IAEA-D4.40.03-CR-1

LIMITED DISTRIBUTION

FIRST 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

Scientific Secretary: Kostas Bourtzis

Vienna International Centre

Vienna, Austria

7–11 October 2019

Contents

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.

One example of how GSS can significantly enhance SIT applicability and efficiency has been their use in the Mediterranean fruit fly, *Ceratitis capitata,* AW-IPM programmes, a technology developed at the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf in conjunction with the Agency's CRP programme. Using irradiation and classical genetic approaches, a series of genetic sexing strains were developed for the Mediterranean fruit fly (medfly). These are currently being used in all mass-rearing facilities producing this pest for large-scale SIT programmes, including the VIENNA-7 and VIENNA-8 strains. These GSS consist of at least two principal components: (a) a Y-autosome translocation, T(Y;A), which is required to link the inheritance of this marker to the male sex and (b) a selectable marker which is necessary for sex separation or female lethality. The latter include a *temperature-sensitive lethal* (*tsl*) gene, which is located on chromosome 5 and has useful biological properties rendering it an integral component of the new generation of medfly GSS currently used in mass rearing facilities and operational programmes worldwide. This is because, in the VIENNA-7 and VIENNA-8 GSS, *tsl*+ (temperature insensitive due to a wild type *tsl* gene) individuals are males (due to Y chromosome linkage) while females are homozygous for the mutant *tsl* allele and can be killed through the incubation of eggs at 34°C for 24 hours. GSS based on pupal colour mutation markers (having the wild type allele linked to the Y chromosome) also exist for other tephritid fruit fly species that include the *white pupae* (*wp*) marker for *Bactrocera dorsalis* and the *black pupae* (*bp*) marker for *Anastrepha ludens* and *Anastrepha fraterculus.*

Despite the importance of the *tsl* marker or other morphological colour markers, GSS have not been developed in many SIT targeted species because the isolation of naturally occurring mutants is a random and labour-intensive process. Furthermore, the process to develop a GSS in one species is not necessarily directly transferable to other species. However, such mutants can now be specifically induced through classical and molecular genetic technologies. If successful, this would open the way to detect and isolate, through gene discovery, orthologous genes in many other SIT targeted species (e.g. fruit flies, mosquitoes, tsetse flies, moths, etc.) and/or induce similar mutations (i.e. *tsl* or other selectable markers or selectable systems) which could then be used for the development of GSS. Thus, the specific objectives of the CRP are: 1) to isolate and characterize marker genes and their associated mutations to be used for generic strategies for the construction of GSS; 2) to develop generic approaches for the construction of GSS for SIT targeted agricultural pests and human disease vectors; and 3) to perform small-scale validation of GSS strains developed with novel generic approaches for both agricultural pests and human disease vectors.

In mosquitoes, two strategies for genetic sexing will be explored. In the first strategy, genes responsible for selectable mutant traits and achieve sex-separation by linking a wild type copy to the male-specific Y chromosome will be identified. The focus will be on genes that determine traits amenable to high-throughput screening and applicable to multiple species, such as a *tsl* or a color marker such as *red-eye*. CRP partners have already developed *tsl*-based strains for *An. arabiensis,* and for *Ae. aegypti* and *Ae. albopictus* strains have been generated exhibiting color-based morphological differences from wild types. In addition, the potential to develop strains based on mutations affecting development at the egg stage will be explored, including desiccation- and

UV-light resistance and egg-shell colour. To enable transferability of systems between species novel methods including NGS sequencing of recombinant pools to identify the genes and the underlying mutations will be developed that determine the mutant traits. Methods will be evaluated for linking desirable functional copies of the mutant genes into Y-chromosomes or M-loci to generate genetic sexing strains. To enable this goal there is a requirement to improve the Y chromosome assembly of *Ae. albopictus*. The second strategy, will explore the use of transgenes that manipulate either sex determination, sexual development or sex chromosome inheritance. In recent years, members of this CRP have made significant progress in the characterization of sex determining pathways including the discovery of the primary sex determining genes of some *Anopheles* and *Aedes* species relevant to this CRP, setting the foundation for manipulating this pathway for genetic sex separation. Sex-converted and female lethal lines have been generated and sex ratio distortion has been achieved by manipulating sex chromosome inheritance. The focus will be on achieving conditional expression of the sex-specific traits, so a genetic sexing strain can be produced and evaluated under small scale laboratory conditions.

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*) 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 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). 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*. 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 *dsx* gene (Jin et al., 2013; Tan et al., 2013).

3. Sex determination: 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*) to determine the female fate in XX embryos. 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), male differentiation results (Pane et al., 2002). This mode of *tra* autoregulation 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). 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).

One of the several *M* factors present in wild populations of *M. domestica*, *Mdmd*, has been recently 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 *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).

4. Recent developments:

4.1. The applications of 'big data' for molecular genetics: The biological 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* and in *Musca domestica*. 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 break-points or map the extent of inversions, which is critical for the stability and fitness of the strains (Franz, 2002). The identification of break-points 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), 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). 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 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, durability 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 the standard method of dsRNA delivery in insects. This system was initially utilized in *C. elegans* (Timmons and Fire 1998; Timmons et al., 2001) but has since applied extensively 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. Thus, until the *tsl* (or *wp*) 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 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).

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, tetracycline-suppressible female-specific embryonic lethality systems have been shown to be highly efficient in producing male-only populations in *A. suspensa* (Schetelig and Handler, 2012). 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 30^{o}C (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.

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). 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). 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). Transient manipulation of *Nix*, a recently discovered male-determining factor in *Aedes aegypti* (Hall et al., 2015), resulted in partial sex conversion. 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., 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.

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.

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.

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.

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.

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.

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.

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.
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., 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.
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.

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 (in press)*.

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.
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.
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.
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.
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.
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.

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.

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.

BOT5013 - Using the Sterile Insect Technique Integrated with Other Suppression Methods for Managing *Bactrocera dorsalis*.

BRA5060 - Using the Sterile Insect Technique to Evaluate a Local Strain in the Control of *Aedes aegypti*.

CHD5007 - Contributing to the Eradication of *Glossina fuscipes fuscipes* to Improve Food and Nutritional Security.

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

CPR5020 - Integrating the Sterile Insect Technique (SIT) for Area-Wide Integrated Pest Management of Tephritid Fruit Flies.

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

ECU5029 - Improving Integrated Fruit Fly Management in Fruit and Vegetable Production Areas.

ETH5021 - Enhancing Livestock and Crop Production Through Continued Consolidated and Sustainable Control of Tsetse and Trypanosomosis.

GUA5019 - Strengthening National Capabilities for the Control of Agricultural Pests Using Nuclear Technologies.

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

LIB5011 – Enhancing area-wide integrated management of fruit flies.

MEX5031 - Using the Sterile Insect Technique to Control Dengue Vectors.

MOR5035 - Implementing the Sterile Insect Technique in the Souss Valley.

OMA5007 – Strengthening Sterile Insect Technique Based Area-wide Integrated Management of Date Palm Pests.

PLW5002 – Improving the Quantity and Quality of Fruits for Exportation and Domestic Consumption Through Area-wide Integrated Pest Management of *Bactrocera* Fruit Flies in Tropical Fruit and Vegetable Production Areas (Phase II).

PAP5001 - Supporting a Feasibility Study on Using the Sterile Insect Technique against the Cocoa Pod Borer.

PHI5033 - Building Capacity in Using the Sterile Insect Technique against Dengue and Chikungunya Vectors.

SEN5037 - Supporting the National Programme to Control Tsetse and Trypanosomosis.

SAF5014 - Assessing the Sterile Insect Technique for Malaria Mosquitos in a South African Setting, Phase II.

SAF5015 - Supporting the Control of Nagana in South Africa Using an Area-Wide Integrated Pest Management Approach with a Sterile Insect Technique Component - Phase I.

SRL5047 – Establishing a National Centre for Research, Training and Services in Medical and Molecular Entomology for Vector-borne Disease Control.

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

THA5052 – Developing sustainable management of fruit flies integrating sterile insect technique with other suppression methods.

UGA5036 - Demonstrating the Feasibility of a Sterile Insect Technique Component as Part of an Area-wide Integrated Pest Management Approach to Increase Livestock Productivity.

