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

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

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

(a) **traits** such as white pupae, black pupae (ebony), temperature-sensitive lethal (tsl), slow development (sd), slow larvae, white eye, red eye, and yellow have been identified as suitable to be used for generic strategies for the construction of GSS. The genes responsible for most of these traits have been isolated and/or exploited as markers (*white pupae*, *black pupae* (*ebony*), *dor*, *tsl*, *white eye*, *cardinal*, *yellow*). Additionally, it has been suggested that genes related to salinity tolerance, desiccation/UV tolerance, auxotrophy, insecticide resistance, and phototaxis may be worth exploring. An auxotrophic mutation known from *Drosophila melanogaster* is being evaluated as a generic selectable marker for GSS development in *Aedes aegypti*;

(b) with respect to the development of **generic approaches** for the construction of GSS for SIT-targeted agricultural pests and human disease vectors, a number of **mutant strains with visual markers** have been established including white pupae strains in *Bactrocera correcta*, *B. oleae*, *B. tryoni*, *Zeugodacus tau* and (new strains for) *Ceratitis capitata* and *B. dorsalis*; black pupae (most likely ebony) strains in *A. fraterculus*, *A. ludens*, *B. tryoni* and *D. suzukii*, while black pupae strains are already available in *Anastrepha fraterculus*, *A. ludens*, and *A. obliqua*; red-eye (cardinal) strains in *Aedes aegypti* (new strains were also developed), while such strains are already available in *Anopheles gambiae, Culex pipiens, Ae. albopictus*, and *Plutella xylostella*; yellow strains in *Ae. albopictus and B. tryoni*, while such strains are already available in *Spodoptera litura (yellow-y);*

(c) three genes were identified as being able to induce **temperature-sensitive lethal phenotypes** and novel *C. capitata* lines with a temperature-sensitive phenotype have been developed. Known *Drosophila* temperature-sensitive lethal mutations were evaluated in *Bactrocera tryoni*. In *An. arabiensis*, EMS-mutagenesis-induced temperature-sensitive strains have been isolated and are currently characterized;

(d) knock-in of a **fluorescent marker protein** on the Y-chromosome of *C. capitata* was attempted by CRISPR-based HDR, but a fluorescent strain was lost after two generations. In codling moths, insertion of a dominant conditional lethal gene into the female-specific W chromosome was successfully achieved. Transgenes have been integrated randomly near the M loci of both *Aedes aegypti* and *Aedes albopictus* using the *piggyBac* transposons;

(e) since the **transfer of genes to sex-determining chromosomes or loci** remains challenging, an inverse strategy is being developed to create artificial male-determining loci and connect them to selective markers;

(f) in parallel, the **sequencing and characterization** of several insect (*A. fraterculus* sp.1, *A. ludens*, *B. dorsalis*, *B. zonata*, *C. capitata*, and *Z. cucurbitae*) genomes and transcriptomes is ongoing, aiming to facilitate the isolation of selectable markers and to identify suitable regions for knock-ins on Y chromosomes of SIT targeted

species. For this purpose, two complementary computational methods for the identification of Y-specific regions in complete genome assemblies and the dynamic evaluation of Y sequence quality and completeness during consecutive assembly steps have successfully been developed. In addition, the improved genome annotations, in combination with advanced transcriptomics have enabled the identification of sex-determining factors in several SIT target species;

(g) new *C. capitata* GSS were developed based on an X-autosome translocation, and quality control analysis indicated that they do not differ from the Y-autosome translocation lines. In addition, this system offers an easy, fast, and generic approach for the genetic refreshment of mass-reared colonies. Quality control analysis was performed on GSS constructed with the newly established medfly *white pupae* mutant lines, and no difference was observed when compared with the original ones, and

(h) a *C. capitata* cage population of XX-only flies (lacking the Y chromosome) having an artificial male determining factor has been established and maintained for twelve generations to further investigate XX fertile male individuals and a masculinization strategy toward the construction of novel GSS.

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 that are considered major pests to agriculture, livestock or human health, 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 the application of SIT continue to increase from FAO/IAEA Member States (MS). Programme efficiency, cost-effectiveness, as well as safety and biosecurity, depend 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: *A. fraterculus* (species complex), *A. grandis, Anastrepha ludens, A. obliqua, A. suspensa, Bactrocera carambolae, B. correcta, B. dorsalis, B. jarvisi, B. oleae, B. tryoni, 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, Diatraea crambidoides, D. saccharalis, Ectomyelois ceratoniae, Grapholita molesta, 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 an 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 which governmental or regional mandates may restrict.

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 efficiency of the SIT in the field and showed that other insect pests could benefit from it. Thus, there is a widely recognized need for the development of sexing systems for SIT programs of other species. In addition, this is a prerequisite for mosquito SIT since females are the transmitting sex of major human pathogens.

2.1. Tephritids: The VIENNA-8 (and VIENNA-7) GSS of *C. capitata* carry the *white pupae* (*wp*), *slow development* (*sd*) (Porras et al., 2020) and *temperature-sensitive lethal* (*tsl*) mutations as well as a Y-autosome translocation that includes wild-type functional copies of these genes. Via an embryonic elevation in temperature, females can be eliminated in an early stage of development (Franz et al. 2021; Augustinos et al. 2017). GSS have been developed for *B. dorsalis* (McCombs & Saul 1995; Isasawin et al., 2012), *B. carambolae* (Isasawin et al., 2014), and *Z. cucurbitae* (McInnis et al., 2004) based on *white pupae* mutations. Similarly, GSS for *A. fraterculus*, *A. ludens* and *A. obliqua* have been developed based on a *black pupae* mutation (Zepeda et al., 2014; Meza et al., 2020). These pupal colour 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 (Fu et al., 2007). Similar to the colour-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 *Cctra*-RNAi transgenic sex-reversion line of *C. capitata* (Saccone et al., 2007) that showed in the last 4 years 100% conversion of XX individuals into fertile XX males (with 50% nontransgenic) was generated, showing a masculinizing maternal effect with dsRNA produced during oogenesis. This transgenic line has been stably maintained for more than five years. The transgene acting as a synthetic maternal masculinizer (MatM) led to a stable cage population of *C. capitata* in which the Y chromosome was excluded. In this population, transgenic XX females (MatM/-) produce only XX males, while non-transgenic XX females (-/-) produce XX female-only progeny. Alternatively, the positive *tra* autoloop can be targeted in the female germline to generate ¨arrhenogenic¨ females, which produce only-male progeny. Many higher dipterans depend on maternal provision of *tra* to engage the *tra* autoloop in the zygote. Depletion of maternal *tra* will prevent zygotic activation of *tra* and male development will follow. Prezygotic repression of maternal *tra* has been observed and documented in the housefly *M. domestica*. For instance, the *Ag* mutation in *M. domestica* acts as a dominant *tra* loop breaker in the female germ line and gives rise to male only progeny (Hediger et al., 2010). Also, transplantations of M carrying germ cells into a female host will give rise to only-male progeny demonstrating that expression of M in the female germline can be used to pre-zygotically block the female promoting activities of *tra* (Dübendorfer and Hediger, 1998 doi: 10.1093/genetics/150.1.221.). In other tephritid species like *A. suspensa*, the transient dsRNA knock-out of *tra* and *tra2* did lead to 98% female sex-reversion, but while those XX males had the advantage of being sterile, their *dstra-2* treated XY siblings were fertile (Schetelig et al., 2012). Thus far *tra-2* function is required for male fertility in XY drosophilids, including the *D. suzukii* pest species, but not in tephritid species that would require an independent male sterility system when *tra-2* suppression is used for sexing.

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 (Kojin et al., 2022). This places unique constraints on any efforts to optimize SIT for mosquitoes, as GSS would significantly contribute to the elimination of females, ideally at any early developmental stage. 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. A GSS for *An. arabiensis* was initially developed by IPCL researchers, utilizing dieldrin resistance (Yamada, 2012). This work was further advanced by a team in South Africa, who, using the IPCL GSS in combination with a local strain of the same species, successfully created a dieldrin-based GSS with a South African indigenous background through a series of backcrosses and outcrosses (Dandalo et al., 2018). However, concerns about low fertility rates, the potential for dieldrin residues in adult males, and the risk of environmental bioaccumulation, led to reservations about the feasibility of using these strains for large-scale operational purposes (Yamada et al., 2015). Despite these challenges, this approach was later applied to develop one of the first GSS for *Ae. albopictus* by linking the rdlR gene, which confers resistance to dieldrin, to the male sex determination gene (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: (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; Lutrat et al., 2022; Ntoyi et al., 2022); (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: Sex conversion approaches have been successfully developed in mosquitoes using the male-determining factor Nix (Aryan et al., 2020; Lutrat et al., 2022; Zhao et al., 2022). 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, by replacing females with converted males. Recently, the targeting of the *A. gambiae* femaleless (*fle*) gene, related to Tra2 splicing factor, led to masculinization and death of genotypic females showing its involvement in female sex determination and dosage compensation (Krzywinska, et al., 2021). The authors showed that *fle* is an evolutionary conserved gene in anophelines and proposed that it could be used as a "universal molecule", could be targeted in genetic control to eliminate females of all malaria vector species. Fle appears to be related to Nix, which has recently been shown to be widely distributed among mosquitoes of the subfamily Culicinae (Biedler et al., 2024). The master regulator of *Anopheles* dosage compensation, 007/SOA, has recently been identified and represents an additional potential target for female lethal GSS (Kalita et al., 2023, Kryzwinska et al., 2023).

2.3. Lepidoptera: The available sexing mechanisms developed for Lepidoptera have been based either on the construction of balanced lethal (BL) strains or W-linked selectable markers. Unfortunately, the use of BL strains for genetic sexing is not easily applicable under mass rearing conditions. Suitable W-linked markers are only available for *Bombyx mori* (Marec and Vreysen, 2019). A GSS with a W-linked dominant conditional lethal mutation (DCLM) would permit the maintenance of both sexes under permissive conditions and the elimination of the female moths under restrictive conditions. However, to date, no DCLM has been identified in Lepidoptera. Alternatively, modern biotechnology methods could be used to introduce a DCLM into the W chromosome. An advantage of this approach is that only female progeny will have the transgene, but not the released males, which will have a fully wild-type genome (Marec et al., 2005). Recently, successful insertion of the DsRed fluorescent marker into the W chromosome of *B. mori* has been achieved using piggy*Bac*-mediated transgenesis, demonstrating that this approach is feasible (Ye et al., 2023). Using another strategy, transgenic sexing strains of *B. mori* and pink bollworm have been made that overexpress tTA in females when raised in the absence of tetracycline in the diet. Sex-specific expression was achieved by using the splicing signals from the pink bollworm *doublesex* (*dsx)* gene (Jin et al., 2013; Tan et al., 2013).

3. Sex determination: Knowledge on sex determination pathways of the SIT-targeted insect species can be very useful for the construction of a GSS (Saccone, 2022). Sex determination is well characterised in *Drosophila melanogaster*, in which two doses of a set of X-linked transcriptional regulators activate the master gene *Sex-lethal* (*Sxl*), which determines the female fate in XX embryos and represses dosage compensation. In XY embryos (males), which contain only one dose of X-linked transcriptional activators, *Sxl* remains inactive. In *Ceratitis capitata*, the *tra/tra-2 > dsx/fru* module of this sex determination pathway is conserved at the structural and functional level (Pane et al., 2002; Bopp et al., 2014). However, the *Sxl* homolog in *C. capitata* (*CcSxl*) is not acting as the upstream regulator of the *tra* homolog (*Cctra*) (Saccone et al., 1998; Zhang et al., 2014). Instead, activation of *Cctra* functional gene products require the presence of maternal *Cctra*, that acts together with the *Cctra-2* gene product (Salvemini et al., 2009), to maintain the epigenetic autoregulatory function resulting in female sexual differentiation. When *Cctra* female activation is prevented by the male determiner (M factor), or artificially by dsRNA, male sex determination and male differentiation results (Pane et al., 2002). This mode of *tra* autoregulation and its embryonic RNAi sensitivity appears to be widely conserved in many other Tephritidae, including *Bactrocera oleae*, *B. tryoni*, *B. jarvisi*, *B. dorsalis*, *B. correcta*, *Anastrepha suspensa*, the Calliphoridae, *Lucilia cuprina*, and the Muscidae, *Musca domestica* (Hediger et al., 2010; Sanchez, 2008; Nagaraju J, and Saccone G., 2010, Laohakieat et al., 2016, 2020; Saccone, 2022). Inactive Cas9 targeting Medfly *tra* without inducing a mutation led to full masculinization indicating that transient repression of *Cctra* transcription during early embryogenesis also affects the establishment of the female-determining *Cctra* autoregulatory loop (Primo et al., 2020). In *L. cuprina*, knock-down of *tra* by Cas9 also interferes with dosage compensation, an effect that could be used for female killing (Williamson et al., 2021).

One of the several M factors present in wild populations of *M. domestica*, *Mdmd*, has been isolated (Sharma et al., 2017). The *Mdmd* gene originated from a duplication of a highly conserved autosomal gene, CWC22, encoding a spliceosome protein, suggesting that *Mdmd* has a direct role in repressing female-specific *Mdtra* splicing. The male-determining factor in *C. capitata* has been previously mapped on the long arm of the Y chromosome (Willhoeft and Franz, 1996). The M factor has been molecularly isolated in the Medfly, named *Maleness-on-the-Y* (*MoY*) and found to induce male-specific splicing of *Cctra* within hours during embryogenesis of XY (Meccariello et al., 2019). *MoY* orthologues are 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*. Interestingly, *MoY* DNA injected into *M. domestica* XX embryos changes the splicing of the Musca *tra* gene, suggesting that *MoY* can act beyond the species barrier. Hence, *MoY* is a promising tool for a tephritid generic transgenic strain sexing by masculinization of XX individuals (Meccariello et al., 2019).

A novel molecular mechanism for male sex determination was revealed in *B. dorsalis* (Peng et al., 2020). An autosomal miRNA, miR-1-3p, showed XY-biased expression in early embryos, and targeting *Bdtra* mRNA led to its male-specific splicing pattern and male sex determination. The male-biased expression of miR-1-3p is likely to be under the direct or indirect control of *BdMoY*. RNA interference as a natural sex determination mechanism has been described only in lepidopteran species (Kiuchi et al., 2014). Still, it is a novelty for dipteran species and its potential evolutionary conservation should be explored.

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 and Ae. albopictus* (Aryan et al., 2020, Hall et al., 2015, Gomulski et al., 2018, Liu et al., 2020, Lutrat et al., 2022. Nix homologs have also been identified in 12 other mosquito species; while most are male-specific, their potential role as a primary sex-determiner has yet to be established (Biedler et al., 2024). *AsuMf*, a male-specific duplication of an autosomal splicing factor in *Armigeres subalbatus*, is required for male development (Liu et al., 2023). In these mosquito species, downstream genes such as *dsx* and *fruitless* (*fru)* have been identified and are regulated by a partially conserved sex-specific alternative splicing mechanism (Scali et al., 2005; Gailey et al., 2006; Salvemini et al., 2011; Salvemini et al., 2013). At the same time, a *transformer* homolog in both species is either absent or remains to be identified. No genetic information is available for the upstream splicing regulators of the *dsx* and *fru* genes, 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). In addition to the sex-determining function, *Masc* also induces dosage compensation (Tomihara et al., 2022). Several recent studies suggest that the role of *Masc* is conserved in Lepidoptera sex determination (Lee et al., 2015; Fukui et al., 2018; Wang et al., 2019; Harvey-Samuel et al., 2020; Deng et al., 2021; Visser et al., 2021; Pospíšilová et al., 2023; Li et al., 2024; Moronuki et al., 2025). However, it is not yet known whether the Fem piRNA-*Masc* sex-determining pathway is conserved in other lepidopteran species having WZ sex determination. For example, deep sequencing analysis identified no female-specific small RNA that mapped onto the *Masc* mRNA in *Ostrinia furnacalis* and Trilocha varians (Fukui et al., 2023; Lee et al., 2024). Recently, a W-linked locus that is a source of small silencing RNAs targeting the *Masc* sequence has been identified in *P. xylostella* (Harvey-Samuel et al., 2022) and Lymantria dispar (Moronuki et al., 2025), suggesting convergent evolution of a *Fem*/*Masc* pathway in this phylogenetically distant species. On the other hand, the presence or absence of the W chromosome plays no role in sex determination of wild silkmoths (*Samia cynthia* ssp.), and their sexual development depends on the Z:A (A = autosome) ratio (Yoshido and Marec, 2023). Similarly, the W chromosome plays no role in sex determination of the butterfly *Bicyclus anynana*, where WZ embryos with a single *BaMasc* copy develop into females, ZZ embryos with 2 different *BaMasc* copies (heterozygotes) develop into males and ZZ embryos homozygous for *BaMasc* die due to failure of dosage compensation. In this butterfly, *BaMasc* itself is thus the primary sex-determining switch that does not require any upstream factor (Van't Hof et al., 2024).

In Hymenoptera, the core *tra*-splicing-autoloop is started by very different means, such as heterozygosity at a complementary sex determining locus in *Apis mellifera* (Gempe et al., 2009) or a transcriptional activator with a parent-of-origin effect in *Nasonia vitripennis* (Zou et al., 2020). The insect sex determination pathway based on sex-specific splicing of *tra* and *dsx* seems ancestral to the Holometabola (Wexler et al., 2019), whereby *tra* serves as transducer of the primary signal and *dsx* as executer for sex determination. However, the primary signal differs widely (Hopkins and Kopp, 2021).

Wexler et al. (2019) isolated *dsx* orthologues in species belonging to three hemimetabolous insect orders, including Hemiptera and Blattodea, and found *dsx* sex-specific splicing regulation in two of them. Interestingly, these orders include major pests such as *Blattella germanica* (L.), and the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), which negatively affect several vegetable crops that are grown in greenhouses. Furthermore, hemipteran species, such as the brown marmorated stink bug *Halyomorpha halys*, a public health pest worldwide, and the green vegetable bug *Nezara viridula*, have been considered as potential candidates for SIT (Bourtzis and Vreysen, 2021).

Recent advances in the Hemiptera genetics of sex determination revealed that nymphal RNAi of the brown planthopper (BPH) *Nilaparvata lugens* - one of the most devastating rice pests in many Asian countries - *tra2* orthologue (*Nltra2*) led to a phenotypic sexual reversal of females into sterile males likely by controlling *Nldsx* splicing (Zhuo et al., 2017; idem, 2018). Zhuo et al. (2021) identified two novel splicing regulators of *Nldsx* in this species, named *Nlfmd* and *Nlfmd2* (female determinant factors), belonging to the hnRNP40 family gene. interestingly, *Nlfmd* produces female-specific and non-sex-specific isoforms via alternative splicing by exon skipping. Female-specific NlFmd is 613 aa long, and the non-sex-specific form is shorter (449 aa). *NlFmd* shows very low similarity to the hymenopteran protein *A. mellifera* Feminizer (AmFem) over a very short region, conserved among *AmFem* and *CcTra* (Hasselmann et al., 2008). Knockdown of *Nlfmd* in female nymphs resulted in masculinization of somatic morphology and *dsx* splicing. The female-specific isoform of *Nlfmd*, *Nlfmd-F*, is maternally deposited and zygotically transcribed. Depletion of *Nlfmd* by maternal RNAi or CRISPR-Cas9 resulted in female-specific embryonic lethality. Knockdown of *Nlfmd2*, also conferred masculinization. Like TRA, the NlFMD protein is an SR-rich protein that does not have a predicted RNA binding domain. The authors suggested that LlTRA2 interacts with NlFMD/TRA like in *Drosophila melanogaster* and possibly other dipteran species.

4. Recent developments:

4.1. The applications of 'big data' for molecular genetics: The community can overcome major bottlenecks in research by the application of next-generation sequencing (NGS) technologies to genetic problems. The available technologies have several 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 by Chromium 10X Genomics (now discontinued) or alternatively TELL-seq and genome scaffolding technologies such as 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 can also 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 and comprehensively analyze early embryo transcription as demonstrated in the olive fly (Bayega et al 2021 (PMID: 33846393). Taken together, these technologies with bioinformatic analysis allow us to produce a wealth of ancillary data that play an increasingly prominent role in the identification of target (marker) genes, including their mode of regulation. An example of these recent developments, both in terms of sequencing chemistry and in its bioinformatic analysis, has been the discovery and subsequent characterization of Y-chromosome sequences, including Y-linked M factors in mosquitoes, *Ceratitis capitata* (*MoY*; Meccariello et al., 2019), *Bactrocera oleae* (Bayega et al., 2020) and in *Musca domestica* (*Mdmd*; Sharma et al., 2017; Li et al., 2024). 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 employed to molecularly identify the loci responsible for many of the GSS-based mutations described above. In addition, a marker-assisted mapping approach was successfully used to help identify the causal genes of both dominant and recessive phenotypes in *Aedes aegypti*, overcoming the significant challenge posed by vast recombination deserts in this species (Chen et al., 2022). Therefore, 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 an inversion that was introduced to reduce recombination. Cytogenetic methods were used to determine the origin and size of the translocated segment, localize translocation breakpoints or map the extent of inversions, which is critical for the stability and fitness of the strains (Franz, 2002). The identification of breakpoints and delimitation of inversions was facilitated by polytene chromosome maps available in most tephritid pests (Stratikopoulos et al., 2008; Drosopoulou et al., 2014). In Lepidoptera with small and numerous holokinetic chromosomes, specific patterns of longer meiotic bivalents in pachytene allowed the identification of sex chromosomes and characterization of radiation-induced chromosome rearrangements (Traut et al., 2007). Cytogenetic research has been greatly accelerated using advanced tools of molecular cytogenetics that are currently available for detailed analysis of insect chromosomes. Various modifications of fluorescence *in situ* hybridization (FISH), such as FISH mapping of repetitive sequences and multigene families (e.g. rDNA and histone genes), genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH) were used for the identification of sex-determining regions to which selectable markers should be linked and for the characterization of DNA content of the Y or W chromosomes (Willhoeft and Franz, 1996; Willhoeft et al., 1998; Fuková et al., 2005), which was relevant to the GSS stability and provided useful data in species with poorly understood karyotypes (Nguyen et al., 2013;

Šíchová et al., 2013). Recent advances in insect genomics has led to the development of new molecular cytogenetic methods required for the construction of high-resolution physical maps, such as BAC-FISH (FISH with bacterial artificial chromosomes as probes) and TSA-FISH (FISH with tyramide signal amplification), which represent an important framework for improving the quality of genome assembly, annotation, and analysis (Nguyen et al., 2013; Carabajal Paladino et al., 2014; Yoshido et al., 2015).

4.3. Genome editing - new tools for modifying genotypes: Genome editing allows the precise modification of genomic DNA sequences *in vivo* and can be achieved using three available technologies – Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). These technologies can be used to induce DNA double-strand breaks (DSBs) at predetermined target locations in the genome. In the case of ZFNs and TALENs, DNA endonuclease domains are attached to proteins whose amino acid sequences have been designed to bind to specific sequences. In the case of CRISPR, the Cas9 protein (or equivalent ones like CPF1) is directed to cut predetermined target locations in the genome by providing 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, Ward et al. 2021). Directed knock-in lines can also be produced by NHEJ (Farnworth et al., 2020). RNA-guided *piggyBac* transposition, which has been successfully employed in human cells (Hew et al., 2019; Rezazade Bazaz et al., 2022), could also be trialled. 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 the '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 enable 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: 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 bind and degrade complementary mRNA sequences. In plants and some invertebrates (e.g. *C. elegans*), the efficacy of RNAi is improved through a combination of signal amplification and systemic spread, such that the entry of one dsRNA or siRNA molecule into a single cell can lead to effective silencing of the target gene throughout the target organism. In most insects, RNAi appears to be cell-autonomous, with no amplification or cell to cell transfer of the gene silencing signal. The lack of a mechanism for amplification and systemic spread of a dsRNA signal in fruit flies and mosquitoes has implications for the development of RNAi as a control tool for insect pests. To achieve effective control, dsRNA/siRNA delivered through the environment (environmental RNAi, eRNAi; Ivashuta et al., 2015) of the pest must somehow be delivered to the appropriate tissue in the target pest at a sufficient dose to produce the necessary level of gene silencing to achieve the desired objective, usually mortality. There is a considerable variation across insect species in their sensitivity to eRNAi, and the evidence to date suggests that this is largely due to the relative uptake, stability, and transport efficiency of dsRNA or siRNA among insects (Ivashuta et al., 2015; Mamta and Rajam, 2017). The effectiveness of eRNAi could be improved by technologies that provide (a) more effective transport across the integument (cuticle or gut), (b) greater protection against degradation by

UV and enzymes, and/or (c) active transport to the target tissues. Microorganisms constitute one of methods for dsRNA delivery in insects. This system was initially utilized in *C. elegans* (Timmons and Fire 1998; Timmons et al., 2001) but has since been extensively applied to insects as well. Viruses are extremely efficient at delivering nucleic acid material into the intracellular environment; however, the *in vivo* application by viruses has not been widely investigated yet, probably due to the many safety issues that accompany their delivery (Kolliopoulou et al., 2017). Nanoparticles have also been used to increase stability and oral uptake efficiency of dsRNA in mosquitoes (Zhang et al., 2010). Liposomes have also been used as a means to protect nucleic acids in aqueous environments, and they were initially tested in various drosophilid species (Whyard et al., 2009). Carrier proteins (Cell-Penetrating Peptides, CPPs) have also been used as delivery systems for dsRNA and have been shown to facilitate the 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.

Feeding larvae of Lepidoptera, Coleoptera, and Hemiptera with *v-ATPase*-specific dsRNAs targeting subunits (e.g., V-ATPase A, B, D, E, H) caused 40–100% mortality. In *Anastrepha fraterculus*, soaking larvae in dsRNA (500 ng/μL) targeting a VMA21 homolog increased mortality from 15% (control) to 25%. For Tephritidae larvae inside fruit, delivery requires transgenic plants. Adult dsRNA feeding is effective, e.g., targeting *BtvATPaseA* in *Bemisia tabaci* (whitefly) caused 97% mortality in six days. Suppressing gut dsRNA nucleases enhances efficacy; in *Bactrocera tryoni*, co-feeding dsRNAs reduced *yellow* mRNA more than *yellow* dsRNA alone (Tayler et al. 2019). In *Ceratitis capitata*, circular dsRNA injection (500 ng) caused ~70% mortality and 50–60% gene silencing, while oral delivery (1.5 μg/fly) caused 15% mortality but reduced mRNA by 48% (Ortola et al. 2024).

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 several 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 for developing a 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), or combinations thereof. 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, among other things like chromosomal complement. For example, it may be possible to transfer sex determination-based GSS components among tephritid species but not to mosquitoes. Or overexpression a sex determination gene may result in sex conversion in one species but be lethal in another - results that are unknowable a priori. In most cases however, these 'generic' approaches for 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 or sex reversal have been successfully developed using several 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 eliminates the need for chromosomal translocations since wild-type alleles can more simply be transposed onto Y-chromosomes for male selection. Temperature-sensitive alleles (e.g. *transformer-2-ts2*) have also been used to generate a population of CRISPR-mutants in *D. suzukii* that, when reared at non-permissive temperatures, result in XY males and XX intersexes that are sterile (Li & Handler 2017). These males are capable of mating, while the intersexes neither mate nor oviposit.

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 necessarily include the introduction of foreign DNA, although initial efforts may include such sequences to aid in strain isolation and validation, for example, using fluorescent markers, which can be omitted in the future by systems such as recombinase-mediated cassette exchange (RMCE) (Schetelig et al., 2019; Horn and Handler, 2005). The success of this approach will depend on the identification of genes underlying suitable selectable traits in target species, how generic specific alleles of these genes will be, and to what extent they are applicable across species targeted for GSS strain development. Its feasibility has now been successfully demonstrated by members of this RCM through the identification of the *white pupae* gene in Medfly, *B. dorsalis* and *Z. cucurbitae*, the creation of a novel *white pupae* phenotype strain in *B. tryoni* (Ward et al., 2021) and the successful generation of mini white pupae rescue constructs that completely recover the mutant background. Conditional lethal mutations, including several *tsl* genes, have now been identified in the Medfly as reported from partners in RCM3 to enable sexing at the embryonic stage. These *tsl* genes are highly conserved in all target insects, so the next step is to induce identical mutations in *tsl* orthologs in these target species and evaluate how generic the transfer of these specific alleles will be. Additionally, rational engineering of conserved *tsl* genes, including those discovered in model species like Drosophila (*shibire*) or yeast (ubiquitin-conjugating enzymes) are being evaluated by partners as another direction to generate GSS with promising results presented in RCM3.

