Institute of Insect Biotechnology

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Justus-Liebig-University Gießen

Characterization of genes used as selectable markers for the development of genetic sexing strains for SIT applications against tephritid pest species using CRISPR/Cas9

INAUGURAL DISSERTATION

zur Erlangung des Doktorgrades (Dr. rer. nat.)

im Fachbereich Agrarwissenschaften, Ökotrophologie
und Umweltmanagement der Justus-Liebig-Universität Gießen

vorgelegt von

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Gießen, 2023

Mit Genehmigung des Fachbereichs Agrarwissenschaften, Ökotrophologie und Umweltmanagement der Justus-Liebig-Universität Gießen

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Tag der Disputation: 05.12.2023

1. Summary

Genetic sexing strains (GSSs) play a crucial role in the sterile insect technique (SIT), a powerful method employed in insect pest control and population management. SIT involves releasing sterile insects into target areas to suppress, eradicate, control, and prevent pest populations. However, in some cases, to ensure the effectiveness of this technique, it is essential to release only sterile males, as the females can still cause damage by laying eggs and/or biting. Male-only releases can be achieved by using GSSs, which are currently based on two selectable markers essential for sex separation and female elimination at the embryonic stage: the yet unknown *temperature-sensitive lethal* (*tsl*) gene and the recently identified *white pupae* (*wp*) gene, which have been used in the most successful GSSs developed thus far, the *Ceratitis capitata* (medfly) VIENNA 7 and VIENNA 8. In this work, I present three studies to narrow down the region where the *Ceratitis capitata tsl* gene is located and to identify *tsl* gene(s) and their causal mutation(s) responsible for the temperature-sensitive lethal phenotype. In the **first** study, I carried out temperature-sensitive lethal tests to determine the temperature sensitivity of twenty-seven *Ceratitis capitata* wild-type, genetic sexing, and *tsl* mutant strains, to assess differences among populations of different origins and to choose reference strains for the following investigations. In the **second** study, I used a combined approach of genomic, transcriptomic, bioinformatic, and cytogenetic data to characterize and analyze the putative *tsl* genomic region in *Ceratitis capitata*. This resulted in the identification of several potential candidate genes within the *tsl* region that could be associated with the temperature-sensitive lethal phenotype. These findings shed light on the underlying genetic mechanisms behind this phenotype and provide valuable insights for further investigations. In the **third** study, one of the potential *tsl* candidate genes previously identified, the *deep orange (Ccdor)* gene, was chosen to be targeted via CRISPR/Cas9 and to assess whether this triggers any temperature-sensitive lethal phenotype. I used CRISPR/Cas9-mediated NHEJ to knock out the *Ccdor* gene and CRISPR/Cas9-mediated HDR to successfully introduce a specific point mutation found in the *tsl* mutant strain. The latter resulted in the isolation of two additional mutations, leading to a temperature-sensitive lethal phenotype during embryonic, larval, and pupal stages. The results of this work represent steps forward for identifying potential markers that can be used to construct new GSSs in tephritids.

1. Zusammenfassung

Die genetischen Geschlechtsselektionsstämme ("Genetic Sexing strains“ = GSSs) spielen eine entscheidende Rolle in der Sterilen-Insekten-Technik (SIT), einer effektiven Methode zur Schädlingsbekämpfung und Populationskontrolle. SIT beinhaltet die Freisetzung steriler Insekten in Zielgebiete, um Schädlingspopulationen zu unterdrücken, auszurotten, zu kontrollieren und zu verhindern. In einigen Fällen ist es unerlässlich, nur sterile Männchen freizusetzen, da die Weibchen noch Schaden anrichten können, indem sie Eier legen und/oder beißen. Männliche Freisetzungen können mit GSSs erreicht werden, die heute auf zwei auswählbaren Markern basieren, die für Geschlechtstrennung und Eliminierung der Weibchen im embryonalen Stadium unerlässlich sind: dem noch unbekannten temperatursensitiven letalen (*tsl*) Gen und dem kürzlich identifizierten *white pupae* (*wp*) Gen, die in den bisher erfolgreichsten GSSs in *Ceratitis capitata* (Mittelmeerfliege) verwendet wurden, den Stämmen VIENNA 7 und VIENNA 8. In dieser Arbeit präsentiere ich drei Studien, um die Region einzugrenzen, in der das *C. capitata tsl*-Gen lokalisiert ist, und um *tsl*-Gene und ihre ursächlichen Mutationen zu identifizieren, die für einen temperatursensitiv letalen Phänotyp verantwortlich sind. In der ersten Studie führte ich temperatursensitive letale Tests durch, um die Temperatursensitivität von 27 *C. capitata* Wildtyp-, GSS- und *tsl*-Mutantenstämmen zu bestimmen, um Unterschiede zwischen Populationen verschiedener Herkunft zu bewerten und Referenzstämme für die folgenden Untersuchungen auszuwählen. In der zweiten Studie verwendete ich einen kombinierten Ansatz aus genomischen, transkriptomischen, bioinformatischen und zytogenetischen Daten, um die mutmaßliche *tsl*-Genomregion in *C. capitata* zu charakterisieren und zu analysieren. Dies führte zur Identifizierung mehrerer potenzieller Kandidatengene innerhalb der *tsl*-Region, die mit dem temperatursensitiven letalen Phänotyp in Verbindung gebracht werden könnten. Diese Ergebnisse werfen Licht auf die zugrundeliegenden genetischen Mechanismen hinter diesem Phänotyp und liefern wertvolle Erkenntnisse für weitere Untersuchungen. In der dritten Studie wurde eines der potenziellen *tsl*-Kandidatengene, das *deep orange* (*Ccdor*) Gen, zur gezielten Bearbeitung mittels CRISPR/Cas9 ausgewählt, um zu überprüfen, ob dies einen temperatursensitiven letalen Phänotyp auslöst. Ich verwendete CRISPR/Cas9-vermitteltes NHEJ, um das *Ccdor*-Gen auszuschalten und CRISPR/Cas9-vermitteltes HDR, um erfolgreich eine spezifische Punktmutation in den *tsl*-Mutantenstamm einzuführen. Letzteres führte zur Isolierung von zwei zusätzlichen Mutationen, die zu einem temperatursensitiven letalen Phänotyp während der embryonalen, larvalen und pupalen Stadien führen. Die Ergebnisse dieser Arbeit stellen Fortschritte bei der Identifizierung potenzieller Marker dar, die zur Konstruktion neuer GSSs in Tephritiden verwendet werden können.

2. Introduction

The ongoing effects of climate change have resulted in an alarming annual temperature increase of approximately 0.1°C (Pörtner *et al*, 2022), which has significant implications for global agriculture and public health. Insect pests and vector-borne diseases are more likely to spread across wider areas due to the changing climate, with some species present throughout the year in regions where they were not previously found. According to estimates by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), the global economy currently loses over $220 billion due to plant diseases and at least $70 billion due to invasive insects (IPPC Secretariat, 2021). Insects also contribute to more than 700,000 annual deaths by spreading infectious diseases, accounting for 17% of all such diseases (WHO & UNICEF, 2017). The persistent use of pesticides in combating these pests, despite been successful, poses a risk to the environment and public health. If we consider the disadvantages, despite their effectiveness, pesticides can lead to issues such as resistance development due to increased usage, in addition to the increase in their prices, and regulatory changes that may restrict the use of certain compounds. As a result, there is an urgent need to develop alternatives, such as the sterile insect technique (SIT) (Knipling, 1955, 1979), to reduce pesticide dependence. Therefore, exploring new strategies and methods to mitigate the risks and contain the impact of insect pests and vector-borne diseases is crucial.

2.1 The Mediterranean fruit fly: *Ceratitis capitata*

The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), also called the "medfly", is a phytophagous insect of the order *Diptera* belonging to the *Tephritidae* family. At the larval stage, the fly develops as carpophagous and polyphagous within the pulp of many fruits. Its presence is documented in more than 260 species of fruits, vegetables, and flowers, including species of strong economic interest (Liquido *et al*, 1991; White & Elson-Harris, 1992; Hancock *et al*, 2000; Copeland *et al*, 2002; Morales *et al*, 2004; Meats & Smallridge, 2007; Sciarretta *et al*, 2018). The species originated in sub-Saharan Africa (Gasperi *et al*, 1991, 2002) and over time has become endemic and stabilized in the rest of Africa, the Mediterranean basin (Robinson & Hooper, 1989), South America (Harris & Lee, 1989; Headrick & Goeden, 1996; Vera *et al*, 2002), Asia and Oceania (Bonizzoni *et al*, 2004). It is currently a cosmopolitan species present all year round in tropical and subtropical regions of all continents (Figure 1) (Malacrida *et al*, 1992; Gasparich *et al*, 1997; Malacrida *et al*, 2007; Gilioli *et al*, 2022).

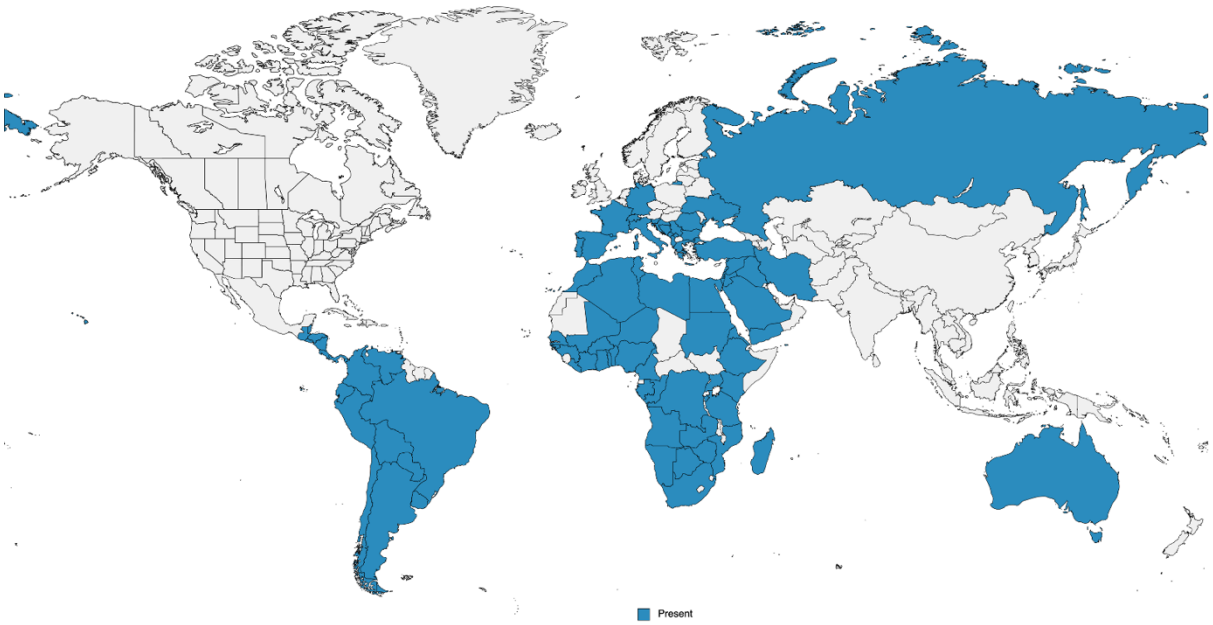

Figure 1. Worldwide distribution map of *Ceratitis capitata*. The map is based on the currently available distribution data retrieved from the EPPO Global Database (https://gd.eppo.int) (accessed on 1 April 2023) and integrated with information from the literature. The figure was created using MapChart (https://www.mapchart.net/index.html).

The life cycle of *Ceratitis capitata*, considered the period between egg laying and the adult stage, can last from approximately 21 to 30 days according to temperatures and climatic conditions. The cycle consists of an embryonic stage, three larval stages, and a pupal stage followed by metamorphosis with consequent eclosing of the adult (Figure 2) (Thomas et al., 2001).

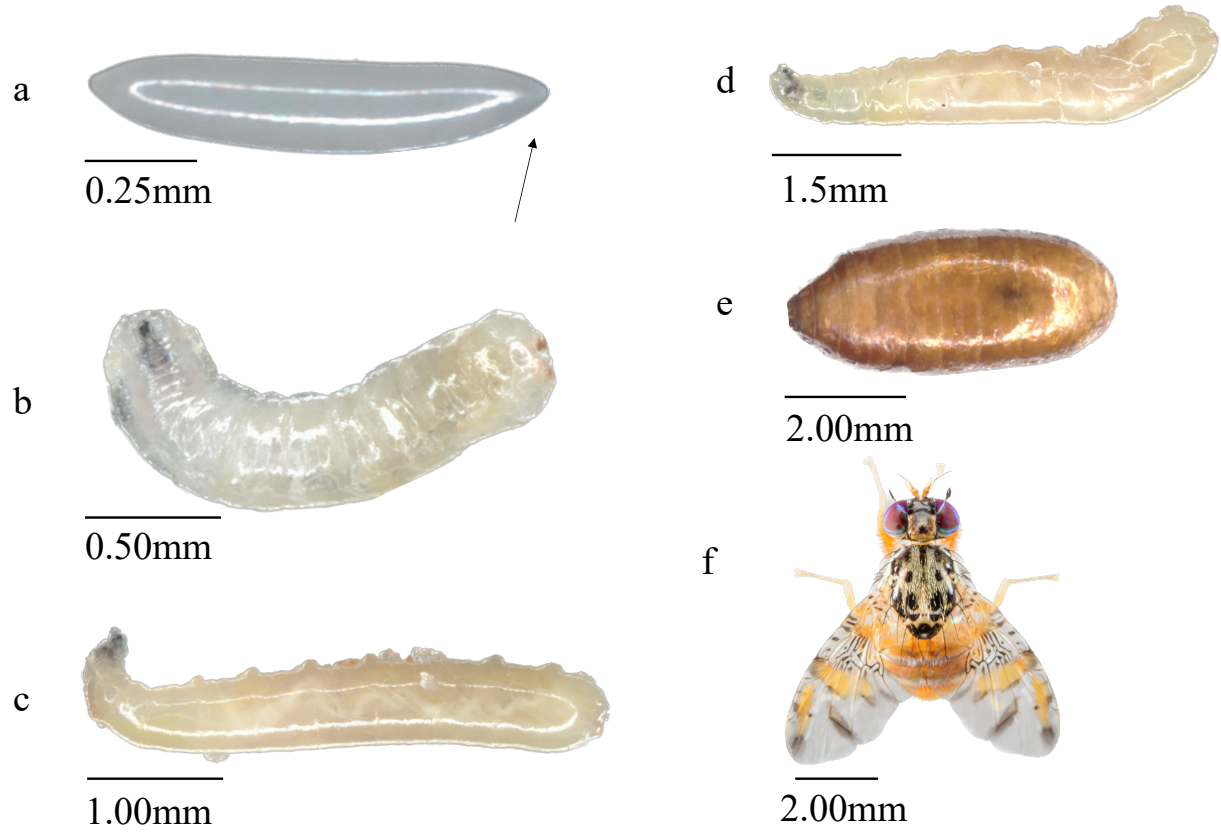

Figure 2. Developmental stages of Mediterranean fruit fly *Ceratitis capitata*. (a) Embryo, (b) 1st instar larva, (c) 2nd instar larva, (d) 3rd instar larva, (e) pupa, and (f) adult male. The black arrow indicates the embryo's posterior pole.

The embryos are curved, cylindrical in shape, white, and shiny. They are approximately 1 mm long, and anterior and posterior poles are distinguished by a micropylar protuberance (Figure 2a). The embryonic stage is followed by three larval stages called "instars" lasting six to ten days (Figure 2b, 2c, 2d). The larvae are light yellow and elongated in shape. The anterior region is narrower and slightly curved toward the ventral region, while the caudal end is flattened. The last instar is usually 7-9 mm, is characterized by eight ventral fusiform zones and is defined as "jumpy larvae" in relation to the particular ability of the larvae to jump and drop onto the ground, in which they reach the pupal stage at a depth of approximately 7-8 cm (Figure 2d; Figure 3) (Thomas et al., 2001). At the end of their development, the larvae transform their exoskeleton into a protective structure in the shape of a brown barrel (puparium) within which they develop with subsequent metamorphosis (Figure 2e, 2f). This stage lasts six to thirteen days when the temperature is between 24°C and 26°C and under optimal humidity conditions. Humidity change may lead to smaller and more fragile puparia (Thomas *et al*, 2001).

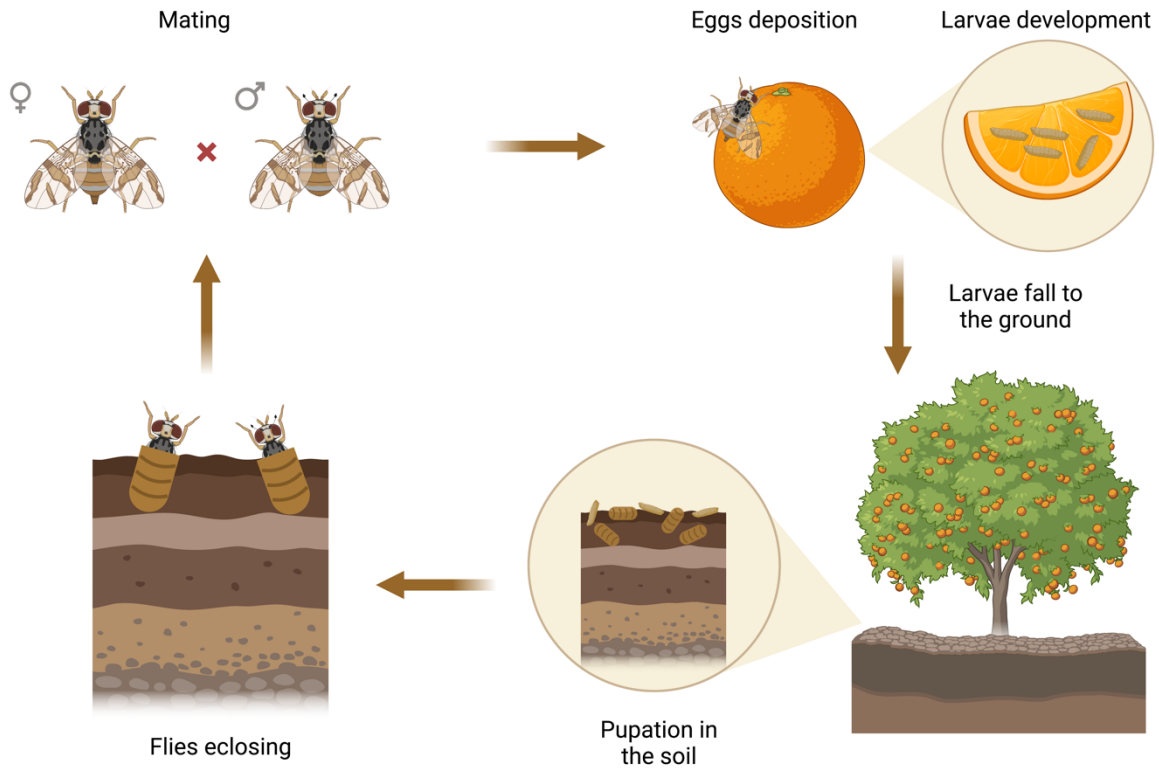

Figure 3. The life cycle of the Mediterranean fruit fly *Ceratitis capitata*. (Clockwise) After mating, female medflies lay eggs by introducing their ovipositor into the fruit. The larval stage takes place within the latter. Upon reaching the third instar, the larvae "jump" from the fruit onto the ground where they carry out the pupal stage. After approximately 21 days, the pupae hatch, allowing the flies to fly and repeat the cycle. The figure was generated using BioRender (https://www.biorender.com).

During the last stage, the puparium breaks, and adults eclose (Figure 3). The adult is of medium size and reaches a length of 4-6 mm. The head is yellowish with a black ocellar plate and brownish antennae. The chest is bright black with cream flecks and covered with dense and short hair. The legs are yellowish, and the wings are broad with a reticulated appearance in the proximal area. They have four transversal bands of a brownish-yellow color. The abdomen has a reddish-yellow color with gray or slate transverse bands (Figure 2g). They exhibit marked sexual dimorphism. The males are characterized by a pair of bristles that start from the external margin of the eyes and have spatula-like ends, while the females are larger than the males and have an abdomen with an ovipositor in the caudal part that can extend up to 1.2 mm during the

act of embryo deposition. Furthermore, females have black hair on the first pair of front legs, while males have a light brown coloration (Figure 4).