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

ZIM5023 - Improving Crop and Livestock Production through the Eradication of Bovine and Human Trypanosomiasis in Matusadona National Park.

RAS5076 - Harmonizing and Strengthening Surveillance Systems to Prevent and Control Exotic and Native Fruit Flies Including the Use of the Sterile Insect Technique.

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

RER5022 - Establishing Genetic Control Programmes for *Aedes* Invasive Mosquitoes.

RLA5070 - Strengthening Fruit Fly Surveillance and Control Measures Using the Sterile Insect Technique in an Area-wide and Integrated Pest Management Approach for the Protection and Expansion of Horticultural Production (ARCAL CXLI).

RLA5074 - Strengthening Regional Capacity in Latin America and the Caribbean for Integrated Vector Management Approaches with a Sterile Insect Technique Component, to Control *Aedes* Mosquitoes as Vectors of Human Pathogens, particularly Zika Virus.

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 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	Biological material is available. QC protocols are available or can

9. Publication of results in a peer reviewed journal.	Papers drafted and submitted.	papers Journal issue with published scientific papers.	be developed. Data for publication available.
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.

FIRST RESEARCH COORDINATION MEETING

Joint FAO/IAEA Division 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

7 - 11 October 2019

Monday, 7 October 2019

08:30 – 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 – 10:00 **Kostas Bourtzis -** "Objectives and activities of the new CRP".

10:00 – 10:30 *Coffee Break*

SESSION I: Presentations by participants (Chairperson: Alfred M. Handler)

10:30 – 11.00 **Philippos A Papathanos** – **"**Methods for generating male-specific rescues of selectable mutant traits for high-throughput insect genetic sexing".

11:00 – 11:30 **Jake Tu –** "Generic markers for developing genetic sexing strains of *Aedes aegypti* and other insects".

11:30 – 12:00 **Hassan Mutasim Mohammed Ahmed** – "Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools".

12:00 – 13:30 *Lunch Break*

13:30 – 14:00 **Jiannis Ragoussis** – "*De novo* genome assembly combined with long-read RNA-seq reveal insights into early embryo development of *Bactrocera oleae*".

14:00 – 14:30 **Kostas D. Mathiopoulos** – "Exploring structure and function of the Tephritid Y chromosome".

14:30 – 15:00 **Alistair C. Darby** – "Sex chromosome genomics"**.**

15:00 – 15:30 *Coffee Break*

15:30 – 16:00 **Simon W. Baxter** – "Genetic characterisation of the *Bactrocera dorsalis* white pupae (wp) locus".

16:00 – 16:30 **Roswitha A. Aumann** – "Analysis of putative *tsl* and *wp* target regions in *Ceratitis capitata*".

16:30 – 17:00 **Antonios Augustinos** – "Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species".

17:00 – 17:30 **Daniel Bopp and Leo W. Beukeboom** – "Towards the identification of a novel paternal male determiner (MI) and the maternal sex determiner Ag in house flies".

Tuesday, 8 October 2019

SESSION I (cont'd): Presentations by participants (Chairperson: Jake Tu)

08:30 – 09:00	**Jaroslaw Krzywinski** – "Targeting sex determination pathway for male-only strains in *Anopheles gambiae* complex".
09:00 – 09:30	**Cyrille Ndo** – "Development of a pupal color- and temperature sensitive lethal-based Genetic Sexing Strain of the malaria vector *Anopheles arabiensis*".
09:30 – 10:00	**Pablo Tortosa** – "Construction of an *Aedes albopictus* genetic sexing strain using a default in eggshell melanization".
10:00 – 10.30	*Coffee Break*
10:30 – 11:00	**Giuseppe Saccone** – "a) Gene targeting of the female determining transformer gene in the Mediterranean fruit fly: an unexpected CRISPR/Cas9 interference effect. b) More on the future of MoY study and how to molecularly screen for tsl genes".
11:00 – 11:30	**Alfred M. Handler** – "Female-specific conditional lethality genetic-sexing strains for improved SIT".
11:30 – 12:00	**František Marec** – "Progress in understanding the genome, sex chromosomes and sex determination in the codling moth, *Cydia pomonella*".
12:00 – 13:30	*Lunch Break*
13:30 – 14:00	**Wei Peng** – "The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii*".
14:00 – 14:30	**José S. Meza** – "Induction and evaluation of generic markers for the development of genetic sexing systems in Anastrepha".
14:30 – 15:00	**Edwin Ramirez** – "Development and evaluation of genetic sexing strains of fruits flies to be used for sterile insect technique applications, as part of AW-IPM programs".
15:00 – 15:30	*Coffee Break*
15:30 – 16:00	**Nidchaya Aketarawong** – "Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera spp*. using bioinformatics and molecular tools".
16:00 – 16:30	**Juan P. Wulff** – "Development of genetic sexing strains for *Anastrepha fraterculus* approaching with generic genomic tools".
16:30 – 17:00	**Angela Meccariello** – "X-shredder in the model and non-model insects".
17:00 – 17:30	Discussion
18.30	Dinner at Centimeter 2 am Spittelberg, Stiftgasse 4 / at the corner with Siebensterngasse, 1070 Vienna

Wednesday, 9 October 2019

SESSION II: Review of the CRP documents (Chairperson: Kostas Bourtzis and Group Leaders - rooms M4, MOE23 and MOE24)

08:30 – 10:00	**Open discussion and composition of the working groups**
10:00 – 10:30	*Coffee Break*
10:30 – 12:00	**Revision of the CRP documents (introduction, contracts) by groups**
12:00 – 13:30	*Lunch*
13:30 – 15:00	**Revision of the CRP documents (introduction, contracts) by groups**
15:00 – 15:30	*Coffee Break*
15:30 – 17:00	**Revision of the CRP documents (introduction, contracts) by groups**

Thursday, 10 October 2019

SESSION III: Review of the individual proposals (Chairperson: Kostas Bourtzis and Group Leaders - rooms M4, MOE23 and MOE24)

08:30 – 10:00	**Revision of individual proposals and planning of the activities to carry out for the 5 years CRP and for the next 18 months**
10:00 – 10:30	*Coffee Break*
10:30 – 12:00	**Revision of individual proposals and planning of the activities to carry out for the 5 years CRP and for the next 18 months**
12:00 – 13.30	*Lunch*
13:30 – 15:00	**Revision of individual proposals and planning of the activities to carry out for the 5 years CRP and for the next 18 months**
15:00 – 15:30	*Coffee Break*
15:30 – 17:00	**Revision of individual proposals and planning of the activities to carry out for the 5 years CRP and for the next 18 months**

Friday, 11 October 2019

SESSION IV: Review of the LFM and drafting the RCM report (Chairperson: Kostas Bourtzis - room M4)

08:30 – 10:00	**Review of the LFM to the entire group and drafting the RCM report**
10:00 – 10:30	*Coffee Break*
10:30 – 12:00	**Drafting of the RCM report**
12:20 – 13:30	*Lunch*
13:30 – 16:00	**Presentation of the RCM report**
16:00 – 16:15	***Closing***

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Methods for generating male-specific rescues of selectable mutant traits for high-throughput insect genetic sexing**.