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 the approach for transfer to other SIT target species. This approach is potentially generic assuming that all insects have an RNAi system and the *dsx* gene is expected to be present and to have the same role in sex determination in all targeted insect species. This would make it a good target for gene silencing. A notable advantage of this approach is that a specially designed GSS may not be required. Diet-mediated delivery of dsRNA could also be a widely applicable mode of delivery although the sensitivity of insects to orally delivered dsRNA is variable (Darrington et al., 2017). Unfortunately, efforts to replicate experimental sex-specific lethality in mosquitoes

have failed in most labs that have attempted to implement orally delivered dsRNA-based sex separation. Attempts from different labs to reproduce already published RNAi-based lethal effects lead by silencing of essential genes to *Aedes* mosquitoes through oral delivery of bacterially produced dsRNA, soaking and microinjections of different larval stages have also not been successful. The reasons for this remain unclear and are thought to include low oral uptake of the dsRNA, issues in delivery of the dsRNAs in the relevant cells/tissues, and ultimately the production of high quality/amount of dsRNAs. Although some partners elected to include this approach at the beginning of this CRP program, unreproducible effects and results in mosquitoes have led all groups to abandon this approach for mosquitoes for the time being (Prates et al. 2024).
Initial studies in *Tephritidae* reported some lethality in *Bactrocera tryoni* and *Ceratitis capitata* following dsRNA treatments (Tayler et al., 2019; Ortolà et al., 2024). A subsequent study in *C. capitata* combined dsRNAs targeting an ATPase gene and two intestinal dsRNA nucleases, reducing dsRNA degradation and achieving 79% mortality after seven days (Volpe et al., 2024). These findings highlight the potential of oral RNAi as a pesticide and pave the way for delivering dsRNAs to the body and ovaries. Ongoing efforts aim to suppress *Cctra* in ovaries, producing masculinized XX progeny and male-only offspring.

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 common sex determination genes and effector genes involved in conserved cell-death pathways. While some of these functional elements are known to be functional between species, it is expected that for most species these specific functional elements will need to be re-isolated and assembled. While orthologous genes and regulatory sequences might be found in more distantly related species, identifying, isolating, assembling and integrating new transgenes into new species may be difficult and time-consuming. Nevertheless, conserved elements of the sex-determining splicing-cascade, such as *tra* or *tra-2*, can be targeted to generate artificially designed "M factors" by RNA interference and dCas9 mediated gene knock down (Pane et al., 2002; Primo et al., 2020) or CRISPR/Cas9 mediated mutagenesis (Aumann et al., 2020; Williamson et al., 2021), which could be made conditional by controlled activation based on site-specific recombination or food supplement-controlled binary expression systems (Eckermann et al., 2014). TRA and TRA-2 proteins from *C. capitata* interact in a yeast two-hybrid assay, supporting a mechanism of female sex determination similar to that described in *Drosophila* (Pane et al., 2002; Pane et al., 2005; Perrotta et al., 2023).

Moreover, tetracycline-suppressible female-specific embryonic lethality systems have been shown to be highly efficient in producing male-only populations in *A. ludens*, *A. suspensa*, *Ceratitis capitata*, *L. cuprina*, and *C. hominivorax* using a female-specifically spliced intron from *tra* (Schetelig and Handler, 2012; Ogaugwu et al., 2013; Schetelig et al., 2016; Yan & Scott 2015; Concha et al., 2016). In addition, a highly conserved dominant temperature-sensitive (DTS) mutant allele of a proteasome 20S subunit gene was created and transformed into *A. suspensa*. This resulted in transgenic lines exhibiting 96-100% pupal lethality when reared at 30ºC (Nirmala et al., 2009). This conditional lethal mutation can be created for a wide variety of insect species and made female-specific using *tra* intron1-splicing for female-lethality at elevated temperatures. In addition, conditional expression of identified M factors serving as primary signals for suppressing the conserved female-determining *tra* splicing positive autoloop could be tried to establish sexing strains.

Approach 5: Altering expression of sex-determining factors.

The genetics of sex determination is not well characterized in most insect species. However, existing data show that the *doublesex* (*dsx*) and *fruitless* (*fru*) genes play a common role in determining whether an organism develops into a male or a female. It has been shown that silencing the female form of the *dsx* in the larval stage results in female lethality in *Aedes aegypti* (Whyard et al., 2015), and CRISPR/Cas9-based knockout of *dsx* can lead to female lethality that can be used for population suppression in a gene drive approach (Kyrou et al., 2018). Considering the conservation of *dsx* and *fru*, this approach may be generic (Salvemini et al., 2011, 2013). Conditional sex conversion could result in twice the number of male progeny by converting females into males (Saccone et al., 2011). Full masculinization was also achieved in the medfly by transient ectopic expression of *MoY* or injection of MOY recombinant protein in XX embryos (Meccariello et al., 2019). Embryonic transient Cas9 interference of Medfly *tra* (without inducing mutation) is an alternative way to induce full masculinization of XX individuals (Primo et al., 2020). If sex conversion is the goal, manipulation of genes upstream in the sex-

determination pathway, either a male determining factor or a *transformer*-like transducer gene, would be needed. One approach is the creation of temperature-sensitive mutant alleles of *transformer-2* using CRISPR/Cas9, such as the *Drosophila suzukii tra-2*^{ts2} allele, resulting in sterile XY males and the conversion of XX females to sterile phenotypic males at non-permissive temperatures (Li and Handler, 2017). A *tra-2*^{ts2} allele for sex conversion was similarly created by CRISPR/Cas9 in *C. capitata* (Aumann et al., 2020) and was used to create *tra-2* knock-outs resulting in XX female to phenotypic male conversion in *A. suspensa* (Li and Handler, 2019). Small RNAs also play a role in sex determination, such as the feminizing factor *Fem* piRNA that acts by suppressing *Masc* in *B. mori* (Kiuchi et al., 2014), and another, albeit converse, role was found for the miRNA-1-3p microRNA that suppresses transformer, resulting in male differentiation, in *B. dorsalis* (Peng et al., 2020). Transient manipulation of *Nix*, the male-determining factor in *Aedes aegypti* (Hall et al., 2015), resulted in partial sex conversion. Transgenic lines that ectopically express *Nix* in both *Ae. aegypti* and *Ae. albopictus* can produce fertile transformed males (Aryan et al., 2020, Lutrat el al 2022; Zhao et al., 2022). In *Ae. aegypti*, masculinized females cannot fly because they lack a second M-locus gene myo-sex that encodes a male-specific flight muscle myosin. In *Ae. albopictus*, converted males (genetic females) are able to fly, but preliminary data indicates that there might be other fitness costs associated with these males. Data presented during RCM3 highlights that masculinization by *Nix* overexpression is significantly affected by position effects. The genomic methods that led to the discovery of *Nix* (Hall et al., 2015) are relatively cost-effective and can be applied to 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 the 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). Expected field performance should also include in some cases, and especially for new lethality systems, potential genetic breakdown as was performed for the Tet-off embryonic lethality system (Zhao et al., 2020). This analysis predicted the frequency of potential resistance to the lethality system under mass-reared conditions. There is a wealth of available literature on this field in addition to the great experience 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 currently used worldwide (FAO/IAEA/USDA, 2019).

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

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

IAEA.org topic(s):
Nuclear technology and applications; Food and agriculture; Insect Pest Control; Sterile Insect Technique.

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

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

Sequencing and bioinformatic efforts
The CRP project supports the sequencing of *Anastrepha fraterculus*, *A. ludens*, *Bactrocera dorsalis*, *B. zonata*, *Ceratitis capitata*, and *Zeugodacus cucurbitae* genomes and transcriptomes for: (a) the discovery of genes suitable as selection markers for the construction of genetic sexing strains; (b) studying the evolution of tephritid genomes and (c) the characterization of the Y chromosome and its evolution. In addition, RNAseq for gene expression and small RNA discovery in lepidopterans and dipterans is carried out.

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
BGD5035 - Validating the Sterile Insect Technique as a Key Component of an Area-Wide Integrated Pest Management Programme Against Aedes aegypti in Dhaka.

BOL5023 - Fruit Fly Control in Bolivia Using Integrated Pest Management Including the Sterile Insect Technique.

BRA5062 – Application of the Sterile Insect Technique for the Control of Aedes aegypti.

BKF5023 - Implementing the Sterile Insect Technique to Reduce Wild Populations of Aedes aegypti and Tsetse.

CMR5026 - Supporting the National Fruit Fly Management Programme.

KAM5011 - Establishing SIT-based Area-wide Integrated Management of Bactrocera zonata and Bactrocera dorsalis.

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

CPR5027 - Demonstrating Feasibility of the Sterile Insect Technique in the Control of the Codling Moth, Cydia pomonella.

CPR5028 - Demonstrating the Feasibility of Applying Area-Wide Integrated Management Strategies Based on the Sterile Insect Technique in the Green Control of Spodoptera litura.

CYP5021 - Preventing the Spread of the Aedes albopictus and Aedes aegypti Mosquitoes.

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

ECU5035 - Assessing the Feasibility of the Sterile Insect Technique to Control the Invasive Vector Mosquito Aedes aegypti and the Mediterranean Fruit Fly at a Pilot Level.

ELS5015 - Integrated Management of Fruit Flies using the Sterile Insect Technique to Establish Areas of Low Prevalence of Fruit Flies.

FIJ5007 - Implementing Pesticide-Free Suppression and Management of Fruit Flies for Sustainable Fruit Production – Phase II.

ISR5022 - Establishing the Sterile Insect Technique Methodology for the Management of the False Codling Moth, Thaumatotibia leucotreta, and Enhancing Integrated Pest Management Against the Peach Fruit Fly, Bactrocera zonata.

JAM5015 - Strengthening National Capacities for the Introduction of the Sterile Insect Technique for Pest Control, Mutation Breeding of Crops and Post-Harvest Treatment of Agricultural Produce Using a Self-Contained Gamma Irradiation Facility.

SWA5004 - Utilizing the Sterile Insect Technique Integrated with Other Suppression Methods for the Management of the False Codling Moth.

MAR5028 - Enhancing National Capabilities on the Suppression of Aedes Albopictus in an Urban Locality Using the Sterile Insect Technique as Part of an Integrated Vector Management Strategy.

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

MOR5040 - Improving the Productivity of Livestock and Crops.

MYA5029 - Improving Fruit Yield and Quality by Using Sterile Insect Techniques as Part of Area-Wide Integrated Pest Management of Fruit Flies in the Mandalay Region.

OMA5009 - Establishing SIT-based Area-wide Integrated Management of Bactrocera zonata and Bactrocera dorsalis.

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

PAN5031 - Validating the Sterile Insect Technique for the Control of the Mediterranean Fruit Fly, Ceratitis capitata.

POR5006 - Integrating the Sterile Insect Technique in the Control of the Invasive Vector Mosquito Aedes albopictus.

PHI5037 - Assessing the Feasibility of the Sterile Insect Technique to Suppress the Aedes aegypti Population.

SRB5006 - Strengthening National Capacity to Integrate the Sterile Insect Technique in the Control of Aedes

Invasive Mosquitoes by Establishing a Mass Rearing Facility.

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

SAF5019 - Testing the Sterile Insect Technique Intervention as a Vector Control Tool against the Primary Malaria Vector, Anopheles arabiensis.

SRL5054 - Using Field Application of the Sterile Insect Technique in a Pre-Operational Trial for the Control of Dengue and Evaluating the Feasibility of the Application of the Sterile Insect Technique for the Control of Melon Fruit Flies.

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

TUR5027 - Implementation of SIT for Suppression and Eradication of Medfly in Turkey.

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

RAF5092 - Enhancing Agricultural Productivity for Improved Food Security in Africa.

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

RAS5095 - Enhancing the Capacity and the Utilization of the Sterile Insect Technique for Aedes Mosquito Control.

RAS5097 - Strengthening and Harmonizing Surveillance and Suppression of Fruit Flies.

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

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

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

RLA5087 - Validating the Sterile Insect Technique for the Control of the South American Fruit Fly (ARCAL).

RLA5092 - Enhancing Regional Capacity for the Adoption of the Sterile Insect Technique as a Component of Mosquito Control Programmes (ARCAL CLXXXVII).

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 papers.	Biological material is available. QC protocols are available or can be developed.

9. Publication of results in a peer reviewed journal.	Papers drafted and submitted.	Journal issue with published scientific papers.	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 2023.	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.
8. Special issue published.	Publication	Special issue published.	Each contract and agreement holder contribute with a paper to the Special Issue.

ACTIVITIES

Contract 23334: Philippos A Papathanos, Hebrew University of Jerusalem (Collaborators: K. Bourtzis, K. Mathiopoulos, A. Darby, M. Schetelig, G. Saccone, I. Ragoussis, F. Marec, J. Krzywinski, C. Ndo, P. Tortosa, N. Windbichler, A. Meccariello)

"Mosquito genetic sexing strains based on RNAi and engineered Y chromosomes" ("Establishing the *Aedes albopictus* molecular editing capabilities toward the construction of GSS")

Brief introduction (1-2 short paragraphs):
Aedes albopicuts, a mosquito that was once endemic to south-east asia, has now become a global nuisance and an epidemiological threat. Despite decades of efforts to manage the spread of this invasive mosquito using traditional methods, successes have been limited. Genetic control technologies have the potential to significantly improve pest managment. Genetic control methods like the SIT work through the mass-rearing and release of sterile males that by mating to wild females reduce the reproductive potential of the pest population. Despite their outstanding local success, genetic control programs are limited in scale, due to the inability to ensure no accidental female-release.Genetic sexing strains (GSSs) can solve this problem. In these strains, a genetic link between sex and selectable trait(s), enable a reliable sex-seperation in masses. The following results describe our attempt to harness the latest advances in genetic editing technologies and insect transgenesis to develop a GSS in *Aedes albopictus*.

During this CRP progress has been achieved in the following areas

We made significant progress towards the development of GSSs in target insect species. With a primary focus on *Ae. albopicuts* we generated using CRISPR genome editing a *yellow* loss-of-function strain and built a neoclassical GSS around this mutation and phenotype, as proof-of-concept for the neoclassical approach. *Yellow* mutants were charactized and found defective in melanin assimilate resulting in a vivid pale phenotype throughout development, with minimal fitness cost to homozygous mutants. To rescue the phenotype mini-*yellow* rescue strains were generated that succesfully recovered cuticle phenotype. Doing so required the development of genetic tools including characterization of ubiquitous promoters to ensure appropriate expression of mini-yellow rescue alleles that were randomly inserted in the genome. To convert these outputs into a GSS following the neoclassical approach we explored methods to functionally link the rescue to maleness. We succesfully identified M-locus specific and unique target sites using genomics data and custom bioinformatic pipelines, however similarly to efforts during this CRP in other target species, significnant engineering obstacles plagued the target delivery of rescues to the M-locus. We therefore explored the use of synthetic masculinization using the Nix M-factor. Combining synthetic nix alleles and mini-yellows we ultimately isolated a GSS line in which pseudomales that are genetic females display the species' typical dark phenotype. In this strain, females can be easily distinguished from males at early developmental stages based on cuticle color. Additionally, males develop significantly faster than sibling yellow females, expanding the naturally occurring protandry. This allows for better temporal sex separation and improved male purity upon pupal recovery. Males of this strain are similar in size and appearance to wild-type males and exhibit comparable reproductive behaviour and competitiveness. Females mated with GSS males demonstrate similar post-mating intolerance for future mating attempts. To make this possible, substantial progress was made in developing a custom CRISPR tools in *Aedes albopictus* which we are now using to streamline and improve genetic editing capabilities. To this end we have characterized endogenous promoters capable of expressing the two CRISPR components, Cas9 proteins (Pol-II promoters), and sgRNA (Pol-III promoters), and tested their activity *in vivo*. Besides work in *Ae. albopictus*, signifacant progress was also made on the development and testing of bioinformatics methods for the characterization of the Y chromosome sequences in various species, for basic biology and for their targeting by CRISPR including *C. capitata, D. suzukkii, B. zonata, B. tryoni*.

Achievements:

1. **Development of selectable traits using CRISPR gene editing in mosquitoes:** CRISPR-based gene editing was used to induce mutations in the ***yellow*** gene of *Ae. albopictus*. ***yellow*** mutants displayed a vivid color phenotype, throughout development.
2. **Mosquito genetic rescue of *yellow* phenotype:** Constructs bearing genetic rescues of *yellow* were generated and tested in transgenic strains in homozygous *yellow* background. A construct expressing the mini-yellow from the regulatory regions of the *hsp83* gene from *Ae. albopictus* was successful in fully rescuing cuticle color, demonstrating the promoter's generic applicability for engineering functional rescues in this insect, when ubiquitous expression is required.
3. **Development of a mosquito *yellow*-GSS:** Strains containing a construct containing the *hsp83*-mini-*yellow* rescue and the dominant *nix* masculinizer were developed and selected for their ability to sex-convert genetic females and rescue *yellow* in a homozygous mutant background. Of all strains, one well-performing strain was isolated and then established as a proof-of-concept strain for the neoclassical approach. This strain was evaluated in small-scale laboratory experiments for insect fitness, compatibility with current sex-separation strategies based on size. Comparative transcriptomics was used to evaluate the degree of female masculinization to deeped our understanding of sex conversion and its potential for generating GSSs.
4. **Y-chromosome genomics and computational genomics tools:** Genomic and transcriptomic datasets were generated in collaboration with multiple members of the CRP consortium for a number of insect pests. Bioinformatic pipelines were developed and tested to use the omics data and identify Y-chromosome specific genes and sequences for the targeted knockin of genetic rescues. This data is also critical in deepening our understanding of the content and function of insect Y-chromosomes.
5. **Funding collaborative research:** Collaborative research projects with multiple members of the CRP consortium were funded from various funding agencies in collaboration with the Papathanos lab. These included EU Horizon Europe funding for the REACT project, DFG-funded research with the Schetelig Lab, VW-funded research with the Wimmer lab, Zaci-funded research with the Baxter lab, BBSRC-funded research with the labs of Windbichler and Meccariello.

Recommendations:

1. Improve methods for the site-specific integration of genetic rescues into the M-locus or Y-chromosomes of target species.
2. Evaluate and/or leverage potential fitness costs like slow development for the generation of GSS in target insect species
3. Continue developing and testing high-throughput selectable traits like *tsl* in target insect species
4. Leverage available CRP omics datasets and develop additional resources like deep RNA-seq to support the identification of naturally occurring high-throughput selectable traits to identify suitable target genes for the high
5. Expand genomic tools and bioinformatics to accelerate the development of genetic sexing in new pest species, allowing for faster, sustainable field applications solutions.
6. Invest in sustaining collaborative and funded research among CRP participants building on successes in current CRP.

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Contract 23335: Alfred Handler, USDA (Collaborators: K. Bourtzis, S. Lanzavecchia, J.S. Meza, M.F. Schetelig)

"Female-specific conditional lethality genetic sexing strains for improved SIT"

Brief introduction (1-2 short paragraphs):
Sterile insect technique (SIT) programs are among the most highly effective biologically-based systems for the control of insect pest populations. SIT depends on the release of overflooding ratios of sterile males into a wild type field population, resulting in non-reproductive females that eventually result in a highly suppressed, if not eradicated, field population. It is realized that conditional genetic systems for generating sterile males-only populations that can be generically applied to a range of related species is a high priority for SIT programs. Thus, the primary research objective for this CRP is to develop new transgenic strains for improved SIT in tephritid fruit fly pest species in the Anastrepha genus, including the Caribbean fruit fly, *A. suspensa*, and the Mexican fruit fly, *A. ludens*. This will include methods and genetic reagents for the more generic use of tetracycline suppressible female-specific conditional lethality systems for genetic-sexing, the discovery and analysis of new gene targets that disrupt male fertility, an evaluation of the genetic stability of conditional lethality systems to avoid survival of genetically-modified insects in the field, an evaluation of transposon-based methods for determining conspecific identity between closely related species, and the development and evaluation of new methods for genetic modifications including gene-editing and transposon-mediated germline tranformation. Notably, these goals will focus on the use of highly conserved genetic systems that, with minor modification, can be used as generic systems for SIT in other related species within the Anastrepha genus, if not in a wider range of tephritid species, and potentially more distantly related dipterans including vectors of disease. Progress in these areas of research, and especially highly conserved methods for genetic-sexing and evaluation of and stabilization of transgenic insects, will broaden the capabilities for achieving efficient and ecologically safe SIT in a large number of related insect pest species.

During this CRP progress has been achieved in the following areas:

- evaluating the genetic breakdown of a transgenic conditional lethality system for insect population control
- use of CRISPR/Cas9-mediated gene editing to mutate exogenous transgenes and native sex determination genes in *Anastrepha suspensa*
- development of new methods for genomic genetic modifications including CRISPR-based gene editing, transposon-mediated germline transformation and recombinase-mediated genomic targeting in Anastrepha and Drosophila pest species
- identification and evaluation of new target genes for disruption of male fertility in Anastrepha and Drosophila pest species

The hAT-family transposable element, hopper, from Bactrocera dorsalis is a functional vector for insect germline transformation

Achievements:

1. The frequency of primary and secondary site lethality reversion in a conditional embryonic lethality system in the *Drosophila melanogaster* model system was determined, and importantly, the frequency and basis for inherent suppression of the cell death lethal effector, potentially resulting in resistance to this population control system in the field, was determined.
2. CRISPR/Cas9-mediated gene editing was achieved in *A. suspensa* to eliminate function in an introduced transgene and the native transformer-2 sex determination gene
3. The mutated *piggyBac* and *hopper* transposable elements were used to define phylogenetic relationships in *Bactrocera dorsalis* complex species, and in particular, conspecific identities
4. The *hopper* transposon, originally discovered in *B. dorsalis*, was successfully used for germline transformation of *D. melanogaster* and *A. suspensa* providing a new vector for insect transformation and proving that the 3,131 bp *hopper* element is functional.
5. Cognates for the *D. melanogaster wampa* and *Prosalpha6T* testis-specific genes were identified and gene expression evaluated in *D. suzukii* and *Prosalpha6T* in *A. suspensa*, providing new targets for male fertility disruption.

Recommendations:

1. Identification of the *deep orange* (*dor*) and *LysRS* gene cognates, that may encode potential sites for *tsl* mutations in *Anastrepha* species
2. Align potential Anastrepha *tsl* amino acid cognate sequences and determine if relevant wild type amino acids exist - if so, initiate CRISPR gene editing to create relevant mutation resulting in amino acid substitution.
3. Identify visible mutations linked to *dor* and *LysRS* for use in GSS
4. Further analysis and gene-editing of known visible mutations linked to the *tsl* in medfly, including *deep orange*, *white pupae*, *white eye*, and *yellow*.
5. Continued evaluation of the *A. ludens nullo-tTA* driver cassette for post-zygotic embryo-specific expression of the tTA driver for female-specific Tet-off lethality GSS.

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Contract 23353 / 25146: Wei Peng, College of Life Sciences, Hunan Normal University (Collaborators: Zhijian Tu, Alfred Handler, Giuseppe Saccone)

"The development of genetic sexing strains for SIT applications in *Drosophila suzukii*"

Brief introduction (1-2 short paragraphs):
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. *D. suzukii* is a polyphagous insect pest which causes significant damage to soft and thin-skinned fruits.
Thus, the molecular mechanism of sex determination in *D. suzukii* need to elucidate, and then a genetically enhanced sterile insect technique by knocking down testis-specific genes can be developed and a masculinization and female-specific lethal or embryonic conditional lethality genetic sexing strains (GSS) can be constructed for sterile insect technique (SIT) applications. This research will shed light on the mechanism of sex setermination in *D. suzukii*, and develop new strategies for large-scale eradication and male-only mass rearing for SIT.

During this CRP progress has been achieved in the following areas:
During this CRP, substantial progress was made in sequencing the mRNA transcriptome from embryos and testes, and studying the function of key genes associated with reproduction in regulating sex determination and spermatogenesis; identifying the sex alternative splicing mechanism of key genes; developing a genetically enhanced sterile insect technique by knocking down testis-specific genes using RNAi techniques; constructing female-specific lethality and masculinization genetic sexing strains (GSS) using CRISPR/Cas9 gene editing technology based on the identification of sex determination key genes in *D. suzukii*.

Achievements:

1. **Investigate the sex-determination cascade genes in regulation of sex determination**: The transcriptome analyses identified 23 sex determination genes in D. suzukii, including Sex-lethal (Sxl), transformer (tra), transformer-2 (tra-2) , doublesex (dsx) and fruitless (fru) which are the sex determining pathway cascade genes, and sisterless A (sisA), scute (sc), runt, deadpan (dpn) and groucho (gro) which encode X-chromosome linked signal elements (XSEs). The temporal expression profile of 23 sex determination genes throughout D. suzukii developmental stages displayed that the highest transcript levels were detected in embryos and the lowest in adults. In addition, fourteen of the identified sex determination genes were differentially expressed between sexes. RT-PCR validation showed Sxl, tra, dsx, and fru genes have sex-specific isoforms in D. suzukii adults, while tra-2 does not, and exon skipping was validated as a common splicing mechanism. The Sxl, tra, tra-2, dsx and fru gene were cloned by using the transcriptome data and rapid-amplification of cDNA ends (RACE) technique. The ORF of these gene are 825, 699, 825, 1095, 2733 bp encoding for 275, 233, 275, 365, 911 amino acids, respectively and exhibits structural features characteristic of known insect sex determination genes. The Sxl and tra genes are highly conserved in structure and sex-specific transcripts for these two genes were detected. To study the function of key genes associated with reproduction in regulating sex determination, we have synthesized the double strand RNA of Sxl, and tra genes and have done the micro-injection into the embryos. The RNAi experiments showed that sexual formation is determined early in the embryo stage and female to male sex reversal was achieved by targeting tra gene. Nearly all XX embryos developed into fully masculinized phenotypic male adults with no apparent female morphology. Upon dissection abnormal hypertrophic gonads were revealed in XX pseudomales but not in the XY males. To study the function of key genes associated with reproduction in regulating sex determination, we have synthesized the double strand RNA of tra-2, dsx and fru genes and have done the micro-injection into the adults. The RNAi experiments showed that *tra-2* and dsx^F genes plays important roles in regulating reproduction of female D. sukuzii, and *fru* gene plays important roles in regulating courtship and mating behaviors of male D. sukuzii.
2. **Investigate the testis-specific genes in regulation of spermatogenesis**: We have also identified numerous spermatogenesis genes such as testis-specific serine kinase 1 (tssk1), testis-specific serine kinase 3 (tssk3), βTubulin 85D (βTub), fuzzy onions (fzo), protamine A (ProtA), spermatocyte arrest (sa) based on the testes transcriptome. We have synthesized the dsRNAs of Tssk1, Tssk3,βTub, fzo, ProtA and sa genes and have done the micro-injection into the embryos. The knockout experiments showed that a reducing of sperm activity and male infertility phenotypes, which indicating that these spermless males can be used

for SIT applications.

3. **Construct the female-specific lethality and masculinization genetic sexing strains (GSS)**: To construct the masculinization genetic sexing strains (GSS) based on targeting sex determination genes, we identified and synthesised the vasa promoter, generating the gene drive D. suzukii transgenic strain expressing Cas9 driven under the vasa promoter. Meanwhile, we identified the U6 promoter in the NCBI genome database. By counting the proportion of individuals with mosaic eyes in G0 flies under the microscope, we can compare the gene editing efficiency of each U6 promoter in D. suzukii. Our results showed that the promoter U6-3 had the highest gene editing efficiency. Based on the identification of vasa and U6-3, we used the CRISPR/Cas9 gene editing technology established a homing-based gene drive system in D. suzukii through targeting the Sxl gene and tra gene. The vasa promoter was used to express Cas9 and the U6-3 was used to express Sxl and tra gRNAs in D. suzukii. The drive component, consisting of Sxl and tra single guide RNA and DsRed genes, was introduced into the female-specific exon of Sxl and tra , which is essential for function in females. In both Sxl and tra targeted locus, gene drive allele could achieve super mendelian inheritance. In terms of phenotypes, we found that 100% of the Sxl knockout females perished during preadult stages with the majority dying during pupal transition. As expected, abnormal development of both the external and internal genitalia was observed in G0 and G1 female adults and produced the male-specific tra and dsx transcript. Interestingly, knocking out tra led to significantly reduced fecundity and fertility in adults of corresponding sex. Moderate transmission rates of the DsRed gene were observed in gene drive males.

Recommendations:

1. Establish and evaluate the rules for the applications of genetic sexing strains (GSS) for sterile insect technique (SIT) before release to the field.

Publications:

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Contract 23358: Ioannis Ragoussis, McGill University (Collaborators: Kostas Bourtzis, Kostas Mathiopoulos, Alistair Darby, Marc Schetelig, Philippos Papathanos)

"Advanced genome assembly and annotation of *Ceratitis capitata* genetic sexing strains"

Brief introduction (1-2 short paragraphs):
Our laboratory has established a combination of genomic sequencing technologies and bioinformatics approaches allowing the generation of high-quality genome assemblies, as well as long read transcriptome sequencing approaches allowing the sequencing of complete cDNA molecules and thus improve the detection of transcript isoforms and genome annotation. The aims of the project were to sequence the genomes of selected strains of insect species within the focus of this CRP and enhance their annotation through transcriptomics.

Aim1: Genome sequencing and assemblies for Egyptll (wild type), Vienna 7 (translocation line w/o inversion), Vienna 8 (translocation line w/o inversion) and D53 (homozygous inversion line), Vienna 7 and Vienna 8 strains in order to support the analysis of the tsl locus.

