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# SECOND RESEARCH COORDINATION MEETING

**Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture**

## Research Coordination Meeting on

## *Generic approach for the development of genetic sexing strains for SIT applications*

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**Summary:**

The application of the Sterile Insect Technique (SIT) in area-wide integrated pest management (AW-IPM) programmes continues to increase in response to requests from Member States. These requests include the development and refinement of SIT packages for programmes to control populations of different insect pests of agricultural, veterinary and human health importance. The development and operational application of such programmes with a SIT component against insect pests and disease vectors continue to reveal research areas where new technologies could further improve efficiency and thus lead to more efficacious programmes.

One such critical area, where important advances need to be made to increase the cost-effectiveness of the technique, or where it is a prerequisite before any SIT application is conceivable, concerns the development of genetic sexing strains (GSS). In SIT programmes against agriculture pests, the release of both sexes is primarily of economic concern; however, in SIT programmes against some insect disease vectors (e.g. mosquitoes), it is an essential prerequisite to release only males since females are blood feeders and may potentially transmit disease even if sterile.

This CRP focuses on the development and evaluation of generic approaches for the construction of genetic sexing strains (GSS) to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors. Significant progress has been achieved so far which can be summarized as follows:

(a) in respect to the isolation of selectable markers to be used for generic strategies for the construction of GSS, several genes have been isolated or exploited as makers including *white pupae*, *black pupae* (*ebony*), *temperature sensitive lethal* (*tsl*), *slow development* (*sd*), *slow larvae, white eye, red eye (cardinal), yellow*. In addition, during RCM2, it was suggested that genes involved in salinity tolerance, desiccation/UV tolerance, auxotrophy, insecticide resistance, and phototaxis may be worth to be explored;

(b) in respect to the development of generic approaches for the construction of GSS for SIT targeted agricultural pests and human disease vectors, a number of mutant strains have been established including white pupae strains in *B. correcta*, *B. oleae*, *B. tryoni*, and (new strains for) *C. capitata*; black pupae (ebony) strains in *B. tryoni* and *D. suzukii,* while black pupae strains are already available in *A. ludens, A. fraterculus, A. obligua*; red-eye (cardinal) strains in *Ae. aegypti*, while such strains are already available in *An. gambiae, Cx. pipiens, Ae. albopictus* and *Plutella xylostella*; yellow strains in *Ae. albopictus*, while such strains are already available in *Spodoptera litura (yellow-y)*. In addition, knock-in of the *white* wild type allele on the Y-chromosome of *C. capitata* was achieved for first time, although the stability needs to be improved since the constructed line was lost after two generations. Insertions were also achieved in the M region of both *Aedes aegypti* and *Aedes albopictus*. In parallel, the sequencing of several insect genomes and transcriptomes is ongoing aiming to facilitate the isolation of selectable markers and to identify suitable regions for knock-ins on Y chromosomes of SIT targeted species, and

(c) in respect to the evaluation of the GSS strains developed with generic approaches, quality control analysis was performed on GSS constructed with the newly established medfly white pupae mutant lines, and no difference was observed when compared with the original ones.

## Background

**Scientific status and problems to be researched**: Insects are the most abundant, speciose and diverse animal group on this planet. Although most insect species are beneficial or harmless, there is a small number of them which are considered as major pests of agricultural, livestock and human health importance and their populations need to be managed. Conventional methods are primarily based on insecticides. However, there are increasing concerns about their negative impact on human and environmental health, as well the inevitable selection of insecticide resistance due to their extensive use. The Sterile Insect Technique (SIT) represents a species-specific, non-polluting and environmentally benign approach that has been extensively used during the last 50-60 years to control populations of insect pests and disease vectors as a component of area-wide integrated pest management (AW-IPM) programmes. Due to its successful use against different target species, the requests of application of SIT continues to increase from FAO/IAEA Member States (MS). Programme efficiency, cost-effectiveness, as well as safety and biosecurity, depends on the availability of genetic sexing strains (GSS) which can allow male-only releases. It is now possible to develop such GSS by both classical and modern biotechnological approaches that are presented later.

*Targeted species*: The pests targeted for SIT applications include species of agricultural, veterinary and human health importance. Potential targeted SIT species of agricultural importance are the following fruit fly species: *Anastrepha ludens, A. obliqua, A. grandis, A. fraterculus* (species complex), *A. suspensa, Bactrocera carambolae, B. correcta, B. dorsalis, B. oleae, B. tryoni, B. jarvisi, B. zonata, Ceratitis capitata, C. fasciventris, C. quilicii, C. rosa, Drosophila suzukii, Zeugodacus cucurbitae* and *Z. tau*. The following moth species are also considered major agricultural pests and potential targets for the SIT: *Cydia pomonella, Grapholita molesta, Ectomyelois ceratoniae, Diatraea saccharalis, D. crambidoides, Pectinophora gossypiella* and *Plutella xylostella*. The following species of human health and veterinary health importance are also considered potential targets for the SIT: *Aedes aegypti, Ae. albopictus, Ae. polynesiensis, Anopheles albimanus, An. arabiensis, An. darlingi, An. gambiae, An. stephensi, Cochliomyia hominivorax, Culex pipiens, Glossina* species, *Lucilia cuprina* and *Musca domestica*.

*1. Genetic sexing methods*: These methods to develop a GSS can be classified into two categories: (a) using classical genetics and (b) molecular engineering methods. The GSS developed several decades ago in the Mediterranean fruit fly, *Ceratitis capitata*, are good examples of classical, sophisticated applications of standard genetic manipulation and successful integration of these strains into operational programmes. For this species, a series of strains bearing a *temperature-sensitive lethal* allele (combined with the *white pupae* marker) were developed by means of irradiation and classical genetics linking the wild type alleles of these genes to the male determining locus and the Y chromosome. Several of these strains (VIENNA-7 and VIENNA-8) have been thoroughly evaluated and are currently being used in mass rearing facilities for large-scale AW-IPM programmes that include a SIT component. In addition to classical genetic approaches for the development of GSS, transgenic approaches transferring at least one non-host DNA sequence into the target genome are being explored. Transgenic insects herein are defined as insects whose genetic material has been altered in a heritable way through the techniques of genetic modification, all of which allow for the combination and/or introduction of foreign genetic material into host insect genomes in a way that does not occur naturally by mating, and/or natural recombination. It should be noted that the regulation of transgenic technology and public acceptance remains a major issue for the implementation of this technology.

*2. Available sexing technologies for application*: Pest control strategies that include an SIT component are currently applied against several insect species. The development of a genetic sexing system in the medfly led to a significant improvement in the cost-effectiveness, and the efficiency of the SIT in the field and showed that other insect pests could benefit from. Thus, there is a widely recognized need for the development of sexing systems for SIT programs of other species, and in addition, this is a prerequisite for mosquito SIT since females are the transmitting sex of major human pathogens.

2.1. Tephritids: The VIENNA-8 (and VIENNA-7) GSS of *C. capitata* carry the *white pupae* (*wp*), *slow development* (*sd*) (Porras et al., 2020) and *temperature sensitive lethal* (*tsl*) mutations as well as a Y-autosome translocation that includes wild-type functional copies of these genes. Via an embryonic elevation in

temperature, females can be eliminated in an early stage of development (Franz 2005; Augustinos et al. 2017). GSS have been developed for *B. dorsalis* (McCombs & Saul 1995; Isasawin et al., 2012) and *Z. cucurbitae* (McInnis et al., 2004) that are based on *white pupae* mutations. Similarly, GSS for *A. fraterculus and A. ludens* have been developed based on a *black pupae* mutation and this mutation has also been isolated in *A. obliqua* (Zepeda et al., 2014). These pupal color markers have the disadvantage that females have to be reared up to the pupal stage before sexing by sorting can be achieved. Transgenic technologies have been used to develop novel sexing systems originally in *Drosophila melanogaster* as a proof-of-principle, and later in several insect pest species. One approach uses an autoregulated tetracycline-suppressible (Tet-off) transcriptional activator (tTA) as a lethal effector that was made female-specific by integration of a sex-specifically spliced gene intron from a *transformer* (*tra*) gene, resulting in female-specific lethality in the absence of dietary tetracycline. Similar to the color-based marker, this system is suboptimal in that the female lethal phase is late in development. A subsequent improvement was made through the development of new Tet-off sexing strains, with lethality acting in early embryogenesis, in *C. capitata*, *A. suspensa*, *A. ludens*, *L. cuprina*, and *C. hominivorax* that also make use of a *tra* intron1 for female-specific lethality (Schetelig & Handler 2012; Ogaugwu et al., 2013; Schetelig et al., 2016; Yan & Scott 2015; Concha et al., 2016). With these systems, female progeny are eliminated during early embryogenesis and most of these systems do not require high dietary concentrations of tetracycline during mass rearing. In addition, a transgenic sex-reversion line of *C. capitata* that shows 95% conversion of XX individuals into fertile males, with 5% intersexes was generated (Saccone et al., 2007). *In vivo* RNAi against *Cctra* driven by a transgene can be effective if the mother deposits dsRNA molecules into the eggs, as revealed by a maternal RNAi effect. Surviving XX male progeny are competitive relative to XY wild-type males. In other tephritid species like *A. suspensa*, the transient dsRNA knock-out of *tra* and *tra2* did lead to 98% of female sex-reversion, but those XX males had the advantage of being sterile, though their XY siblings were fertile (Schetelig et al., 2012).

2.2. Mosquitoes: Female mosquitoes are solely responsible for biting humans and transmitting pathogens, therefore, they must not be released by SIT programs, since they could contribute to local disease transmission. This places unique constraints on any efforts to develop SIT for mosquitoes, i.e. GSS are absolutely required. The first mosquito GSS were developed in the 1970s, using classical genetic approaches involving mutagenesis and chromosomal translocations. These strains relied on the use of insecticide resistance genes which were translocated to the Y chromosome, linking resistance exclusively to males. Using this approach *Anopheles albimanus*, *An. arabiensis* and *An. gambiae* GSS based on dieldrin resistance were developed in the 1970-80s (Kaiser, 1978, Robinson, 1986, Curtis. 1978, Lines, 1985). However, these strains were eventually deemed unsuitable because of high genetic instability, and they are no longer available. Recently, an *An. arabiensis* GSS was developed based on dieldrin resistance by IPCL colleagues, but due to low fertility and concerns over dieldrin residues in adult males and subsequent environmental bioaccumulation, the strain was considered not suitable for field use (Yamada, 2012). Similarly, one of the first GSS was constructed in *Ae. albopictus* by linking *rdlR* gene, conferring resistance to dieldrin, to maleness (Lebon et al., 2018). This strain, together with a second strain obtained using the same approach (Tortosa, personal communication) allow producing >98% of males following dieldrin treatment of larvae and are easily maintained in the insectary. Promising research results and technologies have been reported recently: (a) A first generation GSS has been developed for *Ae. aegypti* in IPCL using classical genetic approaches; (b) Sorting of fluorescent larvae: sex-specifically marked larvae can be sorted by a COPAS sorting machine (Catteruccia, 2005); (c) Female lethality system acting in late larval/pupal stage called ´female-specific RIDL´ (fsRIDL) (Fu et al., 2007); (d) Sex distortion: A "sex-ratio distortion" approach was developed for *Anopheles gambiae*, which destroys X-bearing sperm that resulted in 95-97% male progeny (Galizi et al., 2014) and (e) Sex conversion: Tools for sex conversion have been identified in fruit flies and mosquitoes (Pane et al., 2002; Salvemini et al., 2009; Meccariello et al., 2019, Aryan et al., 2019;Primo et al., 2020). Approaches (d) and (e) are, in theory, more efficient than female lethality, as they could double the total number of male progeny produced per parental population.

2.3. Lepidoptera: The available sexing mechanisms developed for Lepidoptera have been based either on the construction of balanced lethal (BL) strains or W-linked selectable markers. Unfortunately, the use of BL strains for genetic sexing is not easily applicable under mass rearing conditions. Suitable W-linked markers are only available for *Bombyx mori* (Marec and Vreysen, 2019). A GSS with a W-linked dominant conditional lethal mutation (DCLM) would permit the maintenance of both sexes under permissive conditions and the elimination of the female moths under restrictive conditions. However, to date, no DCLM has been identified in Lepidoptera.

Alternatively, modern biotechnology methods could be used to introduce a DCLM into the W chromosome. An advantage of this approach is that only female progeny will have the transgene, but not the released males, which will have a fully wild-type genome (Marec et al., 2005). Recently, transgenic sexing strains of *B. mori* and pink bollworm have been made that overexpress tTA in females when raised in the absence of tetracycline in the diet. Sex-specific expression was achieved by using the splicing signals from the pink bollworm *doublesex* (*dsx)* gene (Jin et al., 2013; Tan et al., 2013).

<u>*3. Sex determination*</u>: Knowledge on sex determination pathways of the SIT-targeted insect species can be very useful for the construction of a GSS. Sex determination is well characterized in *Drosophila melanogaster*, in which two doses of a set of X-linked transcriptional regulators activate the master gene *Sex-lethal* (*Sxl*), which determines the female fate in XX embryos and represses dosage compensation. In XY embryos (males), which contain only one dose of X-linked transcriptional activators, *Sxl* remains inactive. In *Ceratitis capitata*, the *tra/tra-2 > dsx/fru* module of this sex determination pathway is conserved at the structural and functional level (Pane et al., 2002; Bopp et al., 2014). However, the *Sxl* homolog in *C. capitata* (*CcSxl*) is not acting as the upstream regulator of the *tra* homolog (*Cctra*) (Saccone et al., 1998; Zhang et al., 2014). Instead, activation of *Cctra* functional gene products require the presence of maternal *Cctra*, that acts together with the *Cctra-2* gene product (Salvemini et al., 2009), to maintain the epigenetic autoregulatory function resulting female sexual differentiation. When *Cctra* female activation is prevented by the male determiner (M factor) or artificially by dsRNA, male sex determination and male differentiation results (Pane et al., 2002). This mode of *tra* autoregulation and its embryonic RNAi sensitivity appears to be widely conserved in many other Tephritidae, including *Bactrocera oleae, B. tryoni, B. jarvisi, B. dorsalis, B. correcta, Anastrepha suspensa*, the Calliphoridae, *Lucilia cuprina*, and the Muscidae, *Musca domestica* (Hediger et al., 2010; Sanchez, 2008; Nagaraju J, and Saccone G., 2010, Laohakieat et al., 2016, 2020). Dead Cas9 targeting Medfly *tra* without inducing mutation led to full masculinization indicating that transient repression of Cctra transcription during early embryogenesis also affects the establishment of the female-determining Cctra autoregulatory loop (Primo et al., 2020).

In *L. cuprina*, it was recently found that knock-down of tra by Cas9 also interferes with dosage compensation, an effect that could be used for female killing (Williamson et al., 2021: doi: 10.1371/journal.pgen.1009792). The male-determining factor in *C. capitata* has been previously mapped on the long arm of the Y chromosome (Willhoeft and Franz, 1996). Only recently the M factor has been molecularly isolated in the Medfly, named as *Maleness-on-the-Y* (*MoY*) and found to be able to induce male-specific splicing of *Cctra* within hours during embryogenesis of XY (Meccariello et al., 2019). *MoY* orthologues were found widely conserved in Tephritidae species of *Bactrocera* and *Zeugodacus* genera and shown to be functionally conserved in the olive fruit fly *B. oleae* and the oriental fruit fly *B. dorsalis*. Hence, *MoY* is a very promising tool for a tephritid generic transgenic strain sexing by masculinization of XX individuals (Meccariello et al., 2019). Very recently, a novel molecular mechanism for male sex determination has been uncovered in B. dorsalis (Peng et al., 2020). An autosomal miRNA, miR-1-3p, shows XY-biased expression in early embryos and targets Bdtra mRNA leading to its male-specific splicing pattern and male sex determination. The male-biased expression of miR-1-3p is likely under the direct or indirect control of BdMoY. RNA interference as a natural sex determination mechanism has been described only in Lepidopteran species (Kiuchi et al., 2014), but it is a novelty for dipteran species and its potential evolutionary conservation should be explored.

One of the several *M* factors present in wild populations of *M. domestica*, *Mdmd*, has been isolated (Sharma et al., 2017). The *Mdmd* gene originated from a duplication of a highly conserved autosomal gene, *CWC22*, encoding a spliceosome protein, suggesting that *Mdmd* has a direct role in repressing female-specific *Mdtra* splicing. Genetic evidence suggests that a Y-chromosome or M-locus linked *M* factor initiates male development in *Anopheles* and *Aedes* mosquitoes, respectively (Gilchrist and Haldane, 1947, Baker and Sakai, 1979). Recent studies isolated the male-determining factor *Yob*, encoding a novel short protein, in *An. gambiae* (Krzywinska et al., 2016), *Guy1* in *An. stephensi* (Criscione et al., 2013, 2016; Qi et al., 2019), and the primary sex-determiner *Nix* in *Ae. aegypti*, encoding a putative RNA binding protein (Hall et al., 2015), and a putative male-determining gene, *Nix*, in *Ae. albopictus* (Gomulski et al., 2018). In these mosquito species, downstream genes such as *dsx* and *fruitless* (*fru)* have been identified and are regulated by a partially conserved sex-specific alternative splicing mechanism (Scali et al., 2005; Gailey et al., 2006; Salvemini et al., 2011; Salvemini et al., 2013). At the same time, a *transformer* homolog in both species is either absent or remains to be identified. No genetic information

is available for the upstream splicing regulators of the *dsx* and *fru* genes, which are controlled in males by the primary signals *Yob* and *Nix*.