AUTHOR (S): Doron Zaada, Flavia Krsticevic, Yehonathan Alcalay, Philippos A Papathanos

ORGANIZATION: Department of Entomology, Hebrew University, Israel

SHORT SUMMARY OF PAPER

Abstract:

The research program will develop a novel method for sex separation in two target mosquito species, *Anopheles gambiae* and *Aedes albopictus*, based on high-throughput elimination of female larvae. The genetic sexing systems will implement dietary delivery of larvicidal RNAi combined with genetic sexing strains (GSS), built using CRISPR-based gene editing, in which males bear Y-chromosome-linked rescue alleles. To establish these GSS, a significant portion of the project will involve the precise delivery and expression on the Y-chromosome – or M-locus in the case of *Ae. albopictus*, of re-engineered/edited endogenous, autosomal mosquito alleles that act to rescue CRISPR induced recessive mutations. These methods can be rapidly adopted with only minor modifications in the future for any XY heterogametic pest insects, such as fruit flies.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Generic markers for developing genetic sexing strains of *Aedes aegypti* and other insects**

AUTHOR (S): Zhijian Jake Tu[1] and Kostas Bourtzis[2]

ORGANIZATION: [1]Virginia Polytechnic Institute and State University (Virginia Tech); and [2]Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

SHORT SUMMARY OF PAPER

Abstract:

The long-term objectives of this research are 1) to develop genetic sexing strains of *Aedes aegypti* that can be used as a part of an economical, efficient, and environment-friendly method to control dengue, Zika and other arboviral diseases; and 2) to extend the basic information gained from *Aedes aegypti* to other insect pest species of medical or agricultural importance. We firmly believe that integrated pest management (IPM) strategies that build on a combination of approaches will enable the most effective control in different regions of the globe. To contribute towards the aforementioned objectives, we propose the following three specific aims: 1) Systematically identify the causal genes of useful phenotypic markers for developing genetic sexing strains of *Aedes aegypti*. We will integrate modern bulk-segregant analysis (BSA) tools and marker-assisted analysis to identify these genes; 2) Knockout, by CRISPR/cas9, two of the most promising candidate genes in wild-type *Aedes aegypti* to verify the resulting phenotype; and 3) Knock-in a wild-type gene into genomic regions within (or tightly linked to) the M-locus to restore the wild-type phenotype only in males. This will lead to the production of new genetic sexing strains, and establish a method to efficiently link selectable markers to the sex locus. In summary, the proposed research will 1) identify useful marker genes that could be used as generic sexing markers, 2) establish a method to rapidly isolate causal genes that are critical to the development of generic genetic sexing strains, 3) potentially produce genetic sexing strains for *Aedes aegypti*, and 4) develop a new method for sex-linkage of selectable markers without introducing any foreign genes.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

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 propose to identify suitable tsl mutations in the vinegar fly *Drosophila melanogaster* or the baker's yeast *Saccharomyces cerevisiae* and transfer verified mutations by genome editing to the agricultural pest, the cherry vinegar fly *Drosophila suzukii*. In addition, we will identify suitable loci on the Y chromosome of *D. suzukii* for early embryonic expression and place a wild type allele of the tsl gene there to rescue the tsl phenoytpe specifically in males and thereby generate a GGS in *D. suzukii*. Current genome editing tools would 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. Once successful, this strategy could also be used to generate GGS in other agricultural pest species and human disease vectors.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: ***De novo* genome assembly combined with long-read RNA-seq reveal insights into early embryo development of *bactrocera oleae***

AUTHOR (S): Anthony Bayega[1*], Haig Djambazian[1*], Konstantina T. Tsoumani[2], Maria-Eleni Gregoriou[2], Efthimia Sagri[2], Eleni Drosopoulou[3], Penelope Mavragani-Tsipidou[3], Kristina Giorda[4], George Tsiamis[5], Kostas Bourtzis[6], Spyridon Oikonomopoulos[1], Ken Dewar[1], Deanna M. Church[4], Alexie Papanikolaou[7], Kostas D. Mathiopoulos[2#] & **Jiannis Ragoussis[1,8#$]**

ORGANIZATION: McGill University

SHORT SUMMARY OF PAPER

Abstract:

The olive fruit fly (*Bactrocera oleae*) is the most important pest of cultivated olive trees but poorly characterized at a genomics level. We assembled the 480 Mb *B. oleae* genome using short-, mate-pair-, long-, and linked-read sequencing technologies to generate a genome assembly (Genbank accession GCA_001188975.3) with a scaffold N50 of 4.69 Mb and L50 of 30 scaffolds. Short reads from both males and females and the chromosome quotient method enabled identification of Y-chromosome scaffolds which were extensively validated by PCR. Oxford Nanopore long-read RNA-seq of *B. oleae* embryos collected at hourly intervals for the first 6 hours of development yielded a median of 4.5 million total reads/timepoint across six timepoints. The reads showed 98 % alignment rate to the genome. Through our custom data analysis pipeline, we generated a *de novo* transcriptome assembly of the olive fly, based on long-read sequencing data, and identified 11883 genes and a total of 79,810 transcripts. We noticed sex specific isoforms some of which can be found in genes related to sex development. Using internal ERCC RNA standards, we obtained absolute gene expression quantification and we were able to define a set of developmental stage specific transcripts. On a global scale, the first six hours of embryo development were characterized by dramatic transcriptome changes with the total mRNA content per embryo dropping by half from the first hour to the second hour of embryo development. These data provide the first insight into the transcriptome landscape of the developing olive fly embryos. Overall, full-length sequencing of cDNA permitted a detailed characterization of the isoform complexity and the transcriptional dynamics of the first embryonic stages of an economically important insect. Delineating the gene structure, the stage-specific gene expression and the sex-specific alternative splicing can be used to identify new targets which can help suppress fly population in the field and prevent crop damage.

Author affiliations: [1] McGill University and Genome Quebec Innovation Centre, Department of Human Genetics, McGill University, Montreal, Canada; [2] Department of Biochemistry and Biotechnology, University of Thessaly, Biopolis, Larissa 41500, Greece; [3] Department of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece; [4] 10x Genomics, 7068 Koll Center Parkway, Suite 401, Pleasanton, CA 94566, USA; [5] Department of Environmental Engineering, University of Patras, Agrinio, Greece; [6] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria; [7] Hawkesbury Institute for the Environment, Western Sydney University, Richmond NSW 2753, Australia; [8] Department of Bioengineering, McGill University, Montreal, Canada

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

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

AUTHOR (S): Tsoumani K, Gregoriou M-E, Bayega A, Ragoussis J, Mathiopoulos KD

ORGANIZATION: Department of Biochemistry and Biotechnology, University of Thessaly

SHORT SUMMARY OF PAPER

Abstract:

Genetic Sexing Strains (GSS) have significantly enhanced the applicability and efficiency of the classical Sterile Insect Technique (SIT) since they have made possible the release of male insects only. The Y chromosome is of utmost importance in GSS since it is the chromosome where any gene that would provide conditional male viability should be transferred. However, the Y chromosome has been notoriously difficult to analyze due to its heterochromatic and repetitive nature. We have recently completed the sequencing of the entire genome of the olive fruit fly with a combination of short and long read technologies, giving particular emphasis on the assembly of the Y chromosome. Using the CQ method we identified Y-specific scaffolds that totaled 3.9 Mb (which is the expected size of the olive fly Y chromosome) and X-specific scaffolds that totaled 6 Mb. Furthermore, we managed to experimentally validate the Y-specificity of several scaffolds that covered 44% of the Y chromosome. Among those, there was a scaffold harboring *BoMoY* (*Bactrocera oleae Maleness-on-the-Y*), the male determining gene of the olive fly. In combination with the repertoire of early embryonic genes that we are analyzing with the McGill team of Dr Ragoussis, our objective is to understand the interaction of transcription factors in regulatory regions around BoMoY and shed light on the mechanism of masculinization in the olive fly as well as other Tephritids. Such knowledge can help the design of generic male switches that can be applied in GSS. In addition, we will search for other transcription units on the Y, try to understand their function and compare them with the transcription units on the Y chromosomes of other Tephritids in order to elucidate the evolution of that chromosome.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Sex chromosome genomics**

AUTHOR (S): Prof Alistair C Darby and Dr Grant Hughes

ORGANIZATION: Institute of Integrative Biology, University of Liverpool

SHORT SUMMARY OF PAPER

Abstract:

Most genomes which have been sequenced to date have focused on the heterogametic sex and as a result we are missing key information about chromosome regions associated with sex determination. Here we will use newly available high-quality genomic data from male dipterans to discover Y chromosome sequences and find genes linked to sex determination. These are difficult regions of the genome to sequence as they are very repetitive and need long read data (e.g. PACBIO or OXFORD NANPORE) and HiC to resolve them actually.

