6 Sex Ratio Manipulation for Insect Population Control

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6.1 Introduction

Genetic methods for the control of insect populations that pose a burden on human health or are agricultural pests have been in development for over 60 years. During the early 1960s, Knipling and colleagues, supported by the USDA, mass released sexually sterilized males to diminish populations of the screwworm *Cochliomyia hominvorax* over large areas of the USA in what is now known as the sterile insect technique (SIT) (Bushland *et al*., 1955; Krafsur *et al*., 1986, 1987). Even earlier than this, in field trials in Africa, Vanderplank demonstrated that local pest populations can be replaced or even eradicated by taking advantage of post-mating barriers between genetically isolated tsetse fly species (Vanderplank, 1947). The success of these experiments initiated a 'golden age' of insect genetic control (Gould and Schliekelman, 2004). A number of highly successful area-wide programmes were carried out that demonstrated that such strategies are species-specific and environmentally non-polluting and can serve as an alternative to already established methods using insecticides or habitat eradication (Knipling, 1955, 1979; Krafsur *et al*., 1986; Alphey *et al*., 2010). However, for a number of insects the use of SIT-based strategies has been unsuccessful. Particular emphasis is usually placed on the difficulty of sustaining the necessary ratio of sterile to wild males and the migration of wild individuals from neighbouring non-targeted areas (Dietz, 1976; Prout, 1978). Practical issues relating to the fitness of males sterilized by ionizing radiation or chemicals also hampered further advancements. In an attempt to overcome these complications, research has been focused on improving the efficiency of the genetic techniques and to develop systems that can improve the capacity of mass rearing facilities to meet the requirements that are necessary for effective population control. A significant emphasis has been placed on developing systems that ought to be more efficient than SIT, in terms of the sterilizing effect a single released individual imposes on the natural population. During the 1960s to 1970s research focused on the use of natural sterility (hybrid sterility or cytoplasmic incompatibility), translocations, meiotic drive or conditional lethal traits (Whitten, 1985). Although significant progress was made, rarely did this effort translate into truly large-scale implementations due to the difficulty of establishing and maintaining insects with the required characteristics (Schliekelman *et al*., 2005).

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 Transgenic Insects (ed. M.Q. Benedict)

The recent developments of molecular genetic engineering and insect transformation have now re-ignited an academic interest to overcome many of the problems that were previously intractable using standard genetic tools (Handler, 2002; Gould and Schliekelman, 2004). This chapter will focus on the application of contemporary molecular and genetic techniques to manipulate the sex ratio of insect species. To clarify, sex ratio manipulation is said to occur, when within the pool of an individual's fertile offspring, one of the two sexes is overrepresented.

6.2 Overview of Applications and General Principles

In their most typical form, sex ratio distorting (SRD) alleles have been proposed as potential population suppression tools. The reasoning is that in a population of a sexually reproducing organism, induced extinction of one of the two sexes will diminish the population's fertility and could eventually result in the population being driven to collapse. Since the overall 'fertility' of a population is almost always determined by the fertility of its females, which are rate limiting in gamete production, alleles are designed to bias the sex ratio towards male production and to eliminate female offspring. Modelling suggests that the release of SRD alleles can represent a significant improvement to SIT in terms of the potential fitness cost that each individual insect can impose on the population once released (Schliekelman *et al*., 2005). This improvement arises as surviving male offspring help to maintain the allele in the population, even if releases are terminated, until it eventually disappears. Under certain circumstances, SRD alleles could be engineered to display 'invasive' non-Mendelian segregation, resembling naturally occurring selfish genetic elements. Such SRD alleles are designed to increase in frequency in the population once released, surpassing the initial release frequency and further amplifying the effect of release. Compared to SIT, survival of male offspring in the wild can also mitigate density-dependent effects and immigration of wild insects from non-targeted surrounding areas (Foster *et al*., 1988).

Apart from their use in population suppression, SRD alleles have also been proposed as a fundamental technological innovation for the sexing of the laboratory population prior to its release in the natural environment. In such programmes, the release of females provides no benefit, and can in fact undermine the impact of the operation by limiting the dispersal of the released males (Ailam and Galun, 1967). More importantly, when targeting insects like mosquitoes or agricultural pests in which only females are responsible for damage, release of females may not be acceptable as these can further exacerbate disease transmission or economic losses. Segregation of the sexes is particularly advantageous when sexes are differentially sensitive to the sterilization treatment, e.g. females of the screwworm that required more than double the dose of radiation (Hoy *et al*., 1979). Finally, an economic factor also arises by unnecessarily rearing individuals that are subsequently destroyed. This dictates that a system that can effectively eliminate females early in development in the rearing facility would greatly enhance the practicality of any technique that relies on the mass release of modified insects. The success of a programme that uses SRD alleles for population suppression is intimately linked with the effect that sex ratio manipulation has in the context of strain maintenance and rearing in the laboratory, and obviously vice versa. For example if males were engineered to produce only viable male offspring, without some form of conditional repression system to suppress the phenotype in the facility, continual backcrossing to suitable females would be required every generation. Moreover, while a small decrease in fitness could be accepted in the facility if it significantly improved the overall performance of the SRD trait, this cost may not be equally acceptable in field applications.