In Lepidoptera, the chromosomal mechanism of sex determination is the heterogametic WZ type. It was shown by Kiuchi et al. (2014) that the feminizing factor in *B. mori* is a W-encoded small PIWI-interacting RNA named *Fem* piRNA. The authors also showed that the *Fem* piRNA down-regulates the expression of a Z-linked gene, *Masculinizer* (*Masc*), which promotes male development in the absence of a W chromosome. The *Fem* piRNA therefore controls female-specific splicing of the *B. mori doublesex* (*Bmdsx*) gene by down regulating expression of the *Masc* gene (Kiuchi et al., 2014). However, it is not yet known whether the *Fem* piRNA-*Masc* sex-determining pathway is conserved in other lepidopteran species having WZ sex determination. Several recent studies suggest that the role of *Masc* is conserved in Lepidoptera sex determination (Lee et al., 2015; Fukui et al., 2018; Wang et al., 2019; Harvey-Samuel et al., 2020; Deng et al., 2021; Visser et al., 2021).

In Hymenoptera, the core tra-splicing-autoloop is started by very different means, such as heterozygosity at a complementary sex determining locus in *Apis mellifera* [Gempe et al. 2009: OI: 10.1371/journal.pbio.1000222] or a transcriptional activator with a parent-of-origin effect in *Nasonia vitripennis* [Zou et al. 2020: doi: 10.1126/science.abb8949]. The insect sex determination pathway based on sex-specific splicing of tra and dsx seems ancestral to the Holometabola [Wexler et al., 2019: doi: 10.7554/eLife.47490], whereby tra serves as transducer of the primary signal and dsx as executer for sex determination. However, the primary signal differs widely [Hopkins and Kopp, 2021: doi: 10.1016/j.gde.2021.02.011].

## *4. Recent developments*:

4.1. The applications of 'big data' for molecular genetics: The community can overcome major bottlenecks in research by the application of next generation sequencing (NGS) technologies to genetic problems. The available technologies have a number of applications that range from whole genome sequencing to gene expression analysis. Currently, there are two main sequencing platform types (a) "Short read" (50-500 bp) sequencers e.g. Illumina and Ion torrent and (b) "long read" (>5 Kb up to 2 Mb) e.g. PacBio and Oxford Nanopore. There are also a number of applications that leverage the high throughput of the Illumina machines to provide long pseudo-reads of up to 150 Kb, e.g. Chromium 10X Genomics and genome scaffolding e.g. Chromium 10X and HiC. We now have the capability of rapidly obtaining such whole genome sequences from a species, a strain and even a single individual. In addition, using a series of tools we have shown that we have the potential to improve assemblies by integrating linked read and long read data. We are also able to generate haplotype specific assemblies for diploid species using these technologies. Another important development is the application of long read technologies (Nanopore and PacBio) for transcriptome sequencing and assembly, which can be used to enrich genome annotation efforts. Taken together, these technologies with bioinformatic analysis allow us to produce a wealth of ancillary data that play an increasingly prominent role in the identification of target (marker) genes including their mode of regulation. An example of these recent developments, both in terms of sequencing chemistry and in its bioinformatic analysis, has been the discovery and subsequent characterization of Y-chromosome sequences, including Y-linked M factors in mosquitoes, *Ceratitis capitata* (MoY; Meccariello et al., 2019), *Bactrocera oleae* (Bayega et al., 2020) and in *Musca domestica (MdMd*; Sharma et al., 2017*)* These tools in combination with methods described below for genome manipulation, have made it possible to build novel types of GSS in any species targeted. Furthermore, they are currently being employed to molecularly identify the loci responsible for many of the GSS-based mutations described above. Therefore, the access to such tools will likely underpin a new type of capability that will greatly enhance the toolkit available to the SIT community (Papanicolaou et al., 2016; Matthews et al., 2018; Turner et al., 2017; Van't Hof et al., 2016; International Glossina Genome, 2014).

4.2. New era in cytogenetics and chromosome manipulation: In the era of NGS, laser microdissection seems to be a particularly useful tool for preparation of sex chromosome-specific DNA libraries. In insects, this technique was first demonstrated in the codling moth, where it was used for the development of W-chromosome painting probes and for obtaining first sequence information on the composition of this heterochromatic chromosome (Fuková et al., 2007). Using laser microdissection, highly specific X- and Y-chromosome-painting probes were prepared and used for cytogenetic research in the olive fly, *Bactrocera oleae* (Drosopoulou et al., 2012). In the

flour moth (*Ephestia kuehniella*), high-throughput sequencing of laser microdissected sex-chromatin bodies provided the first complex information about the DNA composition of the lepidopteran W chromosome (Traut et al., 2013). Especially in tephritid fruit flies, GSS constructed using classical genetics carry a translocation of an autosomal segment on the Y chromosome and sometimes also an inversion that was introduced to reduce recombination. Cytogenetic methods were used to determine the origin and size of the translocated segment, localize translocation breakpoints or map the extent of inversions, which is critical for the stability and fitness of the strains (Franz, 2002). The identification of breakpoints and delimitation of inversions was facilitated by polytene chromosome maps available in most tephritid pests (Stratikopoulos et al., 2008; Drosopoulou et al., 2014). In Lepidoptera with small and numerous holokinetic chromosomes, specific patterns of longer meiotic bivalents in pachytene allowed the identification of sex chromosomes and characterization of radiation-induced chromosome rearrangements (Traut et al., 2007). Cytogenetic research has been greatly accelerated using advanced tools of molecular cytogenetics that are currently available for detailed analysis of insect chromosomes. Various modifications of fluorescence *in situ* hybridization (FISH), such as FISH mapping of repetitive sequences and multigene families (e.g. rDNA and histone genes), genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH) were used for the identification of sex-determining regions to which selectable markers should be linked and for the characterization of DNA content of the Y or W chromosomes (Willhoeft and Franz, 1996; Willhoeft et al., 1998; Fuková et al., 2005), which was relevant to the GSS stability and provided useful data in species with poorly understood karyotypes (Nguyen et al., 2013; Šíchová et al., 2013). Recent advances in insect genomics has led to the development of new molecular cytogenetic methods required for the construction of high-resolution physical maps, such as BAC-FISH (FISH with bacterial artificial chromosomes as probes) and TSA-FISH (FISH with tyramide signal amplification), which represent an important framework for improving the quality of genome assembly, annotation, and analysis (Nguyen et al., 2013; Carabajal Paladino et al., 2014; Yoshido et al., 2014).

4.3. Genome editing - new tools for modifying genotypes: Genome editing allows the precise modification of genomic DNA sequences *in vivo* and can be achieved using three available technologies – Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). These technologies can be used to induce DNA double-strand breaks (DSBs) at predetermined target locations in the genome. In the case of ZFNs and TALENs, DNA endonuclease domains are attached to proteins whose amino acid sequences have been designed to bind to specific sequences. In the case of CRISPR, the Cas9 protein (or equivalent ones like CPF1) is directed to cut predetermined target locations in the genome by providing it with single-guide-RNAs. Double-stranded breaks in genomic DNA can be repaired either by non-homologous end-joining (NHEJ), resulting in possible disruption of the target sequence through insertion or deletion of nucleotides (creating knockout lines; Meccariello et al., 2017) or by homology-directed repair (HDR) that can be used to insert DNA sequences at the target locus via homologous recombination (HR) producing knock-in lines (Li and Handler, 2017; Aumann et al., 2018; KaramiNejadRanjbar et al., 2018). Directed knock-in lines can also be produced by NHEJ (Farnworth et al., 2020: DOI: 10.1007/978-1-4939-9732-9_11) and tried for by using RNA-guided piggyBac transposition (Hew et al., 2019: doi: 10.1093/synbio/ysz018). Gene editing technologies provide precise mutagenesis capabilities which were previously limited to nonspecific chemical agents - (e.g. EMS) or radiation-based (e.g. X-rays) methods for the creation of insect genotypes needed for effective GSS (e.g. VIENNA-8). CRISPR/Cas9 technology has been limited so far to arthropod species for which an embryonic microinjection protocol exists. However, a recent technical improvement called the ReMOT system (Chaverra-Rodriguez et al. 2018) promises to overcome this technical limitation, allowing GSS development for species for which *in vivo* reverse genetics tools are still not available, for example the tsetse fly. Chemical- and radiation-based mutagenesis must be coupled to large genetic screens designed to detect and recover the desired genetic alterations and these ‘classical’ approaches, while demonstrably effective in some cases, depend on chance occurrences of the mutations or chromosomal rearrangements of interest and can require many person-years of effort to produce desired genotypes. The high precision and accuracy of gene editing technologies enables the creation and assembly of genotypes identical to those created and assembled using ‘classical’ mutagenesis and genetic approaches, but crucially, without necessarily requiring large genetic screens or many person-years of effort. This is a clear benefit of using genome editing technologies for the creation of GSS. Because the organisms produced using gene-editing technologies can be genetically identical to those produced using ‘classical’ approaches, their transition from the laboratory to the field and adoption by end-users could follow current technology transfer strategies for non-transgenic organisms, another potential benefit of using gene-editing technologies.

4.4. New developments on RNAi for pest control: In insects, as in other organisms, RNAi is a powerful tool for experimental studies that aim to determine gene function. This commonly involves the microinjection of dsRNA into the target organism. However, administration through feeding is also possible. The dsRNA is cut by endogenous Dicer proteins into a population of small interfering RNAs (siRNAs), which in turn bind and degrade complementary mRNA sequences. In plants and some invertebrates (e.g. *C. elegans*), the efficacy of RNAi is improved through a combination of signal amplification and systemic spread, such that the entry of one dsRNA or siRNA molecule into a single cell can lead to effective silencing of the target gene throughout the target organism. In most insects, RNAi appears to be cell-autonomous, with no amplification or cell to cell transfer of the gene silencing signal. The lack of a mechanism for amplification and systemic spread of a dsRNA signal in fruit flies and mosquitoes has implications for the development of RNAi as a control tool for insect pests. To achieve effective control, dsRNA/siRNA delivered through the environment (environmental RNAi, eRNAi; Ivashuta et al., 2015) of the pest must somehow be delivered to the appropriate tissue in the target pest at a sufficient dose to produce the necessary level of gene silencing to achieve the desired objective, usually mortality. There is a considerable variation across insect species in their sensitivity to eRNAi, and the evidence to date suggests that this is largely due to the relative uptake, stability and transport efficiency of dsRNA or siRNA among insects (Ivashuta et al., 2015; Mamta and Rajam, 2017). The effectiveness of eRNAi could be improved by technologies that provide (a) more effective transport across the integument (cuticle or gut), (b) greater protection against degradation by UV and enzymes, and/or (c) active transport to the target tissues. Microorganisms constitute one of methods for dsRNA delivery in insects. This system was initially utilized in *C. elegans* (Timmons and Fire 1998; Timmons et al., 2001) but has since been extensively applied to insects as well. Viruses are extremely efficient at delivering nucleic acid material into the intracellular environment; however, the *in vivo* application by viruses has not been widely investigated yet, probably due to the many safety issues that accompany their delivery (Kolliopoulou et al., 2017). Nanoparticles have also been used to increase stability and oral uptake efficiency of dsRNA in mosquitoes (Zhang et al., 2010). Liposomes have also been used as a means to protect nucleic acids in aqueous environments and they were initially tested in various drosophilid species (Whyard et al., 2009). Carrier proteins (Cell-Penetrating Peptides, CPPs) have also been used as delivery systems for dsRNA and have shown to facilitate uptake of dsRNA in the insect gut (Gillet et al., 2017). Furthermore, chemical modifications of siRNAs were shown to improve stability and uptake of these molecules (Joga et al., 2016). Lastly, potato chloroplasts have also been genetically engineered to produce dsRNA, leading to 100% RNAi-induced mortality of Colorado potato beetles that were fed on the modified leaves (Zhang et al., 2015). Given these recent developments, it is conceivable that eRNAi can potentially be used to achieve genetic sexing as part of SIT programs by targeting female-specific transcripts during the developmental stages of the generation to be released (Whyard et al., 2015). Alternatively, eRNAi targeting non-sex specific genes could be useful, if combined with an insect strain expressing male-specifically a recoded, eRNAi insensitive target. This application of eRNAi offers a greater level of control of delivery compared to other eRNAi applications (such as eRNAi pesticides), but unlike these applications it demands near 100% efficacy.

*5. Genetic Sexing Strains for SIT applications - validation in the laboratory*:
Developing large-scale operational SIT programs, regardless of the target species, depends on solving a number of common problems. A major problem is the development of suitable methods, ideally genetic sexing strains that will enable the production of large numbers of male insects in mass-rearing facilities. Despite tangible benefits, a 'generic' approach for the development of GSS, one that can be easily transferred to diverse insect species, is not available. The possibility and feasibility of developing such an approach should be the focus of research activities. There are at least two generic strategies that are currently being considered to developing GSS: 1) the creation of strains that display conditional, female-specific lethal phenotypes, and 2) strains, in which the sex determination pathway itself can be conditionally manipulated leading to sex conversion (female to male). There are many approaches that have been or could be applied to successfully implement these strategies. Of particular interest are those that are the most widely applicable with respect to the number of target species to which the solution could be implemented with a minimum of research and development efforts. Importantly, *the extent of the cross-species transferability of each system will need to be investigated, because gene functions may not be conserved between species*. For example, it may be possible to transfer sex determination-based GSS components among tephritid species but not to mosquitoes. In most cases however,

these ‘generic’ approaches to the development of GSS would reduce research and development time and costs, allowing SIT programs to be more readily developed and implemented.

### Approach 1: Exploiting induced or spontaneous mutations and chromosomal rearrangements.

Genetic sexing strains that show conditional sex-specific lethality have been successfully developed using a number of approaches. The existing Medfly GSS, VIENNA-7 and VIENNA-8, were created by chemical/radiation-induced mutagenesis resulting in strains exhibiting female-specific heat-inducible lethality resulting in male-only survival to adulthood. Females are therefore easily eliminated, by submerging bisexual early embryo collections in waterbaths set at 34°C. In other tephritid species, for example *A. ludens*, selection for spontaneous mutations were exploited for the construction of GSS. Approaches involving mutagenesis and chromosome rearrangement are referred to here as ‘classical genetic’ approaches. In the Medfly, this approach resulted in highly effective GSS; however, it took many years to develop these strains and recapitulating these efforts in other species using the same ‘classical genetic’ approaches may not be practical. A novel molecular approach is using transposon-based insertional mutagenesis that creates mutations by vector insertions, thereby ‘tagging’ mutations that have been selected by a visible or biochemical screen. This allows the straightforward isolation, sequence analysis and genome mapping of the mutated gene for further use in sex-specific selection, and identification of conserved orthologous genes in other species. This approach also eliminates unintended genomic disruption by chemical or irradiation mutagenesis, and also eliminates the need for chromosomal translocations since wild type alleles can more simply be transposed onto Y-chromosomes for male selection.

### Approach 2: CRISPR-induced mutagenesis.

New gene-editing technologies, such as the CRISPR system, will enable the precise and rapid recreation of genetic sexing genotypes. For example, temperature-sensitive lethal alleles, made previously using classical approaches in the VIENNA-7 and VIENNA-8 lines, can now be rationally designed, provided the genetic basis of the phenotype is understood. Furthermore, wild-type rescue alleles can be linked directly to Y chromosomes or M-loci using CRISPR to induce homologous recombination or large chromosomal rearrangements. This strategy, which we call ‘neo-classical’, essentially replicates the ‘classical’ genetic efforts and does not include the introduction of foreign DNA. Success of this approach will depend on the identification of genes underlying suitable selectable traits in target species, how generic specific alleles of these genes will be, and to what extent they are applicable across species targeted for GSS strain development. Its feasibility was recently successfully demonstrated by the identification of the white pupae gene in Medfly, *B. dorsalis* and *Z. cucurbitae*, and the creation of a novel white pupae phenotype strain in *B. tryoni* (Ward et al., 2021). The next step will be to identify conditional lethal mutations, such as the tsl, to enable sexing at the embryonic stage. However, until the *tsl* of the medfly is identified, it will not be clear how generic transfer of this specific allele will be, and the extent of its applicability across species targeted for GSS strain development. Alternatively, rational engineering of *tsl* alleles of conserved genes is another direction to generate GSS.

### Approach 3: Oral delivery of sex-specific lethal dsRNAs.

Conditional sex-specific lethality can also be achieved through the transient manipulation of gene expression using orally delivered double-stranded RNA (dsRNA) that induces the silencing of sex-specific genes or sex-specific isoforms of genes (RNAi) leading to lethality. Recent work has shown that diet-mediated delivery of dsRNA designed to specifically silence the expression of the female isoform of *doublesex* (*dsx*) in larvae of *Aedes aegypti* results in sex-specific lethality of female larvae (Whyard et al., 2015). This is the first time that sex-specific lethality has been linked to Dsx function, and as such further investigation is needed to validate that the approach is transferable to other SIT target species. This approach is potentially generic assuming that all insects have an RNAi system and the *dsx* gene is expected to be present and to have the same role in sex determination in all targeted insect species. This would make it a good target for gene silencing. A notable advantage of this approach is that a specially designed GSS may not be required. Diet-mediated delivery of dsRNA could also be a widely applicable mode of delivery although the sensitivity of insects to orally delivered dsRNA is variable (Darrington et al., 2017). Unfortunately, efforts to replicate experimental sex-specific lethality in mosquitoes have failed in most labs that have attempted to implement orally delivered dsRNA-based sex separation. Attempts from different labs to reproduce already published RNAi-based lethal effects lead by silencing of essential genes to *Aedes* mosquitoes through oral delivery of bacterially produced dsRNA, soaking and microinjections of different larval stages have also not been successful. The reasons for this remain unclear

and are thought to include low oral uptake of the dsRNA, issues in delivery of the dsRNAs in the relevant cells/tissues, and ultimately the production of high quality/amount of dsRNAs.

