sorely lacking. A better understanding of the evolutionary context of the medfly mating system would allow a predictive framework for the effects of altering sex ratios, changing population densities and changing selection pressures to be developed that would ultimately benefit the mass-rearing process. Research into sexual selection in the medfly also has the potential for substantial gains in evolutionary biology. The extensive knowledge of the natural ecology of this species allows us to bridge the gap between studies in the laboratory and the field in a way that is not possible in other genetically well-characterised model organisms such as *D.melanogaster*.

1.6 Outline of Thesis

The work in this thesis was funded by the Natural Environment Research Council (NERC) as part of an industrial 'Collaborative Awards in Science and Engineering' (CASE) studentship with Oxitec Ltd. This was performed under the supervision of Professor Tracey Chapman (principal supervisor, UEA), Professor Luke Alphey (principal supervisor, Oxitec Ltd) and Professor Matt Gage (secondary supervisor, UEA). This work was carried out under FERA licensing (UEA – PHL 170/6543, Oxitec – PHL 247/6840). All experiments were performed by the author unless otherwise stated. The aim of this thesis was to investigate the factors that determine male reproductive success in the Mediterranean fruit fly (*Ceratitis capitata*), and to investigate the application of the transgenic technology 'Release of Insects carrying a Dominant Lethal' (RIDL) towards population control of this agricultural pest.

In Chapter 2 I investigated the relationship between courtship, mating frequency and overall reproductive success. The strength of these relationships was predicted to be altered by the extent of post-mating competition, and this prediction was tested by manipulating the adult sex ratio from male- to female-biased. The results showed that under levels of high male competition males delivered significantly more courtship but gained lower overall paternity than under low competition. Paternity was positively associated with mating frequency, but this

relationship is plastic and was found to be significantly stronger under low competition. This highlights the importance of post-mating processes in lekking species and highlights selection pressures under mass-rearing for SIT. This work was carried out with post-doc Dominic Edward, who contributed to statistical analysis of the experimental data. The manuscript was published in the *Journal of Evolutionary Biology* with co-authors Dominic Edward, Luke Alphey, Matt Gage and Tracey Chapman (2012, DOI: 10.1111/j.1420-9101.2012.02556.x), and can be found in Appendix I.

This approach was extended in Chapter 3 in an investigation of whether male reproductive success in the medfly could be manipulated by establishing selection regimes of altered adult sex ratio. Replicate lines of three selection regimes were established of male-biased, equal sex and female-biased sex ratios, and these lines were propagated for over 50 generations prior to the experiments described. Focal males from each regime were compared for courtship frequency, mating frequency and paternity in two assays of high and low male competition. No evidence for any alteration in mating competitiveness was found for any of the selection regimes.

Chapter 4 describes attempts to establish earlier acting lethality in RIDL genetic constructs. The build-up of tTAV required to induce lethality in current RIDL strains does not occur until late into larval development. Earlier acting lethality, if possible, would have potential advantages both in mass-rearing and in the field. Constructs with embryonic promoters driving tTAV expression were transformed into wild type medfly and characterised for timing of expression. In tandem with this constructs with tetO promoting expression of the apoptotic gene *ReaperKR* were developed. The results from this were the development of lines of medfly with tTAV expression from 48 hours post-zygotic formation. However, despite repeated attempts *ReaperKR* was unable to induce lethality. Possible improvements to future construct development for embryonic lethality are discussed.

In Chapter 5 I detail the results from the life history characterisation of two medfly RIDL strains. These strains demonstrated full functionality of the RIDL construct as well as minimal fitness costs from transgenesis. Comparisons to a traditional GSS showed considerable fitness advantages in terms of longevity and fecundity. Tests against wild-derived males and females also showed strong mating

competitiveness from RIDL males. This chapter also contains the first proof-of-principle experiment to show that RIDL can be used to suppress medfly populations. This work is currently in preparation for submission to *Proceedings of the National Academy of Sciences* with co-authors Martha Koukidou, Polychronis Rempoulakis, Tracey Chapman, Aris Economopoulas, John Vontas and Luke Alphey.

Finally, in Chapter 6 I investigated the relationship between larval diets, gut microbiota and mate choice in medfly, prompted by recent findings in *D.melanogaster* of the role of bacteria in driving assortative mating patterns. The work is also relevant to the application of RIDL strains for control as they employ, under mass-rearing conditions, diets containing tetracycline, which will necessarily modify gut bacteria. Isolating medfly onto two different larval foods generated two distinct populations of gut microbiota, which did not however influence mating preferences. Larval rearing on a food with greater abundance of sugar produced larger males that were more successful at mating with females than males reared on a starch-based larval diet. This effect was trans-generational and persisted for a single generation when offspring of sugar-reared flies were transferred onto starch, suggesting that maternal effects may buffer against nutritional changes in the larval environment.

Chapter 7 is a general discussion of the findings of the thesis and their wider implications both for evolutionary biology and for improvement of RIDL for medfly population control. Male reproductive success is highly context-dependent and the implication of this for the improvement of male mating performance in SIT and RIDL is that a more synergistic approach must be taken to understanding natural selection and sexual selection processes in the medfly. Further development of RIDL also hinges on public and governmental acceptance of genetic modification, and this chapter discusses the potential future for this technology.

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Chapter 2: Variation in adult sex ratio alters the association between courtship, mating frequency and paternity in the lek-forming fruitfly *Ceratitis capitata*

2.1 Abstract

The intensity with which males deliver courtship and the frequency with which they mate are key components of male reproductive success. However, the strength of the relationship between these traits and a male's overall paternity is expected to be strongly context-dependent, e.g. to be altered significantly by the extent of post-mating competition. I tested this prediction in the lekking insect, *Ceratitis capitata* (medfly). I examined the effect of manipulating the sex ratio from male- to female-biased (high and low male competition, respectively) on courtship behaviour, mating frequency and paternity of focal males. Under high male competition, focal males delivered significantly more courtship but gained lower paternity than under lower competition. Paternity was positively associated with mating frequency and small residual testes size. However, the association between mating frequency and paternity was significantly stronger under low competition. I conclude that manipulation of sex ratio significantly altered the predictors of mating success and paternity. The relationship between pre- and post-mating success is therefore plastic and alters according to the prevailing level of competition. The results highlight the importance of post-mating processes in lekking species and illuminate selection pressures placed on insects such as medflies that are mass-reared for pest control.

2.2 Introduction

Fundamental differences between males and females, rooted in anisogamy, lead to the evolution of contrasting reproductive strategies among males and females (Darwin, 1871, Parker *et al.*, 1972). This is evident in the marked sexual dimorphism of traits such as size, plumage colouration, displays, courtship song and pheromone production (Andersson, 1994). Divergence in selection pressures for reproductive traits in males vs. females are thought to arise because of differences in reproductive roles, e.g. stronger competition between males than between females for access to matings and fertilisation opportunities (Johnstone *et al.*, 1996). The basis for differences in reproductive roles has traditionally been thought to be divergence in the relative levels of reproductive investment (often assumed higher in females) and in potential reproductive rates (PRR, assumed to be higher in males; (Bateman, 1948, Parker, 1970, Parker & Partridge, 1998, Chapman, 2006). However, it is now increasingly realised that reproductive roles and strategies are far more flexible than originally perceived, and that PRR is often a poor indicator of the relative level of reproductive investment (Snyder & Gowaty, 2007, Taylor *et al.*, 2008).

Either sex can express mate choice or be subject to intra-sexual competition for mates (Edward & Chapman, 2011). The reproductive strategy adopted by each sex is determined by key factors such as relative reproductive investment patterns, the availability of mates, an individual's capacity to mate with all of the available mates and variation in mate quality. Such factors are important as they alter the benefits and costs of each mating. Developing an understanding of how to predict costs and benefits in both sexes can be very informative. For example, it has long been realised that there are likely to be significant disparities in the costs and benefits of multiple mating in males vs. females, especially in cases where males make little or no investment in offspring care such as in lekking species. Under these conditions, sexual selection for traits in males that increase reproductive success should be very strong (Parker & Simmons, 1996). This is borne out in many lekking species of birds, mammals, amphibians and insects, which often show very marked sexual dimorphism, strong male-male competition and female choice. As a result variation in male reproductive success is often skewed, as relatively few males monopolise

females and father most offspring (Kirkpatrick & Ryan, 1991, Rowe & Houle, 1996, Droney & Hock, 1998).

Males can achieve high reproductive success and father significantly more offspring even under intense competition if they are able to monopolise access to females and their ova. They can do this through increasing their investment in pre-mating traits, such as courtship, which may then lead to higher mating frequency. They can also invest more in post-mating traits such as sperm transfer and mechanisms to promote sperm precedence and prevent female remating (Yuval & Hendrichs, 2000). However, despite the realisation that many traits can influence male reproductive success, until recently few studies had investigated the relative contribution of pre- and post-mating traits to a male's overall paternity (Fricke *et al.*, 2010, Pischedda & Rice, 2012). Hence the relative importance of, and the precise relationship between, pre- and post-mating sexual selection is often unclear. In some species, such as *Tribolium castaneum* flour beetles for instance, there is a positive association between a male's pre- and post-mating reproductive success (Lewis & Austad, 1994). In contrast, in the pond skater *Gerris lacustris*, there appears to be a trade-off between investment in pre- and post-mating traits (Danielsson, 2001). Given these varying patterns the fitness gains of increased investment in either pre- or post-mating traits may not always be straightforward to predict. In addition, perturbation of factors such as the operational sex ratio (the ratio of sexually available males to females (Emlen & Oring, 1977)), could alter the expression, and relationship with fitness, of pre- and post-mating traits (Reuter *et al.*, 2008).

Determining the relative contributions of pre- and post-mating sexual selection and a male's overall reproductive fitness is important for understanding the evolution of mating systems in general, but is also of applied relevance. For example, the identification of traits that are key in promoting success during male-male competition could prove useful in improving husbandry regimes for insects such as the medfly, an agriculturally important pest that is mass-reared for use in biological control programs (Mitchell, 1997). This knowledge could improve existing biological control methods such as the sterile insect technique (SIT, (Knipling, 1955, Dyck *et al.*, 2005)), which relies on matings between released sterile males and wild females, and is thereby dependent on the field mating success of mass-reared insects.

Interestingly, by releasing only males, medfly SIT also significantly changes the male: female sex ratio in program areas.

Here, I conducted an integrated study of the pre- and post-mating traits that influence a male's overall reproductive success. I focused on medfly as it is both an experimentally tractable model for mating behaviour in lekking species and a key target species for field control by SIT. The natural mating system of this species involves lek formation (Aluja & Norrbom, 2000), where males gather for pre-mating competitions in which pheromone signalling and courtship displays occur and females assess potential mates (Yuval & Hendrichs, 2000). The medfly, like several other lekking species (Petrie *et al.*, 1992, Kellogg *et al.*, 1995, Lank *et al.*, 2002, Kraaijeveld *et al.*, 2005), exhibits polyandry (Kraaijeveld *et al.*, 2005). In such species, post-mating male traits are likely to play a significant role in determining reproductive success, yet this area has received little attention. In this study the levels of male-male competition were experimentally manipulated by varying adult sex ratios to create male- and female-biased treatments (generating high and low male competition, respectively). This was done via proximate manipulations of sex ratio, rather than through experimental evolution as has been employed in studies of non-pest fruit flies (e.g. (Snook *et al.*, 2005, Reuter *et al.*, 2008, Crudgington *et al.*, 2009, Crudgington *et al.*, 2010). The aim was to test the effect of sex ratio manipulation on courtship, mating frequency and paternity of focal males. This enabled identification of the traits that most closely predicted a male's pre- and post-mating reproductive success, and to discover how the relationships between these traits and a male's overall paternity varied under different competitive conditions. My prediction was that increasing the level of male-male competition would intensify pre- and post-mating competition, leading to increased sperm competition and potentially uncouple a male's pre-mating reproductive success from the share of paternity gained. A male's pre-mating reproductive success was measured as the frequency of courtship and of mating. Overall reproductive success was measured as paternity gained under competitive conditions over a period of two weeks. Body size and testis size were included as covariates.

2.3 Methods

2.3.1 Fly stocks and culturing

Wild type focal males were from a subculture of the 'Cepa petapa' Moscamed mass-reared factory strain from Guatemala, Central America (Rendon, 1996). To assign paternity, females and competitor males carrying a recessive *white pupae* (*wp*) pupal colour marker were used. Hence pupae fathered by focal wild type males were brown and those by *wp* competitor males were white.

Flies were cultured at 25°C on a 12:12 light: dark cycle and 50% relative humidity, in a controlled environment room. Flies were cultured from eggs collected over a 24 hours period placed in 500g standard larval culture medium (400ml distilled water, 26g brewer's yeast, 24ml 1M HCl, 3ml Formaldehyde (4%), 8ml Nipagin (10% w/v solution in ethanol) and 71g ground carrot flakes) and placed within 17cm x 13cm x 5cm plastic containers lined with sand to a depth of 1cm with a mesh lid. At the third instar larval stage, the larvae 'jump', exit the larval culture medium and pupate in the sand. Development time from egg to adult under these conditions is between 16-18 days.

Virgin flies were obtained by sorting males and females apart within 24 hours of eclosion using light CO_2 anaesthesia, and then stored before use in adult holding cages, with plentiful access to sugar-yeast food (3:1 w/w brewer's yeast: sugar paste) and water. Focal males were marked with a spot of red paint on the dorsal side of the thorax while anesthetised. Pilot experiments had demonstrated no discernible impact of the paint mark on mating frequency ($\chi^2_1 = 0.322$, $P = 0.57$, $N = 200$). Flies entered the experiments when they were fully sexually mature at 5-7 days old. Males were transferred into the mating arenas 1 day prior to the start of the experiments.

2.3.2 Effect of sex ratio on courtship, mating frequency and paternity

Virgin focal wild type males were randomly assigned to one of two groups and placed in 250ml transparent plastic mating arenas as follows. The 'high male competition' group consisted of 50 replicate pots each of one virgin wild type male and two virgin *wp* females with 5 virgin *wp* competitor males. The 'low male competition' group consisted of 50 replicate pots each of one virgin wild type male and 6 virgin *wp* females with one virgin *wp* competitor male. Hence the overall density of flies within each of the pots was the same. Each pot received water through a filter paper wick and sugar yeast food supplied via the lid of a microfuge tube.

The behaviour of each focal male (courtship or mating) was recorded at 30 minute intervals starting at 9am until 12.30pm on alternate days. Courtship was scored if a male was engaged in the emission of pheromone via eversion of the rectal ampulla and/or engaged in wing 'buzzing' (Yuval & Hendrichs, 2000). Mating duration is 1.5-3 hours in this species, therefore sampling at 30 minute intervals ensured that all matings were recorded. A mating was scored if individuals in copula were observed on at least two consecutive observation periods. To ensure equal density and equal exposure to females and competitor males throughout the experiment, the sex ratio conditions were maintained by replacing any dead *wp* males or females with spare virgin individuals of the same age. In total the number of replacements due to deaths were 7 females and 1 male added to 6 cages under low competition; 5 males and 2 females added to 7 cages under high competition. The number of such replacements was therefore low and was not biased towards either treatment. After seven days all *wp* males and females were replaced by 5-7 day old virgins, and the mating assay was continued for another 7 days. This was done to maintain high levels of competition and fertility, so that the reproductive success of the focal males would not be confounded by decreasing fertility of females or competitor males during the experiment.

To measure the paternity of focal males, egg samples were collected from each mating pot on days 1 and 4 of each of the 2 weeks of the experiment. Females laid eggs through the gauze in the lid of each mating pot and these were brushed

lightly, using strips of filter paper, into separate pots containing 150g larval culture media. The two samples of eggs collected per week were placed in excess food (the maximum larval density was 0.5g/egg compared to standard mass-rearing densities of 0.04g/egg (Fay, 1988)). This ensured that all larvae were reared under low density conditions during development. This reduced the potential for differential larval survival given that 2 vs. 6 females were contributing to egg production in the high vs. low competition treatments, respectively. I recorded the number of offspring fathered by the focal males from the 4 samples taken over the 2 weeks.

2.3.3 Associations of morphological covariates with courtship, mating frequency and paternity

I recorded body and testis size of focal males from the high and low male competition treatments as covariates in the analyses and tested for associations with pre- and post-mating reproductive success. Males were frozen at the end of the observation period for subsequent dissection. The wings were first removed and placed into phosphate buffered saline (PBS) and sealed under a cover slip. The wingless males were then transferred to a cavity slide containing 15µl PBS and the abdomen removed in order to dissect out the testes. These organs were then placed in 5µl of PBS, and digital images of the testes and wings recorded using an Olympus BX41 microscope fitted with a JVC KY-F70B digital camera (under x10 magnification for testes x4 magnification for wings). I recorded landmarks on each wing to calculate wing area, measuring along the radial vein, median flexion-line, and the anal fold (Gullan & Cranston, 1994). Testis area was recorded by tracing the perimeter of the recorded images, and areas were then calculated using Image J software (Rasband, 1997).

2.3.4 Data Analysis

Courtship, mating frequency and paternity data were non-normally distributed, and Mann-Whitney Tests were therefore used in initial tests to determine the effect of high and low male competition on these traits. However, in order to include analyses of covariates and test for interactions, I then used generalised linear models (GLMs) with quasipoisson error distributions, to identify significant predictors of courtship, mating frequency or paternity. Included in each model (where appropriate) were courtship and mating frequencies, body size (i.e. wing area) and testes size.

As courtship and mating cannot occur simultaneously the courtship frequency was calculated as the proportion of time spent courting for males that were not mating. For example, if the total number of observations = 60, with 32 of these being mating, then 20 courtship observations would yield a courtship frequency value of 20/ (60-32) = 0.71. There was a highly significant correlation between wing and testis area (mm^2) across males ($r^2 = 0.433$, $P < 0.001$). Therefore I used relative testis size as the covariate for analyses, as calculated from the residuals of a linear regression of testis on body size.

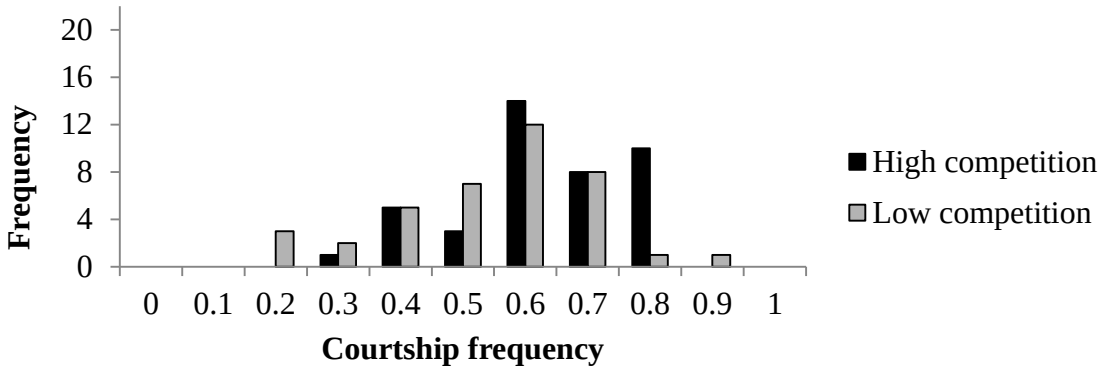
The number of females varied between each pot for the high and low competition treatments, respectively. This required a correction factor to be applied to the mating frequency and paternity data to enable direct comparisons between the reproductive performances of high and low competition focal males. The mating frequency and paternity data for each focal male were therefore divided by the number of females present in each cage (i.e. high competition counts divided by two, low competition counts divided by six). This gave mating frequency and paternity data that were standardised per female. Initial maximal models were constructed to include all main effects and interactions. These models were subsequently simplified to attain the minimal adequate model by stepwise removal of the least significant terms. Terms were removed from the model only if removal did not significantly ($P > 0.05$) influence the fit of the model when comparing previous and reduced models in an analysis of deviance. Analyses were performed using R v2.11.1 (R Development Core Team, 2010) and PASW v18.0.0 (PASW Statistics v.18, 2009).

2.4 Results

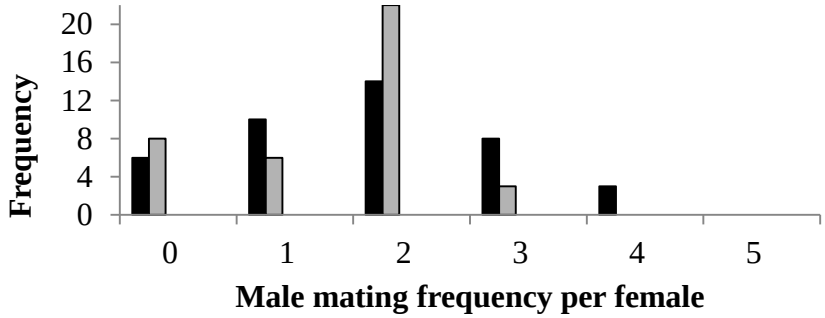
2.4.1 Effect of high vs. low male competition on courtship, mating frequency and paternity

The initial analyses showed that males held under high competition delivered significantly more courtship than did low competition males (u_{79} = 554, P = 0.018; Fig 2.1a). The significant differences in courtship delivery did not translate into differences in mating frequency per female, as there were no significant differences in the number of focal male matings per female (u_{79} = 697.5, P = 0.325; Fig 2.1b). Therefore, the increased courtship activity under elevated male competition did not increase the focal males' matings per female beyond that seen in the low male competition group. In terms of absolute paternity, low competition males (that delivered significantly lower levels of courtship) had significantly higher paternity per female (u_{79} = 558, P = 0.02; Fig 2.1c).

a)



b)



c)

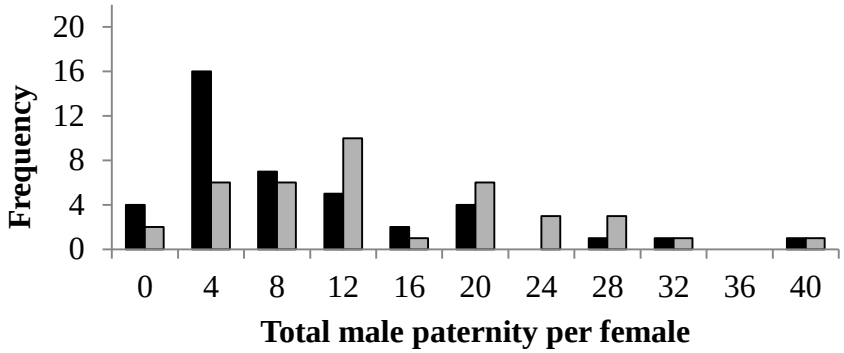


Fig 2.1 Frequency distributions of a) the proportion of time spent courting by focal males under high and low levels of male competition (high competition median = 0.66, S.D. = 0.15, *N* = 41; low competition median = 0.63, S.D. = 0.16, *N* = 39), b) the total number of mating observations per female for focal males (high competition median = 1.41, S.D. = 1.03, *N* = 41; low competition median = 1.35, S.D. = 0.70, *N* = 39), c) the total focal male paternity per female (high competition median = 1.76, S.D. = 0.82, *N* = 41; low competition median = 2.21, S.D. = 0.82, *N* = 39).

2.4.2 Effect of high vs. low male competition on interactions between pre-mating success and paternity

Consistent with the above, the GLM analyses suggested that there was no significant effect of high or low male competition on mating frequency per female (Table 2.1). There was a marginally significant interaction of body size and courtship frequency (P = 0.048), and a marginally non-significant effect of courtship delivery (P = 0.066; Table 2.1). Body size was significantly negatively correlated with mating frequency per female (Table 2.1; r^2 = 0.101, P = 0.013).

Table 2.1 Analysis of the variation in mating frequency per female for focal males held under high or low levels of male competition. Competition level was a fixed effect and courtship and body size were covariates in the analysis. The data were analysed using generalised linear models (GLM) with a quasi-poisson error distribution. Removal of the non-significant interactions had no effect on the overall results of the model.

Factor	Deviance	*F*	*df*	*P*
Competition (high or low)	1.685	0.651	1,75	0.517
Courtship	4.640	1.866	1,77	0.066
Body size	1.090	2.578	1,76	0.013
Competition x Courtship	1.080	0.261	1,72	0.795
Competition x Body size	0.535	0.767	1,73	0.445
Courtship x Body size	1.636	2.010	1,74	0.048

I then examined whether pre-mating success (mating frequency) explained a significant proportion of the variance in post-mating success (i.e. paternity gained per female), and whether this differed across high and low male competition treatments. I found a significant interaction between mating frequency per female and high or low competition (P = 0.030, Table 2.2) in addition to a marginally significant main effect of mating frequency itself (P = 0.050, Table 2.2). The positive relationship between mating frequency per female and paternity per female was therefore significantly stronger under low in comparison to high competition. This is

consistent with the idea that increased levels of competition between males led to increased post-mating competition and hence a breakdown in the association between pre-mating success and paternity (Fig 2.2).

Table 2.2 Analysis of the proportion of variance in post-mating reproductive success (paternity per female achieved by focal males) explained by pre-mating reproductive success (mating frequency per female), for males held under high and low level of competition. Competition level was a fixed effect and mating frequency was a covariate in the analysis. Data were analysed using a generalised linear model (GLM) with a quasi-poisson error distribution.

Factor	Deviance	*F*	*df*	*P*
Competition (high or low)	1.040	0.299	1,77	0.765
Mating Frequency	2.737	1.952	1,77	0.050
Competition x Mating Frequency	5.133	2.205	1,76	0.030

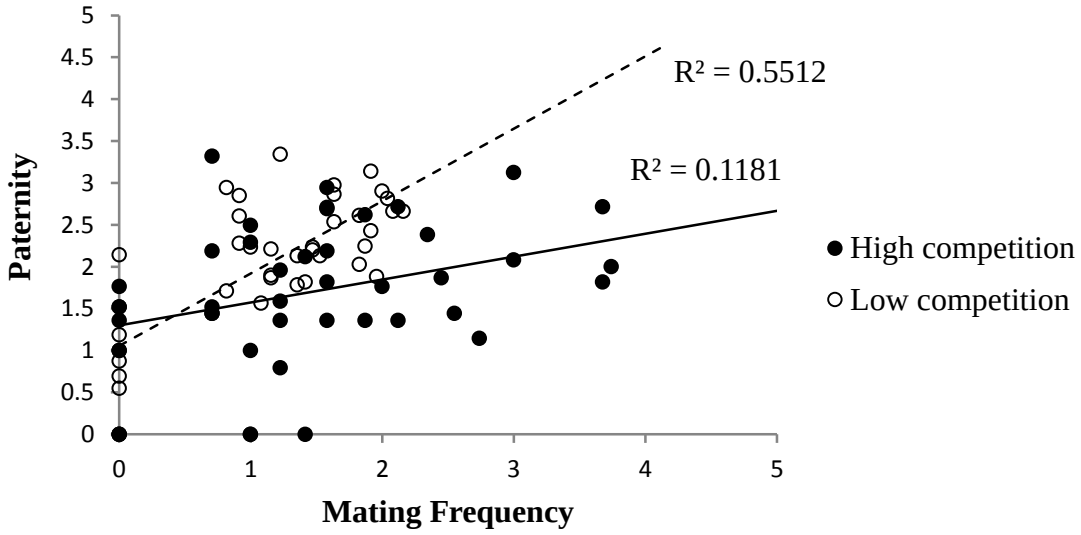


Fig 2.2 Correlations between the number of matings and total paternity per female for focal males held under high (filled circles, solid line, *N* = 41) and low (open circles, dashed line, *N* = 39) levels of male competition.

I also analysed which of the different covariates (courtship, body size, mating frequency and relative testes size) best explained the variation in paternity per female across high and low competition treatments (Table 2.3). Consistent with the analysis above, the most significant predictor of paternity was the interaction between high and low male competition and mating frequency ($P < 0.001$). A significant interaction was also found between mating frequency and testis size ($P = 0.038$; Table 2.3), independent of male competition. Residual testes size also emerged as a significant predictor of paternity ($P = 0.026$), though interestingly this was a negative correlation, with smaller residual testes size associated with increased paternity. There was no significant interaction of testis size with the level of male competition ($P = 0.309$).

Table 2.3 Analysis of the proportion of variation in paternity per female achieved by focal males under high or low levels of male competition due to competition, mating frequency and residual testis size. Competition level was a fixed effect and courtship, mating frequency and residual testis size were covariates in the analysis. Analysis of the variation in mating frequency and residual testis size on paternity achieved by focal males under high and low levels of male competition. Data were analysed using generalised linear models (GLM) with a quasi-poisson error distribution.

Factor	Deviance	*F*	*df*	*P*
Competition (high or low)	0.364	1.327	1,75	0.189
Residual Testis Size	11.065	2.274	1,75	0.026
Mating Frequency	0.236	2.342	1,75	0.022
Mating Frequency x Residual Testis Size	6.789	2.116	1,73	0.038
Competition x Residual Testis Size	5.373	1.024	1,73	0.309
Competition x Mating Frequency	0.661	3.666	1,73	<0.001

2.5 Discussion

The main findings were that manipulation of sex ratio from female to male biased (low to high male competition, respectively) had a significant effect on courtship delivery and offspring production. The strength of the association between mating frequency and the number of offspring fathered was also stronger under low in comparison to high levels of competition.

I found a significant difference in the amount of courtship delivered, with focal males from the high competition treatment exhibiting significantly more courtship behaviour in comparison to low competition males. Courtship delivery therefore exhibited plasticity, with males adjusting their courtship delivery according to the level of competition with males and likelihood of matings with females. This finding complements previous work from experimental evolution in *Drosophila* (Crudgington *et al.,* 2010). It also adds to a growing body of evidence documenting behavioural plasticity in responses to rivals across a wide range of taxa, although the ultimate significance of such plasticity is not yet fully understood (Bretman *et al.*, 2011). Courtship frequency was a marginally non-significant predictor of mating frequency across high or low male competition. This could indicate that while investment in courtship may be necessary in order for females to assess male quality (for instance using morphological measures such as body size or fluctuating asymmetry), it is not itself a reliable predictor of female choice. The idea that female medflies may use courtship to assess the attractiveness of morphological traits has been considered, but results are so far conflicting (Mendez *et al.*, 1999, Hunt *et al.*, 2002). Alternatively, quality of the courtship song itself or the threshold level of courtship needed for males to be competitive may increase as more males are present (Snook *et al.*, 2005). Although the number of matings per female was not significantly different across treatments, males produced more offspring per female under low in comparison to high competition. Under relaxed levels of male-male competition, therefore, focal males gained higher reproductive success per female. The stronger correlation between the number of matings and number of offspring fathered per female under low but not high competition is consistent with the idea

that strong post-mating effects under high competition result from increased sperm competition or cryptic female choice (Zeh & Zeh, 1994).

In terms of the morphological correlates, I found a negative effect of male body size on mating frequency per female. This is in contrast to some previous reports where larger males were reported to mate more frequently (Rodriguero *et al.*, 2002, Anjos-Duarte *et al.*, 2011); however, in such studies, which were comparisons between wild and laboratory flies, any effect of body size may often be an artefact of female preference for wild (and typically larger) males (Rodriguero *et al.*, 2002). Body size may be important in the establishment of territories at lekking sites (Niyazi *et al.*, 2008). The precise mechanism of competition between males for preferred lek positions is not known, although disparities in body size between two competing males may play a role (Kaspi & Yuval, 1999). Under laboratory conditions medflies may not form a natural lek. This could remove the presence of 'hotspots' of female or male activity that may be present under more natural conditions and hence reduce or possibly even reverse the potential role of body size on pre-mating success, as observed in this study.

These results showed a paternity advantage to small residual testis size. It is possible that this reflects greater sperm depletion rates in successful males, or that testis size or sperm production costs (Sella & Lorenzi, 2003) trade off against other life history traits such as growth, lifespan and manoeuvrability. In addition, absolute testis size could be constrained by the size of other reproductive tissues such as the accessory glands (Nijhout & Emlen, 1998), which could themselves be stronger targets of sexual selection (Crudgington *et al.*, 2009). It would therefore be useful to develop an accurate method for measuring short and long male accessory gland size or to quantify seminal fluid production and transfer (Sirot *et al.*, 2009). With such techniques it would then be possible to test for associations of small residual testis size with other reproductive traits in order to understand why small testes did not apparently limit male reproductive success. Evidence from comparative studies shows that testis size is a reliable indicator of the level of promiscuity; the interpretation is that males with larger testes can produce more sperm and can achieve reproductive success even in the face of strong sperm competition (Moller, 1989, Simmons *et al.*, 1993, Moller & Briskie, 1995). However, the medfly typically exhibits low to moderate rates of remating in the wild (e.g. 7.1%; (Bonizzoni *et al.*,

2002), which predicts that wild males should have a small residual testes size. Under selection for increased post-mating competition in the laboratory, males with relatively larger testes, capable of producing large numbers of sperm, should therefore be favoured. However, this was the opposite of what was observed, suggesting that even in the laboratory, factors other than sperm numbers (e.g. seminal fluid proteins, sperm morphology or possibly cryptic female choice) are important in securing paternity.

These findings are broadly relevant to the control of insect pests using methods such as SIT (Knipling, 1955) and the Release of Insects with a Dominant Lethal (RIDL) (Thomas *et al.*, 2000, Gong *et al.*, 2005, Fu *et al.*, 2007, Morrison *et al.*, 2011). In these techniques, the insects are mass-reared, sterilised (under SIT) and released into the wild to mate with wild females. These matings are either sterile (SIT) or pass a dominant lethal gene to offspring (RIDL). Both outcomes lead to a subsequent reduction in the pest population size (Knipling, 1955). Insights into methods for improving SIT-based programs can be gleaned by understanding that the traits which increase male reproductive success are dependent on the relative abundance of potential mates and rivals, and how those traits are affected by selection for mass-rearing (Cayol, 2000). The data suggests that there could be relaxation in sexual selection for large body size under mass-rearing. Selection under mass-rearing may instead focus on rapid development time or early sexual maturation, which could be accompanied by reduced body size. In this case, the screening of lines for body size could indicate the intensity of artificial selection, and help to determine the likely efficacy of a line in SIT or RIDL programs. Changes in selection pressure on body size could also partly contribute to the finding that mass-reared males consistently under-perform in the field against wild type males (Cayol & Zarai, 1999, Lux *et al.*, 2002, Shelly *et al.*, 2007) together with degraded male courtship behaviour under mass-rearing conditions (Briceno *et al.*, 1996, Briceno & Eberhard, 1998, Briceno & Eberhard, 2002).

These findings are also useful in contributing to a better understanding of how selection operates under mass-rearing regimes used for SIT (Cayol, 2000). For example, under natural conditions females visit leks for mating, but then leave in order to oviposit into ripe fruit (Yuval & Hendrichs, 2000). Under high density, confined conditions escape is restricted, and this, together with the presence of many

males, may result in decreased courtship, more frequent mating attempts by males and a higher probability of mating disruption. Exposure of successive generations to these conditions is predicted to focus sexual selection on post-mating processes rather than on pre-mating morphology and behaviour (Reuter *et al.*, 2008). This may have consequences for the efficient implementation of pest control mechanisms such as SIT and RIDL, which rely on maintaining the natural reproductive repertoire of mass-reared males.

These findings are also relevant to the evolution of mating systems in lekking insects. I show that male reproductive success is context-dependent and can be significantly affected by post-mating traits even in lekking species, where the emphasis has traditionally been on pre-mating traits. I found that associations between pre- and post-mating success became uncoupled when male competition increased and the potential for sperm competition and/or female cryptic choice was intensified. This will now allow better predictions for how selection will act on male reproductive traits as the operational sex ratio changes.

2.6 References

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Chapter 3: Male reproductive success does not respond to artificial selection through the manipulation of adult sex ratios in *Ceratitis capitata*

3.1 Abstract

Previous studies have demonstrated that males evolving under increased levels of male-male competition can become more successful in courtship, mating, sperm competition, fertilisation and, possibly as a side effect, can induce greater harm in their mates. In the previous chapter the highly context-dependent relationship between pre- and post-mating male reproductive success under different adult sex ratios was determined. To test whether these effects are altered following adaptation to sex ratio biased conditions I used replicate lines of medfly that had been maintained under male biased, equal sex and female biased adult sex ratios for over 50 generations. I then assessed the implications of this finding for mass-rearing protocols and increasing the efficacy of SIT. I compared the courtship, mating frequency and total paternity of focal males from each sex ratio regime in two assays of high and low male competition. There was no evidence for alterations in mating competitiveness or reproductive success for any of the selection lines under either mating assay. I discuss potential shortcomings in the adult sex ratio selection regimes, and potential explanations for the lack of response to selection that were observed.

3.2 Introduction

In the previous chapter I reported that male reproductive success was highly context-dependent, and can be significantly affected by the expression of post-mating traits under male biased mating conditions.

The emphasis of studies into the medfly mating system has traditionally been on pre-mating traits, which are well characterised (Eberhard, 2000). Less is known about the post-mating processes that determine male reproductive success and how these are altered by the mass-rearing process. Females tend to mate one or few times in the wild (Whittier & Shelly, 1993, Yuval *et al.*, 1996, Kraaijeveld *et al.*, 2005) and, following mating, female receptivity to subsequent matings is significantly lower than for virgin females (Jang *et al.*, 1998). The loss of receptivity following mating is thought to result from the actions of male accessory gland products transferred to the female during mating along with sperm (Jang, 1995, Chapman, 2008).

In SIT-based control programs, it is a well-documented fact that mass-reared males consistently underperform in the field in comparison to wild males (Shelly *et al.*, 1994, Hendrichs *et al.*, 1996, McInnis *et al.*, 1996, Shelly & Whittier, 1996). Mass-reared males seem to be able to find and join leks with the same success rate as their wild counterparts (Shelly *et al.*, 1994). However, mass-rearing causes a drastic reduction in investment in courtship behaviour by males, believed to be brought on by a combination of crowding effects, coupled with increased female availability and receptivity (Rossler, 1975a, Rossler, 1975b, Briceno *et al.*, 1996, Briceno & Eberhard, 1998, Eberhard, 2000, Briceno & Eberhard, 2002, Briceno *et al.*, 2002, Shelly, 2012).