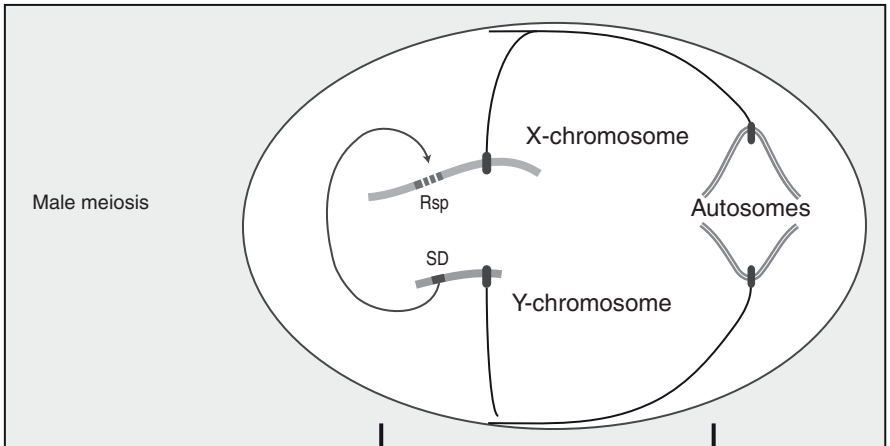
In one of the earliest seminal articles on the subject, Hamilton (1967) discussed how

SRD alleles could be applied to eradicate mosquito populations, as he demonstrated that under certain conditions, non-Fisherian (1:1) sex ratios could arise and be maintained naturally. He considered a population in which males are the heterogametic sex (XY). In such a population, the relative sex ratio is essentially dependent on the frequency of X chromosome sperm to Y chromosome sperm being used in limiting fertilizations. He proved that mutant Y chromosomes that can bias fertilizations in their favour, such that a male only produces sons, gain a selective advantage that allow them to spread within the population. As the Y chromosome spreads, the sex ratio of the population becomes more and more male biased. As a result the population will become smaller and will eventually collapse if the final wild female mates with a male carrying the mutant Y chromosome. This example of sex ratio distortion essentially portrays an invasive Y chromosome meiotic drive system, which interferes with the production of X-bearing sperm. Its main advantage, as far as genetic population control is concerned, is the invasiveness that the mutated chromosome (via the contained mutant allele) gains from effectively eliminating the competing X chromosome during gametogenesis. With time, the mutated Y chromosome becomes more and more abundant, effectively out-competing its wild-type ancestor. In the absence of resistance against the novel mutation, the mutated Y chromosome will eliminate the X chromosome and eventually lead to population collapse due to the lack of females. Because this bias arises through unequal gametogenesis in the parent prior to fertilization (prezygotic), it does not have to result in an overall reduction of fecundity of the male. We will examine naturally occurring meiotic drive systems that result in sex ratio manipulation and highlight how synthetic versions of these are being engineered. Synthetic distorters have the advantage that they would be unaffected by widespread resistance alleles counteracting natural drive systems. Note that prezygotic sex ratio distorters do not necessarily have to be invasive, as insertion of alleles that could eliminate X-bearing sperm on autosomal loci can still be more effective than SIT, even if these distorter loci would eventually be lost (Schliekelman *et al*., 2005).

Distorted sex ratios can also arise through post-zygotic mechanisms, when the survival of one of the sexual fates is selectively diminished, e.g. female-specific killing (FK). Advances in the understanding of the sex determination pathways of target species may result in opportunities to develop novel SRD strains and examples are discussed. Males generated by such sex reversion are often referred to as phenotypic males (PM), since genetically these individuals should be of the other sex given their chromosomal complement.

6.3 Meiotic Drive

Meiotic drive systems, or segregation distorters (SD), a term that also encompasses transmission anomalies that are not strictly meiotic, alter the normal process of meiosis with the consequence that an effective gametic pool with an excess of one allele type is generated (Zimmering *et al*., 1970). Meiotic drive systems basically operate at the level of allele competition and the conflicting alleles are usually described as the driving locus (the SD allele) and its responder locus (the alternative allele). The SD allele can technically only be called a driving locus (that can increase in frequency in a population) when the allele is over-represented amongst the gametes of an individual. If the alternative allele is equally represented in the fertilizing gametic pool, even if these do not result in viable offspring, as would have occurred without an active SD allele, then the effect of segregation distortion will not result in meiotic drive (Fig. 6.1). In this case, as excess recovery of the SD allele in the next generation does not result from a net gain in fertilization events, but what is essentially a fecundity loss for the parent (Lyttle, 1991), and is related to FK strategies discussed in section 6.4. At the population level such an allele may increase in frequency in spite of deleterious



Mature sperm	Y-chr fertile shredded X-chr dead	Y-chr
Gametic *SRD*	no	yes
Offspring *SRD*	yes (females inviable)	yes
Fertility cost to male	yes	no
Meiotic drive	no	yes

Fig. 6.1. Schematic of a Y-chromosome linked segregation distorter locus and the effect of gamete recovery on meiotic drive. A Y-linked segregation distorter (SD) specifically targets and cleaves its responder locus (Rsp) by dsDNA breaks, located on the alternative sex chromosome during male meiosis, leading to shredding of the X chromosome. Meiotic drive of the SD-bearing Y-chromosomes only occurs when shredded X chromosomes are eliminated from the fertilizing gametic pool (gametic sex ratio distortion, SRD). When the X-chromosome-bearing sperm are represented in the gametic pool, while offspring SRD will occur, this will happen at a fecundity cost to the male, as his daughters are inviable.

physiological effects (Hamilton, 1967). When SDs are physically linked to sex determining loci or sex chromosomes, meiotic drive will result in an unequal distribution of sexes in the next generation.