### Approach 4: Sex-specific splicing factors and effectors.

Genetic sexing strains of a number of tephritid species with genotypes resulting in conditional sex-specific lethality have been successfully created using transgenic technologies. These transgenic approaches are fairly generic in that they rely on sex-specific splicing found in genes involved in common sex determination genes and effector genes involved in conserved cell-death pathways. While some of these functional elements are known to be functional between species, it is expected that for most species these specific functional elements will need to be re-isolated and assembled. While orthologous genes and regulatory sequences might be found in more distantly related species, identifying, isolating, assembling and integrating new transgenes into new species may be difficult and time-consuming. Nevertheless, conserved elements of the sex-determining splicing-cascade, such as *tra* or *tra-2*, can be targeted to generate artificially designed "M factors" by RNA interference and dCas9 mediated gene knock down (Pane et al. 2002; Primo et al., 2020) or CRIPSR/Cas9 mediated mutagenesis (Aumann et al., 2020: DOI: 10.1038/s41598-020-75572-x; Williamson et al., 2021: doi: 10.1371/journal.pgen.1009792), which could be made conditional by controlled activation based on site-specific recombination or food supplement-controlled binary expression systems (Eckermann et al., 2014). Moreover, tetracycline-suppressible female-specific embryonic lethality systems have been shown to be highly efficient in producing male-only populations in *A. ludens*, *A. suspensa, Ceratitis capitata*, *L. cuprina*, and *C. hominivorax* using a female-specifically spliced intron from tra (Schetelig and Handler, 2012; Ogaugwu et al., 2013; Schetelig et al., 2016; Yan & Scott 2015; Concha et al., 2016). In addition, a highly conserved dominant temperature-sensitive (DTS) mutant allele of a proteasome 20S subunit gene was created and transformed into *A. suspensa*. This resulted in transgenic lines exhibiting 96-100% pupal lethality when reared at 30ºC (Nirmala et al., 2009). This conditional lethal mutation can be created for a wide variety of insect species, and made female-specific using *tra* intron1-splicing for female-lethality at elevated temperature. In addition, conditional expression of identified M factors serving as primary signals for suppressing the conserved tra splicing autoloop could be tried to establish sexing strains.

### Approach 5: Altering expression of sex-determining factors.

The genetics of sex determination is not well characterized in most insect species. However, existing data show that the *doublesex* (*dsx*) and *fruitless* (*fru*) genes play a common role in determining whether an organism develops to a male or a female. It has been shown that silencing the female form of the *dsx* in the larval stage results in female lethality in *Aedes aegypti* (Whyard et al., 2015) and CRISPR/Cas9-based knockout of *dsx* can lead to female lethality that can be used for population suppression in a gene drive approach (Kyrou et al., 2018). Considering the conservation of *dsx* and *fru*, this approach may be generic (Salvemini et al., 2011, 2013). Conditional sex-conversion could result in twice the number of male progeny by converting females into males (Saccone et al., 2011). Full masculinization was also achieved in the medfly by transient ectopic expression of *MoY* or injection of MOY recombinant protein in XX embryos (Meccariello et al., 2019). Embryonic transient Cas9 interference of Medfly *tra* (without inducing mutation) is an alternative way to induce full masculinization of XX individuals (Primo et al., 2020). If sex-conversion is the goal, manipulation of genes upstream in the sex-determination pathway, either a male determining factor or a *transformer*-like transducer gene, would be needed. One approach is the creation of temperature-sensitive mutant alleles of *transformer-2* using CRISPR/Cas9, such as the *Drosophila suzukii tra-2*$^{ts2}$ allele resulting in sterile XY males and the conversion of XX females to sterile phenotypic males at non-permissive temperatures (Li and Handler, 2017). A tra-2$^{ts2}$ allele for sex conversion was similarly created by CRISPR/Cas9 in *C. capitata* (Aumann et al., 2020: DOI: 10.1038/s41598-020-75572-x), and was used to create *tra-2* knock-outs resulting in XX female to phenotypic male conversion in *A. suspensa* (Li and Handler, 2019). Small RNAs also play a role in sex determination, such as the feminizing factor Fem piRNA that acts by suppressing Masc in B. mori, and another, albeit converse, role was found for the miRNA-1-3p microRNA that suppresses transformer, resulting in male differentiation, in *B. dorsalis* (Peng et al., 2020). Transient manipulation of *Nix*, a recently discovered male-determining factor in *Aedes aegypti* (Hall et al., 2015), resulted in partial sex conversion, and transgenic lines that ectopically express *Nix* produced fertile males (Aryan et al., 2019). The genomic methods that led to the discovery of *Nix* are relatively cost effective and can be applied in other insect species of agricultural and medical importance. Therefore, efforts to discover male-determination factors in these species may lead to new and efficient ways to produce male-only progeny and

facilitate the identification of other key regulators in the sex determination pathway, which may provide new targets of manipulation.

*6. Evaluation guidelines - Quality control of insect strains for SIT applications*:
For the successful development and implementation of a SIT project, it is critical to evaluate the quality of a GSS once it is initially developed as well as to monitor its quality before and after release. The application of quality control analysis as part of SIT programs provides valuable information to improve rearing and release practices for control of target species populations. Evaluation of strains for use in SIT programmes should be conducted by documenting the two most important parameters: a) rearing performance (production and quality control), and b) field performance (field cage or open field). There is a wealth of available literature on this field in addition to the great experience which has been accumulated over half a century of active SIT projects around the globe. The currently available information and experience in SIT projects to control tephritids has resulted in a manual that is currently used worldwide (FAO/IAEA/USDA (2014) Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies, Version 6.0. International Atomic Energy Agency, Vienna, Austria, Vienna - http://www-naweb.iaea.org/nafa/ipc/public/QualityControl.pdf).

## Selected References


Aryan, A., Anderson M., Biedler J.K., Qi, Y., Overcas J.M., Naumenko A., Sharakhova M.A., Mao C., Adelman Z.A. and Tu Z. (2019) *Nix* confers heritable sex-conversion in *Aedes aegypti* and *myo-sex* is needed for male flight. https://doi.org/10.1101/595371.sxdz

Augustinos, A.A., Drosopoulou, E., Gariou-Papalexiou, A., Bourtzis, K., Mavragani-Tsipidou, P. and Zacharopoulou, A. (2014) The *Bactrocera dorsalis* species complex: comparative cytogenetic analysis in support of Sterile Insect Technique applications. *BMC Genet,***15 Suppl 2**, S16.

Augustinos, A.A., Targovska A., Cancino-Martinez E., Schorn E., Franz G., Cáceres C., Zacharopoulou A. and Bourtzis K. (2017) *Ceratitis capitata* genetic sexing strains: laboratory evaluation of strains from mass rearing facilities worldwide. *Entomologia Experimentalis et Applicata*, **164(3)**, 305-317.

Aumann, R.A., Häcker, I. and Schetelig, M.F. 2020 Female-to-male sex conversion in *Ceratitis capitata* by CRISPR/Cas9 HDR-induced point mutations in the sex determination gene *transformer-2*. *Sci Rep*, **10**, 18611.

Aumann, R.A., Schetelig, M.F., Hacker, I. (2018) Highly efficient genome editing by homology-directed repair using Cas9 protein in *Ceratitis capitata*. *Insect Biochem Mol Biol*, **101**, 85–93.

Baker, R.H., Sakai, R.K. (1979) Triploids and male determination in the mosquito, *Anopheles culicifacies*. *J Hered*, **70,** 345–346.

Bayega A, Djambazian H, Tsoumani KT, Gregoriou ME, Sagri E, Drosopoulou E, Mavragani-Tsipidou P, Giorda K, Tsiamis G, Bourtzis K, Oikonomopoulos S, Dewar K, Church DM, Papanicolaou A, Mathiopoulos KD, Ragoussis J. (2020) De novo assembly of the olive fruit fly (Bactrocera oleae) genome with linked-reads and long-read technologies minimizes gaps and provides exceptional Y chromosome assembly. BMC Genomics, **21(1)**, 259.

Betting, J., Seufert, W. (1996) A yeast Ubc9 mutant protein with temperature-sensitive in vivo function is subject to conditional proteolysis by a ubiquitin- and proteasome-dependent pathway. *J Biol Chem* **271**, 25790-25796.

Bopp, D., Saccone, G. and Beye, M. (2014) Sex determination in insects: variations on a common theme. *Sex Dev*, **8**, 20–28.

Catteruccia, F. (2005) An *Anopheles* transgenic sexing strain for vector control. *Nat Biotechnol*, **23**, 1414–1417.

Carabajal Paladino, L.Z., Nguyen, P., Šíchová, J., Marec, F. (2014) Mapping of single-copy genes by TSA-FISH in the codling moth, *Cydia pomonella*. *BMC Genet*, **15**, S15.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y., Peterson, D.R., et al. (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nat Commun*,**9**, 3008.

Concha, C., Palavesam, A., Guerrero, F.D., Sagel, A., Li, F., Osborne, J.A., et al. (2016) A transgenic male-only strain of the New World screwworm for an improved control program using the sterile insect technique. *BMC Biol*, **14**, 72.

Curtis, C.F. (1978) Genetic sex separation in *Anopheles arabiensis* and the production of sterile hybrids. *Bull World Health Organ*, **56**, 453–454.

Darrington, M., Dalmay, T., Morrison, N.I., and Chapman, T. (2017) Implementing the sterile insect technique with RNA interference - a review. *Entomol Exp Appl*, **164**, 155–175.

Deng Z., Zhang Y., Li Y., Huang K., Chen X., Zhang M., Huang J., Ni X., Li X. (2021) Identification and characterization of the masculinizing function of the Helicoverpa armigera Masc gene. *Int J Mol Sci*, **22**, 8650.

Drosopoulou, E., Nakou, I. and Mavragani-Tsipidou, P. (2014) The *Bactrocera oleae* genome: localization of nine genes on the polytene chromosomes of the olive fruit fly (Diptera: Tephritidae). *Genome*, **57**, 573–576.

Drosopoulou, E., Nakou, I., Sichova, J., Kubickova, S., Marec, F. and Mavragani-Tsipidou, P. (2012) Sex chromosomes and associated rDNA form a heterochromatic network in the polytene nuclei of *Bactrocera oleae* (Diptera: Tephritidae). *Genetica*, **140**, 169–180.

Eckermann, K.N., Dippel, S., KaramiNejadRanjbar, M., Ahmed, H.M., Curril, I.M. and Wimmer, E.A. 2014 Perspective on the combined use of an independent transgenic sexing and a multifactorial reproductive sterility system to avoid resistance development against transgenic sterile insect technique approaches. *BMC Genet*, **15(Suppl 2)**, S17.

Ellison, K.S., Gwozd, T., Prendergast, J.A., Paterson, M.C., Ellison, M.J. (1991) A site-directed approach for constructing temperature-sensitive ubiquitin-conjugating enzymes reveals a cell cycle function and growth function for RAD6. *J Biol Chem* **266**, 24116-24120.

Farnworth, M.S., Eckermann, K.N., Ahmed, H.M.M., Mühlen, D.S., He, B. and Bucher, G. 2020 The red flour beetle as model for comparative neural development: genome editing to mark neural cells in *Tribolium* brain development. *Methods Mol Biol*, **2047**, 191-217.

Franz, G. (2002) Recombination between homologous autosomes in medfly (*Ceratitis capitata*) males: type-1 recombination and the implications for the stability of genetic sexing strains. *Genetica*, **116**, 73–84.

Franz, G. (2005) Genetic Sexing Strains in Mediterranean Fruit Fly, an Example for Other Species Amenable to Large-Scale Rearing for the Sterile Insect Technique BT - Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management. in (eds. Dyck, V. A., Hendrichs, J. & Robinson, A. S.) 427–451 (Springer Netherlands). doi:10.1007/1-4020-4051-2_16.

Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L., Condon, G.C., et al. (2007) Female-specific insect lethality engineered using alternative splicing. *Nat Biotechnol*, **25**, 353-357.

Fukova, I., Nguyen, P. and Marec, F. (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. *Genome*, **48**, 1083-92.

Fukova, I., Traut, W., Vitkova, M., Nguyen, P., Kubickova, S. and Marec, F. (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. *Chromosoma*, **116**, 135–145.

Fukui, T., Kiuchi, T., Shoji, K. Kawamoto, M., Shimada, T., Katsuma, S. (2018) In vivo masculinizing function of the *Ostrinia furnacalis Masculinizer* gene. *Biochem Biophys Res Commun*, **503**, 1768–1772.

Gailey, D.A., Billeter, J.C., Liu, J.H., Bauzon, F., Allendorfer, J.B. and Goodwin, S.F. (2006) Functional conservation of the fruitless male sex-determination gene across 250 Myr of insect evolution. *Mol Biol Evol*, **23**, 633–643.

Gilchrist, B.M., Haldane, J.B.S. (1947) Sex linkage and sex determination in a mosquito, *Culex molestus*. *Hereditas*, **33**, 175–190.

Gempe, T., Hasselmann, M., Schiøtt, M., Hause, G., Otte, M. and Beye, M. (2009) Sex determination in honeybees: two deparate mechanisms induce and maintain the female pathway. *PLoS Biol*, **7**, e1000222.

Gillet, F.X., Garcia, R.A., Macedo, L.L.P., Albuquerque, E.V.S., Silva, M.C.M., Grossi-de-Sa, M.F. (2017) Investigating engineered ribonucleoprotein particles to improve oral RNAi delivery in crop insect pests. *Front Physiol*, **8**: 256.

Gomulski LM, Mariconti M, Di Cosimo A, Scolari F, Manni M, Savini G, Malacrida AR, Gasperi G (2018) The Nix locus on the male-specific homologue of chromosome 1 in Aedes albopictus is a strong candidate for a male-determining factor. *Parasites & Vectors*, **11 Suppl 2**

Hall, A.B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V.A., Biedler, J.K., et al. (2015) Sex Determination. A male-determining factor in the mosquito *Aedes aegypti*. *Science*, **348**, 1268–1270.

Harvey-Samuel T., Norman V.C., Carter R., Lovett E., Alphey L. (2020) Identification and characterization of a Masculinizer homologue in the diamondback moth, Plutella xylostella. Insect Mol Biol, **29**, 231–240.

Hediger, M., Henggeler, C., Meier, N., Perez, R., Saccone, G. and Bopp, D. (2010) Molecular characterization of the key switch F provides a basis for understanding the rapid divergence of the sex-determining

pathway in the housefly. *Genetics*, **184**, 155–170.
Hew, B.E., Sato, R., Mauro, D., Stoytchev, I. and Owens, J.B. 2019 RNA-guided piggyBac transposition in human cells. *Synth Biol*, 4, ysz018.
Hopkins, B.R. and Kopp, A. 2021 Evolution of sexual development and sexual dimorphism in insects. *Curr Opin Genet Dev*, **69**, 129-139.
International Glossina Genome, I. (2014) Genome sequence of the tsetse fly (*Glossina morsitans*): vector of African trypanosomiasis. *Science*, **344**, 380–386.
Isasawin S, Aketarawong N, Thanaphum S (2012) Characterization and evaluation of microsatellite markers in a strain of the oriental fruit fly, Bactrocera dorsalis (Diptera: Tephritidae), with a genetic sexing character used in sterile insect population control. *Eur J Entomol*, **109**, 331-338.
Ivashuta, S., Zhang, Y., Wiggins, B.E., Ramaseshadri, P., Segers, G.C., Johnson, S., et al. (2015) Environmental RNAi in herbivorous insects. *RNA*, **21**, 840–850.
Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., Morrison, N.I., Alphey, L. (2013) Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synth Biol*, **2**, 160–166.
Joga, M.R., Zotti, M.J., Smagghe, G., Christiaens, O. (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Front Physiol*, **7**, 553.
Kaiser, P.E., Seawright, J.A., Dame, D.A., Joslyn, D.J. (1978) Development of a genetic sexing system for *Anopheles albimanus*. *J Econ Entomol*, **71**, 766–761.
Karaminejadranjbar, M., Eckermann, K.N., Ahmed, H.M.M., Sanchez, C.H., Dippel, S., Marshall, J.M., et al. (2018) Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management. *Proc Natl Acad Sci U S A*, **115**, 6189–6194.
Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., et al. (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature*, **509**, 633–636.
Kolliopoulou, A., Taning, C.N.T., Smagghe, G., Swevers, L. (2017) Viral delivery of dsRNA for control of insect agricultural pests and vectors of human disease: prospects and challenges. *Front Physiol*, **8**, 399.
Krzywinska, E., Dennison, N.J., Lycett, G.J., Krzywinski, J. (2016) A maleness gene in the malaria mosquito *Anopheles gambiae*. *Science*, **353**, 67–69.
Kuntamalla, P.P., Kunttas-Tatli, E., Karandikar, U., Bishop, C.P., Bidwai, A.P. (2009) *Drosophila* protein kinase CK2 is rendered temperature-sensitive by mutations of highly conserved residues flanking the activation segment. *Mol Cell Biochem* **323**, 49–60.
Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A.K., et al. (2018) A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nat Biotechnol*, **36**, 1062–1066.
Laohakieat, K., Aketarawong, N., Isasawin, S., Thitamadee, S., Thanaphum, S. (2016) The study of the transformer gene from *Bactrocera dorsalis* and *B. correcta* with putative core promoter regions. *BMC Genetics*, **17**, 34.
Laohakieat, K., Isasawin, S., Thanaphum, S. (2020) The transformer-2 and fruitless characterisation with developmental expression profiles of sex-determining genes in Bactrocera dorsalis and B. correcta. *Scientific Reports*, **10**,17938.
Lebon C, Benlali A, Atyame C, Mavingui P, Tortosa P. (2018). Construction of a genetic sexing strain for *Aedes albopictus*: a promising tool for the development of sterilizing insect control strategies targeting the tiger mosquito *Parasit Vectors*, **11**, 658.
Lee, J., Kiuchi, T., Kawamoto, M., Shimo. *Para*ada, T., Katsuma, S. (2015) Identification and functional analysis of a *Masculinizer* orthologue in *Trilocha varians* (Lepidoptera: Bombycidae). *Insect Mol Biol*, **24**, 561–569.
Li, J., Handler, A.M. (2019) CRISPR/Cas9-mediated gene editing in an exogenous transgene and an endogenous sex determination gene in the Caribbean fruit fly, *Anastrepha suspensa*. *Gene*, **691**, 160–166.
Lines JD, Curtis CF. Genetic sexing systems in *Anopheles arabiensis* Patton (Diptera: Culicidae) J Econ Entomol. 1985; **78**, 848–841.
Mamta, B., Rajam, M.V. (2017) RNAi technology: a new platform for crop pest control. *Physiology and Molecular Biology of Plants*, 23, 487-501.
Marec F., Vreysen M.J.B. (2019) Advances and challenges of using the sterile insect technique for the management of pest Lepidoptera. Insects, **10**, 371.
Marec, F., Neven, L.G., Robinson, A.S., Vreysen, M., Goldsmith, M.R., Nagaraju, J., et al. (2005) Development

of genetic sexing strains in Lepidoptera: from traditional to transgenic approaches. *J Econ Entomol*, **98**, 248–259.