As with many animal mating systems, male and female medflies can have very different potential reproductive rates (Bateman, 1948, Parker, 1970, Parker & Partridge, 1998, Chapman, 2006). Female medflies generally mate only a few times (Bonizzoni *et al.*, 2002, Kraaijeveld *et al.*, 2005), and multiple matings have little impact on reproductive potential, while males are thought to mate multiply and their fitness can increase with an increasing numbers of mates. The disparity in male

reproductive success can be exacerbated in lekking species such as medfly, because males can exhibit wide variation in reproductive success, as a small number of males monopolise the females and father most of the offspring (Kirkpatrick & Ryan, 1991, Rowe & Houle, 1996). This in turn can further enhance the disparity between wild males and underperforming mass-reared males (Kirkpatrick & Ryan, 1991, Rowe & Houle, 1996, Droney & Hock, 1998).

Maintaining the reproductive capacity of mass-reared males is clearly important for efficient SIT programs. One factor that is important but has not been investigated in this context is the effect of adult sex ratio. In the previous chapter the effect of altering the phenotypic adult sex ratio was documented to cause significant changes in the predictors of male reproductive success, as well as increasing the importance of post-mating mechanisms with an increasing male biased sex ratio. This issue can be further addressed by investigating the evolutionary effects of varying adult sex ratios on male reproductive performance. In general, sexual selection will act on male traits that maximise reproductive potential, such as those that enhance male performance in male-male competition, sperm competition and eliciting pre-mating or post-mating female choice; even if this results in a reduction of fitness for their mates (Parker, 1979, Wolfner, 1997, Wolfner, 2002, Wigby & Chapman, 2005). By altering the adult sex ratios of lines of medfly over successive generations, it is possible to vary the intensity of sexual selection and sexual conflict, and measure the evolutionary responses of males. Consequences of increasing or decreasing sexual conflict should alter selection pressures on male-male competition and manipulation of females, while also selecting on female resistance. As such it is possible that breeding under male biased conditions could lead to an increased reproductive performance in the field (under SIT programs males are released into extremely male biased sex ratios) and so prove to be a potential husbandry tool.

Experimental evolution has proved an excellent tool for understanding sexual selection (Arnqvist & Rowe, 2005, Ritchie, 2007). By altering the intensity of sexual selection across evolving lines of medfly it should be possible to investigate the mechanisms by which males respond to increasing levels of sexual selection. Previous work by Holland & Rice (1999) in *Drosophila melanogaster* showed that a significant fitness load can be imposed by sexual selection. For example, reductions in the level of male-male competition over time led to a reduction in the amount of

reproductive investment by males. This also resulted in the evolution of males that were more benign to females, and caused reduced mating costs in females.

Building on this experimental evolution approach, studies in flies have subsequently shown that males evolving under increased levels of male-male competition are more successful in courtship, mating, sperm competition, fertilisation and, possibly as a side effect, can induce greater harm in their mates (Hosken *et al.*, 2001, Pitnick *et al.*, 2001, Reuter *et al.*, 2008, Crudgington *et al.*, 2009, Crudgington *et al.*, 2010).

For successful SIT, competitive males that have the capacity to compete for matings with wild females are of key importance (Whittier & Kaneshiro, 1995, McInnis *et al.*, 1996, Shelly & Whittier, 1996). The level of competitiveness of the sterile male in the field can be impaired by a combination of the effects of multiple generations of mass-rearing and sterilisation (Hooper, 1972, Briceno & Eberhard, 1998, Hendrichs *et al.*, 2002, Robinson *et al.*, 2002). It might therefore be thought that it would be an advantage to continually use newly field-derived medfly strains for SIT, as their male mating behaviour will have been less severely compromised by mass-rearing. However inducing females to lay sufficient eggs and cultivating the developing larvae under mass-rearing conditions can take many generations to achieve. Perhaps not surprisingly, older strains are typically more highly fecund (Rossler, 1975b). It is for this reason that well established medfly lines are used for control programs, as above all else, sufficient numbers of males must be reared for release (Knipling, 1979). As such, experimentally evolved lines exposed to high levels of sexual selection could prove to be the solution to this problem, having the potential to produce males which will prove highly competitive when used in SIT programs; simultaneously increasing efficacy and reducing costs.

In this chapter the potential ability of males to respond to either high or low levels of sexual selection/conflict was measured in replicated evolving lines of medfly. Adult sex ratio was varied in 3 x replicated lines of either male- or female-biased lines (3:1 or 1:3 males: females, respectively) to alter levels of sexual selection. Controls were 1:1 adult sex ratio (intermediate levels of sexual selection).

Following 6 years of experimental evolution (48 generations of selection followed by 24 generations in which selection was intermittent) the competitiveness

of males from each replicate line was measured against a standard background by testing focal males from the experimentally evolved lines with standard females and competitor males carrying a recessive *white pupae* (*wp*) pupal colour marker. Two assays on males from all replicates of all regimes were conducted, one at high levels of male competition, one at low levels of male competition (generated by varying phenotypic manipulation of the adult sex ratios in the tests). This was done in order to determine whether any changes in male competitiveness from the selection lines were context-dependent. By measuring frequencies of courtship, mating and paternity it was possible to determine the alterations in investment and success in male reproductive success across the different selection regimes.

3.3 Methods

3.3.1 Selection regimes

Three selection regimes each of male biased, female biased and equal sex ratio lines were set up. These were initiated from the Cepa petapa wild type mass-rearing stock, and comprised 3 replicate cages of 75 females with 25 males (female biased, FB), 3 of 50 males with 50 females (equal sex ratio, ES), and 3 of 75 males with 25 females (male biased, MB). For each generation the required number of males and females were counted out from newly-eclosed (< 24 hour old) adults from each regime using CO2 or ice anaesthesia and placed in adult cages (17 x 13 x 5cm) containing water and sugar / yeast adult food (3:1 w/w brewer's yeast: sugar paste). Eggs were laid through a gauze window in the side of each cage and collected in water troughs below. The adults interacted in those cages for 10-13 days before eggs laid into the water troughs were sampled over two periods of 24 hours (to provide main egg sample and back up egg sample, respectively). For each sample, a standardised volume of eggs was placed onto the top of 500g of standard larval culture medium (400ml distilled water, 26g brewer's yeast, 24ml 1 M HCl, 3ml formaldehyde (4%), 8ml Nipagin (10% w⁄v solution in ethanol) and 71g ground carrot flakes). The larval medium was then placed within (17 x 13 x 5cm) cages with a mesh lid and

containing 1cm of sand. At the third larval instar, the larvae jump from the larval medium and pupate in the sand. Pupae were then sieved from the sand and placed in adult cages containing water and sugar/yeast food until adult eclosion. The process was then repeated. Using this procedure allowed one generation per month. The lines were initiated and selected continuously for 4 years (48 generations) and then maintained under relaxed selection for a further 2 years (24 generations with intermittent selection) before the experiments described here were conducted. During this latter period one replicate of the MB regimes (3) was lost.

3.3.2 Experimental procedure

Virgin flies were obtained by sorting males and females apart within 24 hours of eclosion using light CO_2 anaesthesia, and then stored before use in adult holding cages, with plentiful access to sugar-yeast food (3:1 w/w brewer's yeast: sugar paste) and water. Focal males from selection lines were marked with a spot of red paint on the dorsal side of the thorax while anesthetised. Pilot experiments (on wild type flies) had demonstrated no discernible impact of the paint mark on mating frequency (see section 2.3.1). Flies entered the experiments when they were fully sexually mature at 5-7 days old.

3.3.3 Effect of adult sex ratio selection regimes on courtship, mating frequency and paternity in high and low male competition mating scenarios

Virgin focal males from each of the replicates of the selection regimes were randomly assigned to either the high or low competition assay and placed in 250ml transparent plastic mating arenas as follows. The 'high male competition' assay consisted of 20 replicate pots for each selection line each containing one virgin focal male and two virgin *wp* females with 5 virgin *wp* competitor males (180 pots in total). The 'low male competition' assay also consisted of 20 replicate pots for each selection line; this time with one virgin focal male and 6 virgin *wp* females with one

virgin *wp* competitor male. Hence the overall density of flies within each of the pots was the same. Each pot received water through a filter paper wick and sugar yeast food supplied via the lid of a microfuge tube.

The behaviour of each focal male (courtship or mating) was recorded at 30 minute intervals starting at 9am until 12.30pm every day for five days. Courtship was scored if a male was engaged in emission of pheromone via eversion of the rectal ampulla and/or engaged in wing 'buzzing' (Yuval & Hendrichs, 2000). Mating duration is 1.5-3 hours in this species, therefore sampling at 30 minute intervals ensured that all matings were recorded. A mating was scored if individuals in copula were observed on at least two consecutive observation periods. To ensure equal density and equal exposure to females and competitor males throughout the experiment, the sex ratio conditions were maintained by replacing any dead *wp* males or females with spare virgin individuals of the same age. In total the number of replacements due to deaths was 1 male and 1 female (both *wp*). The number of such replacements was therefore low and was not biased towards either treatment.

To measure the paternity of focal males, I collected egg samples daily from each mating pot. Females laid eggs through the gauze in the lid of each mating pot and these were brushed lightly, using strips of filter paper, into separate pots containing 150g larval culture media. The eggs collected were placed in excess food (the maximum larval density was 0.5g/egg compared to standard mass-rearing densities of 0.04g/egg (Fay, 1988)). This ensured that all larvae were reared under low density conditions during development. This reduced the potential for differential larval survival given that 2 vs. 6 females were contributing to egg production in the high vs. low competition mating assays, respectively. I recorded the total number of offspring fathered by the focal males from the 5 samples taken.

3.3.4 Data Analysis

Courtship, mating frequency and paternity data were non-normally distributed (and could not readily be transformed). The loss of one replicate line also meant the experimental design was unbalanced, and Levene's tests revealed heterogeneity of variances. Therefore, nested ANOVAs and parametric tests were unsuitable for analysing the data. A G-test for heterogeneity for each variable was therefore an appropriate method for analysing the differences in behavioural and paternity data across the treatments and replicate lines (Sokal & Rohlf, 1969). Similar in principle to a Chi square test, G-values are additive, allowing for the partitioning and analysis of the contribution of specific fixed factors to variation in the data as a whole.

As courtship and mating cannot occur simultaneously, courtship frequency was calculated as the proportion of time spent courting for males that were not mating. For example, if the total number of observations = 60, with 32 of these being mating, then 20 courtship observations would yield a courtship frequency value of 20/ (60-32) = 0.71. Analyses were performed using Microsoft Excel (2007).

3.4 Results

3.4.1 High male competition assay

The G analyses showed that when selection line males from male- female-biased and control sex ratio regimes were held under high male competition conditions there was no detectable difference in courtship frequencies across any of the selection lines or replicates ($G_7 = 0.578$, $P = 0.99$) (Fig 3.1). Variation in mating frequency appeared to show a slight trend under high competition, with an increasing number of matings by focal males from male biased selection lines (Fig 3.2). However considering the individual replicates, it is clear that there was considerable variation across replicates within treatments (e.g. ES (3)). As a result there was significantly greater variation in

mating frequency between replicates of the ES regime than across selection regimes (Fig 3.3; Table 3.1).

Paternity was examined in order to determine whether any differences between the lines equated to changes in the fitness in terms of the number of offspring produced by focal males. As above, males from the MB lines produced the most offspring. However variation in paternity was again still mostly explained by variation between replicate lines of the same selection regime, rather than by differences between selection regimes (Fig 3.4; Table 3.2).

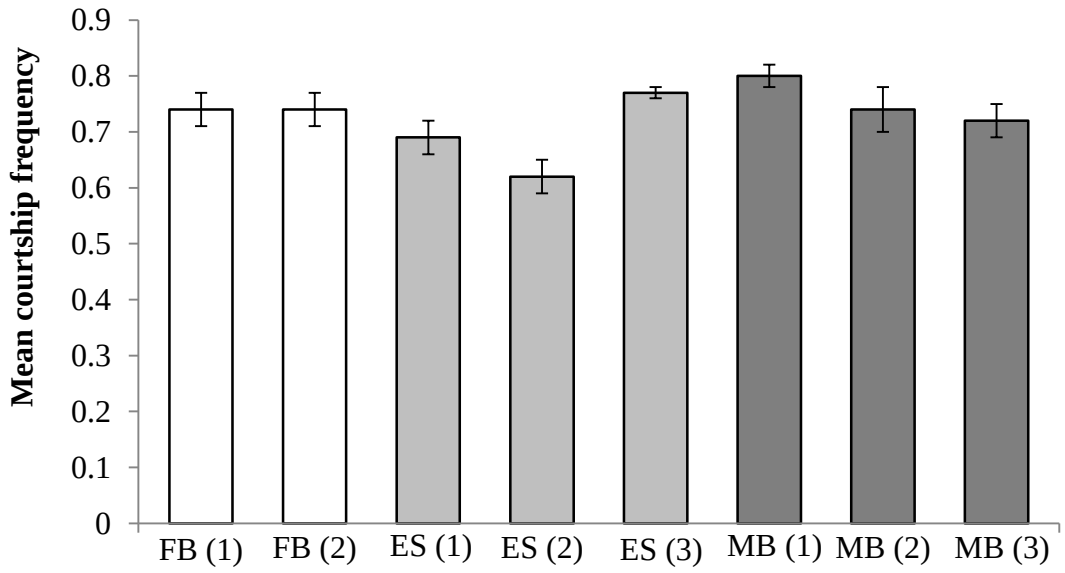

Fig 3.1 Mean (± S.E.) courtship frequencies by focal males from each replicate adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under high levels of male competition (N = 160).

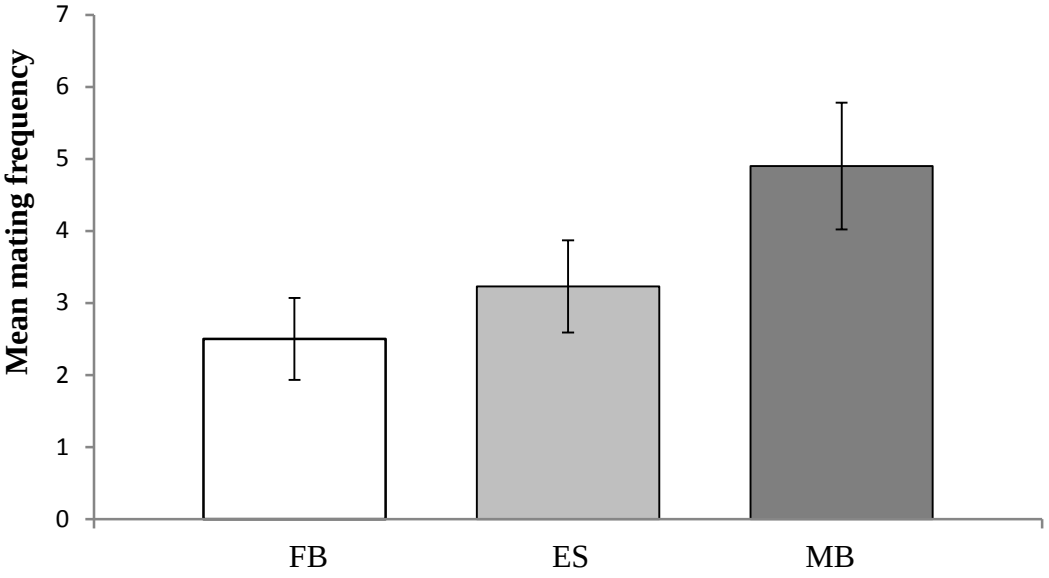

Fig 3.2 Mean (± S.E.) mating frequency of the focal males from selection regimes (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) for adult sex ratio variation under high competition (*N* = 160).

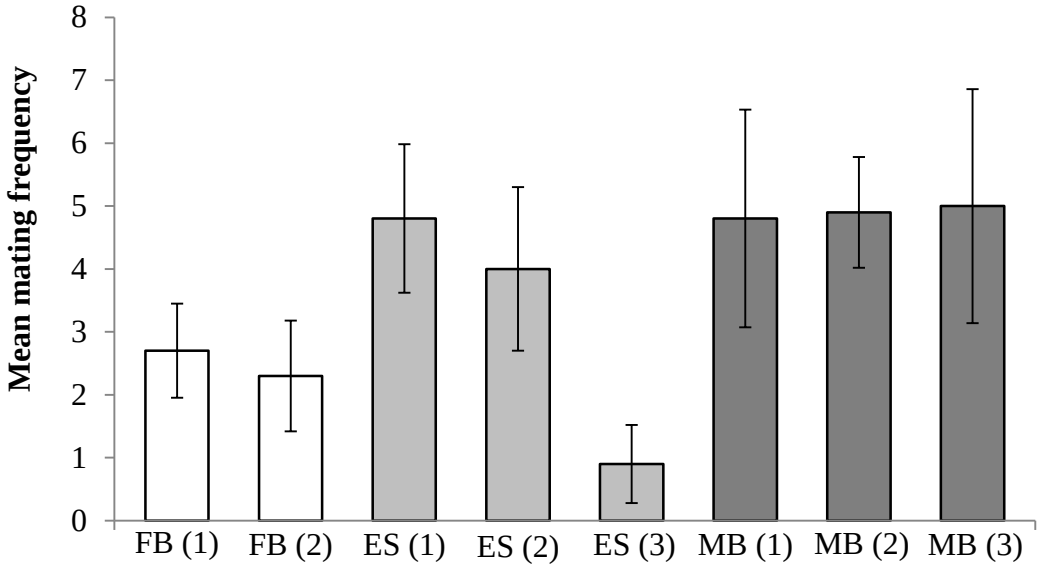

Fig 3.3 Mean (± S.E.) mating frequency by focal males from each replicate of adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under high levels of male competition (*N* = 160).

Table 3.1 G-tests of heterogeneity comparing variation in male mating frequency among selection regimes and replicates when tested under a high competition mating scenario (1 focal male, 5 competitor *wp* males, 2 *wp* females).

Factor – source of variation	***G***	***df***	***P***
Total	107.013	7	3.82E- 20
Selection regimes	42.425	2	6.13E- 20
FB	0.641	1	0.423
ES	63.865	2	6.77E-15
MB	0.082	2	0.48

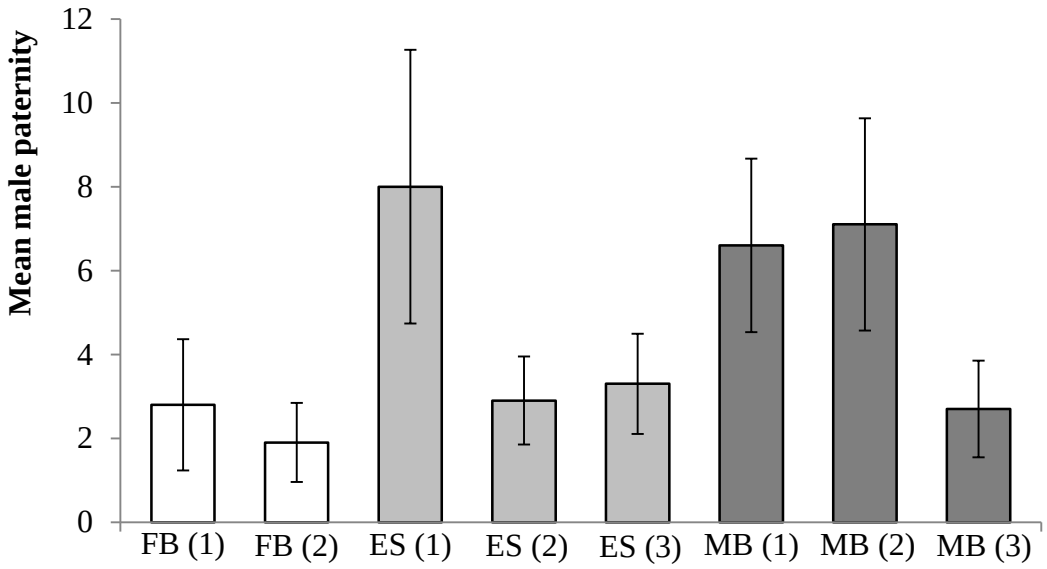

Fig 3.4 Mean (± S.E.) paternity achieved by focal males from each replicate of adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under high levels of male competition (N = 160).

Table 3.2 G-tests for heterogeneity comparing variation in paternity achieved by focal males among selection regimes and replicates of adult sex variation, when tested under a high competition mating assay (1 focal male, 5 competitor *wp* males, 2 *wp* females).

Factor – source of variation	***G***	***df***	***P***
Total	176.17	7	9.61E- 35
Selection regimes	61.955	2	3.52E- 14
FB	3.468	1	0.063
ES	63.496	2	1.63E- 14
MB	47.798	2	4.18E- 11

3.4.2 Low male competition assay

Consistent with the previous results, when the selection line males were held under low male competition there was also no detectable difference in courtship frequencies across the selection lines or replicates (G_7 = 1.318, P = 0.98; Fig 3.5). There was significant variation in mating frequency across the lines; however this could not be attributed to the selection regimes, as differences between replicate lines of the same regime again accounted for significantly more variation (Fig 3.6; Table 3.3).

As before, numbers of offspring produced by focal males were measured to determine the effect of selection of male fitness under a low male competition environment. While there was variation in the amount of paternity achieved by focal males across the dataset, this did not correspond to differences between selection regimes. Most variation was again found between replicates from the same regimes (Fig 3.7; Table 3.4).

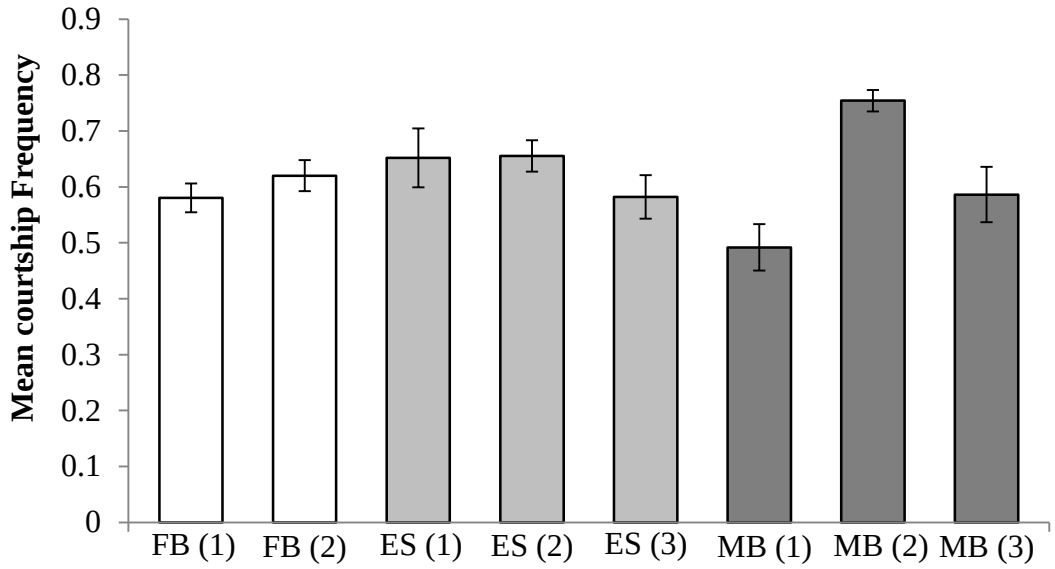

Fig 3.5 Mean (± S.E.) courtship frequencies by focal males from each replicate of adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under low levels of male competition (*N* = 160).

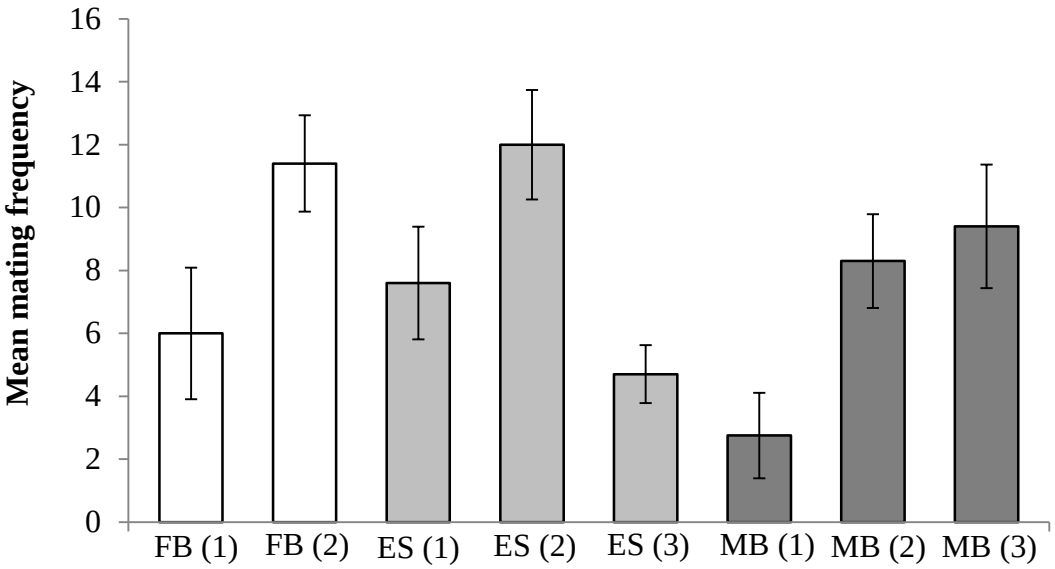

Fig 3.6 Mean (± S.E.) count of mating frequency by focal males from each replicate of adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under low levels of male competition (*N* = 160).

Table 3.3 G-tests for goodness of fit comparing variation in focal male mating frequency among selection regimes and replicates of adult sex variation, under a low competition mating assay (1 focal male, 1 competitor *wp* male, 6 *wp* females).

Factor – source of variation	***G***	***df***	***P***
Total	199.812	7	1.26E- 39
Selection regimes	12.438	2	0.0019
FB	34.077	1	5.30E- 9
ES	66.962	2	2.88E- 15
MB	86.335	2	1.79E- 19

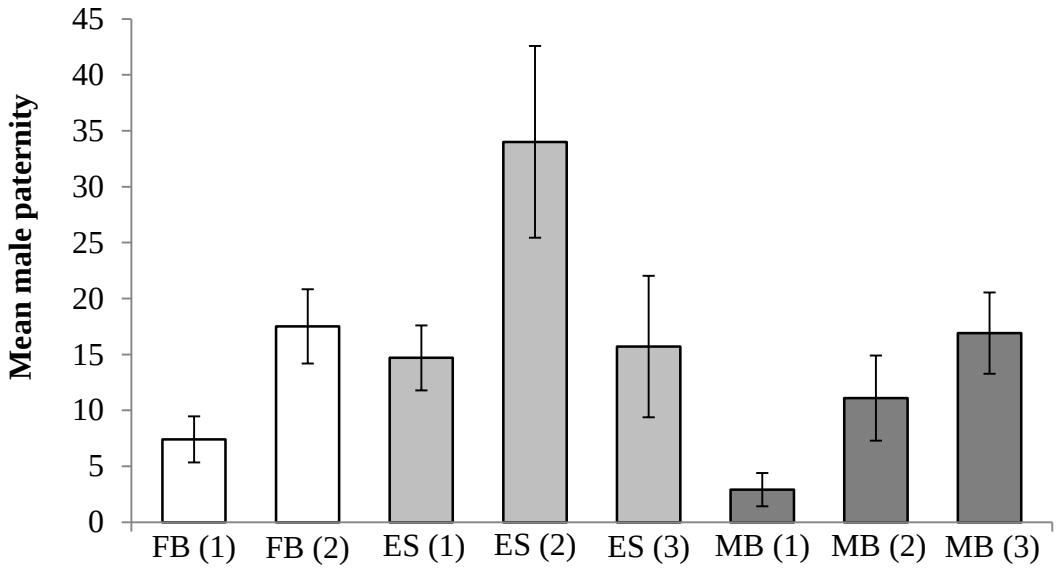

Fig 3.7 Mean (± S.E.) paternity achieved by focal males from each replicate of adult sex ratio variation (FB – Female Biased sex ratio 75:25, ES – Equal sex ratio 50:50, MB – Male Biased sex ratio 75:25) under low levels of male competition (N = 160).

Table 3.4 G-test for goodness of fit comparing variation in paternity achieved by focal males among selection regimes and replicates of adult sex variation when tested under a low competition mating assay (1 focal male, 1 competitor *wp* male, 6 *wp* females).

Factor – source of variation	*G*	*df*	*P*
Total	776.724	7	1.95E- 163
Selection regimes	265.159	2	2.64E- 58
FB	84.345	1	4.16E- 20
ES	206.296	2	1.60E- 45
MB	220.924	2	1.06E- 48

3.5 Discussion

It was predicted that males from lines of the MB selection regime should have experienced significantly greater pressure to compete for successful fertilisation of female ova, and consequently show an increased level of competitiveness compared to the ES and FB lines. By conducting two assays, one under high and one under low competition, it was also possible to investigate whether any resulting differences were context-dependent, i.e. whether males from the male-biased regimes would always be more competitive, or whether they would have an advantage only in a mating scenario which resembled the conditions experienced by them during the selection procedure.

The striking finding from this work however, was the lack of detectable response due to selection regime in male mating behaviour or reproductive fitness. In contrast to the predicted outcomes, and many previous experimental evolution studies in *Drosophila* (Snook *et al*., 2005, Reuter *et al*., 2008, Crudgington *et al*., 2009, Crudgington *et al*., 2010), there were no significant differences between selection regimes in courtship frequency, mating frequency or paternity counts across either regime.

The lack of any trends in courtship or mating frequency differences across both assays is surprising. Multiple generations of hard selection on males from the

male-biased lines had no discernible impact on the ability of focal males to increase their reproductive fitness when compared to males that had experienced normal or reduced levels of male-male competition over time. In the high competition assay there was a non-significant regime effect of mating frequency which was in line with the prediction. However the significant variation between the replicates within the equal sex regime lines was greater than any potential selection regime effect, owing to the very low mating frequency achieved by line ES (3).

Better resolution of any possible effect of experimental evolution on mating frequency in medfly could have been achieved with a higher number of replicate lines per selection regime. Individual replicate effects such as the extremely low mating frequency of ES (3) under high competition therefore have a large impact on the analysis. By having 10 or even 20 replicated lines of each selection regime, fewer flies could be tested from each replicate while still achieving greater statistical power overall. However, beyond the shortcomings in statistical power, it is apparent that any signatures of sexual selection if present are likely to be very small. Other comparable studies have used a similar number of replicates and achieved highly significant results. There are several examples of studies of experimentally evolved populations in *D. melanogaster* that report significant selection responses using the same adult sex ratios and replicate numbers as used here (Wigby & Chapman, 2004, Wigby & Chapman, 2006).

There are several possible explanations for the apparent lack of significant differences between the selection regimes. It is possible that plasticity could swamp any evolutionary signatures of male investment in reproduction, for example. Recent papers have found evidence for changes in *D. melanogaster* behaviour arising from the response of males to their rivals. Males have been consistently shown to invest more heavily in courtship, mating and sperm competition following exposure to rivals (Bretman *et al*., 2010, Bretman *et al*., 2011). It is possible therefore, that medfly males from these selection regimes express plastic responses to their environment that could mask any effects of selection through experimental evolution. Consistent with this, medfly males have been previously shown to be capable of producing context-dependent behaviours to attract females and ward off rivals (Eberhard, 2000).

It is also important to note that the medfly's natural mating system is lek based. As such males have naturally evolved from a system, not of random promiscuity, but from choosy females picking the 'best' males based solely on behaviour and morphology (Shelly *et al.*, 1994, Eberhard, 2000, Robinson *et al.*, 2002, Rodriguero *et al.*, 2002). This highly competitive environment may have resulted in relatively hardwired male behaviour that is not easily moulded by artificial selection for mass-rearing. While mass-reared males may not perform as competitively as their wild counterparts for matings with females, they still join leks and engage in the same courtship behaviours as wild males (Shelly *et al.*, 1994). Medfly populations are placed through a severe genetic bottleneck when establishing mass-rearing populations, as wild flies often fail to reproduce under mass-rearing conditions (Rossler, 1975b). Further adaptation through experimental evolution may be curtailed through a combination of the lack of genetic variation in a well-established mass-rearing strain of medfly and the relatively minor effect of changing the adult sex ratio (compared to the large previous effect of establishing a mass-reared population) in a species of fly which has evolved under intense male competition.

It was evident that the different replicates displayed significant variation in courtship frequency, mating frequency and paternity counts, irrespective of selection regime. These line effects may be the result of genetic drift caused by initial founder effects, low effective population sizes and the long timeframe of the selection protocol (Snook *et al.*, 2009). The foundation of new selection lines may often include genetic sampling effects, coupled with a reduction in the effective population size. With these newly founded populations, these sampling effects coupled with simple genetic drift can lead to the chance depletion of alleles (Willi *et al*, 2006; Hedgecock, 1994). If these effects shift the genetic variation of fitness-related traits then a heterogenous response to selection is to be expected (Keller and Taylor, 2008). The subsequent evolution of these selection lines will then depend not only on the pattern of adult sex ratio, but the availability of genetic variation to respond to the pattern of selection and potentially, additional genetic drift effects due to small effective population sizes (Keller and Taylor, 2008). This could explain the repeatedly poor performance of ES (3), and could account for the lack of selection

regime effects, as drift could account for a much greater amount of the variation between lines than the artificial selection imposed on different regimes.

While in the previous chapter, altering the proximate adult sex ratio lead to a significant alteration in the predictors of male reproductive success, no associated changes were found in the selection lines where the adult sex ratio biases were maintained over successive generations. While it has been possible to develop a better understanding of the context in which male reproductive success is achieved, applying this to developing more competitive males for use in the field has so far failed. However, given that alterations in the adult sex ratio have been found to alter the associations between courtship, mating frequency and paternity, it would seem impossible not to generate artificial male selection based on some key behavioural and morphometric traits.

By addressing the shortcomings in the current experimental design, using greater numbers of replicates while increasing selection pressure over fewer generations, it may be possible to overcome any inertia in the response to increasing sexual selection on males, and assess the mechanisms by which males can adapt to changing selection pressures. In turn this will allow a more comprehensive assessment of the plausibility of generating more competitive lines of male medfly for use in control programs.

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Chapter 4: RIDL construct design for embryonic lethality in the medfly

4.1 Abstract

The development of transgenic technology has the potential to replace the Sterile Insect Technique (SIT) with female-specific conditionally lethal transgenic strains of medfly known as Release of Insects with a Dominant Lethal (fsRIDL). These fsRIDL strains harbour a tetracycline-repressible transactivator (tTA) that causes female lethality in the later larval stages of development. While this lethality is fully repressible and completely penetrant, the build-up of tTA to toxic levels takes time, and the late induction of death leads to wasted resources spent on rearing the females of release generations. Presented here is the testing of the first molecular construct to induce expression of tTA during embryonic development, and the development and testing of constructs carrying a lethal effector gene *ReaperKR* designed to induce more rapid death.

4.2 Introduction

The Sterile Insect Technique (SIT) is a species-specific and environmentally friendly method of insect population control, in which individuals of the target species (usually males) are mass-reared, sterilised (via irradiation) and released in huge numbers (Knipling, 1955, Knipling, 1959, Knipling, 1979, Krafsur, 1998). The released sterile insects compete for matings with individuals from the wild population and successful matings with sterile insects therefore reduce the reproductive potential of the wild population. If continued over a sustained period, with sufficiently large numbers of released insects, the target population will be locally eliminated (Krafsur *et al*., 1987).

The greatest success of SIT was the complete eradication of the New World screwworm, *Cochliomyia homnivorax*, from North and Central America, by the release of irradiated insects (Wyss, 2000). Another target of SIT has been the Mediterranean fruit fly, and SIT has been successfully applied in California and Latin America on a massive scale (Hendrichs *et al.*, 1995, Mitchell, 1997). However despite initial large-scale successes, SIT has not subsequently been adopted widely for insect pest control. Even in successful SIT programs such as with the Medfly, there can be significant flaws with the deployment of traditional SIT, which increase costs and reduce efficacy, as outlined below (Hendrichs *et al*., 2002).

Irradiated insects consistently exhibit dramatic reductions in lifespan and mating competitiveness, which lead to a decrease in their fitness compared to non-irradiated and wild insects (Shelly *et al*., 1994, Lance *et al*., 2000). In the field, medflies have a lek-based mating system where males aggregate to attract mates through the release of pheromones (Yuval & Hendrichs, 2000). It is assumed that this mating system provides a mechanism by which females can choose the ‘best’ of the males available for mating (Whittier & Kaneshiro, 1995). However, sterile irradiated males are significantly less effective at achieving matings in comparison to non-irradiated or wild males (Hendrichs *et al*., 2002, Kraaijeveld & Chapman, 2004). A potentially greater problem occurs because females mated to sterile males are more likely to remate than females mated to fertile males, presumably due to the observed reduction in sperm transfer following sterilisation (Mossinson & Yuval, 2003, Vera

et al., 2003, Kraaijeveld & Chapman, 2004). This will therefore reduce the capacity of release populations to achieve control.

Recent developments in the construction of stable germ-line transformation in non-Drosophilid insects offer the opportunity for refinement of SIT, for example through the creation of transgenics containing conditional lethality as a phenotype. Such a system is the Release of Insects carrying a Dominant Lethal (RIDL, (Thomas *et al.*, 2000). RIDL has significant advantages over SIT, one of which is that with a suitable transformation vector, techniques developed in one species could potentially be used across species covering a wide phylogenetic range. RIDL therefore removes the requirement for irradiation and therefore the associated loss of fitness to the medfly (Gong *et al.*, 2005).

The first insect transformation system to be developed was in *Drosophila melanogaster*, and was derived from the so-called 'P' transposable element (Rubin & Spradling, 1982). This is a class II transposon, 2.9kb in size, which is capable of moving around the genome via a 'cut and paste' mechanism. The ability of the transposase to act in trans (i.e. to catalyse the excision and transposition of any transposon in the genome) allowed the development of a binary vector-helper system for stable transformation (Rubin & Spradling, 1982). In this, the first component is a P element vector incorporating the transgene of interest between inverted terminal repeats (ITR) in place of the original transposase gene, while the second is a helper plasmid containing a P element that provides the transposase but which has been rendered non-autonomous (immobile) by deletion of the 3'ITR (Karess & Rubin, 1984). Co-injection of the vector and helper constructs enables the random transposition of the transgene into the host genome. Stability of the integrated transgene is then achieved after a few cell divisions as the helper plasmids are diluted and eventually lost completely, removing any source of transposase.