We will also talk about our plans to develop the method ReMOT Control (Receptor-Mediated Ovary Transduction of Cargo). While the ReMOT Control technology is ground-breaking, implementation is in its infancy and it has only been employed to knock-out genes in mosquitoes. This revolutionary new method has exceptional promise to provide a flexible approach for the genetic manipulation of a wide variety of non-model arthropods.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Genetic characterisation of the *Bactrocera dorsalis white pupae* (*wp*) locus**

AUTHOR (S): Christopher M. Ward[*], Elisabeth Fung[‡§], Carlos Caceres-Barrios[†], Thu Nguyen[*], Peter Crisp[‡§] and Simon W. Baxter[**]

ORGANIZATION:
[*]School of Biological Sciences, University of Adelaide, Australia
[‡]School of Agriculture, Food and Wine, University of Adelaide, Australia
[§]South Australian Research and Development Institute, Adelaide, Australia
[†]Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria
[**]School of BioSciences, University of Melbourne, Australia

SHORT SUMMARY OF PAPER

Abstract:

Dimorphic color variation can occur in insects and this provides a visual cue to separate individuals according to sex. Gene technology has the potential to engineer Tephritid genetic sexing strains with dimorphic pupal phenotypes for the Sterile Insect Technique (SIT), however, causal color genes have not yet been characterized. Here we report a recessive gene mutation in *Bactrocera dorsalis* that causes a *white pupae* (*wp*) phenotype. We hybridized the *B. dorsalis wp* strain with wild type *B. tryoni* (brown pupae), then performed repeated backcrossing and reselection over twelve generations to produce a *B. tryoni* white pupae introgression line. Whole genome sequencing identified a 2.5 Mb region of the *B. dorsalis* genome that was fixed in the introgression line and controlled the *wp* phenotype. The region contained 81 protein coding genes, including a *major facilitator superfamily* (*mfs*) gene with a 37 bp deletion that truncates the transmembrane protein. Expression of *mfs* in wild type *B. tryoni* occurs almost entirely during pupal development and CRISPR/Cas9 mutagenesis of *mfs* generates mosaic white and tan puparium in injected individuals, providing functional support for a role in pigmentation. Knock-out of *mfs* orthologs may cause white pupae phenotypes in a range of Tephritids, although the construction of a genetic sexing strain will require insertion or translocation of a functional gene to the male Y-chromosome for dimorphic pigmentation.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Analysis of putative *tsl* and *wp* target regions in *Ceratitis capitata***

AUTHOR (S): Roswitha A Aumann, Irina Häcker, Marc F Schetelig

ORGANIZATION: Justus-Liebig-University Gießen, Institute for Insect Biotechnology, Department of Insect Biotechnology in Plant Protection, Winchesterstr. 2, 35394 Gießen, Germany

SHORT SUMMARY OF PAPER

Abstract:

The *temperature sensitive lethal* (*tsl*) and *white pupae* (*wp*) traits are examples of important mutations present in *Ceratitis capitata* utilized for mass rearing that facilitate cost-effective production of male-only populations for sterile releases. While these mutations have been employed in *C. capitata* genetic sexing strains (GSS) for more than 20 years, the specific mutations and mechanisms by which they work are still not known. With new genomic technologies, it should be possible to identify and characterize the genes underlying these traits at the molecular level and understand what specific mutations in the DNA cause the trait. In addition, high-throughput sequencing and bioinformatic techniques make it possible to compare genomes of different species to define synteny and orthology, to understand if a target is present in another species that could be used to facilitate rapid development of robust SIT strains for that species. To transfer tsl/wp to new species of interest, targeted genome editing techniques such as CRISPR/Cas9 make it possible to first confirm the underlying mutations be re-creating *tsl*/*wp* traits in wild type *C. capitata* and then generate comparable mutations in other species to allow direct development of tsl/wp traits. This approach and methods would facilitate direct development of new genetic sexing strains in new species for use in SIT programs.

The medfly is the optimal species to perform this kind of analysis, because, in medfly, a tsl strain is available as well as genomes, transcriptomes, and structural data of the chromosomes. Our first goal, therefore, is the identification of the causative mutations for the *wp* and *tsl* traits in existing GSS. To do so, together with collaborators, we employed methodologies that bring together classical genetics with high-throughput sequencing techniques and emerging genome editing technologies. Using a list of putative causative mutations as a starting point, we have analyzed several possible targets and started their evaluation. The current status of this approach will be presented.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

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:

Temperature sensitive lethal genes (tsl) are considered as a key component of genetic sexing strains developed through classical genetic approaches. In *Drosophila melanogaster*, the model species of Diptera, there are several described tsl genes. There is also availability of genomic data for different Diptera species of economic and medical importance that would benefit from the identification of tsl candidate genes. Isolation and characterization of a tsl gene is a difficult task, since the tsl phenotype can be quite variable, depending on different genetic and environmental parameters. That explains why, even after applied for almost two decades and with the recent availability of a genome assembly, the tsl gene of the medfly VIENNA GSS strains has not been (yet) identified. Moreover, a tsl-based genetic sexing approach needs accompanying morphological markers linked with the tsl gene to allow easy monitoring of the genetic sexing system. Another important concern is genetic recombination that threatens the genetic stability of GSS. In fruit flies, where male recombination is drastically reduced, this is not a major concern, in contrast to mosquitos (such as Aedes) where recombination occurs in both sexes in comparable frequencies.

In this frame, we are working towards the:

a. Identification of candidate tsl genes in different SIT target species, exploiting available knowledge from *D. melanogaster* (and other species), classical molecular tools (such as *in situ* hybridization), and novel tools (bioinformatic analysis).
b. Development of standard protocols to quantify the response to thermal stress. Lessons learnt from the medfly tsl phenotype will be exploited towards the development of thermal stress response protocols for SIT target species of agricultural (priority to *Bactrocera oleae*) and medical importance (priority to *Aedes albopictus*).
c. Support of genetic analysis efforts of the tsl mutation incorporated in medfly GSS. We will do so in collaboration with IPCL, by performing genetic linkage analysis that include the tsl gene and other morphological and molecular markers already mapped on chromosome 5, exploiting classical genetic analysis, *in situ* hybridization (if needed), and novel bioinformatic approaches.

In the availability/identification of appropriate morphological and/or tsl mutations, we will work towards the isolation of the appropriate recombination reducing tools (chromosomal inversions) that will enhance the genetic stability of the resulting strains, with priority on *B. oleae* and *Ae. albopictus*.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Towards the identification of a novel paternal male determiner (M^I) and the maternal sex determiner *Ag* in house flies**

AUTHOR (S): Daniel Bopp[1], Leo Beukeboom[2], Francuski Marcetic Ljubinka[2], Luca Lenzi[1], Laura Medici[1], Elzemiek Geuverink[2], Xuan Li[2] and Claudia Brunner[1]

ORGANIZATION: [1]Institute of Molecular Life Sciences, University of Zurich, Switzerland, [2]Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, Netherlands.

SHORT SUMMARY OF PAPER

Abstract:

Many insect species use the *transformer* gene (*tra*) as the critical switch which instructs female development when ON and male development when OFF. There is a common theme that *tra* is zygotically activated by maternally provided factors, hence eggs are predisposed for female development. Paternally transmitted male factors, on the other hand, can prevent this activation to allow for male development. Though male determining factors normally act in the early zygote there is evidence that male determiners can also evolve to be exclusively expressed in the female germline where they prevent expression of *tra* activating factors. Eggs devoid of these maternal factors will develop into males after fertilization irrespectively of whether paternal male determiners are provided or not. Such a system can be exploited to construct *arrhenogenic* females which only produce male offspring. The house fly *Musca domestica* is an ideal model to investigate this possible application. The sex determining pathway in Musca is polygenic in that many different systems have been found in natural and cultured populations. The spectrum ranges from the use of dominant male determiners at different genomic locations, the use of a female dominant determiner, to an entirely maternal control system. In standard strains Musca uses dominant male factors (*M*) which prevent maternal activation of *tra* in the zygote. *Mdmd*, a paralog of the spliceosomal factor CWC22, was identified to be a male determiner in Musca and appears to be present in different strains at different genomic sites. We are currently investigating a strain where *M* is located on the first chromosome (M^I). Circumstantial evidence suggests that this M^I is structurally different from *Mdmd* and hence may be a novel *M*. Interestingly, the dominant maternal mutation *Ag* maps at a location very close to M^I. *Ag* behaves as an evolved *M* factor which is only expressed in the female germline. Females carrying a copy of *Ag* produce eggs which are devoid of maternal *tra* and, as a result, produce only male progeny. Hence, we consider it possible that *Ag* is a derivative of M^I which lost its somatic function but is expressed in the female germ line and capable of repressing maternal *tra*. We will attempt to isolate M^I and *Ag* molecularly. De novo assemblies of early embryonic transcriptomes prepared from a) mixed males and females b) only females and c) unfertilized eggs of the M^I strain will be compared to identify zygotic male-specific transcripts. Candidates will be validated by RT-PCR and functionally tested by RNAi. Once M^I is identified, we will test its presence in *Ag* females.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Targeting sex determination pathway for male-only strains in *Anopheles gambiae* complex**.