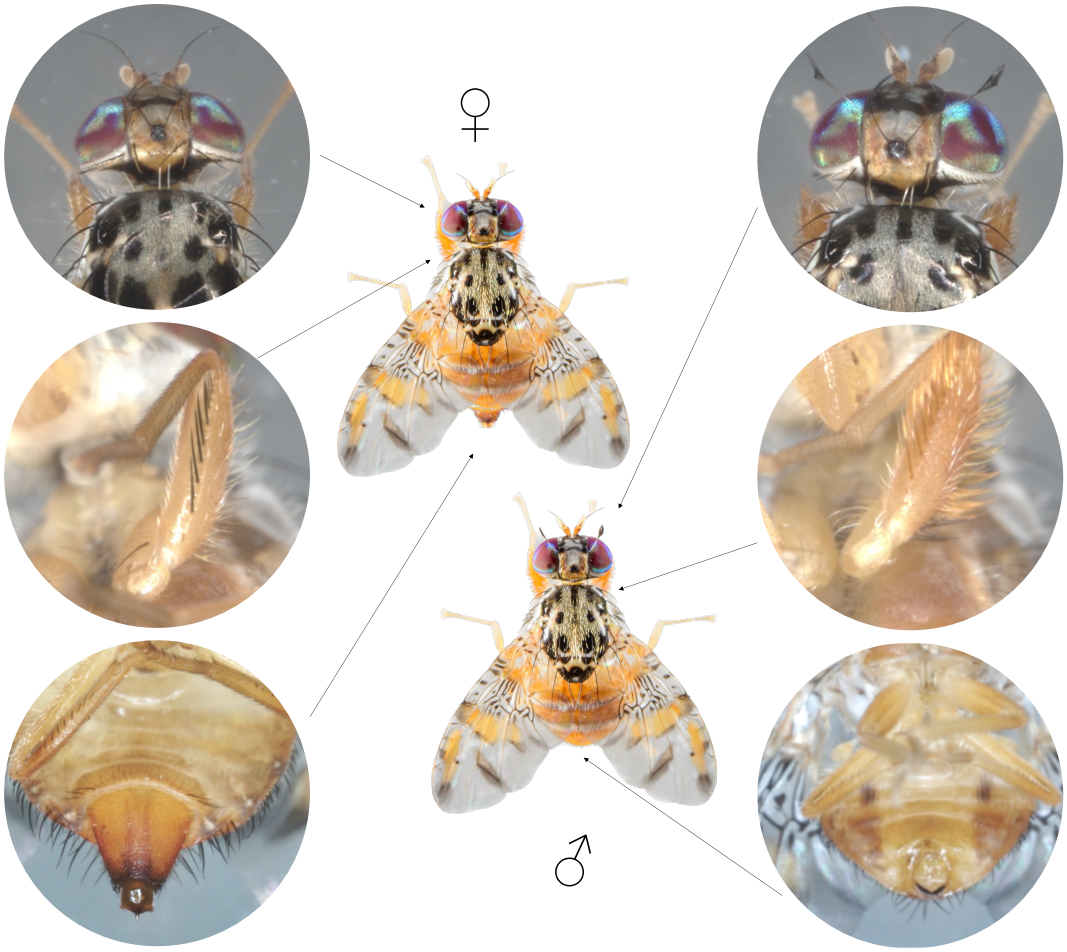

Figure 4. Dimorphisms of the Mediterranean fruit fly *Ceratitis capitata*. Males and females exhibit distinct localized differences. Males possess a pair of bristles on the head, while females have black hair on the first pair of front legs and an ovipositor in the caudal region.

2.2 Sterile Insect Technique (SIT)

Due to the observed insecticide resistance in different populations of tephritids (Hsu *et al*, 2004; Vontas *et al*, 2011; Jin *et al*, 2011, 2016; Ragab El-G, 2018), it became necessary to adopt additional methods to eradicate, suppress, control and prevent their presence. In addition to biological control and synthetic insecticides, one of the most successful methods developed is the sterile insect technique (SIT) (Knipling, 1955, 1979). SIT is a species-specific method used to control insect pests and disease vectors (Enkerlin, 2003; Dyck *et al*, 2021; Klassen & Vreysen, 2021) and is integrated into area-wide integrated pest management (AW-IPM) strategies. AW-IPM can be summarized in three milestones: (1) management of pest populations throughout the ecosystem, (2) long-term strategy requiring continuous implementation, and (3) the use of high-tech technologies (Klassen & Vreysen, 2021). SIT is based on the sterilization of mass-reared insects that are subsequently released in a target area. Despite being irradiated, they remain sexually competitive but are no longer able to produce offspring (Enkerlin, 2003; Dyck *et al*, 2021; Klassen & Vreysen, 2021). Due to the intense sexual aggression of male insects and the reluctance of females to mate more than once, sterile males can mate with wild females, gradually reducing the population over time. The continuous release of sterile males could eventually lead to the eradication, suppression, control, or prevention of the targeted population (Knipling, 1955; Graham, 1985). Recent studies have shown that monogamy is not necessary for the success of SIT (Lance & McInnis, 2021; Whitten & Mahon, 2021). Various species, including tephritids such as *Ceratitis capitata* (Wiedemann) (Steiner & Christenson, 1956; Fisher *et al*, 1985; Smallridge & Hopkins, 2004; Enkerlin *et al*, 2015, 2017; Plá *et al*, 2021; Enkerlin, 2021), *Zeugodacus (Bactrocera) cucurbitae* (Coquillett) (Kuba *et al*, 2020), *Bactrocera dorsalis* (Hendel) (Steiner *et al*, 1965, 1970), *Bactrocera tryoni* (Froggatt) (Fisher, 2020), and *Anastrepha ludens* (Loew) (Jesús Reyes *et al*, 2000; McCombs *et al*, 2009), have been the focus of pilot and eradication programs since the 1960s. For *Ceratitis capitata,* eradication programs have involved the release of sterile males produced through genetic sexing strains (hence GSS) (Caceres, 2002; Rendón *et al*, 2004; Augustinos *et al*, 2017; Franz *et al*, 2021).

Basics of SIT

In the context of SIT, a significant aspect involves sterilizing males before their release (Figure 5). In some species, including *Ceratitis capitata*, the release of both sterile males and females is not as effective as the release of only sterile males (McInnis *et al*, 1994; Rendon *et al*, 2000; Rendón *et al*, 2004). Additionally, in species where sterilized females can still cause crop

damage or transmit diseases despite being unable to reproduce, it is advisable to avoid releasing them altogether. (Lance & McInnis, 2021; Lees *et al*, 2021). To overcome these challenges, GSSs have been developed, which enable early separation of sexes, reduce costs in mass-rearing insects, and effectively address these concerns. (Cáceres, 2002; Rendón *et al*, 2004; Augustinos *et al*, 2017; Franz *et al*, 2021).

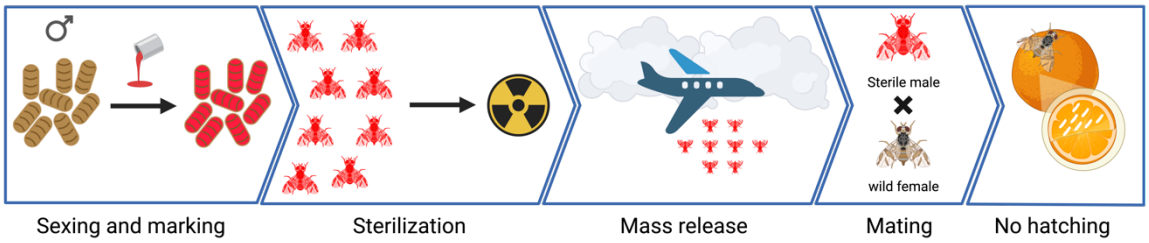

Figure 5. Graphic representation of the sterile insect technique in *Ceratitis capitata*. Following mass production, the males are sorted based on their sex during the embryonic stage and marked during the pupal stage. After emerging as adults, they undergo sterilization and are subsequently released in the SIT application area. Due to their sterility, the released males, when mating with wild-type females, result in the production of nonviable embryos. This contributes to the eradication, suppression, control, or prevention of the target population. The figure was generated using BioRender (https://www.biorender.com).

The GSSs are based on two main components (Franz *et al*, 2021):

- Mutations used as selectable markers for male and female separation.
- A Y-autosome translocation that links the inheritance of these mutations to sex.

For the Mediterranean fruit fly *Ceratitis capitata,* VIENNA 7 and VIENNA 8 GSSs are the two most commonly used (Augustinos *et al*, 2017). They are based on two selectable markers: the *white pupae (wp)* and the temperature-sensitive *lethal (tsl)* genes (Franz *et al*, 2021), both located on the right arm of chromosome 5 (Figure 6) (Kerremans and Franz, 1994; Niyazi et al., 2005; Zacharopoulou et al., 2017) and involved in puparium color and temperature sensitivity, respectively (Franz *et al*, 2021). Recently, an additional marker closely linked to *tsl*, known as *slow development (sd)* (Porras *et al*, 2020), has been identified, and its genetic basis is still unknown.

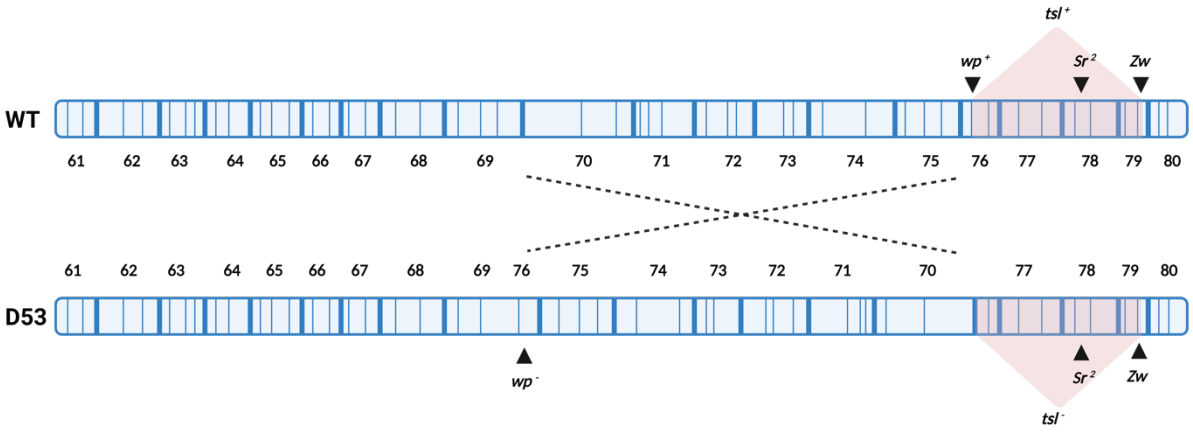

Figure 6. The *Ceratitis capitata* chromosome 5. Schematic representation of the wild-type (above) and D53 (below) medfly salivary gland polytene chromosome map. The putative *tsl* region (shown in red) (Kerremans & Franz, 1994; Niyazi *et al*, 2005) is localized within the *wp* (Ward *et al*, 2021) and *Zw* (Scott *et al*, 1993) genes. It includes the Sr^2 (Niyazi *et al*, 2005) and the yet unknown *sd* (Porras *et al*, 2020) phenotypical markers.

The first generation of GSS was developed in 1977 (Rössler, 1979) by crossing an irradiated male and a wild-type female. This was followed by the isolation of the *wp* mutation and the Y/autosome translocation T(Y;5) to have the wild-type allele of the *wp* gene on the Y chromosome (Robinson & Van Heemert, 1982; Franz *et al*, 2021). The second generation of GSS was developed by the isolation of the lethal recessive mutation *temperature sensitive lethal (tsl)* during a screening of individuals treated with ethyl methane sulfonate (EMS) (Busch-Petersen, 1990) and subsequent translocating it to have both *wp* and *tsl* wild-type alleles linked to the male-determining region (*MoY*) on the Y chromosome (Figure 7) (Meccariello *et al*, 2019; Franz *et al*, 2021). When both alleles of the *wp* gene are mutated, puparia appear white, while in the presence of both mutated alleles of the *tsl* gene, the embryos acquire temperature sensitivity, leading to total lethality upon exposure to 34°C for 24 hours (Figure 7) (Caceres, 2002; Augustinos *et al*, 2017; Franz *et al*, 2021). This is the case for VIENNA 7 and VIENNA 8, where females emerge from white puparia and are temperature sensitive, while males emerge from brown puparia and are temperature resistant. Additionally, VIENNA 7 and VIENNA 8 differ from each other, as they carry a different translocation at positions T(Y;5)52A and T(Y;5)58B, respectively (Fisher & Caceres, 2000; Zacharopoulou *et al*, 2017; Augustinos *et al*, 2017).

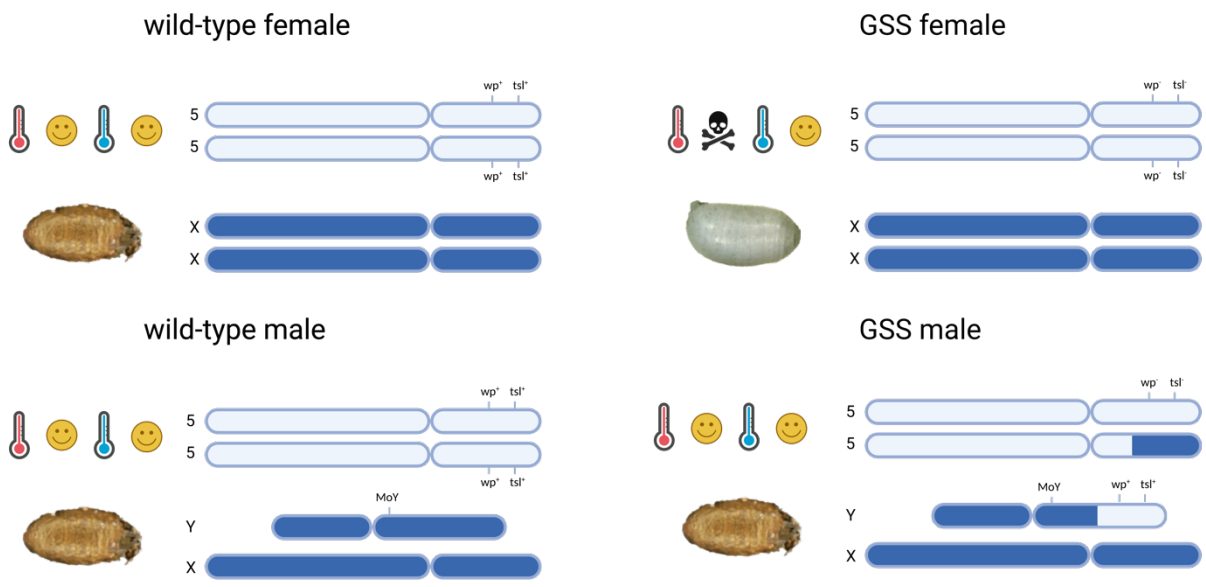

Figure 7. Chromosomal structure of *Ceratitis capitata* wild-type and genetic sexing strains (GSS). Wild-type males and females emerge from brown puparia and are temperature resistant since they are both homozygous for the wild-type allele of the *white pupae* (*wp*) and *temperature sensitive lethal* (*tsl*) genes, which are responsible for pupal color and temperature sensitivity, respectively. GSS females are homozygous for the mutated allele of the *wp* and *tsl* genes, and they emerge from white puparia and die following heat shock. GSS males are heterozygous, as they possess one mutated and one wild-type allele of the *wp* and *tsl* genes, causing them to be temperature resistant and emerge from brown pupae. The figure was generated using BioRender (https://www.biorender.com/).

Similarly, the *wp* mutation was used for the development of GSSs in *B. dorsalis* (McCombs & Saul, 1995; Isasawin *et al*, 2012) and *Z. cucurbitae* (McInnis *et al*, 2004), whereas the black pupae mutation was utilized for *A. ludens*, *A. fraterculus* and *A. obliqua* (Zepeda-Cisneros *et al*, 2014). In these cases, the absence of the *tsl* mutation hinders the elimination of the females at the embryonic stage, making it necessary to rear the flies up to the pupal stage.

Over the years, numerous GSSs have been developed, exhibiting varying fitness levels and temperature sensitivities depending on the specific translocation and rearing conditions to which they were exposed (Augustinos *et al*, 2017). Due to the segregation of the Y-autosome translocation during gametogenesis, GSSs are considered semisterile, and balanced progeny are produced when alternate segregation occurs. Conversely, unbalanced progeny is generated through deletions and triplications during adjacent-1 segregation. Typically, these unbalanced offspring do not survive beyond the embryonic stage, although under certain circumstances, they may reach the pupal or adult stage. This discrepancy in development stages contributes to

variations in productivity levels observed among GSSs (Augustinos *et al*, 2017). The development of stable and functional GSSs in *Ceratitis capitata* has involved strategies to ensure genetic stability and accurate identification of sterile and wild-type insects. One approach was to select translocations where the breakpoints and markers are close to each other, reducing the likelihood of recombination events. A "filter rearing system" was implemented to prevent the accumulation of recombinants (Tanaka *et al*, 1969; Rendón *et al*, 2004), and the In(5)50C;59C inversion on chromosome 5, known as the "D53 inversion," carrying both *wp* and *tsl* markers, was utilized (Figure 6) (Fisher & Caceres, 2000; Augustinos *et al*, 2017). These approaches have proven to be highly effective in maintaining the genetic stability of *Ceratitis capitata* GSSs. An essential aspect of the SIT application involves determining the ratio between sterile and wild insects, as well as accurately distinguishing between wild-type and mass-reared flies prior to release (Dowell *et al*, 2021; Parker *et al*, 2021). For this purpose, physical markers, such as fluorescent dyes, are commonly used to mark sterile insects. However, it is crucial to consider that the utilization of these markers can have various implications, including potential effects on human health, impacts on the competitiveness of the flies (Hagler & Jackson, 2001; Parker *et al*, 2021), and cost issues. To address this problem, morphological markers have been used to construct GSSs. This is the case for the VIENNA 8 - *Sergeant* (*Sr*^{2}) strain, where in addition to *wp* and *tsl* markers, mutant males carry the dominant mutation *Sergeant 2 (Sr*^{2}*)*, which results in three abdominal stripes, whereas wild-type males have only two stripes (Niyazi *et al*, 2005; Rempoulakis *et al*, 2016).

2.3 Generic approach for GSS development

More than two decades of research have been dedicated to the development of stable and functional *Ceratitis capitata* GSSs. The successful implementation of these GSSs in SIT applications (Vargas-Terán *et al*, 2021; Enkerlin, 2021; Simmons *et al*, 2021; Feldmann *et al*, 2021; Lees *et al*, 2021) prompted the adoption of a similar strategy for targeting other species. However, the process of mutagenesis and isolating causal mutations in other species to construct GSSs is often time consuming and may not always be feasible. Consequently, there arose a clear necessity to explore alternative methods (Häcker *et al*, 2021). Hence, the development of a "generic approach" based on the identification of genes that could be used as selectable markers for GSSs, as well as the characterization of *wp* and *tsl* mutations, the identification of orthologous genes in SIT-targeted species (FAO/IAEA, 2019; Franz *et al*, 2021; FAO/IAEA, 2021), and their induction using genome editing approaches such as CRISPR/Cas9 (Doudna & Charpentier, 2014).