Though the P element was responsible for the first steps in insect transgenesis it quickly became apparent that another vector system would be required for non-Drosophilid insects. Despite repeated efforts, the P transformation vector proved unsuccessful for transforming non-Drosophilid insects, and P element excision assays (detecting P element ability to excise from the genome when exposed to transposase) confirmed that the element is immobile outside of the Drosophilidae,

apparently because it requires a particular cellular and genetic environment in which to function (Handler *et al.*, 1993).

At around the same time that the P element was confirmed as non-functional for the creation of non-drosophilid transgenic insects, an alternative transformation system utilising *piggyBac* was being developed (Handler *et al*., 1998). *piggyBac* is another transposable element, first discovered after baculoviruses were found to be capable of acquiring host cell DNA found in *Trichoplusia ni* (Fraser *et al.*, 1983). The *piggyBac* element is completely autonomous; its open reading frame (ORF) encodes a functional transposase, and it has a site specific (TTAA) insertion site (Handler, 2002). The *piggyBac*-mediated transformation system was then developed in a similar manner to the vector-helper system employed during P element mediated transgenesis (Handler *et al.*, 1998). Since then *piggyBac* has been successfully used to transform a wide range of insect species across several orders, including the medfly (Handler *et al*., 1998, Handler, 2002).

As part of the transformation process, it is important to include genetic markers in order to discriminate transformed from non-transformed individuals. Such markers can also be used to discriminate between released and wild insects in SIT or RIDL programs. Fluorescent proteins such as the green fluorescent protein (GFP) are ideal for this purpose (Tsien, 1998). GFP was originally isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). It has proved to be functional in an almost universal range of tissues and species and can be easily detected *in vivo*. However GFP is relatively insoluble and its excitation wavelength can be damaging to living organisms (Heim *et al.*, 1995). Hence, more recently novel fluorescent proteins such as DsRed and ZsGreen have been isolated from non-bioluminescent species of reef-coral (Matz *et al.*, 1999). These proteins are more soluble, can be tested *in vivo* without tissue destruction, can easily be directed to specific intracellular locations, and can be observed as early as 24 hours after DNA delivery (Bourett *et al.*, 2002, Wenck *et al.*, 2003). In addition the use of several different markers allows transformation, and evaluation, of multiple different constructs in tandem. In order to increase visibility and facilitate the screening of transformed insects, promoters that allow ubiquitous expression of the fluorescent marker (e.g. baculovirus promoter IE1 and the Hr5 enhancer (Rodems & Friesen, 1993) are used. Use of the *protamine* promoter enables nuclear localisation of the expressed fluorescent proteins, resulting

in concentrated expression of fluorescence and easier visualisation, identifiable as a 'spotting' pattern that is readily distinguishable from other phenomena such as auto-fluorescence (Fig 4.1).

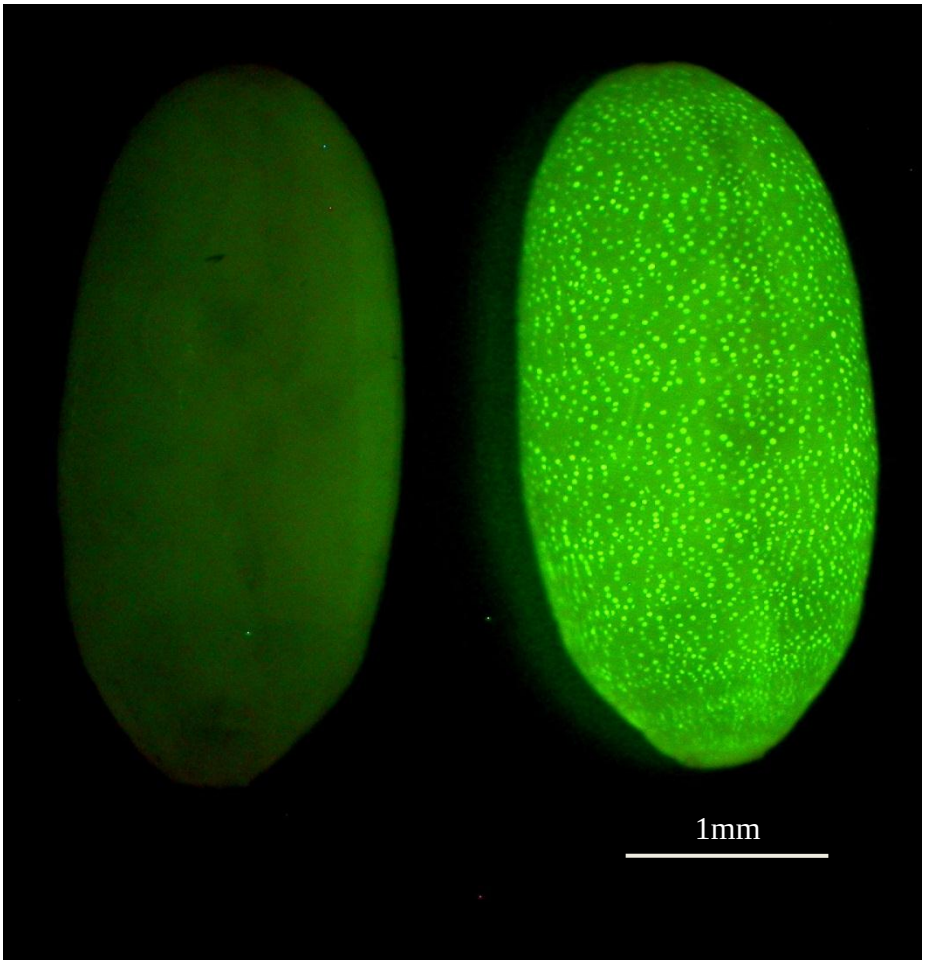

Fig 4.1 **Left** a wild type medfly pupa **Right** a RIDL medfly displaying the ZsGreen fluorescence transformation marker. Visualised with an Olympus SZX12 fluorescence microscope; the clear spotting pattern is due to nuclear localisation of the proteins from the expression of the transformation marker under the control of the *protamine* promoter.

As a result of the improvements in insect transformation and genetic markers detailed above, it has now been possible to implement RIDL as an alternative to SIT. In RIDL, genetic sterility is achieved by using the tetracycline-repressed transactivator tTAV which can be used to drive condition-dependent lethality (Gossen & Bujard, 1992, Thomas *et al.*, 2000). A wide range of pest insects, including the medfly, have been successfully transformed with multiple variants of the RIDL construct including one- and two-component systems (see below) with

autocidal or female-specific (fsRIDL) modes of lethality (Thomas *et al.*, 2000, Gong *et al.*, 2005, Fu *et al.*, 2007, Alphey *et al.*, 2008, Morrison *et al.*, 2009, Labbé *et al.*, 2010, Simmons *et al.*, 2011). To date all published lines of medfly have utilised a one-component, *tTAV* positive feedback loop, which can be altered to produce autocidal or female-specific lethality through the inclusion of introns from the sex-specific splicing *Transformer* (*Cctra*) gene. This ensures female specific expression of *tTAV*, as the primary transcript of the gene in males contains a stop codon and functional tTAV is never produced (Gong *et al.*, 2005, Morrison *et al.*, 2009). The mode of action of an autocidal, one component RIDL system is detailed here (Fig 4.2), and examples of female specific lethal constructs (fsRIDL, OX3864A and OX3647Q strains) are given in the next chapter.

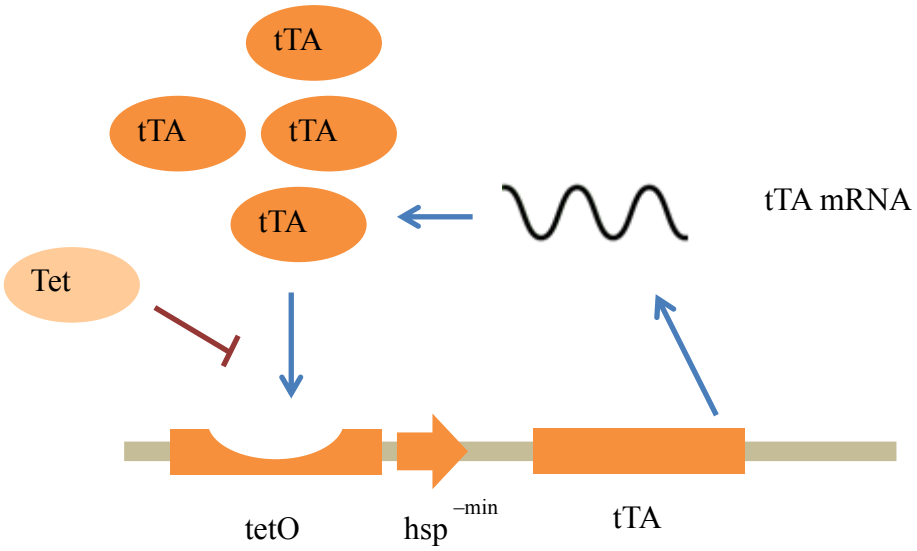

Fig 4.2 Schematic representation of a one-component tetracycline repressible lethal system. In the absence of tetracycline (Tet), basal expression of tTA leads to a positive feedback loop, with expressed tTA binding to tetO and driving expression of a minimal promoter and producing ever increasing synthesis of tTA to an eventually lethal level. Hence in this system tTA is both the driver and effector of lethality. In the presence of Tet, tTA is inactivated and only basal, non-lethal levels of tTA are produced.

Low levels of tTAV in cells are thought to be relatively harmless, however high levels of tTAV are extremely deleterious. The accumulation of tTAV in cells via the positive feedback loop is thought to exert its toxic effect due to transcriptional

'squelching' or interference with ubiquitin-dependent protein degradation, leading to death in the final larval instars (Gill & Ptashne, 1988, Berger *et al.*, 1990, Gong *et al.*, 2005).

Lethality earlier in development could potentially be achieved by the use of a two-component tetracycline-repressible system, comprised of a developmentally regulated promoter, inactive in adults. This could be used to drive expression of *tTAV*, which if combined with a highly toxic effector, could generate conditional embryonic or early larval lethality, much earlier in development than is possible with existing RIDL medfly lines (Fig 4.3).

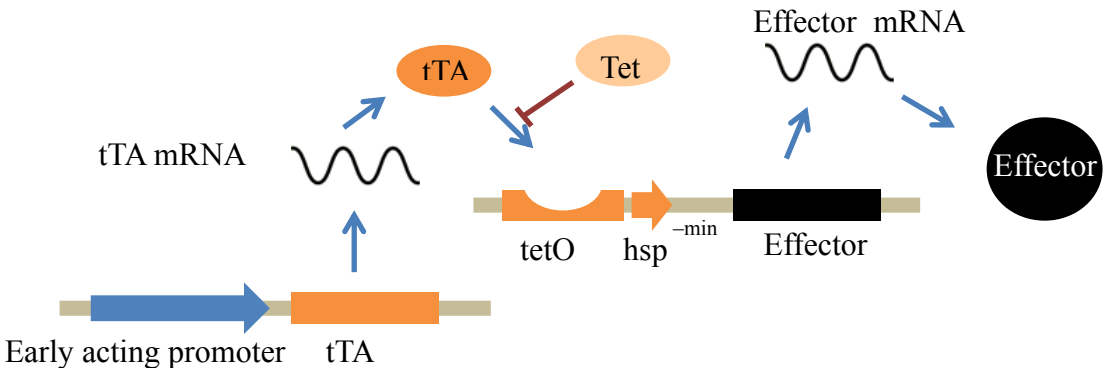

Fig 4.3 **Sc**hematic representation of a two-component tetracycline repressible lethal system. tTA is placed under the control of an early acting promoter (ideally embryo-specific). In the absence of tetracycline (Tet) the tTA produced binds to tetO, driving expression of a lethal effector molecule, resulting in death.

While late-acting lethality is tolerable or even desirable in certain species such as mosquitoes (because increased larval competition may reduce the number of surviving adults (Phuc *et al.*, 2007). In crop pest species such as the medfly, the expression of earlier acting lethality could have several substantial benefits. For example, the majority of crop damage caused by the medfly is due to larval destruction of the fruits, hence embryonic RIDL lethality would reduce the level of damage to fruits to just the small oviposition 'sting' left by females. A female-specific fsRIDL system with embryonic lethality would reduce the costs of larval

rearing for release generations by half because only males would develop. Tighter developmental regulation of the expression of *tTAV* would also result in reduced expression of *tTAV* at the adult stage. Persistent adult expression of *tTAV* can produce modest reductions in lifespan (Gong *et al.*, 2005). In addition using a specific lethal effector gene could substantially reduce the risk of inadvertent female survival off tetracycline (off-tet) with fsRIDL.

This chapter describes work that aims to deliver some of the proposed benefits described above by generating an early acting autocidal embryonic lethal RIDL construct. To do this, early acting expression of tTAV was directed via the use of the promoter region for *DmCG31626*, a protein coding gene originally isolated from *Drosophila melanogaster*. I attempted to use this promoter to drive embryonic lethality via inclusion within the construct of the pro-apoptotic gene *ReaperKR* under control of the tetracycline resistance gene *tetO*.

DmCG31626 has no known molecular function, though the distribution of GYR- and YLP- motifs within it suggests a general role in cuticle assembly. It has a peak of expression at 18-24 hours post-zygotic formation in *D. melanogaster* and expression then drops away in the later larval stages (Cornman, 2010, The modENCODE Consortium, 2010). In this chapter the functioning and timing of this gene was evaluated for its potential use in medfly RIDL, through the testing of several constructs with a *CG31626-tTAV* fused promoter.

ReaperKR is a modified version of the *Reaper* gene, part of a group of pro-apoptotic proteins, the RHG proteins (Hengartner, 2000), that, if deleted, result in a failure of apoptosis during embryo development in *Drosophila* (White *et al.*, 1994). The mode of action by which *Reaper* initiates apoptosis is believed to be by inducing the induction of ubiquitination (whereby ubiquitin is attached to a protein, targeting it for transport to the proteasome and subsequently degradation) of the Inhibitors of Apoptosis (IAPs), which raise the apoptotic threshold by inhibiting caspases (Miller, 1999, Salvesen & Duckett, 2002). It was discovered that this process is a two-way interaction, with IAPs simultaneously stimulating ubiquitination and degradation of the RHG proteins. By converting *Reaper* lysines to arginines, the mutant gene *ReaperKR* was constructed, which demonstrated higher protein stability, as ubiquitin cannot bind to the ReaperKR protein, which increased the induction of apoptosis

(Olson *et al.*, 2003). By including *ReaperKR* as the effector gene in a RIDL construct under the control of an early acting promoter, the aim was to induce high levels of *ReaperKR* expression within 24 hours post-zygotic formation in medfly. The inhibition of ubiquitin conjugation of ReaperKR was then predicted to allow high levels of this pro-apoptotic protein to accumulate rapidly and induce pre- or early-larval death.

4.3 Methods

4.3.1 Rearing conditions

I used the medfly strain TOLIMAN as the wild type (wt) genetic background for the construction of both transgenic lines and for all subsequent testing of strain efficacy. TOLIMAN is a wild type strain originating from Guatemala and reared in the laboratory since 1990, it has been maintained at Oxitec (Oxford, UK) since 2004. Adult fly colonies were kept at 25°C, 30-50% relative humidity and a 13hr light and 11hr dark cycle. Adults were fed on a diet of: 100g yeast hydrolysate, 400g powdered sugar, water was supplied through damp cotton wicks supplemented where appropriate with tetracycline hydrochloride (Sigma-Aldrich) (100µg/ml). Newly hatched larvae were transferred to the surface of larval medium, consisting of a standard yeast-wheatgerm-glucose agar *D. melanogaster* diet again supplemented with tetracycline hydrochloride where appropriate (100µg/ml). L1 larvae were placed onto larval medium at a density of ~ l larva per 0.5g of diet. Pupae were kept between 18- 25°C (depending on requirements) until eclosion.

Post-injection embryos were placed on a nutrient rich apple juice agar plate (0.75g agarose powder, 37.5ml water, 0.6g sucrose, 1ml 10% Nipagin, in ethanol, 12.5ml apple juice).

4.3.2 Maintenance of heterozygote lines

I refer to medfly lines carrying a particular construct by the construct name amended by a letter denoting the 'batch' i.e. line OX4423K is a population of medfly which are heterozygous carriers of the genetic construct OX4423. Heterozygote lines were maintained by backcrossing to the TOLIMAN wild type strain each generation, offspring were reared on the standard larval rearing diet and screened for the presence of the fluorescence transformation marker at the pupal stage with an Olympus SZX12 fluorescence microscope. Upon eclosion, ten males carrying the transformation marker were backcrossed to 30 wild type females, and reared according to standard protocols.

4.3.3 Heterozygote crosses

I established initial experimental lines that contained only half of the final RIDL construct, either the *tTAV* transactivator component or the *tetO* minimal promoter component. Crosses between heterozygote lines were then established to test whether different potential combinations of driver and effector genes would work to generate a useful RIDL strain.

Each cross was performed by setting up a cage with 20 adult flies of one sex carrying a construct with *tTAV* and 20 adult flies of the opposite sex carrying a construct with *tetO*. Approximately 1200 eggs were collected from each cross, of which a quarter will inherit both molecular constructs, produce tTAV which binds tetO and drive expression of a minimal promoter. The minimal promoter drives the expression of whichever effector gene is present within the molecular construct allowing screening for phenotype.

4.3.4 Construct designs

OX4156 contained the green fluorescent marker *ZsGreen* under control of the embryonic cuticular protein promoter gene *CG31626* originally isolated from *Drosophila melanogaster* (Fig 4.4)

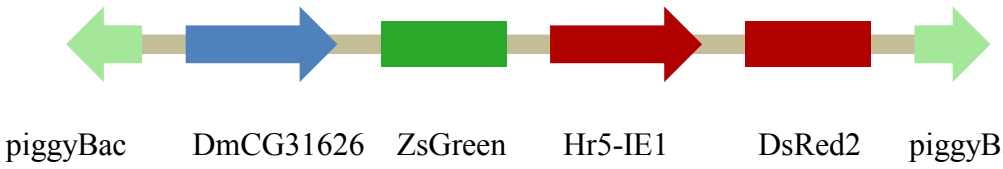

Fig 4.4 The structure of the molecular construct OX4156. The *DmCG31626* promoter drives expression of *ZsGreen* while a separate constitutively active transformation marker *DsRed2* is under the control of the *Hr5-IE1* (an *Hr5* gene enhancer element gene fused with the *Immediate Early 1* baculovirus promoter). The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX4301 utilised the same gene sequences as OX4156 except *ZsGreen* was replaced by the tetracycline-repressible transactivator (*tTAV*) gene, which is under the control of *CG31626* (Fig 4.5)

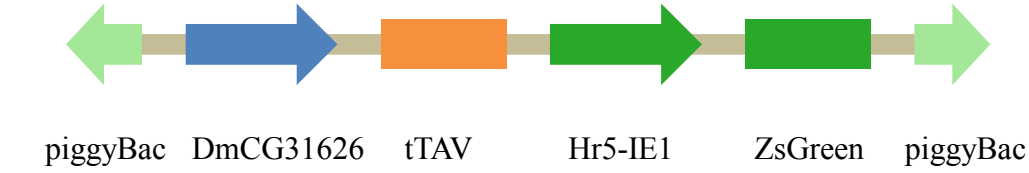

Fig 4.5 The structure of the molecular construct OX4301. The *DmCG31626* promoter drives expression of *tTAV* while a separate constitutively active transformation marker *ZsGreen* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX3867 had the *DsRed2* gene under the control of the tetracycline resistance gene (*tetO*), fused to a *protamine* promoter. In this line expression of the *DsRed2* gene will only occur in the presence of a source of tTAV (Fig 4.6).

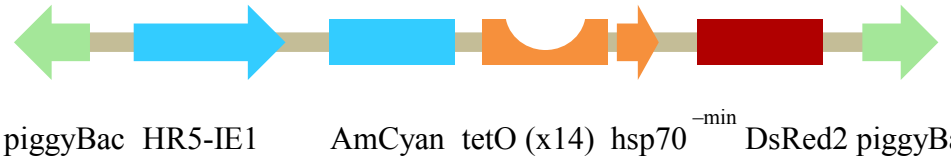

Fig 4.6 The structure of the molecular construct OX3867. There are 14 *tetO* sequences attached to the *hsp70* minimal promoter. In the presence of tTAV *hsp70* upregulates expression of *DsRed2*. A separate constitutively active transformation marker *DsRed2* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX4423 has the *ReaperKR* gene under the control of the tetracycline resistance gene (*tetO*), fused to an *hsp83* minimal promoter. *ReaperKR* expression and resultant lethality of insects carrying the gene will only occur in the presence of a source of tTAV (Fig 4.7).

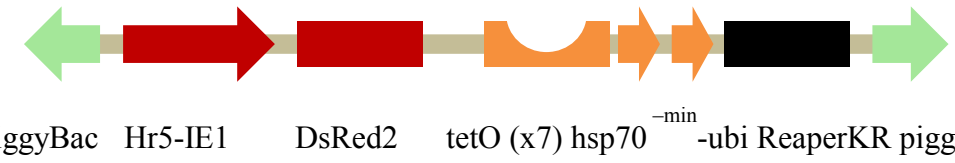

Fig 4.7 The structure of the molecular construct OX4423. There are 7 *tetO* sequences attached to the *hsp70* minimal promoter. In the presence of tTAV, *hsp70* upregulates expression of *ReaperKR*. A separate constitutively active transformation marker *DsRed2* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX4196 has the *tTAV* gene fused to an hsp83 promoter, resulting in ubiquitous expression of tTAV (Fig 4.8)

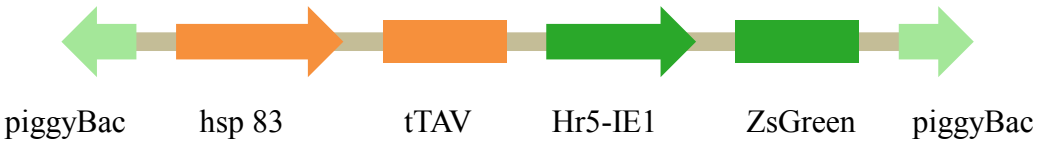

Fig 4.8 The structure of the molecular construct OX4196. The *hsp83* promoter drives expression of *tTAV* while a separate constitutively active transformation marker *ZsGreen* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX3069 is a construct that has the *ReaperKR* gene under the control of the *tetO* gene, fused to an *hsp83* minimal promoter. *ReaperKR* expression and resultant lethality of insects carrying the gene will only occur in the presence of a source of tTAV. This construct has been demonstrated to induce lethality when transformed into *Helicoverpa zea* (Boddie) (the cotton bollworm) (data not shown). It is not suitable for direct use in medfly without modification, as the promoter *OpIE2* used to drive the ubiquitous expression of the transformation marker *DsRed2* does not function in medfly (data not shown) (Fig 4.9).

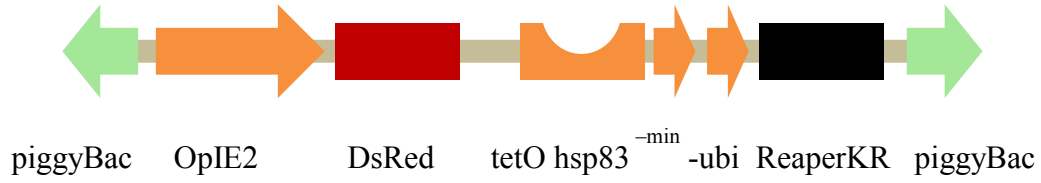

Fig 4.9 The structure of the molecular construct OX3069. There are 7 *tetO* sequences attached to the *hsp83-ubiquitin* minimal promoter. In the presence of tTAV, *hsp83* upregulates expression of *ReaperKR*. A separate constitutively active transformation marker *DsRed* is under the control of the *OpIE2* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

OX4095 is a construct previously used successfully in medfly, and is a candidate for donation of the transformation marker *Hr5-Ie1-DsRed2* to modify OX3069 (Fig 4.10).

Fig 4.10 The structure of the molecular construct OX4095. There are 14 *tetO* sequences attached to the *hsp83-ubiquitin* minimal promoter. In the presence of tTAV, *hsp83* upregulates expression of *Adh*. A separate constitutively active transformation marker *DsRed2* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

4.3.5 OX4536/7 Plasmid construction

Construct OX4536 was made from the modification of ReaperKR construct OX3069 successfully utilised in *H. zea* by removing the marker *OPIE2-dsRed* and replacing it with the *Hr5-IE1-DsRed2* marker from the construct OX4095 (Fig 4.11).

Fig 4.11 The structure of the molecular construct OX4536/7. There are 7 *tetO* sequences attached to the *hsp83-ubiquitin* minimal promoter. In the presence of tTAV, *hsp83* upregulates expression of *ReaperKR*. A separate constitutively active transformation marker *DsRed2* is under the control of the *Hr5-IE1* promoter. The construct is flanked by *piggyBac* sequences that mediate random insertion into the host genome.

Using known nucleotide sequences, it was determined that the restriction enzymes Not-1 and Asc-1 recognition sequences would produce the desired products, and the required complementary 'sticky ends' for subsequent ligation. 1µl of a maxiprep of OX3069 at a concentration of 8.8µg/µl was digested with 1µl Not-1& 1µl Asc1 with 3µl 10x No.4 Buffer and 3µl 10xBSA in 21µl deionised water. I also set up a miniprep of OX4095 at 1µg/ µl in a Not1-Asc1 digest with 12µl of DNA and 10µl of deionised water. Both digests were incubated at 37°C for 3 hours before being stored at -20°C.

1µl of each digest was run on a 1% agarose gel (100mV, 45 minutes) to purify DNA fragments. Plasmid OX3069 produced two fragments of 7.1kb and 1.6kb which were visualised on a transilluminator, the larger fragment was the main plasmid and this band was cut from the gel with a scalpel, while OX4095 also produced two fragments one at 7.7kb and one at 2.4kb, the smaller fragment contained the *Hr5IE1dsRed* marker and was cut from the gel (Fig 4.12).

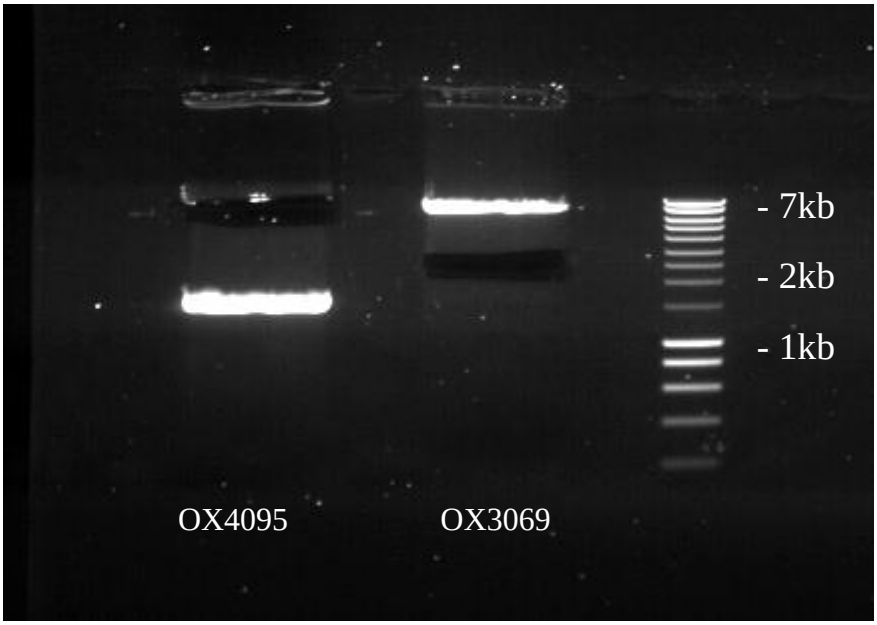

Fig 4.12 Gel purification of the Not1-Asc1 digests for constructs OX3069 & OX4095. OX4095 was cut into two fragments 7.1kb and 1.6kb, the smaller fragment, predicted to contain the *Hr5-IE1* promoter and the *DsRed2* gene, was cut from the gel. OX3069 was cut into two fragments of 7.7kb and 2.4kb, the smaller fragment contained the *OpIE2* and *DsRed* sequence, while the larger band was cut from the gel with the rest of the OX3069 construct.

Gel extraction was performed using the Qiagen® MinElute Gel Extraction Kit, and purified DNA was eluted into 10µl Buffer EB. Semi-quantification of DNA fragments was performed by running 2µl of each sample on a 1% agarose gel, the gel band for the DNA fragment from OX3069 was determined as roughly 10x brighter than the band produced by the DNA fragment OX4095, and the ligation mix was adjusted accordingly

Ligation of the two plasmid fragments was performed with 0.5 µl of DNA from OX3069, 7µl of DNA from OX4095 with 0.5µl of ligase in 2µl of 2x reaction buffer. This mix was incubated at room temperature for 20 minutes before being placed on ice, prior to bacterial cell transformation. Transformation of bacterial cells was carried out with Stratagene XL 10-Gold ultracompetent cells according to the manufacturer's protocol, and incubated in 200µl of transformation medium (2.5µl 1M $MgCl_2$, 2.5µl 1M $MgSO_4$, 40µl filter sterilised 20% glucose, 155 µl LB Medium) at 37°C with vigorous shaking (250rpm) for 1 hour, before setting up two 48-well culture plates. The first was filled with 120µl LB (100µg/ml Ampicillin) in each well, the other plate contained 10µl of a PCR mastermix (1µl DreamTaq buffer, 0.2µl dNTP, 0.2µl Primer hspdiagR GCAGATTGTTTAGCTTGTTCAGC, 0.2µl Primer diagIE1 GCTCCTCGTGTTCCGTTCAAGG, 0.07µl DreamTaq, 8.33µl Water) in each well. Using a pipette with tip, one colony from the plate of transformed bacteria was dipped into one well of each microplate in turn. The LB plate was then sealed with parafilm and placed in a shaking incubator (37°C, 250 rpm). The PCR plate was simultaneously run using the following PCR program: 94ºC - 2 mins, 94ºC - 15 secs, 55ºC- 40 secs,72ºC - 2 mins, (repeat previous 3 steps x 2), 94ºC - 15 secs, 55ºC - 30 secs, 72ºC - 1 min, (repeat previous 3 steps x 29),72ºC - 6 mins, 4ºC – Hold.

When the PCR reaction was finished 2µl from each well was run on a 0.8% agarose gel (120mV, 20min) to screen for positive transformants. Six random positive samples from the equivalent well in the LB plate were inoculated in minipreps overnight (A2, E2, A3, F3, C5, G6) (Fig 4.13).

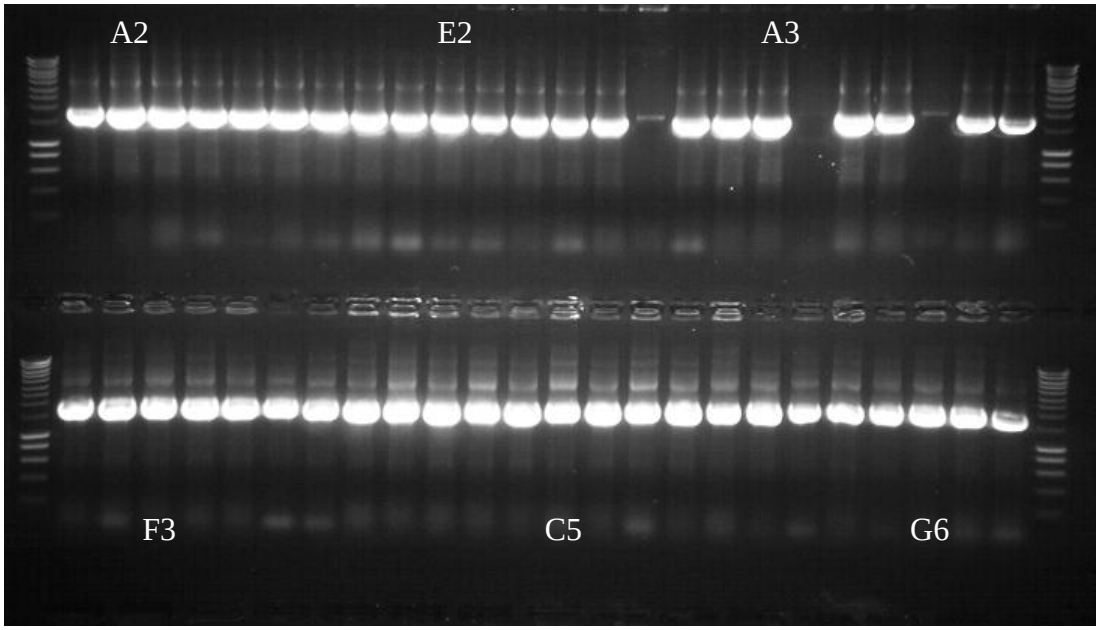

Fig 4.13 Visualisation of PCR screening for positive transformants of the new construct OX4536/7. The 96 well plate was screened for transformants by running 2µl of each sample on a 0.8% agarose gel. I took six randomly chosen positive transformants from wells A2, E2, A3, F3, C5, G6 of the 96 well plate to inoculate into minipreps.

Minipreps were produced by preparing 3ml of LB broth (100µg/ml Ampicillin) in labelled 15ml tubes with 20µl of culture from the selected wells in the microplate, and incubated overnight at 37°C in a shaking incubator (250rpm). Purified plasmid DNA was recovered from 1ml of LB using the GeneJET^{TM} Plasmid Miniprep Kit, eluting the purified DNA into 50µl of elution buffer, and storing at -20°C.

Diagnostics of the plasmid DNA were performed to ensure the integrity of the new construct. 2µl samples of F3 and G6 were cut separately with 0.5µl of restriction enzyme NheI in 15.5µl water and 2µl Fast buffer (with GLS) each. The samples were incubated at 37°C for 2 hours before being run on a 1% gel, in order to visualise the two predicted plasmid fragments of 7.5kb and 2kb. Minipreps from both samples were sent for sequencing to GATC Biotech with Diag IE1 primers to check sequence fidelity.

I inoculated the remaining culture from the minipreps into maxipreps (250ml LB with 100µg/ml Ampicillin) and incubated overnight in a shaking incubator (37°C, 250 rpm). From the maxipreps, I took 700µl and inoculated it into 300µl of 50% Glycerol in a 1.8ml Nunc tube and stored at -80°C; the two stored constructs were named OX4536 & OX4537 respectively.

4.3.6 Preparation of an injection mix for transformation

For the preparation of a micro-injection mix, helper DNA is required to aid transformation. phspBac (Handler *et al.*, 1998) was used, as this is a universal helper expressing the *piggyBac* transposase gene under the Drosophila heat-shock promoter *Dmhsp70* but lacking inverted terminal repeats.

The initial mix was calculated to give concentrations of 600ng/µl of transposon and 300ng/µl of helper DNA in a final volume of 20µl. Then 50µl of 100% ethanol and 2µl of 3M Sodium Acetate pH 5.2 were mixed in and incubated at -20°C for 15 minutes. The mix was then centrifuged at full speed (>13000 x g) for 10 minutes, and the supernatant discarded, taking care not to disturb the pellet. At this point I added 20µl of injection buffer (0.2µl of 0.1M NAH_2PO_4 and 1µl 1M KCl in 18.8µl Endofree water) and the injection mix was left overnight at 4°C for the pellet to dissolve.

4.3.7 Microinjection

½-1 hour pre-blastoderm embryos from newly egg-laying wild type females (5-12 days post-eclosion) were collected in deionised water. The water was poured through a collection pot with a mesh bottom. The eggs were then dechorionated by dipping the pot into a petri dish containing 10% sodium hypochlorite solution followed by gentle swirling for 90 seconds. The pot was then promptly placed in a petri dish of

deionised water, before rinsing with running water 4 or 5 times and placing the pot back in a fresh petri dish of deionised water.

Eggs were then placed on the surface of an agar-apple plate with a paintbrush, ready for microinjection. Glass coverslips with glue were placed on the apple-agar plate, and embryos were laid out in a line along the coverslip with the posterior end arranged to be out of the edge of the glue. Each coverslip was transferred onto a clean slide using a drop of water before being placed into an oven set to 37°C for 3-5 minutes.

The injection mix was spun down in a microcentrifuge at full speed for 5 minutes, and 4µl was loaded onto a needle and mounted onto the microinjector machine (Femtojet Microinjector from Eppendorf). I then injected each embryo with a small amount of DNA (c.60pl). When injecting was complete, each cover slip was removed from its slide and transferred onto a petri dish containing apple juice agar, and with yeast paste smeared around the edges. Petri dishes were then sealed with parafilm and incubated at 24°C until L1's emerged, at which point they were picked and transferred to a standard larval rearing diet, supplemented with tetracycline where appropriate.

4.3.8 Stabilisation of heterozygote lines

Larvae surviving injection were screened at the pupal stage for transient expression of the transformation marker gene, using an Olympus SZX12 fluorescence microscope. Levels of transient expression may indicate the successful uptake of the molecular construct, however only successful transformation of the germ cells allows the establishment of a stable heterozygote line. Successfully eclosed adults were sorted according to sex and combined (either 10 males or 20 females per pool) together with wild type flies of the opposite sex (10 males or 20 females). I collected eggs from these adult cages and reared the larvae according to standard protocols; pupae were later collected and screened for fluorescence. Fluorescence in the generation 1 (G1) offspring indicates inheritance of the molecular construct from the germ cell of the G0 parent, and as such carry the molecular construct in every cell.

Individual fluorescent G1 individuals were set up with wild type flies of the opposite sex, either 1 transgenic male with 6 wild type females or 1 transgenic female with 2 wild type males (transgenic males are preferable at this stage, as one male can be housed with several females and produce a large G2 population for screening). I screened G2 offspring at the pupal stage for strength of fluorescence (clear expression of the transgenic marker, and strong spots of nuclear localisation) and evidence of an autosomal inheritance pattern by screening transformants for sex.