Aim 2. Produce sequencing data data for Bactrocera oleae and generate genome assemblies for Bactrocera zonata in collaboration with the Genetics and Molecular Biology group of the Insect Pest Control Laboratory and other CRP partners, as well as Anastrepha fraterculus, Anastrepha ludens, Bactrocera dorsalis, Bactrocera zonata and Zeugodacus cucurbitae.

Aim 3. Enhance the B. oleae and C. capitata genome annotation by utilizing advanced nanopore long read sequencing approaches to analyze embryos of B. oleae and C. capitata between time 0 post oviposition to 15 past oviposition, at hourly intervals, in collaboration with K. Mathiopoulos, Greece. These time points cover the expression of male determining factors and associated transcriptomic responses.

During this CRP progress has been achieved in the following areas:
We have progressed towards achieving all stated aims of the project. We have applied improved long read technologies, based on Oxford Nanopore Technologies, as well as Hi-C, where possible to generate high quality genome assemblies for all species and strains required by the CRP (see below). In addition, we have analysed early embryo transcription in both *B. oleae* and *C. capitata*. Specifically, for *C. capitata* we have established methodologies and produced data for single, sexed embryos between the hours 0 and 15 post egg laying, at 1h intervals. This work played a critical role for the identification of the *white pupae* mutation, as well as the analysis of the *tsl* locus in C. capitata.

Achievements:

1. Genome assemblies

For genome assembly, we initially deployed a linked red approach based on 10X genomics technology, in combination with the Supernova genome assembler. The methodology showed promising results, however due to legal action vs 10x Genomics the reagents were discontinued and the software development stopped. In parallel, we developed protocols that utilized Nanopore sequencing technology, taking advantage of the continuous improvements in throughput and sequencing data quality. This was applied initially to produce the first high quality genome of *B.oleae* (see Bayega et al 2020).

Data produced for C. Capitata strains using Nanopore technology:

Sample	Total (Gb)
V7 male	76.555
V7 female	51.62
V8 male	24.99
V8 female	96.15
EgII male	47.62
EgII female	52.42
D53 male	31.23
Eg male	7.22

Using these technologies and deep sequencing, we produced assemblies for 8 C. capitata strains We also applied a number of structural variation detection tools to identify a range of indels/deletion/duplication in the *C. capitata* strain genomes and aid the identification of structural abnormalities and identify breakpoints.

This led to publications in collaboration with CRP groups including Ward et al 2021 and Solazzo et al 2024 for the successful identification of the *white pupae* mutation and the analysis of the *tsl* locus respectively.

For Anastrepha fraterculus, Anastrepha ludens, Bactrocera dorsalis, Bactrocera zonata and Zeugodacus cucurbitae assemblies generation, we used long read sequencing polished with short read sequencing and scaffolded using Hi-C (chromatin conformation capture) sequencing. Prior to scaffolding the assembly deduplication was performed to separate a primary assembly and an alternate assembly, and each was then scaffolded independently. The scaffolded assemblies reached N50 length in the range of 60Mb to 120Mb. The scaffolded assemblies were verified with BUSCO and completeness was in the range 97% to 98.5% and had very low duplicated, fragmented and missing orthologs.

The assembly statistics were as follows:

Statistics	*Anastrepha fraterculus*	*Anastrepha ludens*	*Bactrocera dorsalis*	*Bactrocera zonata*	*Zeugodacus cucurbitae*
# contigs	4,973	1,238	588	660	5,140
# contigs (>= 50000 bp)	67	403	58	162	239
Largest scaffold	182,068,020	125,331,998	102,926,020	103,247,910	70,658,899
Total length	793,193,562	720,811,084	537,310,284	600,442,075	374,778,270
Total length (>= 50000 bp)	766,717,392	706,688,108	531,140,571	593,885,298	349,303,203
N50	121,046,886	118,249,840	76,567,395	65,592,266	62,275,020
L50	3	3	4	4	3
GC (%)	37	37	37	36	35
# N's	225,200	231,400	105,800	118,600	134,300
NG50	109,980,515	114,208,257	40,758,100	45,696,414	-
LG50	4	5	8	7	-

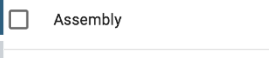
All sequencing data and primary contigs were submitted to NCBI generating a valuable resource for the scientific community:

a) Sequencing data:

Species	NCBI Bioproject	Dataset type	NCBI SRA accessions
Anastrepha fraterculus	PRJNA1065016	Long read WGS	SRR29293661, SRR29293662, SRR29293663, SRR29293664
		Short read WGS	SRR29319412, SRR29319413
		Hi-C	SRR29319415, SRR29319414
Anastrepha ludens	PRJNA1076526	Long read WGS	SRR29307005, SRR29307006, SRR29307007
		Short read WGS	SRR29323014, SRR29323013
		Hi-C	SRR29323016, SRR29323015
Bactrocera dorsalis	PRJNA1076946	Long read WGS	SRR29299427
		Short read WGS	SRR29323025, SRR29323026
		Hi-C	SRR29323028, SRR29323027
Bactrocera zonata	PRJNA1082643	Long read WGS	SRR29299502, SRR29299503
		Short read WGS	SRR29319677, SRR29319676
		Hi-C	SRR29319678
Zeugodacus cucurbitae	PRJNA1070629	Long read WGS	SRR29294704, SRR29294702, SRR29294703
		Short read WGS	SRR29322956, SRR29322955
		Hi-C	SRR29322957, SRR29322958

Contig level assemblies:

Species	Biosample	Bioproject (principal)	Bioproject (alternate)	GenBank assembly (principal)	GenBank assembly (alternate)
Anastrepha fraterculus sp1	SAMN39449916	PRJNA1065016	PRJNA1065015	GCA_037575425.1	GCA_037575645.1
Anastrepha ludens	SAMN39944021	PRJNA1076526	PRJNA1076525	GCA_037783455.1	GCA_037783485.1
Bactrocera dorsalis	SAMN39957830	PRJNA1076946	PRJNA1076945	GCA_037783525.1	GCA_037783465.1
Bactrocera zonata	SAMN40214030	PRJNA1082643	PRJNA1082642	GCA_037783105.1	GCA_037783125.1
Zeugodacus cucurbitae	SAMN39655635	PRJNA1070629	PRJNA1070628	GCA_037783285.1	GCA_037783305.1



A manuscript is in preparation to publish the assemblies and primary comparative analysis of the species genomes.

2.

The early embryo transcription work includes work on the B. oleae embryo development, published in collaboration with the Mathiopoulos group (Bayega at al 2021).

For *C. capitata* embryo work we introduced a number of innovations: a) we isolated single embryos and we performed simultaneous DNA and RNA extraction b) developed a strategy for sensitive embryo sexing using male and female specific PCR assays c) performed cDNA synthesis from low amounts of RNA(individual embryos) d) barcoded and multiplexed library for efficient long sequencing by Nanopore.

In more detail For *C. capitata*, the first 15 hours after egg laying (AEL) have been studied. Starting from 0 hours AEL, embryos were collected by K. Mathiopoulo's group at hourly intervals until 15 hours AEL for a total of 16 timepoints. We received these embryos and randomly selected 15 embryos per timepoint. We completed the extraction of RNA and DNA from all embryos for a total of 255 samples of RNA and 255 samples of DNA derived from single embryos (one timepoint was extracted twice). We established a number of PCR based sexing assays based on genes/regions previously published. These included CcMoY, ITS1, Y114, CcY, and tubulin. The sensitivity of these PCR assays was determined which showed that we could start sexing embryos as early as 4 hours AEL. We performed two PCR reactions on each of the 255 DNA samples to determine sex using primers for CcY and Y114. The PCR amplicons were purified and run on the LabChip to read the profiles and call the sex of each sample. We then compared the performance of the Nanopore cDNA sequencing library preparation protocol (SQK-PCB109) to an in-house lab protocol we developed that uses Panhandle primer design (Bayega et al 2022). We were encouraged to use our Panhandle protocol due to its higher yield in terms of PCR amplicons and better gene-body coverage. We then performed cDNA library preparation on all 240 samples using the Panhandle protocol adding barcodes in the process. Barcodes allowed multiplexing of samples to reduce costs and also reduce technical variability in library preparation and sequencing. We also performed MoY PCR on the first 6 timepoints to confirm the expression of MoY at the expected time. We then pooled cDNA libraries and prepared three sequencing libraries that were sequencing on the Oxford Nanopore Technologies' PromethION sequencing platform using three flow cells.

Sequencing yielded a total of 220 million reads. The reads were processed to remove adapters and poly(A) tails and also to orient them according to strand of origin. Processed reads were aligned to the Ccap_2.1 genome assembly. Reads that did not align to the Ccap_2.1 assembly were aligned to the EGII-3.2.1 assembly which is a more recent and more contiguous assembly. Aligned reads were used to derive a genome-guided transcriptome assembly which contained 22,875 transcripts and 3,879 novel genes, currently missing in the NCBI predicted gene models. These data have already expanded our current known range of gene and isoforms. We have also used the full-length transcript data to improve the annotation NCBI predicted gene models. The previously predicted gene models from NCBI and the novel isoforms and genes were combined, and gene expression measured. Since we added external ERCC RNA standards, we were able to perform absolute quantification of gene expression which we and others have previously shown to yield more accurate quantification of gene expression. For example, we have noticed a novel reduction in abundance of poly(A) transcripts at 2 hours

following oviposition both in the olive fruit fly and medfly. mRNA content drops by over 50% at 2 hours after oviposition and then rebounds at 3 hours after oviposition. We have further used these absolute quantification results to cluster embryos based on their transcriptomic profiles as done in single cell RNAseq. This analysis has allowed us to employ clustering and lineage tracing algorithms developed for single cell RNAseq to perform a molecular pseudo-temporal analysis of gene expression. Through this analysis we have observed that indeed, similarly to Drosophila, medfly embryos undergo a minor and major wave of zygotic genome activation. The early zygotic genes we are uncovering could be important future targets of SIT approaches. The pseudo-temporal data is further shedding light on the maternal to zygotic transition in the medfl. For example, we have learnt that the degradation of maternal transcripts is gradual and occurs at a rate proportional to initial abundance of maternal transcripts such that transcripts high in abundance are degraded faster than those initially abundant at lower concentrations. Further, we have used our sexing data to perform differential gene expression between male and female embryos to identify genes that should enable molecular sexing of early embryos based solely on RNAseq data. This analysis is in progress.

Recommendations:

1. Generate deeper and ultra-long read data in order to achieve more complete Y chromosome genome assemblies for all tephritid species relevant for SIT approaches. This can be achieved using a combination of ultra long DNA fragment sequencing utilising telomere to telomere protocols (T2T) and pore-C, an extension of Hi-C, which utilizes chromatin conformation capture and long reads to improve assemblies and has the potential to improve Y-chromosome assemblies in particular, where the current short read-based Hi-C methodologies are not effective.
2. Generate comprehensive long read-based RNA sequencing data from different tissues and across stages of development for all tephritid species relevant for SIT approach development in order to improve the annotation of the genome and identify mutations in strains aiding SIT approaches.
3. Apply long long read CRISPR/Cas9 targeted sequencing approaches to comprehensively analyse CRISPR/Cas9 edited positions as well as off-target editing events in genome editing approaches.

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2. Ward CM, Aumann RA, Whitehead MA, Nikolouli K, Leveque G, Gouvi G, Fung E, Reiling SJ, Djambazian H, Hughes MA, Whiteford S, Caceres-Barrios C, Nguyen TNM, Choo A, Crisp P, Sim SB, Geib SM, Marec F, Häcker I, Ragoussis J, Darby AC, Bourtzis K, Baxter SW, Schetelig MF. (2021). White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. Nat Commun: 12(1): 491.
3. Bayega A, Oikonomopoulos S, Gregoriou ME, Tsoumani KT, Giakountis A, Wang YC, Mathiopoulos KD, Ragoussis J. (2021). Nanopore long-read RNA-seq and absolute quantification delineate transcription dynamics in early embryo development of an insect pest. Sci Rep. 11(1): 7878.
4. Bayega A, Oikonomopoulos S, Wang YC, Ragoussis J (2022). Improved Nanopore full-length cDNA sequencing byPCR-suppression. Front Genet. 13: 1031355.
5. Sollazzo G, Gouvi G, Nikolouli K, Aumann RA, Djambazian H, Whitehead MA, Berube P, Chen SH, Tsiamis G, Darby AC, Ragoussis J, Schetelig MF, Bourtzis K. (2023). Genomic and cytogenetic analysis of the Ceratitis capitata temperature-sensitive lethal region. G3 (Bethesda): jkad074.

Contract 23360: Nidchaya Aketarawong, Regional R&D Training Center for Insect Biotechnology (RCIB), Department of Biotechnology, Faculty of Science, Mahidol University, THAILAND (Collaborators: Ernst Wimmer, Hassan M.M. Ahemd, Daniel Bopp)

"Development of genetic sexing strains for SIT program of agricultural *Bactrocera* spp. using bioinformatics and molecular tools"

Brief introduction (1-2 short paragraphs):

Bactrocera dorsalis, *B. correcta*, and *B. carambolae* are recorded as destructive agricultural pests on a regional scale. However, due to rapid globalization and global warming, these three species, especially *B. dorsalis*, have become invasive in more areas outside their home ranges. Their damage can cause a loss of fruit products and create trade barriers. Effective and sustainable pest management is required to prevent and control their damage and spread to new areas. The sterile insect technique (SIT) using genetic sexing strains has been proven successful and sustainable, as shown in the case of *Ceratitis capitata* using GSS VIENNA-8. Male individuals of the strain can be separated from females using two sex-linked traits before irradiation and release.

Using a classical genetic approach, genetic sexing strains for these three species have been developed. A genetic sexing Salaya1 strain was developed for *B. dorsalis* based on a brown-white pupal color dimorphism (Isasawin et al., 2012) and was evaluated under long-term mass rearing conditions (Aketarawong et al., 2020). Another genetic sexing Salaya5 strain was developed for *B. carambolae*. This is a proof-of-concept for the development of genetic sexing strains by introgression between *B. carambolae* and its closely related *B. dorsalis* Salaya1 strain (Isasawin et al., 2014). For *B. correcta*, a small colony of genetic sexing strain has been developed, but it needs further refinement (unpublished data). Relying solely on a classical genetic approach, which requires a natural phenotypic selectable marker such as pupal color and a Y-autosome translocation, may limit the development of new or refined genetic sexing strains. To enhance the opportunities, genetic control strategies should be developed and established in parallel.

During this CRP progress has been achieved in the following areas:

During this CRP, three main research aspects were proposed and studied in parallel: (1) Genetic studied on genes related to sex-determination pathway/genes related embryo development; (2) development of genetic control strategies such as CRISPR/Cas9, RNAi, and Tet-off system; (3) screening and evaluating new phenotypic selectable markers. Results from parts (1) and (2) are key components of genetic control strategies, so that these combinations will be possibly used for development of genetic sexing strain. New selectable markers can be used as a component of the classical genetic sexing approach.

Genes in sex-determination pathway (e.g., *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*), and *fruitless* (*fru*)) were successfully studied in *B. dorsalis* and *B. correcta* (Permpoon et al., 2011, Laohakieat et al., 2016, 2020). Meanwhile, four contigs were isolated and characterised from Y-chromosome sequences of B. dorsalis using two Representational Difference Analysis (RDA) libraries (Carraretto et al., 2020). Their characteristics supported that *B. dorsalis* dot-like Y chromosome harbours repetitive sequences, transcribed sequences of a homologue of the PERQ amino acid-rich with GYF domain-containing protein CG11148 gene, and non-LTR retrotransposon-like sequence. FISH data also confirmed that both transcribed and non-LTR retrotransposon-like sequences presented on both the X and Y sex chromosomes of *B. dorsalis*. The three key genes in the sex-determination pathway of *B. carambolae* were molecular characterised and comparative studied to related species, *B. dorsalis* and *B. correcta* (Laohakieat et al., in prep).

Molecular tools such as RNA interference (RNAi) and CRISPR/Cas9 systems were developed. RNA interference of *tra* genes delivered by microinjection and feeding were studied on *B. carambolae* and *B. dorsalis*, respectively. Only RNAi by microinjection demonstrated the knockdown of *tra* gene, leading to female-to-male sex conversion (Laohakieat et al., in prep). Additionally, a CRISPR/Cas9 system was carried out to achieve sequence-specific gene knockout via the non-homologous end joining (NHEJ) pathway. Two independent target sites of the BdorMoY gene were selected for NHEJ-mediated disruption, and three sgRNA cocktails were individually injected into the precellular blastoderm embryos of *B. dorsalis* Salaya1. All G0 brown pupae developed into normal males were individually back-crossed to uninjected white-pupae females. Two out of 18 crosses produced G1 brown-pupae females (pseudofemales) that were confirmed to have the Y-chromosome and a CRISPR-induced deletion in their *BdorMoY* target sequences. These two knockout lines have been maintained to this day (unpublished data).

At least four selectable markers were screened: slow larval, temperature sensitive, eye color and pupal color. First, mature larvae and pupae of the Salaya1 strain were collected and subsequently separated by pupal color. Number of pupae and sex ratio were calculated. Under the appropriate rearing conditions, no significant difference between male and female development was observed (Isasawin and Aketarawong 2020). Second, temperature sensitivity/tolerance was screened in several strains of *Bcatrocera* species. Preliminary data showed that one strain of *B. carambolae* appeared to be temperature-sensitive, while *B. dorsalis* seemed to be tentatively temperature-tolerant (unpublished data). Lastly, the eye color and/or pupal color mutant individuals underwent genetic crossing (mono- and di-hybrid crosses) to understand the mode of inheritance of the mutations. The crossing experiments demonstrated that the eye color and pupal color phenotypes were inherited as simple Mendelian autosomal recessive traits and were unlinked (unpublished data). Molecular characterization of the pupal color gene in *Bactrocera* species is an ongoing project.

Achievements:

1. Three key genes in the sex-determination pathway of *B. carambolae* as candidate target genes for genetic control strategies.
2. The CRISPR/Cas-9 system as a genome editing tool for gene functional analysis and strain development
3. Two *MoY minus* (*MoY*-) pseudofemale lines and transient pseudomales
4. A small colony with double natural mutations is being developed for further characterization as a resource of selectable markers.

Recommendations:

1. Continue effort to develop genetic sexing strains, especially *B. carambolae*, using both classical and genetic control strategies.
2. Enhance the molecular characterization of sex-determining genes to *B. carambolae* from introduced areas.
3. Continue effort to molecular characterize on natural selectable markers in *Bactrocera* spp.
4. Improve the protocol for screening temperature sensitive in *Bactrocera* spp.
5. Develop and set up protocol for dsRNA delivery system.

Publications:

1. Aketarawong N, Isasawin S, Laohakieat K, Thanaphum S (2020) Genetic stability, genetic variation, and fitness performance of the genetic sexing Salaya1 strain for Bactrocera dorsalis, under long-term mass rearing conditions. BMC Genetics, 21(Suppl 2):131. https://doi.org/10.1186/s12863-020-00933-4
2. Carraretto D, Aketarawong N, Di Cosimo A, Mannni M, Scolari F, Valerio F, Malacrida AR, Gomulski LK, Gasperi G (2020) Transcribed sex-specific markers on the Y chromosome of the oriental fruit fly, Bactrocera dorsalis. BMC Genetics, 21(Suppl 2):125. https://doi.org/10.1186/s12863-020-00938-z
3. Isasawin S, Aketarawong N (2020) Study on fruit fly development enhancing the knowledge for pest control using sterile insect technique. The 32nd Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2020), Online conference/ Bangkok, Thailand, 26 November 2020, pp. 622-632.
4. Laohakieat K, Isasawin S, Thanaphum S (2020) The transformer-2 and fruitless characterisation with developmental expression profiles of sex-determining genes in Bactrocera dorsalis and B. correcta. Scientific Reports, 10:17938. https://doi.org/10.1038/s41598-020-74856-6.
5. Laohakieat K, Poonsiri T, Aketarawong N (in preparation). Molecular characterization of *transformer*, *transformer-2*, and *doublesex* genes in the carambola fruit fly, *Bactrocera carambolae*, reveals potential genetic pest management. Insect Science Special Issue.

Contract 23363: Giuseppe Saccone, University of Naples Federico II (Collaborators: Ernst Wimmer, Daniel Bopp, Angela Meccariello, Nidchaya Aketarawong, Sujinda Thanaphum, Siriwan Isasawin, Kamoltip Laohakieat, Kostas Bourtzis, Kostas Mathiopoulos, Simon Baxter, Wei Peng, and Al Handler)

"Sexing by masculinization of XX individuals using a newly identified male determining factor of medfly"

Brief introduction (1-2 short paragraphs): One of the two main approaches to the problem of sexing for SIT prorgrams, is to revert chromosomally XX individuals into adult males, during embryogenesis. This approach is today feasible following two decades of comparative molecular genetic studies performed on the sex determination pathway of *Ceratitis capitata* and later on other pest insect species (Saccone, 2022).
In *Ceratitis capitata*, female sex determination is achieved by maternally deposited Cctra and Cctra-2 mRNAs (and likely proteins) inducing female-specific splicing and a positive feedback loop in the Cctra gene in 5-8h old XX embryos. In XY individuals the male determiner Y-linked MoY gene encoding a 70 aa novel protein represses this Cctra maternal activation, leading to a non-functional Cctra male-specific splicing and male sex determination (Pane et al., 2002; Salvemini et al., 2009; Meccariello et al., 2019). Taking advantage of the molecular isolation of Cctra and of the identification of is novel autoregulatory function with the respect of the Drosophila highly divergent orthologue, many other insect Cctra orthologues have been found by homology which are also able to autoregulate (Saccone, 2022), differently to the Drosophila tra orthologue. The isolation of the male determiner Y-linked MoY in *Ceratitis capitata* led again to find by homology other MoY orthologues in eight Bactrocera and Zeugodacus species (Meccariello et al., 2019). The discoveries that XX reverted males (Cctra or Cctra-2 RNAi) and XY reverted females (Cas9-MoY) are fertile, suggested a remarkable plasticity of Ceratitis capitata to respond to sex determination perturbations.

During this CRP progress has been achieved in the following areas:

During this CRP, a major progress was made in the understanding of the genetics underlying sex determination of Tephritidae species. A collaborative cost-effective effort involving various members of this CRP culminated into the cloning of the master gene for male sex determination in *Ceratitis capitata,* functionally conserved in other Tephritidae species. The use of NGS, long reads PACBIO sequencing, CQ and DE bioinformatic tools, and RNAi/CRISPR/CAS9 functional analyses greatly facilitated the project. Additional MOY orthologues have been identified taking advantage of novel SRA databases published by Zhang et al., 2021, and Alphafold2 modelling led to a MOY structure comprising alpha helixes and beta sheets, which seems to be stable in Molecular Dynamics modelling. Putative biochemical function of MOY is to bind nucleic acids. We have also asked if and confirmed that a transient transcriptional repression of *Cctra* during early XX embryogenesis is sufficient to induce a switch off of the Cctra positive autoregulation, as predicted on the basis of previous sex determination model (Pane et al., 2002). We have also asked if and confirmed that the female-specific CcTRA and the auxiliary CcTRA2 physically interact as in Drosophila, to promote female sex determination.

Having received two years ago a grant from PNRR European funds, we started to explore the use of orally delivered dsRNA molecules to interfere with key genetic functions, including enzymatic and vital ones, as a preliminary step toward the sex determining ones. Following three-day adult feeding using a combination of dsRNA molecules that target the expression of the ATPase vital gene and two intestinal dsRNA nucleases, we observed 79% mortality over seven days, which was associated with a decrease in mRNA levels of the three targeted genes (Volpe et al., 2024). This research illustrates the potential of utilizing molecules as pesticides to achieve mortality rates in Medfly. These novel findings of high effectiveness of oral RNAi in a Tephritidae species opens the road to attempt delivery of dsRNAs from the intestine to the female ovaries and repress female-determining *Cctra* maternal contribution.

Having received one year ago a 24 months grant from PRIN National Italian Ministry of Research and University, we started to explore the use of CLA (Conjugated Linoleic Acid) in the larval and adult food of *Ceratitis capitata* as protection against oxidative stress induced by gamma radiations to improve longevity and quality of the sterile males. Preliminary data showed that wild type adults fed with CLA for 3 days, lived up to 30% longer.

A better understanding of how gene transcription works in the model system *Drosophila melanogaster* is necessary to approach the problems of position effects when a transgene is integrated randomly in the genome and of heterochromatic gene expression silencing when is integrated on Y and X sex chromosomes. For example, SAYP and Bap170, subunits of the SWI/SNF remodelling chromatin complex, have the ability to support enhancer-dependent transcription when artificially recruited to the promoter on a transgene. SAYP and Bap170 appear to collaborate with specific subunits of the Mediator kinase module (e.g., Med12 and Med13) to promote active transcription. Their presence in a locus is necessary for stable recruitment of Med12 and Med13. Their cooperative activity is often local, restricted to regulatory elements where they stabilize the recruitment of other transcriptional activators. Understanding the roles of SAYP and Bap170 provides insights into the mechanisms of chromatin remodelling and transcriptional regulation, with relevance to both normal development and for insect biotechnology aiming to achieve high transgene expression in a difficult chromatin context. With colleagues and experts of Drosophila genetics we uncovered that in addition to the above factors, the Nelf-A protein was found to participate in the process. Nelf-A is a subunit of the Negative Elongation Factor (NELF) complex in *Drosophila melanogaster*, a conserved transcriptional regulatory complex involved in RNA polymerase II (Pol II) pausing during the early stages of transcription elongation. NELF-A interacts with chromatin remodelling factors and histone-modifying enzymes, integrating transcriptional pausing with chromatin dynamics. The cooperation of the factors, independent of enzymatic activities of the complexes they are part of, appears to be a novel mechanism that maintains promoter activity and may be used in many loci of the genome. This knowledge could be useful in future to approach the problem of transgene expression integrated into the Y chromosome of Medfly and other insect species, being the SAYP, Bap170, Med12, Med13 and Nelf-A highly conserved in evolution.

Achievements:

1. Production of Ceratitis XX/XY and XX-only 6-8h old embryos transcriptomics (triplicates).
2. PacBIo genomic sequencing of FAM18 strain of Ceratitis with a shorter Y chromosome and a first Y chromosome assembly.
3. Isolation of *MoY*, the master gene for male sex determination in *Ceratitis capitata* and eight related Tephritidae species (Meccariello et al., 2019)
4. MOY expression in bacteria and in insect *Trichoplusia ni* High FiveTM (Hi5; Sinobiological service) cell lines failed to lead to a high-grade purity necessary for NMR analyses.
5. A synthetic MOY was obtained (GL Biochem (Shanghai) Ltd.) and a polyclonal antibody produced against this artificial MOY. Experiments are still underway.
6. Identification of *MoY* orthologous genes in 10 species of the Ceratitis genus, development of MOY structural model by Alphafod 2 modelling by comparison of 18 OY orthologous proteins, and design of site-specific mutagenesis of MOY to confirm the model.
7. Use of dead Cas9 to transiently repress *Cctra* transcription during early embryogenesis and fully masculinize XX individuals (Primo et al., 2020).
8. A transgenic *Ceratitis capitata* strain carrying a synthetic male determiner (Maternal Masculinizer; MatM) has been characterized molecularly and functionally characterized (Manuscript in preparation in collaboration with Ernst Wimmer, including TET/ON/OFF conditional masculinizing transgenic lines).
9. Proteomic analysis of 15 h old XX/XY and XX embryos and identification of more than 3000 polypeptides, but no TRA, TRA-2, DSX and MOY (unpub. res.; project still going on). Few proteic candidates showing male-bias have been identified.
10. Establishment of a Y-chromosome free cage *Ceratitis* population carrying the MatM transgene and study of sex ratio fluctuations during over 10 generations (manuscript in preparation).
11. Demonstration that the model of CcTRA/CcTRA-2 interaction based on Drosophila previous studies is supported by Yeast Two Hybrid experimental data (Perrotta et al., 2023).
12. Development of a FLAG-*MoY* construct, using a 5 Kb *MoY* genomic region and demonstration that its transient expression in XX embryos leads to full masculinization. Immunoprecipitation and proteomic approaches will be attempted.
13. A shorter fragment of 1.5 Kb *MoY* genomic DNA was cloned and shown to be sufficient for masculinization of XX individuals in a transient expression test.

14. Development of a novel embryonic *Ceratitis capitata* cell line from 24h old XX/XY embryos, transcriptomic analyses and DE of cell line and 24h whole embryos (all key sex determining genes are expressed in the cell line) (manuscript in preparation).
15. Development of a *Ceratitis capitata* biopesticide based on three days co-feeding of a dsRNA mix (targeting dsRNAses and vATPAse) able to induce 80% lethality in seven days (Volpe et al., 2024). Experiments are underway to apply similar strategy to *B. dorsalis*.
16. Use of CLA (Conjugated Linoleic Acid) in the larval and adult food of *Ceratitis capitata* as protection against oxidative stress induced by gamma radiations to improve longevity and quality of the sterile males. Preliminary data showed that wild type adults fed with CLA for 3 days, lived up to 0% longer.
17. Biocontainment facility for *Bactrocera dorsalis* was authorized by Italian Ministry of Agriculture and regional authorities for pest control (Regione Campania) and a colony from La Reunion (France) was established in the Department of Biology (Saccone lab; University of Naples, Italy). CRISPR/Cas9, RNAi and transgenesis experiments recently started toward the development of conditional masculinizing transgenic strains, in collaboration with Ernst Wimmer (Gottingen Univ. Germany).