The progress made in the last decade regarding genomics, transcriptomics, and genome editing plays a fundamental role in the identification of selectable markers, allowing the construction of GSSs even for those species where isolating causal mutations has not been possible (International Glossina Genome, 2014; Papanicolaou *et al*, 2016; Sharma *et al*, 2017; Turner *et al*, 2018; Matthews *et al*, 2018; Bayega *et al*, 2020). Nevertheless, cytogenetics tools, such as *in situ* hybridization, are essential in determining the chromosomal position of genes, locating translocation breakpoints and inversions, assessing the size of translocated segments, and identifying linkages between different species (Willhoeft & Franz, 1996; Willhoeft *et al*, 1998; Fuková *et al*, 2005, 2007; Drosopoulou *et al*, 2012; Traut *et al*, 2013; Šíchová *et al*, 2013; Nguyen *et al*, 2021). These tools are part of the "generic approach" employed in constructing genetic sexing strains for SIT applications, where the primary goal is to produce large quantities of males within mass-rearing facilities, which will then be sterilized and released. For this purpose, several strategies have been proposed, including:

a) constructing GSSs that exhibit conditional female-specific heat-inducible lethal phenotypes, similar to those already established in *Ceratitis capitata* (VIENNA 7 and VIENNA 8) (Franz, 2005; Franz *et al*, 2020).
b) constructing GSSs where the genes involved in sex determination can be manipulated to induce a female-to-male conversion (Sanchez, 2008; Hediger *et al*, 2010; Nagaraju & Saccone, 2010; Salvemini *et al*, 2011, 2013; Laohakieat *et al*, 2016; Meccariello *et al*, 2019; Primo *et al*, 2020; Peng *et al*, 2020; Laohakieat *et al*, 2020).

A fundamental requirement is the possibility of extending this strategy to the greatest possible number of insect pests and disease vectors with the least possible effort in terms of development and costs. Currently, the identification of mutations responsible for the phenotypes already present in GSSs, such as *white pupae (wp)* and *temperature-sensitive lethal* (*tsl*), is considered a feasible strategy for creating GSSs given the great amount of information about them in our possession. However, it is important to note that several other genes/traits that can serve as selectable markers were identified (Robinson, 2002). Following the isolation of the mutations responsible for the wp (Rössler, 1979) and tsl (Franz *et al*, 1994) phenotypes, the investigation for their identification began. While the *wp* gene was recently discovered (Ward *et al*, 2021), the molecular basis of the *tsl* gene is still unknown. Once the latter will also be discovered and their orthologs in other species will be identified, it will be possible to construct GSSs through genomic editing techniques (CRISPR/Cas9) in numerous insects.

2.4 CRISPR/Cas9-induced mutagenesis.

The genome editing technology CRISPR/Cas (clustered regulatory interspaced short palindromic repeats) (Doudna & Charpentier, 2014) is an extremely useful tool to induce mutagenesis in a variety of organisms. Many bacteria and most archaea have very ancient and sophisticated immune systems guided by RNAs encoded by CRISPR loci and CRISPR-associated genes (*Cas*), which are essential for providing immunity against bacteriophage infection and the transfer of genetic material by plasmids (Heler *et al*, 2014; Marraffini, 2015; Mojica & Montoliu, 2016; Jiang & Doudna, 2017). Cas9 in *S. pyogenes* is a large nuclease (1368 amino acids). It cuts 3 bp upstream of the PAM (protospacer adjacent motif) site through its two distinct nuclease domains: a nuclease domain HNH that cuts the DNA strand complementary to the guide RNA sequence (strand target) and a RuvC nuclease domain responsible for cutting the DNA strand complementary to the guide RNA sequence (the nontarget strand) (Figure 8) (Gasiunas et al., 2012; Chen et al., 2014; Heler et al., 2015). Since the first demonstration of its potential for genetic engineering (Jinek *et al*, 2012), the CRISPR–Cas9 system has been rapidly used as a tool for genome manipulation in a broad spectrum of organisms due to its ease of design, ease of use, and high efficiency (Hsu *et al*, 2004). Afterward, exposure to invading genetic elements, coming from phages or plasmids, short fragments of foreign (exogenous) DNA can be integrated stochastically within the CRISPR repeat spaces in the host chromosome as new "spacers" (Amitai & Sorek, 2016), thus providing a genetic record of the infection that allows the host to prevent future invasions (Makarova *et al*, 2006; Barrangou *et al*, 2007). Once integrated, transcription of the CRISPR array and maturation of the precursors-CRISPR transcripts through endonucleotide cleavage produces short CRISPR RNAs mature (crRNA) (Brouns *et al*, 2008). The combined transcription of the CRISPR and tracrRNA loci leads to the formation of a ternary complex formed by tracrRNA, which serves as a scaffold for the binding of the guide with the Cas9 protein and to bind the crRNA. The complex acts as a mechanism of acquired immunity to subsequent infections (Marraffini & Sontheimer, 2008; Garneau *et al*, 2010). Recognition and cleavage of the specific region of DNA, also *in vitro*, requires that Cas9 is assembled with the guide RNA (a native crRNA – tracrRNA or a sgRNA) to form an active complex (Jinek *et al*, 2012, 2014). The guide RNA is a 20 nucleotide sequence complementary to the targeted DNA sequence (Doudna & Charpentier, 2014). Once Cas9 has bound the guide RNA, the complex is active and searches for the target sequence on the DNA and the PAM site adjacent to the target sequence, thus allowing the induction of the double-strand break (Figure 8) (Gasiunas et al., 2012; Jinek et al., 2014). The PAM sequence is crucial for the discrimination between self and nonself-sequences

in vivo (Marraffini & Sontheimer, 2010). Furthermore, a single mutation in the PAM sequence can disable the cleavage activity of Cas9 *in vitro* (Jinek *et al*, 2012) and allows bacteriophages to evade the host immune response (Bikard & Marraffini, 2012; Jiang *et al*, 2013).

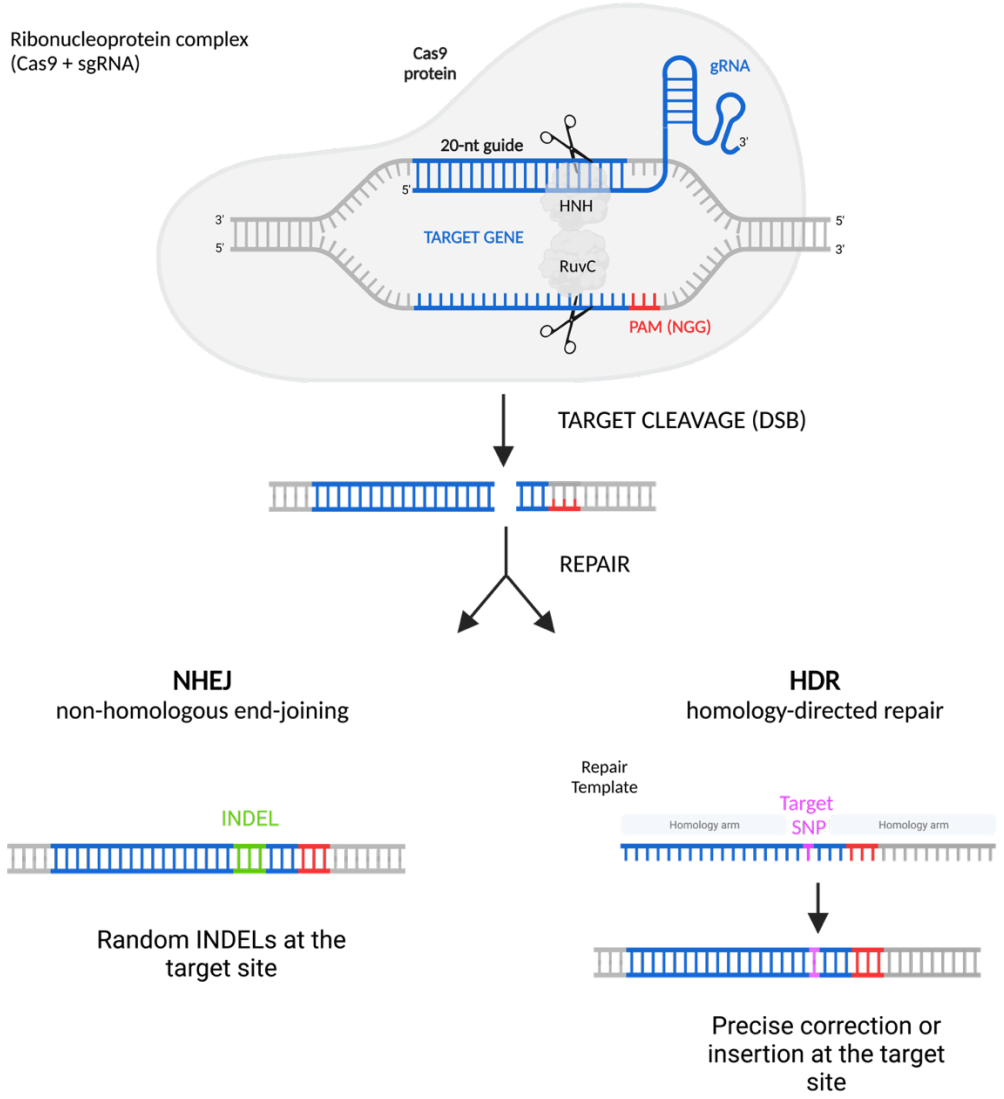

Figure 8. Graphic representation of CRISPR/Cas9 gene editing. When a Cas9 protein and a single gRNA are combined, they form a ribonucleoprotein (RNP) complex. The complex utilizes RNA–DNA base pairing and requires the presence of the PAM site (in red) to cleave genomic DNA at a specific site that is three nucleotides upstream of the PAM site. Cleavage is performed by the nuclease domains HNH and RuvC-like. After cleavage, an intrinsic cellular repair pathway, either NHEJ or HDR, repairs the resulting double-strand break. The NHEJ pathway can be utilized for gene knockouts, which result in random mutations, insertions, or deletions at the target site or targeted genomic rearrangements. The HDR pathway allows for specific gene editing, such as the introduction of single nucleotide substitutions or the insertion/deletion of specific nucleotides. The figure was generated using BioRender (https://www.biorender.com/).

Cas9 uses two nuclease domains, a well-conserved RuvC domain that consists of 3 RuvC motifs and an HNH domain residing in the middle of the protein. Every domain splits a target dsDNA strand at a specific site 3 nucleotides upstream from the sequence PAM "5'-NGG-3'" to produce a DSB (double-strand break) (Gasiunas *et al*, 2012; Jinek *et al*, 2012). The DSB created by Cas9 can then be repaired through different repair systems (Figure 8):

- Nonhomologous end joining (NHEJ) (Lieber *et al*, 2010) is an error-prone process that can be exploited to generate localized mutations, usually small indels, near the cut site indicated by the gRNA. Additionally, in the presence of two or more gRNAs together, NHEJ can be used to obtain deletions of large fragments of DNA, resulting in loss of sequence between the two cuts. This approach is mostly used to study the function of a gene through the generation of small mutations in a coding area or its regulatory region (Meccariello *et al*, 2017; Choo *et al*, 2018; Li & Handler, 2019; Sim *et al*, 2019; Bai *et al*, 2019; Meccariello *et al*, 2019) or to generate functional knockouts (KOs) through the deletion of the entire gene using two gRNAs (Gratz *et al*, 2014).
- Homology directed repair (HDR): allows the integration of foreign DNA sequences with high efficiency in the genome at the target site level (San Filippo *et al*, 2008; Gantz & Akbari, 2018). This mechanism exploits synthetic DNA (donor) with homology to the target sequence. Due to the presence of homology arms from both ends of the cut, single-strand donor DNA can be used to effectively reproduce insertions and deletions or to rebuild a point mutation (Aumann *et al*, 2020; Choo *et al*, 2020; Ward *et al*, 2021), while double-stranded DNA, such as plasmid DNA, can be used as a template, exploiting longer homology arms to promote the insertion of a larger DNA sequence (Gratz *et al*, 2014).

CRISPR/Cas9 has already been applied to several insect species, many of which are of interest to SIT, such as *Ceratitis capitata* (Meccariello *et al*, 2017; Aumann *et al*, 2018; Meccariello *et al*, 2019; Aumann *et al*, 2020; Ward *et al*, 2021), *Bactrocera tryoni* (Choo *et al*, 2018, 2020; Ward *et al*, 2021), *Bactrocera dorsalis* (Bai *et al*, 2019; Zhao *et al*, 2019), *Anastrepha suspensa* (Li & Handler, 2019) and *Zeugodacus cucurbitae* (Paulo *et al*, 2022).

2.5 The *temperature sensitive lethal* (*tsl*) gene: an overview

The release of sterile males is a crucial element in the sterile insect technique application (Dyck *et al*, 2021). Extensive research has been conducted at the IAEA Laboratories in Seibersdorf since the 1980s, resulting in the development of GSSs. The first generation of GSSs relied on the presence of the *wp* mutation, while the second generation incorporated the *tsl* mutation as well. Here, both recessive mutations were combined with a Y/autosome translocation T(Y;5) to place the wild-type allele on the Y chromosome and rescue the wild-type phenotype, resulting in temperature-sensitive females with white puparia and temperature-resistant males emerging from brown puparia. In the presence of this mutation, complete female embryonic lethality was observed following heat treatment at 34°C. Earlier studies utilizing deletion and transposition mapping, along with cytogenetic analysis, provided insights into the genomic location of the *wp* and *tsl* genes in *Ceratitis capitata*. These investigations revealed that both genes are situated on the right arm of chromosome 5 (Kerremans & Franz, 1994). Specifically, the *wp* gene is positioned at 59B on the trichogen polytene chromosome map (Ward *et al*, 2021), while the *tsl* gene is located downstream, likely within the 60C-61B region, close to the *Sergeant-2* gene (Niyazi *et al*, 2005). The successful identification of the *wp* gene (Ward *et al*, 2021) emphasized the importance of identifying the gene(s) and mutation(s) responsible for tsl phenotypes. This would enable the creation of GSSs through genome editing (CRISPR/Cas9) in other significant insect pests. To assess the temperature sensitivity of these strains, several protocols have been developed over time, one of which is the "*temperature sensitive lethal test"* (TSLT) (Franz, 2005; Augustinos *et al*, 2016, 2017), which is essential for temperature sensitivity control in GSS and future strains subjected to CRISPR-induced mutations. Data relating to tests carried out on GSSs have demonstrated the presence of differences even in strains belonging to the same category (Augustinos *et al*, 2017). These can be traced back to various factors, both environmental and genetic. The method of breeding flies is not standardized; therefore, different diets are used as well as numerous years of inbreeding, and the different genetic backgrounds (translocations) can also influence (Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017). Moreover, colonies are often refreshed with new wild material, which can introduce new mutations, thus altering the genetic properties of the strains and reducing the possibility of mass production of males and their use in the application of SIT (Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017).

2.6 Research Objectives.

My **first objective** was to evaluate the temperature sensitivity of twenty-seven *Ceratitis capitata* wild-type, *tsl* mutant, and genetic sexing strains. This endeavor aimed to enhance the implementation of the temperature-sensitive lethal test, identify variations among the tested strains, even within the same category, and select three representative strains, one from each category, as a reference for future investigations (see 3.1). Subsequently, my **second objective** was to investigate the putative region where the *tsl* gene should be located. Using previous cytogenetic studies, it was possible to narrow down the region of interest and deeply examine the genes located within it, their expression levels both in wild-type and *tsl* mutant strains, and the presence of polymorphisms. This resulted in a list of *tsl* candidate genes located within this area (see 3.2). Once one of them was selected, my **third objective** was to knock it out and introduce, via CRISPR/Cas9, one of the point mutations identified in the *tsl* mutant strain into the wild-type background to assess whether this triggers any temperature-sensitive lethal phenotype (see 3.3).

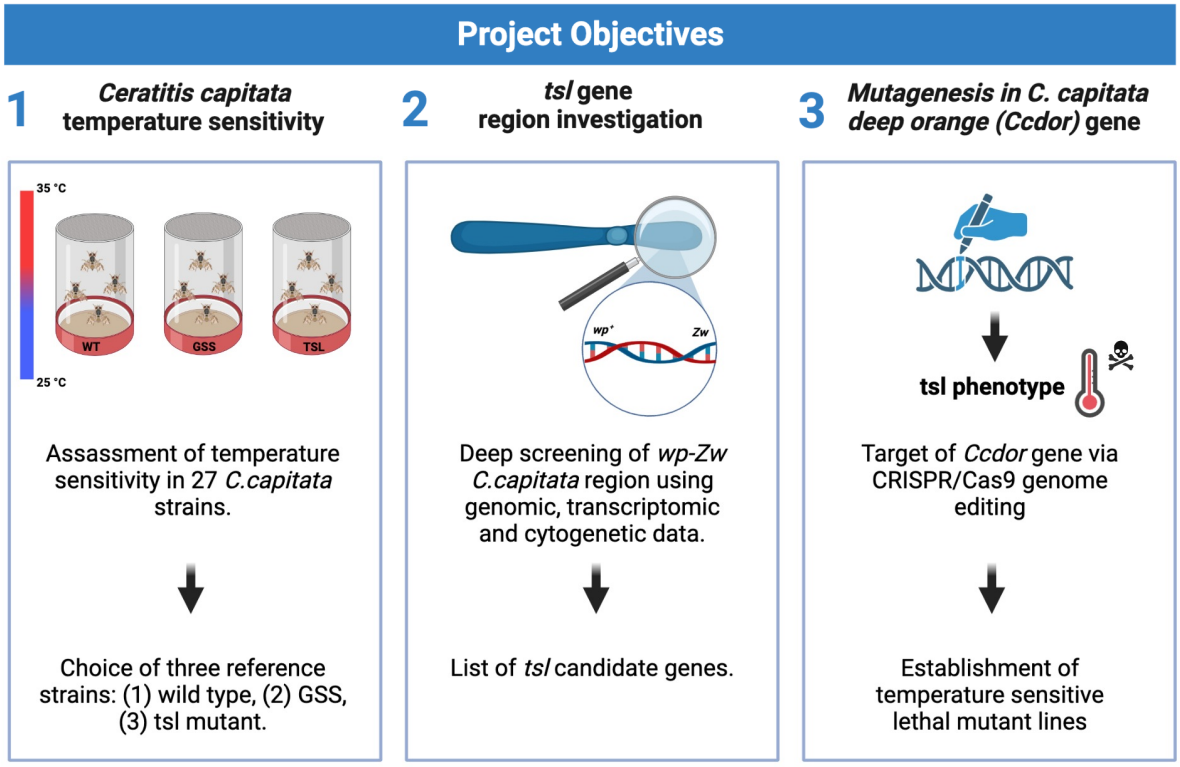

Figure 9. Objectives of this thesis and results obtained. The figure was generated using BioRender (https://www.biorender.com/).

3. Results

The results section of this document incorporates details about three distinct manuscripts that have been published. Each paper is preceded by a page that offers a comprehensive exploration of each work by outlining their primary objective, their specific outcomes, and how these results contribute to the broader theme of the thesis. Furthermore, it provides critical bibliographic information for each manuscript, including the title of the work, a list of authors with an asterisk denoting co-first authors, the journal in which it was published, and a breakdown of author contributions. In addition, the section highlights related presentations, talks, and instances of scientific outreach that are directly connected to each project, showcasing the wider impact and engagement of the research.

The three papers are:

3.1 **Sollazzo G***, Gouvi G*, Nikolouli K, Martinez EIC, Schetelig M, Bourtzis K. (**2022**) Temperature sensitivity of wild-type, mutant and genetic sexing strains of *Ceratitis capitata*. **Insects**; 3(10), 943. https://doi.org/10.3390/insects13100943

3.2 **Sollazzo G**, Gouvi G, Nikolouli K, Aumann RA, Djambazian H, Whitehead MA, Berube P, Che 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: Genes, Genomes, Genetics**, jkad074. https://doi.org/10.1093/g3journal/jkad074

3.3 **Sollazzo G,** Nikolouli K, Gouvi G, Aumann RA, Schetelig MF, Bourtzis K. (**2023**) *Deep orange* gene editing triggers temperature-sensitive lethal phenotypes in *Ceratitis capitata.* **BMC Biology.** In submission.

3.1 Assessment of temperature sensitivity in *Ceratitis capitata* strains

The first aim was to evaluate the temperature sensitivity of twenty-seven *Ceratitis capitata* wild-type, *tsl* mutant, and genetic sexing strains. By doing so, I could determine the most effective application of the temperature-sensitive lethal test and identify any variations among the tested strains, even within the same category. Ultimately, I selected three strains, one from each category, to serve as reference strains in future studies. This study pointed out that the presence of the *tsl* alleles in mutant strains and GSS females results in embryo mortality and reduced pupal recovery and adult emergence rates under high temperatures. However, there is variability in the level of lethality, potentially due to new mutations or genetic background changes affecting the expression of the tsl phenotype. Variability is also observed among wild-type strains, indicating different sensitivities to elevated temperatures.