AUTHOR (S): Elzbieta Krzywinska and Jaroslaw Krzywinski

ORGANIZATION: The Pirbright Institute

SHORT SUMMARY OF PAPER

Abstract:

Novel approaches are urgently needed to successfully respond to the growing threat of infectious diseases transmitted by mosquitoes. Genetic methods of mosquito control are potentially highly effective, yet their use is limited by the lack of high-throughput means of sex separation to generate sufficient numbers of males for operational field releases. We are exploiting the sex determination pathway genes as targets for manipulation to generate conditional female embryo-specific lethality in Anopheles gambiae and its sibling species A. arabiensis. Previously we found that delivery of transcripts of the primary sex determination gene Yob to preblastoderm embryos results in death of females prior to hatching in both species. Recently, we generated A. gambiae transgenic lines that ectopically express Yob in both sexes and produce male-only or strongly male-biased progenies, with surviving females masculinized to a various extent. The lines have been maintained through multiple generations by backcrossing transgenic males with wild-type females. The development of conditionally repressible female-lethal lines is currently in progress in our laboratory.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Development of a pupal color- and temperature sensitive lethal-based Genetic Sexing Strain of the malaria vector *Anopheles arabiensis***

AUTHOR (S): Cyrille NDO, Yacouba POUMACHU, Igor SHARAKOV

ORGANIZATION: OCEAC

SHORT SUMMARY OF PAPER

Abstract:

Background:
Vector control is the cornerstone of malaria prevention strategies using insecticide-based interventions. However, the situation is becoming acute with widespread resistance to insecticides, coinciding with resurgence of malaria cases. Therefore, new and innovative tools to reduce *Plasmodium* transmission are in dire need. This raised considerable interest in using sterile insect technique (SIT) against *Anopheles* malaria vectors. In such SIT programme, one of the challenges to be addressed is elimination/separation of haematophagous vector females from males to be released. The general objective of our work is to develop and evaluate a pupal color- and temperature sensitive lethal-based Genetic Sexing Strain of the malaria vector *Anopheles arabiensis*.
Methods and expected outcomes:
Field wild type *An. arabiensis* were collected in North Cameroon and a colony has been established in insectary. Males will be mutagenized by feeding mosquitoes with10% sucrose solution containing ethyl methanesulfonate during 24h. Treated males will be allowed to mate with virgin females and progenies will be screened for a visible markers and temperature sensitive phenotypes, from F3. Similarly, wild type progenies will also be directly screened. The behavior of the additional tsl and morphological markers isolated will be characterized and those located on the autosome will be translocated to the Y-chromosome by irradiation. The translocated strains isolated in this study and those isolated previously will be further characterized cytogenetically. The GSS will be created by crossing wild type translocated males with tsl females, isolated either in this study or during the previous one. The GSS developed will be then evaluated both in the laboratory and at the semi-field levels.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Construction of an *Ae. albo*pictus genetic sexing strain using a default in eggshell melanization**.

AUTHOR (S): Pablo Tortosa[1,2] & Patrick Mavingui[1,2]

ORGANIZATION:
[1]Unité Mixte de recherche Processus Infectieux en Milieu Insulaire Tropical (UMR PIMIT) Université de La Réunion, CNRS 9192, INSERM 1187, IRD 249. Plateforme de recherche CYROI, 2 rue Maxime Rivière. 97490 Ste Clotilde, Ile de La Réunion, France.
[2]Symbiosis Technology for Insect Control (SymbioTIC). Plateforme de recherche CYROI, 2 rue Maxime Rivière. 97490 Ste Clotilde, Ile de La Réunion, France.

SHORT SUMMARY OF PAPER

Abstract:

The development of the Sterile insect Technique (SIT) and Incompatible Insect Technique (IIT) targeting *Aedes albopictus* suffers from the lack of an efficient and sustainable sexing tool. Although we have recently constructed a Genetic Sexing Strain (GSS) using an insecticide resistance gene as a marker, the acceptability of SIT/IIT relies on a process fully deprived of insecticides. In the present project, we will construct a clean GSS, *i.e.* a mosquito line allowing sex separation without the need of insecticide. We will use a phenotype that we have observed in one of our reference lines and which is characterized by the hatching of white eggs. This phenotype results from defaults in eggshell melanization, a process involving multiple genes and pathways. Of note, white eggs are highly susceptible to desiccation and hence this phenotype is expected to be relevant as a selectable marker. We will construct mosquito lines impaired in egg melanization, hereafter referred as *weg* (white egg) mutants, and use these lines in addition to chromosomal translocation to construct a Genetic Sexing Strain in which female embryos can be killed by desiccation.

FIRST RESEARCH COORDINATION MEETING

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

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **a) Gene targeting of the female determining transformer gene in the mediterranean fruit fly: an unexpected CRISPR/Cas9 interference effect. b) More on the future of *MoY* study and how to molecularly screen for *tsl* genes.**

AUTHOR (S): **Primo[1], P.,** Meccariello[1], A., Gucciardino[1], M., Forlenza[1], F., Perrotta[1], M., S., Buonanno[2], M., Gravina[1], A. Ruggiero[2], A., P., M., Vitagliano[2], L., Giordano[1], E., Salvemini[1], Samson[3], ML, Rabinow[3], L., Robinson[4], M. and **Saccone[1], G.**

ORGANIZATION:
[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]Institute of Molecular Life Sciences and SIB Swiss Institute of Bioinformatics, University of Zurich, 8057 Zurich, Switzerland.

SHORT SUMMARY OF PAPER

Abstract:

a) The Mediterranean fruitfly *Ceratitis capitata (*medfly) is an agricultural pest of high economic impact. We targeted medfly *transformer*, the key female determining gene with the ability to autoregulate, in a way similar to *Sex-lethal* in Drosophila. We injected Cas9-sgRNA RNPs into *Ceratitis* female-only XX embryos which developed into G0 adult XX flies, with up to 50% presenting a completely masculinized phenotype and an exclusively male-specific *Cctra* splicing pattern. However, *Cctra* DNA sequence analysis revealed a lack of gene editing events in both G0 and G1 progenies. We reasoned that *Cctra* transcription was transiently suppressed by an unplanned CRISPR interference (CRISPRi), usually achieved by a defective Cas9 variant (dCas9) unable to cut DNA. This unexpected CRISPRi seems to have caused a masculinization of XX embryos by transient biallelic transcriptional silencing rather than biallelic DNA mutations. We propose that, similarly to dCas9, a wild type Cas9 protein can cause CRISPR interference in autoregulated genes that are activated very early during embryogenesis. **b)** We will discuss our projects to understand how *MoY* functions molecularly in Tephritidae. Furthermore, we will present first steps for a proof of principle of a novel method to quickly screen for putative *tsl* genes, using embryo injections of large genomic *tsl*+ fragments into an *elav* mutant strain of Drosophila.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Female-specific conditional lethality genetic-sexing strains for improved SIT**

AUTHOR (S): A.M. Handler, J. Li, and Y. Zhao

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

SHORT SUMMARY OF PAPER

Abstract:

The sterile insect technique (SIT) provides the most ecologically sound and efficacious means for suppression of insect pest populations, but many current programs are inefficient due to the inability to eliminate females in early development, requiring their rearing, sterilization and release with sterile males. Genetic manipulation of species subject to SIT now allows the use of female-specific tetracycline-suppressible (Tet-off) and dominant temperature sensitive (DTS) conditional lethality strategies to overcome this limitation. Our goal is to develop new transgenic strains for the fruit fly pest species *Anastrepha ludens, A. fraterculus, A. obliqua, A. suspensa* and *Drosophila suzukii* for Tet-off and DTS female-specific lethality for sexing. Notably, both systems will use highly conserved genetic systems for regulatory and sex-specific control and lethal effectors that, with minor modification, can be used as generic systems in other related species. DTS lethality (using the *AsProsβ2*^{1} mutation) has already been demonstrated in *A. suspensa*, and temperature-dependent female-specific lethality will be tested for the first time in additional *Anastrepha* species. Tet-off female-specific embryonic lethality has been achieved in tephritid species, but in *A. ludens*, maternal pre-zygotic lethality resulting in female-sterility also occurred. Thus, new promoter systems that are strictly embryo-specific will be identified and tested for use in *A. ludens*, in addition to *A. fraterculus* and *A. obliqua* and other species where the *serendipity-α* promoter exhibits pre-zygotic activity. The development of two independent female-specific lethality systems for genetic sexing that are highly conserved will broaden the capabilities for achieving efficient sexing, in addition to direct use for population suppression in the field, in a large number of related insect pest species. Importantly, the two systems may also be used together in a stacked redundant female lethality strain, ensuring lethality if either system breaks down due to spontaneous mutations, or is subject to suppression by inherent modifiers in targeted field populations.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Progress in understanding the genome, sex chromosomes and sex determination in the codling moth, *Cydia pomonella***