The phenomenon of meiotic drive was first described in detail in the fly, when workers measuring the fitness of second chromosomes taken from wild populations identified *Segregation Distorter* (*SD*) in

Drosophila melanogaster. *SD* has now become the most intensively studied example of meiotic drive with over 50 years of work elucidating its underlying biology (Sandler and Hiraizumi, 1959; Sandler *et al*., 1959). When *SD* is present in a heterozygous male, *SD*-bearing sperm are typically generated in excess of 95–99% (Hartl *et al*., 1967), as wild-type (SD^{+}) sperm do not achieve proper sperm individualization because they fail to undergo correct histone transitioning and chromatin condensation (Tokuyasu *et al*., 1977). The complex is composed minimally of two major elements, the driving *Sd* gene (note that *Sd* denotes the gene on the *SD* chromosome), and the *Responder* locus (*Rsp*). The *Sd* gene encodes a truncated duplication of a *RanGAP* gene, which mis-localizes in the nuclei of developing sperm. This results in a reduced concentration of nuclear Ran-GTP and disrupts the normal Ran signalling pathway (Merrill *et al*., 1999; Kusano *et al*., 2001, 2002). *SD* chromosomes typically carry numerous modifiers of drive. The most studied is *Enhancer of Segregation Distortion* (*E(SD)*), which is required for full expression of the *SD* phenotype (Brittnacher and Ganetzky, 1984). *Sd* and *E(SD)* are located on chromosome 2L, approximately 1 map unit apart and *Rsp* is located on a heterochromatic region of chromosome 2R. *Rsp* alleles range continuously in sensitivity to *SD* activity from supersensitive (Rsp^{ss}) through standard sensitivity (Rsp^{s}) to total insensitivity (Rsp^{i}). These alleles are not known to be associated with any other discernable phenotypes and only act in *cis* to cause sperm dysfunction: moving Rsp^{s} to a new chromosome makes that chromosome sensitive to distortion (Brittnacher and Ganetzky, 1984). Consistent with the genetic behaviour that *Rsp* functions as a *cis*-acting element (i.e. on the chromosome that it is located on) and not by encoding a diffusible product, *Rsp* sensitivity has been shown to correlate with the number of repeats of a 120-bp sequence (Wu *et al*., 1988), though how this relates to RanGAP function remains unknown (Kusano *et al*., 2003).

Explorations into the molecular details behind *D. melanogaster*'s *SD* and those of other organisms, alongside considerations on their evolutionary stability, have highlighted some basic principles that are thought to be shared by all SD complexes: SDs are minimally composed of the SD and its responder locus (often called target). The SD locus interacts in *trans* with the responder, which in turn exerts its role in *cis*. To become established at all, there must be sufficiently tight linkage between the SD and its target locus to allow for the generation of linkage disequilibrium, with an excess of insensitive and sensitive target alleles in *cis* and *trans*, respectively, to the distorter allele (Prout *et al*., 1973; Lyttle, 1991). Linkage of the SD locus with insensitive responder alleles, guarantees that SD activity does not become autocidal for the SD-carrying chromosome (Charlesworth and Hartl, 1978). Linked modifiers should also evolve linkage disequilibrium, with the SD allele found in coupling with enhancers, like *E(SD)*, and in repulsion with suppressor alleles at the same secondary modifier locus (Lyttle, 1991). Chromosomal rearrangements like inversions and heterochromatin enhance linkage by suppressing crossing over and recombination between these elements (Thomson and Feldman, 1974). The majority of known SDs function during male gametogenesis where gametes that carry the responder allele manifest sperm dysfunction or demise (Lyttle, 1993; Taylor and Ingvarsson, 2003). Sex-linked SD is more common in systems with male heterogamety, and usually it is the X chromosome that drives against the Y. Since recombination between sex chromosomes of heteromorphic males is already greatly reduced or eliminated, sex chromosomes are well suited genomic sites for meiotic drive systems to inhabit, and indeed sex chromosome SDs are over-represented in nature (Hammer, 1991; Lyttle, 1991).

Meiotic drive systems in which the X drives against the Y are not likely of practical use in insect control programmes, though a population could theoretically be brought to collapse by the lack of males. Cases of Y-linked SD in insects occur in culicine mosquitoes. Both mosquito species that have been found harbour these sex ratio

distorters, *A. aegypti* and *Culex pipiens*, actually have homomorphic sex chromosomes where sex is determined by a dominant male-determining allele (*M*) on chromosome 1 (Gilchrist and Haldane, 1947). Males are heterozygous at the sex-determining locus *Mm* and females represent the homozygous *mm* condition. The meiotic drive locus only functions when it is located in *cis* to *M* and is denoted as the M^D gene. M^D acts in *trans* on a responder locus that is proximal to and indistinguishable from *m*. The sensitivity of the *m*-bearing chromosome to M^D varies widely from sensitive (m^s) to insensitive (m^i) (Suguna *et al*., 1977; Wood and Newton, 1991; Cha *et al*., 2006). Subtle enhancers and suppressors of M^D strength have been discovered on all autosomes of *A. aegypti*, which instil further variation in the expression of the male bias (Wood and Ouda, 1987; Wood and Newton, 1991). Cytological studies have shown that the male bias is associated with preferential breakage of chromosomes bearing m^s alleles during the early meiotic stages of spermatogenesis, which results in a decrease in female progeny (Newton *et al*., 1976; Sweeny and Barr, 1978). To maintain linkage disequilibrium, M^D resides in a genomic region of low recombination by associating with the centromere (Newton *et al*., 1974), which displays heterochromatic differences between female- and male-determining loci (Wallace and Newton, 1987; Shin *et al*., 2012). The M^D locus and the linked m^i allele have been discovered nearly worldwide but are not uniformly distributed (Wood and Newton, 1991).