Matthews, B.J., Dudchenko, O., Kingan, S., Koren, S., Antoshechkin, I., Crawford, J.E., et al. (2018) Improved *Aedes aegypti* mosquito reference genome assembly enables biological discovery and vector control. *Nature (in press)*.

McCombs SD, Saul SH (1995) Translocation-based genetic sexing system for the oriental fruit fly (Diptera: Tephritidae) based on pupal color dimorphism. *Ann Entomol Soc Am*, **88**, 695-698.

McInnis DO, Tam S, Lim R, Komatsu J, Kurashima R, Albrecht C (2004) Development of a pupal color-based genetic sexing strain of the melon fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). *Ann Entomol Soc Am*, **97**, 1026-1033.

Meccariello, A., Monti, S.M., Romanelli, A., Colonna, R., Primo, P., Inghilterra, M.G., et al. (2017) Highly efficient DNA-free gene disruption in the agricultural pest *Ceratitis capitata* by CRISPR-Cas9 ribonucleoprotein complexes. *Sci Rep*, **7**, 10061.

Meccariello, A., Salvemini, M., Primo, P., Hall, B., Koskinioti, P., Dalíková, M., Gravina, A., Gucciardino, M.A., Forlenza, F., Gregoriou, M.E., Ippolito, D., Monti, S.M., Petrella, V., Perrotta, M.M., Schmeing, S., Ruggiero, A., Scolari, F., Giordano, E., Tsoumani, K. T., Marec, F., Windbichler, N., Nagaraju, J., Arunkumar, K., Bourtzis, K., Mathiopoulos, K. D., Ragoussis, J., Vitagliano, L., Tu, Z., Papathanos, P.A., Robinson, M. D. and Saccone, G. (2019). Maleness-on-the-Y (MoY) orchestrates male sex determination in major agricultural fruit fly pests. Science 365: 1457–1460. DOI: 10.1126/science.aax1318.

Nagaraju, J., Saccone, G. (2010) How is sex determined in insects? Preface. *J. Genet*, **89**, 269–270.

Nguyen, P., Sýkorová, M., Šíchová, J., Kůta, V., Dalíková, M., Čapková Frydrychová, R., et al. (2013) Neo-sex chromosomes and adaptive potential in tortricid pests. *Proc Natl Acad Sci U S A*, **110**, 6931–6936.

Nirmala, X., Zimowska, G.J. and Handler, A.M. 2009 Characterization of the proteasomebeta2 subunit gene and its mutant allele in the tephritid fruit fly pest, *Anastrepha suspensa*. *Insect Mol Biol*, **18**, 333-40.

Ogaugwu CE, Schetelig MF, Wimmer EA (2013) Transgenic sexing system for *Ceratitis capitata* (Diptera: Tephritidae) based on female-specific embryonic lethality. Insect Biochemistry and Molecular Biology **43**(1): 1-8.

Pane, A., Salvemini, M., Delli Bovi, P., Polito, C., Saccone, G. (2002) The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development*, **129**, 3715–3725.

Papanicolaou, A., Schetelig, M.F., Arensburger, P., Atkinson, P.W., Benoit, J.B., Bourtzis, K., et al. (2016) The whole genome sequence of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), reveals insights into the biology and adaptive evolution of a highly invasive pest species. *Genome Biol*, **17**, 192.

Peng, W., Yu, S., Handler, A.M., Tu, Z.J., Saccone, G., Xi, Z., and Zhang, H. (2020) miRNA-1-3p is an early embryonic male sex-determining factor in the Oriental fruit fly Bactrocera dorsalis. Nature Communications, **11**, 932.

Porras, M.F., Meza, J.S., Rajotte, E.G., Bourtzis, K. and Cáceres-Barrios, C. (2020) Improving the phenotypic properties of the *Ceratitis capitata* (Diptera: Tephritidae) temperature sensitive lethal genetic strain in support of sterile insect technique applications. *Journal of Economic Entomology*, **113**, 2688–2694.

Primo P., Meccariello A., Inghilterra M. G., Gravina A., Del Corsano G., Volpe G., Sollazzo G., Aceto S., Robinson M. D., Salvemini M., Saccone G. (2020) Targeting the autosomal *Ceratitis capitata* transformer gene using Cas9 or dCas9 to masculinize XX individuals without inducing mutations. *BMC Genetics*, **21**, 1471-2156.

Robinson, A.S. (1986) Genetic sexing in *Anopheles stephensi* using dieldrin resistance. *J Am Mosq Control Assoc*, **2**, 93–95.

Saccone, G., Peluso, I., Artiaco, D., Giordano, E., Bopp, D., Polito, L.C. (1998) The *Ceratitis capitata* homologue of the *Drosophila* sex-determining gene *Sex-lethal* is structurally conserved, but not sex-specifically regulated. *Development*, **125**, 1495–500.

Saccone, G., Pane, A., Salvemini, M., De Simone, A., Milano, A., Polito, L.C. (2007) New sexing strains: transforming *Ceratitis capitata* females into males. In: Area-wide Control of Insect Pests: from research to field implementation, pp. 95–102, Vreysen, M.J.B., Robinson, A.S., Hendrichs, J. (Eds.), Springer (ISBN 978-1-4020-6058-8).

Saccone, G., Salvemini, M., Polito, L.C., et al. (2011). The *transformer* gene of *Ceratitis capitata*: a paradigm for a conserved epigenetic master regulator of sex determination in insects. *Genetica*, **139**, 99–111.

Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L.C. and Saccone, G. (2009) *Ceratitis capitata*

*transformer-2* gene is required to establish and maintain the autoregulation of *Cctra*, the master gene for female sex determination. *Int J Dev Biol*, **53**, 109–120.
Salvemini, M., Mauro, U., Lombardo, F., Milano, A., Zazzaro, V., Arca, B., et al. (2011) Genomic organization and splicing evolution of the *doublesex* gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. *BMC Evol Biol*, **11**, 41.
Salvemini, M., D'amato, R., Petrella, V., Aceto, S., Nimmo, D., Neira, M., et al. (2013) The orthologue of the fruitfly sex behaviour gene *fruitless* in the mosquito *Aedes aegypti*: evolution of genomic organisation and alternative splicing. *PLoS One*, **8**, e48554.
Sanchez, L. (2008) Sex-determining mechanisms in insects. *Int J Dev Biol*, **52**, 837–856.
Scali, C., Catteruccia, F., Li, Q. and Crisanti, A. (2005) Identification of sex-specific transcripts of the *Anopheles gambiae doublesex* gene. *J Exp Biol*, **208**, 3701–3709.
Schetelig, M.F., Handler, A.M. (2012) A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochem Mol Biol*, **42**, 790–795.
Schetelig, M.F., Milano, A., Saccone, G., Handler, A.M. (2012) Male only progeny in *Anastrepha suspensa* by RNAi-induced sex reversion of chromosomal females. *Insect Biochem Mol Biol*, **42**, 51–57.
Schetelig, M.F., Targovska, A., Meza, J.S., Bourtzis, K., Handler, A.M. (2016) Tetracycline-suppressible female lethality and sterility in the Mexican fruit fly, *Anastrepha ludens*. *Insect Mol Biol*, **25**, 500–508.
Sharma, A., Heinze, S.D., Wu, Y., Kohlbrenner, T., Morilla, I., Brunner, C., et al. (2017) Male sex in houseflies is determined by *Mdmd*, a paralog of the generic splice factor gene *CWC22*. *Science*, **356**, 642–645.
Sichova, J., Nguyen, P., Dalikova, M. and Marec, F. (2013) Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. *PLoS One*, **8**, e64520.
Stratikopoulos, E.E., Augustinos, A.A., Petalas, Y.G., Vrahatis, M.N., Mintzas, A., Mathiopoulos, K.D., et al. (2008) An integrated genetic and cytogenetic map for the Mediterranean fruit fly, *Ceratitis capitata*, based on microsatellite and morphological markers. *Genetica*, **133**, 147–157.
Tan, A., Fu, G., Jin, L., Guo, Q., Li, Z., Niu, B., Meng, Z., Morrison, N.I., Alphey, L., Huang, Y. (2013) Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*. *Proc Nat Acad Sci U S A*, **110**, 6766–6770.
Timmons, L., Fire, A. (1998) Specific interference by ingested dsRNA. *Nature*, **395**, 854–854.
Timmons, L., Court, D.L., Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene*, **263**, 103–112.
Traut, W., Sahara, K. and Marec, F. (2007) Sex chromosomes and sex determination in Lepidoptera. *Sex Dev*, **1**, 332–346.
Traut, W., Vogel, H., Glockner, G., Hartmann, E. and Heckel, D.G. (2013) High-throughput sequencing of a single chromosome: a moth W chromosome. *Chromosome Res*, **21**, 491–505.
Turner, J., Krishna, R., Van 'T Hof, A.E., Sutton, E.R., Matzen, K., Darby, A.C. (2018) The sequence of a male-specific genome region containing the sex determination switch in *Aedes aegypti*. *Parasit Vectors*, **11**, 549.
Van't Hof, A.E., Campagne, P., Rigden, D.J., Yung, C.J., Lingley, J., Quail, M.A., et al. (2016) The industrial melanism mutation in British peppered moths is a transposable element. *Nature*, **534**, 102–105.
Visser, S., Voleníková, A., Nguyen, P., Verhulst, E.C. and Marec, F. (2021) A conserved role of the duplicated *Masculinizer* gene in sex determination of the Mediterranean flour moth, *Ephestia kuehniella*. *PLoS Genet*, **17**, e1009420.
Ward, C.M., Aumann, R.A., Whitehead, M.A., Nikolouli, K., Leveque, G., Gouvi, G., Fung, E., Reiling, S.J., Djambazian, H., Hughes, M.A., Whiteford, S., Caceres-Barrios, C., Nguyen, T.N.M., Choo, A., Crisp, P., Sim, S.B., Geib, S.M., Marec, F., Häcker, I., Ragoussis, J., Darby, A.C., Bourtzis, K., Baxter, S.W. and Schetelig, M.F. (2021) White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. *Nature Communications*, 12, 491.
Wang, Y.H. Chen, X.E., Yang, Y., Xu, J., Fang, G.Q., Niu, C.Y., Huang, Y.P., Zhan, S. (2019) The *Masc* gene product controls masculinization in the black cutworm, *Agrotis ipsilon*. *Insect Sci*, **26**, 1037–1044.
Wexler, J., Delaney, E.K., Belles, X., Schal, C., Wada-Katsumata, A., Amicucci, M.J. and Kopp, A. Hemimetabolous insects elucidate the origin of sexual development via alternative splicing. *Elife*, 8, e47490.
Whyard, S., Erdelyan, C.N., Partridge, A.L., Singh, A.D., Beebe, N.W., Capina, R. (2015) Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasit Vectors*, **8**, 96.

Whyard, S., Singh, A.D., Wong, S. (2009) Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem Mol Biol*, **39**, 824–832.
Willhoeft, U., Franz, G. (1996) Identification of the sex-determining region of the *Ceratitis capitata* Y chromosome by deletion mapping. *Genetics*, **144**, 737–745.
Willhoeft, U., Mueller-Navia, J., Franz, G. (1998) Analysis of the sex chromosomes of the Mediterraean fruit fly by microdissected DNA probes. *Genome*, **41**, 74–78.
Williamson, M.E., Yan, Y. and Scott, M.J. (2021) Conditional knockdown of transformer in sheep blow fly suggests a role in repression of dosage compensation and potential for population suppression. *PLoS Genet*, **17**, e1009792.
Yamada H, Benedict MQ, Malcolm CA, Oliva CF, Soliban SM, Gilles JR. Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin. Malar J. 2012;**11**:208.
Yan, Y. and Scott, M. J. (2015) A transgenic embryonic sexing system for the Australian sheep blow
Yoshido A., Sahara K., Yasukochi Y. (2014) Silk Moths (Lepidoptera). In: Sharakhov I.V. (ed.) Protocols for Cytogenetic Mapping of Arthropod Genomes. CRC Press, Boca Raton, FL, USA, pp. 219-256
Zepeda-Cisneros, C. S., J. S. Meza-Hernández, V. García-Martínez, J. Ibañez-Palacios, A. Zacharopoulou, A., and G. Franz 2014. Development, genetic and cytogenetic analyses of genetic sexing strains of the Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae). BMC genetics, 15(2), S1.
Zhang, J., Khan, S.A., Hasse, C., Ruf, S., Heckel, D.G., Bock, R. (2015) Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science*, **293**, 860–864.
Zhang, Z., Klein, J. and Nei, M. (2014) Evolution of the sex-lethal gene in insects and origin of the sex-determination system in *Drosophila*. *J Mol Evol*, **78**, 50–65.
Zhang, X., Zhang, J., Zhu, K.Y. (2010) Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). *Insect Mol Biol*, **19**, 683–693.
Zou, Y., Geuverink E., Beukeboom L.W., Verhulst E.C. and van de Zande, L. 2020 A chimeric gene paternally instructs female sex determination in the haplodiploid wasp *Nasonia*. *Science*, 370, 1115-1118.

### Sustainable Development Goals (SDG) which are linked to the proposal:

2. End hunger, achieve food security and improved nutrition and promote sustainable agriculture.
3. Ensure healthy lives and promote well-being for all at all ages.

### IAEA.org topic(s):

Nuclear technology and applications; Food and agriculture; Insect Pest Control; Sterile Insect Technique.

### Nuclear Component

This CRP aims at the development and / or evaluation of genetic sexing strains for use in SIT programmes. The SIT relies on the use of ionizing radiation to sterilize large numbers of insects. Radiation-induced sterility provides a very high level of biosafety and can be used in combination with genetic sexing strains developed and / or evaluated in this CRP. As radiation induces random dominant mutations, there is no possibility of resistance developing to this physical process, a possibility which cannot be excluded with other methods, for example molecular-based approaches.

### Participation of Agency's laboratories

The CRP needs to be supported through adaptive research and development carried out at the IPCL, FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf as part of Projects 2.1.4.1 and 2.1.4.3. This R&D will focus on the isolation and characterization of markers (ideally morphological and / or temperature sensitive lethal), and the evaluation of marker strains and genetic sexing strains for SIT applications developed in the frame of this CRP.

### Sequencing and bioinformatic support for projects.

1) Medfly/Tephritidae *wp tsl* project (Kostas Bourtzis, Marc Schetelig, Alistair Darby, Ioannis Ragoussis, Philippos Papathanos, Nikolai Windbichler, Scott Geib, Simon Baxter, Giuseppe Saccone).
2) Aedes species red-eye project (Kostas Bourtzis, Jake Tu).

3) Anopheles species tsl project (Kostas Bourtzis, Cyrille Ndo, Philippos Papathanos).
4) Genome comparison resources for Tephritidae (including the Y-chromosome) and mosquitoes in order to cover other genomes that may need sequencing (Alistair Darby, Philippos Papathanos, Ioannis Ragoussis, Kostas Mathiopoulos, Kostas Bourtzis, Jake Tu, Giuseppe Saccone).
5) RNAseq for gene expression and small RNA discovery in lepidopterans and dipterans (Kostas Mathiopoulos, Philippos Papathanos, Frantisek Marec, Kostas Bourtzis, Giuseppe Saccone, Ioannis Ragoussis).
6) Anastrepha black pupae project (Kostas Bourtzis, Juan Pedro Wulff, Salvador Meza, Edwin Ramirez, Simon Baxter, Alistair Darby, Ioannis Ragoussis).

## Assumptions

Member States continue to recognize the benefits of developing the SIT package and other genetic and environment-friendly methods for sustainable control of insect pests of agricultural, veterinary and medical importance in AW-IPM programmes and continue to request improved technology and high-quality SIT strains in order to maximise benefit/cost projections.

The demand for area-wide integrated insect pest management approaches, including SIT and augmentative biological control as non-polluting suppression/eradication components, continues to increase, mandating expansion and improvement in cost-effectiveness of these environment-friendly, sustainable approaches.

## Related TC projects

BOL5022 - Reducing Fruit Fly Populations in Different Regions Introducing an Integrated Pest Management Approach Including the Use of the Sterile Insect Technique.

BRA5061 - Using the Sterile Insect Technique to Evaluate a Local Strain in the Control of *Aedes aegypti* (Phase II).

CHI5051 - Implementing Pilot Level of Sterile Insect Technique for Control of *Lobesia botrana* in Urban Areas.

CPR5026 - Applying the Sterile Insect Technique as Part of an Area-wide Integrated Pest Management Approach to Control Two Fruit Flies.

CUB5021 - Demonstrating the Feasibility of the Sterile Insect Technique in the Control of Vectors and Pests.

DOM0006 - Building and Strengthening the National Capacities and Providing General Support in Nuclear Science and Technology.

ECU5031 - Enhancing the Application of the Sterile Insect Technique as Part of an Integrated Pest Management Approach to Maintain and Expand Fruit Fly Low Prevalence and Free Areas.

GUA5021 - Strengthening National Capabilities for the Control of Agricultural Pests Using Nuclear Technologies.

ISR5021 – Assisting in the Development of a Strategy to Counteract *Bactrocera zonata*.

LIB5014 – Supporting Control of Fruit Flies by Establishing a Low Fruit Fly Prevalence Zone.