AUTHOR (S): František Marec[1], Sander Visser[1,2], Renata Kružíková[1,2], Martina Dalíková[1,2], Petr Nguyen[1,2]

ORGANIZATION:
[1] Biology Centre CAS, Institute of Entomology, České Budějovice, Czech Republic
[2] University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic

SHORT SUMMARY OF PAPER

Abstract:

Recently, significant progress has been made in research on codling moth genetics and genomics. We participated in the 'Codling Moth Genome Sequencing Consortium', coordinated by Fang-Hao Wan (Institute of Plant Protection CAAS, Beijing, China). The codling moth genome was sequenced by combination of several advanced technologies, including Illumina, PacBio, BioNano, and Hi-C scaffolding. This approach allowed for a chromosome-level scaffold assembly including the Z chromosome and a portion of the W chromosome without linkage mapping. The chromosome assembly showed a high level of gene synteny with other lepidopteran genomes and confirmed our previous results on the origin of the neo-Z chromosome from fusion of the ancestral Z chromosome with an autosome corresponding to autosome 15 in the silkworm, *Bombyx mori*. The W-chromosome assembly suggests accumulation of transposons, substantial sequence degeneration and the absence of homology between the neo-Z and (neo)-W chromosomes. However, considering the large size of the codling moth W chromosome, the current W-assembly represents only a small fraction of this chromosome which is not sufficient to identify W-specific sequences and a putative female sex determining factor. Therefore, we intend to re-assemble the W chromosome sequence by an alternative approach. In the meantime, we have used the available genomic tools in the codling moth to search for the orthologs of genes involved in the recently discovered sex-determining pathway of *B. mori*. In the transcriptome sequence, we have successfully identified the ortholog of the *Masculinizer* (*Masc*) gene, which promotes male development in *B. mori*, and confirmed its Z-linkage in the codling moth. In addition, we have identified the ortholog of the *B. mori doublesex* gene in the genome assembly. To verify the role of both genes in sex determination of the codling moth, we initiated experiments to determine their alternative expression in female and male embryos. Here we also discuss the importance of new findings for the development of genetic sexing strains in the codling moth and other lepidopteran pests.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii***

AUTHOR (S): Hao Chen, Baoyu Han, Wei Peng*

ORGANIZATION: China Jiliang University

SHORT SUMMARY OF PAPER

Abstract:

Drosophila suzukii Matsumura (Diptera: Drosophilidae), a recently invasive insect pest commonly known as spotted wing Drosophila (SWD), has recently invaded western countries, and it has become an important threat of a wide variety of several commercial soft fruits by causing significant losses in crop yield and quality. In this paper, we will elucidate the molecular mechanism of sex determination in *D. suzukii*, and then develop a masculinization and female-specific lethality or embryonic conditional lethality genetic system for use in SIT. We have applied Illumina sequencing to identify *D. suzukii* sex determination genes and early zygotic genes by analyzing transcripts from early embryonic development stages, which includes the time of sex determination and cellularisation formation. Clusters of gene orthology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations were performed throughout the embryo development to better understand the functions of differential expressed unigenes. We observed that RNA binding and spliceosome pathway were highly enriched and overrepresented during the early stage of embryogenesis. Additionally, transcripts for *Sex-lethal* (*Sxl*), *transformer* (*tra*), *doublesex* (*dsx*) sex determination genes and *serendipity a* (*srya*) cellularisation gene were identified, and expression pattern analysis revealed that the majority of these genes were highly expressed during the embryogenesis. We have identified the sex alternative splicing mechanism of *DsSxl*, *Dstra*, and *Dsdsx* genes in female and male *D. suzukii*. Besides, the role of *Dstra* in sex determination and reproduction was investigated by the CRISPR/Cas9 technology.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

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

AUTHOR (S): José S. Meza, Beatriz Xoconostle, Martha G. Roblero-Roblero, Victor García-Martínez, Jorge Ibañez-Palacios, Olga P. López-Hernández, Maria F. Ruíz-Pérez, Julio C. Coutiño-Montes, Daisy P. Cárdenas-Enriquez, Martha Guillen-Ribera and Arturo Bello-Ribera.

ORGANIZATION: National Program Fruit Flies SADER/SENASICA

SHORT SUMMARY OF PAPER

Abstract:

The "*black pupa*" (*bp*) mutation has been found in three fruit fly pests of economic importance for America, *Anastrepha ludens*, *Anastrepha fraterculus* and recently in *Anastrepha obliqua*, which means that the *bp* marker could be considered as a generic marker for the genus *Anastrepha*. In order to release only sterile males, the recessive marker *bp* has been used for the development of genetic sexing strains (GSS) in *A. ludens* and *A. fraterculus*, using a sorting machine for the separation of black pupa female from the brown pupa male. The cytogenetic analysis of both GSS showed evidence that the location of the *bp* allele is on different chromosomes among these species. The *bp* was located on III-chromosome for *A. ludens* and VI-chromosome for *A. fraterculus*.

However, in this pupal color-based sexing system, a large number of black pupae (females) are wasted, which could mean low cost-effectiveness. Another marker, recently isolated in *A. ludens* was "*slow larvae*" mutation (*sl*), which can offer a solution to this issue. The *sl* marker delays the larval development. Thus, the *sl* marker incorporation in this sexing system allows the separation of the males (pupae) from the females (larvae) during the pupation phase, which makes possible a self-sexing system in the strain and also the use of delayed larvae (females) as host of parasitoids.

Embryonic conditional lethality using temperature sensitive lethal mutations (*tsl*) in early developmental stages are absolutely desired for a genetic sexing system, because they can kill the females in embryo stage reducing rearing cost, the perfect example is the GSS constructed for Mediterranean fruit fly, where is possible to kill the females by heat treatment in eggs stage. In this example, apparently the principle action of *tsl* mutation is the loss of disulphide bond in vital proteins, when the females are exposed to an increase in temperature, unfolded proteins with no disulphide bonds are inactive. In the past, several methodologies to induce *tsl* mutations using ethyl methanesufonate (EMS) had been carry out for *A. ludens* without success, perhaps because the EMS-action is a rare and random event. Currently, the targeted genome editing ability through clustered regularly interspaced short palindromic repeats (CRISPR) and its associated Cas9 endonuclease, has made it possible to have an effective molecular tool for creating heritable and specific germline mutation. Interestingly, the transformer-2 gene functional only in females was edited to produce a temperature sensitive point mutation using CRISPR Cas9 technology, in order to produce male-only at restrictive temperature. Thus, the identification of orthologous genes encoded in *A. ludens* genome would allow assay their potential as targets for the control of this pest using SIT.

As an alternative, a tetracycline suppressible female-specific embryonic lethal system (FSELS) for the Mexican fruit fly was developed. In this system males and females are produced when reared on diet with tetracycline (Tet-diet) and females are eliminated when reared on tetracycline free diet (Tet-free). The FSELS works well for *Ceratitis 44apitate* and *Anastrepha suspensa*, however, in *A. ludens* adult females the system has a secondary

tissue-specific effect on developing oocytes, not observed in both species. The most straightforward explanation for the oocyte-specific sterility is an unexpected sry-a promoter pre-zygotic function in adult females, which was proven by the detection of endogenous sry-a gene expression in virgin females and males up to 10 days post-eclosion. To solve this issue and produce male-only, it was requires feeding newly eclosed adult females on a Tet-diet for up to 5 days, and subsequently transferring them to a Tet-free diet.