The initial period of investigation on the biology of M^D in the 1960s and 1970s was not followed by an in-depth molecular characterization as most strains that were developed to characterize the locus were lost. Fortunately, as *A. aegypti* M^D occurs widely in the wild, a novel round of selection of sex ratio meiotic drivers was successful at identifying a strain that displays a strong male-bias of approximately 85% (Shin *et al*., 2012). This strain (named T37) was used to estimate the recombination frequency between the *M* and the M^D loci at around 5% and multipoint linkage mapping using microsatellite markers and known loci placed the M^D locus within a 6.5 cM interval to facilitate future cloning efforts. T37 is currently being used to investigate the genetic and molecular basis of the M^D mechanism of action and variation in strength, though barriers have to be overcome relating to the incomplete status of the *A. aegypti* genome assembly and lack of suitable markers (Shin *et al*., 2012). During the 1970s trials were initiated to assess its suitability for controlling natural populations of this mosquito using the M^D locus. These experiments revealed the swiftness with which resistance to M^D was selected for in females of cage populations, as predicted by Hamilton, and that the level of distortion ultimately attained was insufficient to achieve effective population control (Hickey and Craig, 1966; Robinson, 1983). However, with the aim of population replacement rather than eradication, M^D was recently proposed as a mechanism to drive desired transgenes into wild populations (Mori *et al*., 2004). Two possibilities were suggested. In the first case, M^D males could be released carrying m^i alleles to which the desired transgene is physically linked. As an initial effect, the population would experience sex ratio distortion, but as homozygous females carrying the released m^i allele would become more abundant, sex ratios would eventually re-stabilize at 1:1 with the transgenes being carried at high frequencies. Alternatively, the transgene could be coupled directly to a strong M^D locus. Progress in this direction is currently limited as the underlying genes that encode M^D function remain unknown. Work in *A. aegypti* is currently also hindered by the lack of high quality genomic data or a reliable system to assemble its highly repetitive genome. Finally, as natural resistance to M^D is already common in nature, only native populations that are highly sensitive could be targeted.

The efforts that have gone into applying M^d to insect control have highlighted the problems that could arise by using naturally occurring distorters for which resistance alleles are already in existence. Also, their

potential to be transferred to other target species is questionable as SD and responder loci are expected to have co-evolved. As a consequence, efforts have intensified to develop entirely synthetic sex distortion strategies. Work in *Anopheles gambiae* mosquitoes, which have heteromorphic XY sex chromosomes, is now being pursued with the long-term goal of inserting on the Y chromosome a transgene that can specifically destroy the X chromosome during male meiosis. The system under consideration relies on the expression of a Y-linked endonuclease that can cleave DNA sequences (15–30 bp) that are uniquely present on the X chromosome (Burt, 2003). Expression of such an endonuclease during male meiosis would lead to recognition and subsequent 'shredding' of the X chromosome, such that X-bearing sperm, which ordinarily give rise to daughters, are eliminated during spermatogenesis. As a result, transgenic males expressing such an endonuclease during meiosis would be developed that only generated viable male offspring. Preliminary work has shown that *A. gambiae* lends itself for the development of such a system on the basis of the genomic organization of its rDNA genes, which are exclusively located on the X chromosome in a tandemly arranged cluster composed of hundreds of copies.

The opportunity arose in the use of the naturally occurring, well-studied homing endonuclease I-*Ppo*I that has evolved to specifically cleave a 29 bp recognition sequence within the peptydil transferase centre of the 28S rDNA gene. To assess whether expression of I-*Ppo*I during spermatogenesis of *A. gambiae* would result in the selective cleavage of the X chromosome, transgenic lines were generated in which expression of I-*Ppo*I was driven from regulatory regions of the spermatogenesis-specific β2-tubulin gene (Windbichler *et al.*, 2008). Given the rarity of Y chromosome integrations, transgenic constructs were initially assessed in autosomal locations. Autosomal integrations would be expected to display distortions in the inheritance of the sex chromosomes if sperm harbouring shredded X chromosomes were incapacitated, though the construct itself, not being bound to the Y chromosome, would not directly benefit from the deviations and would thus not display meiotic drive. Surprisingly, transgenic I-*Ppo*I males induced dominant embryonic lethality in their offspring, which rarely progressed beyond cellularization stage of the embryo. When the underlying sex ratio of the inviable eggs was assessed using markers specific to the Y chromosome, it became clear that underlying the embryonic lethality, sex ratio distortion was actually occurring (90% males). The embryonic lethality phenotype was linked to activity of the I-*Ppo*I endonuclease, carried over in sperm, against the maternal X chromosomes in the fertilized embryos. To address the embryonic lethality and generate true sex ratio distorters, work is now underway that aims to restrict I-*Ppo*I activity to spermatogenesis and eliminate its carry-over effects in embryos. In the meantime, because I-*Ppo*I expression during spermatogenesis essentially leads to male sterility, these strains are now being assessed for their suitability in SIT programmes. Strains harbouring these constructs display good levels of competitiveness when measured by competition assays for mating with limiting females, though there is integration-site dependent variation (Klein *et al.*, 2012). Males of the I-*Ppo*I strains diminish egg-hatching rates with time once introduced in established indoor cage populations and can confer absolute infertility regardless of the strain or member of the *A. gambiae* complex to which they are outcrossed (Klein *et al.*, 2012).