Title: **Temperature sensitivity of wild-type, mutant, and genetic sexing strains of *Ceratitis capitata*.**

Authors: **Sollazzo G. ***, Gouvi G. *****, Nikolouli K., Martinez EIC., Schetelig MF., Bourtzis K.

Status: **published** in Insects (2022).

Contribution:

- Conceptualization: Bourtzis K.
- Data curation: **Sollazzo G.,** Gouvi G., Nikolouli K., Martinez EIC.
- Formal analysis: **Sollazzo G.**, Nikolouli K.
- Investigation: **Sollazzo G.**, Gouvi G., Nikolouli K., Martinez EIC.
- Methodology: **Sollazzo G.**, Gouvi G., Nikolouli K., Martinez EIC., Bourtzis K.
- Supervision: Bourtzis K., Schetelig MF.
- Validation: **Sollazzo G.**, Nikolouli K., Bourtzis K.
- Writing-original draft: **Sollazzo G.**, Nikolouli K., Bourtzis K.
- Writing-review and editing: **Sollazzo G.**, Gouvi G., Nikolouli K., Schetelig MF., Bourtzis K.

Presentation: This work was presented at the 2nd Research Coordination Meeting on the "Generic approach for the development of genetic sexing strains for sterile insect technique applications" of the FAO/IAEA in Vienna, Austria (speaker; 2021 – Talk title: *wp* and *tsl* genes as generic markers for the construction of genetic sexing strains for SIT applications).

insects

MDPI

Article

Temperature Sensitivity of Wild-Type, Mutant and Genetic Sexing Strains of *Ceratitis capitata*

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† These authors contributed equally to this work.

Simple Summary: The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is a major agricultural insect pest species worldwide. The sterile insect technique (SIT), as a component of area-wide integrated management (AW-IPM) programmes, is currently used to control populations of this pest. SIT is based on the mass production and release of sexually sterile insects, ideally males, over a target area. Male-only releases can be achieved by using genetic sexing strains (GSS) such as the medfly VIENNA 8 GSS. Females of this strain are homozygous for a mutation in the *temperature-sensitive lethal* (*tsl*) gene, which kills them when exposed to high temperatures during the embryonic stage. In the present study, we employed a temperature-sensitive lethal test (TSLT) to determine the temperature sensitivity or tolerance of twenty-seven *Ceratitis capitata* wild-type, genetic sexing, and *tsl* mutant strains. Significant differences were detected among the strains studied with respect to egg hatching, pupal, and adult recovery rates. We discussed our findings in the context of SIT applications and climate change.

Abstract: Area-wide integrated pest management (AW-IPM) programmes with a sterile insect technique component (SIT) are used to control populations of insect pests worldwide, including the Mediterranean fruit fly, *Ceratitis capitata*. SIT consists of the mass rearing, radiation-induced sterilization, handling, and release of sterile insects over the target area. Although SIT can be performed by using both sterile males and females, male-only releases significantly increase the efficiency and cost-effectiveness of SIT applications. Male-only releases can be achieved by using genetic sexing strains (GSS). The medfly VIENNA 8 GSS is based on two selectable markers, the white pupae (*wp*) gene, and the temperature-sensitive lethal (*tsl*) genes. The latter allows the elimination of females by exposing embryos to elevated temperatures. This study assessed the temperature sensitivity of twenty-seven medfly strains through a TSLT. Our results indicated significant differences among the strains regarding egg hatching as well as pupal and adult recovery rates due to the presence or absence of the *tsl* mutation and/or the genetic background of the strains. Our findings are discussed in the context of SIT applications, the importance of the *tsl* gene for developing genetic sexing strains, and climate change.

Keywords: Mediterranean fruit fly; sterile insect technique; temperature-sensitive lethal; white pupae; Tephritidae; Diptera

Citation: Sollazzo, G.; Gouvi, G.; Nikolouli, K.; Cancio Martinez, E.I.; Schetelig, M.F.; Bourtzis, K. Temperature Sensitivity of Wild-Type, Mutant and Genetic Sexing Strains of *Ceratitis capitata*. *Insects* **2022**, *13*, 943. https://doi.org/10.3390/insects13100943

Received: 27 July 2022
Accepted: 12 October 2022
Published: 18 October 2022

1. Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824), also called "medfly", is a member of the Tephritidae family in the order Diptera and is considered one of the most harmful species for agriculture worldwide. It is a cosmopolitan, highly polyphagous, and carpophagous species, and its presence has been documented in more than 260 species of fruits and vegetables of major economic interest [1,2]. Medfly's rapid invasion in five different continents and its extensive destructive potential have fueled intensive control programmes aiming to restrict the distribution of the pest in the invaded areas [3,4]. Several methods have been developed to control medfly populations including the sterile insect technique (SIT) [5–7].

As a component of area-wide integrated pest management programmes (AW-IPM), the sterile insect technique has been used to control populations of insect pests and disease vectors worldwide [8]. SIT is based on the mass production and release of irradiation-induced sterile flies over a target area to suppress wild populations of a target species [5,7]. During the last decades, AW-IPM programmes with an SIT component have been developed and implemented to control populations of insect pest species, including medfly, in several parts of the world with the purpose of suppression, prevention, containment, or eradication strategies [9–13].

Several studies have shown that the mass production and release of sterile males only, increase the efficiency and cost-effectiveness of SIT applications significantly. This is because: (a) costs associated with the mass production, handling, release, and post-release monitoring activities are reduced, as there is no production, handling, and release of females, (b) released sterile males are not engaged in mating with sterile females and are only seeking wild females, and (c) avoiding releases of sterile females prevents further damage of fruits and vegetables [14–17]. An efficient and robust way to separate males from females is through developing and using genetic sexing strains (GSS) [17].

Two GSS are currently used in mass rearing facilities to produce sterile males for SIT applications against medfly, namely VIENNA 7 and VIENNA 8 [18]. Both of them have been constructed using classical genetic approaches and carry two recessive selectable markers, the *white pupae* (*wp*) and the *temperature-sensitive lethal* (*tsl*) genes, which are localized on the right arm of chromosome 5 [17]. The females of the VIENNA 7 and VIENNA 8 GSS are homozygous for the mutant alleles of the *wp* and *tsl* genes, emerge from white puparia, and die when exposed as 24-h-old embryos to elevated temperatures (34–35 °C) for 24 h. The GSS males are heterozygous on both genetic loci, with wild-type alleles linked to the Y chromosome and the male determining region through an irradiation-induced translocation. They emerge from brown puparia and survive when exposed to high temperatures [17,19] (Figure 1). The only difference between VIENNA 7 and VIENNA 8 GSS lies in the translocation. VIENNA 7 and VIENNA 8 GSS carry the translocation at position T(Y;5)52A and T(Y;5)58B, respectively (translocation breakpoints determined based on trichogen polytene chromosome maps) [17,19]. Due to chromosomal translocation, GSS are considered semi-sterile presenting reduced productivity, which depends on how the Y-autosome translocation segregates during gametogenesis. Alternate segregation produces genetically balanced progeny, while adjacent-1 segregation produces unbalanced progeny due to deletions and triplications. Progeny with deletions usually die at the embryonic stage. However, those with triplications may survive even up to the pupal or the adult stage, which may explain the difference in the productivity levels observed between different GSS [17].

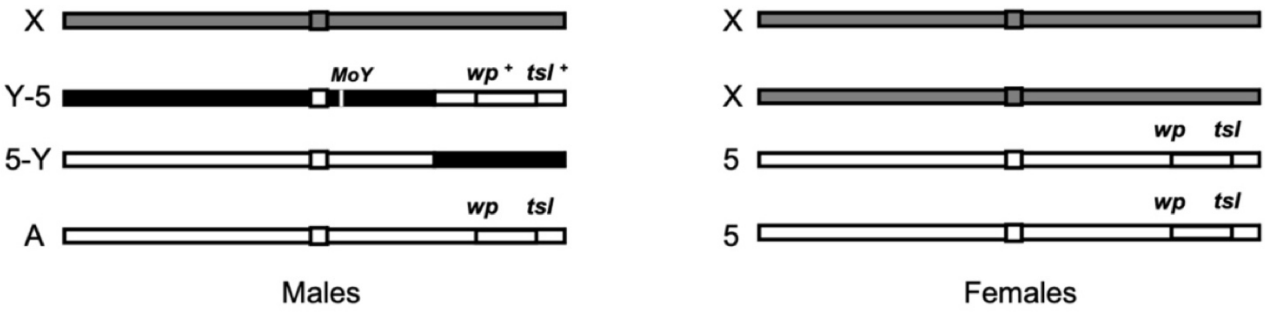

Figure 1. Schematic representation of a *Ceratitis capitata* VIENNA GSS. GSS females are homozygous for the mutant alleles of the *wp* and *tsl* gene markers, while GSS males are heterozygous at both loci with the wild-type alleles of the two markers linked to the male determining region of the Y chromosome (MoY) through an irradiation-induced T(Y;5) translocation.

Recombination phenomena may affect the genetic stability of a GSS and, through the accumulation of recombinants during mass-rearing, can result in the loss of their genetic sexing properties [17,19]. In medfly, this problem has been addressed by: (a) the development and implementation of a "filter rearing system", which removes the recombinants and thus maintains the genetic integrity of the GSS [15,20] and (b) the development of the In(5)50C;59C inversion on chromosome 5 (inversion breakpoints determined based on trichogen polytene chromosome maps), which is also known as "D53 inversion"), that carries the *wp* and *tsl* selectable markers. Inversions are known suppressors of genetic recombination and the incorporation of the D53 inversion in the VIENNA GSS has significantly increased their genetic stability [17,19].

A recent study assessed the genetic stability and rearing efficiency of VIENNA 7 and VIENNA 8 GSS at different facilities worldwide [18]. Despite their common origin, the strains showed differences in egg hatching, pupal recovery, adult emergence rates, and male tolerance to elevated temperatures [18]. The causal factor(s) for these differences could be environmental and/or genetic. The productivity of the GSS may vary in laboratories and mass-rearing facilities due to different rearing practices, including the type of diet and type of translocation and its segregation behaviour during gametogenesis [17,18]. Long-term colony maintenance and inbreeding and the refreshment of mass-rearing colonies with wild genetic material followed by adaptation in the given rearing conditions may have resulted in altered phenotypic properties, with respect to sex separation and male recovery rates upon heat treatment, both being critical for the mass production of high-quality males for SIT applications [17,18]. Thus, in this study, we assess the temperature sensitivity and recovery rates of several wild-type, mutant, and GSS medfly strains under small-scale, laboratory rearing conditions.

2. Materials and Methods

2.1. Ceratitis Capitata Strains and Rearing Conditions

All strains were maintained under small-scale, laboratory rearing conditions at 24 ± 2 °C, 55 ± 10% RH, and 14/10 h light/dark cycle at IAEA Insect Pest Control laboratories (Seibersdorf, Austria). Adults were placed in 20 × 20 × 30 cm cages and fed on yeast: sugar (1:3) with water being provided separately. Larvae were reared on a carrot diet as described previously [21] with minor modifications: 2.5 kg carrot powder (Van Drunen Farms, Momence, IL., USA), 7 kg cooked frozen baby carrots (Ardo, Ardooie, Belgium), 840 g yeast hydrolysate enzymatic (MP Biomedicals^{TM}), 80 g sodium benzoate, water up to 10 L while the pH was adjusted to 4.3–4.4 with 115 ml of hydrochloric acid 32%. As shown in Table 1, twenty-seven strains were used in the present study, classified into three groups: "wild type" strains (No. 1–9), "GSS" (No. 10–20), with females being homozygous and males being heterozygous for the *tsl* mutant allele, respectively, and "mutant" strains

(No. 21–27) with all individuals being homozygous for the *tsl* mutant allele. The GSS strains were constructed by crossing males from the translocation lines (VIENNA 7, VIENNA 8) with females from the *wp tsl* mutant strain. VIENNA 7 and VIENNA 8 GSS have been reconstructed in different years and/or places; this is indicated in their names. Additional characteristics for some of these strains are presented in Table 1 as footnotes.

Table 1. *Ceratitis capitata* strains used in the present study.

	Strain	Group
1	EgII FF21 [a]	wild-type
2	EgII FF26 [a]	wild-type
3	Benakeion Volos FF26	wild-type
4	Seibersdorf (SEIB) FF26	wild-type
5	Argentina FF26	wild-type
6	Benakeion TR 34 FF26 [b]	wild-type
7	Benakeion TR 35 FF26 [b]	wild-type
8	Benakeion TR 34S FF26 [c]	wild-type
9	Benakeion TR 35S FF26 [c]	wild-type
10	VIENNA 8 2010 FF26 [d]	GSS
11	VIENNA 8 2018 FF26 [d]	GSS
12	VIENNA 8 2019 FF26 [d]	GSS
13	VIENNA 8 Sr^2 FF26 [e]	GSS
14	VIENNA 8 "El Pino" FF26	GSS
15	VIENNA 8 Israel FF26	GSS
16	VIENNA 8 Argentina FF26	GSS
17	VIENNA 7 2017 FF26 [d]	GSS
18	VIENNA 7 2018 FF26 [d]	GSS
19	VIENNA 7 2019 FF26 [d]	GSS
20	VIENNA 7 2020 FF26 [d]	GSS
21	*wp tsl* FF21 [a]	mutant
22	*wp tsl* FF26 [a]	mutant
23	*wp tsl* (EgII) FF21 [a]	mutant
24	*wp tsl* (EgII) FF26 [a]	mutant
25	D53-3-28 FF21 [a]	mutant
26	D53-3-28 FF26 [a]	mutant
27	D53-1 FF26	mutant

[a] Strains were kept as parallel cultures in two rooms, FF21 and FF26, at the IPCL under the same rearing conditions for backup purposes. [b] The Benakeion TR 34 and Benakeion TR 35 strains were established with Benakeion strain survivors of TSLT conducted at 34 °C and 35 °C, respectively. [c] The Benakeion TR 34S and Benakeion TR 35S strains were established with Benakeion TR and Benakeion TR 35 strain survivors of TSLT performed at the next generation at 34 °C and 35 °C, respectively. [d] VIENNA 7 and VIENNA 8 GSS, generated in the respective years. [e] VIENNA 8 GSS, carrying the Sr^2 dominant mutation, a homozygous lethal mutation that leads to the expression of a third stripe on the fly's abdomen [22].

2.2. Temperature-Sensitive Lethal Test

The TSLT was performed as previously described [18] with the only difference that the GSS and the mutant strains were tested in all six temperatures (25, 31, 32, 33, 34, and 35 °C) while the wild-type strains were only assessed at 25, 34 and 35 °C ("short TSLT"). Each TSLT was performed with 100 eggs per temperature and replicated three times by collecting eggs over three consecutive days. In total, 1800 eggs (100 eggs × 3 replicates × 6 temperatures) were used for the "TSLT", while 900 eggs were used for the "short TSLT". All eggs were collected within five hours from adult cages containing 5 to 7 days old adults. After collection, eggs were placed on black strips (100 eggs/strip) on top of 90 × 15 mm Petri dishes with larval carrot diet and held at 25 °C for 24 h. After 24 h incubation, each batch was kept at one of the six different temperatures (25, 31, 32, 33, 34, and 35 °C). After the heat treatment, all eggs were left at 25 °C to complete their development. Egg hatching, pupal recovery, and adult emergence rates were determined 5, 15, and 23 days post egg collection, respectively (Figure 2). Egg hatching was calculated for each replicate using the number of collected embryos (100) as a reference and the number of empty eggshells

after five days. Pupal recovery was calculated using the number of pupae obtained divided by the hatched eggs. The adult recovery rate was calculated using the number of eclosed adults divided by the number of pupae.

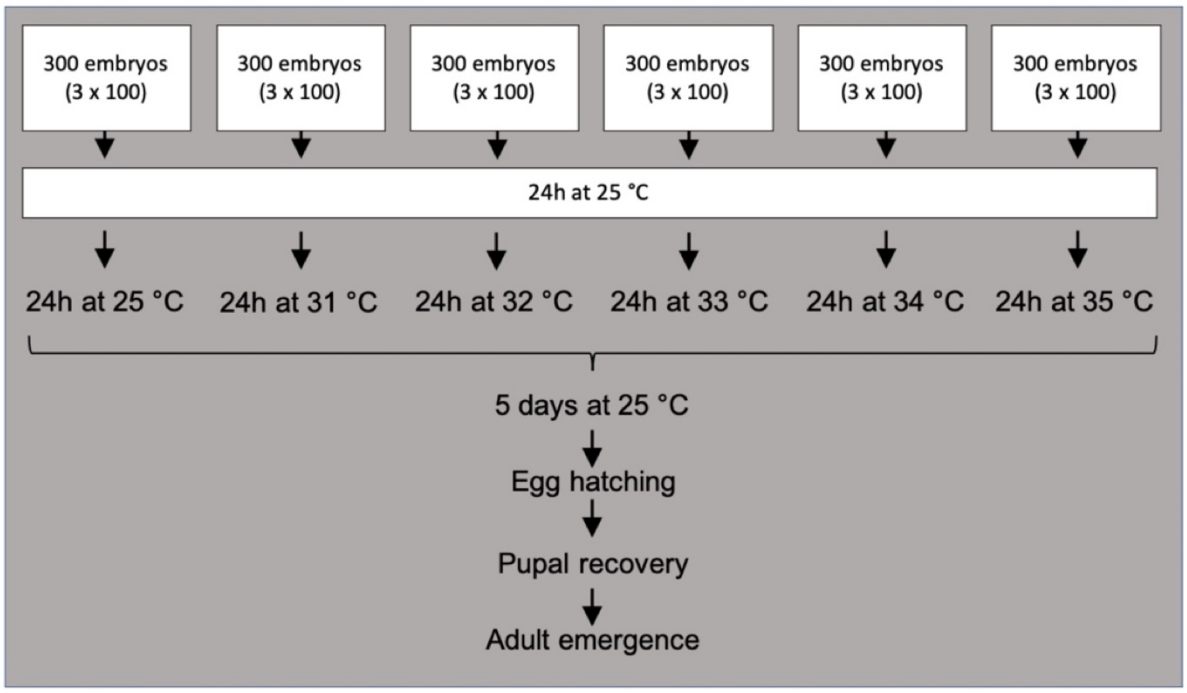

Figure 2. TSLT workflow. For each temperature test, 300 freshly collected eggs were used and incubated at 25 °C for 24 h. After that, heat treatments were conducted for 24 h, and the egg hatching, pupal recovery, and adult emergence rates were determined.

2.3. Statistical Analysis

All statistical analyses were performed using R version 4.0.5 (R Core Team 2021). Egg hatching, pupal recovery, and adult emergence rates represent recovery ratios. Therefore, all datasets were analyzed using a GLM-binomial family and a logit link function [23]. For the recovery rates, the following parameters were measured: a) egg hatching, b) pupae recovery, and c) adult recovery, as the number of adults emerged from the total number of pupae. The generalized linear models (GLM) overdispersion was checked with the DHARMA package [24]. DHARMA tests if the simulated dispersion is equal to the observed dispersion and supports the visual inspection of the residuals. Overdispersion of the GLM-binomial models was addressed with a quasi-binomial model using a logit link function [25]. Analysis of deviance was performed with a Chi-squared test for GLM-Binomial models and an F-test for GLM-quasi-binomial models [26]. Half-normal plots with simulation envelopes were used to inspect the goodness-of-fit of the models [27] visually. The pairwise comparisons of the fitted model estimates were calculated with the 'estimated marginal means' (emmeans) package [28]. For all data, the significance level was set to $\alpha = 0.05$.