4.3.9 Phenotypic expression studies

Lines heterozygous for the transgene constructs were established and crosses between putative heterozygotes were used to determine the expression patterns of inserted genes. Genes such as the embryonic cuticular promoter gene *DmCG31626* were expected to start expression within 48 hours of formation of the zygote. For phenotypic expression studies such as these, embryos were cultured from egg collections made over a one hour period from the standard rearing cages on six separate occasions. I then counted the embryos under an Olympus SZX12 fluorescence microscope and incubated them on wet Whatman filter paper (Fisher Scientific) placed in a petri dish (200 eggs per petri dish, 1200 eggs per line in total). Three of the dishes from each line were then monitored every 12 hours for the appropriate fluorescent marker. The remaining embryos were sealed into the petri dishes with parafilm and incubated for 72 hours. After this time, I unsealed the dishes and placed the L1s under the microscope to assess fluorescence before placing them onto the standard larval rearing diet for further development, developing larvae were screened for fluorescence again at L3, pupation and eclosion.

I tested for lethal effects of the inserted transgenes when crossing tTAV lines with tetO lines by rearing and screening offspring as detailed above and comparing treatments that were supplemented with tetracycline (on-tet) and those without tetracycline (off-tet). Differential survival in the offspring carrying both transformation markers off-tet allowed me to quantify the killing strength of the

lethal effector. Any decrease in survival on-tet would indicate potential 'leaky' expression of the constructs.

4.3.10 Semi quantitative RT-PCR

In order to more accurately quantify the timing of *CG31626* expression in construct OX4301, I performed a semi-quantitative RT-PCR. I collected egg samples at 24 hours and 48 hours post-fertilisation, as well as L1 and L3 larvae, early pupae, late stage pupae and adults from heterozygote construct lines OX4301B, E, H and wild type. All samples were snap-frozen in liquid nitrogen before being stored at -80°C to prevent RNA degradation.

Extraction of RNA from samples was performed utilising 50µg of tissue from embryos at 24 and 48 hours post-fertilisation, and from L1 and L3 larvae, early pupae (1 day since pupation), late stage pupae (6 days since pupation) and adults (24 hours post-adult eclosion). The RNA extraction was carried out with the TRI Reagent® Solution according to the manufacturer's protocol and RNA was stored in 50µl of fresh Milli-Q water. I ran a 1% agarose gel using 2µl of RNA solution to check for RNA presence and non-degradation (Fig 4.14).

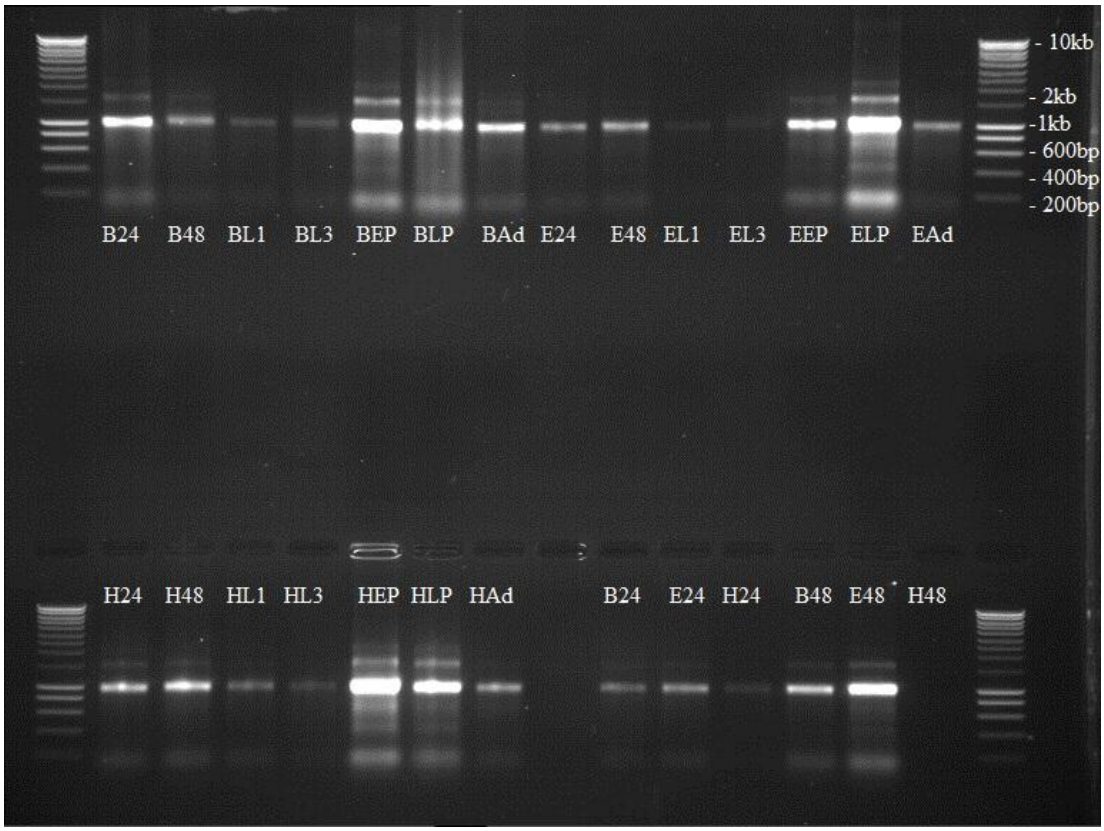

Fig 4.14 Gel screening for the presence and integrity of tTAV mRNA after tissue extraction. A 2µl sample of each RNA extraction was run on a 1% agarose gel in order to confirm the presence and non-degradation of the RNA molecules. The strength of fluorescence for each band was used in conjunction with the results from the absorbance spectrophotometer to determine the amount of RNA present from extraction. First letter denotes line i.e. OX4301B, H or E, followed by 24 and 48 hour embryonic RNA extraction, L1 or L3 (1st or 3rd larval instar), EP (Early Pupa), LP (Late stage pupa) and Ad (Adult fly).

1µl of RNA solution from each sample was also placed on the absorbance spectrophotometer in order to quantify RNA concentration. From this and gel banding the individual volume required to make 1µg of RNA in 17µl of RNase treated water in order to standardise the samples was calculated.

7.5µl of the RNA extract was used with the SuperScript III One-Step RT-PCR System™ with Platinum® *Taq* DNA polymerase protocol. A one step RNA to cDNA conversion and amplification PCR was performed twice with two different sets of primers, Tta-tetdiagR (GCGGAGTTGATGACTTTGGACTTATCC) and Dm31626RTF (GATGACCTCTAGGAAATAACTAACTAATTACCCAC) to amplify tTAV, and CcAdh2RTR (CTTGGAGGTGATGTCGAATTTGGTG), and

CcAdh2RTF (GAAAGCTGTTCGGGCTTCAGGC) to amplify RNA from the housekeeper gene *Adh*. Two control reactions were performed for each primer set. One where Supercript™ III RT/Platinum® *Taq* was replaced by Platinum® *Taq* DNA polymerase to verify absence of genomic DNA. The other with RNase treated water was used instead of template RNA, to act as a negative reaction control.

I removed 5µl of the reaction mix at steps 30, 35 & 40 to be run on a 1% agarose gel and screened for cDNA bands.

4.3.11 Data Analysis

The mean value with standard error of the timings of the phenotypic expression for each molecular construct was determined. The data were non-normally distributed and so a log transformation was applied before running parametric statistical tests. Independent sample *t*-tests and ANOVA were used to compare the mean time of expression (in hours post-zygote formation) between lines. Chi-square tests were used to determine whether the survival of offspring from crosses between different transgenic lines displayed an altered rate of survival as a result of carrying different combinations of the molecular constructs. All analyses were performed using PASW v18.0.0 (PASW Statistics v.18, 2009).

4.4 Results

Direct construction of a working embryonic lethal RIDL system from scratch in *Ceratitis capitata* would be extremely challenging, instead I conducted proof-of-principle experiments to test whether a promoter which switches on during embryonic development could be used to drive expression of a lethality gene. I crossed medfly lines OX4301B, E &H with lines OX4423C & K. The latter two constructs contained the tetracycline-repressible transactivator (*tTAV*) gene under control of the modified *Drosophila melanogaster* embryonic promoter gene *CG31626* and the tetracycline responsive element (*tetO*) driving expression of the lethality gene Reaper KR, respectively.

4.4.1 Embryonic cuticular promoter

Two lines carrying the OX4156 construct, A and J, were tested. The mean time at which ZsGreen first became visible underneath the fluorescence microscope was 48H post-zygotic formation for both lines (Fig 4.15) (OX4156A mean = 47.7 hours, S.E. ± 0.2, N = 567, OX4156J mean = 47.9, S.E. ±0.18, N = 543, t_{1108} = 0.808, P = 0.58), fluorescence persisted throughout the larval instars, but was masked by bleedthrough (visible fluorescence from another marker) of the *DsRed2* transformation marker, and was undetectable at the pupal stage.

Fig 4.15 Screening of the OX4156 construct for ZsGreen fluorescence at 48 hour old embryo and 3rd instar larval stages of development. **Top)** Two embryos both 48 hours post-zygotic formation (to within one hour), top is an OX4156J embryo with ZsGreen visible for the first time, bottom a wild type medfly with no visible fluorescence. **Bottom)** Two 3rd instar larvae are shown, top is an L3 OX4156 larva displaying weak ZsGreen fluorescence (strongest expression remains around the mouthparts), but a strong DsRed2 fluorescence from the transformation marker is visible, bottom a wt L3 larva with no visible fluorescence.

4.4.2 Crosses OX4301 x OX3867F

From the crosses between lines OX4301B, E & H with OX3867F, the resultant eggs were screened for the DsRed2 marker, which was expected to be present in the 25% of progeny inheriting both molecular constructs. Chi square analysis of the transformation markers (ZsGreen and AmCyan) showed no effect of inheriting one or both constructs on normal development or survival (OX4301B x OX3867F, χ^2_1 = 0.592, P = 0.442, Green = 426, Cyan = 439, wild type = 442, Green/Cyan = 423) (OX4301E x OX3867F, χ^2_1 = 0.21, P = 0.924, Green = 430, Cyan = 442, wild type = 433, Green /Cyan = 439) (OX4301H x OX3867F, χ^2_1 = 1.059, P = 0.304, Green = 330, Cyan = 405, wild type = 390, Green/Cyan = 431). DsRed2 fluorescence expression was not present at any point during embryonic development (Fig 4.16) and was first identified at the L1 stage but persisted through to the pupal stage (OX4301B mean = 69.6 hours, S.E. ± 0.35, n = 400, OX4301E mean = 69.4 hours, S.E. ± 0.35, N = 450, OX4301H mean = 68.7 hours, S.E. ± 0.41, N = 405, ANOVA $F_{2,\ 1252}$ = 1.93, P = 0.145), marking a delay of up to a day between visible fluorescence driven by *CG31626* in OX4301 x OX3867F crosses when compared to lines OX4156A & J. This indicated a significant delay in expression as a result of the *tTAV/tetO* system (t-test, $t_{\ 2364}$ = 93.3, P < 0.001).

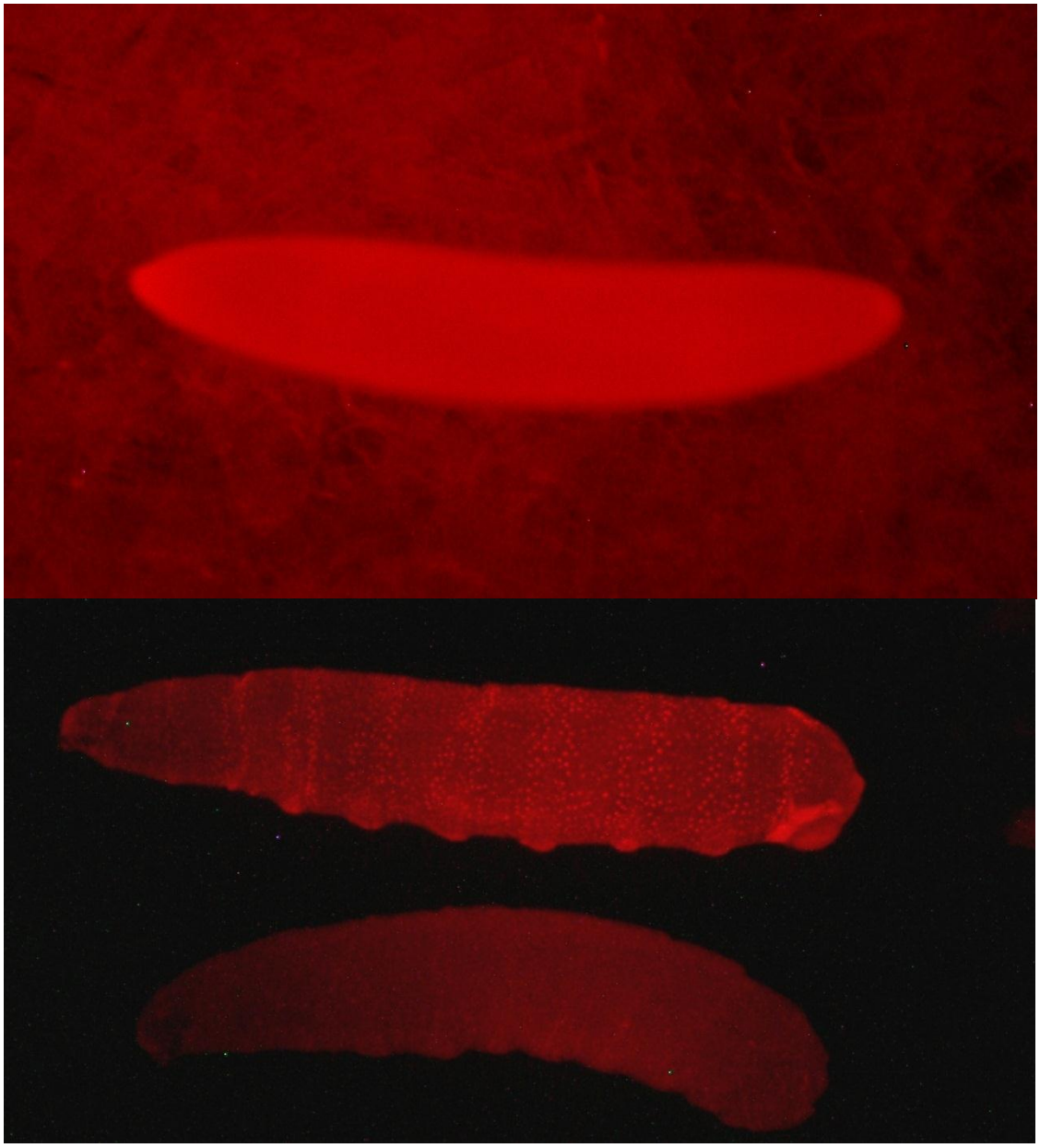

Fig 4.16 Screening of the offspring of OX4301H xOX3867F crosses for DsRed2 transformation marker at 48hour old embryo and 3rd instar larval stage of development. **Top)** One embryo from a cross between OX4301H x OX3867F adults, no embryos showed any fluorescence from the *DsRed2* fluorescence gene at 48H, in contrast to the ZsGreen fluorescence visible in Fig 4.15. **Bottom)** Two L3 larvae, top is from an OX4301H, OX3867F cross, bottom a wt L3. In contrast to OX4156, fluorescence as a result of expression of the *DmCG31626* gene is still strongly visible at the L3 stage, indicating an overall shift in the timing as a result of the *tTA/tetO* system.

4.4.3 Transformation with construct OX4423

A total of 995 pre-blastoderm embryos were injected with the OX4423 *tetO Reaper KR* construct. Injection survivors (346 total, 76 displaying transient expression) were then separated by sex and set up in 25 pools (10 males or 20 females per pool) before crossing to wild type. Nine transgenic lines were continued from the G1 offspring. The heterozygote lines to be continued were created from the G2 pupae collected from each line, and selected on the basis of the strength of fluorescence and a pattern of inheritance that appeared to be autosomal, as with OX4423 C, K, S & X.

4.4.4 Crosses OX4301 x OX4423

Six crosses were established between lines OX4301B, E & H (*CG31626* embryonic expression of *tTAV*) with OX4423 C & K. The resulting eggs were incubated onto wet Whatman paper until the L1 larvae hatched and were screened for fluorescence. Offspring inheriting both copies of the construct should have inherited the *ZsGreen* and *DsRed2* transgenic markers, and when reared on tetracycline, *ReaperKR* lethality should be suppressed in 25% of the progeny. A decrease from this proportion was expected to result from incubating the eggs without tetracycline. However in contrast to the expectation, no lethality was evident at the L1 stage (Table 4.1) or the resultant adult offspring (Table 4.2).

Table 4.1 Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4423 crosses that survived to the first larval instar when incubated without tetracycline.

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4301B x 4423C	0.301	1	0.583	97	102	92	108
4301B x 4423 K	0.585	1	0.444	78	86	90	84
4301E x 4423 C	0.033	1	0.855	65	68	65	65
4301E x 4423 K	0.237	1	0.626	101	107	111	107
4301H x 4423 C	0.120	1	0.729	103	102	99	105
4301H x 4423 K	0.193	1	0.661	65	63	61	66

Table 4.2 Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4423 crosses upon adult eclosion when reared on the standard larval diet without tetracycline.

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4301B x 4423C	0.033	1	0.855	31	30	29	30
4301B x 4423 K	0.317	1	0.573	53	48	49	52
4301E x 4423 C	0.253	1	0.615	73	84	48	47
4301E x 4423 K	0.385	1	0.535	40	35	50	55
4301H x 4423 C	0.571	1	0.450	54	54	66	58
4301H x 4423 K	0.241	1	0.624	51	51	48	50

4.4.5 OX4301 Semi-quantitative RT-PCR

As a result of the lack of lethality induced from the cross between OX4301 and OX4423, I confirmed the timing of the expression of *tTAV* in lines OX4301B, E & H by PCR screening for cDNA fragments from a one-step semi-quantitative RT-PCR. Visualisation of the products produced at cycles 30, 35 and 40 of the PCR protocol, found no visible difference in product between the three tested cycles of the PCR. Transcribed RNA for *Adh* was found to be present at all life stages in all three lines

of 4301, however transcription of RNA from *tTAV* was not found in samples taken at 24 hours post-zygotic formation but was present at 48H onwards (Fig 4.17).

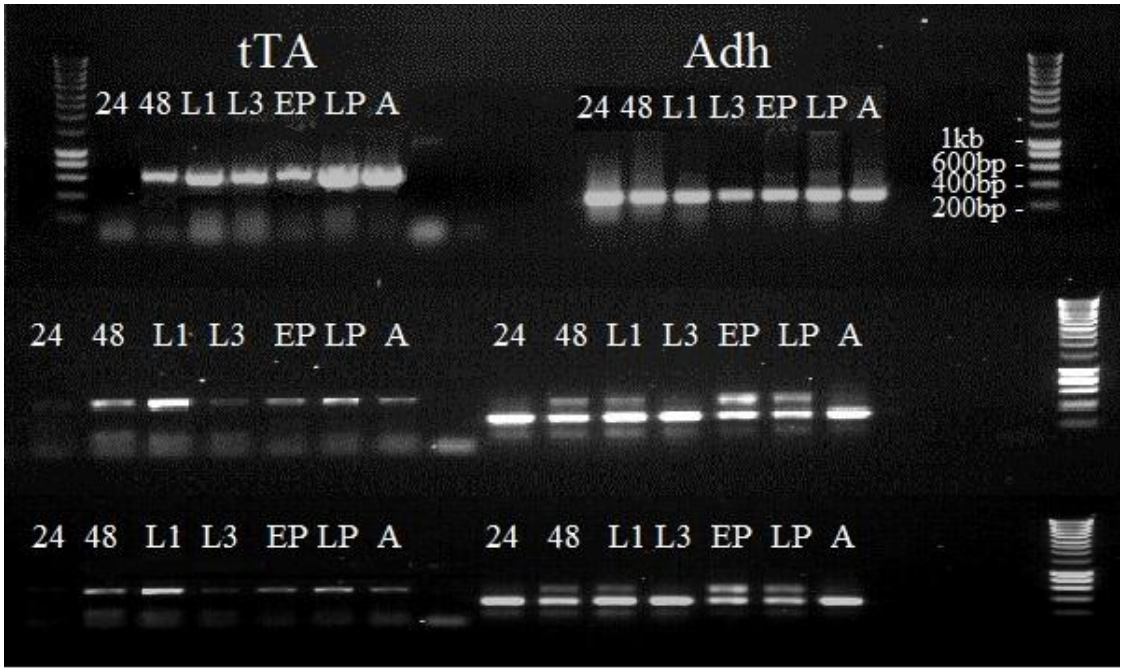

Fig 4.17 One-step RT-PCR screening of pools of OX4301B, E & H offspring for expression of *tTA* and *Adh* across development. From left to right each well contained cDNA from 24 and 48H post-zygotic formation, 1st larval instar (L1), third larval instar (L3), early pupae (1 day old, EP), late pupae (6 days old, LP) and recently eclosed adults. Adh expression was found throughout every life history stage, *tTA* expression was evident at 48H post-zygote formation onwards.

4.4.6 Crosses OX4196J x OX4423

Four crosses were established between lines OX4423 C, K, S & X (*tetO*, *Reaper KR*) and OX4196J (*Hsp83* strongly expressed promoter *tTAV*), in order to test the efficacy of OX4423 to induce lethality under the control of a different promoter construct. Offspring inheriting both copies of the construct should have inherited the *ZsGreen* and *DsRed2* transgenic markers, and when reared on tetracycline, *ReaperKR* lethality should be suppressed in 25% of the progeny. A decrease from this proportion was expected to result from incubating the eggs without tetracycline. However no

lethality was evident from proportions of transgenic markers in the adult offspring (Table 4.3).

Table 4.3 Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4196J x OX4423 crosses upon adult eclosion when reared on the standard larval diet without tetracycline.

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4196J x 4423C	0.021	1	0.885	51	51	48	50
4196J x 4423 K	0.091	1	0.763	49	56	36	45
4196J x 4423 C	0.501	1	0.479	45	50	55	50

4.4.7 Development of construct OX4536/7

To determine if the *ReaperKR* gene could be used to drive lethality in the RIDL system with a different minimal promoter, I produced OX4536/7 from constructs OX3069 (Donor of *hsp83, tetO 7, Reaper KR*) and OX4095 (Transformation marker donor). The restriction digest with NheI produced the predicted fragment sizes, with restriction sites in the predicted locations; this indicated that the molecular construct formed appropriately (Fig 4.18). Data from GATC biotech confirmed the sequence integrity of the new constructs, at this point they were dubbed OX4536 and OX4537 and deemed ready for testing by microinjection into wild type medfly.

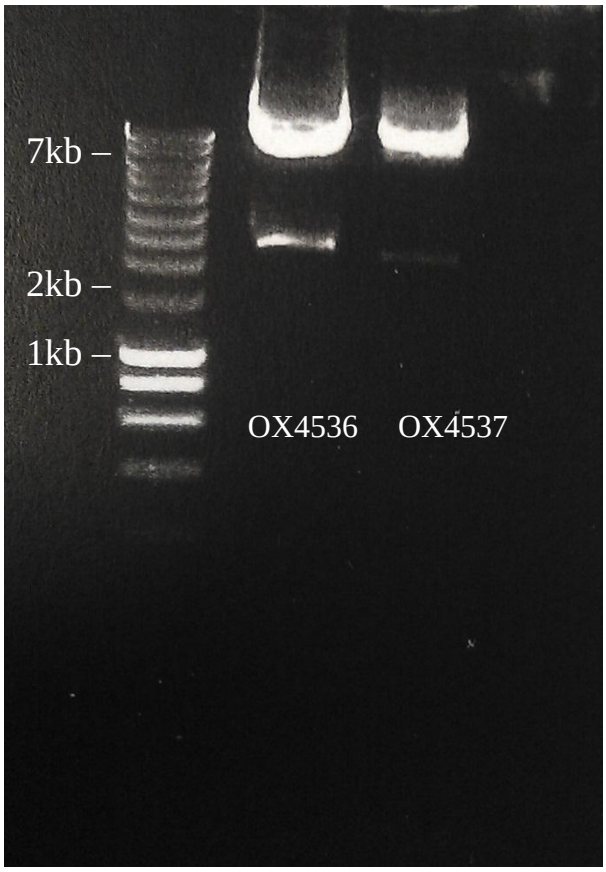

Fig 4.18 Diagnostic of the NheI digest for constructs OX4536 and OX4537. The constructs from microplate wells F3 and G6 were digested with restriction enzyme NheI which produced two gel fragments of 7.5kb and 2kb. The two fragments produced matched the predicted band sizes according to the correct positioning of restriction sites in the new construct.

4.4.8 OX4536 micro-injections

A total of 600 pre-blastoderm embryos were injected with the OX4536 construct. Injection survivors (328 total, of which 141 displayed transient expression) were then separated by sex and set up in 17 pools (10 males or 20 females per pool) before crossing to wild type. I continued twelve transgenic lines from G1 offspring. The heterozygote lines to be propagated were made from the G2 pupae collected from each line, selected on the basis of the strength of fluorescence and a pattern of inheritance that appeared to be autosomal as with OX4536 B, D, Gi, Gii, Hii & I.

4.4.9 Crosses OX4536 x OX4301

Ten crosses were established between lines OX4536B, D, G, I & Q and OX4301B, in order to test the efficacy of OX4423 to induce lethality under the control of a different promoter construct. As before, offspring inheriting both copies of the construct should have inherited the *ZsGreen* and *DsRed* transgenic markers, and when reared on tetracycline, *ReaperKR* lethality should be suppressed in 25% of the progeny. A decrease from this proportion was expected to result from incubating the eggs without tetracycline. There was a significant reduction in the number of L1 larvae carrying the *DsRed2* transformation marker (Table 4.4), however this was not evident at the pupal stage, indicating a delayed onset of expression in the OX4536 construct, and no lethality was evident at the pupal stage of medfly carrying both the *tTAV* and *tetO* constructs (Table 4.5).

Table 4.4a Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4536 crosses at the first larval instar when incubated without tetracycline.

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4301B x 4536B	5.95	1	0.015	39	1	10	3
4301B x 4536D	1.10	1	0.293	17	9	10	10
4301B x 4536G	0.11	1	0.744	15	8	9	6
4301B x 4536I	2.43	1	0.119	31	11	12	10
4301B x 4536Q	0.66	1	0.418	30	2	10	0

Table 4.4b Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4423 crosses at the first larval instar when incubated with tetracycline (100µg/ml).

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4301B x 4536B	12.12	1	0.001	32	0	12	6
4301B x 4536D	10.93	1	0.001	54	9	15	13
4301B x 4536G	2.50	1	0.114	14	5	6	7
4301B x 4536I	6.70	1	0.010	55	18	13	14
4301B x 4536Q	2.03	1	0.278	39	1	6	1

Table 4.5a Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4536 crosses at the pupal stage when reared on the standard larval diet without tetracycline.

Cross	χ^2	*df*	*P*	Green	Red	Wt	Red/Green
4301B x 4536B	2.59	1	0.108	18	14	12	21
4301B x 4536D	0.20	1	0.658	24	19	23	22
4301B x 4536G	1.83	1	0.176	4	8	10	7
4301B x 4536I	0.10	1	0.974	15	20	16	21
4301B x 4536Q	0.75	1	0.506	20	17	20	25

Table 4.5b Results from χ^2 tests on the inherited fluorescence markers carried by offspring of OX4301 x OX4423 crosses at the pupal stage when reared on the standard larval diet with tetracycline (100µg/ml).

Cross	χ^2	*df*	*P*	Green	Red	Wt	Green/Red
4301B x 4536B	0.42	1	0.551	11	11	12	8
4301B x 4536D	1.24	1	0.362	28	21	22	26
4301B x 4536G	0.95	1	0.329	6	4	3	2
4301B x 4536I	0.21	1	0.644	27	31	27	26
4301B x 4536Q	0.08	1	0.784	21	28	21	25

4.5 Discussion

The development of several constructs containing the *DmCG31626* gene has shown that this gene is, in principle, a suitable candidate for driving *Tta* expression at an early developmental stage.

OX4156J demonstrated clearly visible fluorescence during embryonic development, at around 48 H post-zygote, which demonstrates that the promoter gene retained its function when transformed into the medfly. *CG31626* has a natural peak of expression at around 18-24 hours in *D.melanogaster*, which, given the slightly longer life cycle of the medfly, would appear to demonstrate a comparable

developmental expression pathway. As part of OX4301, *CG31626* was incorporated into the *tTAV* signalling pathway, and when medfly lines OX4301 B, H & E were crossed to OX3867F, all three crosses generated fluorescence through the Tet system. This proof-of-principle shows that the OX4301 construct would be capable of driving early expression in a RIDL system. However, an important caveat to this statement is that all three crosses showed a significant delay in the timing of expression of fluorescence driven by *CG31626* when compared to OX4156. Although it is not possible to determine exactly why this delay has occurred, given that all three lines show the same timing of expression for *tTAV* and elicit the same phenotypic response in fluorescence when crossed to OX4156J it is possible to rule out positional effects stemming from the random *piggyBac* mediated insertion. Instead it is likely that the intermediate steps of inducing expression of *tTAV* which is acting in trans in order to drive expression of *DsRed2* through *tetO* is responsible for the later visualisation of fluorescence.

Using semi-quantitative RT-PCR on the lines OX4301B, H & E I found mRNA present for *tTAV* from 48H post-zygotic formation, in line with the timing of fluorescence found in OX4156J. This confirmed that *CG31626* was capable of acting as a promoter to drive *tTAV* expression in the same pattern as *ZsGreen*. Analysing multiple cycles of the PCR reaction found no visible differences in the strength of expression from 48H onwards, further confirming a pre-larval peak of expression for the gene. There were some conflicting findings in the data surrounding the timing of expression for *tTAV* in the OX4301 construct. The presence of mRNA from 48H post-fertilisation conforms to the results from the phenotypic expression crosses with OX3867F if we assume a slight phenotypic delay due to tTAV and *tetO* interactions rather than the promoter. However the phenotypic expression studies confirm that dsRed2 fluorescence is undetectable after larval development, while mRNA for tTAV is still detectable by semi-quantitative RT-PCR. The semi-quantitative RT-PCR failed to discern any differences in gel banding strengths between any of the life history stages of the medfly or between different cycles of the PCR, as such it has little to tell us about the strength of *tTAV* expression between different life stages. A more detailed picture of the pattern of expression would be gained by carrying out a fully quantitative analysis through real-time PCR.

I successfully transformed construct OX4423 into the germ cells of several pre-blastoderm embryos. Given the random insertion of *piggyBac* mediated transformation it is possible for the same construct to display a widely different range of expression strengths and inheritance patterns. Screening of the lines through analysis of fluorescence (strength of temporal and spatial patterns), and checking for autosomal inheritance patterns confirmed multiple suitable lines for further testing (4423 C, K, S & X). While at this stage all lines were being tested on the assumption of appropriate insertional sites, any promising constructs would have this confirmed at a later stage through direct sequencing.

Heterozygote crosses between lines OX4423 C & K with OX4301 B, H & E found no induction of lethality off-tet for those individuals screened for both transformation markers, at any life history stage. This result is extremely surprising given the nature of *Reaper* as a strong pro-apoptotic protein; in addition previous work to generate *ReaperKR* produced even more drastic induction of apoptosis in tested *Drosophila* SL2 cell lines, the mutations to prevent ubiquitination means that even relatively low levels of expression should have serious deleterious effects on organisms. Furthermore *ReaperKR* was used in other RIDL constructs such as OX3069, to successfully transform *H. zea* with the expected lethal results (data not shown). Given the greater phylogenetic distance between *H. zea* and *Drosophila* than between *Drosophila* and medfly and the strong functional conservation of *Reaper* (Bryant *et al.*, 2009), it is unlikely that the failure was due to the non-functioning of the *Reaper* gene itself. There is some evidence that heat shock proteins maintain low inducibility in early embryos across a wide phylogenetic range (Morange *et al.*, 1984, Heikkila *et al.*, 1985, Giudice, 1989), and it has been confirmed that normal hsp83 expression is tightly regulated during the early embryonic stages of the medfly (Komitopoulou *et al.*, 2004, Kalosaka *et al.*, 2009). This low inducibility at the embryonic stages, where *DmCG31626* expression should be at its highest, could lead to a dampening of the expression levels of *Reaper*. However crossing the line to a strong constitutively active promoter line OX4196J also failed to induce lethality, indicating a general problem with OX4423.

After crossing the OX4423 lines to two separate promoter lines, both of which have been proven to work with other *tetO* constructs, I decided that the assemblage of the construct was at fault. Insertional effects should have produced

differential phenotypes between insertion lines, with at least one line producing a reduction in survival for carriers.

OX4536 was proposed as an alternative method to obtain *ReaperKR* functionality in a line of medfly. This construct was made from, and had identical sequences to the pre-mentioned working *ReaperKR* construct OX3069. I switched the promoters driving the transformation marker, as *OpIE2* is non-functional in medfly. OX4423 used *hsp83* the *D.melanogaster* heat-inducible promoter, a popular choice for driving expression of other genes across many insect species. Given the high toxicity of *ReaperKR*, it is important to choose a minimal promoter very carefully. It must be able to warrant extremely low expression levels when the system is 'off', and support high levels of gene expression when the system is 'on'. However the inability of the system to initiate lethality when the system was 'on' indicates that a different promoter may be needed to upregulate expression. OX4536 had minimal promoter *hsp70* in place of *hsp83*, which has slightly higher expression levels, in order to allow me to evaluate whether the functioning of the heat shock promoter used was key to gaining expression of *ReaperKR* in medfly. While OX4536 was successfully transformed into several medfly lines showing favourable expression and inheritance of the transformation marker, crosses between OX4536 and OX4301 lines again failed to induce lethality.

Further development on creating a successful *ReaperKR* construct is necessary before the feasibility of generating an early-acting embryonic lethal RIDL system can be determined. The next logical step for refinement of a *tetO*, *ReaperK*R construct would be to increase the number of *tetO* elements within the construct, in doing so this would in theory maintain the very low levels of 'leaky' expression when the system is 'off' required in order to maintain a line carrying the *Reaper KR* construct, while driving the minimal promoter to upregulate expression of *ReaperKR* when the system is switched 'on'. Early acting lethality of the RIDL construct remains a highly desirable characteristic, and research will inevitably continue to be applied towards perfecting this aim. If successful it has the potential to dramatically reduce costs and improve the efficiency of using RIDL and fsRIDL as pest population control methods for the medfly.

4.6 References

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Chapter 5: Genetic elimination of the Mediterranean Fruit Fly

5.1 Abstract

The Mediterranean Fruit Fly (medfly, *Ceratitis capitata* Wiedemann) is a major, worldwide insect pest that attacks over 300 types of fruits, vegetables and nuts. Current control measures include the use of insecticides, chemical lures and the sterile insect technique (SIT). SIT aims to reduce the reproductive potential of a target population through the mass release of sterilised male insects to mate with wild females. Problems with SIT centre on the poor field performance of released flies, brought about by genetic and environmental effects of mass-rearing and the irradiation process used to achieve sterilization. The development of conditional female-lethal RIDL (Release of Insects carrying a Dominant Lethal) strains for medfly allows potential problems of standard SIT, such as those arising from irradiation, to be mitigated. Here I present life history characterisations and mating competitiveness tests for two medfly RIDL strains, OX3864A and OX3647Q, which exhibit 100% genetic sexing, fluorescent marking and 'genetic sterility' through female-specific lethality. These results show (1) full functionality of RIDL constructs; (2) near equivalency of RIDL and wild type strains for life history characteristics, including longevity and fecundity; (3) a high level of sexual competitiveness of RIDL against both wild type and wild-derived males. I also present the first proof-of-principle experiment on the use of such strains to eliminate medfly populations. Weekly releases of OX3864A males into stable populations of wild type medfly caused a successive decline in medfly numbers, leading to eradication.

5.2 Introduction

The medfly is a major world pest of agricultural importance. Originally native to Africa, it has invaded many other parts of the world including Southern Europe, Australia and the New World tropics, resulting in the widest host range of any pest fruit fly. It is considered the most agriculturally important pest in the world (USDA, 2006). Records have been made of medfly infestation in over 300 types of cultivated and wild fruits, vegetables and nuts, including apple, apricot, bell pepper, coffee, dates, fig, grape, lemon, orange, peach, pear, tomato and walnut.

Methods by which the medfly has been tackled include the use of baited traps (Avery *et al.*, 1994, Ben Jemaa *et al.*, 2010), insecticides (Alemany *et al.*, 2008), biological control (Headrick & Goeden, 1996), and the sterile insect technique (SIT) (Jackson & Lee, 1985, Enkerlin & Mumford, 2001, USDA, 2006). Of these, SIT has garnered the most attention as an environmentally friendly and species-specific method of pest control and has been successfully implemented against many insect species including the medfly (Hendrichs *et al.*, 1983). SIT involves the mass release of sterilised insects into the wild to disrupt normal mating patterns and induce a high frequency of sterile matings (Knipling, 1955). One attractive outcome of SIT is that it can, when effective, lead to the establishment of entirely medfly-free zones. This is advantageous because growers within medfly infested areas otherwise have to abide by costly quarantine compliance regulations in order to export produce beyond the infested zone (Carey, 1992, Krafsur, 1998). However, SIT must of course first prove cost-effective in comparison to other control methods (Hendrichs *et al.*, 1995, Dyck *et al.*, 2005, Wee *et al.*, 2006, Parker & Mehta, 2007).

The success of SIT depends critically upon the ability of released sterile males to find a lek breeding site, to perform courtship and attract wild females, to successfully mate and inseminate females and finally to elicit effective female refractoriness to remating (Eberhard, 2000, Hendrichs *et al.*, 2002, Robinson *et al.*, 2002). The traditional mechanism by which sterility is achieved in SIT released males is through exposure to ionizing radiation; however this has well documented negative impacts on longevity and mating competitiveness (Lux *et al.*, 2002, Robinson *et al.*, 2002, Barry *et al.*, 2003, Kraaijeveld & Chapman, 2004).