Recommendations:

1. Development of conditional masculinizing *Ceratitis capitata* transgenic strains based on maternal *Cctra* repression and zygotic *MoY* ectopic expression should be continued
2. Development of conditional masculinizing *Bactrocera dorsalis* strains based on the same strategy used for *Ceratitis capitata* should be continued taking advantage of the biocontainment facility in Naples (Italy) in collaboration with Ernst Wimmer.
3. Development of an auxotrophic strain of *Bactrocera dorsalis* (in collaboration with Jake Tu and Melanie Hemper) to add another level of biocontainment to the facility.
4. Transfection of the *Ceratitis capitata* cell line MaVoCc2324 with reporter genes; establishment of XX-female only cell line from XX female-only embryos.
5. Investigation of MOY biochemical function by structural biochemistry and by co- immunoprecipitation and proteomics, also using the novel Ceratitis capitata cell line in collaboration with Ernst Wimmer.
6. Exploration of orally supplied a mix of dsRNA molecules (including suppressors of intestinal dsRNAses) at larval and adult stages should continue to investigate effectiveness also in targeting key female determining gene (Cctra and Cctra-2) during ovogenesis to induce masculinization of XX progeny.
7. Exploration of orally supplied CLA to protect irradiated pupae from oxidative stress and improve male quality and longevity.

Publications:

1. 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.
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Participants: Dr Amanda Choo and Ms. Thu Nguyen University of Adelaide. Dr. Peter Crisp, South Australian Research and Development Institute
Project Collaborators: 1. Gene Discovery Team: Owain Edwards (CSIRO), Phil Taylor (Macquarie University), Kostas Bourtzis, Marc Schetelig, Scott Geib, Alistair Darby (D44003 participants);
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"Development of genetic sexing strains of *Bactrocera tryoni* (Queensland fruit fly) for SIT using CRISPR/Cas9 gene editing"

Brief introduction (1-2 short paragraphs):

Genetic biocontrol of horticultural pests using the sterile insect technique requires mass release of factory reared males on a massive scale. Female insects are not required for releases, and their efficient removal can be complex. Genetic sexing strains offer efficient female-exclusion strategies, and they are generally achieved using clear dimorphic traits or with female conditional lethality. Here we use CRISPR/Cas9 mutagenesis to target the genome of horticultural pest *Bactrocera tryoni* (Froggatt, Queensland fruit fly) to create pigment-deficient phenotypes and temperature sensitivity traits that can be utilized to create genetic sexing strains. *Bactrocera tryoni* is a major horticultural pest in Australia that can cause devastating damage to more than 300 species of cultivated fruits and vegetables, imposing significant financial burdens on the horticultural industry. Improved efficiency of the sterile insect technique will have considerable benefit to Australian growers.

The four objectives of this CRP were:

- Objective 1. Develop embryonic lethal temperature sensitive mutations in *B. tryoni* through recreating known *Drosophila melanogaster* mutations in genes *transformer-2*, *RNA polymerase II 215*, *tyrosine hydroxylase* and *shibire*
- Objective 2. Identify the 'White Pupae' gene in *Bactrocera*
- Objective 3. Translocation or insertion of genes onto Y-chromosomes, which is required for male rescue
- Objective 4. Develop phenotypic markers to distinguish sterile released males from wild flies

During this CRP progress has been achieved in the following areas:

All goals of the CRP were achieved.

Objective 1. Development of *B. tryoni* strains with temperature sensitive phenotypes
Temperature sensitive mutations first identified in *Drosophila melanogaster* were recreated in *B. tryoni*, to develop strains with embryo lethality and adult paralysis phenotypes when exposed to elevated temperatures. Single amino acid substitutions in the gene *shibire* were successfully produced with CRISPR/Cas9 including *shibire*^{ts1}, *shibire*^{ts2} and *shibire*^{ts4}. Shibire is an essential GTPase required in endocytosis and synaptic vesicle recycling, and failure to complete this process causes neurotransmitter failure and death. A *shi*^{ts4} mutation was established in *B. tryoni* which showed phenotypes consistent with those observed in *Drosophila*, including adult paralysis at 31°C. The *shi*^{ts4} *B. tryoni* strain also showed temperature dependent lethality at egg, larval and pupal stages when subjected to heat treatment above 31°C. This is the first stable *B. tryoni* temperature sensitive strain generated with targeted mutagenesis and the first demonstration of *shibire* temperature sensitivity in a tephritid species. These phenotypes may assist ongoing efforts to develop a temperature sensitive mutant that can be incorporated into a genetic sexing strain for area-wide management of this fruit fly species. Mutations at different locations in the same gene, *shibire*^{ts1} and *shibire*^{ts2} were also successfully produced. Additional temperature sensitive candidate genes were investigated, including *transformer-2*, *RNA polymerase II 215* and *tyrosine hydroxylase* (Refs 5, 7, 9).

Objective 2. Identify the White Pupae gene
As part of a collaborative effort, the white pupae gene was identified using a combination of genomics, genetic crosses, bioinformatic analysis and functional genomics (Reference 3). The analysis involved generation of a

chromosome level genome assembly of *B. tryoni*.

Objective 3. Development of a *B. tryoni* genetic sexing strain
Genetic sexing strains require the translocation or insertion of a trait onto the male-sex chromosome. We performed irradiation experiments to develop an autosome:Y-chromosome translocation whereby male puparium colour is brown (wild-type) and females that lack the translocation are a grey/white colour. The translocation strain is relatively stable and field trials are required to assess fitness.

Objective 4. Development of *B. tryoni* strains with visible phenotypes
Disruption of genes involved with pigment colour development can cause clear, visible phenotypes. Traits involving colour have applications including robotic sorting of different varieties. We have developed a yellow body mutant strain of *B. tryoni*, which may be a valuable field release strain as it is visibly different to natural populations. Flies caught in monitoring traps can be quickly verified as factory reared flies and not wild type flies. Fitness experiments show minimal fitness costs associated with the trait and indicate strong potential for field application (Ref. 4).

Pupal colour mutations provide a strategy to sort dimorphic colours into distinct classes using robotic seed sorters. We have identified two pupal colour variants, one causing black colouration through mutations in the gene *ebony*, and a second causing a white/grey colour pupae through a mutation in the gene *major facilitator superfamily* (Refs. 3, 10)

Achievements:
1. CRISPR/Cas9 homology directed repair methods established for *Bactrocera tryoni*
2. Temperature sensitive phenotypes created for *shibire*^{ts1}, *shibire*^{ts2} and *shibire*^{ts4}
3. Discovery of the genetic basis of white pupae and black pupae phenotypes in Tephritid flies
4. Development of yellow-body colour mutants to visually differentiate between sterile released flies and wild flies caught in traps.
5. Production of a genetic sexing strain, where males have brown pupae and females have grey-white pupae.

Recommendations:
1. Perform cage-contained outdoor trials using *Bactrocera tryoni* yellow colour mutations, to assess fitness.
2. Perform cage-contained outdoor trials using *Bactrocera tryoni* genetic sexing strain.
3. Develop a yellow-body, white pupae genetic sexing strain via genetic crosses and assess fitness.
4. Determine strategies to sort *Bactrocera tryoni* genetic sexing strain based on puparium colour.
5. Integrate the *shibire*^{ts4} mutation into the genetic sexing strain and seek regulatory approval for field trials.
6. Analyse Y-chromosome sequences to characterise the genetic sexing strain translocation.

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"Establishing the X-shredding sex distortion system in the medfly *Ceratitis capitata*"

Brief introduction (1-2 short paragraphs):
Tropical fruit flies are considered among the most economically important invasive species detected in temperate areas of the United States and the European Union. Eradication and containment campaigns levied against invasive fruit flies in many continents include repeated treatment of large areas with preventive releases of sterilized males (employing Sterile Insect Technique (SIT) technology), and/or an extensive use of pesticides. SIT efficiency, cost-effectiveness, as well as safety and biosecurity, depends on the availability and use of genetic sexing strains (GSS), to remove females from the male-only population that must be released. Despite the importance of sex separation, 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 of developing a GSS in one species is not necessarily directly transferable to other species.
To establish a novel GSS, a Y-chromosome-linked approach has been proposed. 5 different regions were verified as male-specific and corresponding constructs were generated, both for homology-directed repair and non-homologous end joining-mediated knock-ins. Multiple embryo microinjections were performed but no Y-linked GSS has been generated to date. For this, an alternative Y-chromosome-independent GSS strategy has been proposed relying on sex-specific splicing of the *transformer* gene.

During this CRP progress has been achieved in the following areas:
In this CRP we focused on exploring the feasibility of Y-specific integration with a GFP marker. Targets were generated by the Papathanos Lab. Evaluated their male-specificity via PCRs on genomic DNA of Cc males and virgin females. We selected 6 best candidates (Y1; Y2; Y3; 26A; 26B; G3). An additional target was acquired through a literature search focusing on transgenesis in *C. capitata*. This uncovered that Condon and colleagues (2007) had previously observed a piggyBac integration onto the Y chromosome, entitled OX3067C. The published 5' piggyBac-adjacent genomic sequence mapped to a Y-specific region on scaffold 3 and was verified as male-specific through PCR. Attempts at extension of the sequence to include the hypothetical 3' region were unsuccessful, thus the original 438 bp fragment was used for construct design.
We designed the gRNAs, injected each of them alongside Cas9 into WT Cc embryos and assessed gRNAs efficiency via amplicon sequencing. We cloned the plasmids for each target. HDR donor constructs containing Hr5Ie1-eGFP-Sv40 flanked by target-specific homology arms were cloned via Gibson assembly for the 7 targets. The established approach for HDR-mediated knock-in (Meccariello et al., 2024) was carried out with the HDR donor plasmid and a target gRNA-Cas9 protein RNP complex. Multiple sets of traditional HDR injections have been performed into the Benakeion wild-type strain embryos but unfortunately no G1 transgenic flies were found.
We are trying to study another approach to achieve integration on the Y chromosome named NHEJ approach. For the NHEJ approach 3 different types of constructs needed to be cloned, which included a universal donor containing *Hr5Ie1-eGFP-Sv40*, a universal helper containing a gRNA targeting the donor and multiple interchangeable gRNA helpers targeting the Y-specific region of choice. Alongside the 7 unique target gRNA helpers and the two universal plasmids, a tester *white eye* (*we*) target gRNA helper was additionally cloned. This aimed to assess the feasibility of a NHEJ-mediated knock-in in the autosomes since such a 3-plasmid approach has not been tested in the medfly to date.
For the NHEJ approach, a 3-plasmid mix was injected into the eggs of the Cas9-expressing line (OAM-S16), in a similar fashion to the injection set-up used for CRISPaint in *Drosophila* (Bosch et al., 2020). So far, this has only been attempted once for *white eye* and OX3067C each with a total of 600 eggs injected. This injection mix requires additional optimisation as high lethality was observed across the G0 injected individuals, specifically during the larval stages of development. No GFP+ adults were observed for either target across the resulting G1 populations.

Achievements:
1. We selected 6 best candidates on Y chromosome.
2. Tests all gRNAs.

3. Cloned several plasmids.
4. Multiple sets of HDR and NHEJ injections.

Recommendations:

1. Establish injection conditions for the NHEJ approach using the *white eye* target.
2. Similarly to HDR, additional microinjections are necessary to determine the feasibility of NHEJ-mediated Y-chromosome knock-in in the medfly.
3. Showcasing the feasibility of NHEJ in the *we* gene, or any of the Y-chromosome targets, can provide the certainty for the suitability of this HDR-alternative for the use in the medfly. Despite its error-prone nature which may result in imperfect repair of at the integration sites (Yang et al., 2020), NHEJ is still applicable for the Y-chromosome integration as mutations in the target sequence are not regarded as a limiting factor for GFP expression. Therefore, moving forward, the shorter 'YUK' pipeline targets, inaccessible for HDR construct design, could then be subjected to gRNA design for NHEJ-only testing *in vivo*.
4. As Y-linked GSS establishment may not come to fruition, an alternative Y-independent GSS strategy could be explored. Specifically, as insinuated previously (Davydova et al., 2023), a *tra* intron-dependent system based on a phenotypic marker, such as the *white pupae (wp)* can be used (Ward et al., 2021; Zwiebel et al., 1995). Due to the anticipated sex-specific splicing of the target gene, there will be distinct phenotypic outcomes for males and females constituting a GSS, which would allow for easy sex-sorting. The feasibility of such approach is increased compared to the Y-chromosome integration because autosomal HDR of various sizes was proven successful in the medfly in the recent years.

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"Identification and characterization of temperature sensitive lethal genes and response of SIT target species to thermal shock"

Introduction: Medfly VIENNA 7 and 8 GSS are a model for the development of GSS in Diptera. Their success is attributed to the incorporation of two selectable markers, the temperature sensitive lethal and the white pupae mutations, which are genetically linked. Their development took more than two decades and extensive work from different laboratories. Substantial effort was required for the refinement of the protocol that leads to the elimination of females through exposure to elevated temperatures. Among the problems that need to be addressed for the development of tsl-based GSS are: a) The tsl phenotype can be quite variable, due to the nature of the tsl gene used and its interactions with the rest of the genome and the environment; b) Screening for tsl mutations and developing standardized protocols is also laborious; c) Besides the tsl, a morphological marker that will simplify screening is also crucial; d) The two markers need to be closely linked and to be transferred through a single rearrangement event (translocation) in a new chromosomal position, to the same region with the male determining locus. Additional recombination-reducing tools, such as chromosomal inversions, may be needed to reduce genetic recombination, depending on the species. Recent studies point to the possible involvement of reproductive symbionts, such as *Wolbachia*, in the manipulation of host recombination, which is a legitimate scenario from an evolutionary aspect of symbiosis. The recent, artificially-established, relationship of medfly with *Wolbachia*, and the previously suggested utilization of the VIENNA GSS in the development of the combined SIT/IIT application (not as an applied but more as a model for agricultural pests) shows that the symbionts/meiotic recombination interplay needs to be studied, especially if this proves to be an advantage or disadvantage for the genetic stability of genetic sexing strategies. In SIT target species, besides medfly, there are only few studies describing tsl phenotypes or detailed protocols to measure thermal tolerance or sensitivity. Understanding the response of different species to the thermal stress is baseline data needed to identify temperature sensitive or temperature resistant mutations after their isolation or induction. Even when the desirable mutations (or phenotypes) are isolated, it is again laborious and time consuming to link them with the M locus, to develop a genetic sexing strain. In the last few years, there is lot of research focusing on the identification and isolation of the tsl gene that is currently used in the medfly GSSs. The availability of genome assemblies, genetic analysis using modern bioinformatic tools, and classical approaches such as in situ hybridization and targeted genetic mapping, can lead to the identification of this gene or other genes that can be useful for genetic sexing.

During this CRP progress has been achieved in the following areas:

1. *Tsl screening of medfly recently domesticated populations:* Natural populations have been screened in three consecutive years. Although initial results were promising regarding the possibility of tsl-sensitive genotypes being present in these natural populations, screening of next year showed that their thermal response profile was not significantly different from that of the typical resistant strains. These findings suggest that colonization efforts for the identification of specific traits, especially if they are expected to have a fitness cost, should start with single pair crosses for at least two generations. This approach would give the chance to homozygote in advance alleles of interest.
2. *Development of thermal stress response protocols for the tiger mosquito:* Following initial results temperatures of 39 ^{o}C for 6 h, 12 h and 24 h and 41 ^{o}C for 3 h, 6 h, and 12 h were selected to establish the baseline response to thermal stress, as the most prominent combinations to deliver robust response profiles. Such studies need high numbers of synchronized individuals therefore populations need to upscale for few generations prior to repeating the experiments. This may lead to loss of alleles of interest and that's why the alternative approach of firstly making homozygous genotypes using single pair crosses is proposed. This may provide better chances of rescuing genotypes of interest.
3. *Development of new wp/tsl^{+} and wp^{+}/tsl strains through recombination:* Six recombinant lines were developed through this project and are routinely maintained. The percentage of isolated recombinants is in agreement with the small genetic distance estimated between the *wp* and *tsl* loci. All lines exhibit the expected response to the thermal stress. More than 100 GB of sequencing data have been generated and are analyzed.

4. *Development of combinations of the tsl mutation with additional morphological mutations of the 5th chromosome (triple-mutated strains):* Nine triple-mutated recombinant lines were developed through this project and are routinely maintained. The percentage of isolated recombinants is in line with the rather large genetic distance estimated between the *wp-tsl* region and the *y, we,* and *or* mutations. All lines exhibit the expected response to the thermal stress. More than 400GB of sequencing data have been generated and are analyzed.
5. *Development of the Wolbachia-harboring wp tsl strains through crosses and measurement of its effect on the genetic stability of the VIENNA 8 GSS:* Developing of the CC [*w*Cer2] (wp tsl) and the CC [wCer4] (wp tsl) lines was critical for the routine development of the VIENNA 8 GSS that harbor *Wolbachia*. The behavior of the strains has been repeatedly verified regarding hatching, pupation, emergence, and sex ratio at 25, 34, and 35 ^{o}C. These strains are routinely maintained. The effect of *Wolbachia* on the genetic stability of the VIENNA 8 GSS, through its possible implication in genetic recombination has been studied under filtering and no-filtering conditions. Up to now, recombinants appear in small numbers and sporadically for all cages, proving once more the high genetic stability of the VIENNA GSS, but also the absence of any negative effect of *Wolbachia* on it.

Achievements:

1. Development of baseline data for the response to thermal stress of *Ae. Albopctus* L1, where limited knowledge was available.
2. Development of tools for the genetic analysis of the *tsl* mutation and the 5th medfly chromosome: the development of new lines through genetic crosses and screening for rare recombinants allowed to place the *tsl* mutation in new genetic backgrounds and develop strains with multiple mutations for the 5th chromosome. A large amount of sequencing data has been collected and can support future genetic studies. Initial analysis verified that these lines can add support to accumulating evidence pointing to the 'true' *tsl* gene and at the same time eliminate other possible candidates.
3. Development of *Wolbachia*-harboring medfly strains: the development of these strains, and the availability of VIENNA 8 GSS with *Wolbachia*, which constitute a unique system, is providing evidence for the effect (or not) of reproductive symbionts on the genetic stability of the GSS strains through the manipulation of recombination. This is something that has not been considered/addressed before. This system is also excellent for laboratory studies and modeling on the possible development of combined SIT/IIT approaches for agricultural pests, where the need for perfect sexing strategies is even more evident.

Recommendations

1. The *tsl* mutation is placed in 15 new combinations, where part of the genome has been replaced through recombination. These lines can be screened for characteristics that are generally attributed to the *tsl* mutation, such as slow development and reduced hatching. Analysis of the NGS data gathered will be supportive of this work.
2. Focusing on these lines, we plan to test whether the *tsl* mutation can be correlated with increased sensitivity to other kinds of stresses, such the cold and the desiccation stress.
3. The possible manipulation of genetic recombination by *Wolbachia* is something that intrigues our group. We plan to continue the monitoring of the *Wolbachia*-harboring VIENNA 8 GSS but at the same we will focus on more appropriate strains for such studies, since recombination in males is rear in medfly. We plan to introduce *Wolbachia* in strains harboring multiple morphological mutations of the 5th chromosome and measure recombination in infected and uninfected lines.
4. Collection of baseline data for the *Ae. albopictus* response to thermal stress has been performed to some extent. We plan to continue this work with the recently domesticated natural populations. Provided that we will secure funding/manpower, we plan to revisit natural populations but starting with single pair crosses this time, to have better chances of making homozygous *tsl* sensitive and resistant lines.
5. The recent identification of the *cardinal* gene in *Ae. aegypti* makes worth trying inducing this mutation in *Ae. albopictus*. Apart from the recent biotechnological approaches and considering that recombination is not suppressed in *Aedes* males, isolation of recombination-reducing inversions could be beneficial for the development of genetic sexing strategies, as shown in *Ae. aegypti*.
6. Collaboration with developing prototypes to explore natural dimorphisms or phenotypes that are artificially linked with sex. Our primary focus is in medfly and *Ae. albopictus* at different developmental stages. More specific targets are to move towards the automation of color-based sorting at pupal stage (based on the *wp* mutation of the medfly), the automation of eye color-based sorting at the pupal stage of *Ae. albopictus* (focusing

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Planned submission for the special issue: Ch. Chondrogiannis, M. Kamilari, M.E Grigoriou, K. Bourtzis, A, Augustinos (planned): Genetic analysis of recombinants between the *wp - tsl* in support of tsl genes identification.

Contract 23378: Kostas Mathiopoulos, University of Thessaly (Collaborators: Jiannis Ragoussis, Philippos Papathanos, Antonios Augustinos, Giuseppe Saccone, Alistair Darby, Alexie Papanicolaou, Marc Schetelig, Frantisec Marec, Kostas Bourtzis, Simon Baxter, Antigone Zacharopoulou, Elena Drosopoulou)

"Exploring structure and function of the tephritid Y chromosome"

Brief introduction (1-2 short paragraphs):
Genetic Sexing Strains (GSSs) 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 GSSs 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. To address this gap, our work initiated and extended on the development and testing of novel computational methods to detect Y-linked sequences in Tephritid assemblies. The constant optimization resulted in two novel Y-detection methods, KAMY and R-CQ, tailored for the detection of Y-linked elements from the Tephritidae Y chromosomes. In addition, a manual curation workflow was described that allowed the empirical assessment of chromosomal origin, Y assembly quality and expression state. Experimental procedures were used to validate our approach of analysing the Y. Overall, the results from the experimental validation proved the accuracy and usefulness of the manual curation process and set a basis on which the experience acquired on a dataset could improve the computational predictions. In parallel, additional Tephritid assemblies gradually became available and were subjected to sex chromosome analysis using KAMY and R-CQ.

Our final pool of Tephritidae Y sequences included *B. oleae* (3.9 Mb), *C. capitata* (26.8 Mb), *B. dorsalis* (4.6 Mb) and *B. zonata* (16.8 Mb). A comparative analysis was initiated for the two closest species, *B. dorsalis* and *B. zonata*, which also included the highest quality Y chromosome assemblies. No significant synteny conservation was observed across the two neither on the annotated gene level nor on raw sequence.

The problematic annotation of Y-linked genes is a known constraint; therefore, the manual curation approach was extended on transcribed units using appropriate RNA-seq data for *B. oleae* and *C. capitata*, both of which are reared in our lab. The expression of Y-linked transcribed regions was profiled and coupled with RNAi experiments that employed microinjections of dsRNA in insects.

In addition, the assessment of Y regions from the olive fruit fly also suggested an erroneous and problematic assembly state for that chromosome, on which a novel Y-oriented long-read assembly pipeline was developed. This effort resulted in an initial draft assembly of the olive fruit fly and the novel high-contiguity assembly of *B. zonata*.

During this CRP progress has been achieved in the following areas:
new computational methods for sex chromosome detection have been developed and employed on Tephritid genomes, that appear to have both a high specificity for Tephritid sex chromosomes and a generic applicability over the model species *D. melanogaster*. Such improvements provided the appropriate framework for the thorough characterization of the Y chromosome's sequence and the understanding of the computational constraints linked to these elements. Such considerations set the basis for achieving improvements over a Y-oriented genome assembly approach, that during initial development, it significantly advanced the completeness and structure resolvance of *B. zonata'*s Y chromosome. Such results provide both the evidence and the appropriate tools for considering the re-use of existing genomic resources for the refinement of Y assemblies. Finally, we successfully detected and validated the Y-linkage of novel transcription units on the Y chromosomes of Medfly and olive fruit fly, which comprise the first discovered Y elements of those species apart from the Tephritid maleness factor.

Achievements:
1. Development and benchmarking of two improved Y detection methods (KAMY & R-CQ).
2. Refinement of the *B. zonata* Y chromosome using the developed Y detection methods and selected assembly tools.
3. Experimental validation of Y linked genomic regions from *C. capitata* and *B. oleae*.
4. Annotation and experimental validation of two novel Y-linked genes (PGY-23 & PGY-35) on the Y chromosome of *B. oleae*.

Recommendations:

1. Focus on insects with a smaller Y chromosome. Since a large Y chromosome (such as that of the medfly) most likely relates to the presence of large repetitive areas and extensive heterochromatinization, it may be wise to switch efforts of Y-integrations towards insects with smaller or dot-like Ys, such as *B. dorsalis* or *B. oleae*.
2. Develop strategies to improve gene expression in the Y chromosome. Such strategies may include from finding less heterochromatic regions in the Y, to insulators (e.g., CTCF) and chromatin modifiers (SARs/MARs), to epigenetic modifiers (via CRISPR/dCas9 fused with epigenetic modifiers), to (Y-specific) promoters and enhancers (probably coupled with dCas9-transcriptional activators).
3. Develop Male-Specific Markers: Focus on identifying and validating additional Y-linked markers specific to the Tephritid species studied. This will enhance the precision of sexing processes, improving laboratory efficiency in SIT programs and other pest control applications.
4. Pilot CRISPR-Based Interventions: To understand the function of Y chromosome transcriptional units we have been doing RNAi-based silencing of several of them. Given the advancements in gene editing, selected male-specific reproductive genes on the Y chromosome could also be targeted through CRISPR-editing, providing insights into practical applications.

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Contract 23379: Frantisek Marec, Biology Centre CAS, Institute of Entomology (Collaborators: Marc F. Schetelig and Roswitha Aumann, University of Giessen, Germany)

"Development of generic strategies for the construction of genetic sexing strains in pest Lepidoptera"

Brief introduction (1-2 short paragraphs):
SIT programmes against lepidopteran pests rely on bisexual releases. Male-only releases are not possible because a convenient genetic sexing system to produce male-only progeny is not yet available for any Lepidoptera pest (Marec and Vreysen, 2019). We addressed the development of generic strategies for the construction of genetic sexing strains (GSS) in Lepidoptera in two ways. First, we re-examined the approach for the construction of GSS elaborated by Marec et al. (2005), which is based on the insertion of a dominant conditional lethal (DCL) gene into the female-specific W chromosome. However, in contrast to the above work, which proposed to do this using transgenesis, we intended to insert the selectable gene into the W chromosome using CRISPR/Cas9 genome editing. We have previously identified a candidate DCL gene, namely a dominant cold-sensitive mutation N^{60g11} of the *Notch* gene of *Drosophila melanogaster*, isolated an orthologous sequence in the codling moth (*Cydia pomonella*) and designed a mutant version of this *CpNotch* sequence encoding a truncated protein. Second, we studied the molecular mechanisms of sex determination in selected pest species (i) to verify whether the sex-determining pathway discovered in the silkworm *Bombyx mori* (Kiuchi et al., 2014) is conserved in other Lepidoptera and (ii) to determine whether the identified sex-determining genes can be used for the development of GSS. This research was also mainly conducted on codling moth, which is a key pest of pome fruit and walnut orchards in temperate regions of the world and is an example of a successful and long-term SIT application.

During this CRP progress has been achieved in the following areas:
Our research has paved the way for the construction of GSS in Lepidoptera species using advanced molecular genetic methods, such as CRISPR/Cas9 genome editing. We have developed a gene construct for CRISPR/Cas9 knockin that, when inserted into the codling moth W chromosome, has the potential to eliminate female offspring during embryogenesis. Although we were not able to demonstrate the successful insertion that would allow us to establish codling moth mutant lines and test the applicability of this approach for the development of GSS, this construct contains restriction endonuclease cloning sites between each sequence so that the construct can be modified by cloning and adapted for other lepidopteran pests.

Our research on codling moth sex determination provided functional evidence for the essential role of the *Cydia pomonella Masculinizer* (*CpMasc*) gene in male development and supported the hypothesis that this masculinizing function is conserved in Lepidoptera. The results obtained contribute to our understanding of the function of the *Masculinizer* gene in Lepidoptera in general and provide a basis for further investigation of genes involved in the sex-determining pathway in Lepidoptera. Knowledge of this pathway in key pest species could greatly facilitate the development of generic GSS to improve the applicability of SIT for the control of Lepidoptera pests. However, an upstream feminizing factor or other genes involved in codling moth sex determination remain to be identified.