3. Results

3.1. Egg Hatching at 25 °C, 34 °C, and 35 °C

Statistically significant differences were detected among the strains for egg hatching at the rearing temperature of 25 °C ($F = 24.851$, $df = 26$, $p < 2.2 \times 10^{-16}$) (Figure 3; Table S1). Regarding the wild-type strains, egg hatching ranged from 85.11 ± 2.76% for Benakeion TR 34S to 98 ± 1% for EgII FF26 at 25 °C. In the case of GSS, egg hatching at 25 °C had

its lowest value at 65.22 ± 4.12% for VIENNA 8 2010 and reached up to 92 ± 2.40% for VIENNA 8 Israel. The respective values for the mutant strains were 86.22 ± 2.91% for wp tsl FF26 and 96.11 ± 4.04 for D53-3-28 FF26. Significant differences were also observed among all strains when embryos were exposed for 24 h at both 34 °C ($F = 48.767$, $df = 26$, $p < 2.2 \times 10^{-16}$) and 35 °C ($F = 40.615$, $df = 26$, $p < 2.2 \times 10^{-16}$) (Figure 3). Heat exposure at 34 °C had moderate effects on egg hatching for the wild-type strains and more pronounced ones in the case of the GSS and mutant strains. Mutant strains had a hatching rate of zero or close to zero at 34 or 35 °C, which manifests the thermosensitivity of the *tsl* mutant allele present in these strains, except for the D53-3-28 FF21 and D53-3-28 FF26 strains. Similarly, recovery of females was reduced in the GSS as they were homozygous for the *tsl* mutant allele (Table S1). Pairwise comparisons of each strain are shown in Table S2.

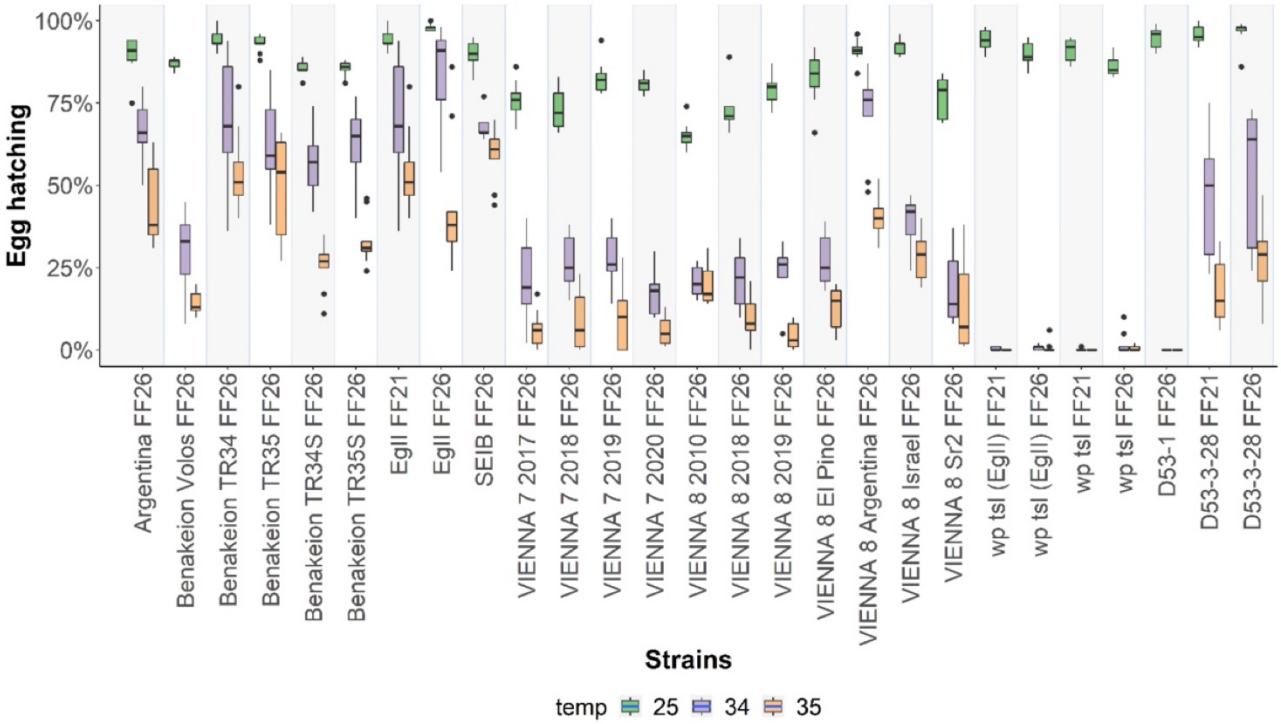

Figure 3. Egg hatching rates of twenty-seven *Ceratitis capitata* wild-type, mutant, and GSS strains. Egg hatching rates of strains reared at 25 °C without heat-shock treatment and after 24 h heat-shock treatment at 34 °C and 35 °C are shown. Dots represent outliers, while dashes represent the absence of variance in the data.

3.2. Pupal Recovery at 25 °C, 34 °C, and 35 °C

Pupal recovery rates were assessed based on the hatched larvae for all strains evaluated. Statistically significant differences were detected among all strains tested in respect to pupal recovery at 25 °C ($F = 12.944$, $df = 25$, $p < 2.2 \times 10^{-16}$) and ranged between 50.11 ± 9.1% for VIENNA 8 2018 and 93.22 ± 3.53% for D53-3-28 FF21 (Figure 4; Table S1). The low pupal recovery of the GSSs was attributed to their semi-sterility. Significant differences were also observed when embryos were exposed for 24 h at both 34 °C ($F = 6.241$, $df = 24$, $p = 5.011 \times 10^{-14}$) and 35 °C ($F = 5.5303$, $df = 22$, $p = 2.266 \times 10^{-11}$) (Figure 4; Table S1). The highest pupal recovery rates among wild-type strains were observed in EgII FF26 (59.78 ± 13.54%), while the lowest was observed in Benakeion Volos (10.56 ± 5.41%), when embryos were exposed at 34 °C. VIENNA 8 Argentina and VIENNA 8 2018 had the highest and lowest pupal recovery at 34 °C among the GSSs, respectively, while in the case of the *tsl* mutant strains, heat-shock at 34 °C had detrimental effects in all strains with

0 or close to 0 pupal recovery (Table S1). As mentioned above, D53-3-28 FF21 and D53-3-28 FF26 were the only *tsl* mutant strains that exhibited an abnormal pattern and behaved unlike their mutant classification. Pairwise comparisons of each strain are shown in Table S3, indicating statistically significant differences among the wild-type strains, GSS, and mutant strains.

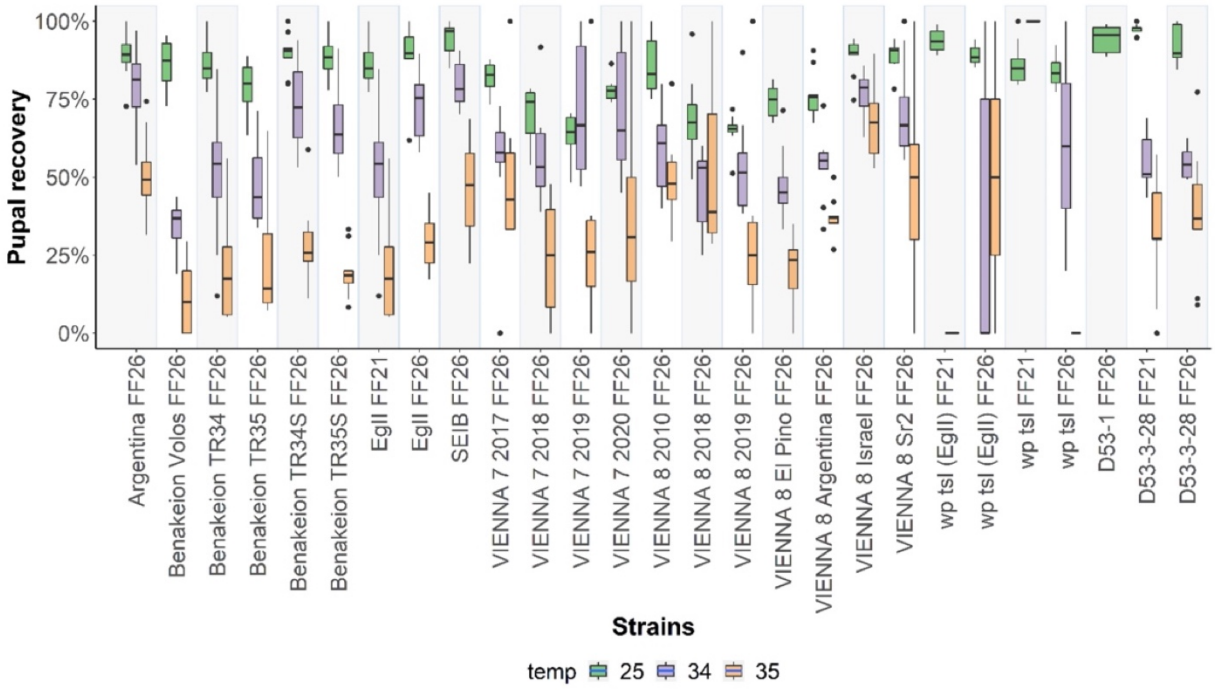

Figure 4. Larval to pupal recovery rates of twenty-seven *Ceratitis capitata* wild-type, mutant, and GSS strains. Pupal recovery rates of strains reared at 25 °C without heat-shock treatment and after 24 h heat-shock treatment at 34 °C and 35 °C are shown. Dots represent outliers, while dashes represent the absence of variance in the data. In the case of *wp tsl* (EgII) FF21, *wp tsl* FF21, and D53-1 FF26, the missing data are due to undefined ratios (denominator was 0).

3.3. Adult Recovery at 25 °C, 34 °C, and 35 °C

Similar to what was observed for egg hatching and pupal recovery, statistically significant differences were also found among the strains studied with respect to adult recovery at 25 °C ($F = 6.937$, $df = 25$, $p < 2.2 \times 10^{-16}$; Figure 5; Table S1). Significant differences were also detected in the adult emergence when embryos were exposed for 24 h to both 34 °C ($F = 2.5586$, $df = 26$, $p = 0.0002887$) and 35 °C ($F = 2.5149$, $df = 21$, $p = 0.0006646$) (Figure 5; Table S1). Pairwise comparisons of each strain are shown in Table S4.

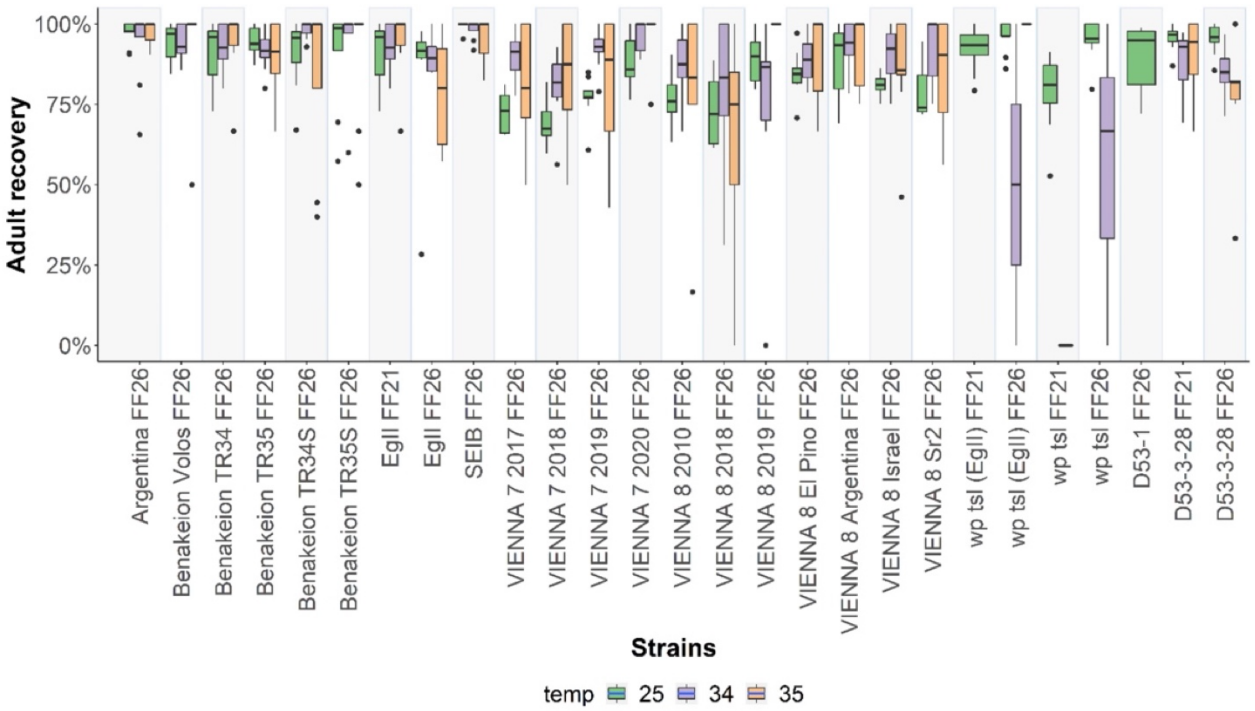

Figure 5. Pupal to adult recovery rates of twenty-seven *Ceratitis capitata* wild-type, mutant, and GSS strains. Adult recovery rates of strains reared at 25 °C without heat-shock treatment and after 24 h heat-shock treatment at 34 °C and 35 °C are shown. Dots represent outliers, while dashes represent the absence of variance in the data. In the case of *wp tsl* (EgII) FF21, *wp tsl* FF21, *wp tsl* FF26, and D53-1 FF26, the missing data are due to undefined ratios (denominator was 0).

3.4. Temperature-Sensitive Lethal Tests at Additional Temperatures

Heat exposure of 24 h old GSS and *tsl* mutant embryos at 34 °C and 35 °C showed differential sensitivity among the strains (Table S1). Due to this variability, we investigated the thermal response of these strains (GSS and mutant) at three additional temperatures, 31 °C, 32 °C, and 33 °C, to define the point at which heat sensitivity is expressed in each strain. Interestingly, egg hatching between 31 °C and 32 °C did not show any significant decrease, except for the strains VIENNA 7 2017 (df = 5, $p = 0.0137$), VIENNA 8 Sr^2 (df = 5, $p = 0.0377$), and wp *tsl* FF21 (df = 5, $p = < 0.0001$), where the difference was statistically significant. When the temperature was increased to 33 °C, embryos presented higher sensitivity in most cases, apart from the strains VIENNA 7 2019, VIENNA 8 Sr^2, and VIENNA 8 Argentina, which showed no significant decrease in egg hatching. The results indicated that the reduced fertility at the embryonic stage starts at 31 °C for all the GSS and *tsl* mutant strains except for VIENNA 8 Israel, VIENNA 8 Argentina, and D53-3-28 FF26, where exposure to 33 °C is required (Figure 6, Table S5).

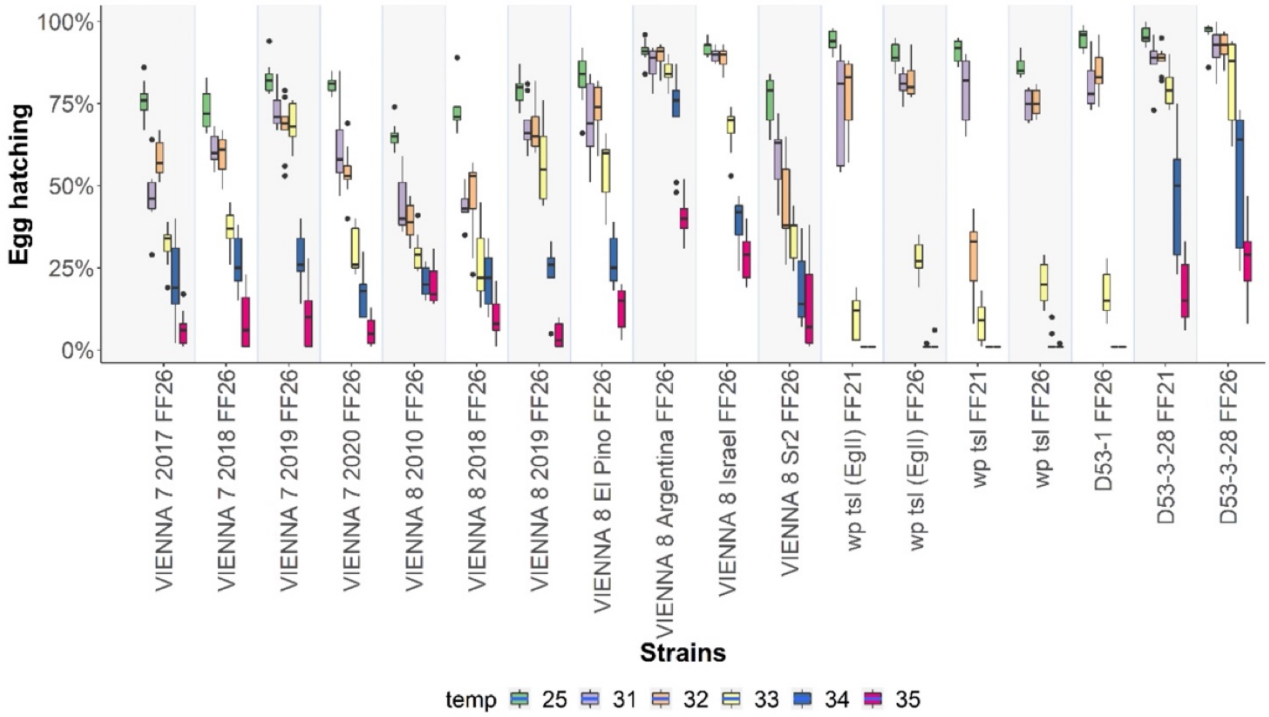

Figure 6. Egg hatching rates of *Ceratitis capitata* GSS and mutant strains of the TSLT at six different temperatures are shown. Dots represent outliers, while dashes represent the absence of variance in the data.

4. Discussion

The *temperature-sensitive lethal (tsl)* gene plays a crucial role in the successful implementation of SIT applications against the agricultural pest, *Ceratitis capitata*, as its engineered presence in the genetic sexing strains VIENNA 7 and VIENNA 8 allows the robust and efficient sex separation at the embryonic stage, thus resulting in the release of sterile males-only individuals [17,26,28]. As the function of the *tsl* gene is critical as a selectable marker for sex separation, its genetic stability should be monitored regularly to ensure the overall stability and robustness of the GSS [18]. The present study applied to different wild-type, GSS and *tsl* mutant strains temperature-sensitive lethal tests and confirmed, with some exceptions discussed below, the presence and stability of the *tsl* gene marker in several genetic sexing strains and *tsl* mutant strains that are routinely used in SIT-related research and applications.

Temperature-sensitive lethal tests were performed on eleven GSS. Our results indicated that the thermal exposure of embryos reduced egg hatching, pupal recovery, and adult emergence. As female embryos were homozygous for the *tsl* allele and sensitive to elevated temperatures, lethality was observed mainly in females. In contrast, lethality was rescued in male embryos carrying a wild-type, tsl^{+} allele. Moreover, a frequent application of "TSLTs", in addition to the "filter rearing system" is strongly recommended due to the possibility of having recombinants, which, being white puparia and resistant to elevated temperatures, can accumulate under mass-rearing conditions and lead to the loss of the sexing properties and the breakdown of the genetic sexing strain [17,18].

It is also important to note that the GSS strains tested in the present study: (a) are products of two different translocations (VIENNA 7 and VIENNA 8), (b) may include additional markers such as Sr^{2} [24], (c) may have been (re)constructed at different time points and (d) may have been introgressed into local genetic background depending on the region of SIT applications. These factors may have affected their recombination rates due to the

distance between the translocation break point and the selectable markers [17] and their response to elevated temperatures [18]. Our results showed significant temperature sensitivity at temperatures lower than 34 °C and 35 °C resulting in female lethality and reduced male recovery. This is important because, in the context of operational SIT programmes, reduced male recovery rates would increase the cost of the applications. SIT facilities may decide to change the strain opting for a strain with higher fitness at 25 °C and improving the overall performance of their strain by enriching it with a local genetic background. In addition, if complete female lethality can be achieved at a lower temperature, the recovered males will have experienced less stress and may be of higher biological quality. Bottlenecks, long-term inbreeding, accumulation of spontaneous mutations, and introgression into local genetic backgrounds may have played a role in modulating the tsl phenotype, the rearing efficiency, and the characteristics of the genetic sexing strains. Several studies have reported that prolonged mass-rearing leads to laboratory adaptation, which sequentially can result in loss of genetic diversity and significant changes in alleles [29–32]. These alterations can affect various phenotypes and life-history traits. Therefore, they should be continuously monitored to verify that genetic diversity, biological quality, and competitiveness are maintained and that the production of high-quality sterile insects for SIT applications is assured [33,34]. Towards this goal, periodic refreshments of mass-reared colonies with wild or partially inbred material have been proven to greatly value mass-rearing facilities [29,31,35]. Infusion of the mass-reared colony with "fresh" flies can limit genetic bottlenecks and maintain the genetic diversity and biological quality of the mass-reared insects.