As the location of the *A. gambiae* rDNA genes being located exclusively on the X chromosome is not universal, the identification of suitable native sequences that are unique to the X chromosome will be a prerequisite to explore this approach in other target species. Ideally target sequences should be present in multiple copies on the X chromosome. With the recent advances in the endonuclease re-engineering using a number of platforms including zinc-finger nucleases (ZFNs), TALENs or homing endonucleases (HEGs) it may now be feasible to design an endonuclease to target any

sequence (Gao *et al.*, 2010; Morbitzer *et al.*, 2011; Stoddard, 2011; Li *et al.*, 2012; Pan *et al.*, 2012; Schierling *et al.*, 2012; Xiao *et al.*, 2013). The availability of promoters that drive expression of such nucleases during male meiosis is another requirement and the *β*2-tubulin promoter has already been shown to drive expression of transgenes in a number of insects (Catteruccia *et al.*, 2005; Smith *et al.*, 2007; Scolari *et al.*, 2008; Zimowska *et al.*, 2009).

6.4 Sex-Specific Lethality

Historically, female killing (FK) systems have been developed as a complementary technology for SIT programmes that allow sexing of the release generation. It should be noted that the unwanted sex can also be separated from the other based on unique, sex-specific morphological or developmental features although sex-specific lethality systems have generally been favoured, since the unwanted sex is typically eliminated early in development, decreasing rearing and distribution costs (Robinson, 1983). Lethal systems also have the advantage that sex-specific elimination can be performed in a high-throughput manner at the level of a population (e.g. en masse treatment of embryos) with the treatment of a compound or by changing the laboratory environment (e.g. temperature). Sexing that relies on morphological discrimination must occur at the level of the individual insect and requires labour-intensive steps or automation. Morphological discrimination is also typically insect specific and relies on the availability of naturally occurring polymorphisms between the sexes. In a typical example, female pupae of the *Aedes* mosquito can be separated very effectively from males on the basis of size, females being significantly larger if reared in optimal conditions (Bellini *et al.*, 2007). On the other hand, pupae of the anopheline mosquitoes are not as amenable to size separation, with some exceptions including *Anopheles quadrimaculatus* and *Anopheles albimanus* (Mark Benedict, personal communication).

Traditionally, FK strains were generated by translocating naturally occurring mutations to the Y chromosome that conferred either resistance to chemicals such as insecticides, or heat-sensitivity. Loci conferring insecticide resistance, isolated from field populations, have been most extensively used, especially for mosquitoes (Lines and Curtis, 1985; Robinson, 1986, 2002; Shetty, 1987). In what is paradoxically the only benefit of the evolution resistance, using such strains, larvae are treated early in development with a discriminating dose of insecticide so that susceptible females are killed but males carrying the translocated resistance locus on their Y chromosome survive. Sexing strains were developed by translocation for nearly 20 species and were especially pioneered in the silkworm, the Mediterranean fruitfly, Australian sheep blowflies and a number of mosquito species (Robinson, 2002). For two of these, the mosquito *A. albimanus* and the Mediterranean fruitfly *Ceratitis capitata*, the sexing strains have been developed sufficiently to mass-rear at levels integrating the SIT. Currently, only the *Ceratitis* strain has been used in truly large-scale release operations over extended periods and it serves as a demonstration to the value of robust sex-separation methods (Franz, 2005). Strains are still being developed using translocation techniques, for example the novel sexing strains generated by translocation for *A. arabiensis* (Yamada *et al.*, 2012). The use of translocations to move naturally occurring polymorphisms to sex chromosomes is usually a laborious and often serendipitous task with low success rate. Strains that are developed often suffer from unexpected fitness costs that are not immediately evident under laboratory conditions (Robinson, 2002). These disadvantages aside however, there are a number of benefits that support the continued use of translocations. Primarily, suitable animals are not considered genetically modified organisms (GMOs) as no foreign gene has been added to the genome, thus simplifying regulatory approval and conferring wider acceptability. Also as translocations can move large

regions of genome, the resistance phenotype does not have to be dependent on the activity of a single open reading frame and simple selection for the trait implies that *a priori* knowledge of the underlying genes involved is not necessary.

More recently, substantial efforts targeting insects that are amenable to genetic transformation have gone into the development of transgenic constructs to engineer genetic sexing systems. Transferring transgenic constructs across species is expected to be more straightforward, as only a few of the components that generate the trait are expected to work in a species-specific manner, e.g. regulatory elements or miRNAs.

To demonstrate the feasibility of transgenic tools, efficient sex-specific negative selection systems were developed in transgenic *D. melanogaster* strains based on the conditional expression of a toxic gene product over a decade ago (Heinrich and Scott, 2000; Thomas *et al*., 2000). In both cases, transcriptional control elements from the female-specific yolk protein 3 gene were used to drive expression of a tetracycline-repressible transcription factor (tTA) in the female fat body. In the absence of tetracycline in the growth medium, tTA activated the expression of a cytotoxic gene, here either the apoptotic *hid* or an activated *Ras* mutant, which were under the transcriptional control of tetracycline-responsive elements (tRE). When both components were brought together in the absence of tetracycline only males survived. Adding tetracycline to the diet rescued female viability. Since lethality is the default outcome in the absence of repression, this system (termed female-RIDL® for release of insects carrying a dominant lethal) has the additional advantage that it can also be used directly for population suppression, as transgenic female offspring born in the natural environment would die. Surviving transgenic sons meanwhile remain in the population and add to the suppression effect by maintaining the allele in the population. Modelling has shown that with density-dependence and assuming that released and wild-type males are equally fit, such repressible FK constructs are more effective than comparably sized releases of males in SIT, especially if alleles are released on multiple loci in homozygous males (Schliekelman *et al*., 2005; Black *et al*., 2011). Work is now underway to transfer these engineered alleles to other insects including species of agricultural importance such as *C. capitata* (Fu *et al*., 2007), the olive fruit fly *Bactrocera oleae* (Ant *et al*., 2012), the Mexican fruit fly *Anastrepha ludens*, the Caribbean fruit fly *Anastrepha suspense* (Schetelig and Handler, 2012), the pink bollworm *Pectinophora gossypiella* (Morrison *et al*., 2012), the diamondback moth *Plutella xylostella* (Martins *et al*., 2012) and species of public health concern including *A. aegypti* and *A. albopictus* (Fu *et al*., 2010; Labbe *et al*., 2012). (For a more detailed discussion on this technology please refer to Chapter 10, this volume.)