MAR5026 - Sustaining the Suppression of Aedes albopictus in a Rural Area with Possible Extension to An Urban Dengue-Prone Locality through Integrated Vector Management Strategy.

MEX5032 - Scaling up the Sterile Insect Technique to Control Dengue Vectors.

MOR5038 - Strengthening the Sterile Insect Technique.

PLW5003 – Facilitating Sustainability and Ensuring Continuity of Area-wide Pest Management - Phase III.

SAF5017 - Assessing the Sterile Insect Technique for Malaria Mosquitos - Phase III.

SEY5012 - Establishing Area-wide Integrated Pest Management by Using the Sterile Insect Technique in Combination with Other Control Methods on the Suppression of the Melon Fly.

SUD5038 - Implementing the Sterile Insect Technique for Integrated Control of *Anopheles arabiensis*, Phase II.

TUR5026 - Conducting a Pilot Program on Integrated Management of Aedes aegypti Including Sterile Insect Technique.

URT5035 - Implementing the Sterile Insect Technique as Part of Area-wide Integrated Pest Management for Controlling Invasive Fruit Fly Populations.

VIE5021 – Integration of the Sterile Insect Technique with Other Suppression Methods for Control of *Bactrocera* fruit flies in Dragon Fruit Production.

RAF5074 - Enhancing Capacity for Detection, Surveillance and Suppression of Exotic and Established Fruit Fly Species through Integration of Sterile Insect Technique with Other Suppression Methods.

RAS5082 - Managing and Controlling Aedes Vector Populations Using the Sterile Insect Technique.

RAS5090 - Advancing and Expanding Area-wide Integrated Management of Invasive Pests, Using Innovative Methodologies Including Atomic Energy Tools.

RER5026 - Enhancing the Capacity to Integrate Sterile Insect Technique in the Effective Management of Invasive Aedes Mosquitoes.

RLA5082 - Strengthening Food Security through Efficient Pest Management Schemes Implementing the Sterile Insect Technique as a Control Method.

RLA5083 - Enhancing Capacity for the Use of the Sterile Insect Technique as a Component of Mosquito Control Programs.

RLA5084 - Developing Human Resources and Building Capacity of Member States in the Application of Nuclear Technology to Agriculture.

INT5155 - Sharing Knowledge on the Sterile Insect and Related Techniques for the Integrated Area-wide Management of Insect Pests and Human Disease Vectors.

**LFM-Logical Framework Matrix Input**:

**Overall Objectives**:

The main objective of this CRP is the development and evaluation of generic approaches for the construction of genetic sexing strains (GSS) to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors.

**Specific Objectives**:

1) To develop generic strategies for the construction of GSS for SIT applications
2) To assess the efficiency, applicability and the range of the species transferability of the generic approaches
3) To evaluate, at small scale, GSS developed through the generic approaches

**Outcomes**:

1) Generic strategies for the development of GSS for SIT applications developed
2) The efficiency, applicability and the range of species transferability of the generic approaches assessed
3) GSS developed through the generic approaches evaluated at small scale

**Outputs**:

1) Markers to be used for generic strategies for the development of GSS for SIT applications against targeted agricultural pests identified (at least two markers)
2) Markers to be used for generic strategies for the development of genetic sexing strains for SIT applications against targeted disease vectors identified (at least two markers)
3) Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted agricultural pests evaluated (at least two strains)
4) Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted disease vectors evaluated (at least two strains)
5) GSS based on generic approaches for SIT applications against targeted agricultural pests developed (at least two strains)
6) GSS based on generic approaches for SIT applications against targeted disease vectors developed (at least two strains)
7) GSS developed based on generic approaches for SIT applications against targeted agricultural pests at small scale evaluated (at least two strains)
8) GSS developed based on generic approaches for SIT applications against targeted disease vectors at small scale evaluated (at least two strains)
9) Publication of results in a peer reviewed journal

**Activities**:

1. Selecting participants and awarding contracts and agreements
2. Organizing the first RCM.
3. Organizing the second RCM.
4. Evaluation of the mid-term CRP.
5. Organizing the third RCM.
6. Organizing the fourth RCM.
7. Final evaluations.
8. Publish the results of the CRP in a special issue of an international journal.

**LOGICAL FRAMEWORK**:

| **Narrative Summary** | ***Objective Verifiable Indicators*** | ***Means of Verification*** | ***Important Assumptions*** |
|---|---|---|---|
| ***Overall Objective***<br><br>The main objective of this CRP is the development and evaluation of generic approaches for the construction of genetic sexing strains (GSS) to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors. | N/A | N/A | Requests by Member States in the area of insect pest and disease vector control using the SIT are increasing. To transfer this nuclear technology to Member States, the availability of genetic sexing strains for an efficient, cost-effective, safe and biosecure implementation at large scale is an essential precondition. Biological material is available. |

| | | | |
|---|---|---|---|
| **Specific Objectives** | | | |
| 1. To develop generic strategies for the construction of GSS for SIT applications | At least two generic strategies for the construction of GSS developed. | Reports and / or published papers. | Generic strategies for the construction of GSS can be developed. |
| 2. To assess the efficiency, applicability and the range of the species transferability of the generic approaches | The efficiency and the range of the applicability of at least two generic approaches assessed. | Reports and / or published papers. | Assessing the efficiency and the range of the applicability of the generic approaches is possible. |
| 3. To evaluate, at small scale, GSS developed through the generic approaches | At least two GSS developed through the generic approaches evaluated. | Reports and / or published papers. | Protocols for the evaluation of GSS developed through the generic approaches are available or can be developed. |
| **Outcomes** | | | |
| 1. Generic strategies for the development of GSS for SIT applications developed | Protocols and approaches determined | Data collected | Facilities and resources available. |
| 2. The efficiency, applicability and the range of species transferability of the generic approaches assessed | Tools and protocols developed | Data collected | Facilities and resources available. |
| 3. GSS developed through the generic approaches evaluated at small scale | Tools and protocols developed | Data collected | Facilities and resources available. |

| | | | |
|---|---|---|---|
| **Outputs** | | | |
| 1. Markers to be used for generic strategies for the development of GSS for SIT applications against targeted agricultural pests identified (at least two markers). | At least two markers identified. | Reports and or published papers. | Biological material is available. Protocols are available or can be developed. |
| 2. Markers to be used for generic strategies for the development of genetic sexing strains for SIT applications against targeted disease vectors identified (at least two markers). | At least two markers identified. | Reports and or published papers. | Biological material is available. Protocols are available or can be developed. |
| 3. Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted agricultural pests evaluated (at least two strains). | At least two strains evaluated. | Reports and or published papers. | Biological material is available. QC protocols are available or can be developed. |
| 4. Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted disease vectors evaluated (at least two strains). | At least two strains evaluated. | Reports and or published papers. | Biological material is available. QC protocols are available or can be developed. |
| 5. GSS based on generic approaches for SIT applications against targeted agricultural pests developed (at least two strains). | At least two strains developed. | Reports and or published papers. | Biological material and tools are available. Protocols are available or can be developed. |
| 6. GSS based on generic approaches for SIT applications against targeted disease vectors developed (at least two strains). | At least two strains developed. | Reports and or published papers. | Biological material and tools are available. Protocols are available or can be developed. |
| 7. GSS developed based on generic approaches for SIT applications against targeted agricultural pests at small scale evaluated (at least two strains). | At least two strains evaluated. | Reports and published papers | Biological material is available. QC protocols are available or can be developed. |
| 8. GSS developed based on generic approaches for SIT applications against targeted disease vectors at small scale evaluated (at least two strains). | At least two strains evaluated. | Reports and or published papers | Biological material is available. QC protocols are available or can be developed. |
| 9. Publication of results in a peer | Papers drafted and | Journal issue | Data for publication available. |

| | | | |
|---|---|---|---|
| reviewed journal. | submitted. | with published scientific papers. | |
| ***Activities*** | | | |
| 1. Selecting participants and awarding contracts and agreements. | Proposals evaluated and 9 Research Contracts, 12 Research Agreements and 1 Technical Contract awarded. | Signed contracts and agreements. | Suitable proposals submitted, funding available and approval of Contracts and Agreements by CCRA-NA committee. |
| 2. Organising the first RCM. | 1st RCM held 2019. | Participants' activities and logical framework revised. | Contracts and Agreements signed by counterpart organisations. |
| 3. Organising the second 2nd RCM. | 2nd RCM to be held 2021. | Participants and RCM Progress Reports. | Progress satisfactory. |
| 4. Evaluation of the mid-term CRP. | Mid-term CRP evaluation presented to CCRA | Mid-CRP report | Progress satisfactory. |
| 5. Organising the third RCM. | 3rd RCM to be held 2022. | Participants and RCM Progress Reports. | Progress satisfactory and mid-CRP evaluation approved by CCRA-NA committee. |
| 6. Organise the fourth RCM. | 4th RCM to be held 2024. | Participants and RCM Final Reports | Final reports are submitted to the Agency. |
| 7. Final evaluations | Final CRP evaluation approved by CCRA | Final CRP evaluation | Progress satisfying. |
| 6. Special issue published. | Publication | Special issue published. | Each contract and agreement holder contribute with a paper to the Special Issue. |

**SECOND RESEARCH COORDINATION MEETING**

**Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture**

**"Generic approach for the development of genetic sexing strains for SIT applications"**

**IAEA Headquarters, Vienna International Center, Austria**
**Room M4**

**18 - 22 October 2021**

***Project Officer: Kostas Bourtzis***

**Monday, 18 October 2021**

| | |
|---|---|
| 08:00 – 09:00 | Identification and registrations at the VIC Gate (next to subway station U1); Carry the passport and grounds passes to be obtained |
| 09:00 – 09:10 | **Rui Cardoso-Pereira -** Opening of the meeting. |
| 09:10 – 09:30 | Introduction of participants, administrative announcements. |
| 09:30 – 09:45 | **Kostas Bourtzis -** "Current status of the CRP and future challenges". |
| 09:45 – 10:30 | *Coffee Break* |

**SESSION I: Presentations by participants (Chairperson: Philippos Papathanos)**

| | |
|---|---|
| 10:30 – 11.00 | **Germano Sollazzo and Maria-Eleni Gregoriou** – **"***White pupae* and *temperature sensitive lethal* genes as generic markers for the construction of genetic sexing strains for SIT applications". |
| 11:00 – 11:30 | **Roswitha A. Aumann –** "Towards generic GSS design: analysis of *white pupae* and a *tsl/sd* candidate gene in *Ceratitis capitata*". |
| 11:30 – 12:00 | **Angela Meccariello –** "Efficient generation of autosomal transgenes using CRISPR/Cas9 knock-in in *Ceratitis capitata*". |
| 12:00 – 13:00 | *Lunch Break* |
| 13:00 – 13:30 | **Ernst A. Wimmer** – "Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools". |
| 13:30 – 14:00 | **Antonios Augustinos** – "Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species". |
| 14:00 – 14:30 | **Kostas D. Mathiopoulos** – "Exploring structure and function of the tephritid Y chromosome". |
| 14:30 – 15:00 | **Jiannis Ragoussis (virtual)** – "Nanopore long-read RNA-seq and absolute quantification delineate transcription dynamics in early embryo development of *Bactrocera oleae*". |
| 15:00 – 15:30 | **Alistair C. Darby** – "Working towards functional genomics in tsetse"**.** |
| 15:30 – 16:00 | *Coffee Break* |
| 16:00 – 16:30 | **Alfred M. Handler (virtual)** – "New target genes for male sterility in *Drosophila suzukii* |

and *Anastrepha suspensa*".

16:30 – 17:00 **Silvia B. Lanzavecchia (virtual)** – "Development of genetic sexing strains for *Anastrepha fraterculus* approaching with generic genomic tools".

17:00 – 17:30 **José S. Meza (virtual)** – "Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*".

17:30 – 18:00 **Edwin Ramirez (virtual)** – "Development and evaluation of genetic sexing strains of fruits flies to be used for sterile insect technique applications".

**Tuesday, 19 October 2021**

**SESSION I (cont'd): Presentations by participants (Chairperson: Marc Schetelig)**

08:30 – 09:00 **Simon W. Baxter (virtual)** – "Developing *Bactrocera tryoni* genetic sexing strains".

09:00 – 09:30 **Nidchaya Aketarawong (virtual)** – "Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera spp.* using bioinformatics and molecular tools".

09:30 – 10:00 **Wei Peng (virtual)** – "The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii*".

10:00 – 10.30 *Coffee Break*

10:30 – 11:00 **Giuseppe Saccone** – "Investigating male sex determination and embryonic genetic functions of Medfly to develop novel sexing strains: *Drosophila* as resource and an *in vivo* test tube".

11:00 – 11:30 **Doron Zaada** – **"**Neo-classical approach in the construction of genetic sexing strain in *Aedes albopicuts*".

11:30 – 12:00 **Muhammad Misbah ul Haq** – "Collection and up-scaling of *Aedes aegypti* and *Ae. albopictus* from different climatic and topographic regions of Pakistan for initial screening to hunt natural *tsl* mutation in wild populations".

12:00 – 13:00 *Lunch Break*

13:00 – 13.30 **František Marec** – **"**Progress in the development of generic strategies for the construction of genetic sexing strains in the codling moth".

13:30 – 14:00 **Daniel Bopp** – "Examining pre-zygotic interference strategies for male-only production in higher dipterans".

14:00 – 14:30 **Julien Cattel (virtual)** – "Optimization of the dieldrin selection for the genetic sexing strain in *Aedes albopictus*: reducing the toxicity and quantification of residues in resistant males".

14:30 – 15:00 **Jake Tu (virtual) –** "Marker-assisted mapping uncovers the causal gene of the *red-eye* (*re*) mutation in *Aedes aegypti*: towards a neoclassical approach to sex separation".

15:00 – 15:30 **Célia Lutrat (virtual)** – "Development of transgenic sexing strains for *Aedes albopictus* and *Aedes aegypti* mosquitoes and proof-of-concept of a combination system allowing sorting of non-transgenic males".

15:30 – 16.00 *Coffee Break*

16:00 – 16:30 **Jaroslaw Krzywinski** – "Toward the development of a repressible genetic sexing system in the African malaria mosquito, *Anopheles gambiae*".

16:30 – 17:00 **Thabo Mashatola** – "Development of temperature sensitive alleles to eliminate females during mosquito mass production: steps towards development of the sterile insect technique against *Anopheles*".

17:00 – 17:30 **Cyrille Ndo (virtual)** – "Screening for morphological visible markers and additional temperature sensitive strains of *Anopheles arabiensis*".

17:30 – 18:00 General discussion

**Wednesday, 20 October 2021**

**SESSION II: Working groups: discussion, planning, and coordination of work plans (Chairperson: Kostas Bourtzis and Group Leaders - rooms M4 and A2411)**

08:30 – 10:00 Open discussion and composition of the working groups

10:00 – 10:30 *Coffee Break*

10:30 – 12:00 Working groups: discussion, planning, and coordination of work plan

12:00 – 13:30 *Lunch*

13:30 – 15:00 Working groups: discussion, planning, and coordination of work plan

15:00 – 15:30 *Coffee Break*

15:30 – 17:00 Working groups: discussion, planning, and coordination of work plan

**Thursday, 21 October 2021**

**SESSION III: Review of the CRP documents and drafting the report (Chairperson: Kostas Bourtzis and Group Leaders - rooms M4 and A2411)**

08:30 – 10:00 Revision of the CRP documents (introduction, individual proposals) including planning of the activities to carry out for the next 18 months

10:00 – 10:30 *Coffee Break*

10:30 – 12:00 Revision of the CRP documents (introduction, individual proposals) including planning of the activities to carry out for the next 18 months

12:00 – 13.30 *Lunch*

13:30 – 15:00 Drafting RCM report and preparation of the list of achievements for the mid-term evaluation

15:00 – 15:30 *Coffee Break*

15:30 – 17:00 Drafting RCM report and preparation of the list of achievements for the mid-term evaluation

**Friday, 22 October 2021**

**SESSION IV: Compiling the RCM report (Chairperson: Kostas Bourtzis - room M4)**

08:30 – 10:00 Compiling RCM report and the list of achievements for mid-term evaluation

10:00 – 10:30 *Coffee Break*

10:30 – 12:00 Compiling RCM report and the list of achievements for mid-term evaluation

12:20 – 13:30 *Lunch*

13:30 – 16:00 Presentation of the RCM report, and decision on the date and place of next RCM.

16:00 – 16:15 ***Closing***

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER:

**White pupae and temperature sensitive lethal genes as generic markers for the construction of genetic sexing strains for SIT applications**

AUTHOR (S):

Germano Sollazzo[1,2], Maria-Eleni Gregoriou[1], Roswitha A Aumann[2], Katerina Nikolouli[1], Georgia Gouvi[1], Ioannis Ragoussis[3], Alistair Darby[4], Marc F Schetelig[2], Kostas Bourtzis[1]

ORGANIZATION:

1. Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Laboratories, 2444 Seibersdorf, Austria
2. Justus-Liebig-University Gießen, Institute for Insect Biotechnology, Department of Insect Biotechnology in Plant Protection, Winchesterstr. 2, 35394 Gießen, Germany
3. McGill University Genome Centre, McGill University, Montreal, Quebec, Canada
4. Centre for Genomic Research, Institute of Integrative Biology, The Biosciences Building, Crown Street, Liverpool, L69 7ZB, United Kingdom

SHORT SUMMARY OF PAPER

*Abstract:*

The VIENNA 7 and VIENNA 8 genetic sexing strains (GSS) of the Mediterranean fruit fly *Ceratitis capitata* are the most successful GSS constructed so far, and they are currently used world-wide in sterile insect technique (SIT) applications against this major agricultural pest. These GSS are based on three genetic markers: *white pupae* (*wp*), *temperature-sensitive lethal* (*tsl*) and, more recently, *slow development* (*sd*) genes, the identification of which can set the ground for the development of a generic approach for the construction of GSS in other insect pests and disease vectors.