Analysis of four different *wp tsl* strains indicated that they fully expressed the temperature-sensitive lethal phenotype. In contrast, out of the three inversion lines studied, only one of them (D53-1) behaved like a typical *tsl* mutant strain; however, the other two lines (having the same origin but kept for a long time in separate rearing rooms) demonstrated a significant number of survivors at both 34 °C and 35 °C. All three strains contain the D53 inversion, which covers parts of chromosome 5 [In(5)50C;59C]. Chromosomal inversions are known suppressors of genetic recombination, and therefore, we would expect that the D53 inversion, which resides in the *wp tsl* genomic region, would suppress recombination in that region [17]. The left breakpoint of the inversion is located between the *wp* and *tsl* loci, being closer to the *wp* locus [19,36]. Therefore, recombination phenomena could be possible as the *tsl* locus is outside the borders of the D53 inversion. A revertant mutation at the *tsl* gene combined with genetic recombination could explain the presence of survivors at elevated temperatures, thus creating *wp* tsl^+ individuals. Mutation(s) on secondary genetic loci might also affect the expression of the tsl phenotype. The latter may require exposure to higher temperatures to achieve complete lethality, as recently shown for another medfly strain carrying the *tsl* mutation [37].

Differences were detected among the tested wild-type strains at elevated temperatures 34 °C and 35 °C. For example, the hatching rate of the "Benakeion Volos" strain was low at both temperatures compared to the wild-type strain EgII FF26 suggesting that temperature-sensitive lethal alleles (not necessary of the same *tsl* locus) may be present in this strain, and they could be potentially harnessed for the establishment of novel temperature-sensitive lethal strains. On the other hand, the "Seibersdorf" and "Argentina" strains exhibited high eclosion rates, suggesting they are more tolerant at high temperatures. This property could be helpful for SIT applications in regions with a warmer climate.

Moreover, climate change increases the need to characterize such mutations and is not to be underestimated [38]. In combination with the invasive character of *C. capitata*, climate change favoured the shift of its geographic range into new areas [39,40]. On the other hand, climate change can severely affect an AW-IPM programme because temperature changes can influence the sterile males and male mating competitiveness. Rising temperatures could lead to the death of sterile males released during SIT. However, it should be noted that *Ceratitis capitata* can adapt to such stressful conditions [41,42]. It has been observed that virgin sexually mature males prefer a warmer temperature than females (±1.7 °C), also keeping an optimal mating propensity [41]. Since the global surface annual temperature

has increased at an average rate of 0.1 °C, almost double compared to 20 years ago, and increases of 1.5 °C and 2-4 °C are expected by 2050 and 2100, respectively [43], *C. capitata* may still have room for adaptation over the next years. This may speed up the life cycle, increase the number of generations per year, and expand its geographic range. In addition, it is essential to note that climate change has also resulted in the unpredictable appearance and frequency of weather phenomena such as storms, heavy rains, and heat and cold waves. Those may impact IPM programmes with an SIT component, especially during the release of sterile males, since such phenomena may affect their longevity, flight ability, and mating performance.

5. Conclusions

The temperature-sensitive lethal tests on twenty-seven *Ceratitis capitata* strains resulted in significant differences. The homozygous lethal *tsl* alleles in mutant strains and GSS females trigger embryonic lethality and subsequent reduction in pupal recovery and adult emergence rates when embryos are subjected to elevated temperatures. However, variability was observed in the lethality levels, which could be due to novel mutations resulting in revertant alleles or changes in the genetic background, which might influence the expression levels and/or the penetrance of the tsl phenotype. Variability was observed among the wild-type strains suggesting that some may be more sensitive to elevated temperatures than others. In addition, some of the *tsl* mutant strains may express the tsl phenotype at lower than usual temperatures (less than 34 °C). The results of the present study indicate that TSLTs should frequently be carried out with strains used in SIT-related projects, both in research labs and operational programmes. In addition, the thermal tolerance of GSS males used in SIT field applications should be tested because high temperatures occur more frequently due to climate change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13100943/s1, Table S1: Egg hatching, pupal recovery, and adult emergence rates at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains. Table S2: Pairwise comparisons of egg hatching at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains. Table S3: Pairwise comparisons of pupal recovery at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains. Table S4: Pairwise comparisons of adult recovery at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains. Table S5: Temperature pairwise comparisons of egg hatching at 25 °C, 31 °C, 32 °C, 33 °C, 34 °C and 35 °C for GSS and *tsl* mutant *Ceratitis capitata* strains.

Author Contributions: Conceptualization, K.B.; Data curation, G.S., G.G., K.N. and E.I.C.M.; Formal analysis, G.S. and K.N.; Investigation, G.S., G.G., K.N. and E.I.C.M.; Methodology, G.S., G.G., K.N., E.I.C.M. and K.B.; Supervision, M.F.S. and K.B.; Validation, G.S., K.N. and K.B.; Writing—original draft, G.S., K.N. and K.B.; Writing—review and editing, G.S., G.G., K.N., M.F.S. and K.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Insect Pest Control Subprogramme of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in the manuscript.

Acknowledgments: This study benefitted from discussions at meetings for the Coordinated Research Project D44003, "Generic approach for the development of genetic sexing strains for SIT applications", funded by the International Atomic Energy Agency (IAEA). GG would like to thank George Tsiamis for his support and encouragement during this study. The authors thank Nikos Papadopoulos and Gustavo Taret for providing insect populations.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

Table S1: Egg hatching, pupal recovery, and adult emergence rates at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains.

Strain	Temperature (°C)	Egg hatching* (%)	Pupal recovery* (%)	Adult emergence* (%)
EgII FF21	25	93.89	86.39	90.96
	34	67.78	52.46	92.50
	35	54.78	22.52	95.50
EgII FF26	25	98.00	89.00	87.52
	34	83.67	72.64	89.76
	35	44.44	32.25	84.50
Benakeion Volos FF26	25	87.33	86.01	93.64
	34	29.89	35.32	93.68
	35	14.22	12.50	93.75
Seibersdorf (SEIB) FF26	25	89.78	93.81	99.47
	34	67.67	79.15	98.34
	35	59.22	47.09	94.82
Argentina FF26	25	89.67	88.85	97.21
	34	67.11	76.99	91.83
	35	43.78	49.75	97.96
Benakeion TR 34 FF26	25	93.89	86.39	90.96
	34	67.78	52.46	92.50
	35	54.78	22.52	95.50
Benakeion TR 35 FF26	25	92.89	79.31	94.27
	34	62.78	48.32	90.84
	35	48.56	26.32	89.57
Benakeion TR 34 S FF26	25	85.11	89.69	90.39
	34	55.89	71.17	98.32
	35	25.89	27.04	77.78
Benakeion TR 35 S FF26	25	85.44	88.43	90.15
	34	61.78	65.65	95.89
	35	33.00	19.87	94.92
VIENNA 8 2010 FF26	25	65.22	85.35	76.25
	31	44.22	66.83	72.18
	32	39.56	64.33	92.14
	33	29.44	72.08	87.43
	34	21.90	59.82	89.31
	35	19.89	49.72	76.40
VIENNA 8 2018 FF26	25	72.78	68.85	71.84
	31	43.89	48.10	78.42
	32	45.33	50.49	76.70
	33	25.44	60.26	88.41
	34	21.40	50.47	79.63
	35	9.33	52.38	81.82
	25	79.22	64.66	89.59

VIENNA 8 2019 FF26	31	68.33	67.32	90.34
	32	67.44	70.68	88.11
	33	56.89	66.99	91.84
	34	22.00	56.36	84.68
	35	4.33	25.64	100.00
VIENNA 8 Sr^2 FF26	25	76.44	89.53	78.41
	31	58.33	72.76	78.80
	32	45.00	71.85	82.82
	33	35.00	74.92	83.47
	34	16.60	73.49	89.34
	35	13.33	56.67	77.94
VIENNA 8 Guatemala "El Pino" FF26	25	82.56	74.56	85.02
	31	69.67	70.33	83.22
	32	73.89	66.62	87.81
	33	56.22	67.39	85.34
	34	26.50	44.53	88.98
	35	12.89	24.14	89.29
VIENNA 8 Israel FF26	25	92.00	88.53	81.31
	31	89.67	75.71	78.56
	32	88.89	80.25	75.70
	33	67.11	69.21	86.36
	34	37.20	76.08	89.40
	35	28.44	66.02	84.62
VIENNA 8 Argentina FF26	25	91.00	76.56	88.52
	31	86.89	74.42	87.97
	32	89.89	72.81	91.51
	33	84.89	66.36	94.67
	34	68.20	52.49	91.62
	35	40.22	37.85	91.24
VIENNA 7 2017 FF26	25	76.11	81.61	72.45
	31	46.56	69.93	78.84
	32	58.00	67.05	78.29
	33	31.89	74.22	85.92
	34	19.20	60.94	90.60
	35	6.67	45.00	77.78
VIENNA 7 2018 FF26	25	72.78	69.31	68.72
	31	60.67	58.06	77.92
	32	59.56	56.53	74.59
	33	37.00	66.67	83.33
	34	25.80	54.65	80.14
	35	8.56	36.36	85.71
VIENNA 7 2019 FF26	25	82.89	63.00	76.81
	31	72.78	56.64	87.60
	32	67.89	64.48	85.53
	33	69.33	54.33	86.73
	34	26.30	64.64	91.76
	35	10.44	28.72	77.78
VIENNA 7 2020 FF26	25	81.22	78.11	87.39
	31	60.78	57.59	85.40

	32	54.22	67.83	76.74
	33	29.56	81.20	95.37
	34	16.80	64.88	97.25
	35	5.89	33.96	94.44
wp/tsl FF21	25	91.33	86.25	79.13
	31	79.33	50.70	48.62
	32	28.78	50.97	57.58
	33	8.89	26.25	28.57
	34	1.00	10.00	0.00
	35	1.00	0.00	0.00
wp/tsl FF26	25	86.22	83.63	95.07
	31	74.78	69.99	63.91
	32	75.44	68.48	48.17
	33	20.44	39.13	88.89
	34	2.30	26.09	50.00
	35	1.22	0.00	0.00
wp/tsl (EgII) FF21	25	94.33	94.94	93.30
	31	75.78	75.37	51.75
	32	78.44	79.32	54.64
	33	10.11	25.27	39.13
	34	1.00	0.00	0.00
	35	1.00	0.00	0.00
wp/tsl (EgII) FF26	25	89.56	88.96	95.82
	31	80.56	76.83	72.17
	32	82.00	80.35	74.03
	33	27.56	62.50	80.00
	34	1.10	18.18	50.00
	35	1.56	7.14	100.00
D53-3-28 FF21	25	95.78	97.33	95.47
	31	88.33	90.94	96.68
	32	88.56	88.83	96.05
	33	79.56	84.08	92.19
	34	42.10	54.39	89.08
	35	17.67	32.08	90.20
D53-3-28 FF26	25	96.33	93.08	95.17
	31	91.56	91.99	91.42
	32	92.33	93.14	93.02
	33	82.44	73.99	89.07
	34	51.70	54.35	85.77
	35	26.33	37.97	78.89
D53-1 FF26	25	95.11	94.39	89.36
	31	81.33	67.21	89.43
	32	84.33	70.36	91.39
	33	17.11	23.38	58.33
	34	1.00	0.00	0.00
	35	1.00	0.00	0.00

* The values are presented as mean ± standard deviation.

Table S2: Pairwise comparisons of egg hatching at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII_FF21	25 - 34	0.326	0.0724	Inf	4.503	<.0001
	25 - 35	0.539	0.0929	Inf	5.801	<.0001
	34 - 35	0.213	0.1123	Inf	1.897	0.0579
EgII_FF26	25 - 34	0.158	0.0507	Inf	3.121	0.0018
	25 - 35	0.791	0.123	Inf	6.43	<.0001
	34 - 35	0.633	0.1312	Inf	4.823	<.0001
Benakeion Volos FF26	25 - 34	2.783	0.124	Inf	22.468	<.0001
	25 - 35	3.728	0.138	Inf	26.936	<.0001
	34 - 35	0.944	0.12	Inf	7.867	<.0001
Seibersdorf (SEIB) FF26	25 - 34	0.283	0.0356	Inf	7.95	<.0001
	25 - 35	0.416	0.0414	Inf	10.046	<.0001
	34 - 35	0.133	0.0499	Inf	2.67	0.0076
Argentina FF26	25 - 34	0.29	0.0565	Inf	5.126	<.0001
	25 - 35	0.717	0.0859	Inf	8.342	<.0001
	34 - 35	0.427	0.0968	Inf	4.415	<.0001
Benakeion TR 34 FF26	25 - 34	0.326	0.0724	Inf	4.503	<.0001
	25 - 35	0.539	0.0929	Inf	5.801	<.0001
	34 - 35	0.213	0.1123	Inf	1.897	0.0579
Benakeion TR 35 FF26	25 - 34	0.392	0.072	Inf	5.442	<.0001
	25 - 35	0.649	0.0938	Inf	6.917	<.0001
	34 - 35	0.257	0.1131	Inf	2.271	0.0231
Benakeion TR 34 S FF26	25 - 34	0.421	0.0546	Inf	7.703	<.0001
	25 - 35	1.19	0.0969	Inf	12.281	<.0001
	34 - 35	0.77	0.1063	Inf	7.242	<.0001
Benakeion TR 35 S FF26	25 - 34	0.324	0.0559	Inf	5.807	<.0001
	25 - 35	0.951	0.0933	Inf	10.2	<.0001
	34 - 35	0.627	0.1023	Inf	6.127	<.0001
VIENNA 8 2010 FF26	25 - 34	1.9605	0.108	Inf	18.185	<.0001
	25 - 35	2.0221	0.109	Inf	18.558	<.0001
	34 - 35	0.0616	0.117	Inf	0.526	0.5986
VIENNA 8 2018 FF26	25 - 34	1.222	0.129	Inf	9.468	<.0001
	25 - 35	2.066	0.205	Inf	10.062	<.0001
	34 - 35	0.844	0.236	Inf	3.575	0.0004
VIENNA 8 2019 FF26	25 - 34	1.18	0.106	Inf	11.103	<.0001
	25 - 35	2.96	0.281	Inf	10.522	<.0001
	34 - 35	1.78	0.298	Inf	5.974	<.0001
VIENNA 8 Sr^2 FF26	25 - 34	1.428	0.231	Inf	6.192	<.0001
	25 - 35	1.746	0.276	Inf	6.334	<.0001
	34 - 35	0.318	0.35	Inf	0.911	0.3625
VIENNA 8 Guatemala "El Pino" FF26	25 - 34	1.1	0.108	Inf	10.17	<.0001
	25 - 35	1.86	0.169	Inf	10.988	<.0001
	34 - 35	0.76	0.196	Inf	3.874	0.0001

VIENNA 8 Israel FF26	25 - 34	0.853	0.0591	Inf	14.423	<.0001
	25 - 35	1.174	0.0745	Inf	15.747	<.0001
	34 - 35	0.321	0.0932	Inf	3.449	0.0006
VIENNA 8 Argentina FF26	25 - 34	0.234	0.0475	Inf	4.935	<.0001
	25 - 35	0.816	0.0855	Inf	9.544	<.0001
	34 - 35	0.582	0.093	Inf	6.258	<.0001
VIENNA 7 2017 FF26	25 - 34	1.28	0.16	Inf	7.986	<.0001
	25 - 35	2.47	0.307	Inf	8.044	<.0001
	34 - 35	1.19	0.34	Inf	3.502	0.0005
VIENNA 7 2018 FF26	25 - 34	0.996	0.132	Inf	7.557	<.0001
	25 - 35	2.167	0.253	Inf	8.577	<.0001
	34 - 35	1.171	0.277	Inf	4.222	<.0001
VIENNA 7 2019 FF26	25 - 34	1.08	0.122	Inf	8.842	<.0001
	25 - 35	2.1	0.222	Inf	9.482	<.0001
	34 - 35	1.02	0.249	Inf	4.108	<.0001
VIENNA 7 2020 FF26	25 - 34	1.53	0.111	Inf	13.781	<.0001
	25 - 35	2.62	0.202	Inf	13.015	<.0001
	34 - 35	1.09	0.228	Inf	4.797	<.0001
wp/tsl FF21	25 - 34	42.2	61.06	Inf	2.583	0.0098
	25 - 35	42.2	61.06	Inf	2.583	0.0098
	34 - 35	1	1.18	Inf	0	1
wp/tsl FF26	25 - 34	25	31.96	Inf	2.522	0.0117
	25 - 35	25	31.96	Inf	2.522	0.0117
	34 - 35	1	1.18	Inf	0	1
wp/tsl (EgII) FF21	25 - 34	56.255	84.191	Inf	2.693	0.0071
	25 - 35	42.154	61.06	Inf	2.583	0.0098
	34 - 35	0.749	0.927	Inf	-0.233	0.8156
wp/tsl (EgII) FF26	25 - 34	34.3	47.06	Inf	2.577	0.01
	25 - 35	34.3	47.06	Inf	2.577	0.01
	34 - 35	1	1.18	Inf	0	1
D53-3-28 FF21	25 - 34	26.51	47.38	Inf	1.834	0.0667
	25 - 35	105.72	198.1	Inf	2.487	0.0129
	34 - 35	3.99	4.39	Inf	1.257	0.2088
D53-3-28 FF26	25 - 34	20.87	38.59	Inf	1.643	0.1004
	25 - 35	69.14	130.18	Inf	2.25	0.0245
	34 - 35	3.31	3.35	Inf	1.186	0.2356
D53-1 FF26	25 - 34	77.8	136.66	Inf	2.48	0.0132
	25 - 35	77.8	136.66	Inf	2.48	0.0132
	34 - 35	1	1.18	Inf	0	1

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.