Transgenic sexing systems have also been generated by selecting *for* males rather than *against* females. For the most part, selection has been based on the tissue specific expression fluorescent proteins either from promoters that express in male gonads (Catteruccia *et al*., 2005; Smith *et al*., 2007; Scolari *et al*., 2008; Zimowska *et al*., 2009) or by placing the transgene on the Y chromosome (Condon *et al*., 2007). High throughput sexing is then achieved using an automated fluorescence sorter (Catteruccia *et al*., 2005; Marois *et al*., 2012). Progress on engineering systems based on conditional selective survival of males has been slow though potentially suitable alleles have been known for some time. In fact, in the first example of *A. gambiae* transgenesis in the late 1980s, workers used neomycin resistance to select for stable germline transformation (Miller *et al*., 1987; Sakai and Miller, 1992) and more recently resistance to puromycin has also been developed for this mosquito (E. Marois and E. Levashina, personal communication). To express the antibiotic resistance allele specifically in males, the use of ubiquitous male-specific promoters, male-specific alternative splicing, or placement of the transgene on to the Y chromosome are conceivable. Positive and negative selection systems could be also combined to generate transgenic strains that permit selection of

either of the sexes as required depending on the chemical added to the rearing environment. As an example a simple construct is shown in Fig. 6.2. The construct is inserted on the Y chromosome, either by random integration of a transposable element, or phiC31 mediated site-specific integration when suitable docking sites are available. Three transgenes are needed: a puromycin resistance cassette, a tetracycline-inducible lethal cassette (here positive feedback of the tetracycline transactivator) and a transformation marker. Transgenic strains harbouring this construct on their Y chromosome would permit killing of females in the presence of puromycin or alternatively killing of males in the presence of tetracycline. Selection for either of the sexes by exposure to chemicals could be a highly useful technological innovation, for applications such as SIT but also for experimental purposes.

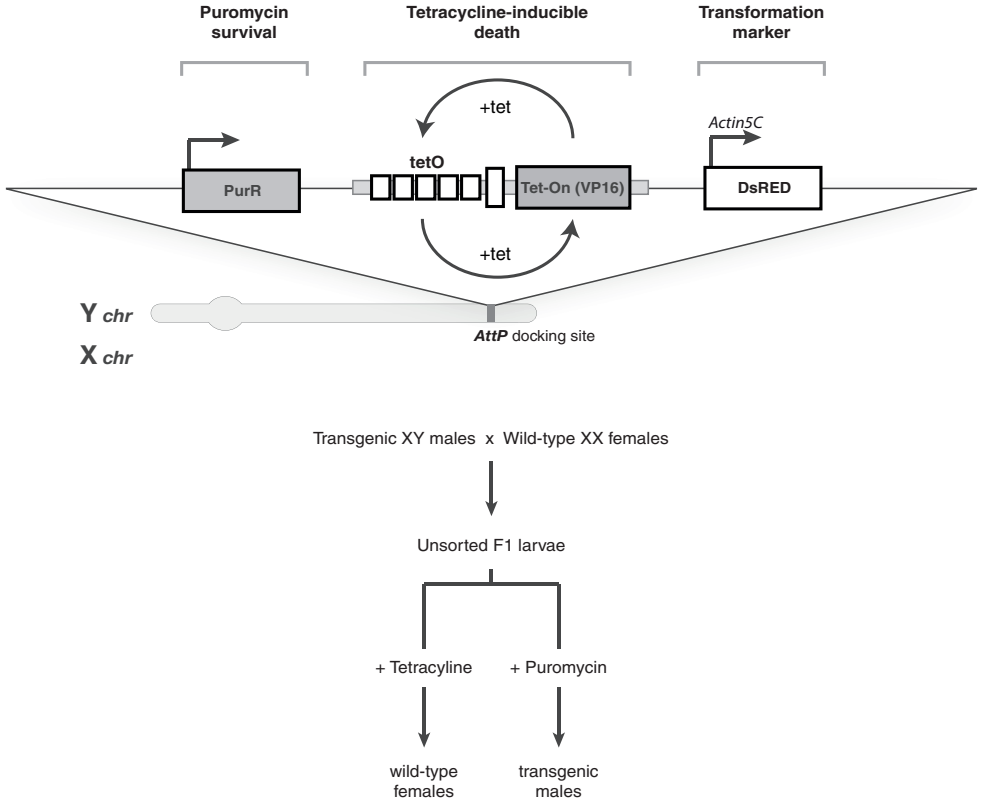


Fig. 6.2. Example of a simple double positive-negative selection system linked to the Y chromosome for efficient sexing technologies. Such a transgenic construct would require three transgenes, a transformation marker (here Actin5C:DsRED), a positive selection system (here puromycin) and a negative selection system (here tetracycline). The puromycin resistance cassette would provide resistance to males in rearing environments containing puromycin and the tetracycline-inducible negative selection would lead to expression of a toxic gene product in the presence of tetracycline (tet-on). Here the tetracycline-induced toxin is a positive-feedback loop of the synthetic transcriptional activator (Tet-on-VP16) binding to its own regulatory region leading to a toxic accumulation of the protein. As the cassette is placed on the Y-chromosome, females never encounter the transgenes, eliminating the need for sex-specific regulatory elements or for crossing to a wild-type strain.