This project is organized in three main topics:
1). Regarding to the generic markers for *Anastrepha* species and the evaluation of the different genetic sexing systems, we propose two objectives.
a). To compare sexing systems based on pupal color (Tapachula-7 strain) vs a combination among pupal colour and self-sexing system (Tapachula/slow-7 strain) in *A. ludens*.
b). To develop a GSS black pupa colour based for *A. obliqua*.

2). Regarding to know if the alteration (mutation) of proteins involved in the embryo protection to thermal treatments can induce *tsl* mutations, we propose two objectives.
a). Investigate the gene expression in *A. ludens* embryos exposed to thermal treatments.
b). Investigate the possible induction of *tsl* mutations by CRISPR-Cas9, in genes encode proteins expressed in *A. ludens* embryos.

3). To improve the tetracycline suppressible female-specific embryonic lethal system (FSELS) in *A. ludens*, we propose two objectives.
a). Assessment the FSELS testing different embryonic promoters from embryonic genes, such as nullo and spitting image (*spt*), in *A. ludens*.
b). Assessment the FSELS in *A. obliqua*.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **Development and evaluation of genetic sexing strains of fruits flies to be used for sterile insect technique applications, as part of AW-IPM programs**.

AUTHOR (S): Cristian Morales, Edwin Ramírez

ORGANIZATION: Medfly Program – Guatemala

SHORT SUMMARY OF PAPER

Abstract:

The Mexican Fruit Fly, *Anastrepha Ludens* (Loew) and Mediterranean fruit fly, *Ceratitis capitata* (Wied.) are some of the most relevant agricultural pests due to the damage produced to many species of fruit-producing plants of agronomic importance. Control programs have been established to eradicate these pests using the Sterile Insect Technique (SIT), a combination of methods and strategies of Integrated Pest Management under the Area Wide approach with positive economic, social and environmental consequences.

The use of new strains implies a series of advantages for the control programs, which could mean a reduction of costs; however, in contrast there may be a biological cost in the ability or performance of sterile males in the field. Therefore, when trying to use these novel strains in control programs, it will be important to have data from field trials that supports the feasibility of their use. During the evaluation, it is proposed to measure performance through survival and reproduction, analysing parameters such as fertility, larval production, development rate, emergence of adults, proportion of males and sexual competitiveness. We have identified that the SIT for both species, *A. ludens* and *C. capitata*, have areas of potential improvement, thus could increase the efficiency of their mass rearing, as well as their performance in the Integrated Pest Management under the Area Wide Approach. So, we expect to find better strains that allow the release in the field of highly competitive sterile males for an adequate induction of sterility in wild populations.

We have planned to induce mutations to achieve new rearrangements in chromosomes that could generate stable strains, with high fecundity and high performance. Specifically, we will work on:

1) applied protocols for crosses and lines selection, exploring the possibility of finding selective thermal sensitivity for *A. ludens* females; 2) for *C. capitata* we will develop evaluations of strains with a shorter development time of females. We will evaluate the potential of a new medfly GSS VIENNA 8 which does not show the expression of the slow gene. This study will help understand the relevance of the slow gene, in terms of efficiency (savings in space, energy, hand labour and others). It will also open the possibility to produce a new GSS VIENNA 8 strain where males and females can be produced in all larvae collections and understand its implications in the mass rearing process.

FIRST RESEARCH COORDINATION MEETING

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

Vienna, Austria

7-11 October 2019

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, Siriwan Isasawin, 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:

Genetic sexing strain (GSS) is a key component for sustainable and effective SIT program. Male individuals of the strain can be separated from females, using sex-link traits, before irradiation and release. This action can help to reduce damage caused by females as well as cost in mass-rearing and/or in field performance. Available genetic-based techniques including 1) classical genetic sexing strain, 2) transposase mediated random integration, 3) RNA interference, and 4) genome editing using CRISPR-Cas9, were proved to apply for sex sorting or sexing.

Two *Bactrocera* spp., *B. dorsalis* and *B. carambolae*, were constructed using classical genetic approaches for the first generation of genetic sexing Salaya 1 and Salaya 5 strains, respectively. At least Salaya 1 strain could be scaled up from clean colony to the other production lines at the mass-rearing facility. This strain comprises a character that may be theoretically developed for the second generation of GSS. In the meantime, genes and/or regulatory regions involved in early embryogenesis or sex determination pathway have been studied using bioinformatics and molecular tools. This knowledge will be a part of the RNA interference as well as the conditional lethality system for construction of GSS. In addition, exploration of RNAi delivery system will be parallel done.

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

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

AUTHOR (S): Wulff, J.P.; Segura, D.F.; Rivarola, M.; Conte, C.A.; Giardini M.C.; Scannapieco, A.C.; Milla, F.H.; Crippa, G.R., Bachmann, G.E.; Cladera, J.L. and Lanzavecchia, S.B.

ORGANIZATION: Instituto de Genética "E. A. Favret" (IGEAF), Instituto Nacional de Tecnología Agropecuaria (INTA).

SHORT SUMMARY OF PAPER

Abstract:

The fruit fly *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae) is one of the most destructive quarantine pest in the South American region. It is considered a complex of cryptic species with at least eight morphotypes described. Studies of mating compatibility, genetic analysis and taxonomy support the presence of only one biological entity of this complex in Argentina, named Brazilian-1 morphotype or *A. fraterculus sp1*. Efforts to control wild populations are coordinated by governmental initiatives using toxic baits and trapping in an integrated pest management approach against *A. fraterculus*. In this context, the development of species-specific control methods such as the sterile insect technique (SIT) is strongly recommended. The generation of genetic sexing strains (GSS) has greatly improved the SIT efficiency reducing production costs by the separation of sexes at early stages of the developmental time. Current GSS systems in *Ceratitis capitata* and *Anastrepha ludens* are used in SIT strategies. In the case of *A. fraterculus*, this classical approach is currently under evaluation. We carried out several studies to perform a complete genetic evaluation *A. fraterculus sp1* present in Argentina, which includes: a characterization and purification of strains carrying karyotypic variants; analysis of the genetic variability of wild and laboratory populations using microsatellite markers recently developed in our laboratory; and a gene expression analysis based on *A. fraterculus* full transcriptome information.
Whole-genome approaches have been explored in *Bactrocera tryoni*, *C. capitata*, *B. cucurbitae* and *B. oleae* among others Tephritidae species, providing information of genes and molecular mechanisms involved in main phenotypic and behavioral traits. These data represent the bases for future studies on the development of generic tools to produce GSS employing new technologies. Transgenesis have been applied to generate sexing systems in insects. Significant advances in this area have been made with the development of transgenic sexing strains of *C. capitata*, *A. suspensa*, *A. ludens*, *Lucilia cuprina and Cochliomyia hominivorax*. The use of these new technologies in *A. fraterculus* represents a challenge and an alternative for the generation of female-lethal sexing strains or male-specific marking system in support to the development of a species-specific control method against this pest. Furthermore, the novel gene editing (*e.g.* CRISPR/Cas9) has been proven to be an effective method for creating heritable and specific germ line mutations with unlimited potential for applications on pest management strategies, including *A. fraterculus.* Therefore, our main goals of this research proposal are: 1) to characterize the *A. fraterculus sp1* genome and provide gene markers/ sex-specific genes for the development of genetic sexing strains (GGS) and, 2) to generate and evaluate *A. fraterculus* GSS for SIT implementation by using new technologies (CRISPR/Cas9, transgenesis).

FIRST RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Vienna, Austria

7-11 October 2019

TITLE OF WORKING PAPER: **X-shredder in the model and non-model insects**

AUTHOR (S): Angela Meccariello, Barbara Fasuló, Philippos A. Papathanos and 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 pests 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. A novel genetic control strategy has been generated in *Anopheles gambiae* using CRISPR/Cas9 to cut the X chromosome in multiple places during spermatogenesis. This approach, named "X-shredder", results in a male-biased sex ratio in the population over the course of few generations. Here, we show that the X-shredder can be transferred in the model and non-model insects *Drosophila melanogaster* and *Ceratitis capitata*, respectively. The aim of this project is to apply the X-shredding system to suppress pest insect populations considered to be an agricultural and economic burden.