Table S3: Pairwise comparisons of pupal recovery at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII_FF21	25 - 34	0.499	0.121	Inf	4.13	<.0001
	25 - 35	1.345	0.25	Inf	5.378	<.0001
	34 - 35	0.846	0.272	Inf	3.112	0.0019
EgII_FF26	25 - 34	0.203	0.0632	Inf	3.215	0.0013
	25 - 35	1.015	0.1833	Inf	5.537	<.0001
	34 - 35	0.812	0.1894	Inf	4.288	<.0001
Benakeion Volos FF26	25 - 34	0.89	0.123	Inf	7.209	<.0001
	25 - 35	1.93	0.345	Inf	5.585	<.0001
	34 - 35	1.04	0.366	Inf	2.841	0.0045
Seibersdorf (SEIB) FF26	25 - 34	0.17	0.0467	Inf	3.639	0.0003
	25 - 35	0.689	0.0964	Inf	7.149	<.0001
	34 - 35	0.519	0.1038	Inf	5	<.0001
Argentina FF26	25 - 34	0.153	0.0615	Inf	2.487	0.0129
	25 - 35	0.59	0.1268	Inf	4.65	<.0001
	34 - 35	0.437	0.1348	Inf	3.239	0.0012
Benakeion TR 34 FF26	25 - 34	0.511	0.123	Inf	4.164	<.0001
	25 - 35	1.345	0.251	Inf	5.35	<.0001
	34 - 35	0.833	0.274	Inf	3.043	0.0023
Benakeion TR 35 FF26	25 - 34	1.411	0.285	Inf	4.95	<.0001
	25 - 35	2.373	0.328	Inf	7.227	<.0001
	34 - 35	0.962	0.327	Inf	2.946	0.0032
Benakeion TR 34 S FF26	25 - 34	0.231	0.0595	Inf	3.885	0.0001
	25 - 35	1.199	0.2086	Inf	5.749	<.0001
	34 - 35	0.968	0.2143	Inf	4.516	<.0001
Benakeion TR 35 S FF26	25 - 34	0.298	0.0609	Inf	4.888	<.0001
	25 - 35	1.493	0.2144	Inf	6.965	<.0001
	34 - 35	1.195	0.2203	Inf	5.426	<.0001
VIENNA 8 2010 FF26	25 - 34	0.342	0.0986	Inf	3.467	0.0005
	25 - 35	0.54	0.1242	Inf	4.351	<.0001
	34 - 35	0.199	0.1537	Inf	1.292	0.1965
VIENNA 8 2018 FF26	25 - 34	0.379	0.155	Inf	2.441	0.0147
	25 - 35	0.261	0.205	Inf	1.274	0.2026
	34 - 35	-0.117	0.247	Inf	-0.474	0.6354
VIENNA 8 2019 FF26	25 - 34	0.338	0.157	Inf	2.147	0.0318
	25 - 35	1.597	0.378	Inf	4.221	<.0001
	34 - 35	1.26	0.394	Inf	3.193	0.0014
VIENNA 8 Sr^2 FF26	25 - 34	0.191	0.0684	Inf	2.8	0.0051
	25 - 35	0.457	0.1151	Inf	3.973	0.0001
	34 - 35	0.266	0.1313	Inf	2.026	0.0427
VIENNA 8 Guatemala "El Pino" FF26	25 - 34	1.237	0.153	Inf	8.099	<.0001
	25 - 35	2.221	0.233	Inf	9.54	<.0001
	34 - 35	0.983	0.252	Inf	3.909	0.0001
	25 - 34	0.146	0.0481	Inf	3.036	0.0024

VIENNA 8 Israel FF26	25 - 35	0.293	0.0699	Inf	4.195	<.0001
	34 - 35	0.147	0.0806	Inf	1.826	0.0679
VIENNA 8 Argentina FF26	25 - 34	1.047	0.169	Inf	6.199	<.0001
	25 - 35	1.68	0.202	Inf	8.324	<.0001
	34 - 35	0.632	0.198	Inf	3.185	0.0014
VIENNA 7 2017	25 - 34	1.032	0.178	Inf	5.787	<.0001
	25 - 35	1.628	0.281	Inf	5.791	<.0001
	34 - 35	0.596	0.302	Inf	1.973	0.0485
VIENNA 7 2018	25 - 34	0.217	0.0956	Inf	2.27	0.0232
	25 - 35	0.655	0.2376	Inf	2.758	0.0058
	34 - 35	0.438	0.2499	Inf	1.753	0.0797
VIENNA 7 2019	25 - 34	-0.0644	0.101	Inf	-0.636	0.5251
	25 - 35	0.7276	0.311	Inf	2.338	0.0194
	34 - 35	0.792	0.318	Inf	2.49	0.0128
VIENNA 7 2020 FF26	25 - 34	0.14	0.0818	Inf	1.708	0.0876
	25 - 35	0.833	0.272	Inf	3.062	0.0022
	34 - 35	0.693	0.2813	Inf	2.464	0.0137
wp/tsl FF21	25 - 34	14.8	17.99	Inf	2.22	0.0264
	25 - 35	14.8	17.99	Inf	2.22	0.0264
	34 - 35	1	1.03	Inf	0	1
wp/tsl FF26	25 - 34	11.9	13.73	Inf	2.139	0.0325
	25 - 35	11.9	13.73	Inf	2.139	0.0325
	34 - 35	1	1.03	Inf	0	1
wp/tsl (EgII) FF21	25 - 34	44.4	75.15	Inf	2.239	0.0252
	25 - 35	44.4	75.15	Inf	2.239	0.0252
	34 - 35	1	1.03	Inf	0	1
wp/tsl (EgII) FF26	25 - 34	18.9	24.4	Inf	2.278	0.0227
	25 - 35	18.9	24.4	Inf	2.278	0.0227
	34 - 35	1	1.03	Inf	0	1
D53-3-28 FF21	25 - 34	29.86	64.79	Inf	1.566	0.1174
	25 - 35	77.34	168.89	Inf	1.991	0.0465
	34 - 35	2.59	2.54	Inf	0.971	0.3314
D53-3-28 FF26	25 - 34	10.66	15.45	Inf	1.631	0.1028
	25 - 35	20.73	30.22	Inf	2.079	0.0376
	34 - 35	1.95	1.86	Inf	0.694	0.4876
D53-1 FF26	25 - 34	39.5	64.25	Inf	2.263	0.0236
	25 - 35	39.5	64.25	Inf	2.263	0.0236
	34 - 35	1	1.03	Inf	0	1

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.

Table S4: Pairwise comparisons of adult recovery at 25 °C, 34 °C and 35 °C for twenty-seven *Ceratitis capitata* strains.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII_FF21	25 - 34	-0.204	0.488	Inf	-0.418	0.6762
	25 - 35	-0.745	0.933	Inf	-0.799	0.4246
	34 - 35	-0.542	0.99	Inf	-0.547	0.5844
EgII_FF26	25 - 34	-0.224	0.569	Inf	-0.393	0.6943
	25 - 35	0.252	0.853	Inf	0.295	0.7679
	34 - 35	0.475	0.901	Inf	0.528	0.5977
Benakeion Volos	25 - 34	-0.00761	0.679	Inf	-0.011	0.9911
	25 - 35	-0.01878	1.575	Inf	-0.012	0.9905
	34 - 35	-0.01117	1.681	Inf	-0.007	0.9947
Seibersdorf (SEIB) FF26	25 - 34	1.16	1.137	Inf	1.018	0.3087
	25 - 35	2.33	1.066	Inf	2.188	0.0286
	34 - 35	1.17	0.843	Inf	1.393	0.1637
Argentina FF26	25 - 34	0.912	0.627	Inf	1.454	0.1461
	25 - 35	-0.54	1.29	Inf	-0.419	0.6753
	34 - 35	-1.452	1.262	Inf	-1.15	0.25
Benakeion TR 34 FF26	25 - 34	-0.19	0.487	Inf	-0.391	0.6961
	25 - 35	-0.745	0.931	Inf	-0.801	0.4234
	34 - 35	-0.555	0.988	Inf	-0.562	0.574
Benakeion TR 35 FF26	25 - 34	0.506	0.381	Inf	1.328	0.1843
	25 - 35	0.65	0.494	Inf	1.317	0.1879
	34 - 35	0.145	0.526	Inf	0.275	0.7831
Benakeion TR 34 S FF26	25 - 34	-1.83	0.971	Inf	-1.885	0.0594
	25 - 35	0.989	0.741	Inf	1.334	0.1822
	34 - 35	2.819	1.15	Inf	2.452	0.0142
Benakeion TR 35 S FF26	25 - 34	-0.936	1.05	Inf	-0.894	0.3713
	25 - 35	-0.713	2.16	Inf	-0.329	0.7418
	34 - 35	0.223	2.32	Inf	0.096	0.9232
VIENNA 8 2010 FF26	25 - 34	-0.88387	0.479	Inf -	1.845	0.065
	25 - 35	-0.00869	0.415	Inf	-0.021	0.9833
	34 - 35	0.87519	0.591	Inf	1.48	0.1389
VIENNA 8 2018 FF26	25 - 34	-0.054	0.11	Inf	-0.49	0.6242
	25 - 35	-0.1301	0.128	Inf	-1.015	0.3103
	34 - 35	-0.0761	0.154	Inf	-0.494	0.6215
VIENNA 8 2019 FF26	25 - 34	0.443	0.434	Inf	1.019	0.308
	25 - 35	-16.675	3492.268	Inf	-0.005	0.9962
	34 - 35	-17.117	3492.268	Inf	-0.005	0.9961
VIENNA 8 Sr^2 FF26	25 - 34	-0.8367	0.384	Inf	-2.181	0.0292
	25 - 35	0.0274	0.383	Inf	0.072	0.9428
	34 - 35	0.8642	0.514	Inf	1.682	0.0926
VIENNA 8 Guatemala "El Pino" FF26	25 - 34	-0.404	0.385	Inf	-1.049	0.2942
	25 - 35	-0.3842	0.732	Inf	-0.525	0.5996
	34 - 35	0.0198	0.803	Inf	0.025	0.9803
	25 - 34	-0.686	0.366	Inf	-1.875	0.0608

VIENNA 8 Israel FF26	25 - 35	-0.234	0.387	Inf	-0.607	0.5441
	34 - 35	0.452	0.484	Inf	0.934	0.3503
VIENNA 8 Argentina FF26	25 - 34	-0.0407	0.0467	Inf	-0.871	0.3835
	25 - 35	-0.0303	0.0663	Inf	-0.457	0.6474
	34 - 35	0.0104	0.0676	Inf	0.154	0.8776
VIENNA 7 2017 FF26	25 - 34	-1.299	0.331	Inf	-3.928	0.0001
	25 - 35	-0.286	0.472	Inf	-0.605	0.5452
	34 - 35	1.013	0.561	Inf	1.806	0.071
VIENNA 7 2018 FF26	25 - 34	-0.553	0.242	Inf	-2.288	0.0221
	25 - 35	-0.781	0.516	Inf	-1.514	0.1301
	34 - 35	-0.228	0.551	Inf	-0.414	0.6789
VIENNA 7 2019 FF26	25 - 34	-1.2133	0.356	Inf	-3.411	0.0006
	25 - 35	0.0989	0.534	Inf	0.185	0.8531
	34 - 35	1.3122	0.615	Inf	2.134	0.0328
VIENNA 7 2020 FF26	25 - 34	-1.619	0.842	Inf	-1.924	0.0544
	25 - 35	-0.897	1.457	Inf	-0.616	0.538
	34 - 35	0.722	1.664	Inf	0.434	0.6643
wp/tsl FF21	25 - 34	NA				
	25 - 35					
	34 - 35					
wp/tsl FF26	25 - 34	2.96	1.93	Inf	1.533	0.1254
	25 - 35	NA				
	34 - 35					
wp/tsl (EgII) FF21	25 - 34	NA				
	25 - 35					
	34 - 35					
wp/tsl (EgII) FF26	25 - 34	3.13	2.98	Inf	1.051	0.2935
	25 - 35	-13.43	5013.62	Inf	-0.003	0.9979
	34 - 35	-16.57	5013.62	Inf	-0.003	0.9974
D53-3-28 FF21	25 - 34	0.0693	0.0367	Inf	1.886	0.0593
	25 - 35	0.0568	0.0706	Inf	0.805	0.4208
	34 - 35	-0.0124	0.0779	Inf	-0.159	0.8734
D53-3-28 FF26	25 - 34	0.0988	0.0393	Inf	2.516	0.0119
	25 - 35	0.19	0.0847	Inf	2.244	0.0248
	34 - 35	0.0911	0.0918	Inf	0.993	0.3208
D53-1 FF26	25 - 34	NA				
	25 - 35					
	34 - 35					

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.

Table S5: Temperature pairwise comparisons of egg hatching at 25 °C, 31 °C, 32 °C, 33 °C, 34 °C and 35 °C for GSS and *tsl* mutant *Ceratitis capitata* strains.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
VIENNA 8 2010 FF26	25 - 31	0.3886	0.0568	Inf	6.847	<.0001
	31 - 32	0.1115	0.0708	Inf	1.576	0.115
	32 - 33	0.2952	0.0839	Inf	3.517	0.0004
	33 - 34	0.3433	0.1053	Inf	3.259	0.0011
	34 - 35	0.0491	0.1184	Inf	0.414	0.6787
VIENNA 8 2018 FF26	25 - 31	0.5057	0.0847	Inf	5.97	<.0001
	31 - 32	-0.0324	0.1039	Inf	-0.312	0.7552
	32 - 33	0.5775	0.134	Inf	4.309	<.0001
	33 - 34	0.171	0.1692	Inf	1.011	0.3122
	34 - 35	0.8319	0.241	Inf	3.451	0.0006
VIENNA 8 2019 FF26	25 - 31	0.1479	0.0512	Inf	2.888	0.0039
	31 - 32	0.0131	0.0585	Inf	0.224	0.8228
	32 - 33	0.1702	0.0669	Inf	2.543	0.011
	33 - 34	0.8493	0.1182	Inf	7.186	<.0001
	34 - 35	1.7255	0.3016	Inf	5.721	<.0001
VIENNA 8 Sr^2 FF26	25 - 31	0.27	0.0907	Inf	2.98	0.0029
	31 - 32	0.26	0.1249	Inf	2.078	0.0377
	32 - 33	0.251	0.1575	Inf	1.596	0.1105
	33 - 34	0.647	0.2254	Inf	2.868	0.0041
	34 - 35	0.318	0.297	Inf	1.072	0.2836
VIENNA 8 Guatemala "El Pino" FF26	25 - 31	0.1697	0.0541	Inf	3.138	0.0017
	31 - 32	-0.0588	0.0597	Inf	-0.985	0.3246
	32 - 33	0.2733	0.0716	Inf	3.818	0.0001
	33 - 34	0.7131	0.1242	Inf	5.743	<.0001
	34 - 35	0.7598	0.2061	Inf	3.687	0.0002
VIENNA 8 Israel FF26	25 - 31	0.02569	0.0187	Inf	1.377	0.1684
	31 - 32	0.00871	0.0203	Inf	0.428	0.6683
	32 - 33	0.28104	0.0325	Inf	8.638	<.0001
	33 - 34	0.53711	0.0592	Inf	9.066	<.0001
	34 - 35	0.32129	0.0836	Inf	3.841	0.0001
VIENNA 8 Argentina FF26	25 - 31	0.0462	0.028	Inf	1.652	0.0984
	31 - 32	-0.0339	0.0287	Inf	-1.182	0.2374
	32 - 33	0.0572	0.0302	Inf	1.897	0.0578
	33 - 34	0.1647	0.0421	Inf	3.907	0.0001
	34 - 35	0.5822	0.0766	Inf	7.596	<.0001
VIENNA 7 2017 FF26	25 - 31	0.492	0.0788	Inf	6.237	<.0001
	31 - 32	-0.22	0.0892	Inf	-2.464	0.0137
	32 - 33	0.598	0.1102	Inf	5.426	<.0001
	33 - 34	0.407	0.1576	Inf	2.583	0.0098
	34 - 35	1.158	0.2743	Inf	4.221	<.0001
VIENNA 7 2018 FF26	25 - 31	0.182	0.059	Inf	3.086	0.002
	31 - 32	0.0185	0.0672	Inf	0.275	0.7833
	32 - 33	0.476	0.09	Inf	5.287	<.0001

	33 - 34	0.3192	0.1227	Inf	2.602	0.0093
	34 - 35	1.1451	0.2136	Inf	5.361	<.0001
VIENNA 7 2019 FF26	25 - 31	0.1301	0.0466	Inf	2.794	0.0052
	31 - 32	0.0695	0.0562	Inf	1.237	0.2162
	32 - 33	-0.0211	0.0585	Inf	-0.36	0.7187
	33 - 34	0.9028	0.1058	Inf	8.531	<.0001
	34 - 35	0.9901	0.2039	Inf	4.857	<.0001
VIENNA 7 2020 FF26	25 - 31	0.29	0.0537	Inf	5.397	<.0001
	31 - 32	0.114	0.07	Inf	1.63	0.1032
	32 - 33	0.607	0.1031	Inf	5.886	<.0001
	33 - 34	0.573	0.1559	Inf	3.674	0.0002
	34 - 35	1.04	0.2629	Inf	3.958	0.0001
wp/tsl FF21	25 - 31	0.141	0.0351	Inf	4.016	0.0001
	31 - 32	1.014	0.0973	Inf	10.421	<.0001
	32 - 33	1.175	0.2099	Inf	5.598	<.0001
	33 - 34	2.185	0.615	Inf	3.553	0.0004
	34 - 35	0	0.8279	Inf	0	1
wp/tsl FF26	25 - 31	0.14241	0.0296	Inf	4.814	<.0001
	31 - 32	-0.00888	0.0342	Inf	-0.26	0.795
	32 - 33	1.30568	0.0862	Inf	15.155	<.0001
	33 - 34	2.12389	0.2777	Inf	7.649	<.0001
	34 - 35	0.69315	0.461	Inf	1.504	0.1327
wp/tsl (EgII) FF21	25 - 31	0.1867	0.0548	Inf	3.407	0.0007
	31 - 32	-0.0346	0.0656	Inf	-0.527	0.5982
	32 - 33	2.0488	0.2577	Inf	7.95	<.0001
	33 - 34	2.3136	0.8842	Inf	2.617	0.0089
	34 - 35	0	1.1978	Inf	0	1
wp/tsl (EgII) FF26	25 - 31	0.1059	0.0228	Inf	4.643	<.0001
	31 - 32	-0.0178	0.0259	Inf	-0.687	0.4923
	32 - 33	1.0905	0.0643	Inf	16.948	<.0001
	33 - 34	3.2108	0.3649	Inf	8.799	<.0001
	34 - 35	-0.3365	0.4705	Inf	-0.715	0.4745
D53-3-28 FF21	25 - 31	0.08091	0.0305	Inf	2.651	0.008
	31 - 32	-0.00251	0.0372	Inf	-0.068	0.9461
	32 - 33	0.10717	0.0452	Inf	2.371	0.0177
	33 - 34	0.5454	0.0868	Inf	6.281	<.0001
	34 - 35	0.95937	0.1756	Inf	5.464	<.0001
D53-3-28 FF26	25 - 31	0.04856	0.0337	Inf	1.44	0.1498
	31 - 32	-0.00846	0.0387	Inf	-0.218	0.8272
	32 - 33	0.11328	0.0503	Inf	2.25	0.0245
	33 - 34	0.41903	0.0952	Inf	4.404	<.0001
	34 - 35	0.72226	0.1766	Inf	4.089	<.0001
D53-1 FF26	25 - 31	0.1565	0.0267	Inf	5.858	<.0001
	31 - 32	-0.0362	0.0325	Inf	-1.115	0.2648
	32 - 33	1.595	0.113	Inf	14.11	<.0001
	33 - 34	2.8397	0.5136	Inf	5.529	<.0001
	34 - 35	0	0.7092	Inf	0	1

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.

3.2 Analysis of the *wp-Zw* genomic region: putative *tsl* gene location.

In a second study, we examined the region where the *tsl* gene is expected to be located. In this study, using genomic, transcriptomic, bioinformatic, and cytogenetic data, it became feasible to reduce the area of interest and investigate the genes situated within it, their levels of expression in both wild-type and *tsl* mutant strains, and the mutations they show in the mutated strain. Our findings suggest that this region contains 561 genes, with 322 of those having mutations. By comparing gene expression, we identified 32 differentially expressed genes. Through a combination of text mining and genetic comparative analysis, we discovered 33 orthologous genes associated with heat sensitivity in *D. melanogaster*. These results helped to compile a list of genes that, when mutated, may be involved in temperature-sensitive lethal phenotypes in *Ceratitis capitata*.

Title: **Genomic and cytogenetic analysis of the *Ceratitis capitata* temperature-sensitive lethal region.**

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Status: **published** in G3: Genes, Genomes, Genetics (2023)

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Presentation: This work was presented at the 2nd Research Coordination Meeting on the "Generic approach for the development of genetic sexing strains for sterile insect technique applications" of the FAO/IAEA in Vienna, Austria (speaker; 2021).

G3, 2023, 13(6), jkad074
https://doi.org/10.1093/g3journal/jkad074
Advance Access Publication Date: 29 March 2023
Investigation

Genomic and cytogenetic analysis of the *Ceratitis capitata temperature-sensitive lethal* region

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Abstract

Genetic sexing strains (GSS) are an important tool in support of sterile insect technique (SIT) applications against insect pests and disease vectors. The yet unknown *temperature-sensitive lethal* (*tsl*) gene and the recently identified *white pupae* (*wp*) gene have been used as selectable markers in the most successful GSS developed so far, the *Ceratitis capitata* (medfly) VIENNA 8 GSS. The molecular identification of the *tsl* gene may open the way for its use as a marker for the development of GSS in other insect pests and disease vectors of SIT importance. Prior studies have already shown that the *tsl* gene is located on the right arm of chromosome 5, between the *wp* and *Zw* loci (*tsl* genomic region). In the present study, we used genomic, transcriptomic, bioinformatic, and cytogenetic approaches to characterize and analyze this genomic region in wild-type and *tsl* mutant medfly strains. Our results suggested the presence of 561 genes, with 322 of them carrying SNPs and/or insertion–deletion (indel) mutations in the *tsl* genomic region. Furthermore, comparative transcriptomic analysis indicated the presence of 32 differentially expressed genes, and bioinformatic analysis revealed the presence of 33 orthologs with a described heat-sensitive phenotype of *Drosophila melanogaster* in this region. These data can be used in functional genetic studies to identify the *tsl* gene(s) and the causal mutation(s) responsible for the temperature-sensitive lethal phenotype in medfly, and potentially additional genes causing a similar phenotype.