Release of Insects carrying a Dominant Lethal (RIDL) is an alternative to SIT (Thomas *et al.*, 2000, Alphey, 2002, Alphey & Andreasen, 2002, Alphey *et al.*, 2008, Morrison *et al.*, 2009) that removes the need for ionizing radiation. One version of RIDL, on which I focus here, involves the mass release of insects carrying a female-specific lethal transgene (fsRIDL) (Fu *et al*., 2007, Fu *et al*., 2010, Valdez de *et al*., 2011). As with SIT, wild females that mate with engineered RIDL males produce significantly fewer viable female offspring than those mating with wild males, thereby decreasing the reproductive potential of the wild population. If sufficient numbers of females mate with fsRIDL males then the population will collapse. Prototype fsRIDL strains of medfly have already been developed (Fu *et al.*, 2007, Morrison *et al.*, 2009) in which 100% female-specific lethality at the pre-pupal stage is observed. This lethality can be switched off to enable females to develop normally during mass-rearing by providing a chemical repressor (tetracycline) in the diet; if reared in the absence of the repressor only males survive.

OX3864A and OX3647Q are the first fully developed fsRIDL strains for this important agricultural pest. I describe the testing of these two new fsRIDL strains of medfly, culminating in successful elimination of wild type (wt) medfly populations in large cage trials using strain OX3864A. OX3864A provides repressible, fully penetrant female-specific lethality, together with a dominant fluorescent marker. Based on a series of tests of life history, survival, fecundity and fertility, this strain of fsRIDL medfly displays no significant fitness depression from carrying transgenes, and exhibits a high level of mating competitiveness. In addition I demonstrate a proof-of-principle, that periodic release of OX3864A males can eradicate a wild type medfly population.

5.3 Methods

5.3.1 Medfly stocks

The medfly strain TOLIMAN was used as the wild type (wt) background for the construction of both transgenic lines and for all subsequent testing of life history traits prior to the experiments in Crete. TOLIMAN is a wild type strain originating from Guatemala and reared in the laboratory since 1990. It has been maintained at Oxitec (Oxford, UK) since 2004.

tsl is a genetic sexing strain derived from the TOLIMAN wild type in which females are selectively killed by exposure to high temperatures, Genetic sexing is achieved by exposure of eggs to 34°C for 24 hours, though female survival may still be compromised until the temperature is below 23°C, a *wp* marker is closely associated with *tsl* and can be used to easily detect female viability (Franz *et al*., 1994, Caceres, 2002).

OX3864A and OX3647Q utilise the tetracycline repressible transactivator (*tTA*) as both the transactivator and lethal effector via a positive feedback loop (Gong *et al*., 2005). Female specific lethality was achieved by introduction of an alternatively spliced, sex-specific intron from the medfly *transformer* gene (*Cctra*) in the *tTA* open reading frame (Fu *et al*., 2007). Only females produce functional tTA, which initiates a lethal tTA positive feedback loop in the absence of tetracycline or alternative analogues, while males produce truncated and non-functional tTA (Fig 5.1, Chapter 4 and Fu *et al*., (2007)).

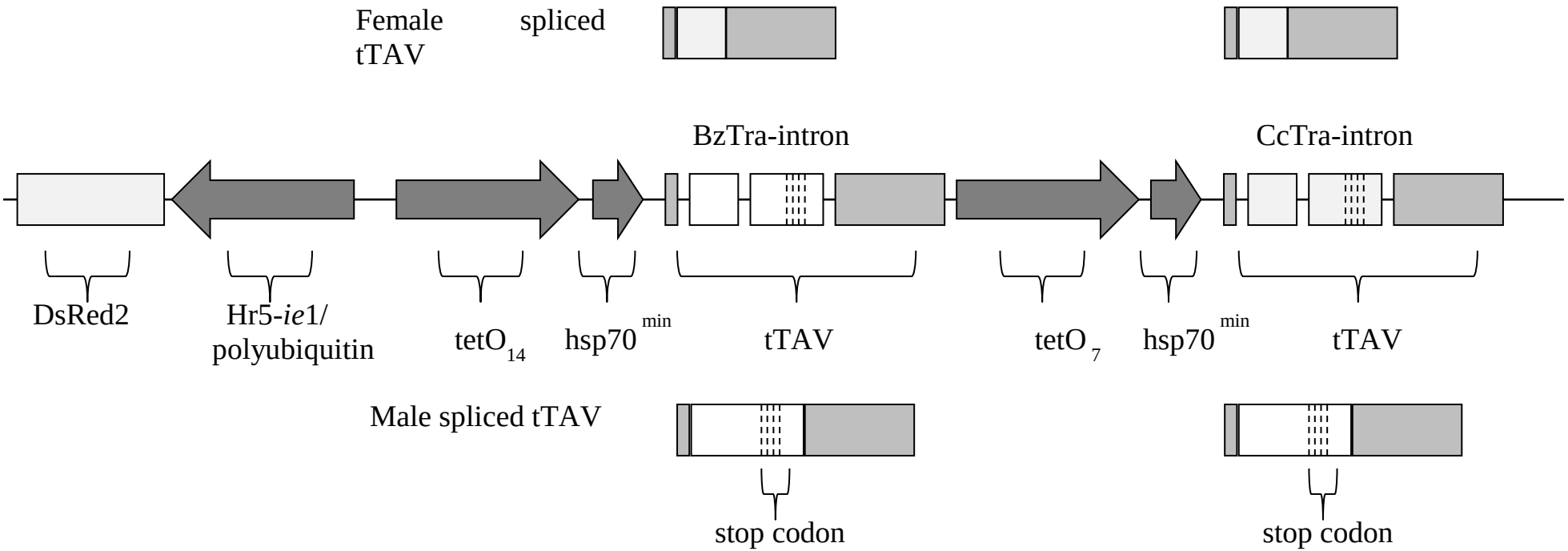
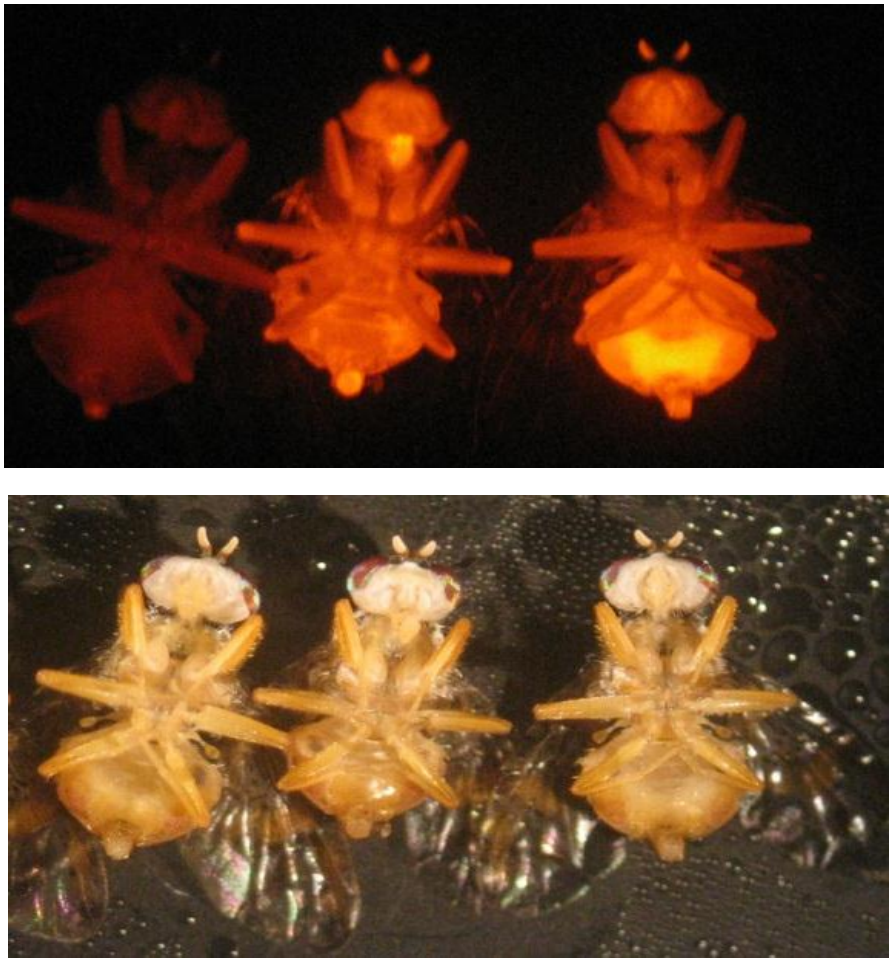


Fig 5.1 Key elements of transgenic constructs: OX3864 and OX3647 use a *tetO-tTA* positive feedback system to give tetracycline-repressible lethality (Thomas *et al*., 2000), here rendered female-specific because of the addition of the sex-specific alternative splicing *BzTra* and *CcTra* introns (Fu *et al*., 2007). Only females produce splice variants encoding functional tTAV protein. OX3864 and OX3647 differ in the promoter used for *DsRed2*. OX3864 utilised HR5 IE1, while OX3647 used *ubi-p63E* (the polyubiquitin promoter region).

Both strains carry a fluorescent marker to allow discrimination of RIDL and wild type medfly (Fig 5.2). The difference between these constructs relates to the promoter used for the fluorescent marker, which was *Hr5-IE1* for OX3864 and *ubi-p63E* for OX3647 (Gong *et al*., 2005).



WT **OX3647Q** **OX3864A**

Fig 5.2 (**Top**) Red fluorescence produced by *DsRed2* renders the RIDL males easily and reliably distinguishable from wild type, (**bottom**) same adults shown under a bright-field illumination.

5.3.2 Insect Rearing at Oxitec

Adult fly colonies were kept at 21°C (*tsl* female survival may be compromised at 25°C), 50% R.H. and a 13hr light and 11hr dark cycle. Adults were fed on a diet of 100g yeast hydrolysate and 400g powdered sugar. Water was supplied through damp cotton wicks and supplemented where necessary with tetracycline hydrochloride (Sigma-Aldrich) (100µg/ml). Newly hatched larvae were transferred to the surface of larval medium, consisting of a standard yeast-wheat germ-glucose agar *D. melanogaster* diet (ASG). For 'on-tet' rearing this was supplemented with tetracycline hydrochloride to a final concentration of 100µg/ml. L1 larvae were placed onto larval medium at a density of ~ l larva per 0.5g of diet. Pupae were kept between 18-25°C (depending on requirements) until eclosion.

5.3.3 Insect Rearing at the University of Crete

This was conducted as described above, except that experiments were conducted at 25°C, and the larval diet was substituted for an altered medium consisting of 550ml distilled water, 162g sugar, 81g brewer's yeast, 6g citric acid, 5g sodium benzoate and 242g wheat bran. For 'on-tet' rearing this diet was supplemented with tetracycline hydrochloride to a final concentration of 100µg/ml.

5.3.4 Tests for longevity

Longevity tests were performed at 21°C and R.H. = 50% for each line within 6 replicate plastic cages (9cm x 9cm x 9cm), each containing 30 males and 30 females of the same genotype. Three of each of the 6 cages were randomly assigned to a 'stress' test of no food and no water, while the remaining cages had an *ad libitum* supply of both. Each cage was assessed daily for dead flies, which were then sexed and removed. These tests continued until each cage went extinct.

5.3.5 Lifetime female fertility and egg hatching rates

From the non-stressed cages described above, eggs were collected over 24 hour periods, and counted under a stereo-microscope. The egg samples were then incubated on wet Whatman filter paper (Fisher Scientific) and sealed into a Petri dish with parafilm (200 eggs per Petri dish, 600 eggs per line in total). 72 hours after egg collection, Petri dishes were unsealed and examined under a dissection microscope in order to count the number of empty vs. unhatched egg casings.

5.3.6 Adult eclosion rates

300 pupae from each line were kept singly and monitored for eclosion. Adults were checked for sex and visible deformity before recording. Uneclosed or partially eclosed pupae casings were counted and then discarded.

5.3.7 Mating competition and remating tests

Mating competitiveness tests were carried out according to FAO/IAEA/USDA guidelines (2003). Adult OX3864A, OX3647Q, TOLIMAN wild type were obtained from larvae reared off-tet at low density (1 larva/0.5g larval medium).

Field cages (1.25m tall with a base of $0.5m^2$) were constructed inside a greenhouse at the Zoology Department, Oxford University (Oxford, UK), with small orange trees (~1m in height) placed inside, experiments took place during August (sunrise 06.00) utilising natural light and a stable temperature and humidity (25°C, 50% R.H.). These tests used wild type males and females, and OX3864A or OX3647Q males reared on a tetracycline free (‘non-tet’) larval diet (Gong *et al*., 2005). 30 males from either OX3864A or OX3647Q were placed together with 30 wild type males at 06:30, and 30 females introduced 30 minutes later. Mating couples were removed and the copulation initiation time was recorded. The mating experiments ended 9 hours after initiation (16:00) or whenever all females had mated, whichever was sooner. Mated males were examined for the *DsRed2* fluorescent marker under a

fluorescent microscope. Tests were performed with 10 replicates for each line and 162 and 174 couples assessed for OX3864A and OX3647Q respectively. In all replicates a minimum of 20% of the females mated, which demonstrated that the cages used provided a suitable mating environment (FAO/IAEA/USDA, 2003). The relative sterility index (RSI) was used as a measure of male sexual competitiveness (McInnis *et al*., 1996), which also measures the potential reproductive isolation of the fsRIDL lines from the wild type.

Mated females from the previous experiments were then placed into one of two types of new cage per treatment. New cages contained either females that had initially mated an OX3864A male (N = 40) together with wild type and OX3864A males (in a 1:1:1 ratio), or females that had initially mated a wild type male (N = 40) together with wild type and OX3864A males (again in a 1:1:1 ratio). This process was repeated with the OX3647Q experiment. The experiment was run for 3 days, with cages observed for 9 hours daily for matings. Each couple was removed during mating (OX3864A: 15 mated pairs, OX3647Q: 24 mated pairs) and the male assessed for genotype.

For mating competitiveness tests with wild-derived flies, pupae were recovered from infested oranges gathered from insecticide-free orange orchards in Heraklion province, Crete. Virgin wild-derived adults were separated by sex immediately after eclosion and reared at 25ºC (50% R.H.) for 10-13 days until sexual maturity. All flies were allowed to adjust to natural light and temperature conditions of the glasshouse for a minimum of 24 hours prior to the start of the experiment, and each experiment began one hour after sunrise and lasted for a minimum of 9 hours. Mating tests were performed in green-house facilities at the University of Crete. OX3864A mating competition tests were performed in 7 replicates with 89 couples assessed.

5.3.8 Caged suppression of stable wild type populations

The protocol for the caged suppression experiment was based on methods introduced by de Valdez *et al*. (2011). Stable populations of wild type medfly were established in four large greenhouse-based field cages (each $8m^3$ and containing a 1.5m tall

lemon tree), at the University of Crete, Heraklion, Crete, utilising natural light and a stable temperature and humidity (around 25°C, 50% R.H., for temperature variations see Fig 5.6). Cages contained three food and water sources and two oviposition pots filled with deionised water, each with two 40cm^2 egg laying surfaces.

Wild type populations were established over an 8 week period by introducing a fixed number of pupae to each cage per week (200 in week 1, 300 in week 2, 180 in week 3 and 230 thereafter weeks 4-8). Pupal additions for the first 4 weeks originated from a wild type stock colony; thereafter all pupal additions were from eggs caught in the oviposition pots. Egg numbers were determined daily. Adult numbers in each cage were calculated as the difference between the numbers of uneclosed pupae and dead individuals and the total pupae added.

At week 7, cages were randomly divided into treatment or control. From week 8 onwards, treatment cages received weekly additions of 1700 OX3864A pupae reared off-tet (resulting in ~1,500 adult males per week) (an approximate ratio of 5 OX3864A males to 1 wild type male in week 8) and a weekly recruitment ratio of roughly 15:1 (OX3864A to wild type males). Once OX3864A introductions began (week 8), the pupal return to a treatment cage was made proportional to its respective rate of pupae production, with the control cages providing a stable weekly pupal return coefficient. For example, in week 16 the mean number of pupae recovered from the control cages was 300. Because returns to the control cages were set at a constant 230 per week, the number of pupae to return to all cages is set by a coefficient of (230/300 = 0.76). While in the same week one of the treatment cages produced a pupal output of 126. The pupal return for this cage (using the coefficient), was therefore 96 (126 × 0.76). This methodology allowed for a dynamic pupal return that was dependent on egg production and pupal survival. Female specific RIDL lethality is achieved at the pre-pupal stage which is essential to this calculation. Female lethality after pupation would lead to a disproportionate return of wild type pupae to the experimental cages (i.e. of 100 pupae, 75 would be expected to eclose, with a 2:1 wt:RIDL ratio).

5.3.9 Data Analysis

Longevity was compared using Cox's model of Proportional Hazards. Comparisons of fecundity and fertility were made using repeated measures ANOVA analysis on total daily egg production per cage and the estimated mean daily egg production per female and egg-first instar larva hatch rates (a Greenhouse-Geiger correction was used for egg production per female to control for non-sphericity in the repeated measures ANOVA). Comparisons of the eclosion rates and male proportions among lines were made using ANOVA with a Tukey HSD *Posthoc* test. For repeated measures analyses, pairwise comparisons were made with a Bonferroni correction. R_0 (net reproductive rate value per female), G (spanning the peak of fertility from one generation to the next) and *r* (an index of fitness per female) for comparison of the fertility of each line were calculated by adaptating the Euler equation (Gotelli, 2008). Pairwise comparisons of each RSI (Relative Sterility Index) to wild-derived or wild type flies were performed using t-tests. Proportions were first arcsin transformed for analysis. Comparisons of remating rates in wild type females were performed using Fisher's exact tests. The Cox's Proportional Hazards model was run utilising the open source statistical package R (R Development Core Team, 2010), all other tests were performed with the statistical package PASW v18.0.0 (PASW Statistics v.18, 2009). Means ± standard errors are given throughout.

5.4 Results

5.4.1 Life History Analysis

These tests measured the performance of two fsRIDL lines of medfly (OX3864A and OX3647Q) and a temperature sensitive female lethal *tsl* strain (currently used in SIT to produce male-only releases). These strains were compared to the TOLIMAN wild type (wt). This is the genetic background from which both fsRIDL lines (and the *tsl* line) were derived and therefore provides an appropriate comparator against which to test for any side effects of transformation.

5.4.2 Tests for longevity

Flies held under the stress conditions had, as expected, significantly reduced life spans compared to those provided with full food and water, with all flies dead within

6 days (Log Rank Test $\chi^2_1 = 1307$, $P < 0.001$; Fig 5.3a). With full food and water, there were significant effects on survival of genotype ($\chi^2_3 = 15.6$, $P < 0.001$; Fig 5.3b. Sex had no significant effect on longevity ($\chi^2_1 = 0.17$, $P = 0.68$), and therefore the survival data for both sexes were combined to make the comparison between lines. Under stress conditions, OX3647Q showed higher survival in both sexes in comparison to the wild type (wt = 4.1 days ± 0.054; OX3647Q = 4.38 days ± 0.066; Cox's Proportional Hazards: $z = -2.1$, $P = 0.035$) but significantly lower survival under full food, non-stressed conditions (wt = 18.9 days ± 0.52, OX3647Q = 13.7 days ± 0.53; $z = 5.92$, $P < 0.01$). There was no significant difference in the average lifespan of OX3864A and *tsl* flies in comparison to the wild type for either treatment, (stressed treatment: OX3864A = 4.13 days ± 0.055; $z = 0.59$, $P = 0.55$, *tsl* = 4.13 days ± 0.064; $z = 0.53$, $P = 0.33$; full food, non-stressed treatment: OX3864A = 17.2 days ± 0.53; $z = 1.13$, $P = 0.26$, *tsl* = 17.0 days ± 0.52; $z = 0.7$, $P = 0.49$).

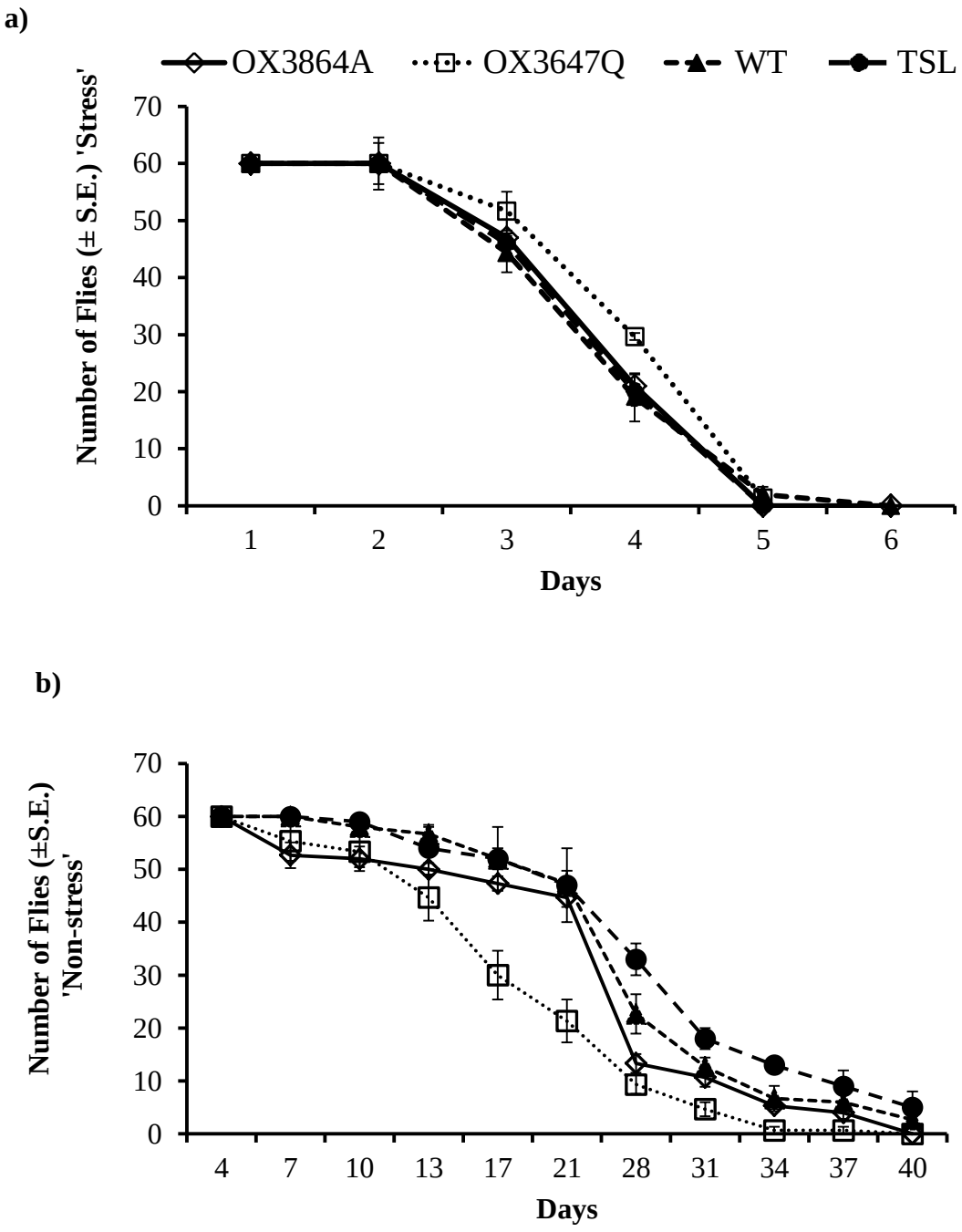


Fig 5.3 a) 'Stress test': male and female adult survival combined, without food or water (*N* =180) over 6 days post-eclosion. OX3647Q showed a significant increase in survival relative to wt. b) Male and female adult survival combined, with food and water 'non-stressed' (*N* = 180). OX3647Q showed significantly reduced survival relative to wt. No significant difference was observed in survival between wt, *tsl* and OX3864A across either treatment. All values are mean (± S.E.)

5.4.3 Lifetime female fecundity

To compare fecundity across the lines, five egg collections were made at regular intervals from the non-stressed control cages described above. Each collection comprised of eggs laid over a 24 hour period. From this the average egg production over the lifetime of each cage was calculated. Per-cage total egg production declined significantly over time (Repeated Measures ANOVA: $F_{1.6, 12.9} = 253.04$, $P < 0.001$) and there was a significant effect of genotype ($F_{4.8, 12.9} = 5.19$ $P = 0.008$). Pairwise comparisons with a Bonferroni correction revealed that significantly fewer eggs were produced over a cage's lifetime in both RIDL and *tsl* lines in comparison to the wild type (wt mean egg production = 4315 ± 48.51, OX3864A = 3470 ± 226, $P < 0.014$; OX3647Q = 2593 ± 147, $P < 0.001$; *tsl* = 2465 ± 93.29, $P < 0.001$). OX3647Q and *tsl* strains also produced significantly fewer eggs than OX3864A (OX3647Q vs. wt, $P < 0.001$; *tsl* vs. wt, $P = 0.005$, Fig 5.4a).

By recording daily mortality it was also possible to estimate egg production per female over time. Consistent with the above, this also showed that fecundity declined significantly with time (Repeated Measures ANOVA: $F_{1.9, 15.2} = 131.85$, $P < 0.001$). However, there was no significant effect of genotype on the extent of this decline ($F_{5.7, 15.2} = 2.19$, $P = 0.104$, Fig 5.4b). Supporting this, a one-way ANOVA on the number of eggs laid at peak fecundity (day 10), also revealed no significant differences in egg laying per female between any of the lines ($F_{3, 8} = 0.029$, $P = 0.97$).

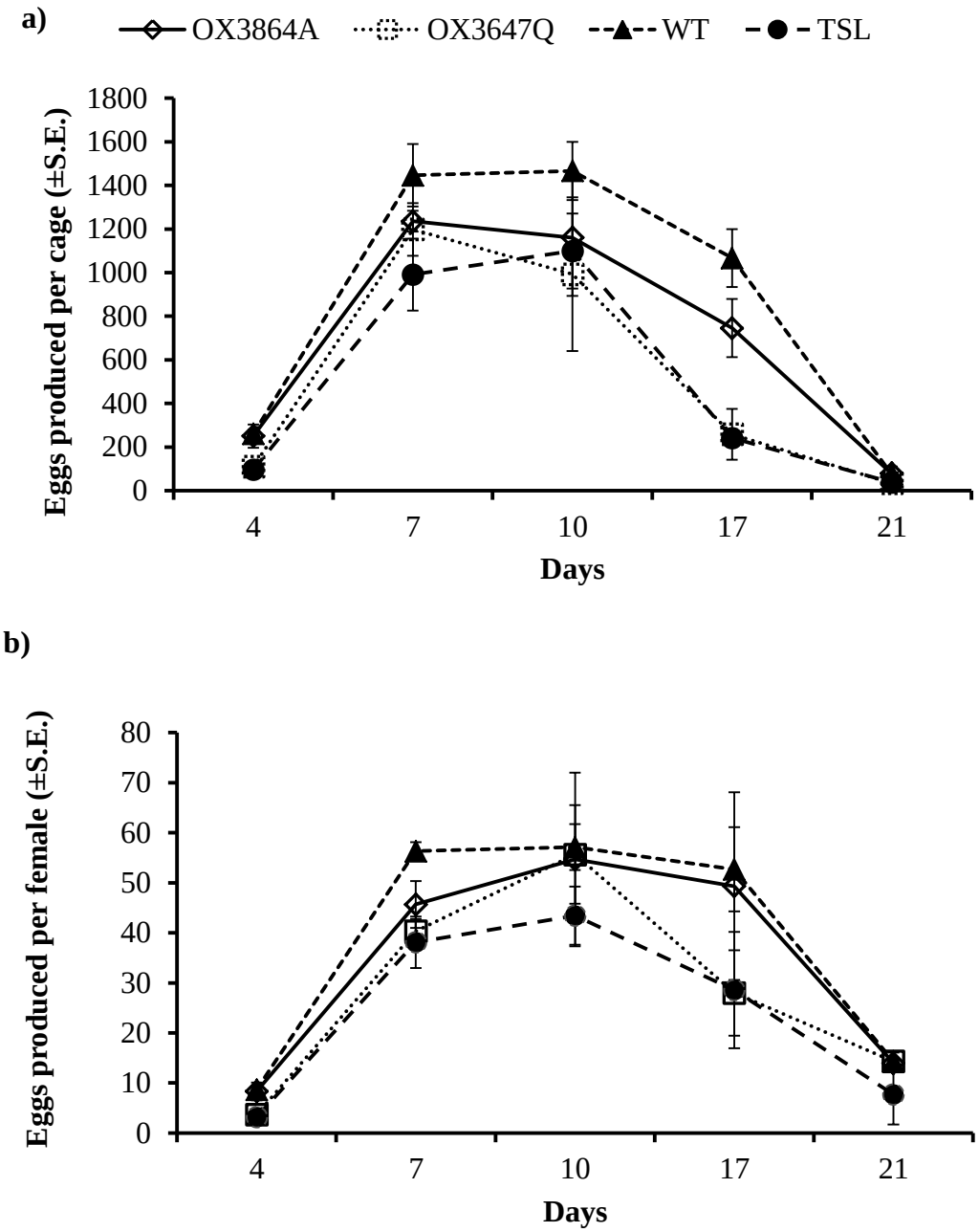


Fig 5.4 a) Female productivity from a cage of 30 females: average egg production from a cage of 30 females over 3 weeks. Wt lines produced significantly more eggs than the other three lines, and OX3864A produced significantly more eggs than *tsl* and OX3647Q. b) Individual average lifetime female fecundity: no significant difference was found between the strains in the average number of eggs laid per female, differences in egg production between cages of different genotypes is due to differential female survival not fecundity. All values are mean (± S.E.)

5.4.4 Egg hatching rates

Egg hatching rates were estimated for all lines from a known number of eggs collected over a 24 hour period on 5 occasions spread over the experiment (days 4, 7, 10, 14, 17 & 21 post-adult eclosion; total eggs sampled per line = 1800). L1 larvae and unhatched eggs were counted 72 hours after egg collections. There was a significant effect of maternal age on egg hatching rates (Repeated Measures ANOVA $F_{5, 40} = 207.3$, $P < 0.001$), as well as a significant effect of genotype ($F_{15, 40} = 4.52$, $P < 0.001$, Fig 5.5). Pairwise comparisons with a Bonferroni correction showed that OX3647Q and *tsl* strains had mean egg hatching rates that were significantly lower than the wild type (wt = 89.56 ± 0.84; OX3647Q = 79.11 ± 0.84, $P < 0.001$; *tsl* = 78.33 ± 0.84, $P < 0.001$; OX3864A = 87.11 ± 0.84, $P = 0.247$).

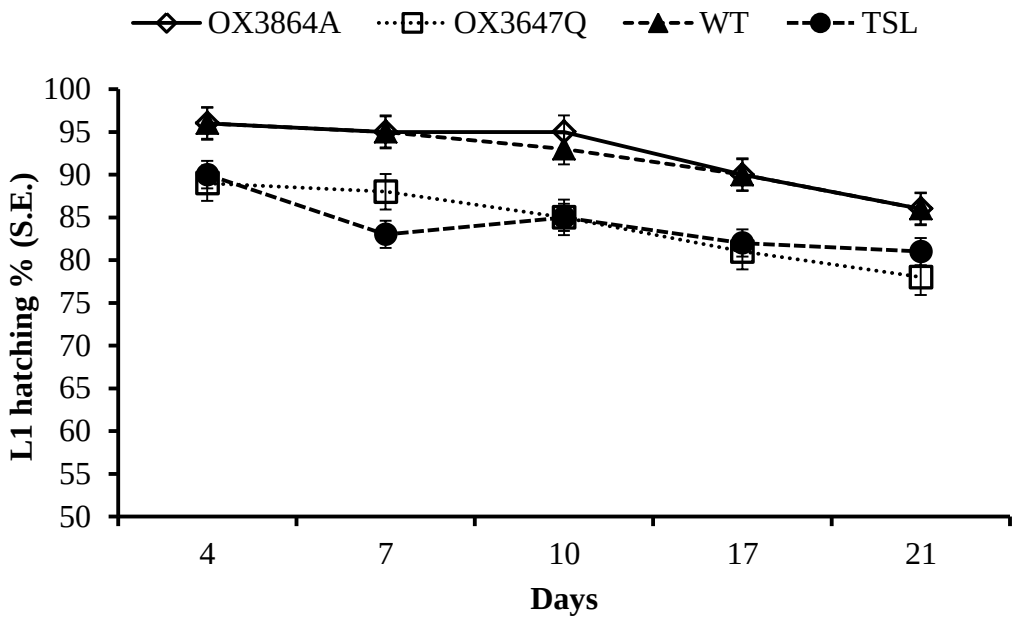


Fig 5.5 Hatching rates of first instar larvae (L1) from eggs. *tsl* and OX3647Q had significantly reduced egg hatch rates relative to wt, OX3864A did not differ significantly from wt. All values are mean (± S.E.)

5.4.5 Adult eclosion rates

Both the number of adults eclosing and the sex ratio of emerging adults were examined for 300 pupae per line (3 replicates of 100 each). There was a significant difference in eclosion rates between lines (ANOVA: $F_{3, 10} = 9.89$, $P < 0.001$). A Tukey HSD *post hoc* test revealed that this was mostly attributable to a significantly lower adult eclosion rates in OX3647Q in comparison to the wild type (wt = 86.1% ± 0.69; OX3647Q = 75.7% ± 2.43, $P < 0.01$; OX3864A = 84.7% ± 0.91, $P = 0.9$; *tsl* = 81.2% ± 1.04, $P = 0.25$). There was a significant effect of genotype on the adult sex ratio ($F_{3, 10} = 5.06$, $P = 0.036$), which Tukey HSD *post hoc* tests showed was attributable to a difference in the sex ratio of males to females in OX3647Q but not in the other lines (wt = 47% ± 1.8; OX3647Q = 54% ± 1, $P = 0.035$; OX3864A = 55% ± 2.3, $P = 0.055$; *tsl* = 50% ± 1.5, $P = 0.83$).

5.4.6 Fitness indices

From the individual life history components the net reproductive rate value per female (R_0) and average generation times (G) (spanning the peak of female fertility from one generation to the next) were calculated for each line. From these estimates, an index of fitness (r) per female was then derived. The r value for the wild type was 0.195, which equates to each female contributing on average 0.195 females per day to the next generation. The other lines had lower indices of fitness (OX3864A: $r = 0.187$, OX3647Q: $r = 0.176$, *tsl*: $r = 0.165$, Table 5.1).

Table 5.1 Indices of Fitness for strains OX3864A, OX3647Q, wild type and *tsl*. From the rate of deaths recorded for each line a survivorship schedule was calculated. This combined with female fertility gives an average R_0 value for each line, which is the number of females reaching reproductive age which can be estimated to be produced from a single progenitor female. The generation time (G) calculates the average peak of fertility for each line and the time in days between these peaks in two generations. *r* is the index of fitness, calculated from the Euler equation (Gotelli, 2008) and the life history data and represents the intrinsic rate of population growth in females produced per day from a single progenitor female.

	WT	OX3864A	OX347Q	TSL
Net Reproductive Rate (R_0) of Females	267.6	183.7	113.1	133.1
Generation time in days (G)	32	32.1	35.6	36
Index of fitness (*r*)	0.195	0.187	0.176	0.165

5.4.7 Mating competitiveness of OX3864A and OX3647 males with wild type TOLIMAN flies

The ability of OX3864A and OX3647 males to compete against wild type males for matings with wild type females was tested with a relative sterility index (RSI) as a measure of male sexual competitiveness (McInnis *et al*., 1996), which also measures the potential reproductive isolation of the fsRIDL lines from the wild type. RSI ranges between 0 and 1. A RSI of 1 would represent 100% of matings by transgenic males, 0 would indicate 100% with the wild type and 0.5 equal numbers of matings with each (FAO/IAEA/USDA, 2003). The results showed that neither transgenic strain showed a significant reduction in competitiveness relative to wild type males (t-test: OX3864A: RSI 0.46 ± 0.08, t_{18} = -2.09, N = 162, P = 0.05; OX3647Q: RSI 0.47 ± 0.09, t_{18} = -1.72, N = 174, P = 0.1).

There were no significant differences in female re-mating frequency between females initially mated with either wild type or fsRIDL males (Fisher's Exact: OX3864A: χ^2_1 = 0.82, N = 40, P = 0.775; OX3647Q: χ^2_1 = 0, N = 40, P = 1) and for those females that did re-mate, the genotype of the second male had no effect on

remating frequency (OX3864A: $\chi^2_1 = 0.58$, $N = 15$, $P = 0.4$; OX3647Q: $\chi^2_1 = 0.17$, $N = 24$, $P = 0.5$).

5.4.8 Mating competitiveness of OX3864A males with wild-derived medfly

Seven replicates were performed in total to test the mating competitiveness of OX3864A males with wild-derived medflies. A total of 89 couples were analysed with the mean RSI value of OX3864A flies being 0.45 ±0.13, (t-test: $t_{12} = -0.9$, $N = 89$, $P = 0.38$), which gave no evidence for a significant difference in mating competitiveness between OX3864A and wild-derived males.

5.4.9 Suppression of stable caged populations of wild type medfly using OX3864A

Dramatic decreases in weekly egg production were observed by 7 weeks post-RIDL release (PR) in treatment cages compared with a continued stable rate of egg production in control cages (Fig 5.6). This was due to the proportion of returned progeny carrying the OX3864A transgene increasing in treatment cages, resulting in a rapid decline in the female population (Fig 5.6). Transgene frequency in the treatment cage populations was monitored by screening the returning pupae (chosen from all the pupae produced at random) for the presence of the dominantly expressed DsRed2 fluorescent marker. The frequency of the transgene in the returning progeny of the treatment cages was at 100% by week 8 PR (Fig 5.6), with both cages considered extinct by week 14 PR (extinction defined as zero egg production for two consecutive weeks).