6.5 Manipulation of Sex Determination Mechanisms

Sex ratios can also be distorted by interfering with the regulatory pathways that orchestrate sex determination. Similarly to FK, sex reversion by manipulation of the sex determination genes to phenotypically alter the sexual fate of an individual has been proposed both as a method to suppress wild populations in the field and as a system to eliminate females from the release generation. The principal benefit of this technique over the FK strategy is that like prezygotic manipulation, sex ratio manipulation does not come at a cost of reduced fecundity by eliminating half of the progeny. Instead of killing females, they are converted to phenotypic males that can contribute to the spread of the allele, which, with additional releases, build up the frequency of the allele in subsequent generations, thereby increasing the level of suppression, though this crucially depends on phenotypic males (PMs) being fertile. Indeed modelling has shown that, assuming PM are as fertile as normal genotypic males, such alleles should be more effective in population suppression than both FK and SIT (Schliekelman *et al*., 2005).

Sex conversion relies on a concrete understanding of the genes involved in sex determination in the targeted organism. The hierarchical organization of the sex determination pathway in insects is believed to adhere to a similar theme: at the top of the pathway a primary signal leads to the activation of the key gene, which then recruits a conserved double-switch gene that acts diversely in males and females to orchestrate sexual differentiation. In *D. melanogaster*, where sex determination has been most extensively studied, the primary signal can be thought of as the ratio of X chromosomes to autosomes (X:A ratio), where a ratio of 1 (2X:2A) initiates female development and a ratio of 0.5 (1X:2A) initiates male development (Cline, 1993). Thus, XX, XXY, and XXYY flies are females, while XY and XO flies are males, and flies with more than two copies of an X chromosome are unable to survive. In females both X chromosomes remain active, while males compensate for having half the X chromosomes as females, by roughly doubling the expression levels of X-linked genes, in a process known as dosage compensation. The X:autosome signal is mediated by the interaction of four X-linked genes known as the numerators (*sisterless A* (*sisA*), *sisterless B* (*sisB*), *sisterless C* (*sisC*) and *runt* (*run*)), with a major autosomal gene, the denominator *deadpan* (*dpn*) (Cline and Meyer, 1996). Their interaction regulates the expression of the master switch gene *Sex lethal* (*Sxl*) early in post-zygotic development. A double dose of the X-linked nominators in females initiates expression of *Sxl* from its early promoter, leading to a burst of SXL protein in female embryos. Later in development, *Sxl* is expressed in both sexes. In males, all *Sxl* transcripts include a translation-terminating third exon. In females, sex-specific splicing is dependent on the early accumulation of SXL, which then acts to splice primary mRNAs of its own gene. This results in a positive feedback loop that establishes and maintains sexual memory (Cline, 1984; Bell *et al*., 1991). The activation of *Sxl* in females results in the appropriate splicing of the key gene *transformer* (*tra*), which in turn regulates the alternative splicing of the *doublesex* (*dsx*) and *fruitless* (*fru*) genes, to produce sex-specific transcription factors that ultimately control most aspects of sexual differentiation and behaviour (Shearman, 2002). Since the pathway is turned off in males, a default male-specific isoform of *dsx* and *fru* is produced instead.

Substantial effort has gone into elucidating similar details of the sex determination pathway in other insects, mostly on the basis of homology to the *Drosophila* model. What has emerged has been the understanding that the evolution of the sex-determining cascade occurs from the 'bottom up' (Wilkins, 1995). Genes at the bottom of the cascade represent older, more ancestral members of the pathway that are more highly conserved between related species. Upstream genes are recruited by frequency-dependent selection for the minority sex at each step, to reverse the

sexual choice of the gene they precede (for a theoretical analysis of this model see Pomiankowski *et al*., 2004). Predictably therefore, *dsx* has been identified in all Diptera, Lepidoptera and Hymenoptera examined (Shukla and Nagaraju, 2010). In most of these insects *dsx* is sex-specifically spliced into one male-specific and one female-specific isoform, like *Drosophila*, whereas in the housefly *Musca domestica*, the honeybee *Apis mellifera*, the silkmoth *Bombyx mori* and the mosquito *A. aegypti dsx* is spliced to produce more than two isoforms (Ohbayashi *et al*., 2001; Hediger *et al*., 2004; Cho *et al*., 2007; Salvemini *et al*., 2011; Shukla *et al*., 2011). While *dsx* is well conserved at the bottom of the pathway, genes upstream are more divergent. *tra* orthologues have been identified in the jewel wasp *Nasonia vitripennis*, *M. domestica*, *C. capitata*, the Australian sheep blowfly *Lucilia cuprina*, *B. oleae*, the West Indian fruit fly *Anastrepha obliqua*, the Caribbean fruit fly *Anastrepha suspensa*, *Glossina morsitans* and *A. mellifera* and in each case translation-terminating male exons are the basis of an autoregulatory splicing mechanism (Inoue and Hiroyoshi, 1986; Pane *et al*., 2002; Lagos *et al*., 2007; Ruiz *et al*., 2007; Hasselmann *et al*., 2008; Concha and Scott, 2009; Beukeboom and van de Zande, 2010; Hediger *et al*., 2010). While clear orthologues of *sxl* have been identified in a number of other insects, sex-specific splicing and a role in sex determination are only conserved within the genus *Drosophila* (Sanchez, 2008). The primary signals that initiate sex determination also display significant natural diversity. One of the most common primary signals among insects is a dominant male-determining gene, as in humans. In *C. capitata,* and *A. gambiae*, the presence or absence of the Y chromosome determines sex (Baker and Sakai, 1979; Willhoeft and Franz, 1996; Krzywinski *et al*., 2004). *Aedes* and *Culex* mosquitoes have a non-recombining sex-determining region located on chromosome 1 (Craig and Hickey, 1967). In *M. domestica* the male-determining gene has been found linked to either of the autosomes (I-V) or the X or Y chromosomes in isolated populations from different parts of the world (Sakai and Miller, 1992; Kozielska *et al*., 2008). In Hymenoptera sex is determined by a haplodiploid mechanism in which males emerge from unfertilized eggs and females from fertilized eggs (Heimpel and de Boer, 2008).