Previous (cyto)genetic analysis has indicated that all three genes are located on the right arm of chromosome 5. In a recent collaborative effort, the *white pupae* (*wp*) gene was identified in *C. capitata*, *Bactrocera dorsalis* and *Zeugodacus cucurbitae*. This allowed the induction of white pupae mutations in other species of SIT importance, some of which will be reported in this presentation.

In parallel, there are ongoing efforts to identify the other two genes used as selectable markers in the VIENNA GSS of *C. capitata*, the *tsl*/*sd* genes. An integrated approach based on genetic, cytogenetic (including *in situ* hybridization), genomic, transcriptomic, and bioinformatic analysis allowed us to identify a limited number of *tsl*/*sd* candidate genes. We selected and targeted one of them *via* marker-less CRISPR mutagenesis, and the currently available data of this effort will be presented.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**
**18-22 October 2021**

TITLE OF WORKING PAPER:
**Towards generic GSS design: analysis of *white pupae* and a *tsl/sd* candidate gene in *Ceratitis capitata***

AUTHOR (S):
Roswitha A Aumann[1], Germano Sollazzo[1,2], Katerina Nikolouli[2], Georgia Gouvi[2], Flavia Krsticevic[3], Ioannis Ragoussis[4], Alistair Darby[5], Kostas Bourtzis[2], Marc F Schetelig[1]

ORGANIZATION:
5. Justus-Liebig-University Gießen, Institute for Insect Biotechnology, Department of Insect Biotechnology in Plant Protection, Winchesterstr. 2, 35394 Gießen, Germany
6. Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Laboratories, 2444 Seibersdorf, Austria
7. Hebrew University of Jerusalem, Department of Entomology, Robert H Smith Faculty of Agriculture, Food and Environment, 7610001, Rehovot, Israel
8. McGill University Genome Centre, McGill University, Montreal, Quebec, Canada
9. Centre for Genomic Research, Institute of Integrative Biology, The Biosciences Building, Crown Street, Liverpool, L69 7ZB, United Kingdom

SHORT SUMMARY OF PAPER

*Abstract:*

*White pupae, temperature-sensitive lethal* and *slow development* (*wp, tsl, sd*) are the successfully used and long-sought mutations in the *C. capitata* genetic sexing strains (GSS) VIENNA 7 and VIENNA 8. Identifying the genes causing these distinct phenotypes could allow the possibility of implementing a 'generic approach' to develop GSS for SIT applications in several pest insects using minimally invasive technologies such as CRISPR/Cas9.

Together with collaborators, we were recently able to identify a metabolite transporter encoding gene as causal for the white pupal shell. Extensive genome sequencing, comparative genomics, cytogenetics, transcriptomic analysis, and functional CRISPR/Cas9 knockouts were necessary to find and unambiguously identify *white pupae* in the Mediterranean fruit fly. Interestingly, the CRISPR/Cas9 mediated deletion of a single amino acid in the Major Facilitator-like superfamily domain causes the same vibrant phenotype as the natural mutation, an 8 kb large insertion of an LTR (putatively BEL/Pao) retrotransposon.

The classical and modern genetic toolset was subsequently used to identify putative *tsl* and *sd* candidate genes. So far, the slower development rate of the homozygous GSS females, compared to the heterozygous GSS males, was thought to be due to a pleiotropic effect of the *tsl* gene. However, Porras et al. recently showed that it is due to a different gene (*sd*), closely linked to the *tsl* gene (Porras et al., 2020). We selected and targeted a first *tsl/sd* candidate via marker-less CRISPR-HDR mutagenesis. The established mutants are currently analysed, and the first results of this project will be presented.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Efficient generation of autosomal transgenes using CRISPR/Cas9 knock-in in *Ceratitis capitata*.

AUTHOR (S): Angela Meccariello & Nikolai Windbichler

ORGANIZATION: Imperial College London

SHORT SUMMARY OF PAPER

*Abstract:*

Insect disease vectors and agricultural pests have major negative health, societal and **economic impacts**. Currently, agricultural pest control strategies are based on the use of pesticides and on the Sterile Insect Technique (SIT). Heavy use of pesticides is harmful to human health and the environment. SIT involves mass rearing, sorting and release of sterilized male flies inducing a local suppression of the wild population. However, it has some limitations, as its efficiency is decreased by random events of recombination, it is laborious and not cost-effective. The improvement of genetic engineering tools in recent years is allowing for the development of advanced genetic control strategies using Crispr/Cas9. Here we show a genetic Crispr-toolkit developed for *C. capitata* has enabled the production of several gene-edited and GM lines. This potential toolkit could be used to develop non-transgenic genetic sexing strains to improve the existing Sterile Insect Technique.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER:

**Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools.**

AUTHOR (S):

**Hassan M. M. Ahmed** & **Ernst A. Wimmer**

ORGANIZATION:

Georg-August-University Göttingen, Dept. of Developmental Biology, Johann-Friedrich-Blumenbach-Institute for Zoology and Anthropology, GZMB, Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany

SHORT SUMMARY OF PAPER

*Abstract:*

To generate genetic sexing strains (GSS) that are based on a temperature-sensitive lethal (tsl) mutation, which represents a selectable marker responsible for female killing, and a rescue of this situation by a wild type allele of that genetic locus translocated to the Y chromosome, which allows only the males to survive at the non-permissive temperature, we are evaluating tsl mutations of highly conserved proteins that were generated in the baker's yeast *Saccharomyces cerevisiae* by transferring such mutations by genome editing to the agricultural pest, the cherry vinegar fly *Drosophila suzukii*. In this respect the *D. suzukii* homologs of CkII-alpha, UBC2, UBC3, and UBC9 have been isolated and for the genes *Ds_cKII-alpha*, *Ds_UBC2*, and *Ds_UBC3*, suitable targets for genome editing to generate the temperature sensitive mutations D212N or P>S, respectively, have been identified and validated. To generate visible GSS based on body or pupal color, we isolated the genes *Ds_ebony* (*e*) and *Ds_black* and designed gRNAs to mutate exon I of *e* by HDR repair.

In collaboration with Dr. Windbichler, London, UK, and Dr. Papathanos, Rehovot, Israel, we identify suitable loci on the Y chromosome of *D. suzukii* for gene expression. These loci would then be used to place a wild type allele of a tsl gene or a body/pupal color gene to rescue the tsl or body color phenoytpe specifically in males and thereby generate a GGS in *D. suzukii*. Current genome editing tools should make it possible to create the tsl allele in a way to resemble a classical mutant, which could also come to existence by mutagenesis, and to introduce a wild type copy of that gene onto the Y chromosome to resemble a small translocation, which could also come about by chromosomal breaks and rearrangements as induced by classical mutagenesis approaches.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species

AUTHOR (S): Ch. Chondrogiannis**, A. Augustinos**

ORGANIZATION: Department of Plant Protection Patras, Institute of Industrial and Forage Crops, Hellenic Agricultural Organization 'Demeter', Patras, Greece

SHORT SUMMARY OF PAPER

*Abstract:*

During the first two years of the project, an insectary has been established in the Department of Plant Protection Patras, using resources allocated from this project and HAO-DEMETER, consisting of three rearing rooms, a washing room, and a working room (~40 sqm total). More than 40 medfly strains have been colonized, most of them related to the purposes of this project.

Regarding the setup of the tsl test for medfly and the fast screening of tsl phenotypes, after the analysis of several laboratory strains, it was evident that a single test of 25, 34, and 35$^{o}$ C is enough to reveal the presence of the typical 'tsl' phenotype. Focusing on strains kindly provided by IPCL, harbouring morphological markers of the 5$^{th}$ chromosome, the wp, or, we wp, h wp, 45-el wp, or wp, we ye, and ye wp strains presented the typical 'tsl resistant' phenotype (with no or detrimental effect of elevated temperatures on hatching, pupation, and emergence). Wild-type strains such as Benakeion, SEIB, and Egypt II, presented the tsl resistant phenotype as well. On the other hand, only the wp tsl strain presented the tsl-sensitive phenotype, as expected.

Following the understanding that the laboratory strains tested presented the typical tsl-resistant or tsl-sensitive phenotype, series of crosses were performed to generate new combination of strains through recombination between the tsl locus and morphological markers of the 5$^{th}$ chromosome. Following two generations (crossing and backcrossing), and the setup of approximately 200 isomale families in F3, we managed to generate the following lines: a) one (1) wp line (among 29 screened) that is tsl resistant; b) two (2) wp$^{+}$ lines (among 28 screened) that are tsl-sensitive; c) nine (9) triple-mutated or wp tsl lines (among 20 screened) and, d) seven (7) triple-mutated ye wp tsl lines (among 22 screened). Although 6 putative triple-mutated we wp tsl lines were identified (among 13 screened), we did not manage to keep any of them for follow up experiments. Representative lines are kept as separate inbred families and have presented the expected tsl phenotype four three consecutive generations.

In respect to the identification of tsl sensitive or tsl resistant medfly natural populations, in collaboration with University of Thessaly (Professor Nikos Papadopoulos), we have screened four natural populations that have been colonized relatively recently. Tsl screening has been performed for three generations, to avoid any environmental- or generation-related effect. Our results do not show any clear difference in respect to their response to the thermal stress. Additional populations are being collected and will be screened.

We tried to colonize both *Bactrocera oleae* and *Aedes albopictus* in our insectary. Efforts to directly colonize the olive fruit fly from the wild have been unsuccessful, with limiting factors being the reduced percentage of hatching and inability of L1 to feed on different artificial or semiartificial diets. Regarding *Ae. albopictus*, a Greek population supplied by the Benaki Phytopathological Institute (Dr. Antonios Michaelakis) has been introduced in our insectary. However, due to delays in identifying a reliable blood source that can routinely supply as with bovine or pig blood in our area, thermal response experiments have not been initiated. Several

Greek *Ae. albopictus* populations are expected from Benaki Phytopathological Institute and will be used in thermal response experiments.

For the next year, we are planning the following activities:

a. Tsl screening of additional Greek medfly populations, covering different geographic areas.

b. Generation of additional triple mutated strains for chromosome 5, to support mapping of the medfly tsl locus relatively to available morphological markers (45-el wp tsl and we wp tsl).

c. Use of the lines generated for genetic mapping purposes.

d. Establishment of one olive fruit fly population (already old-domesticated) and generation of a new one though enrichment from wild material. Thermal stress experiments will be performed at least on the old-domesticated population.

e. Finalize the identification of a reliable blood source for *Ae. albopictus* and initiation of thermal response experiments.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Exploring structure and function of the tephritid Y chromosome

AUTHORS: Tsoumani KT[1], Rallis D[1], Papanicolaou A[2] and Mathiopoulos KD[1]

ORGANIZATION: [1]Department of Biochemistry and Biotechnology, University of Thessaly, Greece

[2]Hawkesbury Institute for the Environment, Western Sydney University, Australia

SHORT SUMMARY OF PAPER

*Abstract:*

The olive fruit fly, *Bactrocera oleae,* remains the most destructive pest of the olive cultivations worldwide. Given the shortcomings of traditional control approaches, effective population management requires new technological solutions. Such promising solutions may stem from modifications of the classical SIT through the generation of a Genetic Sexing Strain (GSS) strain using the CRISPR-Cas9 system. This technology requires, however, a molecular characterization of the sex-specific Y chromosome. Even though sequencing efforts resolved some of the *B. oleae* Y genomic sequence, characterisation is far from complete. This is primarily due to the repetitive, heterochromatic, and gene poor nature of Y chromosomes in general and limitations in the current approaches. Here, through manual curation we benchmarked current computational approaches and developed new ones the ensemble of which allowed us to accurately characterise Y chromosome sequence, identify misassemblies – including the MoY neighborhood - and define Y-chromosome regions that are amenable to CRIPSP experiments. Through a thorough manual curation of all candidates, our new method was able to identify 93% of the true Y contigs without the need for computationally expensive whole genome alignments. As this approach can be used in other species, this capability allows us to identify CRISPR insertion sites and explore the genetic history of the Y chromosome in Tephritid flies.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER:
Nanopore long-read RNA-seq and absolute quantification delineate transcription dynamics in early embryo development of *Bactrocera oleae*

AUTHOR (S):
Anthony Bayega,[1] Spyros Oikonomopoulos,[1] Maria-Eleni Gregoriou,[2] Konstantina T. Tsoumani,[2] Antonis Giakountis,[2] Yu Chang Wang,[1] Kostas D. Mathiopoulos[2] and Jiannis Ragoussis[1,3]

ORGANIZATION:
1. McGill Genome Centre, Department of Human Genetics, McGill University, Montréal, Québec, Canada
2. Laboratory of Molecular Biology and Genomics, Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece
3. Department of Bioengineering, McGill University, Montréal, Québec, Canada

*co-leads

SHORT SUMMARY OF PAPER

*Abstract:*

The olive fruit fly, *Bactrocera oleae*, is the most important pest for the olive fruit but lacks adequate transcriptomic characterization that could aid in molecular control approaches. We apply nanopore long-read RNA-seq with internal RNA standards allowing absolute transcript quantification to analyze transcription dynamics during early embryo development for the first time in this organism. Sequencing on the MinION platform generated over 31 million reads. Over 50% of the expressed genes had at least one read covering its entire length validating our full-length approach. We generated a de novo transcriptome assembly and identified 1768 new genes and a total of 79,810 isoforms; a fourfold increase in transcriptome diversity compared to the current NCBI predicted transcriptome. Absolute transcript quantification per embryo allowed an insight into the dramatic re-organization of maternal transcripts. We further identified *Zelda* as a possible regulator of early zygotic genome activation in *B. oleae* and provide further insights into the maternal-to-zygotic transition. These data show the utility of long-read RNA in improving characterization of non-model organisms that lack a fully annotated genome, provide potential targets for sterile insect technic approaches, and provide the first insight into the transcriptome landscape of the developing olive fruit fly embryo.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Working towards functional genomics in tsetse

AUTHOR (S): Darby A.C., Beliavskaia, A. Makepeace B.L. and Bell-Sakyi L.

ORGANIZATION: Department of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool

SHORT SUMMARY OF PAPER

*Abstract:*

The Tsetse flies (genus Glossina) are the sole vector of African Trypanosomiasis causes nagana in animals with an estimated loss to livestock production of $4.5 billion annually in Sub-Saharan Africa. Economic losses are both direct (reduced productivity of diseased animals, the death of animals and cost of treatment), but also indirect (deriving from the loss of cattle for traction, reducing the area of land that can be opened for arable agriculture). In addition, there is a human disease cost deriving from sleeping sickness. The World Health Organization (WHO) estimates that 60 million people in Africa are at risk of contracting sleeping sickness (about 40% of the continent's population). These provide exceptional economic, social and public health imperatives for effective control of trypanosomiasis.

The presentation will provide an update on the tsetse male genome assembly using long read sequencing. Along with progress in genetic manipulation and the characterisation of a putative tsetse cell line.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: New target genes for male sterility in *Drosophila suzukii* and *Anastrepha suspensa.*

AUTHOR (S): A.M. Handler, K. Tariq, R. Furlong and Q. Xia

ORGANIZATION: USDA-ARS, Center for Agricultural, Medical and Veterinary Entomology, Gainesville, Florida

SHORT SUMMARY OF PAPER

*Abstract:*

In an effort to identify new genes that can act as targets for male sterility in fruit fly pest species, a gene necessary for male fertility in *Drosophila melanogaster* has been isolated in the spotted-wing drosophilid, *Drosophila suzukii*, and the Caribbean fruit fly, *Anastrepha suspensa*. *Wampa* is a recently characterized component of the outer axonemal dynein arm in *D. melanogaster*, that results in male sterility due to loss of flagellar motility in null mutants, that are otherwise viable with no other apparent morphological defects. An in silico Blastn search for *wampa* in *D. suzukii* resulted in identification of a coiled-coil domain-containing protein having 88% nucleotide identity to *Dm-wampa*, with a Blastp peptide identity of 96%. RT-PCR and qPCR analysis of *Ds-wampa* showed testis-specific expression for 7 days after adult eclosion, with no evident expression in female ovaries or adult male and female carcasses. The *A. su*spensa genome has not been sequenced or assembled, but high identity protein cognates in other sequenced tephritid species, ranging between 83% and 85% peptide identity, were used to find consensus *wampa* cognate motifs that could be used for PCR amplification in *A. suspensa*. A partial consensus *wampa* sequence was subsequently amplified that, similar to *Ds-wampa*, exhibited testis-specific expression for 10 days after adult eclosion, with no evident expression in female ovaries or adult male and female carcasses. Further functional analysis of the *Ds-wampa* and *As-wampa* cognates will include CRISPR-Cas9 NHEJ knock-out mutations that will assess their importance to male fertility, and their potential use as targets to create sterile male populations.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Development of genetic sexing strains for *Anastrepha fraterculus* approaching with generic genomic tools

AUTHOR (S): Conte CA[1], Giardini MC[1], Scannapieco AC[1], Rivarola M[2], Rivera Pomar R[3], Wulff JP[1], and Lanzavecchia SB[1].

ORGANIZATION: [1] Instituto de Genética (IGEAF), Instituto Nacional de Tecnología Agropecuaria (INTA). [2] Instituto de Biotecnologia (IB), INTA, IABIMO-CONICET, Buenos Aires, Argentina. [3] Universidad Nacional del Oeste de Buenos Aires (UNOBA).