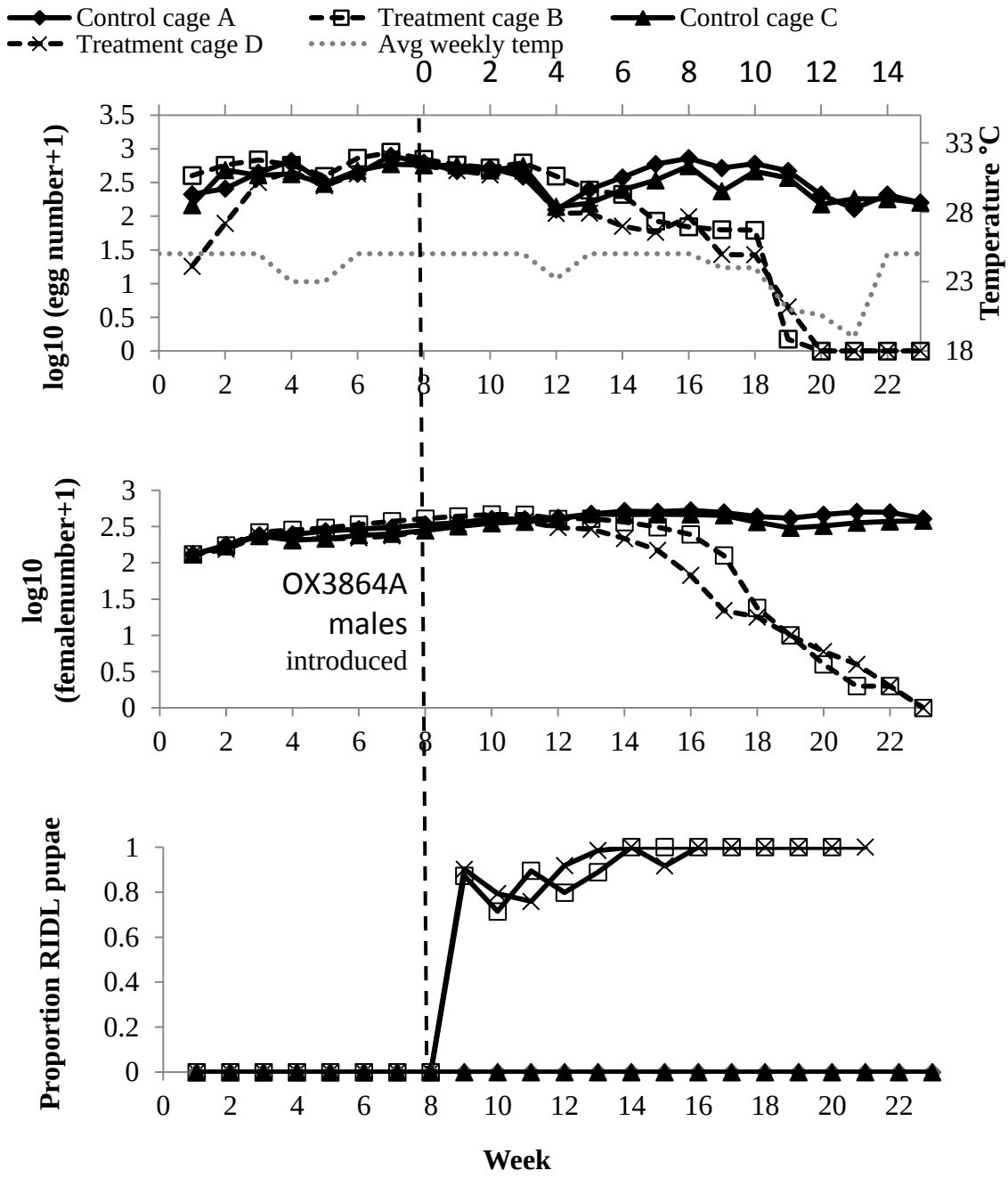


Fig 5.6 Changing medfly population dynamics through introduction of OX3864A males

a) Average daily egg production for each week in treatment and control cages. Weeks 0-8 constituted the population stabilisation period (230 pupae/week). Introductions of 1700 OX3864A pupae into each treatment cage commenced from week 8 onwards, in addition pupal return to each treatment cage was made proportional to weekly egg production with the control cages providing a weekly egg-pupae proportion coefficient. Six weeks after OX3864A male introductions there was a clear reduction in egg production in the treatment cages, continuing until extinction of the wild type population in both treatment cages by week 22. The dotted line denotes the average weekly daytime temperature (Celsius); taken from daily midday temperature readings. b) Numbers of females in treatment and control cages. c) Proportion of progeny returned to each of the treatment cages displaying the *DsRed2* fluorescent phenotype, this reached 100% in both treatment cages by week 17

5.5 Discussion

The two homozygous lines of fsRIDL medfly tested, OX3864A and OX3647Q, had high fitness compared to a classic genetic sexing strain *tsl* and equal mating competitiveness with the wild type TOLIMAN strain from which they were derived. In addition OX3864A displayed equal mating competitiveness with wild-derived males and proved capable of population suppression in proof of principle cage trials. By comparison at each stage with the wild type medfly from which both lines originated and with the genetic sexing strain *tsl*, it was possible to investigate any negative impacts on performance that arose during strain development.

Each line (OX3864A, OX3647Q, *tsl* and wt) was first tested for longevity under both 'stressed' (no food and water) and 'non-stressed' (plentiful food and water) conditions, in line with published guidelines (FAO/IAEA/USDA, 2003). No significant impact of sex was found on longevity. The lack of an effect on female survival in the transgenic strains is especially important as it shows that there is no evidence for any adverse 'leaky expression' of the female specific lethal insert. Neither *tsl* nor OX3864A differed significantly in survival from wild type across stressed or non-stressed full food treatments. OX3647Q, however, differed significantly in mean survival from wild type under both treatments, dying sooner than wild type in the presence of food and water (> 5 days earlier) and slightly later under stressed conditions (< 0.5 days).

Tests of fecundity revealed that all strains displayed reduced egg production over the course of their lifetime when compared to the wild type. However, the magnitude of this effect was greater for the OX3647Q and *tsl* strains, which were significantly less fecund than OX3864A. Analysis of age-specific egg production showed no significant differences between any of the strains. This indicates that differences in egg production over the lifetime of the cages were due to differential adult survival not fecundity. When comparing egg hatching rates between lines, OX3647Q and *tsl* had a significantly reduced egg to first larval instar egg hatching ratio compared to wild type. This reduction was not seen in the OX3864A strain.

Pupal to adult eclosion rates did not differ significantly between wild type, OX3864A or *tsl*, however there was a significant reduction in overall eclosion along

with a decreased proportion of female eclosions in OX3647Q, which could be due to a slight leaky expression of the fsRIDL transgene in the presence of dietary tetracycline.

The life history analysis shows that OX3864A displayed elevated fertility and survival in comparison to the *tsl* strain, and exhibited life history characteristics that were comparable with the TOLIMAN wild type. Hence, almost no negative impacts of transgenesis were found for this strain. OX3647Q was less robust than wild type, but was competitive with the classical *tsl* genetic sexing strain. This is most clearly summarized in Table 5.1, which combined the separate data from survival, fecundity and fertility to give a basic index of fitness for each line. OX3864A had a similar overall fitness value to the wild type line, which exhibited the highest *r* fitness value overall. Both transgenic lines demonstrated superior fitness overall in comparison to *tsl*. Note that these experiments also likely underestimate fitness differences as the experiments were conducted at a temperature permissive for *tsl*. Rearing strains at higher temperatures is predicted to further increase *r* values (through a reduction of the generation time, G) relative to *tsl*.

Mating competitiveness of both transgenic strains against wild type was high, with an RSI of 0.46 (± 0.08) for OX3864A and 0.47 (± 0.09) for OX3647Q. Hence neither strain was found to be significantly different from wild type in tests on over 200 mating couples. No significant difference in mating competitiveness between the lines was detected and there was therefore no evidence for negative impacts of transgenesis as measured by this crucial parameter.

Mating to OX3864A and OX3647Q males did not significantly affect the remating behaviour of wild type females relative to mating with a wild type male. This suggests that there was no reduction in the ability of transgenic males to induce female refractoriness to remating. In contrast, previous work has shown that female remating is significantly higher following initial matings to irradiated (as in SIT) as compared to non-irradiated males (Kraaijeveld & Chapman, 2004). Overcoming this deleterious effect by use of an fsRIDL strain could represent a significant improvement.

Some negative effects appear to arise from the presence of the transgene, potentially from insertion effects, but these could also be due to inbreeding during

the construction of the strain (Catteruccia *et al*., 2003, Marrelli *et al*., 2006). In principle, further work could distinguish between these causes, for example out-breeding and re-deriving the strain might reduce inbreeding but not insertion effects. However, overall both fsRIDL strains showed good competitiveness and line OX3864A was selected for the next phase of assessment based on its consistently higher fitness in the first phase of tests.

Competitiveness of the OX3864A males against wild-derived males for matings with wild-derived females was high, with OX3864A males obtaining 45% of the observed matings (RSI 0.45± 0.13). This was not significantly different from the number of observed matings of wild-derived males, so there was no evidence for a difference in mating competitiveness between OX3864A and wild-derived medfly. The minimum RSI value proposed for a viable SIT strain in tephritid flies is 0.2 (FAO/IAEA/USDA, 2003), hence this requirement has been far exceeded in the OX3864A fsRIDL strain tested here. This strain displays better mating competitiveness than traditional genetic sexing strains such as the *tsl* line Vienna-7 (RSI ca. 0.3) (Shelly & McInnis, 2003). This equates to RIDL males achieving almost twice as many matings with wild females compared to traditional SIT lines.

It was also shown that weekly releases of OX3864A into a stable population of wild type medfly can eradicate a target population. Numbers of females in caged populations of wild type declined rapidly following the introduction of fsRIDL males - as these males mated with wild type females they passed on a copy of the female-lethal transgene to their progeny. This resulted in a sharp increase in the ratio of OX3864A to wild type males, which continued as the population declined. This further increased the proportion of progeny carrying the transgene and thus the efficacy of each RIDL release, despite a constant number of homozygous transgenic releases each week. Both OX3864A treated cages showed a reduction in egg production 7 weeks post-release, and were extinct by week 14. This trial used the TOLIMAN wild type strain, rather than wild-derived flies (the logistics of establishing new artificially reared populations of wild-derived flies were prohibitive). However the results on the efficacy of OX3864A mating competitiveness shown above suggest that OX3864A would be similarly effective against wild flies.

In conclusion, fsRIDL appears to present an effective alternative to the irradiated *tsl* strains currently employed in SIT. Weekly release of OX3864A males caused rapid collapse to a stable caged population, providing further evidence for the potential of OX3864A to be utilised as a medfly population control method.

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Chapter 6: Larval diet alters associations with commensal gut bacteria and induces trans-generational effects on body size and male mating success in *Ceratitis capitata*

6.1 Abstract

Reproductive isolation is a key step in the process of speciation. Recent work by Sharon *et al* (2010) reported highly surprising significant pre-mating reproductive isolation between populations of *Drosophila melanogaster* reared for just one generation on either sugar or starch medium. Furthermore, this isolation was maintained over several generations, but was abolished by treatment with antibiotics, suggesting a key role for commensal gut bacteria. Here I tested for similar associations in the medfly. My aim was to investigate in more detail the underlying basis of any differences between lines maintained over generations on different larval food types. The results showed that the flies maintained as larvae on diets containing either a starch or sugar base harboured distinct populations of gut microbiota. However, there were no significant within line mating preferences, hence no evidence for reproductive isolation. Instead, I found that males reared on a sugar diet had a significantly slower development time, greater male body mass and a significant mating advantage in comparison to starch-reared flies. These phenotypes persisted when offspring of sugar-reared flies were transferred onto starch. This suggests the existence of trans-generational maternal effects that could act as a buffer against nutritional changes in the larval-rearing environment.

6.2 Introduction

All animals are associated with a large consortium of non-pathogenic microbes (Savage, 1977, Hooper & Gordon, 2001). In most instances the relationship between microbes and their hosts is loose and flexible. The predominance of microbes is in the gut where a complex, comprehensive and ever-changing microbial community exists, which in insects has shown to be shaped both by host genotype and diet (Savage, 1977, Chandler *et al.*, 2011). Single gene manipulations, e.g. down-regulation of the *caudal* gene in *Drosophila*, can alter the composition of the gut microbiome, allowing rare microbes to become more abundant (Ryu *et al.*, 2008). On an evolutionary scale, closely related species often share similar bacterial communities (Hosokawa *et al.*, 2007, Noda *et al.*, 2007, Chandler *et al.*, 2011). However diet can have an even greater effect on the gut microbiome than host species identity. For example, a recent study on 11 natural *Drosophila* species found that while there was evidence that the composition of the microbiome showed taxonomic restriction, host diet had a significantly greater effect on the composition of the gut bacterial community (Chandler *et al.*, 2011). This is the pattern expected if the guts of organisms are readily colonised by bacteria living on the ingested food.

Despite the flexible and non-obligatory relationship between host and gut bacteria, their relationship can be complex. Gut microbes can have surprising effects on host metabolism, immunity, mate choice, which will ultimately influence to some extent their mutual evolution (Mueller *et al.*, 2012). Functional studies have shown that disruptions to the gut bacteria community can have serious detrimental health impacts (Hooper, 1972, Ben Ami *et al.*, 2010). Knowledge gleaned from the study of microbial ecology is hence becoming increasingly important in medicine and human health, as drug developers and clinicians start to exploit therapies that directly target gut microbiota (Lemon *et al.*, 2012, Mueller *et al.*, 2012).

The relationship between gut microbes and hosts can be described as mutually beneficial, a symbiotic relationship whereby the host gains access to more resources or protection against pathogens while the gut bacteria enjoy a plentiful food supply and a relatively safe and stable environment. However, this relationship has the potential to become exploitative if the bacteria attempt to influence their host

into providing more food, a better environment and potentially aid in their proliferation, for example through the secretion of molecules that modify host physiology or behaviour (Heijtz *et al.*, 2011).

Examples of bacterial secretions that can influence host behaviour are found across a wide range of organisms. Female apple maggot flies, for example, are attracted to bacteria that colonise the oviposition 'stings' made into fruits. Such signals from bacteria can override responses to host pheromones that normally function to deter other females from ovipositing in the same fruit. The bacterial signals therefore promote aggregated oviposition among females (MacCollom *et al.*, 1992), which may enhance bacterial transmission (Judd & Borden, 1992, Dìaz-Fleischer *et al*., 2000, Lam *et al*., 2007). Other examples come from the hindgut bacteria (*Enterobacter*, *Pantoea*, *Klebsiella* spp.) of the desert locus *Schistocerca gregaria* and the cockroach *Periplaneta Americana*, which are reported to alter host pheromone synthesis in a way that enhances social interactions and may therefore promote bacterial proliferation (Cruden & Markovetz, 1987, Dillon & Charnley, 2002, Dillon & Dillon, 2004). Manipulation of the host by gut bacteria is not limited to invertebrates, and studies in mice have demonstrated some remarkable effects, e.g. bacterial-induced phenotypic changes in bone mass development, obesity levels, innate immunity, anxiety and feeding levels (Dubuquoy *et al.*, 2003, Heijtz *et al.*, 2011, Mestdagh *et al.*, 2011, Sjögren *et al.*, 2012).

Recently, it was reported that the mechanism underlying the evolution of reproductive isolation in a classic laboratory study of speciation through ecological adaptation (Dodd, 1989), was due to differentiation of the gut microbiome (Sharon *et al.*, 2010). In the original speciation experiment (Dodd, 1989) a population of *Drosophila pseudoobscura* was split into 4 replicates each and placed onto two different diets, four replicates onto a standard sugar food and four onto starch food. These populations were maintained for one year (10 generations), during which time several changes in allele frequencies were recorded between the populations. For example, an increase in the frequency of the '*fast*' allele of the *Amylase* (*Amy*) gene was reported in the starch flies, associated with adaptive divergence to the different diets. Most interestingly, rapid adaptation to the different media resulted in significant and replicated behavioural isolation. Flies from each diet showed a significant preference for mating with individuals maintained on the same diet over

individuals maintained on the other diet (assortative mating). It was proposed that this behavioural isolation came about due to a pleiotropic by-product of ecological adaptation to the different diets.

This experiment was recently repeated in *Drosophila melanogaster* (Sharon *et al.*, 2010). Surprisingly, in this experiment it was reported that after just one generation, flies maintained on the diets containing either a sugar or starch base showed the same patterns of significant positive assortative mating as reported previously by Dodd (1989). This unexpectedly rapid occurrence of pre-mating isolation was found to be associated with a change in the gut microbiota of the flies maintained on separate foods. A causal role for the gut microbiota in assortative mating was then suggested by the application of an antibiotic treatment, which removed mating preferences and hence the pre-mating reproductive isolation. This was then confirmed by supplementing antibiotic-treated flies with *Lactobacillus plantarum* bacteria. This fully restored the assortative mating for diet, and the pre-mating reproductive isolation. The bacterial-induced mating preference was maintained for at least one generation after switching diets, suggesting that the bacteria are at least partially capable of vertical transmission (Sharon *et al.*, 2010). A proposed mechanism was through bacterial manipulation of host cuticular hydrocarbons (CHCs). It was proposed that this could be a direct effect of bacterial secretions, or indirect, via alterations by the gut bacteria of food substrates used as CHC precursors (Ringo *et al.*, 2011). CHCs are used by many insects as short-range pheromones and are important in mate choice decisions (Gleason *et al.*, 2009, Everaerts *et al.*, 2010). Consistent with a role for pheromones, several differences in CHCs were found between individuals maintained on sugar vs. starch diets, and the levels of CHCs were also significantly reduced by antibiotic treatment (Sharon *et al* (2010).

Sharon *et al*. (2010, see also Ringo *et al.*, 2011) proposed that in the wild, fly populations living on different host foods would be, at least partially, geographically isolated. In combination with a bacteria-induced mating preference this could reduce population interbreeding. Over time genetic drift in the host genome could reinforce this assortative mating affect, increasing sexual isolation between populations and acting as a precursor to speciation.

The gut bacteria-induced reproductive isolation (RI) found in *D.melanogaster* is striking in its similarity to the effects documented in many species as a result of infection by *Wolbachia*. *Wolbachia* are intracellular bacteria which are very common and infect an extremely wide range of arthropod taxa, with estimates of infection rates ranging anywhere from 25-70% across insect species (Hilgenboecker *et al.*, 2008). *Wolbachia* are intra-cellular alpha-proteobacterial endosymbionts that are transmitted vertically through host eggs and which have the capacity to alter diverse aspects of host biology (Werren, 1997, Stouthamer *et al.*, 1999, Stevens *et al.*, 2001, Bourtzis & Miller, 2003, Hilgenboecker *et al.*, 2008, Werren *et al.*, 2008). The most common effect of *Wolbachia* on their hosts is cytoplasmic incompatibility (CI). CI is a phenomenon that results in sperm and eggs being unable to form viable offspring, it results from selection associated with the maternal inheritance pattern of *Wolbachia* (Bourtzis & Miller, 2003, Werren *et al.*, 2008, Saridaki & Bourtzis, 2010). Although the molecular basis of CI remains unknown it results from arrested development in zygotes formed from the fusion of infected sperm and uninfected eggs. The reciprocal cross is not affected. The CI benefits *Wolbachia* because infected females are significantly more productive than uninfected females (Werren, 1997, Werren *et al.*, 2008). CI can also arise when gametes containing incompatible strains of *Wolbachia* fuse (Werren, 1997, Presgraves, 2000, Zabalou *et al.*, 2008).

CI confers a strong selective advantage to *Wolbachia*, increasing its spread in the population and selecting against hosts who are either uninfected or carry an incompatible strain of *Wolbachia* (Werren, 1997, Werren *et al.*, 2008). It has been predicted that this will most likely give rise to, or reinforce, RI between populations infected with different strains of *Wolbachia* (Coyne, 1992, Hurst & Schilthuizen, 1998, Telschow *et al.*, 2005). Evidence to support this comes from *Nasonia* wasps, where *Wolbachia* infection is the prime candidate for the driver of speciation between *Nasonia giraulit* and *Nasonia longicornis* (Brucker & Bordenstein, 2012a, Brucker & Bordenstein, 2012b). Given the strong fitness consequences for bacteria and hosts associated with *Wolbachia* compatibility, it is perhaps not surprising that mate choice is influenced significantly by the presence of *Wolbachia*. In one study, the removal of *Wolbachia* from an infected strain using antibiotics resulted in decreased levels of mate discrimination by 50% between thirty year old selection lines of *Drosophila melanogaster* that had been diverging for toxin tolerance

(Koukou *et al.*, 2006). In the *D.paulistorum* species complex, females from sympatric populations of multiple subspecies carry incompatible strains of *Wolbachia* and avoid mating with males from different subspecies. In doing so they improve their fitness but also enhance RI (Miller *et al.*, 2010). This effect can be reversed, and hence random mating between subspecies restored, by treatment of the host with antibiotics (Miller *et al.*, 2010). The mate preference is therefore directly linked to the presence or absence of *Wolbachia* rather than as a result of a co-evolutionary response by the host. Recent work shows that *Wolbachia* can be present in the olfactory neurons of *D.melanogaster*, *D. simulans* and *D. paulisotrum*, and may upregulate the expression of key proteins and enzymes involved in CHC synthesis such as *fatty acid binding protein* and *delta-9-desaturase* (Peng *et al.*, 2008, Albertson *et al.*, 2009, Chao *et al.*, 2010, Miller *et al.*, 2010). The tight association between *Wolbachia* in the antennal lobe and CHC synthesis could potentially affect olfactory-mediated behaviour and mate choice (Peng *et al.*, 2008, Miller *et al.*, 2010).

The evolutionary explanation for, and significance of, *Wolbachia*-influenced host mating preferences is relatively easily understood. The benefit for the *Wolbachia* stems from manipulating host mating decisions in a manner that leads to increased bacterial transmission. Hosts on the other hand will minimise fitness costs by avoiding the decreased productivity of incompatible matings. Vertical inheritance of the bacteria promotes strong coevolution between hosts and bacteria. However, for extracellular commensal gut bacteria the significance of the link with host mate preferences is more problematic. Gut bacteria are often only loosely associated with their hosts and are not generally expected to show tight coevolution with them. Hence the evolutionary significance of strong effects of gut bacteria on host mating preferences is hard to understand. In order for coevolution to occur in these loose relationships, commensal gut bacteria must ensure fidelity of the host to a particular food and a mechanism to ensure their transmission to offspring. In a species such as *D. melanogaster*, food use can vary widely across an individual's lifetime. Therefore the predicted transient associations between gut bacteria and *D. melanogaster* seem unlikely to represent strong drivers of assortative mating. The wider implications of these findings in an ecological context have therefore yet to be determined.

Currently, the phenomenon of gut microbe-induced mate choice has been reported exclusively in *D. melanogaster*. It is therefore unknown whether assortative mating between hosts driven by extracellular symbionts could be more widespread. One complication is that experiments designed to investigate the effect of *Wolbachia* on assortative mating have often used antibiotics for removal of *Wolbachia* from the host system (Koukou *et al.*, 2006, Miller *et al.*, 2010). However, this treatment also alters gut microbiota, an effect that is usually overlooked in the interpretation of such experiments. In the investigation by Sharon *et al.* (2010), the abundance of *Wolbachia* in the host was significantly higher on the starch food. However it was ruled out as a driver of the mating assortment effect because both groups of flies contained the same strain of *Wolbachia,* and add-back experiments with *Lactobacillus plantarum* to antibiotic-treated flies reintroduced assortative mating without the presence of *Wolbachia*. *L. plantarum* appeared to induce assortative mating in this experiment. However the effect of bacterial add-back on CHC levels was not reported. Further studies on the influence of gut bacteria on host mating preferences are therefore sorely needed.

The potential for gut bacteria-induced assortative mating is predicted to be of particular interest in the medfly. Until recently associations between the Mediterranean fruit fly and bacteria received little scrutiny (Marchini *et al.*, 2002). However, it is now known that medfly guts contain a relatively simple, discrete community of bacteria, concentrated in the esophegeal bulb. This community is dominated by *Enterobacteriaceae* (*Klebsiella*, *Enterobacter, Pectobacterium, Citrobacter* and *Pantoaea* spp) (Behar *et al.*, 2008b, Ben-Yosef *et al.*, 2008a, Ben-Yosef *et al.*, 2008b). However mass-reared medflies also have increased levels of the potentially pathogenic *Pseudomonas* spp (Ben Ami *et al.*, 2010). It has been previously demonstrated that gut bacteria are capable of altering host behaviour and fitness in medfly and are at least partially capable of vertical transmission (Behar *et al.*, 2005, Behar *et al.*, 2008a, Behar *et al.*, 2008b, Ben-Yosef *et al.*, 2008b, Ben Ami *et al.*, 2010). There may therefore be the potential for tighter evolutionary relationships between gut bacteria and medflies than previously appreciated.

Effects of gut bacteria on mate choice could have serious implications for mass-rearing protocols in SIT/RIDL programs. ‘Sterile’ males consistently perform less well than wild males for matings with wild females during SIT programs

(Hendrichs *et al.*, 1996, Krafsur, 1998, Hendrichs *et al.*, 2002). It is therefore possible that a significant proportion of this lack of competitiveness is due to the incompatibility of the gut microbiomes between mass-reared males and wild females (Behar *et al.*, 2008b, Ben Ami *et al.*, 2010). This raises the possibility that incompatibility between lab-reared males and wild females could be reduced through bacterial 'add-back' of commensal gut bacteria lost during laboratory adaptation. Investigations into gut microbiota and assortative mating in natural populations are readily achievable as the ecology of the medfly has been extensively studied (Dìaz-Fleischer & Aluja, 2000). Interestingly, it appears that although the medfly is a suitable host for *Wolbachia* and can be experimentally infected, the presence of natural *Wolbachia* infections has been recorded only once in a wild population from southern Brazil (Rocha *et al.*, 2005, Sarakatsanou *et al.*, 2011). Hence *Wolbachia* is unlikely to be an important driver of assortative mating in the medfly. This allows gut microbe-host interactions to be studied without the confounding influence of *Wolbachia* infection.

In this chapter one population of medfly was split into three replicates of each of two treatments reared on either sugar-based (ASG) or starch-based (Starch) larval diets. After five generations of rearing these lines were tested for differences in the adult gut microbiome, and for any effects of larval diet on mating preferences (with and without gut microbes). In addition, larval diet effects on correlates of fitness such as development speed and body mass were recorded, to determine the relative effect of nutrition and gut microbes on mating success.

6.3 Methods

6.3.1 Rearing conditions

The wild type (wt) TOLIMAN medfly strain was used to establish replicated lines of flies maintained on two different larval diets. Larvae were cultured on modified versions of the diets used by Sharon *et al.* (2010), either i) sugar-based 'ASG' medium (1% agar, 7.4% sugar, 6.7% maize, 4.75% yeast, 2.5% Nipagin (10% in ethanol), 0.2% propionic acid) or ii) 'Starch' (S) medium (1.5% agar, 3% starch, 5% yeast, 0.5% propionic acid). Each regime was maintained in three allopatric replicates. Adults emerging from each of the replicates from the two larval food regimes were maintained in groups of 30 males and 30 females in plastic cages (9cm x 9cm x 9cm) held at 25°C, 50% relative humidity. Adults from all lines received the same standard adult diet (*ad libitum* access to sugar-yeast food; 3:1 w/w brewer's yeast: sugar paste and water). Each replicate was maintained under these conditions for four generations prior to the mating tests described below.

To test for any effects of the two different larval food regimes on adult mating success, eggs were collected from the fourth generation of selection, and the emerging adults split into four different larval food treatments. Flies were maintained either on the same food as their parents (parental food/ focal food: ASG/ASG or S/S) or switched to the alternative food (ASG/S or S/ASG; Fig 6.1). In order to test for the contribution of gut bacteria to any differences in mating preferences, a further set of treatments was created. Groups of randomly selected adults from each treatment were given a cocktail of antibiotics. For these groups eclosing adults were maintained on the standard adult sugar-yeast food with access to water supplemented with a spectrum of antibiotics $^{(Ab+)}$ to target a wide range of bacteria: 50µg/ml kanamycin, 100 µg/ml ampicillin, 100 µg/ml streptomycin, 25 µg/ml chloramphenicol, 50 µg/ml apramycin, 50 µg/ml hygromycin and 100 µg/ml tetracycline. The full set of 8 treatments was therefore as follows: ASG/ASG, ASG/ASG $^{Ab+}$, ASG/S, ASG/S $^{Ab+}$, S/S, S/S^{Ab+}, S/ASG, S/ASG^{Ab+}.

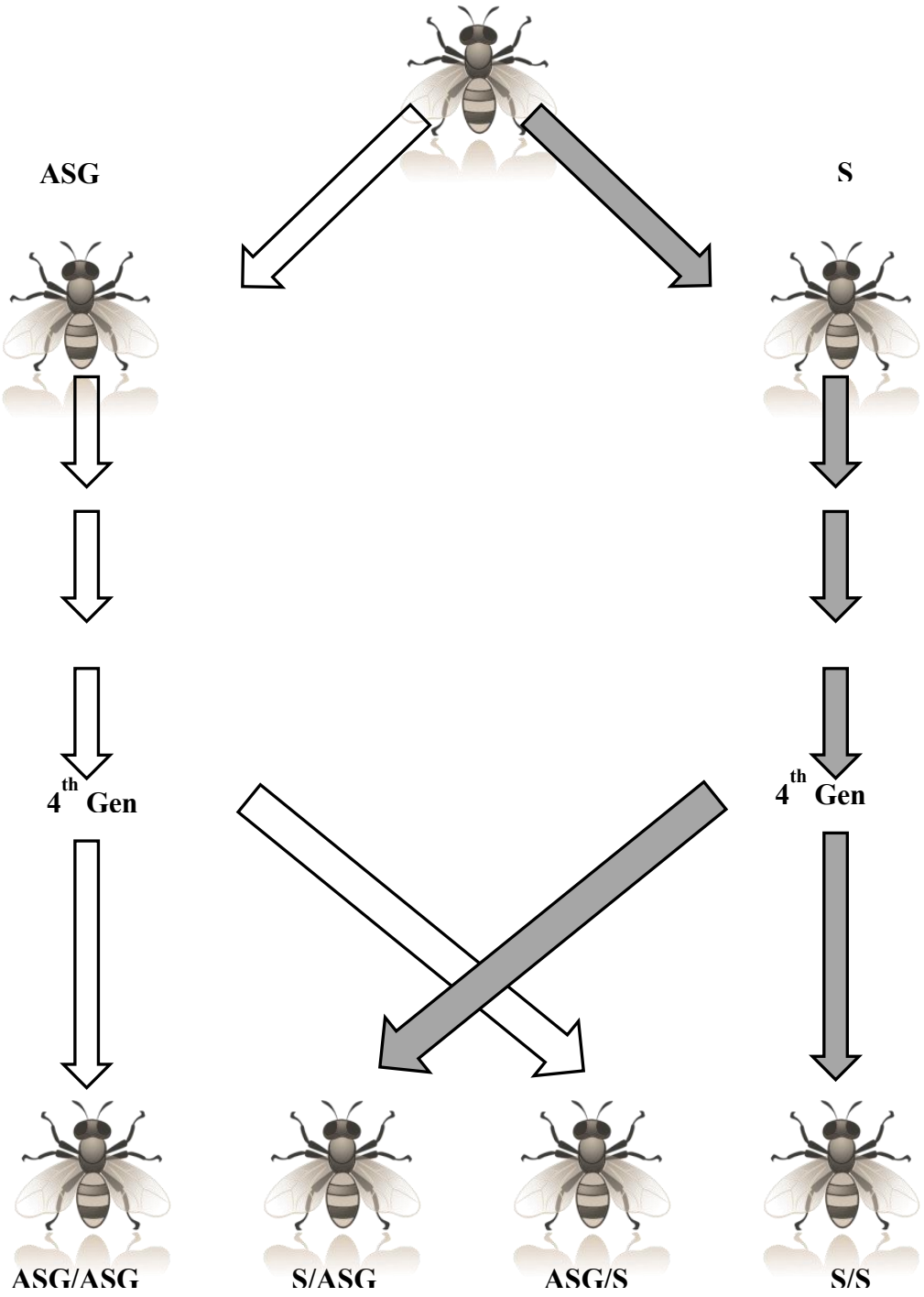

Fig 6.1 Schematic representation of rearing procedure. Wild type flies were used to initiate two larval diet lines (maize-based 'ASG', or Starch (S) food; 3 replicates of each). Flies were reared for four generations on ASG or S food. At the fifth generation, each treatment was sub-divided into two and maintained on the same larval diet as over the four previous generations, or switched onto the alternative diet. Each treatment was then further divided into two, with one group being exposed to an antibiotic cocktail $^{(Ab+)}$, and the other left untreated.

6.3.2 Extraction and sequencing of 16SrDNA from the gut bacteria of medflies reared on the different larval diets

Culture-dependent bacterial isolation

Virgin flies from each treatment in the fifth generation of rearing were collected at 7 days post-eclosion and frozen at -20°C in 20% glycerol solution, prior to analysis. Flies were washed twice in 2.5% bleach and twice in sterile RO water to remove external bacteria. The entire gut was dissected under a Leica-M26 dissecting microscope in a cavity slide containing sterile phosphate buffer saline (PBS). Five guts from flies from the ASG and Starch lines were homogenised in an eppendorf with a sterilised pestle in 1ml of 20% glycerol. Eight serial dilutions were made from 10^{-1} to 10^{-8}. 200µl of each solution was then pipetted and spread onto pre-prepared 200ml Lysogeny Broth (LB) agar plates (with 50µg/ml of nystatin and cyclohexamide to prevent fungal contamination) and then incubated overnight at 30°C to assess bacterial growth. Individual colonies of bacteria that grew were picked from the 10^{-8} plates using a bacterial loop and then inoculated into 10ml LB flasks. Flasks were then placed in a shaking incubator at 30°C and 200rpm overnight.

The DNA from the bacterial cells in each flask was then extracted and purified using the QIAprep® Miniprep kit. Purified plasmid DNA was eluted into 50µl of sterile water. DNA was then quantified by using a Nanodrop (Thermoscientific 2000c). Approximately 80ng of 16S rDNA was then amplified by PCR (BIORAD DNA Engine) in a 35µl reaction (1µl 10x buffer solution, 2.5µl 10mM dNTPs, 1.5µl $MgCl_2$, 2.5µl DMSO, 1.25µl 20mM Forward primer, 1.25µl 20mM Reverse primer, 35.5µl H_2O/DNA). Universal bacterial primers 533F (GTGCCAGCMGCCGCGGTAA) (Hugenholtz *et al.*, 1998) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991) for small-subunit rDNA (16S) amplification (1000bp) were used. The PCR protocol included an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1min, 45°C for 45s and 72°C for 90s, with a final extension step of 72°C for 5min. Reaction products were checked for purity on a 1% agarose gel, gel bands were cut and purified with the QIAquick® Gel Extraction Kit, DNA was eluted into 30µl of sterile RO water, and again quantified via Nanodrop. 420ng DNA was included in the sequencing reaction

mix, which contained 1.5µl 5x sequencing buffer, 1µl 3.1 Sequencing Big Dye mix, 0.5µl 533F/1492R, 7µl DNA (60ng/ul). The sequencing reaction comprised an initial denaturing step of 96°C for 1 min followed by 26 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4min with a final step of 60°C for 10s. The resulting samples were sent to The Genome Analysis Centre (TGAC™) for DNA sequencing.

6.3.3 Clone library analysis – *culture-independent bacterial isolation*

The clone library method allows the analysis and assessment of relative abundance of gut bacterial species for which culturing may not be possible, by cloning 16S ribosomal DNA fragments from PCR into plasmid vectors. These can subsequently be transformed into *E.coli* host cells. This 'culture-independent' method of bacterial amplification can generate hundreds of *E.coli* colonies each carrying a unique fragment of 16S rDNA, which can then be sequenced.

Fifteen medfly guts each were taken from 5th generation flies at 5 days post-eclosion, for each of the four treatments: ASG-reared flies, ± fed antibiotics for five days and Starch-reared flies ± fed antibiotics for five days. DNA was extracted according to the Qiagen DNeasy® Blood & Tissue kit protocol, with modifications to increase bacterial DNA yield. Tissue was homogenised in a tissue-lyser (MP Fastprep®-24) for 3 minutes with 200µl enzymatic lysis buffer and 40µl lysozyme. The samples were then incubated at 37°C for 2 hours before undergoing three freeze/thaw cycles (placed in liquid nitrogen for 5 min, incubated at 65°C for 10min). Samples were then incubated at 70°C for 30 minutes with 200µl buffer AL (without ethanol) and 25µl proteinase K, before following the standard spin-column DNA extraction protocol.

DNA was precipitated in ethanol by adding to each 200µl elution, 20µl 3M NaAc pH 5.2 and 500µl 100% ethanol. Samples were then vortexed and incubated at -20°C for 30 min. The samples were then centrifuged at 14,000 rpm for 20 minutes, the supernatant discarded and the pellet washed with 500µl 80% ethanol. Pellets were allowed to air dry for 3minutes before being re-suspended in 10µl of sterile RO water.

16S rDNA was amplified and gel purified using the same primers and methods as detailed above. One 16S rDNA amplicon from each treatment was cloned into the pCR™II-TOPO® vector using the TOPO® TA Cloning Kit. Clones were then transformed chemically into One Shot® Mach1™-T1^{R} chemically competent *E.coli* cells, incubated in S.O.C. (Super Optimal broth with Catabolite repression, a nutrient rich growth medium) for 1 hour and then plated onto LB agar plates in two volumes (200µl and 50 µl) to ensure at least one agar plate was obtained with well-spaced colonies. Each plate contained 50µg/ml kanamycin and was coated with X-gal. The pCR™II-TOPO® plasmid confers kanamycin resistance to cells, allowing a screen for successful transformation via X-Gal digestion, which normally produces a blue precipitate in the presence of β-galactodsidase. However a successful cloning event disrupts the formation of β-galactodsidase, preventing the hydrolysis of X-Gal and resulting in the production of white, not blue colonies. Agar plates were spread then incubated overnight at 37°C, and 100 white colonies from each plate were picked for inoculation into 10ml LB flasks and incubated overnight at 37°C and 200rpm, before purifying plasmid DNA with the QIAprep® Miniprep kit. Blue/white colour screening was checked by picking three blue and three white colonies at random for a restriction analysis with restriction enzyme EcoRI. This was to confirm that the presence of PCR fragments in plasmids was indeed associated with the appropriate colony colour. Restriction samples were run on a 1% gel to screen for the presence or absence of the appropriate 16S rDNA gel band fragment.