Achievements:
1. We have developed two gene constructs, one with a fluorescent marker and a selectable gene and the other with a fluorescent marker only, to be inserted into the codling moth W chromosome using CRISPR/Cas9. We have also developed all other prerequisites for the use of CRISPR/Cas9 knockin in codling moth and optimised the microinjection of the constructs into the eggs.
2. In the codling moth, we have identified orthologs of *Bombyx mori Masculinizer* and *doublesex* genes, *CpMasc* and *Cpdsx*, and demonstrated their role in sex determination and sexual development, respectively.
3. We have developed a method that allows us to efficiently extract small RNAs and mRNAs from individual codling moth eggs using the mirVana miRNA Isolation Kit (Ambion, Life Technologies) and simultaneously extract their DNA and determine the sex of the embryos by PCR.
4. We have generated sex-specific small RNA-Seq and mRNA-Seq libraries from codling moth embryos at the stage of onset of sex determination. These sequence libraries are available to search for sex-determining factors and genes involved in sex determination or important for sexual development.

Recommendations:

1. Continue efforts to insert the prepared gene constructs into the codling moth W chromosome using the CRISPR/Cas9 method and complete the development of genetic sexing strains in this species.
2. Modify the prepared gene construct and test this approach in another pest species where egg microinjection is more feasible.
3. Generate sex-specific small RNA-Seq libraries of codling moth embryos at different developmental stages and analyse them for a primary sex-determining factor.
4. Generate sex-specific mRNA-Seq libraries from codling moth embryos at different developmental stages and perform differential expression analysis to identify genes potentially involved in sex determination. Then select candidate genes that show sex-specific or strongly sex-biased expression in both the available 12 hpo (hours post oviposition) libraries and the newly obtained libraries and investigate their function.

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Contract 23381: Marc F. Schetelig, Roswitha A. Aumann, Irina Häcker; Justus-Liebig-University Gießen, Department of Insect Biotechnology in Plant Protection (Collaborators: Kostas Bourtzis, Philippos Papathanos, Alistair C. Darby, Alfred M. Handler, Germano Sollazzo, Peter Crisp, Ioannis Ragoussis, Simon Baxter, Lucas Prates)

"Evaluation of putative *tsl* and *wp* targets in the Mediterranean fruit fly"

Brief introduction (1-2 short paragraphs):

Due to climate change and globalized trade, the global spread of invasive agricultural pests, including the Mediterranean fruit fly and the Spotted wing drosophila, has increased. This problem threatens food security, contributes to significant economic losses, and undermines current pest control measures, many of which depend on environmentally harmful pesticides.

New pest management technologies that go beyond traditional pesticide use are needed to address this challenge. Classical genetics can produce traits useful for genetic sexing or inducing sterility in pest fruit flies, but this approach is inefficient. Transgene-based methods remain constrained by GMO regulations. The targeted approach and results described here have the potential to bypass some of these limitations and support the development of genetic sexing systems suitable for large-scale pest suppression programs.

During this CRP progress has been achieved in the following areas:

During this CRP, substantial progress was made in dissecting and applying genetic approaches to develop robust genetic sexing tools for the Mediterranean fruit fly (medfly). Specifically, several putative *temperature-sensitive lethality* (*tsl*) and *white pupae* (*wp*) loci were localized and validated, enabling a clearer understanding of how these genetic variants could be used to generate efficient genetic sexing strains. Building on recently refined genomic technologies, candidate *tsl* mutations were targeted and introduced into defined genomic positions using CRISPR/Cas9-mediated genome editing. These engineered mutations were systematically analyzed, confirming their functional impact under elevated temperatures and establishing their efficacy for genetic sexing applications.

Key to these efforts was the integration of classical cytogenetics, genomic sequencing, and in situ hybridization mapping. A range of chromosomal markers was established on chromosome 5, including genes such as *dor*, *LysRS*, *halfway*, *slipper*, and various annotated loci (e.g., LOC101450909, LOC101451089, LOC101458780, LOC101455867, *peroxiredoxin*), greatly enhancing the resolution of genetic mapping. By comparing the temperature sensitivity thresholds of wild-type, mutant, and existing genetic sexing strains, it was possible to confirm the functional relevance of these genes and variants to tsl phenotypes.

Parallel efforts were directed toward identifying and characterizing *wp* mutations. Applying newly developed bioinformatic pipelines enabled researchers to pinpoint the underlying genetic changes responsible for the white pupae phenotype. This was strengthened by successfully demonstrating that a knock-out of the *wp* gene could produce the reliable, easily visible white pupal phenotype. Combined with the *tsl* markers, these *wp* mutations can be harnessed to create efficient genetic sexing strains that require minimal manual sorting.

In addition to the direct genetic characterization of medfly strains, a broader comparative genomics approach has been employed. By examining similar loci across related species, insights were gained into how certain genetic traits can be transferred or adapted to new target pests. Such evolutionary comparisons pave the way for the streamlined development of robust genetic sexing strategies in other invasive pests of agricultural importance.

The *dor* and *LysRS* genes have then been successfully manipulated to establish temperature-sensitive lethal (tsl) traits in the Mediterranean fruit fly (medfly). Specific point mutations in both genes induced embryonic, larval and pupal lethality under heat stress, demonstrating recessive phenotypes with some maternal effects. The LysRS was identified as the tsl gene in medfly that is successfully used since decades in Vienna 7/8 sexing strains. This innovation will enable precise genetic sexing by selectively eliminating female embryos via heat treatment, if rescued from the Y chromosome, making it a powerful tool for the Sterile Insect Technique (SIT). The LysRS-based genetic approach should also be scalable to other pest species due to the genes high conservation, highlighting its potential for widespread applications in sustainable pest control.

These advancements culminated in the successful collaboration of 17 research groups, including several CRP participants, which jointly secured EU Horizon Europe funding through the REACT project. This partnership aims to leverage the knowledge gained during the CRP to rapidly transfer genetic sexing strain (GSS) technology

to *Bactrocera* species, another group of economically significant pests. Overall, the results generated during the CRP have clarified the genetic foundations of key traits such as *tsl* and *wp,* and have set the stage for their practical application in sustainable, field-ready pest management strategies and programs after successfully transferring them to novel pest insects.

Achievements:

1. **Identification of Candidate Genes:** *Temperature-sensitive lethal* (e.g. *dor* and *LysRS*) and *white pupae* (*wp*) genes were identified in multiple insect species and mapped to chromosomal locations.
2. **Functional verification of *white pupae*:** Successful knockouts of the ***white pupae*** *(wp)* gene consistently produced the white pupae phenotype in several Tephritid species, demonstrating the gene's generic applicability for engineering this trait across multiple insect species.
3. **Functional verification of temperature-sensitive lethal genes:** CRISPR/Cas9 was successfully used to engineer candidate tsl phenotypes in genes like *dor* and *LysRS*. Subsequent tests of these mutant lines confirmed that they effectively displayed *tsl* phenotypes under elevated temperatures (34-36°C), and identified *LysRS* as the long-sought *tsl* gene in medfly.
4. **Comparative Genomics Insights:** The CRP consortium generated **datasets** for several key pests, which were used to identify novel genes and characterize chromosome structures. These findings will serve as a foundation for the future development of Genetic Sexing Strains (GSS), but the actual generation of GSS remains a subsequent goal.
5. **Consortium Formation for Technology Transfer:** Collaboration among 17 groups led to securing of EU Horizon Europe funding (REACT project; https://react-insect.eu/) to adapt genetic sexing systems to *Bactrocera* species, broadening the impact of these findings.

Recommendations:

1. Use comparative genomics to assess the transferability of *tsl* and *wp*-based sexing strategies to other key pest species.
2. Leverage CRP datasets to identify additional genes complementing *tsl* and *wp* for improved genetic sexing systems.
3. Expand genomic tools and bioinformatics to accelerate the development of genetic sexing in new pest species, allowing for faster, sustainable field applications solutions.
4. Collaborate with regulators and stakeholders to establish guidelines for field deployment of genome-edited lines.

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19. Yan Y, Aumann RA, Haecker I, Schetelig MF (2023) CRISPR-based genetic control strategies for insect pests. Journal of Integrative Agriculture. 22:651-668.
20. Sollazzo G, Nikolouli K, Gouvi G, Aumann RA, Schetelig MF, Bourtzis K (2024) *Deep orange* gene editing triggers temperature-sensitive lethal phenotypes in *Ceratitis capitata*. BMC Biotechnol. 1;24(1):7.
21. Yan Y and Schetelig MF (2024) Protocol for genetic engineering in *Drosophila suzukii* using microinjection. STAR Protocol 14;5(3):103248
22. Yan Y, Ahmed HMM, Wimmer EA, Schetelig MF (2024) Biotechnology-enhanced genetic controls of the global pest *Drosophila suzukii*. Trends in Biotechnology Sep 25:S0167-7799(24)00249-X
23. Aumann RA, Gouvi G, Rehling T, Sollazzo G, Bourtzis K, Schetelig MF (2025) Engineered temperature-sensitive lethality in *Ceratitis capitata. submitted.*
24. Yan Y, Zhao, Schwirz J, Borghesi C, Liu C, Liu B, Qian W, Wan F, Schetelig MF (2025) The *transformer* gene controls sexual development in *Drosophila suzukii. Insect Science (Special Issue, this CRP)*
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Contract 23396: Edwin Ramírez, MEDFLY/PROGRAM (Collaborators: Carlos Cáceres, Salvador Meza, Scott Geib, Cristian Morales)

"Development and evaluation of genetic sexing strains of fruit flies to be used for sterile insect technique applications, as part of AW-IPM programs"

Brief introduction (1-2 short paragraphs):
The knowledge generated in this CRP will be invaluable for pest control programs that use SIT. We have identified areas for potential improvement in the SIT for both species, *A. ludens* and *C. capitata*, such as the efficiency of mass rearing and the performance of strains in AW-IPM with an SIT component. Our goal was to develop new genetic sexing strains for *A. ludens* and *C. capitata* or improve existing ones. Complementary research was essential in determining the following variables: a) yield and quality, b) stability of the genetic sexing mechanism, and c) field performance.
We aimed to identify improved strains of *A. ludens* that facilitate early separation of males and females in their life cycle, enabling the release of highly competitive sterile males for effective induction of sterility in wild populations. To achieve this, we selected *A. ludens* lines originating from mutagen-treated flies (EMS) and searched for a tsl gene. After further selection, we developed line 166-10, which demonstrated 100% mortality under these conditions. The optimal heat treatment for this line involves exposing eggs to 38°C for 24 hours. In parallel, we evaluated multiple lines for genetic sexing system (GSS) development, inducing mutations to create new chromosome rearrangements. The best lines from the GSS GUA10 were F167, F274, F60, F254, F44, F55, F121, and from GSS TBP7, the best lines were F10A, F12, and F97. On the other hand, we evaluated the mating and remating behavior of wild *Ceratitis capitata* females with two types of males (Vienna 8/Toliman INV-D53 and fluorescent Vienna 8 1260) to provide practical insights on optimal irradiation ages and the effect of male pupa size.

During this CRP progress has been achieved in the following areas:
During this CRP, great progress was made. We found a new line (166-10) that shows a total lethality (100%). For the effective heat treatment, it was necessary to define: 1) the optimal age of the egg to avoid the maternal effect, 2) the duration (in hours) of the heat treatment, and 3) the effective temperature. In this way, we now know that line 166-10 shows a 100% mortality when its eggs are exposed to temperatures of 38 degrees Celsius for 24 hours.

In addition, we developed a series of evaluations focused on the selection of the best lines of GSS A. ludens; for this purpose, mutations were induced to achieve new rearrangements in chromosomes. From the GSS GUA10, the better lines were: F167, F274, F60, F254, F44, F55, F121, and from the GSS TBP7 lines: F10A, F12 and F97. As a result, the San Miguel Petapa Mass Rearing Plant already have these ten lines with a stable genetic sexing mechanism, an adequate performance in the laboratory (rearing), and a good performance in the field.

For *C. capitata* we utilized the distinctive fluorescence of Vienna 8 1260 males in offspring to assess female remating tendencies following initial mating with irradiated Vienna 8/Toliman INV-D53 males, which had been irradiated at different ages or sizes. We found that irradiation at the adult stage (0 to 72 hours post-emergence) was more effective, resulting in fewer fluorescent offspring compared to males irradiated at the pupal stage (72 to 24 hours pre-emergence). Regarding male pupa size, the data did not indicate a significant impact on sexual performance, suggesting the need for further investigation into additional factors, such as hypoxia, that may influence male sexual behavior.

Achievements:
1. Induce mutations to create new chromosomal rearrangements that lead to stable strains with high fecundity and performance, particularly for the Mexican fruit fly (A. ludens), using improved genetically sexed lines. Currently, new, better lines of genetically sexed strains are available for use.

2. Implement crossing and line selection protocols to explore selective thermal sensitivity in A. ludens females. Notably, Line 166-10 exhibited 100% lethality when subjected to heat treatment during the egg stage, making it a candidate for investigating translocations related to sexing mechanisms.

3. For *Ceratitis capitata*, we utilized the unique fluorescent traits of Vienna 8 1260 males to gather valuable data for mass rearing and field applications, discovering that irradiating adult males yields better results.

Recommendations:

1. For A. ludens, future research on line 166-10 should prioritize applying low doses of irradiation to males andconducting crosses to develop a line with thermal sensitivity specifically translocated to females.
2. The ten new lines from GSS GUA10 and TBP7 A. ludens should remain available to meet any mass rearing demands, as they feature a stable genetic sexing mechanism and demonstrate strong performance both in the laboratory and in the field.
3. Regarding male pupa size of C. capitata, the data did not indicate a significant impact on sexual performance, suggesting the need for further investigation into additional factors, such as hypoxia, that may influence male sexual behavior.

Publications:

1. Ramírez-Santos, E., Rendon, P., Gouvi, G., Zacharopoulou, A., Bourtzis, K., Cáceres, C., Bloem, K. (2021). A novel genetic sexing strain of *Anastrepha ludens* for cost-effective sterile insect technique applications: Improved Genetic Stability and Rearing Efficiency. *Insects*, 12, 499. https://doi.org/10.3390/insects12060499.

Contract 23398: Cyrille Ndo, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC) (Collaborators: Igor Sharakov, Alistair C. Darby, Philippos Papathanos, Pablo Tortosa, Kostas Bourtzis)

"Development of a temperature sensitive lethal-based genetic sexing strain of the malaria vector *Anopheles arabiensis carrying a morphological visible marker*"

Brief introduction (1-2 short paragraphs):

Malaria remains a major health problem in the tropics, with an estimated 263 million clinical cases occurring and 597 000 deaths occurred in 2023 as a result of severe malaria**.** The fight against malaria focuses on two main aspects including prevention through vector control based on the use of insecticides or insecticide-treated tools such as long-lasting impregnated mosquito nets (LLINs) to prevent mosquito bites, and management of cases using antimalarial drug therapy. Unfortunately, the development and spread of resistance to the insecticides in Anopheles vectors and of resistance to drugs in Plasmodium parasites could seriously compromise malaria control operations. Is this therefore unanimously acknowledged that, in order to achieve malaria elimination in endemic settings, new and innovative tools to reduce malaria transmission, which can integrate with and enhance current malaria control strategies, are needed.

The Sterile Insect Technique (SIT) is one of the vector control tools being investigated. For SIT programmes against malaria vectors, only males must be released since females are hematophagous and can transmit the disease. Therefore, one of the crucial steps in the development and implementation of SIT programme in Anopheles mosquitoes is sex separation and female elimination. Indeed, an efficient sex separation and female elimination system will definitely guarantee the release of males only as well as it will allow reducing rearing cost. Sex separation and female elimination must ideally take place as early as possible during the development (eg: eggs or L1) as this minimizes rearing cost and would overall facilitate the handling and processing of male only-based sterile releases. Additionally, the method developed for sex separation and female elimination should ideally be conditionally lethal for females because they are needed to maintain the colony and for male production. This work aimed to Develop a temperature sensitive lethal-based genetic sexing strain of the malaria vector *Anopheles arabiensis* carrying a morphological visible marker.

During this CRP progress has been achieved in the following areas:

During this CRP, mutagenesis of wild type male *Anopheles arabiensis* mosquitoes was achieved using ethylmethane methanesulfonate (EMS) and this induced temperature sensitivity allowing selecting five additional TLS strains. Their L1 were sensitive to heat with 100% mortality achieved following exposure at 41°C for 3 hours. Moreover, the embryos were also sensitive to heat with 0 to 1.33 % hatch rate observed after heat exposure, indicating high mortality was achieved.

In addition, the life history traits of temperature sensitive strains isolated were further characterized and this showed some slight differences compared to the control (non-mutagenized mosquitoes). A significant reduction in fecundity and fertility was observed in all TSL stains (mean egg per female: 34 to 52; mean egg hatch rate: 54.31% to 68.72%) compared to the wild type (mean egg per female:93; mean egg hatch rate:93.25%). In the same vein, slight differences were observed in fecundity and fertility between the TSL strains. Regarding adult longevity, the TSL strains showed slight reduction of longevity (mean: 12-16 days) compared to the control (mean: 18 days).

Furthermore, the inheritance pattern of the TSL alleles was assessed. The F1 isofemale progenies of the colony obtained from crossing between the TSL males and wild type females showed heat tolerant phenotypes, while progenies of the colony obtained from crossing between wild type males and TSL females returned similar results. These results showed that TSL alleles are recessive, and their inheritance is not linked to sex but is more likely autosomal.

Throughout the project period, the *An. arabiensis* wild colony and the *An. arabiensis* temperature sensitive colonies established were maintained at the insectary of OCEAC. The temperature sisitive phenotype was maintained by exposing isofemale L1 larvae to heat at each two generations and selecting for isofemale families showing 100% mortality.

Achievements:

1. Isolation of five *Anopheles arabiensis* tsl strains following mutagenesis using EMS further demonstrating

that this method is efficient in inducing temperature sensitive phenotype in mosquitoes.
2. Characterization of the fitness (larval development, fecundity, fertility, longevity) of the five tsl strains isolated which exhibited characteristics that can allow to maintain it in the insectary for future SIT related works.
3. Determination of the inheritance pattern of the tsl, which was recessive and autosomal.
4. Establish that the embryos are also sensitive to heat, with very low egg hatch rate following heat treatment.

Recommendations:
1. Consider using gene editing approaches to create a morphological marker in Anopheles mosquitoes.
2. Engage more discuss with member country on the possibility of using genetic modified mosquitoes for SIT application.
3. Use genomic approaches to better understand mechanisms responsible of temperature sensitivity in Anopheles and other mosquitoes of medical importance.

Publications:
None.

Contract 23402: Lanzavecchia Silvia, Wulff Juan Pedro, Laboratory of Insects of Agronomic Importance, IGEAF, INTA Castelar, Buenos Aires, Argentina. [Collaborators: (a) Participants of the CRP:Jiannis Ragoussis, Kostas Bourtzis, Al Handler (b) Other collaborators: Diego F. Segura (IGEAF-IABIMO, INTA, Argentina), Maximo Rivarola IB-IABIMO, INTA, Argentina], Rolando Rivera Pomar (CREG-UNLP, Argentina)]

"Development of genetic sexing strains for *Anastrepha fraterculus* approaching with generic genomic tools"

Brief introduction (1-2 short paragraphs):

The South American fruit fly *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae) is one of the most destructive quarantine pests in several countries of the 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*. 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*, genetic sexing strains have been developed at IPCL FAO-IAEA (Seibersdorf, Vienna, Austria) using a classical approach (Meza et al. 2020). From the generated GGSs, the most genetically stable and productive strain (named Af-GSS-89) was imported to Argentina and is currently being tested at the Santa Rosa mass rearing facility (ISCAMEN, Mendoza, Argentina). We carried out several studies to perform a complete genetic evaluation of *A. fraterculus* sp1 present in Argentina, which included: a cytogenetic characterization of wild and laboratory population 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, Zeugodacus cucurbitae* and *Bactrocera oleae* among others Tephritidae species, providing information on 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. Therefore, our main goals of this research proposal are: 1) to characterize *A. fraterculus sp*1 (both a wild population and a black pupae mutant strain from Vacaria, Brasil) genome to identify relevant gene markers; 2) to improve GSS previously generated by classical methods using classical and new technologies approaches (CRISPR- Cas9, transgenesis).

During this CRP progress has been achieved in the following areas:

During this CRP, significant progress has been made in developing a genetically homogeneous strain of *A. fraterculus* sp. 1 (named AfX1Y5w2 strain) after eight generations of sibling crosses. This strain exhibited a unique sex chromosome configuration (X1X1 for females, X1Y5 for males) and was associated with a strain of the *Wolbachia* sp. endosymbiont (wAfraCast2). Whole-genome sequencing was conducted on individual female and male adult flies of AfX1Y5w2 strain, and a de novo genome assembly was generated for each sex. The quality of the obtained data enabled the assembly of the nuclear genomes at the chromosome level and the complete assembly of the mitochondrial genome. These achievements provide a detailed characterization of genome features, including the annotation of gene-coding regions, as well as the identification and distribution of repeats, ribosomal RNA clusters, and simple sequence repeats. This baseline information was used to propose a set of candidate genes, which were localized in the *A. fraterculus* sp. 1 female and male genomes, to generate GSS via gene editing in support of the SIT strategy and to develop alternative biocontrol methods using RNA interference. Additionally, metagenomic analysis provided microbial nucleotide sequences and the whole genome assembly of *Wolbachia* sp. (wAfraCast2 strain) infesting *A. fraterculus* sp. 1 potentially useful for comparisons among morphotypes and related species.

This study was performed with a collaboration of six research groups and a draft manuscript including main achievements is being produced to be published.

Achievements:

1. Chromosome-scale genomes of both female and male *A. fraterculus* sp. 1.

2. Complete mitochondrial genome of *A. fraterculus* sp. 1.
3. A set of candidate target genes for gene editing (develoment of GSSs for SIT) and RNAi-based alternative biocontrol strategies.
4. Complete genome assembly of the *Wolbachia* strain *w*AfraCast_2.

Recommendations:

1. Improve the assembly of X and Y chromosomes through advanced bioinformatics and transcriptomic analysis of early embryos.
2. Use NGS technologies to analyze SNP variation in proposed target genes for gene editing and RNAi.
3. Generate mutant strains of *A. fraterculus* sp. 1 with visible phenotypic alterations using gene editing techniques, by testing two different CRISPR/Cas9-gRNA delivery methods: (a) embryo microinjections and (b) injections of adults (DIPA-CRISPR).
4. Apply the RNAi technique for functional analysis of candidate genes and the development of novel biocontrol strategies.

Publications:

1. Máximo Rivarola, Claudia A. Conte, Berube Pierre, Chen Shu-Huang; M. Cecilia Giardini, Alejandra C. Scannapieco, Fabián H. Milla, María C. Soria, Romina M. Russo, Juan P. Wulff , Haig H Djambazian, Rolando R. Pomar, Alfred M. Handler, Kostas Bourtzis, Ioannis Ragoussis, Silvia B. Lanzavecchia. Chromosome-scale genome assembly of the South American fruit fly, *Anastrepha fraterculus* sp. 1. In preparation.

Contract 23403: Alistair C. Darby, University of Liverpool (Collaborators: Grant Hughes, Jiannis Ragoussis, Philippos Papathanos, Simon Baxter, Marc F. Schetelig, Roswitha A. Aumann, Al Handler, Kostas Bourtzis, Scott Geib, Lesley Bell-Sakyi, Grant Hughes)

"Genomic analysis to support the development of genetic sexing strains for SIT and CRISPR-CAS"

Brief introduction (1-2 short paragraphs):
This proposal will use comparative genomics between of dipteran species to help and identify genetic markers and sex specific genes. We will also have a focus on resolving sex chromosomes. Most of the genomes that have been produced have focused on the heterogametic sex and as a result we are missing key information about these are important chromosomes 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. We will then look for orthologous genes in other species to provide novel genes targets in species that have not been sequenced to the same quality and standard. A collaborator Lesley Bell-Sakyi has produced a "putative" tsetse cell line which we plan to test candidate guide RNAs for tsetse. Working in collaboration with partners in Liverpool and the CRP, we will explore using the new transformation technology ReMOT Control approach which allows rapid, efficient, and cost-effective mutagenesis, given that embryo microinjection is challenging. We will test its application on *Ceratitis capitata*, the Mediterranean fruit fly to extend ReMOT Control to new forms of genetic manipulation, and then identify peptides to allow the approach to be applied to a broad range of species. We have produced a first draft chromosome level assembly of *Glossina morsitans morsitans* and *C. capitata* using PACBIO and HiC completing Aim 1.

During this CRP progress has been achieved in the following areas:
1) Generation, assembly, and annotation of male genomes of *Ceratitis* and *Glossina* using genomic and transcriptomic data.
2) Identification of Y chromosome data for *Ceratitis*.

Achievements:
1) Generation, assembly, and annotation of male genomes of *Ceratitis* and *Glossina* using genomic and transcriptomic data.
2) Identification of Y chromosome data for *Ceratitis*.

Publications:

1. Kayla M Hardwick, Awino Maureiq Edith Ojwang', Francesca Stomeo, Solomon Maina, Gladys Bichang'a, Paul-André Calatayud, Jonathan Filée, Appolinaire Djikeng, Caitlin Miller, Leah Cepko, Alistair C Darby, Bruno Le Ru, Sarah Schaack (2019). Draft genome of *Busseola fusca*, the maize stalk borer, a major crop pest in Sub-Saharan Africa. Genome biology and evolution 11: 2203-2207.
2. Christopher M Ward, Roswitha A Aumann, Mark A Whitehead, Katerina Nikolouli, Gary Leveque, Georgia Gouvi, Elisabeth Fung, Sarah J Reiling, Haig Djambazian, Margaret A Hughes, Sam Whiteford, Carlos Caceres-Barrios, Thu NM Nguyen, Amanda Choo, Peter Crisp, Sheina B Sim, Scott M Geib, František Marec, Irina Häcker, Jiannis Ragoussis, Alistair C Darby, Kostas Bourtzis, Simon W Baxter, Marc F Schetelig (2021). White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. Nature communications 12, 1-12.
3. Eric R Lucas, Alistair C Darby, Stephen J Torr, Martin J Donnelly (2021) A gene expression panel for estimating age in males and females of the sleeping sickness vector *Glossina morsitans*. PLOS Neglected Tropical Diseases 15(9): e0009797.
4. Germano Sollazzo, Georgia Gouvi, Katerina Nikolouli, Roswitha A. Aumann, Haig Djambazian, Mark A. Whitehead, Pierre Berube, Shu-Huang Chen, George Tsiamis, Alistair C. Darby, Jiannis Ragoussis, Marc F. Schetelig & Kostas Bourtzis (2023) Genomic and cytogenetic analysis of the *Ceratitis capitata* temperature-sensitive lethal region. G3, 2023, 00(0), jkad074

Contract 23466: Daniel Bopp, University of Zurich, Switzerland (Collaborators: Leo Beukeboom, Ernst Wimmer, Hassan Mutasim Mohammed Ahmed, Giuseppe Saccone)

"Analysis of a monogenic housefly strain and testing potential of establishing a maternal based sex determining mechanism in pest insects for generating strains in which females produce only male progeny conditionally"

Brief introduction (1-2 short paragraphs):
In many insect species eggs are predisposed for female development due to presence of maternal factors which activate the female-promoting gene *transformer* (*tra*) in the early zygote. This self-regulatory feedback loop guarantees a continuous and uninterrupted transmission of the female fate from one to the next generation. To implement the male fate, it is necessary to break the loop to silence *tra*. Typically, this is achieved by dominant Male factors (M) which are paternally transmitted and prevent the zygotic activation of the *tra* loop. Surprisingly, this level of control appears to have diversified extensively as different genes can be employed to act as dominant *tra* loop breakers.

Houseflies provide an excellent model for studying the evolutionary diversification of these M loci. These flies possess several recently evolved proto-Y chromosomes, each containing a male-determining locus (M). Besides the use of paternally transmitted M factors which act post-zygotically, it is conceivable that the *tra* loop can also be interrupted pre-zygotically. Depletion of maternal *tra* during oogenesis would predispose these eggs for male development. The impact of the maternal genotype on sex determination is best documented in the case of the *Ag* strain of *Musca domestica*. The *Ag* mutation in Musca has a dominant maternal effect which causes depletion of maternal *tra* products that are normally deposited into eggs. *Ag* has been suggested to be a germline-specific allele of the Male determining factor (*MI*) located on chromosome I. In order to better understand the mechanism of maternally based only male production, our key objective was to identify and characterize *MI* and *Ag* molecularly.

During this CRP progress has been achieved in the following areas:
- Identification of a potential MI candidate (SAM I)
- Comprehensive structural analysis of SAM I
- Functional analysis of the SAM I based on silencing (RNAi) and targeted disruption (CRISPr/CAS9)
- A potential link of this MI candidate to Ag

Achievements:
1. A differential expression analysis of early mixed sex embryos versus female embryos of the MI strain recovered a set of 588 male enriched sequences. Amongst these we found one candidate which is unique to MI males and named this candidate SAM-1 (Simultaneous Alternative M factor on chromosome 1).
2. Silencing by dsRNA injections or targeted disruption by CRISPR/Cas9 both lead to a significant reduction in MI male fertility and partial feminization of the testes as revealed by presence of nurse cell like cysts and substantial amount of female spliced Mdtra. These results all agree with a pivotal role of SAM-1 in preventing expression of active female tra in MI males.
3. SAM I location on chromosome I has been verified and its genomic surrounding has been analysed. Chromosome quotient analysis showed that both the candidate itself and the flanking regions appear to be of Y- linked nature. This suggests some level of differentiation around the candidate during early proto-Y formation.
4. SAM-1 is also present in genomic reads from monogenic females, which carry the maternal tra repressor *Ag* and produce exclusively male progeny. When reads of these females are aligned to SAM-1, the coverage suggested that only one copy of this sequence is presented consistent with the presence of only one dominant Ag locus. This observation supports the idea that Ag, mapped at the same chromosomal location, is a derived allele of MI that is only active in the female germline.