SHORT SUMMARY OF PAPER

*Abstract:*

The generation of Genetic Sexing Strains (GSSs) has greatly improved the efficiency of the Sterile Insect Technique (SIT) at lower production costs by the separation of sexes at early stages of development. Succesful SIT approches with the use of classical GSSs have been implemented to control tephritid species in the American continent (e.g. *Ceratitis capitata* and *Anastrepha ludens*). In the case of *A. fraterculus,* a classical GSS has been developed and is currently under evaluation. In addition, transgenesis has been applied to create sexing systems in insects. Significant advances in this area have been achieved for several tephritid species, such as, *C. capitata, A. ludens, A. suspensa, Bactrocera oleae* and *B. tryoni*. Novel gene editing technologies (e.g. CRISPR/Cas 9) have proven to be an efficient method to generate heritable and specific germ line mutations (e.g. *C. capitata*, *A. suspensa*, *B. tryoni*, *B. oleae*) with almost unlimited potential to improve current pest management strategies. Our main goals are to provide baseline information of *A. fraterculus* genome and to apply transgenesis/gene editing technologies to produce GSSs for this pest species. We have performed a complete characterization of *A. fraterculus* sp. 1 infesting fruits of economic importance in Argentina by using genetics, cytogenetics and a transcriptome approach. During the last period, a pure *A.fraterculus* sp. 1 strain carrying *w*AfraCast2 (*Wolbachia* strain) and 100% $X_1X_1/X_1Y_5$ (sex cromosomes) has been stablished in our lab and F5 descendants stored for genome analysis. Short-term objectives are: 1) obtain female/male draft genome sequences, 2) start bioinformatic analyses and obtain a preliminary characterization of specific genomic regions of the Y chromosome, and 3) set up new assays of *A.fraterculus* early embryo microinjections for transgenesis and CRISPR/Cas 9.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*

AUTHOR (S): José S. Meza, Victor García-Martínez, Jorge Ibañez-Palacios, Martha Roblero-Roblero, Maria F. Ruíz-Pérez, Julio C. Coutiño-Montes, Daisy P. Cárdenas-Enriquez, Martha Guillen-Ribera.

ORGANIZATION: National Program Fruit Flies SADER/SENASICA

SHORT SUMMARY OF PAPER

*Abstract:*

In order to increase the self-separation system of the Tapachula/slow-7 sexing strain (Tap/slow-7), the pure *slow larvae* (*sl*) mutation was crossed with wild-type insects for 4 generations, always using hybrid females and wild-type males. Subsequently, *sl* mutants (identified by pupal depigmentation and adult) with a longer larval stage were isolated to link it to the *black pupa* (*bp*) mutant and reconstruct Tap/slow-7. During the rebuilding process, the larval stage was allowed to remain in the larval diet for up to 18 days (typically 10 days), the strain with a longer larval stage was isolated and a new Tap/slow-7 was obtained. During the evaluation of the sexual self-separation of this new version of Tap/slow-7, no more sexual separation was observed, the most plausible explanation is that the recombination events that occurred during the crossing, took place between the two genes that they control the characters of depigmentation and slow larval development. So, we conclude that the *sl* mutation, which has two traits, depigmentation and slow larval development could be controlled by at least two relatively linked genes.

The *bp* and *sl* loci are reported to be found on mitotic chromosome 2 and the frequency of recombination between them is 44% in females, meaning that the two loci are relatively distant, but no recombination was detected in males. However, when these two markers where used in genetic sexing strains, that use the same translocation like Tap-7 and Slow-7, respectably (Meza et al, 2019), it was observed a certain percentage of male recombination in both strains.

We know that in Tap-7 the *bp* allele is found within the fragment translocated to the Y chromosome very close to the break point and the percentage of male recombination is 0.012% (recombination between the break point and the marker). For Slow-7, we hypothesized that the *sl* allele is in the non-translocated fragment distant from the same breakpoint, perhaps further away than the *bp* allele, because the total recombination is greater (0.152%) than Tap-7. Looking in more detail at the sex ratio of the recombinant insects, there is more wild-type females in both strains, but males appear with the same frequency in both GSS. In the evaluation of genetic stability for both GSS, where recombinant individuals are not eliminated, in contrast to expected the aberrant accumulation begins from the sixth generation and increases rapidly in GSS Tap-7 with a clear trend of accumulation of recombinant *bp* males, while that GSS Slow-7 both aspects, destruction and the sex ratio of recombinant insects remain constant at low levels of recombination over generations. So, we assume that the breakdown in a GSS depends not only on the distance between the marker and the breaking point, but also on the fitness of the selected marker and the fraction in which the marker is located; in the translocated or non-translocated fraction.

On another hand, we are working to induce translocation in *Anastrepha obliqua*, using the *black pupa* as marker in order to develop the first GSS for this specie. The irradiation dose used ranged from 20 to 25 grays and the age of the pupae at the time of irradiation was two days before emergence. Eight hundred seventy-five males were screened in four trials without success. The next trials will be carried out at lower irradiation doses (10 and 15 grays).

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Development and evaluation of genetic sexing strains of fruits flies to be used for sterile insect technique applications

AUTHOR (S): Edwin Ramírez, Cristian Morales

ORGANIZATION: Medfly Program – Guatemala

SHORT SUMMARY OF PAPER

*Abstract:*

Aware of the positive implications on the use of new and better strains for any control program, we have developed protocols for the search and evaluation of novel strains of *Ceratitis capitata* (Wied.) and *Anastrepha ludens* (Loew). We have focused on selecting the best lines based on cost reduction criteria in mass rearing and the good performance of sterile males in the field. We expect to find better strains that would allow the release in the field of highly competitive sterile males for an adequate induction of sterility in wild populations.

Mutations have been induced to achieve new rearrangements in the chromosomes that could generate genetically stable strains, with high fecundity and high yield. For the Mexican Fruit Fly, *A. Ludens*, three repetitions have been performed using GUA10 females (275 individual crosses per repetition, with a total of 825) and two repetitions using the original TBP7 females (275 individual crosses, with a total of 550). For treatment using GUA10 the following families were selected: F167, F274, F60, F254, F44, F55, and F121. For treatment using TBP7 the following families were selected: F10A, F12, and F97. These new families are pending to be reared on a scale (medium) that shows their genetic stability (level of recombination), yield, dosimetry, and finally, if it is convenient, mating competitiveness of the males under field conditions. On the other hand, we have continued to select lines coming from flies that have been treated by means chemical mutagenesis (EMS) to explore the possibility of finding thermal sensitivity. From the black pupae family 166 (previously reported), which showed partial lethality (50%) due to heat treatment in the egg stage, 500 individual pairs were made to identify individuals with total lethality (100%). Of the 500 pairs, only one line was found and selected, 166-10, which showed a 94% lethality with respect to its control (without heat treatment). Currently the selection of more lines continues.

For *C. capitata*, we have performed evaluations of wild female copulations with two types of males (Vienna 8 / Toliman INV-D53 and Vienna 8 1260) with the focus of taking advantage of the distinctive traits (fluorescence) of Vienna 8 1260 males in the offspring to generate practical information on optimal irradiation ages. The partial results have shown that the age of irradiation of the males exerts an influence on the quality of the copulation of the females and on their tendency to remating.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Developing *Bactrocera tryoni* genetic sexing strains

AUTHOR (S): Simon Baxter[1], Zoey Nguyen[1], Elisabeth Fung [2,3], Anzu Okada[3], Ngoc Mai Han Nguyen[4], Peter Crisp[2,3] and Amanda Choo[2]

ORGANIZATION: [1]University of Melbourne, [2]University of Adelaide, [3]South Australian Research and Development Institute, [4]Macquarie University

SHORT SUMMARY OF PAPER

*Abstract:*

The Queensland fruit fly, *Bactrocera tryoni*, is a polyphagous tephritid pest of ripening fruits in Australia. Ongoing efforts to supress population outbreaks with the sterile insect technique have been successful, however, modelling predicts that male only releases would be more efficient than the current bi-sex sterile release strategy. Here we aim to develop genetic sexing strains to facilitate male only sterile releases. Heritable mutations producing white or black pupae colour phenotypes have been used to develop genetic sexing strains in several tephritids, which usually have brown pupae. The approach requires females to express the autosomal recessive pupae mutation, while males express the wild type brown phenotype, thanks to an autosomal translocation to the Y-chromosome which rescues normal pupal development. Recessive mutations in a *major facilitator superfamily* transporter gene are known to cause "white pupae" phenotypes, and an unknown genetic mechanism causes "black pupae" phenotypes. First, we will discuss progress in developing a white pupae genetic sexing strain of *B. tryoni* and potential to sort pupae using this phenotype with an automated seed sorter. Second, we use CRISPR/Cas9 gene editing to generate a novel *B. tryoni* black pupae mutant strain and assess fitness costs associated with the phenotype.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera* spp. using bioinformatics and molecular tools

AUTHOR (S): Nidchaya Aketarawong, Kamoltip Laohakieat, Sujinda Thanaphum

ORGANIZATION: Regional R&D Training Center for Insect Biotechnology (RCIB), Department of Biotechnology, Faculty of Science, Mahidol University, Thailand

SHORT SUMMARY OF PAPER

*Abstract:*

The Salaya1 strain is a genetic sexing strain for *Bactrocera dorsalis*. It was proved to be ready for industrial scale and sterile insect technique (SIT) programs because its rearing profile is consistent and comparable to other pupae-color based genetic sexing strains (GSSs). Sex-ratio distortion was also observed and possibly used for construction of the GSS based on the different rates of development between males and females. Therefore, larvae and pupae of the Salaya1 strain were separately collected and counted. Male-biased sex ratios were detected at larval, pupal, and adult stages, while a few cases of female bias were detected at only adult stage. However, the ratio between total males and females was not significantly different. This result inferred that development of male and female larvae of Salaya1 strain may be similar at the appropriate rearing condition. Further studies have been focused on development between males and females at various temperatures.

Meanwhile, four key genes, *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*), and *fruitless* (*fru*), in the sex-determination pathway were completely studied and published for *B. dorsalis* and *B. correcta*. The RNAi knockdown of *tra* gene in *B. dorsalis* confirmed that *tra* controls sex-specific splicing of *tra* itself, *dsx*, and *fru* transcripts. Moreover, expression profiles of sex-determining genes in different developmental stages of both species revealed that the *Maleness-on-the-Y* (*MoY*) gene expresses in fertilized eggs containing an XY sex chromosome at the early embryogenesis and disappears in pupal stage. The heterogeneous pattern of *tra* transcripts were detected in both sexes and became the sex-specific pattern in the later stages of embryos until adulthood. The male-specific *dsx* transcript was observed in the beginning stage of gastrulation of embryos, while male-specific *fru* transcript firstly appeared in the male larvae. These knowledges have been transferred to study the related fruit fly species.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: The Development of Genetic Sexing Strains (GSS) for SIT Applications in *Drosophila Suzukii*

AUTHOR (S): Dan Deng, Shisi Xing, Qing Liu, Zongzhao Zhai, Wei Peng*

ORGANIZATION: Hunan Normal University

SHORT SUMMARY OF PAPER

*Abstract:*

The transcriptome analyses identified 23 sex determination genes in *Drosophila suzukii*, a major devastating and invasive crop pest, including *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*) , *doublesex* (*dsx*) and *fruitless* (*fru*) which are the sex determining pathway cascade genes, and *sisterless A* (*sisA*), *scute* (*sc*), *runt*, *deadpan* (*dpn*) and *groucho* (*gro*) which encode X-chromosome linked signal elements (XSEs). The temporal expression profile of 23 sex determination genes throughout *D. suzukii* developmental stages displayed that the highest transcript levels were detected in embryos and the lowest in adults. In addition, fourteen of the identified sex determination genes were differentially expressed between sexes. RT-PCR validation showed *Sxl*, *tra*, *dsx*, and *fru* genes have sex-specific isoforms in *D. suzukii* adults, while *tra-2* does not, and exon skipping was validated as a common splicing mechanism. The *Sxl*, *tra*, *tra-2*, *dsx* and *fru* gene were cloned by using the transcriptome data and RACE technique. The ORF of these gene are 825, 699, 825, 1095, 2733 bp encoding for 275, 233, 275, 365, 911 amino acids, respectively and exhibits structural features characteristic of known insect sex determination genes. The RNAi experiments showed that sexual formation is determined early in the embryo stage and female to male sex reversal was achieved by targeting *tra*, *tra-2* and *dsx* genes. Silencing of *tra* and *dsx* reduced the expression of *vitellogenin* (*Vg*) gene and decreased fecundity with decreased ovary length, ovariole length and ovariole number.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

**TITLE OF WORKING PAPER:** Investigating male sex determination and embryonic genetic functions of Medfly to develop novel sexing strains: Drosophila as resource and an in vivo test tube.

**AUTHOR (S):** Primo[1], P., Perrotta[1], M., S., A. Ruggiero[2], A., Vitagliano[2], L., Ruvo[2], M., Barra[1,2], G., Giordano[1], E., Salvemini[1], M., Rabinow[3], L., Ahmed[4, 5] HMM, Wimmer[4], E., and Saccone[1], G.

**ORGANIZATIONs:**

[1]Department of Biology, University of Naples "Federico II", Napoli, Italy.
[2]Institute of Biostructures and Bioimaging (IBB), CNR, Naples, Italy.
[3]Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115.
[4]Department of Developmental Biology, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Göttingen Center for Molecular Biosciences, Georg-[6]August-University Göttingen, 37077, Göttingen, Germany.
[5]Department of Crop Protection, Faculty of Agriculture-University of Khartoum, P.O. Box 32, 13314, Khartoum North, Khartoum, Sudan.

**SHORT SUMMARY OF PAPER**

**Abstract**

As a proof of principle of a functional screen for Medfly *tsl* putative genes, we planned to use a Drosophila *tsl* mutant strain, *elav*, encoding a neuronal RNA-binding protein, and perform embryos injections of a BAC genomic *tsl+* fragment (100 kb long) to confirm a transient functional rescue. We first confirmed that the $elav^{ts1}$ stock was inviable at 25ºC, by placing vials of flies at this non-permissive temperature. Adults did not die immediately and some even lived long enough to lay a few eggs. Some of these survived long enough to hatch as larvae and a small number survived to adulthood. However, no F1 adults lived long enough to propagate a viable stock at 25ºC. Adults were also allowed to lay eggs at the permissive temperature of 18ºC. After 24 hours of egg-laying, vials were cultured in parallel at 18ºC and 25ºC. Larvae and a small number of adults were observed following continuous culture at 25ºC. A transient heat-shock of 1 hr at 37 ºC were also applied to 24 hour embryo collections. Again, larvae and a small number of adults were observed in the vials. Therefore, under these conditions $elav^{ts}$ does not offer the proper temperature-sensitivity required to test the ability of a BAC to rescue *ts*-induced embryonic lethality. We started to explore alternative Drosophila embryonic *tsl* mutant strains to be used within the next months. Medfly XX-only embryos injections of recombinant Medfly synthetic MOY protein induced phenotypic and molecular intersexuality, confirming the maintenance of function of the synthetic molecule. A first series of Medfly XX-only injections of recombinant BdMOY and BoMOY proteins led to mild transient shift of *Cctra* splicing toward male-specific one, and in XX/XY embryos not phenotypic effects at adult stages. A first series of embryos injections of a *MoY* transgenic vector (PiggyBac based) led to a dozen adult flies which were crossed and the subsequent progeny was screened for transgenics. No fluorescent individuals in $F_1$ were detected. We attempted to integrate by Cas9 a dsRED transgene into *MoY* locus to obtain detectable XY females to be maintained by crossing them with XX reverted males (by *Cctra* RNAi). We obtained few XY reverted females but lacking integration of the tramsgene.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Neo-classical approach in the construction of genetic sexing strain in *Aedes albopicuts*

AUTHOR (S): Doron SY Zaada, Flavia Krsticevic & Philippos A Papathanos

ORGANIZATION: Faculty of Agriculture, Food and Environment - the Hebrew University of Jerusalem

SHORT SUMMARY OF PAPER

*Abstract:*

Early female elimination prior to release is a desirable feature in many genetic control systems and a mandatory prerequisite in mosquitoes. Genetic sexing strain is an efficient solution, but its construction using random mutagenesis and translocations is laborious and serendipitous. On the other hand, modern approaches that involve transgenic strains hold great promise for mosquito control but often encounter public opposition.

Using novel bioinformatic approaches and modern genetic editing tools we aim to construct a non-transgenic, genetic sexing strains for the Asian Tiger mosquito *Aedes albopictus*. By a) identifying unique male-specific genomic regions; b) generation of site-specific loss-of-function recessive mutations, and c) linking functional copy of the mutated gene to maleness.

Using male vs female WGS data we have identified unique, male-specific regions along the recently published assembly. We have confirmed these region's existence both in WT and laboratory strains. We have used the CRISPR/Cas9 system to generate a vivid recessive phenotypic mutation that we are currently trying to recover from within male-specific regions.

We believe that our bioinformatic pipeline and guided engineering approach could be easily adopted for the construction of novel, non-transgenic genetic sexing strains for any heterochromatic (XY) insect pest.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Collection and up-scaling of *Aedes aegypti* and *Ae. Albopictus* from different climatic and topographic regions of Pakistan for initial screening to hunt natural tsl mutation in wild populations.

AUTHOR (S): Muhammad Misbah ul Haq

ORGANIZATION: Nuclear Institute for Food and Agriculture (Pakistan Atomic Energy Commission) Pakistan.