Each of the 200 samples for sequencing was prepared in a 4µl solution containing 400ng DNA, and sent for sequencing at Source Bioscience. Reactions were carried out with the M13R primer (AGGAAACAGCTATGACCAT).

6.3.4 Mating tests under choice conditions

For mating tests, virgin male and female flies were taken from each treatment by sorting males and females apart on ice within 24 hours of eclosion. To test for assortative mating by larval diet type and whether this was altered by antibiotic treatment, I placed groups of four 7 day-old males and females together in a mating

chamber as shown in the table below (Table 6.1). Six sets of fully reciprocal test groups were established, each comprised of one ASG/ASG or ASG/S male and female and one S/S or S/ASG male and female, with (Ab+) or without antibiotic treatment, in a 250ml transparent plastic mating arena.

Table 6.1 Mate choice test set up

Test	Male 1	Female 1	Male 2	Female 2
ASG/ASG v S/S	ASG/ASG	ASG/ASG	S/S	S/S
ASG/ASG$^{(Ab+)}$ v S/S$^{(Ab+)}$	ASG/ASG$^{(Ab+)}$	ASG/ASG$^{(Ab+)}$	S/S$^{(Ab+)}$	S/S$^{(Ab+)}$
ASG/S v S/S	ASG/S	ASG/S	S/S	S/S
ASG/S$^{(Ab+)}$ v S/S$^{(Ab+)}$	ASG/S$^{(Ab+)}$	ASG/S$^{(Ab+)}$	S/S$^{(Ab+)}$	S/S$^{(Ab+)}$
ASG/ASG v S/ASG	ASG/ASG	ASG/ASG	S/ASG	S/ASG
ASG/ASG$^{(Ab+)}$ v S/ASG$^{(Ab+)}$	ASG/ASG$^{(Ab+)}$	ASG/ASG$^{(Ab+)}$	S/ASG$^{(Ab+)}$	S/ASG$^{(Ab+)}$

To enable identification, one male and one female from the same population in each mating test were marked with a spot of red paint on the dorsal side of the thorax, while anaesthetised on ice. The two females were introduced into each 250ml

transparent plastic mating arena the night before each mating test; the males were introduced the following morning at 8.30am. Twenty cages were set up for each replicate cross, with a total of 60 cages analysed per treatment. Each mating arena was observed from male introduction, the identity of the first male and female to mate was recorded along with time of male introduction and the time that mating started and ceased. Cages were monitored until 12.30pm or when the first mating pair ceased mating.

6.3.5 Body mass and eclosion time

To measure the effect of larval diets on correlates of fitness the body mass and rate of development were measured for flies maintained on the different larval diets. The dry weights of individuals used in the mating tests were measured by freezing individuals at -20°C for 24 hours followed by desiccation at 25°C for 24 hours. Using the same method, separate measures of body mass were also made from 100 adult males and 100 adult females from each replicate at 7 days post-eclosion.

Eclosion times were measured on 100 pupae collected from each replicate. For this, the number of flies emerging at 12 hour intervals was recorded from day 20 post egg-collection.

6.3.6 Data analysis

Analyses were performed using Rv2.11.1 (R Development Core Team, 2010) and PASW (PASW Statistics v.18, 2009). All means are reported with standard errors (±). The effects of parental and focal diet on mating success and choice were analysed using G-tests. Comparisons of body mass and eclosion rates were analysed with a nested ANOVA, to include a test for replicate effects. *Posthoc* Tukey HSD tests were used for pairwise comparisons between groups. Mating success and mating latency data were non-normally distributed. To test the effect of larval food

and antibiotic treatment on male mating success and latency generalized linear models (GLMs) with quasi-binomial and quasi-poisson error distributions were used. A linear mixed effect model was initially used to assess whether replicates within treatments had a significant effect, prior to running GLMs. GLMs were run with larval diet, parental larval diet and antibiotic treatment as fixed factors with measures of male-female weight difference, male weight and relative male weight difference between focal male and competitor. Models were simplified to attain the minimally adequate model by stepwise removal of the least significant terms using Analysis of Deviance. Sequencing reaction reads were analysed on Finch TV v1.4.0 (Geospiza Inc) for quality and read length.

6.4 Results

6.4.1 Effect of larval diet regime on composition of gut bacteria

Isolation of culture-dependent bacteria

Direct culturing produced plates with several colonies across all sets of plates. However all colonies were of a uniform size, consistency and colour, indicative that the bacteria were likely to be from the same species. The least diluted plates where colonies had not formed a uniform lawn (i.e. the 10^{-6} dilution plates) had 50-100 colonies each. 5 colonies each were picked from these for sequencing. The five colonies sequenced from the plate with ASG flies revealed a monoculture of *Enterobacter hormaechei*. The colonies sequenced from Starch flies gave four sequences matching *E. hormaechei* and one unidentified *Enterobacter spp.* The almost uniform nature of the bacteria growing on both sets of plates indicated that direct culturing was unlikely to provide an accurate representation of the medfly gut microbiota. Many species present may therefore be unculturable, and this method could distort comparisons of species identities and abundances.

Isolation of culture-independent bacteria

Analysis of the clone library allowed for culture-independent analysis of medfly gut microbiota. 16S rDNA PCR amplification of gut extractions produced strong gel bands (800-1000bp) in all samples of ASG and Starch reared flies. By comparison the gel bands produced for antibiotic treated samples (Ab+) were extremely weak; indicating that the antibiotic cocktail given to the medfly had been successfully ingested and had significantly reduced the microbiotic load (Fig 6.2). Clone libraries were not constructed for antibiotic treated samples, as it would not be possible for those samples to ascertain an accurate representation of the alterations to the microbiota.

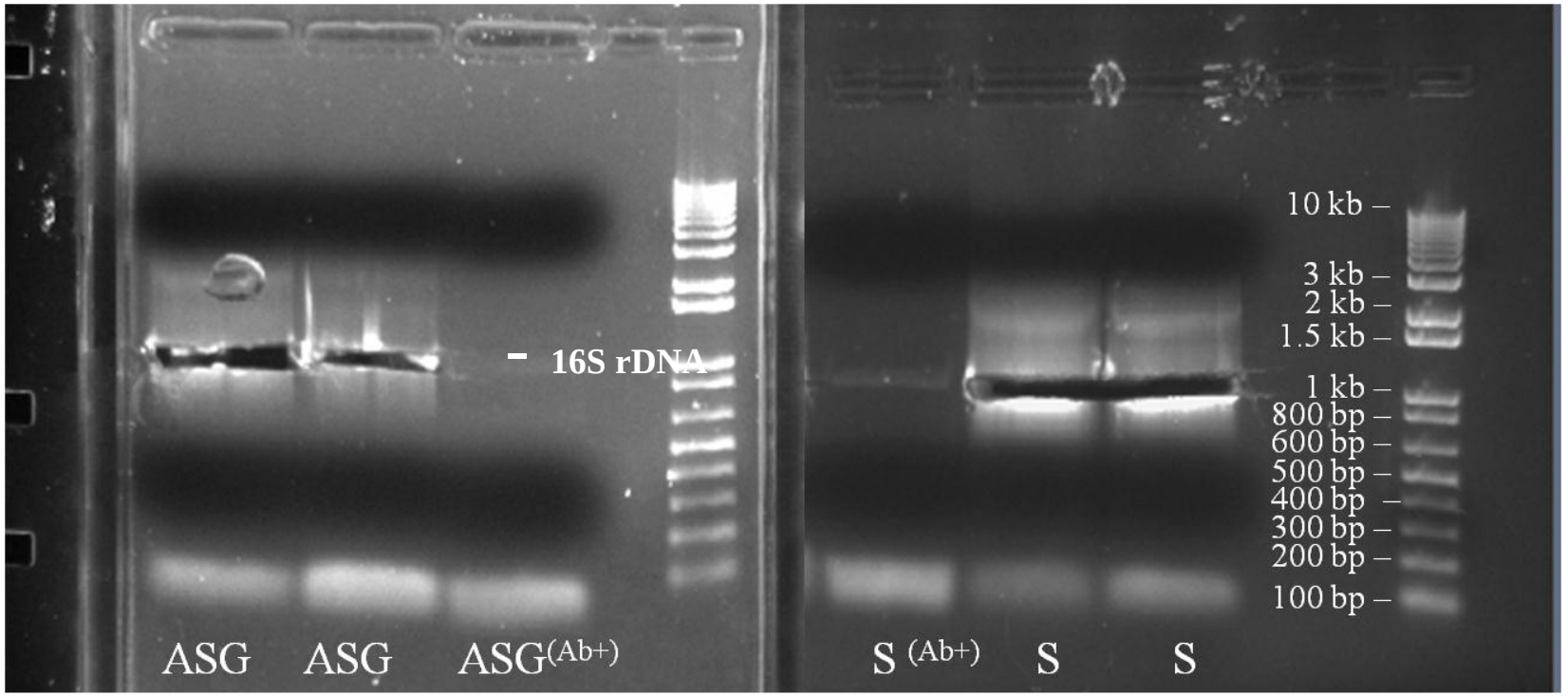

Fig 6.2 Gel imaging of 16S rDNA PCR amplification (800-1000bp) of bacteria isolated from medfly gut extractions. (Ab+) denotes antibiotic treated gut extractions, gel bands were much fainter indicating successful reduction of gut bacteria in these samples. Non-antibiotic treated samples were cut from the gel and purified for cloning.

The clone library analysis produced several hundred predominantly white bacterial colonies, indicating efficient vector cloning (Fig 6.3). EcoRI restriction analysis was used to confirm that blue/white colony screening correlated to the presence or absence of PCR product. Of the six colonies chosen for restriction analysis, a gel band was visible in all three white colonies, no band was visible in two of the three blue colonies, in the third a faint band was visible (Fig 6.4).

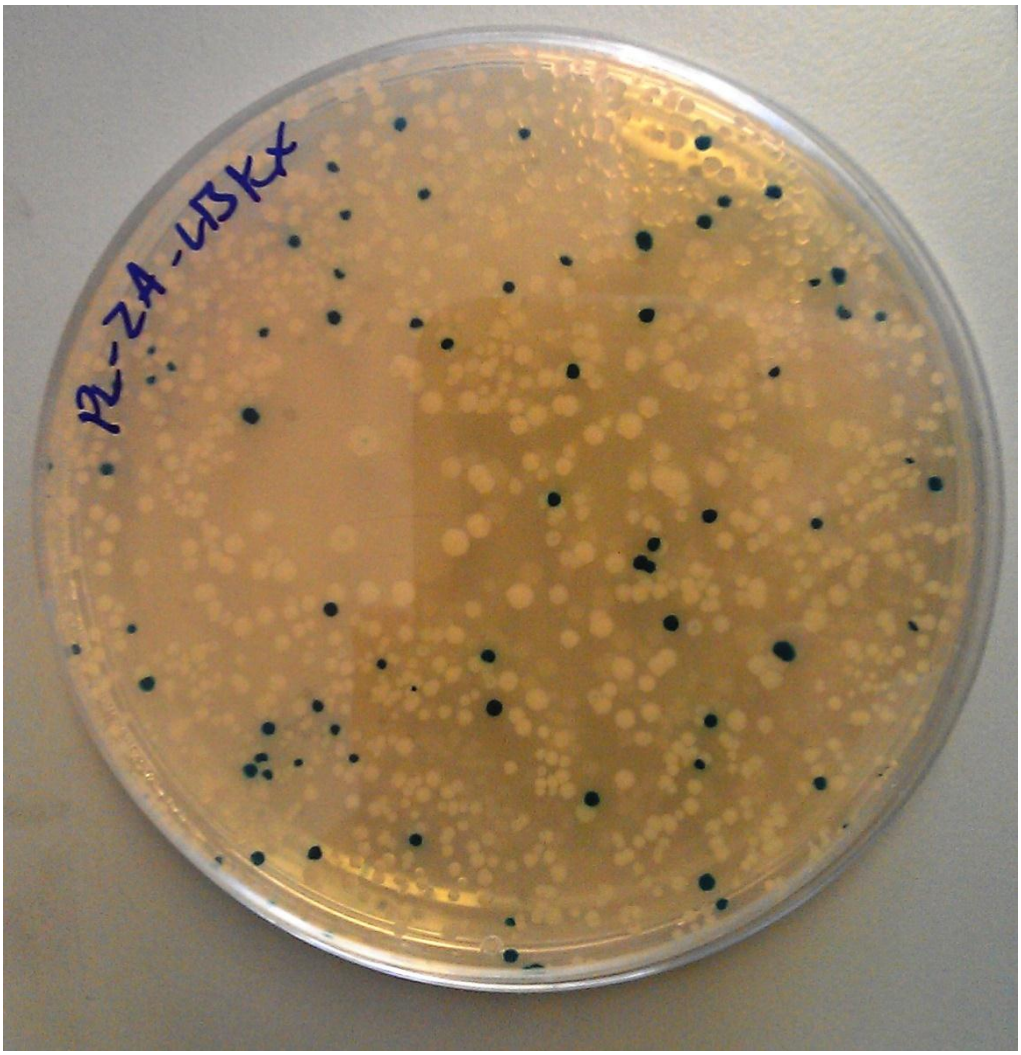

Fig 6.3 An example of successfully transformed colonies through blue/white screening. 200ml LB Agar plate with 50µg/ml Kanamycin coated with 50µl X-gal. Plate 2A had 50µl of S.O.C. medium with transformed *E.coli* spread onto the plate before being incubated overnight at 37°C. Blue-white screening allows the selection of successful cloned transformants, X-gal leaves a blue precipitate when hydrolysed by β-galactosidase, successful cloning of the 16S rDNA PCR fragment into the pCR™II-TOPO® plasmid disrupts the LacZ operon and no precipitate forms, resulting in the production of a white colony.

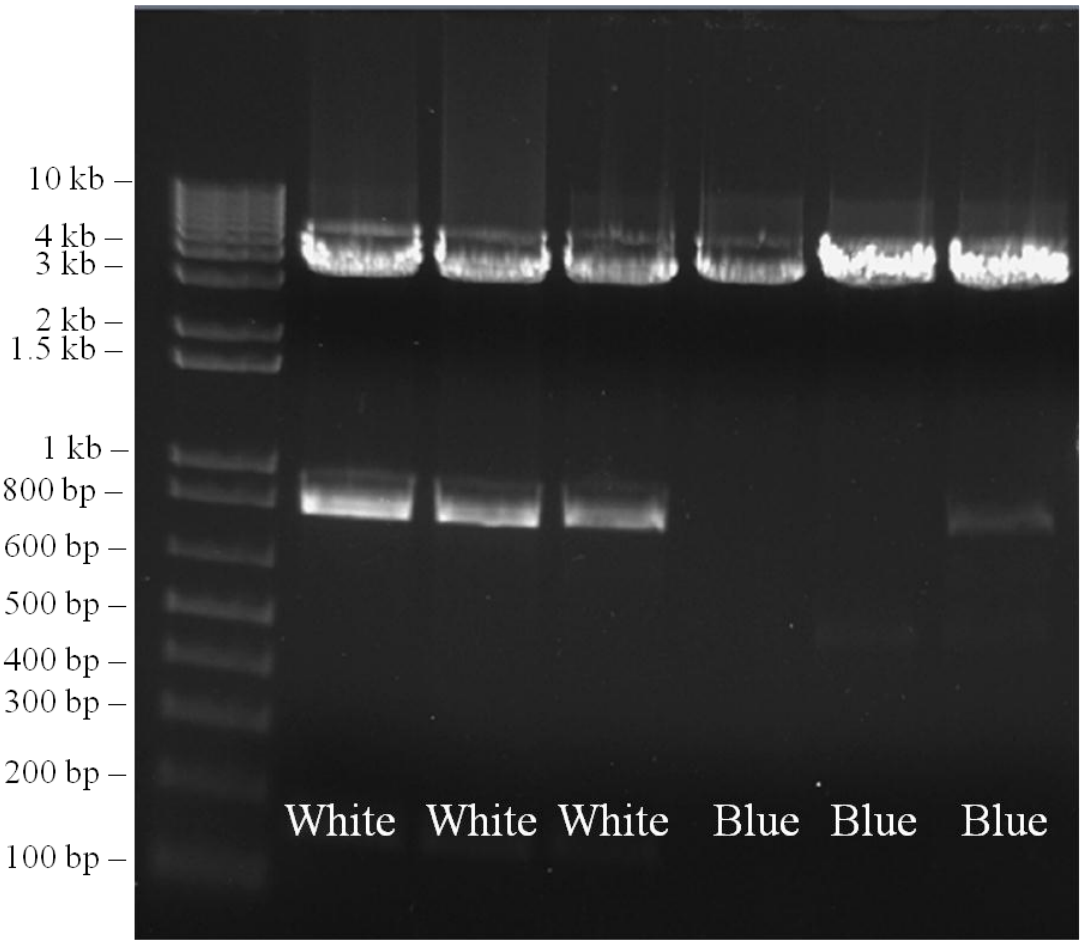

Fig 6.4 Gel imaging of restriction analysis, left to right are gel bands produced from three white colonies and three blue colonies picked from plate 2A. Plasmid DNA was isolated from bacteria and cut by incubating with restriction enzyme EcoRI, liberating an 800 bp fragment if the plasmid contained a cloned insertion. Each sample was loaded onto a gel, the largest band produced in all samples is the 3956bp pCR™II-TOPO® plasmid. A strong band is present in the samples from white colonies at 800bp; there is no band in two of the three blue colony samples and a very faint band present in one blue colony sample. This indicates that blue-white screening successfully eliminates plasmid vectors without the cloned insertion.

Sequencing of the 16S rRNA gene sequences from the ASG and Starch reared flies produced 187 high quality sequences (92 from ASG, 94 from Starch), each with greater than 400 non-gap characters, to ensure high quality reference alignment with database searches. NCBI nucleotide BLAST (Basic Local Alignment Search Tool (Altschul *et al.*, 1997)) revealed that three bacterial orders made up all of the sequences identified within the dataset. These were Enterobacteriaceae, Actinomycetales and Lactobacillales. Medfly reared on the ASG diet contained 62% Enterobacteriaceae, 37% Lactobacillales and 1% Actinomycetales, while flies reared

on the Starch diet contained 81% Enterobacteriaceae and 19% Lactobacillaes (Table 6.2, Fig 6.5) A G-test indicated that there was a significant difference in the relative abundance of bacterial species between the two groups of flies (G_2 = 10.874, P = 0.004). Full data, with the closest database match, species name and percentage homologies are given in Appendix II.

Table 6.2 Bacterial communities in adult *Ceratitis capitata* grown as larvae on ASG or Starch media.

Bacteria Genera	ASG %	Starch %
Enterobacter sp	14	36
Serratia sp	28	45
Providencia sp	16	-
Citrobacter sp	-	2
Klebsiella sp	1	4
Pantoea sp	3	2
Enterobacteriaceae	**62**	**81**
Actinomycetales	**1**	**-**
Enterococcus sp	37	19
Lactobacillales	**37**	**19**

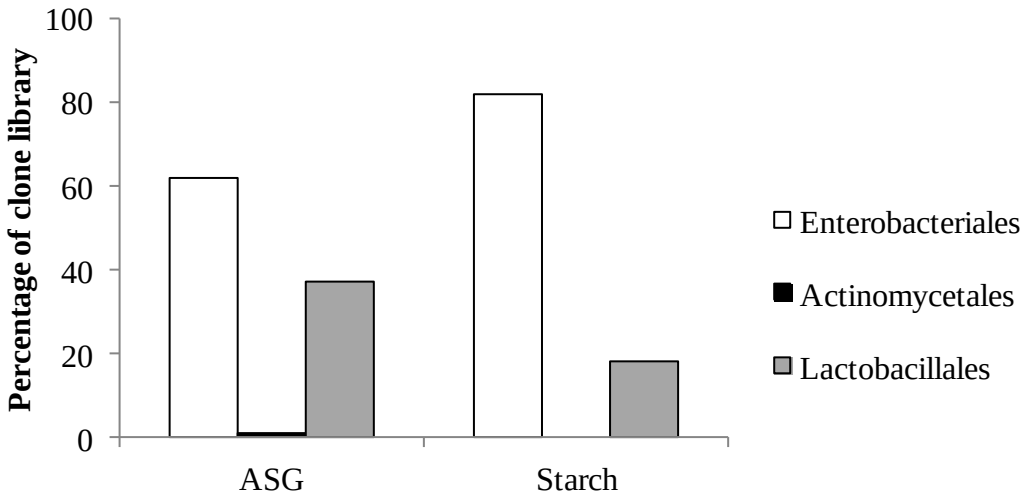

Fig 6.5 The proportions of different bacterial genera found in flies reared on ASG or Starch food

6.4.1.1 Enterobacteriaceae

The Enterobacteriaceae represented 71.5% (134 out of 187) of the sequences, and comprised the dominant order of bacteria in medfly guts from both diets. This family of Gram-negative bacteria is commonly associated with plant and animal hosts. They are often found as free-living associates of *D. melanogaster* and medfly, and almost every wild and laboratory population contains some Enterobacteriaceae (Behar *et al.*, 2005, Chandler *et al.*, 2011).

The genera of Enterobacteriaceae associated with the two populations of medfly comprised of *Enterobacter*, *Serratia*, *Providencia*, *Pantoea*, *Citrobacter* and *Klebsiella*. While *Serratia* and *Providencia* are commonly associated with the intestinal tract in animals (Janda & Abbott, 2006), they are regarded as potential pathogens and are known to elicit an immune response in *Drosophila* (Lazzaro *et al.*, 2006). *Enterobacter*, *Pantoea*, *Citrobacter* and *Klebsiella* spp are frequently associated with the medfly gut. These bacteria are reported to be present at all developmental stages, and can also be horizontally transmitted (Bourtzis & Miller, 2003, Behar *et al.*, 2008a). In contrast to *Serratia* and *Providencia*, these four genera of Enterobacteriaceae are considered non-pathogenic and are potentially beneficial to the host, as all have the capacity to fix nitrogen, hence increasing the host's potential range of protein sources (Behar *et al.*, 2005).

6.4.1.2 Lactobacillales

Lactobacillales are another widespread family of bacteria associated with animal hosts; they are gram-positive, acidophilic and usually found on nutrient-rich resources (Ljungh & Wadstrom, 2009). Only one genus of the family, *Enterococcus* was detected in these samples; it is a common inhabitant of insects. In previous studies of *Drosophila* this genus was found exclusively in wild-caught populations and has not previously been reported amongst the gut bacterial community of medfly (Bourtzis & Miller, 2003, Behar *et al.*, 2008b, Ben Ami *et al.*, 2010, Chandler *et al.*, 2011).

6.4.1.3 Actinomycetales

Detected at a presence of 1% in ASG-reared flies and absent in Starch-reared flies, this is a family of Gram-positive bacteria. It has been reported to be present in < 1% of the *Drosophila* microbiome, but this is its first detection in the medfly (Bourtzis & Miller, 2003, Behar *et al.*, 2008b, Ben Ami *et al.*, 2010, Chandler *et al.*, 2011).

Although this was only a limited analysis of the gut microbiota, it demonstrated that the gut bacteria in the ASG and Starch populations were notably different by the fifth generation of selection, with a shift towards a more Enterobacteriaceae-dominated microbiota in the flies reared on a Starch diet.

6.4.2 Effect of larval diet on mate choice

None of the mating tests showed any evidence that dietary treatment (and any associated changes in gut microbiota) significantly influenced mating success (Table 6.3). Antibiotic treatment also had no significant effect on mate choice (Antibiotics: G_5 = 3.995, P = 0.55, No Antibiotics: G_5 = 0.419, P = 0.995).

Table 6.3 Comparison of the number of females mating first on both food types in the six multiple choice mating crosses, analysed with a G-test for heterogeneity. Dietary treatments are indicated as 'Parent food/Offspring food'. Treatment names denote whether flies were maintained on the same food or different larval diet to that on which they were maintained for the previous four generations. (Ab+) indicates whether adults were treated with antibiotics. There was no significant variation across replicates ($P < 0.05$).

	ASG ♀	**Starch ♀**			
Mating choice test			***G***	***df***	***P***
Total counts across all tests	186	173	4.414	11	0.956
ASG/ASG v S/S	29	29	0	1	1
$ASG/ASG^{(Ab+)}$ v $S/S^{(Ab+)}$	31	28	0.153	1	0.696
ASG/S v S/S	25	33	1.107	1	0.293
$ASG/S^{(Ab+)}$ v $S/S^{(Ab+)}$	31	30	0.016	1	0.898
ASG/ASG v S/ASG	36	23	2.888	1	0.089
$ASG/ASG^{(Ab+)}$ v $S/ASG^{(Ab+)}$	34	30	0.25	1	0.617

In addition, no assortative mating for diet was detected. The number of matings between males and females from the same food (homotypic) vs. males and females from different foods (heterotypic) were not significantly different from random across any treatments (Table 6.4). Antibiotic treatment also had no significant effect on assortative mating (Antibiotics: $G_5 = 3.789$, $P = 0.58$, No Antibiotics: $G_5 = 1.261$, $P = 0.939$).

Table 6.4 Comparison of heterotypic and homotypic mate pairings in the six sets of mating crosses, with heterogeneity G-test analysis. Parent food/Offspring food denotes whether flies were maintained on the same food or switched to the opposite larval diet to that on which they were raised for the previous 4 generations. (Ab+) indicates whether adults were treated with antibiotics. Heterogeneity of G across all replicates $P < 0.05$ indicated no significant effect of replicates.

Mating choice test	Heterotypic	Homotypic	*G*	*df*	*P*
Total counts across all tests	179	180	5.05	11	0.929
ASG/ASG v S/S	26	32	0.622	1	0.43
$ASG/ASG^{(Ab+)}$ v $S/S^{(Ab+)}$	34	25	1.378	1	0.24
ASG/S v S/S	32	26	0.622	1	0.43
$ASG/S^{(Ab+)}$ v $S/S^{(Ab+)}$	32	29	0.148	1	0.701
ASG/ASG v S/ASG	30	29	0.017	1	0.896
$ASG/ASG^{(Ab+)}$ v $S/ASG^{(Ab+)}$	36	38	2.263	1	0.132

The results did though show a significant effect of dietary treatment on male mating success. ASG-reared males (ASG/ASG and S/ASG) were significantly more likely to be the first males to pair with females than were males from the Starch treatment. This effect was strongly significant across all replicates, in 57/58 matings, ASG-reared males were the first to mate ($G_1 = 70.3$, $P = 5.09 \times 10^{-17}$).

In order to discount the possibility that dietary effects were overriding any effect of bacterial differences on mating outcomes, mating tests were also run with flies from populations which had been reared for four generations on the separate diets before all being placed on the same larval diet (either ASG or Starch) for the final generation. In tests where flies were reared on a larval diet of ASG, there was no effect of parental treatment on 'first matings'. However, in tests carried out with a final generation of Starch rearing, there was a significantly greater number of 'first matings' achieved by males with ASG-reared parents (Fig 6.6, Table 6.5).

To test whether the addition of antibiotics affected mating preferences, fly populations were reared on adult food supplemented with antibiotics (kanamycin, ampicillin, streptomycin, chloramphenicol, apramycin, hygromycin and tetracycline).

Following treatment, fly mating preferences were tested using the same protocol as before and were found to be unchanged by antibiotic treatment (Table 6.5).

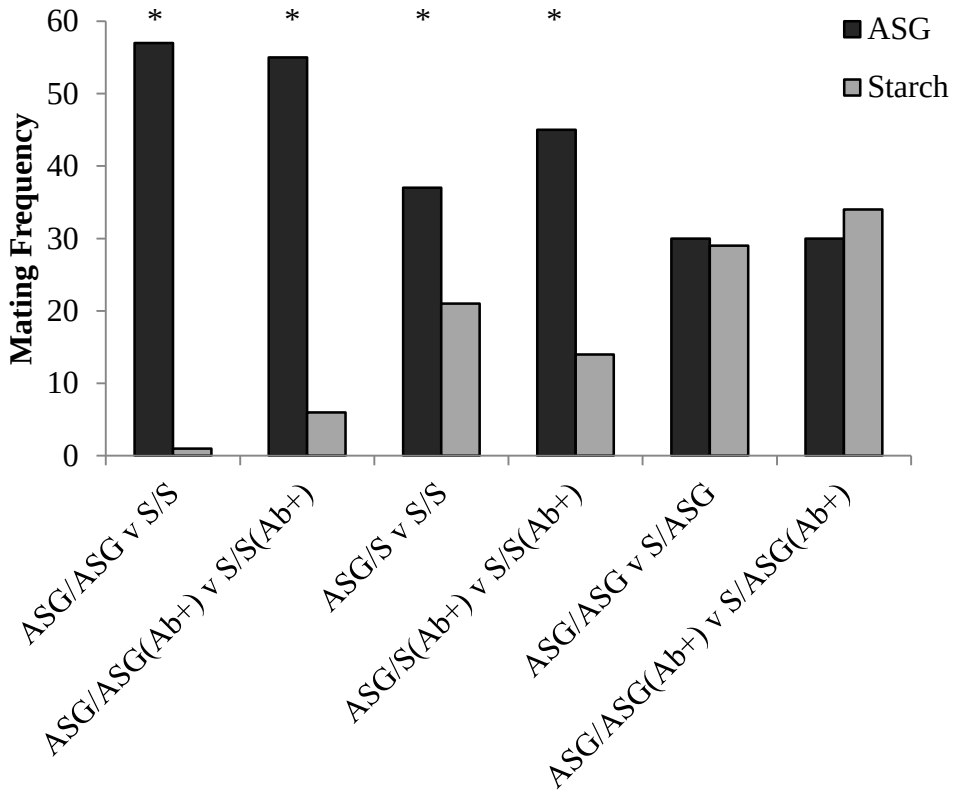

Fig 6.6 Number of males that achieved the first mating in the mating tests across the different larval food treatments. Black bars denoted frequency of first matings by ASG males, grey bars denote frequency of first matings by Starch males (*) denotes significant differences from expected values of first matings under random mating within mating test according to heterogeneity G-test analysis (*N* = 359).

Table 6.5 Comparison of the number of first mating pairs across the six sets of mating crosses, using with heterogeneity G-test analysis. Parent food/Offspring food denotes whether flies were maintained on the same food or switched to the opposite larval diet to that on which they were maintained for the previous four generations. (Ab+) indicates whether adults were treated with antibiotics. Heterogeneity of G across all replicates $P < 0.05$ indicated no significant effect of replicates.

	A ♂		S ♂				
Mating choice test	**A ♀**	**S ♀**	**A ♀**	**S ♀**	***G***	***df***	***P***
Total counts across all tests	130	124	56	49	144.714	11	1.79E-25
ASG/ASG v S/S	25	32	0	1	70.302	1	5.09E-17
$ASG/ASG^{(Ab+)}$ v $S/S^{(Ab+)}$	27	28	4	2	52.539	1	4.22E-13
ASG/S v S/S	20	17	9	12	4.472	1	0.034
$ASG/S^{(Ab+)}$ v $S/S^{(Ab+)}$	21	24	10	4	17.135	1	3.5E-5
ASG/ASG v S/ASG	18	12	18	11	0.017	1	0.896
$ASG/ASG^{(Ab+)}$ v $S/ASG^{(Ab+)}$	19	11	15	19	0.25	1	0.617

6.4.3 Effect of larval diet on male mating success

There was no significant difference between replicate lines in mating success or latency to mate (Linear Mixed-effects model; Analysis of Deviance, all $P > 0.990$); hence the replicate data were pooled for GLMs used to identify the significant predictors of mating success. The GLM analysis showed a significant interaction between males with parents reared on an ASG larval diet and male body weight. This was associated with a greater number of matings in mating choice tests. There was also a significant effect of focal male diet on mate success, as offspring with Starch-reared parents had an increased mating success when reared on an ASG larval diet (Table 6.6).

Table 6.6 Analysis of the proportion of variance in mating success by males explained by diet and body weight analysed by Generalized Linear Models (GLM) with a quasi-binomial error distribution. Larval rearing diet, larval rearing diet of parents, relative weight difference between mated male and mated female (MFW), male weight (MW) and the relative weight difference between focal male and competitor male (RMW) were all included in the initial model. Larval diet and parental diet were fixed effects while MFW, MW and RMW were covariates.

Dependent variable	**Source**	**Deviance**	***F***	***df***	***P***
Mate success	Larval Diet	707.83	10.49	1,698	$1.2e^{-3}$
	Parent x MFW	715.78	18.45	1,698	$1.99e^{-5}$
	Parent x MW	731.12	33.82	1,698	$9.22e^{-9}$
	Parent x RMW	705.46	8.12	1,698	0.0045

6.4.4 Mating Latency

The analysis of mating latency showed a significant delay in mating in males and females that were treated with antibiotics (Fig 6.7). There was a significant interaction between antibiotic treatment and the relative weight differences between flies (Table 6.7). Mating latency was negatively correlated with relative male body weight in the non-antibiotic treated flies. However in the antibiotic treatment, mating latency was uncorrelated with relative male body weight (Fig 6.8). There was also a marginally significant (P = 0.047) interaction between antibiotic treatment and the relative weight difference between males and females.

Table 6.7 Analysis of the proportion of variance in mating latency explained by antibiotic treatment and body weight, analysed by Generalized Linear Models (GLM) with a quasi-poisson error distribution. Antibiotic treatment (AB), relative weight difference between mated male and mated female (MFW), male weight (MW) and the relative weight difference between focal male and competitor male (RMW) were all included in the initial model. Antibiotic treatment was a fixed effect while MFW, MW and RMW were covariates.

Treatment	Deviance	*F*	*df*	*P*
MW	1821	15.662	1,351	$9.165e^{-5}$
AB x RMW	1846.6	20.199	1,351	$9.492e^{-6}$
AB x MFW	1755	3.983	1,351	0.047

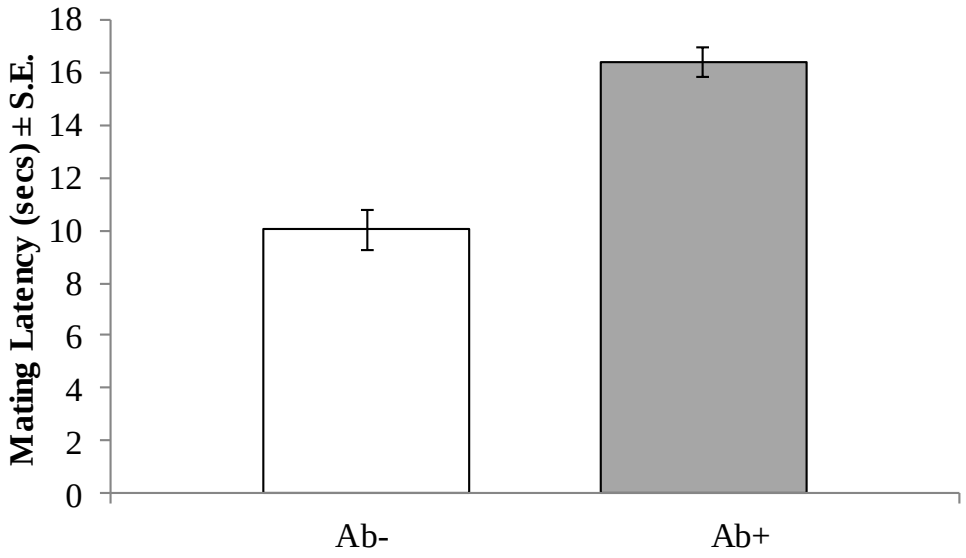

Fig 6.7 Mean mating latency (seconds ± SE) for males across antibiotic (grey bar, *N* = 194) and non-antibiotic (white bar, N = 165) treated groups. Antibiotic treatment had a significant effect on mating latency across all larval rearing treatments (see Table 6.9) Ab- denotes no antibiotic treatment, Ab+ is antibiotic treated flies.

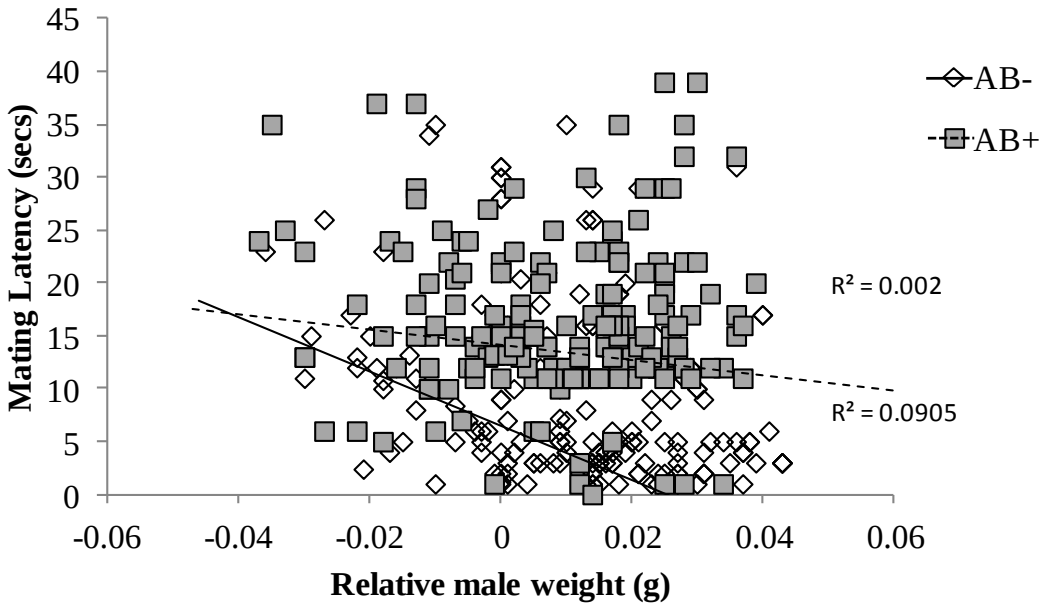

Fig 6.8 Correlations between male mating latency in seconds and relative weight difference between the mated and competitor males. No antibiotic (Ab-), open diamonds, solid line, N = 165; antibiotic treatment (Ab+), filled squares, dashed line, N = 194.