Recommendations:
1. The location of SAM I within the intron 4 of the BuGZ gene requires further investigation. It is conceivable that this peculiar arrangement affects the BuGZ gene *in cis* suggesting a potential participation of BuGZ as an additional component of the MI locus.

2. A detailed comparison of the SAMI sequences and flanking sequences in MI males and Ag females may reveal regulatory elements responsible for changes in expression.
3. Our functional tests have failed yet to show complete male-to-female reversal as would be expected when M is disrupted. Thus, a new targeting strategy and a better understanding of how SAM-1 works will be needed to test its necessity for proper male development.

Publications:

1. Li X, Visser S, Son JH, Geuverink E, Kıvanç EN, Wu Y, Schmeing S, Pippel M, Anvar SY, Schenkel MA, Marec F, Robinson MD, Meisel RP, Wimmer EA, van de Zande L, Bopp D, Beukeboom LW. Divergent evolution of male-determining loci on proto-Y chromosomes of the housefly. Nat Commun. 2024 Jul 16;15(1):5984. doi: 10.1038/s41467-024-50390-1

Contract 23468: Zhijian Jake Tu, Department of Biochemistry, Virginia Polytechnic Institute and State University; Collaborators:Kostas Bourtzis, Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

"Generic markers for developing genetic sexing strains of *Aedes aegypti* and other insects"

Brief introduction (1-2 short paragraphs):
Integrated pest management (IPM) strategies that build on a combination of approaches will enable the most effective control in different regions of the globe. In the context of vector control, genetic strategies could be implemented to either reduce vector populations or replace competent vector populations with disease-resistant populations, both of which could reduce the transmission of vector-borne infectious diseases. Mass release of a vector species requires that females must be removed before release. 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. To contribute towards the aforementioned objectives, we propose the following three revised specific aims: 1) Systematically identify the causal genes of useful phenotypic markers for developing genetic sexing strains of *Aedes aegypti*. We will integrate 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) further develop methods to produce non-transgenic males by taking advantage of transgene-marked m-chromosomes.

During this CRP progress has been achieved in the following areas:
We decided to explore two sex-linked markers, red-eye and ebony, to accomplish the objectives described in Aims 1 and 2. The causal gene for the red-eye phenotype was unknown prior to our study. To develop a generic genetic sex-separation strategy, it is critical to be able to efficiently identify the causal gene of the selected marker for applications in a wide range of species. This was challenging in Ae. aegypti due to suppressed recombination in large regions including the sex locus. To overcome this difficulty, we developed a marker-assisted-mapping (MAM) strategy to readily screen for and genotype only the rare but informative recombinants, drastically increasing both the resolution and signal-to-noise ratio (Chen et al., 2022). Using MAM, we mapped the sex-linked spontaneous red-eye (re) mutation and subsequently determined, by CRISPR/Cas9-mediated knockout, that cardinal is the causal gene of re, which is the first forward genetic identification of a causal gene in Ae. aegypti. These results provide the molecular foundation for using gene-editing to develop versatile and stable genetic sexing methods by improving upon the current re-based genetic sexing strains (Koskinioti et al., 2021; Misbah-ul-Haq et al., 2022). We also knocked out ebony, a previously sex-linked marker (Li et al., 2017) and confirmed its phenotype. However, this marker may not be suitable for developing sexing strains due to its incomplete penetrance and fitness cost.

We made efforts to develop new sex separation strategies that enable robust sex separation and the production of non-transgenic males for release from a single line of Aedes aegypti. For example, by crossing individuals with transgene-marked, sex-linked recessive lethal alleles, we produce a line in which all non-transgenic individuals are males, and all marked transgenic individuals are females. We also produced a single line in which non-genetically-modified males can be separated from transgenic males and females that can produce more non-GM males, generation over generation. These new methods are compatible with diverse applications including releases where non-transgenic or non-GM males are preferred. These marked m-chromosomes allow the introduction of various features including counter-selection and they will not be inherited by the non-GM males. These lines have been maintained in the laboratory for multiple generations, and we are testing the competitiveness of the non-transgenic males produced by these new lines. We have also demonstrated that counterselection (Matinyan, Karkhanis et al. 2021) could be used to remove transgenic individuals. We have shown that A. aegypti that contains the fcu1 transgene can be killed using 5-fluorocytosine.

Achievements:
1. We developed a marker-assisted mapping method and identified the causal gene for the sex-linked red-eye phenotype, which provided the molecular foundation for developing versatile/generic and stable genetic sexing methods.

2. We developed new sex separation strains that enable efficient sex separation and the production of non-transgenic males from a single line of Aedes aegypti.

Recommendations:

1. The non-transgenic or non-GM males produced by the new methods should be tested in mass-rearing conditions to further assess the stability and competitiveness of these lines.
2. Existing inversions near the sex-locus (e.g., Misbah-ul-Haq et al., 2022) could be integrated with the aforementioned methods to enhance the stability of these lines.

Publications:

1) 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. (2020). *Nix* alone is sufficient to convert female *Aedes aegypti* into fertile males and *myo-sex* is needed for male flight. Proc Natl Acad Sci U S A. 117 (30), 17702-17709.
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Contract 23469: Arturo Rivera Bello, Moscafrut Program AGRICULTURA/SENASICA México. (Collaborators: Jose S. Meza, Al Handler, Edwin Ramirez)

"Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*"

Brief introduction (1-2 short paragraphs):
To improve the SIT application in the Mexican fruit fly, *Anastrepha ludens* control, a genetic sexing system (GSS) based on the pupal color is being used in Mexico and the south of USA, that allows release males exclusively. This GSS uses a *black pupa* marker, which also was found in *A. fraterculus* and *A. obliqua*. Here we propose developing a similar GSS for the second economic importance pest in Mexico, *A. obliqua* and for *A. fraterculus*. On the other hand, to improve the GSS for *A. ludens* by exploring other markers such as slow larval development and to search for candidates for a generic temperature-sensitive lethal mutation.

During this CRP progress has been achieved in the following areas:
After the incorporation the *slow larva* mutant (*sl*) as a second mutation in the *Anastrepha ludens* Tap-7 GSS, which give origin to a new GSS named Tap/slow-7, this new GSS allowed 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. We focused on determining the potential use of mass rearing of the Tap/slow-7 and after several generations in adaption to the mass rearing condition, the new Tap/slow-7 was compared to the Tapachula-7 genetic sexing strain (Tap-7) currently in use, that only carry the *bp* mutation and share the same translocation. The fitness of the new Tap/slow-7 was lower than that Tap-7 and the genetic stability of Tap/slow-7 was weak, resulting in high recombination between the *bp* and *sl* alleles and accumulation of *bp* females throughout the generation and the self-sexing system collapsed.

A new *A. ludens* mutation called necrotic body (*nb*) was isolated, which was not linked to any previously isolated mutation. This new mutation was used to translocate another chromosome of *A. ludens*, which resulted in the fourth translocated chromosome, thus providing a powerful genetic tool for mapping any new marker obtained from this species.

The induction of a GSS for *A. obliqua* based on black pupa color was started, and a new mutation was isolated that, because it affects pupal shape, we named pupal sphere (*sp*). This marker was an autosomal recessive mutation and genetic linkage analysis showed that the *sp* mutation was located on the same chromosome as the black pupa mutation. Finally after testing thousands of males with different irradiation doses (10 to 35 Gy) to induce a Y-autosomal translocation, using *bp* mutation, the highest dose (35 Gy) produced for first time a GSS for *A. obliqua*, where males emerge from normal brown pupa and the females from black pupa.

On another hand, in order to search for candidates for a generic temperature-sensitive lethal mutation, we performed a comparative analysis of the egg transcriptome after exposure to high temperature (35°C) and for that it was analysed the expression levels of genes encoding heat shock proteins (Hsps); a family of proteins produced in response to exposure to stressful conditions. We identified two genes encoding members of the Hsp70 family with a significant up-regulated differential expression in eggs exposed to 35°C, with 6- and 3-fold increases in the level of expression to eggs at 25°C.

Achievements:
1. The *slow larva* mutant of *A. ludens* was included in the Tap-7 GSS, to give origin to Tap/slow-7 GSS.
2. A comparative analysis between the Tap-7 and Tap/slow-7 was performed under mass rearing condition. Resulting the Tap/slow-7 with lower fitness and weak genetic stability.
3. In *Anastrepha obliqua* the *sphere pupa* marker was isolated and studied, resulting in an autosomal and recessive mutation linked to the *black pupae* mutation.
4. The first GSS for *A. fraterculus* based on pupal color was developed.
6. The first GSS for *A. obliqua* based on pupal color was developed.
7. Six RNA-Seq libraries from *A. ludens* embryos exposed to thermal stress at 35°C and control conditions at 25°C.
8. *De novo* transcriptome annotated using multiple databases (UniprotKB, InterPro, GO, KEGG)

9. Identification of 126 genes significantly induced in embryos subjected to 35°C, representing diverse functional categories associated with key biological pathways related to thermosensation and response to heat stress.
10. Characterized two key genes, Al(2)efl and AlHsc70-2, members of the Hsp70 family, which demonstrated significant upregulation under thermal stress at 35°C, as validated through semi-quantitative PCR.
11. Chromosomal Localization of Al(2)efl and AlHsc70-2 in the *A. ludens* genome and genetic structure characterization.

Recommendations:

1. The next phase focuses on continuing inducing new translocations to develop additional GSS lines with enhanced potential for mass-rearing, while scaling up and validating the T(Y;A)-22 and T(Y;A)-364 lines. This includes evaluating their genetic stability, recombination rates, and biological performance under mass rearing conditions. Mating competitiveness with wild populations will also be assessed to ensure field effectiveness. Additionally, cytogenetic and molecular analyses will be conducted to understand recombination mechanisms and monitor chromosomal integrity. These efforts aim to refine the existing GSS lines and integrate the most stable and efficient options into SIT operations

2. If all GSS of *A. obliqua* based on puparium color emerge male from black pupae, a genetic inversion could be carried out in order to avoid this type of recombination.

3. The validated candidate genes Al(2)efl and AlHsc70-2 hold significant potential for advancing genetic sexing strain (GSS) development in *A. ludens*. These thermosensitive genes, identified through transcriptomic analysis, are functionally essential and show homology to key stress-response genes in D. melanogaster. The chromosomal proximity of AlHsc70-2 to the bp (ebony) marker on chromosome 2 offers an opportunity to integrate both a visual marker and a thermosensitive trait into a single GSS design. The next steps involve CRISPR-Cas9-mediated knockdown of Hsc70-2 in wild-type and bp mutant strains to evaluate thermal sensitivity and embryonic or larval lethality at elevated temperatures. Additionally, fitness costs associated with Hsc70-2 knockdown in bp mutants should be assessed under standard conditions to ensure strain viability for mass-rearing. Once validated, chromosomal translocations of the bp marker and modified Hsc70-2 region to the Y chromosome should be pursued using irradiation and crossing protocols or by exploring CRISPR-based targeted translocation techniques if feasible. This strategy builds on existing findings and provides a clear path forward, integrating genetic modifications and functional evaluations to construct a robust GSS strain. If successful, this approach will significantly enhance the efficiency and scalability of SIT programs for *A. ludens*.

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Contract (Agreement) 23470: Ernst A. Wimmer, Hassan M.M. Ahmed, 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. (Collaborators: Philippos A. Papathanos, Kostas D. Mathiopoulos, Marc F. Schetelig, Antonios Augustinos, Giuseppe Saccone)

"Generation of foreign DNA-Free sexing strains based on temperature sensitive lethals and Y-chromosomal rescue by genome editing tools"

Brief introduction (1-2 short paragraphs):
Successful genetic sexing strains (GSS) can be 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. In this project, we tried to transfer suitable tsl mutations identified in the baker's yeast *Saccharomyces cerevisiae* by genome editing to the agricultural pest, the cherry vinegar fly *Drosophila suzukii*. In addition, we started to identify suitable loci on the Y-chromosome of *D. suzukii* for transgene integration and expression. Current genome editing tools should make it possible to create the tsl allele in a way to resemble a classical mutant, which could also come to existence by mutagenesis, and to introduce a wild-type copy of that gene onto the Y-chromosome to resemble a small translocation, which could also come about by chromosomal breaks and rearrangements as induced by classical mutagenesis approaches.

To generate tsl-based GSS for the invasive fruit pest *D. suzukii*, we started designed engineering of highly conserved proteins. Based on yeast screens and rational construction of alleles for orthologs or related paralogs, several genes had been made temperature sensitive by single amino acid substitution in yeast. These genes code for highly conserved proteins such as ubiquitin-conjugating enzymes. Interestingly, we identified that the range of the permissive temperature and the non-permissive temperature for these tsl mutations are suitable for sexing in sterile insect technique approaches. For genes encoding E2 ubiquitin-conjugating enzymes, a highly conserved proline, which stabilizes a turn in the enzyme, had been mutated systematically to serine (P>S) in different yeast paralogs to generate tsl alleles in each case (Ellison et al. 1991; Betting and Seufert 1996). Interestingly the targeted amino acid is highly conserved throughout the eukaryotes. Once those specific mutations have been introduced in *D. suzukii*, their tsl phenotype will need to be evaluated at different temperatures. Then gene fragments need to be identified, which are sufficient to rescue the tsl alleles at the non-permissive temperature. This identification will be informed by comparative genomics data on synteny and phylogenetic sequence conservation.

To introduce rescuing gene fragments for the tsl mutations by CRISPR/Cas9-induced HDR to create a translocation without the introduction of heterologous DNA, first suitable Y-chromosomal loci have to be identified. Therefore loci on the *D. suzukii* Y chromosome will be examined for their suitability to express a fluorescent marker driven by different stage specific promoters. After integration, the rescue of the tsl alleles will have to be evaluated and finally a *D. suzukii* GSS could be generated by combining the homozygous tsl allele with Y-chromosome bearing the rescue fragment. The GSS will then have to be evaluated for fitness and male competitiveness.

During this CRP progress has been achieved in the following areas:

To examine translocation of rescuing fragments onto the Y-chromosome and evaluate expression at those sites, we have improved on the CRISPR/Cas9 genome editing technology as well as insect transgenesis approaches in *D. suzukii* and have identified functional germ line and early embryonic promoters (Ahmed et al., 2019; 2020). In addition, germline-specific Cas9 driver constructs using *Ds_nanos* and *Ds_vasa* regulatory elements were constructed in *piggyBac* transposon vectors to generate fly lines that have improved genome editing rates. Moreover, recombinase-mediated cassette exchange (RMCE) vectors have been functionally verified in *D. suzukii* and *piggyBac* jump-starter lines have been generated to facilitate removal of *piggyBac* insertions, that are used for first marking the genome editing events. Furthermore, to target *piggyBac* integrations to specific loci (especially on the Y-chromosome) an RNA-guided hyperactive transposase (*dCas9-mhyBase*) has been constructed.

To generate tsl mutations in the cherry vinegar fly, we have identified *D. suzukii* orthologs of the yeast *UBC2*, *UBC3*, and *UBC9* genes. These genes have been cloned, mutated versions have been generated by PCR mutagenesis, and potential gRNAs were identified and functionally evaluated. The mutated versions will be used to replace the wild type allele using CRISPR/Cas9 genome editing. Into UBC2, the P>S mutation has already been successfully introduced by HDR bringing along a transformation marker, which can be seamlessly removed by flanking *piggyBac* inverted terminal repeat (ITR) by an additional step to leave behind the genome-edited allele.
Based on the sequence information of the *D. suzukii* Y-chromosome provided by Philippos A. Papathanos's group, we started to introduce attP docking sites into Y-unique Kmers (YUKs) around the genes Kl3, Kl2, and ARY. Regions spanning those genes were PCR amplified and verified by sequencing. Three gRNAs were chosen upstream of ARY and a repair template is currently being generated for CRISPR/Cas9-induced HDR.

Achievements:
1. Generation of additional molecular tools to improve genome editing in *D. suzukii*.
2. Improvement on transposon- and site-specific-recombination-based transgenesis approaches in *D. suzukii*.
3. Identification, cloning, and mutagenesis of suitable genes to generate tsl mutations.
4. Identification of unique Y-chromosomal sites close to active genes for targeted gene integration.

Recommendations:
1. Establishment of additional strategies to generate tsl genes or proteins by transfer or design.
2. Examination of different strategies to target gene integration on Y chromosomes or male-determining gene loci based on CRISPR/Cas9 (HDR, NHEJ) gene editing or RNA-guided transposases.
3. Development of improved strategies to identify potential integrations on Y chromosomes or male-determining gene loci.

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Contract 23823: Thabo Mashatola, Witness Ramashia, Lizette L Koekemoer and Givemore Munhenga (Collaborators: Cyrille Ndo, Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), Kostas Bourtzis, Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Hanano Yamada, IPCL, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture).

"Development of Temperature Sensitive Alleles to Eliminate Females during Mosquito Mass Production: Steps Towards Development of the Sterile Insect Technique against *Anopheles*"

Brief introduction (1-2 short paragraphs):
The goal of this research was to develop temperature-sensitive alleles for use in the SIT against Anopheles mosquitoes, particularly *An. arabiensis*, to aid in malaria vector control. The project aimed to identify and isolate visible or conditional lethal mutations as selectable markers, with the ultimate goal of translocating these mutations to the sex-determining chromosome. This would create a genetically stable and operationally feasible genetic sexing strain (GSS) for mosquito mass production and release. Key objectives included screening laboratory colonies for temperature sensitivity, genetic mapping of lethal alleles and morphological markers, and using irradiation to induce translocations, ultimately establishing an effective GSS system.

Progress has been made in understanding the thermal limits for *An. arabiensis* survival and the effects of mutagens such as ethyl methanesulfonate (EMS) and gamma radiation in inducing temperature-sensitive lethal alleles and morphological mutations. Although challenges in isolating families carrying the desired mutations were encountered, efforts were shifted to refining protocols for developing GSS and exploring the genetic and molecular characterization of new mutant lines (gene editing). Additionally, the research expanded to include development of transgenic strains and investigation of the feasibility of sorting and releasing non-transgenic males, further advancing the potential of SIT for vector control.

During this CRP progress has been achieved in the following areas:
1. **Mutagenesis with gamma irradiation and EMS**: Successful optimization of mutagen concentrations showed that higher doses of gamma irradiation and EMS led to reduced fecundity and fertility in *An. arabiensis*, with EMS inducing more pronounced morphological mutations compared to gamma irradiation. These findings highlight the potential of these mutagenesis methods for creating conditional mutations necessary for developing a GSS for vector control.
2. **Iso-family studies**: The research involved assessing hundreds of iso-females from both EMS-treated and control groups. The EMS treatment resulted in significantly lower egg hatching rates and fecundity, with promising indications of potential *tsl* mutations, though a fully functional GSS was not achieved.
3. **Transgenic strain development**: The development of a fluorescent-based transgenic sexing strain (KWAG-AY2) demonstrated high efficiency in sexing and favorable life-history traits, such as better survival rates and higher flight ability in males compared to the reference strains, showcasing its potential for mass rearing in the SIT program.
4. **Microinjection trials**: Embryonic microinjections with CRISPR Cas-9 constructs (ebony and cardinal genes) showed initial success in hatching and recovering larvae. However, no CRISPR-mediated genetic modifications were detected in the adult progeny, highlighting the need for further refinement of the microinjection and CRISPR techniques to achieve the desired genetic alterations for GSS development.

Achievements:
1. Successfully established relationship between mutagen concentration and reproductive metrics.
2. The creation of the KWAG-AY2 fluorescent-based transgenic sexing strain.
3. Successful recovery of larvae following CRISPR-based genetic modifications.

Recommendations:
1. **Refinement of mutagenesis protocols**: Given the promising results with gamma irradiation and EMS, it is recommended to further optimize the concentrations and exposure durations of these mutagens to enhance

mutation rates while minimizing negative impacts on mosquito fitness. Additional research should focus on fine-tuning the balance between mutagen effectiveness and biological viability for developing conditional mutations suitable for GSS.

2. **Further investigation into *tsl* mutations**: While potential *tsl* mutations were identified in iso-family studies, a fully functional GSS was not developed. It is recommended to continue isolating and breeding for *tsl* mutations, particularly those with stable inheritance patterns, to establish a robust GSS for mass production. Further heat-shock screening and inbreeding strategies may be needed to increase mutation detection rates and improve the system's functionality.
3. **Optimization of transgenic strain development**: The fluorescent-based KWAG-AY2 transgenic strain demonstrated high sexing efficiency and favorable life-history traits. It is recommended to continue developing and testing this strain under field conditions, focusing on evaluating its long-term genetic stability, reproductive performance, and compatibility with the SIT. Also, investigate the possibility of combining this strain with another fluorescent strain that can allow sorting and release of non-transgenic males. Finally, addressing potential risks and regulatory challenges associated with transgenic strains should be prioritized.
4. **Improvement of CRISPR microinjection efficiency**: Despite promising recovery of larvae, no successful CRISPR events were detected in the G0 adults. Future efforts should focus on refining the microinjection process, optimizing CRISPR Cas-9 constructs, and improving the efficiency of genetic modification. This may involve exploring alternative gene editing techniques, enhancing delivery methods, or utilizing more efficient CRISPR systems to achieve targeted genetic modifications for GSS development. Also test out other gene editing methods such as RNA interference.
5. **Collaboration and resource sharing**: To accelerate progress, it is recommended to continue collaborating with other research institutions and stakeholders involved in insect vector control programs. Sharing resources, data, and methodologies will help overcome technical challenges, improve experimental designs, and facilitate the scaling up of successful GSS development strategies for use in operational vector control programs.

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2. Mashatola et al. 2025. Recent advances in sexing strategies for *Anopheles arabiensis*: Implications for genetic vector control strategies and malaria eradication. In preparation.

Contract 24085: Muhammad Misbah ul Haq (Collaborators: Antonios Augustinos, Katerina Nikolouli, Kostas Bourtzis)

"Hunt for naturally existing *tsl* mutation in *Aedes aegypti* and *Ae. albopictus* for construction of more robust genetic sexing strain (GSS) for SIT"

Brief introduction (1-2 short paragraphs):
The SIT program for mosquitoes relies on the mass production and release of sterilized males, with the critical goal of ensuring zero release of sterilized females, as only females feed on blood and are responsible for pathogen transmission. However, sex separation in mosquito SIT programs remains a significant challenge, requiring improvements to enhance efficiency. Developing a technique that can reliably and efficiently eliminate females from the production system at the earliest possible stage would greatly improve the SIT program. Such a technique would reduce operational costs and minimize the risk of accidentally releasing females into the environment. Construction of suitable Genetic Sexing Strains (GSS) can be the one of the solutions for this issue

In this project, we aimed to identify naturally occurring temperature-sensitive lethal (*tsl*) mutations in wild populations of *Ae. aegypti* and *Ae. albopictu*s to develop a genetic sexing strain (GSS) in which males are heat-resistant while females are heat-sensitive and can be selectively eliminated for efficient sex separation. Wild populations of *Ae. aegypti* and *Ae. albopictus* were collected from diverse geographical regions across Pakistan. Area-wise mass and iso-male families were established in the laboratory and scaled up for thermal screening. A thermal screening protocol was developed to identify heat-resistant strains that could potentially harbor *tsl* mutations. Different developmental stages were subjected to thermal stress to identify the presence of *tsl* mutations in the wild populations. If these *tsl* mutations are found and confirmed, their integration with existing GSSs based on phenotypic markers like Red-eye GSS/Inv , Red-eye GSS/Inv35, White-eye GSS etc. will significantly enhance the efficiency of sex separation in Aedes mosquitoes for mass-rearing purposes. The cost and labour will also be reduced at mass scale.

During this CRP progress has been achieved in the following areas:
The project aim was to identify naturally occurring temperature-sensitive lethal (*tsl*) mutations in wild populations of *Aedes aegypti* and *Ae. albopictus* from diverse regions of Pakistan. The progress made so far is as following:

1. Collection and Colony Establishment.
Eggs and larvae were collected from 10–12 climatic and topographic zones across Pakistan using ovitraps and larval inspections in collaboration with district dengue vector surveillance teams. Wild mass colonies of both Aedes species were established and upscaled in the laboratory for thermal screening and detection of *tsl* mutation. Similarly, 100 iso-male colonies from various locations of both Aedes species were established for *tsl* screening.

2. Setting up of Thermal Screening protocols.
First-instar larvae (L1), fourth-instar larvae (L4), and pupae were exposed to temperatures ranging from 27°C to 41°C for durations of 2 to 24 hours to determine optimal conditions for tsl mutation detection. Initial screenings identified 39°C and 40°C (for 3 and 5 hours) as suitable conditions for extensive testing. All the mass and iso male colonies were screened out on the above-mentioned thermal stress regime and identification of potential heat-resistant strains possibly having tsl mutation.

3.Identification of potential heat-resistant mass family.
Among the tested colonies, the *Ae. aegypti* colony "Koh Aedes Aeg" exhibited significantly higher resistance at 39°C and 40°C based on L4 and pupation rates when L1 and L4 larvae were exposed respectively. However, inconsistencies were noted at 41°C, suggesting it is unsuitable temperature for evaluating tsl mutations. Exposure of pupae to the same thermal regime revealed that male pupae had higher heat tolerance compared to female pupae, but further investigation is required to confirm these findings.

4.Iso-Male Family Screening and bracketing a potential iso-male family.
Iso-male families of both Aedes species were established and subjected to a similar thermal screening regime. One iso-male family (iso-male aegypti-7) demonstrated slightly higher and repeatable survival under thermal stress, though the results remain inconclusive. These families will undergo additional testing in future experiments.
Overall, the project has made significant progress in identifying potentially heat-resistant strains, with "Koh

Aedes Aeg" showing promise. However, further research is required to confirm the presence of tsl mutations and establish a reliable GSS.

Achievements:

1. Collection, establishment and upscaling of wild mass and iso-male colonies from 10-12 different climatic and topographic regions of Pakistan for *tsl* detection and SIT application.
2. Determination of thermal (temperature and duration) protocol for screening of Aedes strains for detection of *tsl* on the basis of thermal resistance or sensitivity.
3. Identification of a heat-resistant wild mass family named "Koh Ae. aegypti" having potential of having natural *tsl* mutation for construction of *tsl* based *Ae. aegypti* GSS.

Recommendations:

1. Single pair screening.

Screen individual pairs of *Aedes aegypti* and *Aedes albopictus* collected from diverse climatic zones to identify *tsl* mutations. Use the identified mutations to establish homozygous populations for further study and application.

2. Introgression of identified tsl in Red-eye GSS/Inv35-PAK

introgression of already identified *tsl* by other CRP members into Pakistani Red-eye GSS/Inv35-PAK to develop GSS for SIT application in Pakistan.

3. Screening of Additional Populations

Expansion of the geographical range of collections to include regions with extreme climatic conditions to maximize genetic diversity and potential for discovering *tsl* mutations to strengthen the dataset especially of *Ae. albopictus*.

4. Behavioral and Reproductive Studies.

Characterize the Red-Eye GSS with and without inversion in terms of mating competitiveness, reproductive fitness, and other biological attributes under laboratory, semi-field, and field conditions to assess its suitability for Sterile Insect Technique (SIT) applications.

5. Confirmation of *tsl* Mutations in Identified Strains:

Investigating the genetic basis of observed resistance to confirm whether it aligns with a *tsl* mutation.

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4. Misbah-ul-Haq, M.; Carvalho, D.O.; Duran De La Fuente, L.; Augustinos, A.A.; Bourtzis, K. (2022). Genetic Stability and Fitness of Aedes aegypti Red-Eye Genetic Sexing Strains With Pakistani Genomic Background for Sterile Insect Technique Applications. Front. Bioeng. Biotechnol., 10, doi:10.3389/fbioe.2022.871703.
5. Misbah-ul-Haq M, Augustinos AA, Carvalho DO, Duran de la Fuente L, Bourtzis K. The Effect of an Irradiation-Induced Recombination Suppressing Inversion on the Genetic Stability and Biological Quality of a White Eye-Based *Aedes aegypti* Genetic Sexing Strain. *Insects*. 2022; 13(10):946. https://doi.org/10.3390/insects13100946.

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

9 to 13 December 2024

Monday, 9 December 2024 (M building: M6)

08:00 – 09:00 Identification and registrations at the VIC Gate (next to subway station U1); Carry the passport and grounds passes to be obtained.

09:00 – 09:10 **Rui Cardoso Pereira** - Welcome statement.

09:10 – 09:20 **Kostas Bourtzis** - Goals of the meeting.