SHORT SUMMARY OF PAPER

*Abstract:*

Aedes mosquitoes are responsible for the transmission of numerous viral infections in Pakistan and around the globe. Non-conventional vector control method like SIT has gained lot of attention in recent years for its control. But the major obstacle in successful mosquito's SIT program is sex separation at early life stages of Aedes at mass rearing facilities. An excellent example for this purpose is development of of GSS for SIT purposes are the VIENNA 7 and VIENNA 8 of the Mediterranean fruit fly, which are based on a color and a thermal lethality mutation linked to the sex. To achieve the same, we have collected *Aedes albopictus* and *Ae. aegypt* from different topographic and climatic regions of Pakistan to hunt natural tsl mutation in wild populations of these two species to construct tsl based GSS that can address sex separation issue. The collection was done via ovi-traps and larval collections from potential breeding places in urban and rural settings. The ovi-traps were designed by cutting 1.5-liter plastic beverages bottles into two parts and each part was wrapped with black paper to make it more attractive for adult female mosquitoes. A strip of filter paper was encircled on the inner side of the bottle and half filled with water for keeping the filter paper moist. The traps were monitored regularly for presence of eggs. The collected egg papers were dried at room temperature for 1-2 weeks and afterwards used for colony establishment. The larval collection was done in coordination with district dengue surveillance teams of health department which were working for dengue control program in four provinces of Pakistan. Overall, from twelve different locations of Pakistan, 13 Aedes species were collected. Out of which 11 were *Ae. aegytpi* and 2 were *Ae. albopictus* colonies. Collected samples of eggs or larvae ranged from 13 to 289 per location. Each egg and larval sample were properly labelled with location name, altitude, longitude and latitude. Due to small sample size, 6 colonies were lost whereas 7 colonies (5 *Ae. aegypti* and 2 *Ae. albopictus*) were established and up-scaled in mosquito lab. Four colonies were started with eggs collected from ovi-traps and remaining were started from larval collection. Adults emerged were kept in 30 x 30 x 30 cm adult plastic cages and provided 10% sugar solution and were biweekly blood fed on alive mice and pigeons. Eggs cups having moist filter papers were placed after 48 to 72 hours of blood feeding for eggs collections. Multiple egg collections were done per each colony to upscale the colony and for initial tsl screening. For initial tsl screening, L1 larvae were exposed to different temperatures ranging from 37- 41 $^{o}$C for various time intervals ranging from 2 to 24 hrs in water baths. Afterwards, L3-4 larvae were counted to record the survival and further pupal and adult emergence. The strains with high %survival will be considered potential resistant whereas strains with low %survival will be recorded as sensitive to heat. This activity is yet to be completed and was delayed due to difficulty in collection and up scaling of colonies due to COVID-19 lockdown and restrictions.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Progress in the development of generic strategies for the construction of genetic sexing strains in the codling moth

AUTHOR (S): Frantisek Marec[1], Kristyna Pospisilova[1,2], Arjen E. van't Hof[1], Atsuo Yoshido[1], Sander Visser[1,3], Renata Kruziková[1,2], Martina Daliková[1,2], Petr Nguyen[1,2]

ORGANIZATION:
[1] Biology Centre CAS, Institute of Entomology, Ceske Budejovice, Czech Republic
[2] University of South Bohemia, Faculty of Science, Ceske Budejovice, Czech Republic
[3] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands

SHORT SUMMARY OF PAPER

*Abstract:*

In this project, we have two main goals and to achieve them we use the codling moth, *Cydia pomonella*, as a model species. First, we focus on the development of genetic sexing strains by inserting a selectable gene into the W chromosome of the codling moth. Second, we study genes involved in sex determination of the codling moth to find new possibilities for genetic sexing. We intend to achieve the first goal by genome editing using the CRISPR/Cas9 method. For this research, we have available the genomic and transcriptome sequences of codling moth males and females. We have also identified two sufficiently long W-specific sequences (CpW2-*Eco*RI and CpW5-long), confirmed their W-specificity by PCR and extended them by sequencing and assembly of the W-derived BAC clone containing these sequences. In these sequences, we have identified more than 200 potential sgRNA target sequences, required for targeted mutagenesis. In addition, we optimized injections into codling moth eggs. When injecting solutions into eggs 3–4 hours after oviposition (hpo), we achieved 16% survival in hatched larvae, which is still low compared to the silkworm, *Bombyx mori*, but it allows us to get results. In sex determination research, we have identified and characterized *Cpdsx* and *CpMasc* genes in the codling moth, orthologs of *B. mori doublesex* gene and *Masculinizer* gene, respectively. However, by trascriptome analysis we found multiple splice variants of the *CpMasc* gene. Using earlier developed PCR-sexing and qRT-PCR, we characterized embryonic expression of these splice variants within time window of 8–24 hpo and identified two male-specific *CpMasc* variants with a peak of expression at 12 hpo. Then, by using RNAi approach, we injected two designed siRNAs into codling moth embryos to knockdown the male-specific *CpMasc* transcripts. One of these siRNAs successfully reversed male-specific splicing of *Cpdsx* to female-specific splicing in male embryos. Our results thus clearly suggest that *CpMasc* plays a major role in male sex determination of the codling moth.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: EXAMINING PRE-ZYGOTIC INTERFERENCE STRATEGIES FOR MALE-ONLY PRODUCTION IN HIGHER DIPTERANS

AUTHOR (S): SAMUEL JUNG[1], CLAUDIA BRUNNER[1], LAURA MEDICI[1] LEO BEUKEBOOM[2], DANIEL BOPP[1]

ORGANIZATION: [1]UNIVERSITY OF ZÜRICH SWITZERLAND, [2]UNIVERSITY OF GRONINGEN, THE NETHERLANDS

SHORT SUMMARY OF PAPER

*Abstract:*

Sexual differentiation in insects is initiated by a short cascade of gene regulatory events during early development which fixes the sexual identity of the zygote irreversibly. The *transformer* (*tra*) gene plays a pivotal role acting as the main switch in the pathway. When ON, cells will be assigned a female fate, when OFF, cells will follow the male pathway. Hence, coordinated ON/OFF regulation of *tra* at the individual level is critical to the correct implementation of the sexual phenotype. In higher dipterans zygotic activation of *tra* requires presence of maternally contributed *tra* products to engage a positive feedback loop which secures continued expression of active *tra* throughout female development. Male development normally follows from inheritance of a paternally transmitted *M* factor which prevents maternal activation of the *tra* loop in the zygote. We propose that, when the maternal supplement of *tra* is already abolished in the egg, the resulting zygote will develop into a male irrespective of whether *M* factor is present or not. Based on this proposition we would like to investigate the potential of introducing *M* factors into the female germ line to prevent expression of maternal *tra* products. As a result, we expect that these ¨arrhenogenic¨ females produce male-only progenies. As a proof of principle, we are currently studying a housefly strain in which females carrying the dominant *Ag* factor produce only males. The finding that eggs laid by *Ag* females do not contain maternal *tra* products (Hediger et al 2010) suggests that *Ag* acts like a *M*-factor which is exclusively active in the female germ line. The *Ag* mutation maps to the same location on chromosome I where one of the many autosomal M factors is located. It is thus conceivable that *Ag* is a germline-specific derivative of this $M^I$ factor. Since $M^I$ is different from the previously identified *M* factor, *Mdmd* (Sharma et al. 2017), we are currently trying to identify this novel *M* factor by testing candidate genes which are present and transcribed only in male individuals of the $M^I$ strain. Once identified, it will be interesting to examine whether and, if yes, to what extent *Ag* and $M^I$ are structurally related and how they differ in the regulation of *tra* temporally and spatially. We hope that understanding how *Ag* functions in the germ line will help us to devise a new strategy for male-only production based on pre-zygotic rather than post-zygotic intervention.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Optimization of the dieldrin selection for the genetic Sexing Strain in *Aedes albopictus*: reducing the toxicity and quantification of residues in resistant males

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SHORT SUMMARY OF PAPER

*Abstract:*

The mass production of mosquitoes at an industrial scale requires efficient sex separation methods which can be mechanical, genetic or based on an artificial intelligence system. Compared with others methods, the genetic sexing approach offers the advantage of being faster and applicable at the larvae stages, limiting the cost and space due to rearing of females. We recently developed a GSS in *Aedes albopictus* based on sex linkage of the *rdl* gene with the M locus, leading to dieldrin resistance in males only. Such strategy has already been developed on *Anopheles arabiensis*, in which it has been shown that dieldrin ingested by larvae could be detected in adults and bioaccumulated in predators, raising the question of its use at a large scale. In this context, we performed experiments aiming at decreasing dieldrin concentration and the time of exposure but still maintaining a low and stable percentage of females (around 1%). We show that the level of dieldrin exposure originally used induces toxicity and kills 60% of resistant males. This level of toxicity could be easily modulated by modifying the dose and/or time of exposure allowing to recover nearly all resistant males. We quantified through GS-MS-MS the residues of dieldrine in resistant male adults exposed to increasing doses of dieldrin. Dieldrin toxicity on larvae is positively correlated with the dieldrin quantity found in adults. Interestingly we show that in samples of males with lower exposure to dieldrin, detection of the insecticide is close to the limit of quantification. More precisely, we detect less than 8.5 pg of dieldrine per mosquito, a thousand times less than the residues actually measured in *An. Arabiensis*. Altogether, presented data confirm that dieldrin exposure protocol can be adjusted to reach a compromise between male toxicity, efficient sex sorting and the level of dieldrine residues measured in adults. Finally, we discuss the introgression of this GSS with the *Wolbachia* incompatible lines that we recently constructed to implement IIT pilot trials.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: **Marker-assisted mapping uncovers the causal gene of the red-eye (*re*) mutation in *Aedes aegypti*: towards a neoclassical approach to sex separation**

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SHORT SUMMARY OF PAPER

*Abstract:*

*Aedes aegypti* is a major vector of arboviruses that cause dengue, chikungunya, yellow fever and Zika. Although recent success in reverse genetics has facilitated rapid progress in basic and applied research, integration of forward genetics with modern technologies remains challenging in this important species, as up-to-47% of its chromosome is refractory to genetic mapping due to extremely low rate of recombination. Here we describe the development of a marker-assisted-mapping (MAM) strategy to readily screen for and genotype only the rare but informative recombinants, drastically increasing both the resolution and signal-to-noise ratio. Using MAM, we mapped a transgene that was inserted in a >100 Mb recombination desert and a sex-linked spontaneous red-eye (*re*) mutation just outside the region. We subsequently determined, by CRISPR/Cas9-mediated knockout, that *cardinal* is the causal gene of *re*, which is the first forward genetic identification of a causal gene in *Ae. aegypti*. MAM does not require densely populated markers and can be readily applied throughout the genome. This study also provides the molecular foundation for using gene-editing to develop versatile and stable genetic sexing methods by improving upon the current *re*-based genetic sexing strains. If time permits, we will also discuss other recent progresses towards expanding the toolbox for genetic sex-separation of *Ae. aegypti*.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

**TITLE OF WORKING PAPER: Development of transgenic sexing strains for *Aedes albopictus* and *Aedes aegypti* mosquitoes and proof-of-concept of a combination system allowing sorting of non-transgenic males**

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SHORT SUMMARY OF PAPER

*Abstract:*

Chemical control of *Aedes albopictus* and *Aedes aegypti* disease vector mosquitoes is costly, unsustainable and increasingly ineffective due to the emergence of insecticide resistance. Genetic control, including the Sterile Insect Technique, represents an interesting alternative but is limited by a slow, error-prone, and wasteful sex-separation step. Better understanding of mosquito sex determination and sex loci is crucial for developing control tools, especially genetic sexing strains. In *Aedes aegypti*, *Nix*, located within the M-locus, is the primary gene responsible for masculinization and *Nix*-expressing genetic females develop into fertile, albeit flightless, males. In *Ae. albopictus*, *Nix* has also been implicated in masculinization, but its role remains to be further characterized.

In this work, we established *Ae. albopictus* transgenic lines ectopically expressing *Nix*. Several are composed exclusively of genetic females, with transgenic individuals being phenotypic and functional males due to the expression of the *Nix* transgene. Their reproductive fitness is marginally impaired, while their flight performance is similar to controls. These results show that *Nix* is sufficient for full masculinization in *Ae. albopictus*. Moreover, the *Nix* transgene is linked to a fluorescence marker allowing efficient automated sex sorting. Interestingly, we also observed that transgenesis constructs carrying *Nix* together with a fluorescence reporter preferentially land near the endogenous M-locus. This "homing" phenomenon allowed us to develop additional genetic sexing strains with a fluorescence marker linked to the endogenous M-locus.

Altogether, we present two genetic sexing strains for each vector species based on fluorescence markers linked to the m and M sex loci, obtained either by targeted CRISPR-Cas9 genome editing, piggyBac random integration or piggyBac homing. These four strains allow automated purification of transgenic males. Furthermore, we show how combining these sexing strains may allow the mass production of non-transgenic males in pilot experiments. Scaling-up would allow 100,000 neonate male larvae to be sorted in under 1.5 hour with 0-0.1% female contamination, resulting in males with similar survival and flight ability as laboratory-reared wild-type males.

Such Genetic Sexing Strains should enable a major upscaling of genetic control programs against these two medically important vectors and a choice between the transgenic or non-transgenic male release options.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER:
Toward the development of a repressible genetic sexing system in the African malaria mosquito, *Anopheles gambiae*

AUTHOR (S):
Elzbieta Krzywinska, Aqib Ali, Alex Gidley, Jaroslaw Krzywinski

ORGANIZATION:

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SHORT SUMMARY OF PAPER

*Abstract:*

Interference with expression or splicing of the insect sex determination pathway genes can lead to death, sex reversal, or intersexual phenotypes in females. The sex determination genes are thus attractive tools/targets that could be exploited for control of pest and vector populations. In the major malaria vectors *Anopheles gambiae* and *A. arabiensis*, sex is determined by the Y chromosome-linked gene *Yob*, which triggers male development and activates dosage compensation. Previously, we generated *A. gambiae* transgenic lines that ectopically express *Yob* from autosomal loci and produce male-biased or male-only progenies. Deficiency of females resulted from an abnormal overexpression of the X chromosomes, a dominant trait requiring continuous backcrossing of transgenic males with wild-type females to maintain the lines. In an effort to overcome that requirement we created tet-off constructs to generate transgenic lines with conditionally repressible female lethality. In total, we established 18 lines, of which 5 had a transgene on the Y chromosome. All autosomal lines, in the absence of doxycycline, produced male-biased progeny, with surviving females masculinized to a various extent, and usually developing more slowly than the wild-type females. However, contrary to the expectation, exposure to the antibiotic of eggs and larvae in water and adult females in sugar solution and blood prior to oviposition did not restore normal female development in the lines tested to date. Potential causes include insufficient delivery of doxycycline at the critical moment during embryonic female development and/or leakiness of the tet-off system. Analysis of the lines and work on improvement of the system is ongoing.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications."**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Development of Temperature Sensitive Alleles to Eliminate Females during Mosquito Mass Production: Steps Towards Development of the Sterile Insect Technique against Anopheles.

AUTHOR (S): Thabo Mashatola[1,2], Lizette L Koekemoer[1,2] and Givemore Munhenga[1,2]

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SHORT SUMMARY OF PAPER

*Abstract:*

The South African mosquito SIT project targeting the malaria vector *Anopheles arabiensis* is now at an advanced stage. Numerous technical aspects of the technology been optimised in preparation of a small-scale pilot trial. However, one of the most critical elements of the technology that remains outstanding is having an efficient sex separation mechanism to exclude female mosquitoes from the production line before sterilisation by irradiation and field releases. Females have to be excluded because, unlike their male counterparts, they can transmit diseases. Furthermore, releasing sterile males only increases the efficiency of the SIT program since they will concentrate primarily on mating with wild females. Although some sex separation strategies are available for *An. arabiensis*, none are efficient and applicable under an operational setting. The use of classical genetics to establish a genetic sexing strain (GSS) in *An. arabiensis* has been proposed. This approach requires a visible or conditional lethal mutation as a selectable marker linked to the sex-determining locus. Temperature-sensitive lethal (*tsl*) mutations and any inheritable phenotypic traits have been identified as possible selectable markers. Our team is identifying and isolating these selectable markers and then utilising irradiation to translocate them to the sex-determining chromosome. This sex linkage of a selectable temperature sensitivity gene will result in conditions that kill females but support males' production. During the last CRP meeting, we set objectives to screen different *An. arabiensis* laboratory colonies for temperature sensitivity, establish the association between insecticide-resistance target site mutations and *tsl* and their potential for use as selectable markers, determine the inheritance pattern and genetic mapping of temperature sensitivity lethality alleles and use mutagens to induce both *tsl* mutations and phenotypic/morphological markers. During the upcoming RCM2 meeting, I will present results and progress on the objectives that were set during the last session. Additionally, I will highlight our future plans to establish more temperature sensitivity lethality mutations using EMS and irradiation. Ultimately, once a temperature sensitivity lethality based GSS line is established, its reproductive and physiological fitness will be assessed together with its genetic stability over several generations.

**SECOND RESEARCH COORDINATION MEETING**

**On "Generic approach for the development of genetic sexing strains for SIT applications"**

**Vienna, Austria**

**18-22 October 2021**

TITLE OF WORKING PAPER: Screening for morphological visible markers and additional temperature sensitive strains of *Anopheles arabiensis*

AUTHOR (S): Cyrille Ndo[1], Yacouba Poumachu[1] & Kostas Bourtzis[2]

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SHORT SUMMARY OF PAPER

*Abstract:*

Our general objective is to develop and evaluate a pupal color- and temperature sensitive lethal-based Genetic Sexing Strain of the malaria vector *Anopheles arabiensis*. The work carried out so far aimed to isolate visible markers, and additional TSl colonies.

To this end, wild type *An. arabiensis* was collected in Sahelian zones of Cameroon and colonized in the insectary. Males were then mutagenized by feeding mosquitoes with 10% sucrose solution containing ethyl methane sulfonate during 24h. Treated males were allowed to mate with virgin females that were subsequently blood-fed and allowed to lay eggs. The fecundity and eggs fertility of mutagenized males were evaluated by counting number of eggs laid by a female and number of eggs that hatched, respectively. Progenies (eggs, larvae, pupae and adults) were screened for visible markers, over 3 generations. Moreover, from F3 temperature sensitive phenotypes were screened by assessing mortality at 24h after exposition of L1 larvae to heat at 41°C for 3hours.

Overall, mutagenesis didn't affect male survival which was similar to the control (90-91%). In contrast, mutagenesis decreased male fecundity and fertility, with more profound impact observed in mosquitoes that were exposed to the higher dose (0.001M) of the mutagen. No morphological visible marker was successfully isolated. By contrast, two additional TSL colonies have been established and are under characterization.

**D44003-CR-2**
**Second Research Coordination Meeting on Generic Approach for the Development of Genetic Sexing Strains for SIT Applications**
**Vienna, Austria**
**18 to 22 October 2021**

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