6.4.5 Body mass and development time of flies raised on different larval diets

Larval food and parental diet treatment had a significant effect on male mass (nested ANOVA $F_{4, 135} = 49.578$, $P < 0.001$, Fig 6.9) with ASG/ASG males (0.123g ± 0.004) being significantly heavier than S/S flies (0.098g ± 0.002), mean difference = -0.025, ± 0.004, Tukey HSD $P < 0.001$). Males from populations which had been reared on ASG for four generations but switched to Starch-rearing for the 5th generation (ASG/S) had a greater body mass than S/S flies, and were not significantly different to ASG/ASG flies 0.121g ± 0.003, difference to S = 0.023 ± 0.004, Tukey HSD $P < 0.001$, difference to ASG/ASG = -0.002 ± 0.004, Tukey HSD $P = 0.97$. No such pattern was detected in reverse, as S/ASG males were indistinguishable in body mass from ASG/ASG males 0.121g ± 0.003, difference = -0.002 ± 0.004, Tukey HSD $P = 1$). The effect of food on body size did not last for more than one generation as the male offspring of the flies switched to Starch-rearing from ASG (ASG/S/S) had a body mass which was not significantly different to males from a Starch-rearing background, (0.096g ± 0.003, difference = -0.002 ± 0.004 $P = 0.989$). For females,

there was no effect of treatment on body mass (nested ANOVA $F_{4, 135} = 0.619$, $P = 0.659$, Fig 6.9).

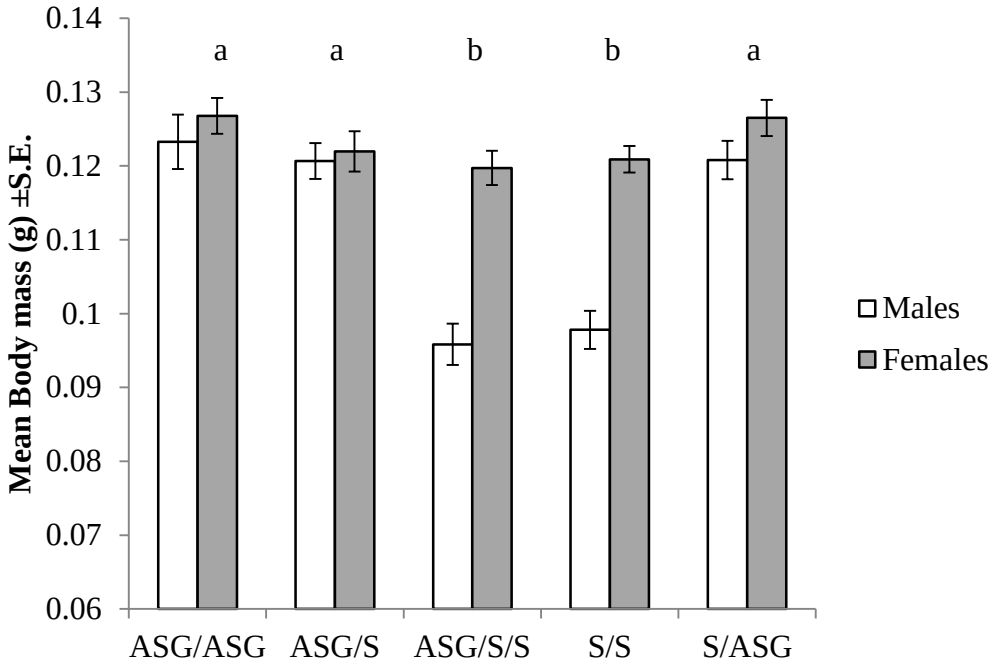

Fig 6.9 Mean (± S.E.) male and female body weight across six different larval rearing treatments. There was no significant difference in male weight for ASG/ASG flies or ASG/S. However ASG/S/S flies had a body mass that was significantly lower than for ASG-reared flies and the same as for Starch-reared flies (male groups which differ significantly in body mass are identified by separate letter annotations i.e. a, b,). There was no significant effect of treatment on female body mass.

Development time (mean number of days from egg collection to pupal eclosion for males and females combined) was significantly different across the four dietary treatments (nested ANOVA $F_{3, 1188} = 349.682$ $P < 0.001$). Flies reared on a larval diet of ASG had the slowest development times regardless of the diet on which their parents were raised (ASG/ASG 23.3 days ± 0.043 from egg to adult eclosion, S/ASG = 23.2 days ± 0.043), while ASG/S flies were faster to eclose (mean = 22.7 days ± 0.043), $P < 0.001$), though still took significantly longer to develop than did flies raised continually on Starch (21.7 days ± 0.043 $P < 0.001$, Fig 6.10).

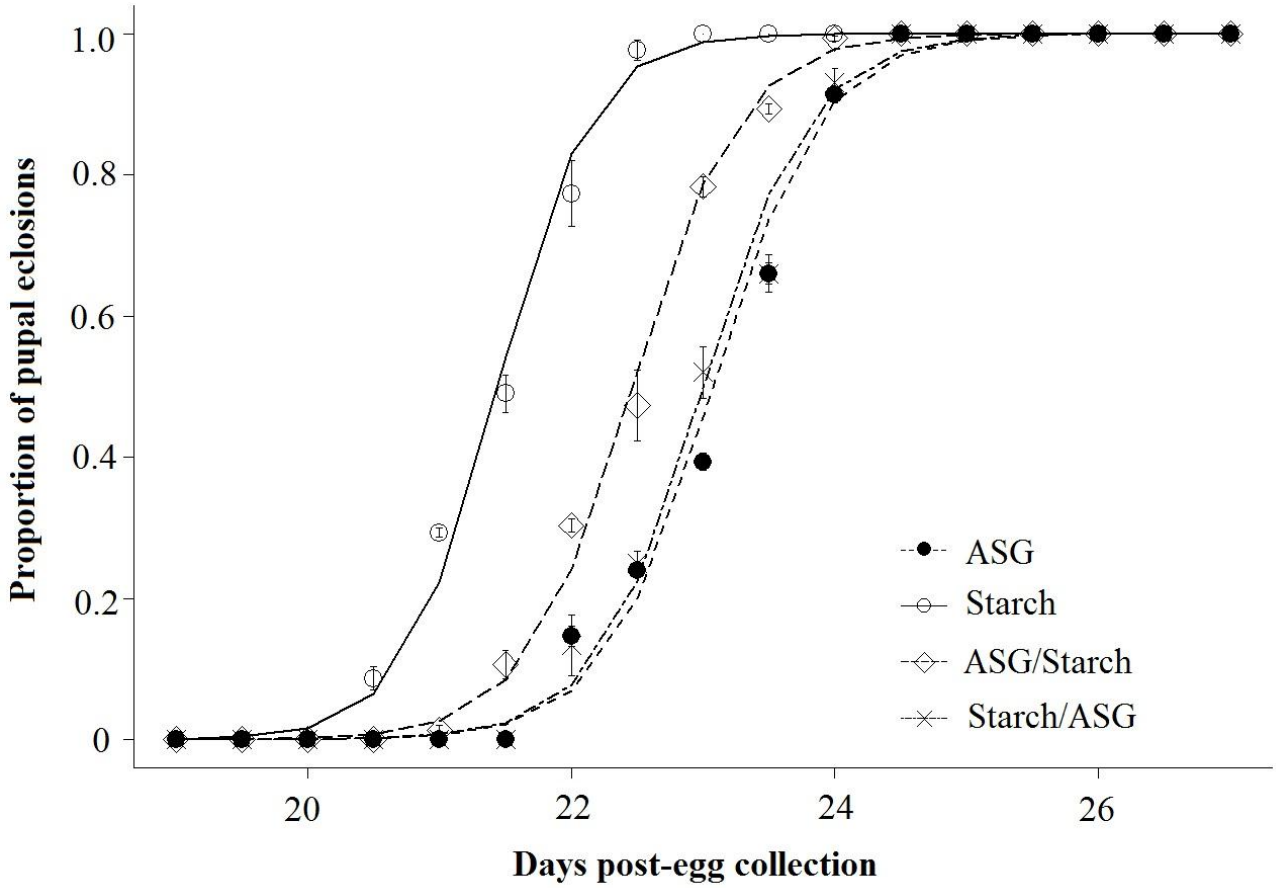

Fig 6.10 Proportion of pupae eclosing per day of 3 replicates of 100 randomly sampled pupae from each of four larval rearing treatments. A regression line was fitted to each distribution. ASG-reared flies (ASG/ASG, Starch/ASG) took an average of 1.5 days longer for adult eclosion than did Starch-reared flies, while Starch-reared flies with ASG parents (Starch/ASG) took an average of 1 day longer to eclose than did Starch-reared lines.

6.5 Discussion

The propagation of replicated populations of medfly lines from a single progenitor stock on ASG and Starch larval diets resulted in a significant change in the composition of gut bacterial communities. Flies reared on the ASG diet had 18% more Lactobacillale bacteria in their gut than flies reared on Starch food, and fewer Enterobacteriaceae. This is consistent with reports that Lactobacillales are primarily associated with nutrient rich resources. In line with this idea, ASG has a greater availability than the Starch diet of sugars, and could represent a better substrate for the growth of these gut microbes (Ljungh & Wadstrom, 2009).

Interestingly previous analyses using Suicide Polymerase Endonuclease Restriction (SuPER) PCR (designed to amplify low abundance DNA fragments) detected a minor, but stable, bacterial community of *Pseudomonas* at about 0.005% of the total medfly bacterial population (Behar *et al.*, 2005, Behar *et al.*, 2008a, Behar *et al.*, 2008b). *Pseudomonas* is known to be pathogenic to the nematode *C.elegans* and *Drosophila* (Apidianakis *et al.*, 2005, Hilbi *et al.*, 2007), and may have important effects on the life history of the medfly. However, I did not detect *Pseudomonas* in this study, indicating that the clone library approach, and sequencing of c.100 colonies per treatment will not provide resolution to detect bacterial species present at < 1% of the total gut microbiota.

In many species of holometabolous insect, there is a complete disconnect between the bacteria associated with the larval and adult life stages, as, prior to pupation, many larvae will eject the entire contents of their gut. This process does not seem to occur in medfly, although the guts of newly eclosed adults are depleted in bacteria (Lauzon & Potter, 2008). It has also been reported that medfly often do not feed for the first 24 hours post-eclosion during the period in which the peritrophic membrane forms. This may allow bacteria that are carried over from the pupal stage to recolonise the adult gut (Behar *et al.*, 2009). My results are consistent with these findings - despite a shared adult food source, flies with ASG or Starch-based larval diets had divergent proportions of key gut microbes, which would not be possible without at least some carry-over of the larval gut microbiota.

Despite the alterations in larval diet and in the adult gut bacteria, no assortative mating was detected. Hence there was no evidence for effects of gut microbes on mate choice, in contrast to the findings in *D. melanogaster* (Sharon *et al.*, 2010). The lack of an effect on mate choice by alterations to the gut microbiota is perhaps not surprising. The medfly is highly polyphagous, and geographically isolated wild populations show little or no reproductive isolation over years of study, despite likely exposure to a much wider range of microbes than engineered here (Cayol & Zarai, 1999, Cayol *et al.*, 2002, Lux *et al.*, 2002, Behar *et al.*, 2009). However, in Sharon *et al.* (2010), the observed assortative mating effect was attributed to a single species of bacteria *Lactobacillus plantarum*, which was not found in the medfly gut. It remains to be demonstrated whether exposure to this or other bacteria associated with different medfly populations might be capable of inducing mate choice behavioural changes in their hosts. Ringo *et al*. (2011), in a commentary on their findings in *D. melanogaster* (Sharon *et al.*, 2010), pushed for a synergistic view of microbiota and hosts, whereby each evolves together as a single unit of evolution, a 'hologenome' (Zilber-Rosenberg & Rosenberg, 2008). While the failure to replicate a bacteria-induced assortative mating effect in medfly does not rule out the potential for the gut microbiota to play a wider role in the process of reproductive isolation and speciation, it does not yet widen the taxonomic distribution of this reported effect beyond *D. melanogaster*. In addition, the evolutionary pressures and mechanisms that could drive such an effect in non-obligate symbionts remain to be discovered. Therefore, the wider evolutionary significance of gut bacteria associated effects on host mate choice remains unclear.

There was a significant increase in mating latency for flies treated with antibiotics in this experiment. Previous reports suggest an association between antibiotic treatment and a reduction in the abundance of CHCs (Miller *et al.*, 2010, Sharon *et al.*, 2010). This has been attributed to an interaction between bacteria and pheromone profiles, though it has not been conclusively shown that this is not a side-effect of the antibiotics themselves. In the current study, while antibiotic treatment had no effect on mate choice, it appeared to delay the onset of mating, which may be due to a dampening of the natural pheromones; this would need to be confirmed by pheromone extraction and Gas Chromatography-Mass Spectrometry (GC-MS).

Despite the lack of bacterial or dietary influence on assortative mating, there was a striking effect of larval rearing diet on male and female development time, and male body weight and mating success. Many previous studies have demonstrated the positive effect of male body mass on male reproductive success in this species, as well as the dramatic impacts of diet on male size, fertility and longevity (Churchillstanland *et al.*, 1986, Arita & Kaneshiro, 1988, Yuval *et al.*, 1998, Kaspi *et al.*, 2002). Female weight appeared not to differ significantly across different treatments, which fits with previous reports where female size is significantly less affected by larval diet, with females often showing a greater effect of dietary regime on protein reserves and ovarian development (Kaspi *et al.*, 2002).

In addition to the benefit of ASG rearing on males, male offspring of the flies reared on 'good' larval food, such as ASG/S flies, maintained significant differences in body weight and mate success over males reared for multiple generations on Starch. This trans-generational effect lasted for only one generation, as flies exposed to two generations of Starch rearing no longer displayed any body mass effects of their ASG heritage. There are three possible mechanisms that could underlie these trans-generational effects: 1) larval-bacterial interactions, 2) egg provisioning, 3) epigenetic inheritance.

1) Changes in protein and sugar concentrations of larval diets can have an impact on development time and adult male body size. Previous studies that have documented these changes have mostly ignored correlated changes in gut microbiota, despite it being understood that gut bacteria can dramatically affect the amount of food resources available to the host (Kaufman & Klug, 1991, Behar *et al.*, 2005, Behar *et al.*, 2008b). Within this experimental design, gut bacteria were cleared from the host post-adult eclosion, as such it is possible that the diet based effects observed were at least partially caused by gut microbiota. In the wild, female medflies have been observed to deposit many bacteria into fruits along with their eggs when ovipositing (Behar *et al.*, 2008a). It is possible that ASG females leave their offspring with more beneficial bacteria than their Starch counterparts, buffering the effects of transfer to a 'poor' diet and enabling developing larvae to access more of the proteins and sugars present in the larval substrate. In order to test this hypothesis, comparisons would need to be made between antibiotic treated, bacterial add-back substrates and control larval diets.

2) Female body size was not significantly affected by larval diets, however previous reports indicate that in medfly increased protein/sugar levels during larval development may result in greater ovarian development, rather than necessarily affecting female body size (Kaspi *et al.*, 2002). Egg laying rates were not monitored in this experiment; therefore the effect of nutrition on female fecundity is unknown. Observed less frequently is that females may vary in how much they provision their eggs with resources, often visible as variation in egg size (Gwynne, 1988). An increase in the provisioning of nutrients to eggs could explain the carry-over of differential male body size from ASG to one generation on Starch. An analysis of the size and contents of eggs laid from females reared on different larval media would be needed to assess this as a potential mechanism of trans-generational inheritance of the altered male phenotype.

3) Epigenetics is the process of modifying gene expression or phenotype without a change in the underlying DNA sequence. Epigenetic changes are associated with the post-translational modification of histones, which are thought to be mitotically but not meiotically stable, with epigenetic information therefore being erased at the start of each generation (Bonasio *et al.*, 2010). Recently this view has come to be challenged, as heritable transmission of small interfering RNAs (siRNAs) can maintain changes to gene expression across generations (Burton *et al.*, 2011). The mechanisms behind this process indicate that nuclear RNAi factors promote the maintenance of heritable silencing signals, but transcription is required to maintain multigenerational inheritance, such that a phenotype may persist into the F1 generation after an environmental change, but will not be evident in the F2 generation.

The correlated changes in development time and body mass between ASG and Starch lines were maintained in the offspring of ASG flies, even when transferred to the Starch diet, but only for a single generation. This fits well with observations that might be expected from siRNA inheritance, as without the environmental cues from ASG food, siRNA transcription ceases along with the changes to larval development so that the F2 generation receive no benefit. It raises the exciting possibility of investigating epigenetic processes and inheritance in this important pest insect. Further work could analyse the many expected differences in

the transcriptome between these two populations, and whether siRNAs can be detected in the eggs of medfly.

To conclude, though altered larval diets change the gut microbiota of adult flies, there was no evidence that the effects of diet on male body size and mating success were mediated by gut bacteria. Changes in nutrition are expected to result in later larval development and influence mating success. However the trans-generational effect is surprising and the mechanism by which it works is unknown.

6.6 References

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Chapter 7: General discussion of the thesis

In this chapter I give an overview of the main findings of the thesis, with ideas for further work based on my findings, followed by a discussion of the wider implications. In the latter section I discuss the role of context-dependent male reproductive success and the implications of this for studies of reproductive success, mass-rearing and assessment of RIDL/SIT lines. I also discuss the potential hurdles that must be overcome in terms of political and public resistance to the use of GM, before RIDL can be brought into the field.

7.1 Main findings

7.1.1 Variation in adult sex ratio alters the association between courtship, mating frequency and paternity in the lek-forming fruit fly *Ceratitis capitata* (Chapter 2)

In this chapter I investigated the role of altering adult sex ratio on the association between pre- and post-mating male reproductive success. I showed that there was a significant context-dependent association between pre-mating traits such as courtship frequency and mating frequency and post-mating reproductive success. Under high male competition, males delivered significantly more courtship but gained lower paternity than males under low competition. Paternity was positively correlated with mating frequency and small residual testes size; however the association between mating frequency and paternity was significantly stronger under low competition. The relationship between pre- and post-mating success is therefore plastic and alters according to the level of male-male competition. This result is important because it highlights the importance of post-mating processes in lekking species.

These results showed a significant mating advantage to males with a reduced body size. This is in contrast to previous reports showing an advantage of large body

size (Churchillstanland *et al*., 1986, Rodriguero *et al*., 2002, Anjos-Duarte *et al*., 2011) or no association with body size (Arita & Kaneshiro, 1988, Whittier *et al*., 1994, Whittier & Kaneshiro, 1995). Thus, whether there is a consistent role for size in male mating success remains unclear. It is worth noting that comparing across the studies above may introduce many potentially confounding factors such as the use of wild vs. mass-reared flies, or flies of different origins etc. It is probably not co-incidental that the only previous report to find a mating advantage to a smaller male body size was in a laboratory-based mating assay (Arita & Kaneshiro, 1988). Such tests performed under relatively crowded conditions in which the mating arena is restricted in size may result in an advantage to smaller males through greater manoeuvrability. This result has potential implications for mass-rearing, which typically takes place under extremely high density. The sexual selection for large males in the wild may therefore be completely relaxed or even reversed under mass-rearing conditions in which there are no lekking sites and limited opportunities for males to aggressively compete for prime 'hotspots' of female activity (Shelly *et al*., 1994). Further studies focusing exclusively on the effect of male body size as a factor in mating success are needed to resolve this issue. Tests would need to take place in both lab and field-cage settings under varied densities and sex ratios in order to confirm that size-biased matings are context-dependent.

There was also a significant paternity advantage to small residual testes size, a surprising result that could signify a trade-off with other life history traits such as overall body size, lifespan or manoeuvrability (Sella & Lorenzi, 2003). Alternatively testis size could be constrained by selection on the size of other reproductive tissues such as accessory glands (Nijhout & Emlen, 1998). Accessory gland secretions are known to induce female refractoriness to re-mating in medfly (Jang, 1995), though the mechanisms by which these products work and the effect of accessory gland size itself are currently unknown. Further work is needed to both accurately measure the short and long male accessory glands as well as to quantify accessory gland production and transfer during copulation (Sirot *et al*., 2009).

The most striking finding in this experiment was the breakdown in the correlation between pre- and post-mating success under increased levels of male competition. In lekking species such as the medfly, the key determinants of a male's reproductive success are usually thought to be success in pre-mating competitions.

However, my results show that post-mating reproductive success can vary widely under different contexts and is equally, if not more important, to measure in lekking species. The results show the context-dependent nature of male reproductive success, in that the characteristics that predict successful males in one environment may be unreliable in a different environment. Elucidation of these predictors will allow a better understanding for how selection will act on male reproductive success as the operational sex ratio changes, and may have implications for mass-rearing regimes used for SIT/RIDL. Successive generations of exposure to mass-rearing conditions can degrade male courtship behaviour (Briceno & Eberhard, 1998). My results show that males may be undergoing a shift towards sexual selection on post-mating processes in mass-rearing facilities, which will have serious consequences when such strains are released into the wild for population suppression.

7.1.2 Male reproductive success does not respond to artificial selection through the manipulation of adult sex ratios in *Ceratitis capitata* (Chapter 3)

In Chapter 2 I showed the potential for different adult sex ratio regimes to alter the sexual selection pressures on male reproductive traits, shifting the focus from pre- to post-mating processes as the levels of male-male competition increased. In Chapter 3 I built on this work to test whether these effects could also be observed following the establishment of long-term adult sex ratio selection regimes. However, after 50 generations of selection there was no evidence for alterations in mating competitiveness or reproductive success for any of the selection lines.

The selection experiment used three replicate lines for each regime of male-biased, equal sex and female-biased adult ratios. There was generally significant variation between replicates; hence any smaller signal due to selection regime could not be detected. One solution to this problem would have been to use increased numbers of replicates for each selection regime, to give greater statistical power to resolve small effects. However, this would not have resolved the problem of large variation between replicates *per se*. Such variation may indicate that the replicates were subject to significant levels of genetic drift. Genetic drift occurs in populations

due to the random fluctuations in reproductive success by certain individuals, this can cause changes in allelic frequencies over time separate from any natural selection of the genes involved. In small, reproductively isolated population, this can cause large changes in the frequencies of alleles between populations (Masel, 2011). Despite this, the lack of any selection regime effect was still surprising, because related studies conducted on similar scales with similar numbers of replicates and sample sizes have revealed a number of different responses to selection on adult sex ratio (Wigby & Chapman, 2004, Crudgington *et al.*, 2009).

In chapter 2 I observed significant alteration in the predictors of male reproductive success with changes in the proximate adult sex ratio. The prediction was therefore to see similar effects upon evolutionary manipulation of adult sex ratio. However this did not occur, similar-scale manipulations in sex ratio over many generations did not lead to the expected divergence in pre- and post-mating characteristics between lines. It is well known that substantial inadvertent selection can take place in mass-rearing, leading to alterations in male courtship behaviour and eroding a male's mating success with wild females. This is separate from initial 'founder effects' or 'bottlenecking' that occurs by selecting for individuals that will breed in captivity (Briceno *et al*., 1996, Briceno & Eberhard, 1998, Briceno & Eberhard, 2002). Initially therefore we expect sufficient genetic variation in male courtship to enable adaptation to the mass-rearing environment. However it is possible that the mass-rearing process in the TOLIMAN base strain used as the progenitor for the selection lines subsequently eroded genetic variation minimising adaptation to selection regimes.

The mass-rearing process selects for divergence from the phenotypes expressed in wild males and females. For example, mass-rearing selects for faster and more synchronised development (Carey, 1982, Cayol, 2000). As a result of this and the crowded conditions, females are selected for high egg production and viability. As a result the benefits of mate choice in mass-reared females may not be expressed and indeed opportunities for mate choice may be limited. This presumably explains why mass-reared females tend to show increased acceptance of male courtship attempts (Calkins, 1991, Briceno & Eberhard, 2000, Briceno & Eberhard, 2002). Mass-reared males are simultaneously selected for shorter courtship durations and, as suggested by the results in chapter 2, increased investment in sperm

competition (Briceno *et al*., 1996, Briceno & Eberhard, 1998). It is possible that, under this suite of strong selection pressures, insufficient genetic variation is then available for responding to relatively weak selection for altered adult sex ratio.

The effect of mass-rearing on genetic variation in mass-reared populations has been extensively debated in the context of whether it is legitimate to use sexual selection models in SIT release decision making (Lance & McInnis, 1993, Boake *et al*., 1996). The results from Chapter 2 suggest that phenotypic manipulation of adult sex ratio results in the expression of sufficient variation upon which selection could potentially act. Yet it apparently does not do so. Further investigation is therefore required ideally with a greater number of selection line replicates (10 or 20, which would also further explore the contribution of genetic drift) and more intense selection pressure (e.g. more extreme sex ratios, see Reuter *et al* 2008, or more extreme mating system manipulation, e.g. monogamy vs. polyandry). This would better address the issue of whether it is plausible to generate strains containing more competitive males for use in control programs.

7.1.3 RIDL construct design for embryonic lethality in the medfly (Chapter 4)

In chapter 4 I developed several new lines of transgenic medfly in order to test whether a RIDL system with earlier acting lethality than existing strains could be created. Current strains utilise a one-component *tTA* positive feedback system, tTA build up in the cells of the flies induces toxicity at high levels leading to death. Ingestion of tetracycline prevents tTA binding to the tet responsive element (*tetO*), and prevents lethality. However, in the absence of tetracycline, build-up of the toxic tTA can still take time and lethality may be expressed later in development, e.g. at the third larval instar (Gong *et al*., 2005). I focused on whether the development of a two-component RIDL system using an early acting promoter to drive *tTA* expression and with a tet responsive element driving expression of a lethal product, could be used to more effectively induce embryonic lethality. The embryonic promoter *DmCG31626* did induce expression of *tTA* from 48 hours post-zygotic formation in construct OX4301. Two constructs containing *ReaperKR* (Olson *et al*., 2003), a

strong driver of apoptosis, were generated (OX4423 and OX4536). Each had a minimal promoter derived from heat shock protein genes *hsp70* and *hsp83*, respectively. However, neither constructs induced lethality in crosses with OX4301, nor with a constitutively active *tTA* construct OX4196.

The inability to induce lethality from *ReaperKR* expression is surprising. It drives apoptosis strongly and induces lethality in other insects such as *D. melanogaster* and the cotton bollworm (*Helicoverpa zea*), and has been used successfully in previous genetic constructs made by Oxitec. A successful *ReaperKR* construct might feasibly be generated in future work if more tet responsive elements (*tetO*) were placed in front of the *hsp* minimal promoter. This could increase the sensitivity of the construct to tTA levels, and increase expression levels of *ReaperKR* when flies are reared in the absence of tetracycline.

7.1.4 Genetic elimination of the Mediterranean Fruit Fly (Chapter 5)

I characterised life history traits for two conditional female-lethal RIDL strains (OX3864A and OX3647Q). These strains exhibit genetic sexing, fluorescent marking and could in principle be used for population control via ‘genetic sterility’, i.e. through the induction of female-specific lethality. OX3647Q displayed a reduced fitness compared to wild type, but generally had higher fitness than a classical genetic sexing strain *tsl*. OX3864A performed more favourably. Its life history characteristics were comparable to the wild type strain from which it was derived, making it significantly superior to existing genetic sexing strains. The mating competitiveness of both transgenic strains was high, with neither strain displaying any reduction in mating ability compared to wild type.

OX3864A was tested further due to its favourable rearing characteristics in the glasshouse tests. In field cage trails in Crete, this strain was set up in mating competition tests with wild-derived males and females. In small field cages, the mating competitiveness of the OX3864A strain was not significantly different to wild-derived males. I also showed that weekly releases of OX3864A males into stable populations of wild type medfly eradicated the target population. In conclusion

I showed that the OX3864A strain is a viable alternative to, and a potentially significant improvement on, the use of current genetic sexing strains in SIT for population control.

7.1.5 Larval diet alters associations with commensal gut bacteria and induces trans-generational effects on body size and male mating success in *Ceratitis capitata* (Chapter 6)

In the final data chapter, I described how I tested the hypothesis that splitting a population of medfly into two allopatric groups maintained on altered larval diets would alter their gut microbiota and mate choice decisions. Recent work has shown the extensive influence that specific gut microbes can have on host life history characteristics and behaviour (Dillon & Dillon, 2004, Sharon *et al*., 2010, Heijtz *et al*., 2011, Sjögren *et al*., 2012). A study in *D. melanogaster* reported that lines of flies separated onto two different foods developed a strong mating preference for flies derived the same food type, an effect which was completely removed by antibiotic treatment (Sharon *et al*., 2010). In the chapter 6 study I attempted to demonstrate this effect in medfly. This was to test the idea that the poor mating competitiveness of mass-reared flies in comparison to their wild counterparts (Eberhard, 2000, Briceno & Eberhard, 2002, Briceno *et al*., 2002) could be at least partly explained by effects of their divergent gut microbiota on mate choice.

I found significant variation between the populations reared on different diets in terms of the relative abundance of gut bacterial families. However, this did not influence any aspect of mate choice or male reproductive success in comparison to antibiotic treated flies. Antibiotic treatment produced a significant delay in the onset of mating. However it is unclear whether this is due to the removal of gut microbes or a side-effect of the antibiotics themselves. Interestingly, I showed that a sugar-based larval diet gave males a significant mating advantage and increase in body mass over males reared on a starch-based larval diet. This effect persisted even after a single generation of starch rearing, so that offspring of sugar-reared flies maintained an increased body mass and mating success rate.

There was no evidence that the effects of diet on body size and mating success were mediated via gut microbiota, as they also occurred in antibiotic treated flies. Changes in nutrition are expected to alter development and influence mating success (Kaspi *et al*., 2002, Shelly *et al*., 2002, Shelly & McInnis, 2003). However, the trans-generational effect is surprising, and the mechanism by which it works is unknown. Here I list three possible mechanisms. 1) Larval-bacterial interactions. The divergent gut microbe communities on different diets allow differential accumulation of nutrients by developing larvae; these are at least partially vertically transmitted to the next generation, providing a buffer against a nutrient poor (Starch) larval diet. 2) Females reared on the sugar-based diet produce larger and/or better provisioned eggs, which improve the development of their offspring. 3) Epigenetic inheritance produces a temporary change in the expression of genes that are responsible for body growth and development.

7.2 Implications and future directions – what makes a successful male?

Studies of the sexual behaviour of medfly have been carried out for decades, and the full repertoire of male and female sexual traits have been extensively characterised (Dìaz-Fleischer & Aluja, 2000, Eberhard, 2000, Robinson *et al*., 2002). Despite this work, there are few syntheses. Many reports are robust in their own right but give conflicting predictors for what characteristics are associated with increased male reproductive success. For example, as noted above, male body size is reported to have a positive association with mating success, no influence or to be negatively associated (Churchillstanland *et al*., 1986, Arita & Kaneshiro, 1988, Whittier *et al*., 1994, Whittier & Kaneshiro, 1995, Rodriguero *et al*., 2002, Anjos-Duarte *et al*., 2011). Another example is the symmetry of the superior frontal orbital setae, which is associated with increased male mating success in some reports (Mendez *et al*., 1999, Rodriguero *et al*., 2002) but not in others (Hunt *et al*., 2002). However, one consistency is that male mating success is generally significantly related to the level of male sexual activity. Males that perform more courtship and court the most number of females achieve most matings. However, this relationship is weak and is

unlikely to be the sole determinant of mating success (Whittier *et al*., 1994, Briceno & Eberhard, 2000, Lux *et al*., 2002). Courtship frequency is sexually selected and interacts significantly with nutritional status (Briceno & Eberhard, 2002, Briceno *et al*., 2002). For example, high protein diets appear to increase a male's ability to attract females to leks, and increase courtship frequency and mating success in males (Yuval *et al*., 1998, Shelly *et al*., 2002, Shelly & McInnis, 2003). However high protein-fed males also suffer significantly higher mortality under 24 hour starvation conditions in comparison to sugar-only fed males (Kaspi & Yuval, 2000). As such the optimal protein levels needed in order to balance longevity vs. courtship success is unknown. Finally the composition of the gut microbiota is also reported to have significant effects on longevity, health and reproduction. The ingestion of particular bacteria (i.e. *Klebsiella oxytoca*), increases male sexual competitiveness (Ben Ami *et al*., 2010, Gavriel *et al*., 2011). However, there is considerable debate as to the relative costs and benefits of a gut bacterial 'load' on general health and longevity. One report found a significant increase in longevity when flies had the entire gut microbiota removed via antibiotic treatment, but this effect was not evident when flies were maintained on a sugar-only diet (Ben-Yosef *et al*., 2008a). Other studies have found that *Enterobacter spp* may play a key role in protein acquisition for medfly and their presence and activity can increase host longevity (Behar *et al*., 2005, Behar *et al*., 2008, Ben-Yosef *et al*., 2008b). Energetic status, courtship frequency, nutritional status and composition of the gut microbiome are therefore likely part of a complex inter-dependent framework that determines male reproductive success through a combination of natural and sexual selection. The bacteria-host dynamic is currently severely under-researched, almost nothing is known about the effects of adaptation to mass-rearing by the host and exposure to standardised diets has on the gut microbiota. Further work into the bacteria-host relationship and the impact of mass-rearing is likely to produce significant gains in understanding for sexual selection theory. Practical interest stems from the impact this could have on SIT and RIDL. Both techniques have the potential for severe effects on the health of the gut microbiota through the use of irradiation and antibiotics, respectively (Thomas *et al*., 2000, Lauzon & Potter, 2008, Ben Ami *et al*., 2010).

In summary consistent, global determinants of male reproductive success have not yet been identified, and yet female choice of males is non-random (Whittier *et al*., 1994, Whittier & Kaneshiro, 1995, Lance *et al*., 2000). There is increasing evidence; some significant elements of it gained through the research reported in this thesis, that the answer to this paradox is that the determinants of male reproductive success are context-dependent.

7.2.1 Implications for future studies into male reproductive success

The extensive literature on mating behaviour in medfly often fails to fully consider the context in which the different studies have taken place. As noted above, this may in part explain some of the conflicting results. The most dramatic and consistent changes in male mating behaviour result from the capture of wild strains and their colonisation into the mass-rearing environment (Briceno & Eberhard, 1998, Eberhard, 2000, Briceno & Eberhard, 2002). It is well known that these changes result from more than 'founder effects', 'genetic bottlenecks' or 'genetic drift'. Many of the changes are adaptive, predictable adjustments in behaviour in response to the very different and intensely competitive conditions consistently found across multiple mass-reared strains (Briceno & Eberhard, 1998, Pereira *et al*., 2007). This shows that male characteristics which increase reproductive success are not absolute, but dependent on the individual's environment. My work in chapter 2 demonstrated that alterations in adult sex ratios can significantly alter the likely sexual selection pressures on males, and whether traits that increase reproductive success are dependent on the availability of mates and levels of competition.

Sexual selection research is increasingly incorporating the role of the sexual conflict environment in mating studies, as it becomes increasingly apparent that different genotypes can gain different levels of fitness depending upon the availability of females, the presence of rivals, the population density and the balance between sexual selection and natural selection pressures (Crudgington *et al*., 2010, Rodríguez-Muñoz *et al*., 2010, Brommer *et al*., 2011). Research in the medfly needs

to incorporate these advances in order to make further progress in determining what makes a successful male.

7.2.2 The future of RIDL in the medfly

My work in this thesis demonstrates the potential for RIDL to surpass existing genetic sexing strains and SIT in suppressing target populations of medfly. RIDL strains show excellent rearing qualities and high mating competitiveness, with few negative effects of transgenesis. In addition, the caged suppression trial experiments showed that it is possible, in principle, to use the female specific dominant lethality of the RIDL flies as a population suppression mechanism. Field cage mating experiments showed no difference in mating success between RIDL males and wild-derived males; so that in initial tests the OX3864A RIDL strain surpasses the performance of existing SIT strains (Shelly & McInnis, 2003). However, my work, in conjunction with the existing literature, has highlighted the context-dependent nature of male reproductive success. The mating competitiveness tests with RIDL males in this thesis have taken place in fairly artificial environments at a relatively high density. In the wild leks usually consist of 3 to 4 males, under SIT conditions this can rise to between 8-16 males (Shelly *et al*., 1994, Shelly & Whittier, 1996). As such, the mating tests carried out with line OX3864A represent the extreme end of male density and is likely to far exceed female density in the wild. Therefore, while these initial assessments indicate high competitiveness of RIDL strains, confirmation will only be possible with the use of RIDL strains in controlled release studies. In any future RIDL suppression program, the success of released males will depend on their ability to mate successfully under very different density and sex ratio conditions to those that have been conducted in the laboratory. Once released, male reproductive success in wild insect populations will become dependent both on natural and sexual selection (Rodríguez-Muñoz *et al*., 2010).

A substantial hurdle that must be overcome before release trials can take place is any public and political opposition to the use of genetic modification technology. RIDL technology has so far been trialled in *Aedes aegypti* mosquitoes in

open field releases (Harris *et al*., 2011). This mosquito is responsible for transmitting dengue fever, generally regarded as the most medically important arthropod-borne viral disease in the world, with 50-100 million cases each year and no commercial vaccine (WHO-TDR, 2006). However, there has been significant opposition to these trials from special interest advocacy groups such as GeneWatch UK (Enserink, 2010, Wallace, 2010). There are also stringent regulatory issues that must be dealt with in each country where RIDL technology is intended to be used (Convention on Biological Diversity, 2003). Whether these obstacles will be overcome to introduce RIDL as a commonplace technique to combat Dengue is currently unknown, and this will also have implications for the use of RIDL in the medfly. Only with the approval to carry out open field trials with the medfly will it be possible to truly test whether the benefits of RIDL over existing SIT make it a viable alternative for pest population control.

7.3 References

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Appendix I. Variation in adult sex ratios alters the association between courtship, mating frequency and paternity in the lek-forming fruitfly *Ceratitis capitata*. Journal of Evolutionary Biology. Online Early View. 2012.