SESSION I: Presentations by participants (Chairperson: Katerina Nikolouli)

09:20 – 10:00 **Amanda Amorim Da Silva Cardoso, Chrysanthi Ioannidou, Katerina Nikolouli, Giovanni Petrucci**, Mariaeleni Grigoriou, Chujia Chen, Austin Compton, Zhi Gong, Kostas Mathiopoulos, Zhijian Tu, Marc Schetelig & Kostas Bourtzis - "Neoclassical genetic sexing strains for SIT: evaluation of selectable markers in tephritids and mosquitoes".

10:00 – 10.30 *Coffee Break & Group Photo*

10.30 – 11:00 Roswitha A. Aumann, Lucas Prates, Germano Sollazzo, Kostas Bourtzis & **Marc Schetelig –** "Markers, rescue constructs, and sex-linkage: evaluating components for neoclassical GSS".

11:00 – 11:30 Amanda Choo, Thu Nguyen, Elisabeth Fung, Sheina Sim, Scott Geib, Dani Paulo, Anzu Okada, Kostas Bourtzis, Peter Crisp & **Simon W. Baxter** – "Advancing Bactrocera fruit fly control using genetics".

11:30 – 12:00 Hassan M. M. Ahmed, Chun Yin Leung & **Ernst A. Wimmer** – "Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools".

12:00 – 13:00 *Lunch Break*

13:00 – 13:30 Venetia Karathanasi, Gerasimos Giannatos & **Antonios Augustinos** – "Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species".

13:30 – 14:00 Serafima Davydova, Jonathan Mann & **Angela Meccariello –** "GSS generation: Y-dependent and Y-independent approaches" (virtual presentation).

14:00 – 14:30 Dimitrios Rallis & **Kostas D. Mathiopoulos** – "Exploring the structure and function of Tephritid Y chromosomes".

14:30 – 15:00 **František Marec**, Kristyna Pospisilova, Kristina Pinkrova, Tereza Vajnarova, Atsuo Yoshido & Arjen E. van't Hof – **"**Summary of work on the development of genetic sex strains and the study of sex determination in the codling moth, *Cydia pomonella*".

15:00 – 15:30 *Coffee Break*

15:30 – 16:00 Fernan R. Perez Galvez, Richard Furlong & **Alfred M. Handler** – "Target genes for male sterility, sexing and visible markers in *Drosophila suzukii* and *Anastrepha suspensa*".

16:00 – 16:30 Máximo Rivarola, Claudia A. Conte, Berube Pierre, Chen Shu-Huang; M. Cecilia Giardini, Alejandra C. Scannapieco, Fabián H. Milla, María C. Soria, Romina M. Russo, Juan P. Wulff, Haig H Djambazian, Rolando R. Pomar, Alfred M. Handler, Kostas Bourtzis, Ioannis Ragoussis & **Silvia B. Lanzavecchia** – "Chromosome-scale genome assembly of Argentine *Anastrepha fraterculus sp. 1*: providing tools for classical and innovative species-specific pest control strategies".

16:30 – 17:00 **Edwin Ramírez**, Pedro Rendón & Cristian Morales – "Development and evaluation of genetic sexing strains of fruit flies to be used for sterile insect technique applications" (virtual presentation).

17:00 – 17:30 General discussion

Tuesday, 10 December 2024 (M building: Press room)

SESSION I (cont'd): Presentations by participants (Chairperson: Simon Baxter)

08:30 – 09:00 **Nidchaya Aketarawong**, Kamoltip Laohakieat, Sujinda Thanaphum & Thanalai Poonsiri – "Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera spp.* using bioinformatics and molecular tools".

09:00 – 09.30 Dan Deng, Shisi Xing, Wen Wen, Xiu Bao & **Wei Peng** – "The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii*".

09:30 – 10:00 **José S. Meza**, Daisy P. Cárdenas-Enriquez, Victor García-Martínez, Jorge Ibañez-Palacios & Brenda Torres Huerta – "Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*".

10:00 – 10:30 *Coffee Break*

10:30 – 11:00 Anthony Bayega, Spyros Oikonomopoulos, Dimitris Rallis, Dionisia Mavritsakis, Antonia Spanomitrou, Kostas D Mathiopoulos & **Jiannis Ragoussis** – "Long-read RNA-seq delineates temporal transcriptional dynamics in multiplexed and sexed single medfly embryos".

11:00 – 11.30 **Giuseppe Saccone**, Serena Aceto, Sarah Maria Mazzucchiello, Gennaro Volpe, Dora Baccaro, Mattia Farracchio, Stefania Liguori, Domenico De Falco, Fulvio Bertolotto, Ennio Giordano, Marco Salvemini, Luciana Esposito & Luigi Vitagliano – "Evolution of the Y-linked MoY male determining factor in *Ceratitis* and *Bactrocera* genera: structural and functional predictions".

11:30 – 12:00 Ece Kivanc, Ivan Spöcker, Samuel Jung, Claudia Brunner, Elzemiek Geuverink, Leo Beukeboom & **Daniel Bopp** – "Functional and genomic studies of SAM-1, a novel male determiner candidate on chromosome 1 of the common housefly *Musca domestica*".

12:00 – 13:00 *Lunch Break*

13:00 – 13:30 **Thabo Mashatola**, Witness Ramashia & Givemore Munhenga – "Developments of *Anopheles arabiensis* genetic sexing strains: Progress, challenges, and future directions for Sterile Insect Technique-based malaria vector control".

13:30 – 14:00 **Cyrille Ndo** – "Development of a temperature sensitive lethal-based genetic sexing strain in the malaria vector *Anopheles arabiensis*: achievements and challenges".

14:00 – 14:30 **Muhammad Misbah ul Haq** & Abdul Aziz – "Hunt for naturally existing tsl mutation in *Aedes aegypti* and *Ae. albopictus* for construction of more robust genetic sexing strain (GSS) for SIT".

14:30 – 15:00 *Coffee Break*

15:00 – 15:30 **Melanie Hempel**, Austin Compton, Atashi Sharma, Azadeh Aryan, Wanhao Chi, Xiaoxi Zhuang, Mark Potters & Zhijian Tu **–** "Differential and conditional elimination of homomorphic sex chromosomes in *Aedes aegypti* for population control".

15:30 – 16:00 Doron S. Y. Zaada, Or Toren, Denys Gildman, Shira Kehat, Daniella A. Haber, Vytautas Mackevičius, Eric Marois, Irina Häcker, Marc F. Schetelig, Yael Arien1 & **Philippos Aris Papathanos –** "Genetic sexing in *Aedes albopictus* using a synthetic M locus" (virtual presentation).

16:00 –17:00 General discussion

18:00 – 21:00 Group dinner

<u>Wednesday, 11 December 2024 (M Building: Press room, MOE67 and MOE68 rooms)</u>

SESSION II: Final CRP report and new CRP proposal

08:30 – 09:30 **All**: Open discussion on progress made and outcomes achieved.

09:30 – 10:00 **Kostas Bourtzis**: Drafting the Final CRP Report (assignment of tasks).

10:00 – 10:30 *Coffee Break*

10:30 – 12:00 **All**: Drafting the Final CRP Report.

12:00 – 13:00 *Lunch*

13:00 – 15:00 **All**: Drafting the Final CRP Report.

15:00 – 15:30 *Coffee Break*

15:30 – 16:30 **All**: Progress made on drafting the Final CRP Report (assignment of tasks to complete and review the report).

16:30 – 17:00 **Kostas Bourtzis**: Special Issue – Publication Plan.

<u>Thursday, 12 December 2024 (M Building: Press room, MOE67 and MOE68 rooms)</u>

SESSION II (cont'd): Final CRP report and new CRP proposal

08:30 – 10:00 **All**: Brainstorm for a new CRP proposal – Identification of gaps of knowledge.

10:00 – 10:30 *Coffee Break*

10:30 – 11:30 **All**: Brainstorm for a new CRP proposal – Identification of gaps of knowledge.

11:30 – 12:00 **Kostas Bourtzis**: Assignment of tasks for the new CRP proposal.

12:00 – 13:00 *Lunch*

13:00 – 15:00 **All**: Drafting the new CRP proposal.

15:00 – 15:30 *Coffee Break*

15:30 – 17:00 **All**: Drafting the new CRP proposal.

Friday, 13 December 2024 (M Building: Press room, MOE67 and MOE68 rooms)

SESSION II (cont'd): Final CRP report and new CRP proposal

08:30 – 10:00 **All**: Drafting the new CRP proposal.

10:00 – 10:30 *Coffee Break*

10:30 – 12:00 **All**: Progress made on drafting the new CRP proposal (assignment of tasks to complete and review the proposal).

12:00 – 13:00 *Lunch*

13:00 – 13:30 **All**: Presentation of the Final CRP Report.

13:30 – 14:00 **All**: Presentation of the new CRP proposal.

14:00 – 14:15 ***Closing***

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

9-13 December 2024

TITLE OF WORKING PAPER: Neoclassical Genetic Sexing Strains for SIT: Evaluation of Selectable Markers in Tephritids and Mosquitoes

AUTHORS **(presenting authors in alphabetical order): Amanda Amorim Da Silva Cardoso**[1,2], **Chrysanthi Ioannidou**[1,3], **Katerina Nikolouli**[1], **Giovanni Petrucci**[1,4], Mariaeleni Grigoriou[1,5], Chujia Chen[6,7], Austin Compton[7,8], Zhi Gong[9], Kostas Mathiopoulos[5], Zhijian Tu[6,7,8], Marc F. Schetelig[4], Kostas Bourtzis[1]

ORGANIZATION:
[1]Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Laboratories, 2444 Seibersdorf, Austria
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[9]Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan, China

SHORT SUMMARY OF PAPER

Abstract:

Sex separation in insect mass rearing for SIT is often achieved using Genetic Sexing Strains (GSS). In GSS, a genetic marker with a dominant phenotype is expressed differentially between males and females, allowing for easy separation. In a classical GSS, females are homozygous for a recessive mutant phenotype, while males are heterozygous with a dominant phenotype. While successful, these strains present several issues, including genetic instability and semi-sterility. Moreover, their development requires the identification of natural mutants of the marker, which is random in nature and not available in all insect species.

Advances in genome editing have enabled more precise and efficient development of GSS. In addition, the recent discovery of the *wp* and *tsl* genes in *Ceratitis capitata* highlights the effectiveness of a generic approach in aiding the development of sexing systems for insect pests and disease vectors. Knockout lines expressing the *wp* phenotype have been developed in various species, including *Bactrocera dorsalis*, *B. correcta*, *B. oleae*, *B. zonata*, *Zeugodacus tau*, and *Aedes aegypti*. Similarly, the *ebony* gene, which is involved in pigmentation, has been targeted in *Anastrepha* species and *Ae. aegypti*, resulting in distinct phenotypes like black pupae and darker larval heads, respectively. In *Ae. aegypti*, the recent discovery of the gene responsible for the red-eye phenotype paved the way for inducing the same mutation in other disease vector species. Another promising marker, the *levy* gene, which induces temperature-sensitive lethality in *Drosophila melanogaster*, has shown heat-sensitive phenotypes in *Ae. aegypti*.

Identifying orthologous genes across species combined with novel genome editing approaches holds significant potential for constructing innovative GSS in diverse species. By developing GSS with new selectable markers, SIT programs can achieve more stable, effective, and scalable pest and vector control solutions.

TITLE OF WORKING PAPER:
Markers, rescue constructs, and sex-linkage: evaluating components for neoclassical GSS

AUTHOR (S): **Roswitha A. Aumann[1], Lucas Prates[1], Germano Sollazzo[1,2,3], Kostas Bourtzis[2], Marc F. Schetelig[1]**

ORGANIZATION:
[1] Justus-Liebig-University Giessen, Department for Insect Biotechnology in Plant Protection, Giessen, Germany
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[3] Imperial College London, Department of Life Sciences, South Kensington Campus, London, UK

SHORT SUMMARY OF PAPER

Abstract:

Traditional methods for controlling insect pest populations are often ineffective or environmentally harmful, underscoring the need for innovative solutions like the Sterile Insect Technique (SIT). SIT programs involve releasing sterile insects into the wild, and their efficiency and cost-effectiveness can be greatly enhanced by producing male-only populations for release. Such populations can be achieved using genetic sexing strains (GSS), driving growing interest in the development of novel GSS for SIT target insect pest species. Advances in genetic and genomic technologies offer promising avenues for creating a generic approach to develop GSS to enhance SIT programs without relying on transgenic modifications.

Our research focuses on the Mediterranean fruit fly (medfly), an invasive pest that causes significant economic losses, for which a highly functional GSS has been developed based on spontaneous or chemically induced mutations that produce two distinct phenotypes: white puparia and temperature-sensitive lethality (tsl).

We identified the gene responsible for the white puparia phenotype, known as *white pupae* gene (Ward et al., 2021), and investigated two highly promising candidate genes for the tsl phenotype: *deep orange* (Sollazzo et al., 2024) and *Lysyl-tRNA synthetase (LysRS)*. Our findings confirmed a link between the original medfly tsl phenotype and the *LysRS* gene. By employing CRISPR/Cas9-mediated homology-directed repair (HDR), we engineered specific mutations in *LysRS*, conclusively demonstrating its role in the tsl phenotype. This discovery provides a molecular basis for temperature-sensitive lethality, facilitating the development of more efficient GSS.

Towards developing a generic approach for GSS design, we created a *mini-white pupae* (*mini-wp*) and a *mini-LysRS* rescue construct and integrated them at various autosomal positions in the respective mutant strains. The successful rescues of these phenotypes from different genomic locations confirms the robustness and versatility of the *mini-gene* systems in generating viable, selectable markers for GSS development.

Furthermore, we attempted the integration of the *mini-wp* cassette into the Y chromosome through remobilization experiments and HDR targeting of selected Y loci. Although integration into the Y chromosome poses technical challenges, our efforts represent a step toward developing male-specific expression systems, crucial for eliminating females in SIT programs.

Our findings advance the creation of non-transgenic GSS in medfly, offering an environmentally friendly and potentially regulation-compliant approach to pest management.

TITLE OF WORKING PAPER: **Advancing Bactrocera fruit fly control using genetics**

AUTHOR (S): Amanda Choo[1], Thu Nguyen[2], Elisabeth Fung[3], Sheina Sim[4], Scott Geib[4], Dani Paulo[4], Anzu Okada[1], Konstantinos Bourtzis[5], Peter Crisp[3] & Simon Baxter[2]

ORGANIZATION: *[1]School of Biological Sciences, University of Adelaide, Australia, [2]School of BioSciences, University of Melbourne, Australia, [3]South Australian Research and Development Institute, Adelaide, Australia, [4] U.S. Department of Agriculture, Pacific Basin Agricultural Research Center, Hilo, USA, [5]Insect Pest Control Laboratory, Department of Nuclear Sciences and Applications, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Austria*

SHORT SUMMARY OF PAPER

Abstract:

The Queensland fruit fly, *Bactrocera tryoni*, is a serious Australian horticultural pest that can be controlled using the sterile insect technique (SIT). Sterile factory reared males are released and mate with wild females that produce inviable embryos. A major challenge of SIT is removal of factory females, which hinder release efficiency. Here we use CRISPR/Cas9 genome editing to develop multiple *B. tryoni* pupal colour and temperature sensitive mutants and use one to create a genetic sexing strain to visually identify males. Mutations created in the pigmentation gene *ebony* resulted in a black puparium and a dark adult body colour, however, fitness costs were relatively high. This phenotype was akin to the *black pupae* genetic sexing strain used for SIT against the Mexican fruit fly, and genome sequencing subsequently confirmed *ebony* as the causal gene. Next, a previously described *white pupae* gene was used to create a *B. tryoni* genetic sexing strain. Low dose X-ray radiation translocated part of chromosome 5, including a wild-type copy of *white pupae*, to the male Y-chromosome. This produced a strain where male pupae are brown, and females pale grey. Finally, we developed a temperature sensitivity phenotype via inducing mutations in the dynamin gene *shibire*, which may also benefit sex separation. This research can potentially improve SIT efficiency through mechanical sorting and removal of female pupae.

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, Chun Yin Leung** & **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, several strategies could in principle be applied:

A) Transfer of a known tsl allele working in sexing strains of another species: e.g. tsl alleles of the gene deep orange from the Medfly *Ceratitis capitata*.

B) Design of tsl alleles based tsl mutations of highly conserved proteins that were generated in the baker's yeast *Saccharomyces cerevisiae* by transferring such mutations using genome editing.

C) Generate tsl alleles of genes in the sex determination pathway – such as *Sex lethal* (*Sxl*) or *tra* – by adding heat-sensitive N-degrons or inteins.

Whereas strategy C) would only require editing at the targeted sex determination gene locus, the other strategies A) and B) will require in addition a rescue by a wild type allele of the targeted genetic locus translocated to the Y chromosome, which allows only the males to survive at the non-permissive temperature. In this respect, we are identifying suitable loci on the Y chromosome of *D. suzukii* for gene expression in collaboration with Dr. Papathanos, Rehovot, Israel. Promising loci would then be used to place a wild type allele of a tsl gene or to rescue the mutant phenotype specifically in males and thereby generate a GGS in *D. suzukii*. Current genome editing tools should make it possible to create the tsl allele in a way to resemble a classical mutant, which could also come to existence by mutagenesis, and to introduce a wild type copy of that gene onto the Y chromosome to resemble a small translocation, which could also come about by chromosomal breaks and rearrangements as induced by classical mutagenesis approaches. Therefore, strategies A) and B) as well as potentially also the N-degron approach of C) could generate foreign DNA-free neo-classical sexing strains.

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

AUTHOR (S): V. Karathanasi, G. Giannatos, **A. Augustinos**

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

SHORT SUMMARY OF PAPER

Abstract:

The research performed in the frame of this project can be summarized in the following:

- *Separating the wp and tsl mutations in medfly through recombination:* The *wp* and *tsl* loci are physically close on the 5th chromosome. By performing suitable crosses, we managed to develop six (6) lines where these mutations have been separated through recombination. Started from homozygous *wp/tsl* and wild type (EgyptII and Seib) strains, we developed two (2) *wp* and four (4) *tsl* strains, which are stable for more than 35 generations now, presenting the expected sensitivity/resistance to thermal stress. In collaboration with IPCL, sequencing analysis has been performed and data analysis is ongoing. Additional phenotypic characters that are considered to be associated with the *tsl* mutation, including delayed development and reduced hatching, are also being analysed and will be followed up beyond this project.
- *Development of medfly strains combining additional morphological markers of the 5th chromosome and tsl:* Availability of such strains can facilitate the genetic analysis of the 5th chromosome and place the *tsl* mutation in new genetic backgrounds. Through independent recombination events, three (3) *we wp tsl*, three (3) *ye wp tsl*, and three (3) *or wp tsl* strains have been created. These strains exhibit the expected sensitivity to thermal stress. In collaboration with IPCL, sequencing analysis has been performed and data analysis is ongoing. Additional phenotypic characters that are generally considered to be associated with the *tsl* mutation, including delayed development and reduced hatching, are also being analysed and will be followed up beyond this project.
- *Thermal response of recently domesticated medfly populations:* Five recently domesticated populations have been established in our insectary and tested. There were differences among them in respect to the response to the thermal stress but not the difference observed between the 'typical' tsl-resistant and tsl-sensitive strains. This has not been further followed (through, for example, individual crosses) due to the extensive workload needed.
- *Aedes albopictus sensitivity to thermal stress:* Our initial thermal response assays in L1 pointed to 39 ^{o}C – 41 ^{o}C and time exposure ranging between 8 h – 24 h as the best starting point for the identification of sensitive and resistant genotypes in the tiger mosquito. Four populations from Greece have been collected, colonized, and used in thermal response assays. Our results are not conclusive yet, but it seems that we need a combination of two temperatures (39 ^{o}C and 41 ^{o}C) and two different time exposures to the selected temperatures (8 h and 24 h) to create a robust thermal response profile for each population. Research on this is continuing beyond this project.
- *Interaction between Wolbachia, tsl phenotype, and genetic stability of the medfly VIENNA GSSs:* Symbiotic profile can interact not only with fitness and response of the host to different stresses (such as increased temperatures) but also with the rate of recombination, as recently suggested in *Drosophila*. *Wolbachia* was introduced to the VIENNA 8 GSS and the VIENNA 8 GSS (*w*Cer2] VIENNA 8 GSS (*w*Cer4] were developed. After keeping these strains under filtering for more than 25 generations now and without filtering for more than 20 generations, it seems that there is no negative effect on genetic stability and/or response to the typical 'tsl' thermal stress. Research on this will go on beyond this project, both in respect to the effect of *Wolbachia* (and other symbionts) on the behaviour/stability of the VIENNA 8 GSS but also targeting the possible effect on recombination rates in other, more suitable, medfly strains.

TITLE OF WORKING PAPER: GSS generation: Y-dependent and Y-independent approaches

AUTHOR (S): Serafima Davydova, Jonathan Mann, Angela Meccariello

ORGANIZATION: Imperial College London

SHORT SUMMARY OF PAPER

Abstract:

Tropical fruit flies are considered among the most economically important invasive species detected in temperate areas of the United States and the European Union. Eradication and containment campaigns levied against invasive fruit flies in many continents include repeated treatment of large areas with preventive releases of sterilized males (employing Sterile Insect Technique (SIT) technology), and/or an extensive use of pesticides. SIT efficiency, cost-effectiveness, as well as safety and biosecurity, depends on the availability and use of genetic sexing strains (GSS), to remove females from the male-only population that must be released. Despite the importance of sex separation, 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 of developing a GSS in one species is not necessarily directly transferable to other species.

To establish novel GSS, a Y-chromosome-linked approach has been proposed. 5 different regions were verified as male-specific and corresponding constructs were generated, both for homology-directed repair and non-homologous end joining-mediated knock-ins. Multiple embryo microinjections were performed but no Y-linked GSS has been generated to date. For this, an alternative Y-chromosome-independent GSS strategy has been proposed relying on sex-specific splicing of the *transformer* gene.

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

AUTHOR (S): Rallis D, Mathiopoulos KD

ORGANIZATION: Department of Biochemistry and Biotechnology, University of Thessaly, Greece

SHORT SUMMARY OF PAPER

Abstract:

Heteromorphic sex chromosomes comprise fascinating genomic elements due to their unique sequence composition that results from the distinct processes acting on them during their evolution. Especially for Y chromosomes, it is common for male-determining factors to reside on them, hence limiting their presence only to the male sex, providing a unique area for the establishment and regulation of sex-specific genetic characteristics. The exploration of these dynamics becomes particularly important in pest species that exert their destructive action through only one of the two sexes. In these cases, the sex-limited nature of Y chromosomes puts them in the core of alternative and species-specific population containment methods that manipulate the reproductive dynamics of a local population. Tephritids comprise such a family of insects, with many of their members containing heteromorphic sex chromosomes while their female-oviposition habits render them important agricultural pests. However, the sequence, content and structure of Tephritidae Y chromosomes has been poorly studied due to intrinsic challenges in reconstructing and identifying sequences of the Y. It is common for these elements to accumulate repeats, obtain a highly heterochromatic character and become gene poor, all of which comprise burdens against the trivial characterization of their sequence and composition. Therefore, despite the efforts to exploit the Y in Genetic Sexing Strains for Sterile Insect Technique (SIT) applications against Tephritidae species, this was only partly achieved in a vague, random and species-limited manner. In our work, we employed advanced genomic resources to develop and describe novel methods for assembling, identifying and analyzing Y chromosomes, towards providing the framework for their generic and efficient use in control approaches like the SIT. We used two Tephritidae pest species as models, *Bactrocera oleae* and *Ceratitis capitata*, over which we developed two novel Y-detection methods. These were benchmarked for their efficiency against previously existing methods, where they presented a greater applicability over different Y chromosomes. This provided 3.9 Mb and 26.8 Mb of Y chromosome sequence from *B. oleae* and *C. capitata* respectively, over which we detected putative functional elements. Specifically, two previously uncharacterized genes were annotated on the Y of *B.* oleae, PGY-23 and PGY-35, while we managed to achieve partial silencing through RNAi technology for PGY-35. In addition, we identified multiple partial copies of the genes *imp-4* and *cytosolic aminopeptidase* for which we also achieved silencing of the respective autosomal copies towards inferring a putative regulatory role of the Y amplification. Our initial datasets were further complemented with novel assemblies from the related Tephritidae species *Bactrocera dorsalis* and *Bactrocera zonata*, for which we identified 4.6 Mb and 16.8 Mb of respective Y sequence. An initial comparative analysis among those two closely related Tephritids indicated a large conservation of syntenic regions across the autosomes, which however could not be replicated for the Y chromosome. Finally, the detection of transcription units on the *B. oleae* Y allowed the initial analysis of expression regulation elements and revealed a putative 12 bp regulatory box with possible male specific role.

TITLE OF WORKING PAPER: Summary of work on the development of genetic sex strains and the study of sex determination in the codling moth, *Cydia pomonella*

AUTHOR (S): **Frantisek Marec**[1], Kristyna Pospisilova[1,2], Kristina Pinkrova[1,2], Tereza Vajnarova[1,2], Atsuo Yoshido[1], Arjen E. van't Hof[1]

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

SHORT SUMMARY OF PAPER

Abstract:

Our research on codling moth has two main objectives. First, we aim to develop a generic approach for the construction of genetic sexing strains in Lepidoptera based on the insertion of a dominant conditional lethal mutation (DCLM) into the W chromosome using CRISPR/Cas9 knock-in. Second, we study the molecular mechanisms of sex determination to find new options for genetic sexing. For CRISPR/Cas9, we have two constructs: (i) CpW-DsRedT3-CpNotch, which contains a fluorescent marker gene *DsRedT3* and a truncated allele of the *CpNotch* gene to act as a selectable DCLM, and (ii) CpW-DsRedT3 without the mutant *CpNotch* allele. We also designed sgRNA and verified that it cleaves the corresponding target sequence of the W chromosome using the Cas9 in vitro cleavage assay. We injected the modified CpW-DsRedT3 construct into nearly 3 thousand eggs. In the initial experiments, the survival rate of the injected eggs was very low, but after optimising the microinjection conditions, we achieved a survival rate of about 9% to adulthood. However, none of the females that developed from the injected eggs and mated with untreated males showed signs of successful CRISPR/Cas9 knock-in, as their eggs did not exhibit DsRedT3 fluorescence. Regarding the second objective, we successfully completed the study on the function of the *Masculinizer* gene (*CpMasc*) and demonstrated its essential role in male sex determination. To search for an upstream feminizing factor, we obtained sex-specific small RNA-Seq and mRNA-Seq libraries from early embryos. However, screening of the small RNA-Seq datasets revealed no candidate feminizing piRNA, suggesting that either sex determination in codling moth does not rely on piRNA or that screening of a wider window of embryonic expression is required to identify relevant piRNAs. Through differential expression analysis of mRNA-Seq libraries, we have identified about 100 sex-specific or sex-biased genes, but without further investigation, no conclusion can be made about their role in sex determination. In *Bombyx mori*, sex-specific splicing of *doublesex* (*Bmdsx*) is mediated by the male-specific isoform of the *Bombyx* homolog of IGF-II mRNA binding protein, $BmIMP^{M}$. We have identified a codling moth ortholog *CpImp*. However, no male-specific splice variant of *CpImp* was found in codling moth transcriptomes. Our results suggest that the *B. mori* sex-determining pathway is only partially conserved in the codling moth.

TITLE OF WORKING PAPER: Target genes for male sterility, sexing and visible markers in *Drosophila suzukii* and *Anastrepha suspensa*

AUTHORS: Fernan R. Perez Galvez, Richard Furlong, and Alfred M. Handler

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

SHORT SUMMARY OF PAPER

Abstract:

Previously we identified cognates for two genes necessary for male fertility in *Drosophila melanogaster*, *wampa* and *Prosalpha6T*, in the spotted-wing drosophilid, *Drosophila suzukii*, and the Caribbean fruit fly, *Anastrepha suspensa*. Highly elevated testis-specific gene expression of both genes, similar to *D. melanogaster*, was observed in *D. suzukii*, but only the *As-wampa* cognate exhibited sex-specific testis expression in *A. suspensa*, while the putative *As-Prosalpha6T* gene exhibited non-sex-specific constitutive expression in both adult males and females. Further structural and transcriptional analysis of the putative *As-Prosalpha6T* cognate indicated that the gene isolated is actually the non-sex-specific *Prosalpha6* isoform. Its expression in adult male testes, however, indicates that the transition to the *Prosalpha6T* isoform that occurs in *D. melanogaster* testes does not occur in *A. suspensa*, and possibly other tephritid species where WGS annotations only identify the *Prosalpha6* subunit alpha-1 cognate. It is known, however, that sterile null mutants for *Prosalpha6T* in *D. melanogaster* can be rescued by testis-specific ectopic expression of *Prosalpha6*, suggesting that cognates of *Prosalpha6* may assume this role for fertility in tephritid male spermatocytes. For *Ds-wampa*, a CRISPR-Cas9 HDR knock-out was successfully created *D. suzukii*, though four sibling G1 lines were all recessive lethal, and it remains to be determined if *Ds*-Wampa has a vital larval/pupal function previous to spermiogenesis in *D. suzukii*. Additional CRISPR-Cas9 mutagenesis studies performed in the *A. suspensa* genes for the *white pupae* (*wp*) marker and *deep orange* (*dor*) that encodes the *tsl* mutation used for sexing in medfly (Sollazzo et al., 2024. *BMC Biotechnol* 24, 7). An *A. suspensa wp*$^{-}$ mutant line was created, while initial *dor* HDR gene-editing resulted in marker fluorescence in several G0 larvae and pupae, none survived possibly due to targeting exon 4, known to effect pupal lethality in medfly. New target sites will be tested, including those within exon 6 now known to include the *tsl* site in medfly. To expand the number of pest species amenable to control by genomic manipulation, the cornsilk fly, *Euxesta eluta*, was transformed with high efficiency for the first time with an IE1-DsRed marked *piggyBac* vector indicating that germ-line transformation and gene-editing methodologies may be used routinely in this and other cornsilk fly species.