D44003-CR-1
First Research Coordination Meeting on Generic approach for the development of genetic sexing strains for SIT applications
Vienna, Austria
7 to 11 October 2019

List of Participants

(as of 2019-09-30)

S. No.	Authority	Personal Details
1	**Argentina**	Mr Juan Pedro WULFF Instituto Nacional De Technologia Agropecuaria (INTA) Rivadavia 1439 CIUDAD AUTOMOMA DE BUENOS AIRES ARGENTINA Tel:+54 (11)44500805 3898 Email:wulff.juan@inta.gob.ar
2	**Australia**	Mr Simon BAXTER ARC Future Fellow School of Biological Sciences The University of Adelaide 5005 ADELAIDE, SA AUSTRALIA Tel: Email:simon.baxter@adelaide.edu.au
3	**Cameroon**	Mr Cyrille NDO Organisation de Coordination pour la lutte contre les endémies en Afrique Centrale (OCEAC) 2.0 Rue du Centre Pasteur P.O.Box 288 YAOUNDÉ CAMEROON Tel:+237 (22)232232 Email:cyrndo@yahoo.fr; contact@oceac.org
4	**Canada**	Mr Ioannis RAGOUSSIS McGill University and Genome Quebec Innovation Centre 740 Dr. Penfield MONTREAL CANADA Tel:+1 (514)398 6508 Email:ioannis.ragoussis@mcgill.ca
5	**China**	Mr Wei PENG China Jiliang University No. 258, Xueyuan Road 310018 HANGZHOU CHINA Tel: Email:pengwei@cjlu.edu.cn

6	**Czech Republic**	Mr Frantisek MAREC Department of Genetics; Institute of Entomology; Biology Centre ASCR Branisovska 31 370 05 CESKE BUDEJOVICE CZECH REPUBLIC Tel: Email:marec@entu.cas.cz
7	**France**	Mr Pablo TORTOSA Institut de recherche pour le développement (IRD) Representation de La Reunion, CS41095 Parc Technologique Universitaire 2 rue Joseph Wetzell 97495 SAINTE CLOTILDE CEDEX FRANCE Tel:+262 (262)483350 Email:pablo.tortosa@univ-reunion.fr
8	**Germany**	Mr Hassan AHMED Justus-von-Liebig-Weg 11 Department of Developmental Biology AG Wimmer 2 OG 37077 GOETTINGEN LOWER SAXONY GERMANY Tel:+49 551 39 7037 Email:hmutasi@biologie.uni-goettingen.de
9	**Germany**	Ms Roswitha AUMANN Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) Project Group "Bioresources" Winchesterstrasse 2 35394 GIESSEN GERMANY Tel: Email:roswitha.aumann@agrar.uni-giessen.de
10	**Germany**	Mr Marc SCHETELIG Institute for Insect Biotechnology Winchesterstrasse 2 GIESSEN GERMANY Tel:+49 (641)9939504 Email:marc.schetelig@agrar.uni-giessen.de

11	**Germany**	Mr Germano SOLLAZZO Justus-Liebig-University Giessen Institute for Insect Biotechnology Winchesterstr. 2 35394 GIESSEN GERMANY Email:germano.sollazzo@gmail.com
12	**Greece**	Mr Antonios AVGOUSTINOS Institute of Industrial and Forage Crops Hellenic Agricultural Organization DEMETER Department of Plant Protection P.O Box 5099 26442 PATRAS GREECE Tel: Email: antoniosaugustinos@gmail.com
13	**Greece**	Mr Kostas MATHIOPOULOS Department of Biochemistry and Biotechnology University of Thessaly Ploutonos 26 LARISSA GREECE Tel:+30 2410-565284 Email:kmathiop@bio.uth.gr
14	**Greece**	Ms Konstantina TSOUMANI Department of Biochemistry and Biotechnology University of Thessaly Ploutonos 26 LARISSA GREECE Tel: Email:ktsoumani@hotmail.com
15	**Guatemala**	Mr Edwin Mauricio RAMIREZ SANTOS Medfly Program Guatemala 16 Calle 3-38 Zona 10 01010 GUATEMALA GUATEMALA Tel:+502 6631 7826 Email:eramireztoo@gmail.com

16	**Israel**	Mr Yehonatan ALCALAY Department of Entomology of the Robert H. Smith Faculty of Agriculture Food and Environment Hebrew Univerisity REHOVOT ISRAEL Tel: Email:yehonatanal@mail.huji.ac.il
17	**Israel**	Mr Philippos PAPATHANOS Department of Entomology of the Robert H. Smith Faculty of Agriculture Food and Environment Hebrew Univerisity REHOVOT ISRAEL Tel: Email:p.papathanos@gmail.com
18	**Italy**	Mr Pasquale PRIMO via Napoli, 52 FRATTAMINORE ITALY Tel: Email:P.Primo@iaea.org
19	**Italy**	Mr Giuseppe SACCONE Department of Biology University of Naples Federico II Complesso Universitario di Monte S. Angelo Via Cinthia Building 7, room 0F-01 80126 NAPOLI ITALY Tel: Email:giuseppe.saccone@unina.it
20	**Italy**	Mr Luigi VITAGLIANO University of Sannio BENEVENTO ITALY Tel: Email:luigi.vitagliano@unina.it

21	**Mexico**	Mr Jose Salvador MEZA HERNANDEZ Subdirector de Sexado Génetico, Programa Moscafrut Acuerdo SAGARPA-IICA Camino a los cacahotales s/n. C.P. 30860 TAPACHULA CHIAPAS MEXICO Tel:0054 964 6435030 Email:jose.meza@iica-moscafrut.org.mx
22	**Netherlands**	Mr Leo BEUKEBOOM University of Groningen Institute of Evolutionary Life Sciences P.O. Box 11103 9700 CC GRONINGEN NETHERLANDS Tel: Email:l.w.beukeboom@rug.nl
23	**Switzerland**	Mr Daniel BOPP Institute of Molecular Life Sciences University of Zurich Winterthurerstrasse 190 8057 ZURICH SWITZERLAND Tel:+41 (44)63548691 Email:daniel.bopp@imls.uzh.ch
24	**Thailand**	Ms Nidchaya AKETARAWONG Department of Biotechnology; Faculty of Science; Mahidol University 272 Rama VI, Ratchathewee BANGKOK THAILAND Tel: Email:nidchaya.akt@mahidol.ac.th
25	**Thailand**	Kamoltip LAOHAKIEAT Department of Biotechnology, Faculty of Science, Mahidol University 272 Rama VI Road, Phayathai, Ratchathewee 10400 BANGKOK THAILAND Tel:+66 2 201 5310 Email:kamoltip.lao@gmail.com
26	**Thailand**	Mr Sujinda THANAPHUM Department of Boitechnology, Faculty of Science, Mahidol University 272 Rama VI Road 10400 BANGKOK RATCHATHEWE THAILAND Tel:+66 +662-880-2673 (H) or +668-1433-3963 (M) Email:sujinda.tha@mahidol.ac.th
27	**United Kingdom**	Mr Alistair DARBY Functional and Comparative Genomics University of Liverpool LIVERPOOL UNITED KINGDOM Tel: Email:acdarby@liv.ac.uk; Alistair.Darby@liverpool.ac.uk

28	**United Kingdom**	Mr Jaroslaw KRZYWINSKI The Pirbright Institute Ash Road, Pirbright GU24 0NF WOKING UNITED KINGDOM Tel: Email:jaroslaw.krzywinski@pirbright.ac.uk
29	**United Kingdom**	Ms Angela MECCARIELLO Imperial College Road SW72AZ LONDON UNITED KINGDOM Tel: Email:meccarielloangela@gmail.com
30	**United Kingdom**	Mr Mark Andrew WHITEHEAD University of Liverpool Paddockwray, Burton CHESHIRE UNITED KINGDOM Tel: Email:hlmwhite@student.liverpool.ac.uk
31	**United States of America**	Mr Alfred M. HANDLER USDA, ARS, Center for Medical, Agricultural and Veterinary Entolomology 1700 SW 23rd Drive Gainesville, Florida UNITED STATES OF AMERICA Tel: Email:al.handler@usda.gov
32	**United States of America**	Mr Max SCOTT North Carolina State University 2500 Stinson Dr. RALEIGH NC UNITED STATES OF AMERICA Tel: Email:mjscott3@ncsu.edu
33	**United States of America**	Mr Zhijian TU 303 Fralin Virginia Tech BLACKSBURG UNITED STATES OF AMERICA Tel: Email:jaketu@vt.edu