Outside of the *Drosophila* model system, transient injection of double-stranded RNA targeting the *tra* gene has been shown to lead to sex conversion in *C. capitata*, *B. oleae*, *A. suspensa*, and *L. cuprina* (Pane *et al*., 2002; Lagos *et al*., 2007; Concha and Scott, 2009; Schetelig *et al*., 2012). These proof-of-principle experiments have opened the door for attempts to generate stable transgenes that interfere with the expression of sex determination genes. Inhibition of female developmental pathways using transgenic constructs that express miRNAs or long dsRNA that target female-specific splice forms are expected to act dominantly as maleness is often the default setting of the pathway (Schliekelman *et al*., 2005). A genetic system could be developed, based on the RIDL system discussed in section 6.4, that uses the tetracycline repressible transactivator system (tTA) to conditionally express zygotically in the early embryo a miRNA that is engineered to target *tra* or some other female-specific isoform of sex determining genes. In the presence of tetracycline, the miRNA would not be expressed allowing for the production of both males and females, allowing the generation of a homozygous strain and simplifying large-scale production and rearing. However, in the absence of tetracycline (i.e. a release into the wild) the miRNA would be expressed, resulting in 100% of the progeny to be males (50% genotypic XY males, 50% phenotypic fertile XX males). However, of critical importance will be the fertility status of the phenotypic XX males. In *Drosophila*, XX males are infertile because they lack genes on the Y chromosome that are required for male fertility (Hackstein and Hochstenbach, 1995). Unlike *Drosophila*, the *Ceratitis* and *Bactrocera* phenotypic males that were generated using transient RNAi were fertile. This suggests that for some organisms it may be feasible to produce fertile XX males

based on the expression of a single transgene. On the other hand, dissection of the *Anastrepha* pseudo-males revealed abnormal hypertrophic gonads, suggesting that in this species the generation of XX fertile males may be not be feasible. The fertility status of the *Lucilia* males was not reported. Overall, the possibility of directly interfering with the expression of genes of the sex determination pathway is a recent area of research, as the technologies and genomic tools required are relatively novel.

6.6 Conclusions

We have discussed three methods for using sex ratio manipulation and their application to insect population control: segregation distortion (SD), female killing (FK) and sex reversion to make phenotypic males (PM). As we have discussed, a detailed theoretical comparison of their relative field performance has shown that these methods can be more effective than SIT (Schliekelman *et al*., 2005). In scenarios of repeated releases of males, one SD insect achieves the same population suppression effect as 1.5–20 PM insects, 2–70 FK insects and 16–3000 SIT males. Moreover, this advantage improves as the size of the released population increases. The SD system that was modelled here was not an invasive allele, as it was not assumed that it would be placed on the Y chromosome, where it would directly benefit from drive. An allele that functions in this way is likely to be much more effective than an autosomal one.

Overall, female killing using the RIDL system, developed by the UK-based biotech firm Oxitec, is at the most advanced stage of development. Its foremost benefit is that it is the simplest system to transfer to novel species, requiring only germline transformation and the availability of regulatory elements that allow expression of the toxic transgene in a female-specific manner. Open field trials have been initiated to target a number of important insect species, most notably the *A. aegypti* mosquitoes in the Cayman Islands (Harris *et al*., 2011, 2012), Malaysia (Lacroix *et al*., 2012) and Brazil. Their releases are currently utilizing transgenic strains that develop bisexual lethality in the field, though female-specific killing strains have been developed and would be expected to improve efficiency.

Synthetic segregation distorters and sex reversion of females to phenotypic males are naturally more complex to engineer. Sex reversion requires insight into the insects' sex determination pathway, transcriptome and genome sequences and the availability of methodologies to interfere with the expression of genes that specify femaleness. Synthetic segregation distorters are possibly the hardest to engineer.

In the mid-1980s Chris Curtis, the most well-known and prolific advocate of applying SIT-based genetic control of mosquitoes, argued that 'there may be a danger that the intellectual appeal of recombinant DNA, transposable elements etc. may lead applied entomologists to waste time on baroque schemes, without thinking whether their aims could be achieved more simply and quickly by old-fashioned selection, translocations etc.' (Curtis, 1985). However, 2012 also saw the first publication describing the release of transgenic mosquitoes using the RIDL system in the Cayman Islands, achieving an 80% reduction in the overall population of the target mosquito (Harris *et al*., 2011, 2012). In a number of agricultural pests recent advances in sex reversion have been shown in proof-of-principle experiments. The first genetically sterile strain of *A. gambiae* is currently being assessed for its suitability in mass-releases of SIT programmes (Windbichler *et al*., 2008; Klein *et al*., 2012). Ultimately the future of insect population control may lie in the combination of contemporary molecular biology, transgenic techniques with classical genetics.

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