TITLE OF WORKING PAPER: **Chromosome-scale genome assembly of Argentine *Anastrepha fraterculus* sp. 1: providing tools for classical and innovative species-specific pest control strategies**

AUTHOR (S): Máximo Rivarola[1], Claudia A. Conte [2], Berube Pierre [3], Chen Shu-Huang [3]; M. Cecilia Giardini, [2], Alejandra C. Scannapieco [2], Fabián H. Milla [2], María C. Soria [2], Romina M. Russo[2], Juan P. Wulff [4], Haig H Djambazian[3], Rolando R. Pomar[5], Alfred M. Handler[6], Kostas Bourtzis[7], Ioannis Ragoussis[3], Silvia B. Lanzavecchia[2]

ORGANIZATION: [1] Instituto de Biotecnología IABIMO-CONICET, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina. [2] Instituto de Genética gv al IABIMO-CONICET, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina. [3] McGill Centre, Montreal, Canada. [4] North Carolina State University. NCSU, USA. [5] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina. [6] U.S. Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608, USA. [7] Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria.

SHORT SUMMARY OF PAPER

Abstract:

Anastrepha fraterculus morphotype 1 is a major pest affecting fresh fruit production in Argentina and neighboring countries. To manage wild populations of this pest, integrated pest management (IPM) strategies are employed, which include chemical treatments and trapping. Additionally, environmentally-friendly control methods, such as the sterile insect technique (SIT), are currently being developed. Genetic sexing strains (GSS) of *A. fraterculus* sp. 1 have been established and are undergoing evaluation. Our study aims to provide genomic insights into this morphotype, supporting both SIT and innovative control methods, including genetic engineering and RNA interference. Using high-fidelity long-read and short-read sequencing technologies, we analyzed high molecular-weight DNA samples from an inbred strain of *A. fraterculus* sp. 1, resulting in chromosome-scale genome assemblies for both female and male samples. The female genome was assembled into 58 scaffolds (760 Mb), while the male one into 68 scaffolds (750 Mb). BUSCO analysis indicated remarkable completeness, with 98.8% and 98.7% for the female and male genome, respectively. Synteny analysis confirmed the assembly of five complete autosomes and facilitated the accurate assembly of the X and Y chromosomes. Gene prediction revealed 22,362 coding genes, with repetitive regions constituting 46% of female and male genomes. The mitochondrial genome was fully assembled and annotated, and nucleotide sequences of associated microorganisms, including symbiotic bacteria, were identified. This research provides a comprehensive genomic characterization of *A. fraterculus* sp. 1 and highlights potential targets for gene editing and RNAi-based biocontrol strategies.

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

AUTHOR (S): Edwin Ramírez, Pedro Rendón, Cristian Morales

ORGANIZATION: Medfly Program – Guatemala

SHORT SUMMARY OF PAPER

Abstract:

As outlined at the start of this CRP, our primary focus was to enhance the Sterile Insect Technique (SIT) by improving the performance of sterile males released in the field for the control of *Ceratitis capitata* and *Anastrepha ludens*, as well as identifying better lines of genetically sexed strains.

For *C. capitata*, as detailed in previous reports, we developed crossing protocols for wild females mating with two different male strains (Vienna 8 / Toliman INV-D53 and Vienna 8 1260). The goal was to leverage the distinctive fluorescent traits of Vienna 8 1260 males in the offspring, which would provide valuable insights into the age of the males at the time of irradiation, as well as their body size. While the effect of male age at irradiation has been previously reported, we conclude that irradiating sterile males close to emergence or shortly after emergence appears to preserve their sexual performance, resulting in better quality sperm transfer and minimizing the likelihood of wild females remating. This suggests that irradiating males at or near their emergence stage, or at adult age, leads to the strongest reproductive suppression.

Regarding male size, we assessed whether it impacts mating success and sperm transfer. Unlike other studies, we did not vary dietary conditions; instead, we selected males directly from the rearing colony without exposure to different food types. We evaluated males of sizes 4, 5, 6, 7, 8, and 9 from the Vienna 8 D53 strain. Most sizes showed no significant effect on egg-to-fluorescent pupa conversion, with only size 6 showing a notable difference. However, the data do not support the conclusion that male size significantly influences sexual performance, particularly in terms of preventing remating by wild females.

For *Anastrepha ludens*, we identified the 166-10 line, which demonstrates complete lethality (100%) when heat treatment is applied to the eggs. To optimize the effectiveness of this heat treatment, we focused on defining three key parameters: 1) the optimal age of the eggs to avoid maternal effects, 2) the duration (in hours) of the heat treatment, and 3) the effective temperature. In recent months, our efforts have been directed toward attempting to translocate thermal sensitivity. The protocol for this translocation was as follows: 1) low doses of irradiation were applied to wild males, 2) irradiated wild males were crossed with females from the 166-10 line, 3) F1 males from these crosses were then mated with females from the 166-10 line, 4) approximately 1,050 families (pairs) were established, 5) eggs from each family were collected and subjected to heat treatment, with the expectation of identifying a positive family (defined by 35-50% hatching and 100% male offspring). Three repetitions of this protocol were conducted in an effort to achieve translocation, but none resulted in the formation of a positive line. Further studies will continue, with the aim of achieving a successful outcome in the future.

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[1,2], Kamoltip Laohakieat[1,2], Sujinda Thanaphum[1,2], Thanalai Poonsiri[3]

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SHORT SUMMARY OF PAPER

Abstract:

Bactrocera carambolae (the carambola fruit fly) is one of the most destructive agricultural insect pests, particularly in Southeast Asia and South America. Three orthologous genes (i.e., *tra*, *tra-2*, and *dsx*) were isolated and characterized. Their structures and functions were highly conserved compared to *B. dorsalis* and *B. correcta*; however, their expression profiles were slightly different. RNA interference (RNAi) of the *Bcartra* gene resulted in phenotypic males (94.12%). These flies were characterized as either males or pseudomales using two approaches: observation of their progeny after single-pair mating with wild-type females and detection of *MoY* sequences in their genomic DNA. The expression patterns of sex-determining genes revealed male-specific splicing of the *tra*, *tra-2*, and *fru* genes in pseudomales. The results supported the relative 1:1 ratio between males and pseudomales. Additionally, the expression profiles of genes related to zygotic and sex determination were studied in fertilized and unfertilized embryos, larvae, pupae, and adults of *B. carambolae*. The results showed that these genes in *B. carambolae* expressed relatively late compared to the closely related species, *B. dorsalis*. The interactions among proteins BcarTRA, BcarTRA-2, and BdorRBP1 (from database) and *Bcartra* pre-mRNA sequences were also predicted and interpreted using the Alphafold and PISA programs, respectively. The results supported the interaction between BcarTRA and BcarTRA-2 as well as the interaction of both proteins with the *Bcartra* pre-mRNA sequences at the putative TRA/TRA-2 binding sites.

CRISPR/Cas9 was used to knock out the *MoY* gene in *B. dorsalis* Salaya 1, a genetic sexing strain based on pupal color, resulting in the production of brown-pupae pseudofemales. These pseudofemales were successfully maintained as new strains under ABSL2 conditions for over a year and will be used for further analysis of gene expression profiles in female flies with an XY background.

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

AUTHOR (S): Dan Deng, Shisi Xing, Wen Wen, Xiu Bao, Wei Peng*

ORGANIZATION: Hunan Normal 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. To study the function of key genes associated with reproduction in regulating sex determination, we have synthesized the double strand RNA of *Sex-lethal* (*Sxl*) and *transformer* (*tra*) genes and have done the micro-injection into the embryos. The RNAi experiments showed that sexual formation is determined early in the embryo stage and female to male sex reversal was achieved by targeting *tra* gene. Nearly all XX embryos developed into fully masculinized phenotypic male adults with no apparent female morphology. Upon dissection abnormal hypertrophic gonads were revealed in XX pseudomales but not in the XY males. We have also studied the function of *testis-specific serine kinase 1* (*tssk1*) gene in the spermatogenesis of *D. suzukii*. We have knock-downed the expression of *tssk1* using RNA interference, resulting in significant disruptions in spermiogenesis, decreased sperm motility, and hindered development of eggs. The female-specific lethality and masculinization genetic sexing strains (GSS) were constructed by targeting *Sxl* and *tra* genes. The drive component, consisting of *Sxl* and *tra* single guide RNA and DsRed genes, was introduced into the female-specific exon of *Sxl* and *tra*, which is essential for function in females. In both *Sxl* and *tra* targeted locus, gene drive allele could achieve super mendelian inheritance. In terms of phenotypes, we found that 100% of the *Sxl* knockout females perished during preadult stages with the majority dying during pupal transition. As expected, abnormal development of both the external and internal genitalia was observed in G0 and G1 female adults and produced the male-specific *tra* and *doublesex* (*dsx*) transcript. Interestingly, knocking out *tra* led to significantly reduced fecundity and fertility in adults of corresponding sex. Moderate transmission rates of the DsRed gene were observed with line that expressed Cas9 with two nuclear localization sequences from the *D. suzukii vasa* promoter. Our results not only confirm the conserved function of *Sxl* and *tra* gene in sex determination but also provide a sustainable and environmentally friendly means for control of *D. suzukii* populations by constructing female-specific lethality and masculinization genetic sexing strains (GSS).

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

AUTHOR (S): José S. Meza, Daisy P. Cárdenas-Enriquez, Victor García-Martínez, Jorge Ibañez-Palacios, Brenda Torres-Huerta.

ORGANIZATION: National Program Fruit Flies SADER/SENASICA1

SHORT SUMMARY OF PAPER

Abstract:

The genetic sexing strains (GSSs) *Anastrepha obliqua* T(Y; A)-22 and T(Y; A)-364 were isolated using the recessive *black pupa* (*bp*) marker. The translocation in T(Y; A)-22 was achieved through irradiation at 35 Gy, while ethyl methanesulfonate at 40 mM was used for T(Y; A)-364. Both strains are maintained in a colony using a genetic filter system, recording their phenotype and fertility. The T(Y; A)-22 strain, in its F12, reached a maximum eggs hatch rate of 22.33%, with 11.09% of males exhibiting the black pupa phenotype and no wild-type females. In contrast, T(Y; A)-364 displayed a maximum eggs hatch rate of 20.67%, with 15.96% black pupa males and a production of 6.87% wild-type females. The increase in fertility and the stable recombination found over generations in T(Y; A)-22 suggest that this strain could be used for mass-rearing purposes. Additionally, a cytogenetic analysis of mitotic chromosomes in the T(Y; A)-22 strain was conducted. We identified two breakpoints on different chromosomes: one on pair number 2, characterized by its greater length, with a break occurring in the middle of one of the homologs; the other, a smaller breakpoint, was observed on an autosome that remains unidentified due to its similarity in length with different chromosomes.

On the other hand, we evaluated the effect of thermal stress on *A. ludens* eggs using differential expression analysis (RNA-Seq). We identified 432 overexpressed genes (log2FC ≥ 1.2) in eggs exposed to 35°C compared to those maintained at 25°C. Among the genes identified in the differential expression analysis (DEA), two members of the Hsp70 family (*Al(2)efl* and *AlHsc70-2*) showed significantly increased expression in eggs exposed to 35°C, with 6-fold and 3-fold increases, respectively. In *Drosophila melanogaster*, these proteins are known to be involved in supporting the tertiary structure of proteins and in the folding of newly synthesized proteins. Allele phenotypes include lethal, abnormal size, and reduced size classes. *Al (2)efl* and *AlHsc70-2*, along with five genes upregulated or downregulated in eggs exposed to 35°C, were selected for amplification via RT-PCR. As a result, 7 of the 8 genes were successfully amplified. Amplification conditions for the genes Nlaz-PC, 14-3-3 zeta, Cyp4g15-PC, Cyt-c-p, l(2)efl (Ta = 65.8°C), and Hsc70-2 were standardized for verification via RT-qPCR.

TITLE OF WORKING PAPER: Long-read RNA-seq delineates temporal transcriptional dynamics in multiplexed and sexed single medfly embryos

AUTHOR (S): Anthony Bayega[1], Spyros Oikonomopoulos[1], Dimitris Rallis[2], Dionisia Mavritsakis[3], Antonia Spanomitrou[3], Kostas D Mathiopoulos[2], **Jiannis Ragoussis[1,4]**

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SHORT SUMMARY OF PAPER

Abstract:

Long-read RNA sequencing has great potential to improve genomic characterization of non-model organisms due to its ability to yield full-length genes. Coupled with absolute gene expression quantification, dynamics of development orchestrated at transcript level can be elucidated with high precision. The resolution of this precision can be further improved by studying organisms as close as possible to their basic entities, single cells for example or single embryos. Here, we collected developing embryos of the Mediterranean fruit fly (medfly, Ceratitis capitata) at hourly timepoints for the first 15 hours of development. The medfly is an organism of huge economic importance in agriculture due to its wide host range and destructive capacities of fruits including apples, pear, citrus, olives, etc. We simultaneously isolated total RNA and genomic DNA from single embryos and sexed the embryos using Y-specific PCR assays. The RNA, spiked with external ERCC standards to aid in absolute quantification, was used to perform Nanopore long-read RNA-seq. We developed a genome-guided transcriptome assembly based on full-length transcripts and identified a total of 22,875transcripts comprising 3879novel genes, missed in the current NCBI predicted gene models. We show that indeed, the absolute quantification of gene expression performs superiorly to relative quantification in highly dynamic systems such as developing embryos. Further, we used unsupervised clustering and lineage tracing algorithms to group and accurately place embryos along a pseudo-temporal development trajectory. We show that medfly embryos undergo successive waves of zygotic genome activation. We discover a dramatic reorganization of maternally deposited mRNA occurring within the first 3 hours of egg laying followed by maternal-to-zygotic transition. We finally identify modules of temporal syn-expression and elucidate the biological role of these modules. Together, these results provide the first detailed look at early embryo development in the medfly and should aid in future control efforts of this pest.

TITLE OF WORKING PAPER:

Evolution of the Y-linked *MoY* male determining factor in Ceratitis and Batrocera genera: structural and functional predictions

AUTHOR (S):
Saccone[1], G, Aceto[1], S., Mazzucchiello[1], S.M., Volpe[1], G., Baccaro[1], Farracchio[1], M., Liguori[1], S., De Falco[1], D., Bertolotto[1], F., Sangle[1], H. V., Giordano, E., Salvemini[1], M., Esposito[2], L., and Vitagliano[2], L.

ORGANIZATION:
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SHORT SUMMARY OF PAPER

Abstract:

The Y-linked *MoY* gene is sufficient and necessary for *Ceratitis capitata* male sex determination, as previously shown by RNAi, CRISPR/Cas9 and transient gain-of-function experiments (Meccariello et al., 2019). MOY is able to promote either directly or indirectly male-specific non-productive splicing of the female-determining *Cctransformer* pre-mRNA. *MoY* orthologues are necessary for male sex determination also in the olive fly (*B. oleae*) and the oriental fruit fly (*B. dorsalis*) and shown to be conserved in few other Bactrocera species. The MOY protein is very short (70 aa) and lacks similarity to any other protein hitherto characterized, leaving open the questions concerning its structure and function. Preliminary attempts to gain insights into the structural properties of MOY proteins by using predictive approaches based in machine-learning techniques and a limited number of sequences did not provide convincing results. In this scenario, we downloaded the genomic Illumina reads of the following 10 Ceratitis species: *Ceratitis cosyra, C. ditissima, C. fasciventris, C. pallidula, C. punctata, C. querita, C. quilicii , C. quinaria, C. rosa,* and *C. rubivora* from the NCBI SRA archive. We assembled the raw reads and selected by BLAST the contigs containing putative ORFs of MOY. The 10 Ceratitis MOY orthologues showed sequence identity ranging between 84% and 70%, and a conserved short length ((67-70 aa). The *MOY* gene tree topology (Maximum Likelihood phylogenetic tree) agrees with the previously defined species phylogeny. We used AlphaFold2 to perform *de-novo* structure prediction of all the investigated sequences (the ten "novel" contigs and the eight previously known Bactrocera MOY orthologs). Preliminary predictions highlighted the occurrence of a two-stranded β-sheet on the N-term in almost all tested sequences. Based on this observation, we are currently carrying out structure-to-function analysis to gain insights into the biochemical role of these proteins.

TITLE OF WORKING PAPER: FUNCTIONAL AND GENOMIC STUDIES OF *SAM-1*, A NOVEL MALE DETERMINER CANDIDATE ON CHROMOSOME I OF THE COMMON HOUSEFLY *MUSCA DOMESTICA*

AUTHOR (S): ECE KIVANC[1], IVAN SPÖCKER[1], SAMUEL JUNG[1], CLAUDIA BRUNNER[1], ELZEMIEK GEUVERINK[2], LEO BEUKEBOOM[2], DANIEL BOPP[1]

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

SHORT SUMMARY OF PAPER

Abstract:

Houseflies provide an excellent model for studying the evolutionary diversification of primary sex-determining loci. These flies possess several recently evolved proto-Y chromosomes, each containing a male-determining locus (*M*). The majority of these *M* loci harbor the male-determining gene *Mdmd*, a neo-functionalized duplication of the spliceosomal factor CWC22. However, a notable exception is the *M* locus on chromosome I (*MI*), which lacks the *Mdmd* gene, suggesting the presence of a structurally distinct male-determining factor.

Differential expression analysis in early embryos led to the identification of a male-specific transcript, present only in *MI* males. This transcript is absent in females and in males carrying the *Mdmd* gene. This male-specific transcript, which we named Simultaneous Alternative M Factor 1 (*SAM-1*), is a strong candidate for the *MI* male-determining factor. Interestingly, *SAM-1* was also detected in genomic reads from monogenic females, which carry the maternal *tra* repressor *Ag* and produce exclusively male progeny. This observation supports the idea that *Ag*, mapped at the same chromosomal location, is a derived allele of *MI* that is only active in the female germline.

In the current study we investigate the organization and function of *SAM-1* further, as it could offer a promising model for constructing a monogenic strain in which females produce only male offspring. *SAM-1* is transcribed from a region within intron 4 of the *BuGZ* gene. Targeted silencing of *SAM-1* through dsRNA injections or CRISPR/Cas9-mediated disruption results in a significant reduction in *MI* male fertility and partial feminization of the testes. These results are consistent with a critical role of *SAM-1* in proper male differentiation. However, there is no conclusive evidence yet that *SAM-1* alone accounts for all the functions of *MI*.

It is possible that the presence of *SAM-1* within intron 4 interferes with normal expression or function of *BuGZ*. We hypothesized that *BuGZ* is needed to sustain the positive feedback loop of the female promoting *tra* gene in houseflies (*Mdtra*). To test this hypothesis, we silenced various splice variants of the *BuGZ* gene in female embryos and monitored the activity of *Mdtra*. Silencing *BuGZ* in female embryos led to the emergence of male-specific isoforms of both *Mdtra* and *Mddsx*. The cis-presence of *SAM-1* appears to reduce *BuGZ* expression, supporting the idea that *SAM-1* influences *BuGZ* expression in a cis-regulatory manner.
Our current model proposes that the *MI* sex-determining system works by reducing the expression of *BuGZ* below a threshold needed to uphold the *tra* loop.

TITLE OF WORKING PAPER: Developments of *Anopheles arabiensis* genetic sexing strains: Progress, challenges, and future directions for Sterile Insect Technique-based malaria vector control

AUTHOR (S): Thabo Mashatola[1,2], Witness Ramashia[1,2] and Givemore Munhenga[1,2]

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SHORT SUMMARY OF PAPER

Abstract:

Anopheles arabiensis is a principal malaria vector in South Africa and much of sub-Saharan Africa, playing a significant role in the transmission of malaria. Current vector control methods have proven insufficient to tackle the widespread impact of this species, underscoring the need for innovative solutions. The Sterile Insect Technique (SIT) presents a promising strategy for controlling *An. arabiensis*; however, its success relies on the efficient separation of male mosquitoes from females prior to irradiation and field release. Females must be excluded from mass rearing, as they are capable of blood feeding and transmitting *Plasmodium* parasites. Current sex separation methods for *An. arabiensis* are inadequate to meet the large scale demands of SIT programs. To address this, the development of genetic sexing strains (GSSs) using classical and transgenic approaches is underway. Progress includes the identification of insecticide resistance (e.g., dieldrin) and morphological traits (such as body colour variations) in combination with temperature-sensitive lethal (*tsl*) mutations as selectable markers. Additionally, CRISPR-Cas9 gene-editing technology is being explored to induce mutations in various genes, which could serve as additional selectable markers. Molecular and genetic characterization of mutant lines is ongoing to ensure the stability and effectiveness of these markers. Furthermore, the development of two transgenic *An. arabiensis* sexing strains, one with a fluorescent marker linked to the X chromosome and the other to the Y chromosome, has been developed and this will allow for the sorting and release of non-transgenic male mosquitoes. These advancements provide a robust foundation for the operational phase of the SIT project and are key steps towards the successful completion of current pilot trials. Ultimately, they lay the groundwork for large-scale malaria control efforts aimed at reducing the transmission of Plasmodium and mitigating the burden of malaria in affected regions.

TITLE OF WORKING PAPER: Development of a temperature sensitive lethal-based genetic sexing strain in the malaria vector *Anopheles arabiensis*: achievements and challenges

AUTHOR (S): Cyrille NDO

ORGANIZATION:
Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé-Cameroon

SHORT SUMMARY OF PAPER

Abstract:

Vector control using insecticide-based interventions is the cornerstone of malaria prevention strategies. However, the situation is becoming worse with the escalation and spread of insecticide resistance in major malaria vectors across the African continent. Therefore, new and innovative tools that can help reducing Plasmodium transmission and achieving malaria elimination are in dire need. This raised considerable interest in using the sterile insect technique (SIT) against Anopheles malaria vectors. This project aimed at developing and evaluating a pupal color- and temperature sensitive lethal-based Genetic Sexing Strain (GSS) of the malaria vector *Anopheles arabiensis*.

Mutagenesis of wild males *An. arabiensis* was achieved using ethyl methanesulfonate which induced temperature sensitivity. Five temperature sensitive lethal strains (TSL-strains) were isolated, maintained in the insectary and are available for future development of a TSL-based GSS. Characterization of life history traits of these strains revealed some slight differences compared to the control (non-mutagenized mosquitoes). A significant reduction in fecundity and fertility was observed in all TSL stains compared to the wild type mosquitoes. Regarding adult longevity, the TSL strains showed slight reduction of longevity. Crossing experiments indicated that the TSL are located on the autosome and are recessive. Both embryos (eggs) and larvae of the TSL strains were heat-sensitive, with more that 98% mortality achieved after heat exposure, compared to less that 20% in the control (wild type).

Failure to isolate a morphological visible marker represents a major challenge towards the development of the TSL-based GSS.

TITLE OF WORKING PAPER: Hunt for naturally existing tsl mutation in Aedes aegypti and Ae. albopictus for construction of more robust Genetic Sexing Strain (GSS) for SIT

AUTHOR (S): Muhammad Misbah ul Haq, Abdul Aziz

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

SHORT SUMMARY OF PAPER

Abstract:

This study aimed to identify naturally existing temperature-sensitive lethal (tsl) mutation in wild populations of *Aedes aegypti* and *Aedes albopictus* collected from diverse geographical regions across Pakistan. The first step involved the collection of eggs and larvae using ovitraps and larval inspections in collaboration with district dengue vector surveillance teams. Populations were gathered from varied climatic zones, and new colonies were established in the laboratory for further testing. Due to limited initial collections, the colonies were expanded to the second generation before exposing first-instar larvae to temperatures ranging from 39°C to 41°C for periods of 2 to 24 hours. Afterwards, the larvae were subjected to thermal stress at 40°C for durations of 3 to 5 hours in water baths to screen for heat resistance potentially linked to the tsl mutation.

The results from thermal screening showed variability in survival rates, with *Ae. aegypti* demonstrating comparatively greater resistance to heat than *Ae. albopictus*. Wild populations collected from different regions also exhibited differing levels of heat tolerance, with two to three *Ae. aegypti* colonies showing relatively higher resistance at 40°C for 3 to 5 hours. However, inconsistencies were noted during re-screening, as these resistant colonies did not consistently survive subsequent heat exposure. Parameters such as larval developmental time, pupation rates, pupal weight, adult emergence and sex ratios were recorded but did not significantly vary once larvae survived the initial thermal shock.

Additionally, iso-male families of both Aedes species were established and subjected to similar thermal screening conditions. Though one or two iso-male families of both Aedes species demonstrated slight and repeatable survival under thermal stress, the overall results were inconclusive. These iso-male families will be further re-tested in future experiments to validate any potential heat resistance.

In conclusion, while some mass families, particularly *Ae. aegypti*, showed initial signs of heat resistance, the lack of consistent and reproducible results suggests that true heat-resistant traits may be rare or difficult to identify in these populations. Further research, including the development and testing of more iso-male families, is under process to confirm whether temperature-sensitive lethal mutations exist in these Aedes species.

TITLE OF WORKING PAPER: Differential and conditional elimination of homomorphic sex chromosomes in *Aedes aegypti* for population control

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SHORT SUMMARY OF PAPER

Abstract:

Aedes aegypti mosquitos are the major vector for multiple viruses that cause diseases including dengue, yellow fever, and Zika. In contrast to other insect species, *Ae. aegypti* have *homomorphic sex-determining chromosomes* where sex is determined by a small (1.3 Mb), dominant, male determining locus (M-locus). Within this M-locus resides *Nix*, the male determining factor that is required and sufficient for male sex development. Recombination between homologous regions of *Ae. aegypti* sex chromosomes presents a challenge in the development of genetic sexing strains for mass release programs. We have developed new ways to achieve separation of the non-biting males from females, a critical bottleneck affecting genetic control programs. One approach is a system of Differential Elimination of Marked sex chromosomes (DeMark), which uses recessive lethal alleles to produce exclusively non transgenic males for release. We are developing lines that use selection or counterselection to conditionally select for or against males or females depending on the rearing conditions. These technologies may be applied individually or multiplexed to cater to different genetic biocontrol measures for the purpose of maximizing disease prevention while improving efficiency and cost effectiveness.

TITLE OF WORKING PAPER: Genetic sexing in *Aedes albopictus* using a synthetic M locus.

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SHORT SUMMARY OF PAPER

Abstract:

Genetic control holds great promise for mosquito population suppression. However, area-wide deployment of many approaches requires an effective, scalable and inexpensive method to separate females prior to the mass-release of males. Genetic sexing strains (GSS) can theoretically solve this problem by genetically linking sexual identity with one or more selectable traits. In the Asian Tiger mosquito, *Aedes albopictus,* 'Nix' is the primary signal for sex determination and acts as a dominant masculinizer converting a genetic female into a fertile male. This feature was recently leveraged to engineer a fluorescent-marker based GSS.

To expand this genetic framework in Ae. albopictus, here we develop selectable traits based on the CRISPR mutagenesis of endogenous genes followed by their functional rescue in males using 'Nix'-encoding transgenes. As a proof-of-concept we chose the highly-conserved 'yellow' gene. We generated and subsequently transformed 'yellow' homozygous females with a construct containing a copy of Nix and a mini-yellow rescue, to generate a well-performing yellow-GSSs in which females are phenotypically yellow. Males of the yellow-GSS, which are genetic females lacking the ancestral sex-.determining M locus, are phenotypically wild type (dark), enabling easy visual sex separation throughout ontogeny. Interestingly, we observed that *yellow* mutants and GSS females take longer to complete larval development. We show that this phenotype can be readily exploited for large-scale sexing by extending the natural range of differences in development rates between the sexes. Given the role of 'yellow' in embryonic cuticle hardening, GSS females lay mosquito eggs that are sensitive to long-term desiccation, providing also a useful barrier to gene flow from the unintentional release of contaminating females. We evaluate the potential compatibility of yellow-GSS with size-based sex separation methods, given their now established presence in programs involving the mass-release of Aedes males. In the context of sex separation genetics, this study extends the phenotypic concept of "slow development" to include in populo competing genotypes of mutants alleles with mild-but-sufficient fitness costs, that are privately rescued in males.

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

9 to 13 December 2024

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