Keywords: Mediterranean fruit fly, sterile insect technique, genetic sexing strain, white pupae, Tephritidae

Introduction

The Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) is a major agricultural insect pest in almost all continents (Harris and Lee 1989). The sterile insect technique (SIT) is an environment-friendly, species-specific method, which is used as a component of area-wide integrated pest management strategies for the population control of medfly as well as other insect pests and disease vectors (Knipling 1955; Enkerlin 2003; Dyck *et al.* 2021; Klassen and Vreysen 2021). SIT is based on the mass production, sterilization by irradiation, and systematic release of sterile insects over an area to suppress, locally eradicate, contain, or prevent the (re)establishment of the SIT-targeted insect pest populations (Enkerlin *et al.* 2017; Dyck *et al.* 2021; Klassen and Vreysen 2021). Although many, and successful, SIT programs have been based on bisexual releases, male-only releases are more efficient and cost-effective, as has been shown in medfly, and can be achieved using genetic sexing strains (GSS) (Hendrichs *et al.* 1995; Rendon *et al.* 2004; Franz *et al.* 2021).

VIENNA 7 and VIENNA 8 are the two most commonly used GSS in SIT applications against medfly (Augustinos *et al.* 2017). They are based on two selectable markers, the *white pupae* (*wp*) and the *temperature-sensitive lethal* (*tsl*) genes (Franz *et al.* 2021). Irradiation-induced translocations have linked the wild-type alleles of these genetic loci with the male-determining region (*MoY*) on the Y-chromosome (Meccariello *et al.* 2019; Franz *et al.* 2021), and cytogenetic analysis identified the translocation breakpoints of VIENNA 7 and VIENNA 8 GSS on trichogen polytene chromosomes at T(Y; 5)52A and T(Y; 5)58B, respectively (Zacharopoulou *et al.* 2017; Franz *et al.* 2021). Therefore, males of these GSS are heterozygous for these loci, emerge from brown puparia and are resistant to elevated temperatures. At the same time, females are homozygous for the mutant alleles of the *wp* and *tsl* genes, emerge from white puparia and are sensitive (die) when exposed at high temperatures, 34°C or 35°C (Caceres 2002; Augustinos *et al.* 2017; Franz *et al.* 2021).

More than 20 years of research and development efforts were required to construct and validate VIENNA 8 GSS, the most successful and high-quality GSS developed so far. Although there has been an increase in requests by FAO and IAEA Member States to develop and implement the SIT against different insect

Received: December 22, 2022. **Accepted:** March 16, 2023

plant pests, livestock pests, and disease vectors, for most of them, there is no sexing system which could be used in support of such applications. Therefore, a "*generic approach*" is needed for the prompt development of GSS, which will not depend on the random discovery or induction of mutations which could be used as selectable markers for sex separation. The suggested concept is based on the discovery of genes which are useful markers for genetic sexing strategies, the characterization of the causal mutations, the identification of the orthologous genes in SIT-targeted species, and finally the induction of the same or similar mutations using genome editing approaches (FAO/IAEA 2021).

The availability of genomes, transcriptomes, genome editing, and functional genetics tools for many of the SIT-targeted insect pest species and disease vectors can greatly support the concept of the "*generic approach*" (Sim *et al.* 2019; FAO/IAEA 2021). However, the (cyto-)genetic characterization of the successfully used medfly GSS, and especially the molecular identification of its marker genes (*wp, tsl*) are key factors to facilitate the concept. Genetic studies and in situ hybridization experiments allowed the mapping of several genes and the establishment of linkage groups (Brown *et al.* 1989; Konsolaki *et al.* 1990; Tolias *et al.* 1990; Rina and Savakis 1991; Zacharopoulou *et al.* 1992; Scott *et al.* 1993; Zwiebel *et al.* 1995; Gariou-Papalexiou *et al.* 2002; Stratikopoulos *et al.* 2008). Mapping the genes *white*, *yellow*, PS_2a, *Pgd*, *S36*, *S38*, *Vg1*, *Vg2*, *Sxl*, and *Zw* on chromosome 5 in medfly indicated synteny to the *Drosophila melanogaster* X chromosome (Gariou-Papalexiou *et al.* 2002; Zacharopoulou *et al.* 2017). Earlier studies, which were based on deletion and transposition mapping combined with cytogenetic analysis, suggested that the medfly *wp* and *tsl* genes are located at position 59B and 59B–61C of the trichogen polytene chromosome map, respectively (Kerremans and Franz 1994). The chromosomal inversion D53 was also found in the vicinity of these genes as it spans the region 50B–59C of the medfly chromosome 5 [on trichogen polytene chromosome map; (Zacharopoulou *et al.* 2017; Franz *et al.* 2021)]. Inversion D53, which has been used as a recombination suppressor in VIENNA 8 GSS to enhance its genetic stability, played a major role in identifying the gene responsible for the white puparium phenotype. Comparative genomic analysis of strains with and without the inversion led to the identification of the right breakpoint of this chromosomal alteration. Combined with comparative transcriptomic analysis, it resulted in the discovery of the *wp* gene located inside the inversion, close to its right breakpoint (Ward *et al.* 2021). In situ hybridization analysis localized the *wp* gene on position 76B of the salivary gland polytene chromosomes, which corresponds to position 59B of trichogen polytene chromosomes, thus confirming the results of the deletion mapping experiments (Ward *et al.* 2021). The discovery of the *wp* gene and the mutations responsible for the white puparium phenotype in medfly and two closely related tephritid species (the Oriental fruit fly *Bactrocera dorsalis* and the melon fly *Zeugodacus cucurbitae*) allowed the induction of CRISPR/Cas9 mutations in the orthologous *wp* gene of the Queensland fruit fly *Bactrocera tryoni* and the establishment of the respective mutant line, thus providing proof-of-concept for the "*generic approach*" (Ward *et al.* 2021).

Although the *wp* gene is a useful selectable marker, it only allows sex separation at the pupal stage. In contrast, the *tsl* gene can be used to separate males and females earlier in development, at the embryonic stage. Therefore, the discovery of this gene would provide a powerful tool for the generic approach to develop GSS for SIT applications. Unlike the white pupae phenotype for which we had functional proof that it is due to a single-copy gene (Ward *et al.* 2021), such evidence is not available for the tsl phenotype; it is unknown whether it is due to a single (protein coding or not) gene or multiple genes, closely linked or not. The *wp* mutation was isolated during an irradiation experiment (Rössler 1979) and it was recently discovered that the white pupae phenotype is due to the insertion of a transposable element (Ward *et al.* 2021). On the other hand, the *tsl* mutation was isolated during an ethyl methanesulphonate (EMS) screen (Busch-Petersen 1990). Both irradiation and EMS mutagenesis screens may induce numerous and various types of mutations, from single nucleotide substitutions to large chromosomal rearrangements, making the detection of a mutation associated with a certain phenotypic trait a rather challenging task. Based on all the currently available genetic and cytogenetic information, the *tsl* gene(s) is located downstream of *wp*, in the region 59B–61C of the trichogen polytene chromosome map, which most likely corresponds to the area 76B–78C of the salivary gland polytene chromosome map (Kerremans and Franz 1994; Niyazi *et al.* 2005; Franz *et al.* 2021). As the *glucose-6-phosphate 1-dehydrogenase* gene (also known as *Zw*) has been located, by in situ hybridization, on position 79C of the polytene chromosome map (Scott *et al.* 1993), the *tsl* gene is certainly located inside the genomic region between *wp* and *Zw* (hereafter *tsl* genomic region), which corresponds to 76B–79C on the map of the medfly polytene chromosome 5. In situ hybridization has been used in this study to localize candidate *tsl* genes as well as to validate the assembly of the *tsl* genomic region. The present study uses cytogenetic, genomic, transcriptomic, and bioinformatic approaches to characterize the medfly *tsl* genomic region and to identify polymorphisms that could potentially be associated with the tsl phenotype toward the discovery of the *temperature-sensitive lethal* (*tsl*) gene.

Materials and methods

Ceratitis capitata strains and rearing conditions

Two wild-type strains, Egypt II (EgII) and Benakeion, the VIENNA 7 GSS, and the *wp tsl* mutant strain of medfly were used in the present study. All four strains were kept under standard laboratory conditions at 24 ± 2°C, 55 ± 10% RH, and 14/10 h light/dark cycle. Adults were fed on yeast and sugar (1:3) with water being provided separately, while larvae were reared on a carrot diet as described previously (Sollazzo *et al.* 2022).

Transcriptomic analysis

Ceratitis capitata Benakeion and *wp tsl* strains were used in a preliminary experiment to estimate the time required for eggs to be exposed at 34°C for the expression of the tsl phenotype (lethality). Egg collections were performed from cages at their fifth to eighth oviposition day. Eggs were counted and placed on black filter papers (three replicates of 100 eggs per filter paper) on top of ca. 50 g of carrot diet, in 90 × 15 mm Petri dishes. The latter were kept at 25°C for 24 h and then incubated for 30, 60, 120, 240, 360 min and 24 h at 34°C. Control plates remained at 25°C. Egg hatching was estimated five days after the egg collection. Based on the results, two time points (60 and 120 min) were chosen for the transcriptomic analysis and 150 eggs were placed per filter paper. Three replicates per strain, temperature and time point were performed. Eggs were kept at 25°C for 24 h and then incubated for 60 and 120 min at 34°C, while the control plates were left at 25°C for 60 min. RNA extraction was performed after the end of each exposure period. Total RNA was extracted by homogenizing each pool of 150 eggs in liquid nitrogen and using the RNeasy Micro kit (QIAGEN).

mRNA was isolated using the NEBNext polyA selection and the Ultra II directional RNA library preparation protocols from NEB

and sequenced on the Illumina Novoseq 6000 at the Centre for Genomic Research, Institute of Integrative Biology, University of Liverpool, UK, using dual indexes as 150 bp paired-end reads (library insert 500 bp). Individual libraries were sequenced to provide >1 million paired-end reads per sample. Each replicate was then assembled separately using Trinity (Haas *et al.* 2013). The assembled transcripts from Trinity were mapped to the Ccap 3.2.1 genome (accession GCA_905071925.1) using minimap (Li 2018) (parameters -ax splice: hq -uf). The Illumina reads were mapped with STAR (Dobin *et al.* 2013). The freely available open-source software Integrative Genomics Viewer (IGV) v 2.6 (Robinson *et al.* 2011) was used to view all data at a genomic and gene level as well as to manually search variants/polymorphisms inspecting gene-by-gene the whole region. The annotation of the Ccap 3.2.1 genome was performed with Funannotate (v1.6.0) (Palmer and Stajich 2020). The annotation was generated during a project to identify *white pupae* genes of several tephritids (Ward *et al.* 2021) (see ENA bioproject PRJEB36344). Genomic repeats were identified using RepeatModeler (v.1.0.11) (Smit *et al.* 2008), and softmasked using RepeatMasker (v4.0.7) (Smit *et al.* 2013). The Funannotate pipeline comprises of multiple steps; (1) "train"—where a genome-guided transcriptome is generated using Trinity (v2.8.5) (Grabherr *et al.* 2011), (2) "predict"—which runs ab initio and evidence-based gene prediction tools, followed by consensus-based gene model selection, and (3) "update"—which is used to refine UTRs and gene models.

Differential expressed (DE) genes analysis between the wild-type and *wp tsl* mutant strains was assessed using the *edgeR* (Robinson *et al.* 2010) package in the Degust public server (https://degust.erc.monash.edu-v4.2-dev, Powell) with the False Discovery Rate cutoff set at 0.05 and abs logFC at 1.

Genomic analysis and detection of polymorphisms

Genomic DNA from one male and one female of the *wp tsl* mutant strain were extracted using ExtractMe DNA tissue kit (Blirt, Poland) following the manufacturer's recommendations, while genomic DNA for the VIENNA 7 female was extracted using phenol/chloroform (Saccheri and Bruford 1993). The libraries for the *wp tsl* strain were prepared using an Illumina TruSeq Nano DNA Kit (Illumina, USA). They were sequenced on an Illumina HiSeq X platform resulting in 150-bp paired-end reads (Macrogen, Korea). The libraries for the VIENNA 7 GSS were prepared using an Illumina TruSeq PCR-free kit (Illumina, USA) and were sequenced on an Illumina HiSeq 400 platform resulting in 150-bp paired-end reads (Centre for Genomic Research, Liverpool). Raw fastq NGS reads were imported in Geneious Prime 2022.1.1 and subjected to trimming using BBDuk Adapter/Quality Trimming Version 38.37 with default settings (plug-in available on Geneious Prime 2022.1.1). The *wp tsl* trimmed reads were mapped to the reference sequence (*wp–Zw* region) extracted from Ccap 3.2.1 genome (accession GCA_905071925.1) using the "*Bowtie2*" (*v. 2.4.5—© Ben Langmead*) Geneious Prime 2022.1.1 plug-in with default parameters (*Alignment type: end to end; High sensitivity*). The obtained consensus contig for the *wp tsl* strain was used for polymorphisms calling.

A putative *tsl* polymorphism must be homozygous for the *tsl* allele in the *tsl* mutant strains (*wp tsl* males and females, and VIENNA 7 females). For this purpose, the Geneious Prime 2022.1.1 tool "*Find Variations/SNPs*" was used and polymorphisms meeting the following criteria were considered: (1) located inside and outside the coding sequence (Geneious Prime 2022.1.1 function), (2) had a minimum variant frequency of 0.80%, (3) had a minimum variant *P*-value of 10^{-6} (where *P*-value represents the probability of a sequencing error resulting in observing bases with at least the given sum of qualities), and (4) had a minimum strand bias *P*-value of 10^{-5} when exceeding 65% strand bias. Polymorphic regions found were classified as insertion, deletion, and SNPs. SNPs found in coding regions were classified as transition or transversion and as synonymous or nonsynonymous according to the nature of the nucleotide and amino acid change, respectively (Geneious Prime function).

For 10X genome sequencing, high molecular weight DNA was prepared as follows: 20 individuals of each sex and strain (EgII and *wp tsl*) were pooled, ground in liquid nitrogen and DNA was extracted using the QIAGEN Genomic tip 100/G kit (Qiagen, Germany). To remove smaller DNA molecule, we performed a size selection with 0.75% agarose SAGE BluePippin cassette (SAGE, MA, USA). The DNA samples were then processed into Linked-Read libraries using the single molecule barcoding kit from 10× Genomics Technologies. The method is based on gel beads containing DNA primers that contain part of the Illumina sequencing adapter (P5 side), bead barcode sequence, and a random sequence. DNA was joined with beads in an oil-buffer emulsion using a microfluidic system. The system was calibrated to produce a ratio close to 20:1 DNA per bead. The emulsion is then isothermally amplified using the bead primers. This process links the barcode to the synthesized strands. We then break the emulsion and perform cleanup, SPRIselect, end-repair, A-tailing, and ligation of the second part of the adapter containing library index and Illumina P7 sequence. After library QC and normalization, sequencing was performed on Illumina HiSeq X in paired-end with 150 cycles pooling two libraries per lane, yielding on average 200 million read pairs per library. The demultiplexed fastq files were assembled into contigs using the supernova assembler (version v2.1.1, 10X Genomics). Different molecules or GEM barcode fractions were tested, ranging from 0.4 to 1, with raw coverages varying between 56X and 120X. The representative assembly for each sample was chosen based on the highest N50. Optimal parameters were determined to be between 0.8 and 1 for barcode fraction, with raw coverage between 100X and 120X. Transcriptomic and genomic data used in the present study have been deposited to NCBI under BioProject No. PRJEB57574.

SNPs found in DE genes, and the medfly orthologs of the *D. melanogaster* heat-sensitive genes (see below) were confirmed through MEGABLAST searches against the EgII and *wp tsl* mutant strain 10X Genomics data using Geneious Prime 2022.1.1 BLAST tool. 10X Genomics data were previously set as a database and results were shown as "*Hit table including Query-centric alignment*". Furthermore, a maximum of 100 hits per read was allowed with an E-value of 1e-100 and a scoring (match–mismatch) of 1–2.

Drosophila melanogaster heat-sensitive genes and *Ceratitis capitata* orthologs

As the medfly temperature-sensitive lethal (tsl) phenotype is essentially a heat-sensitive phenotype since it is being expressed at elevated temperatures, we compiled a list of *Drosophila melanogaster* (*Dm*) temperature-sensitive genes by searching FlyBase (www.flybase.org) using the key word "*heat sensitive*". Protein sequences were downloaded using FlyBase "*Sequence Downloader*" tool, imported in Geneious Prime 2022.1.1 and organized based on chromosomal location. Their medfly ortholog genes in the *tsl* genomic region were identified *via* blastp analysis (using default settings) and, using the reference sequence of this region (*wp–Zw* region), extracted from *C. capitata* 3.2.1 genome (accession GCA_905071925.1) as a database. The detected *Dm* heat-sensitive

gene orthologs in the medfly *wp–Zw* region were screened for polymorphisms using the *wp tsl* consensus contig.

In situ hybridization analysis

As reported previously, salivary glands from third-instar larvae were used as a source for polytene chromosome preparations for in situ hybridization analysis (Zacharopoulou 1990; Gouvi *et al.* 2022). The dissection of the glands was performed in 45% acetic acid, and the glands were then transferred on a cover slip in a drop of 3:2:1 fixative solution of glacial acetic acid/water/lactic acid. Once the glands became transparent, they were squashed. The quality of the polytene chromosomes was then checked in a phase contrast microscope and, if satisfactory, they were flattened via an overnight incubation at −20°C. Next day, the slides were frozen in liquid nitrogen, and the coverslip was removed with a razor blade. The final steps included dehydration of the polytene chromosome preparations in absolute alcohol, air-drying, and storage at room temperature (RT) until their use. DNA probes were prepared using Platinum Green Hot Start PCR Master Mix (2X; Thermo Fisher), while amplicon purifications were made with Zymo DNA Clean and Concentrator kit (Zymo Research, Orange, California). The primers used for the PCRs probes synthesis are presented in Supplementary Table 1. The probes were labeled with digoxigenin using the Dig DNA labeling Kit by Roche, according to the manufacturer's instructions. The DNA probes (120 ng in 15 µl final volume) were denatured for 10 min at 95°C, immediately put on ice and then mixed with 2 µl Hexanucleotide Mix 10×, 2 µl dNTP Labelling Mix and 1 µl Klenow enzyme labeling grade. The reactions were placed overnight at 37°C and were ended with the addition of 2 µl of 0.2 M EDTA (pH 8.0), 5 µl of distilled water, 25 µl 20× SSC, and 50 µl of HD formamide. The probes were stored at −20°C until their use.

In situ hybridization was performed as described in Ward *et al.* (2021). Before hybridization, stored chromosome preparations were hydrated subsequently for 2 min each in the following solutions: 70, 50, 30 ethanol, and then placed in 2× SSC at RT for 2 min. The stabilization of the chromosomes was done in 2× SSC at 65°C for 30 min, followed by denaturation in 0.07 M NaOH for 2 min, and washed in 2× SSC for 30 s. The slides were then dehydrated for 2 min in 30, 50,70, and 95% ethanol solutions, respectively, and air dried. The hybridization was performed on the same day by adding 15 µl of denatured probe (boiled for 10 min and ice-chilled). The slides were covered with siliconized coverslips, sealed, and incubated at 45°C overnight in a box laid out with wet Whatman paper to keep the preparations moist. After incubation, the coverslips were floated off in 2×SSC, and the slides were washed in 2×SSC for 3 × 20 min at 53°C, followed by one wash for 5 min in Buffer 1 (100 mM Tris–HCl pH 7.5/1500 mM NaCl). Slides were then placed for 30 min into Blocking Solution (Blocking reagent 0.5% in Buffer 1) followed by an additional wash for 1 min in Buffer 1. The antibody mix was then added to each slide and incubated in a box with wet Whatman paper for 45 min at RT. Two 15 min washes in Buffer 1 were followed by one wash for 2 min in Detection Buffer (100 mM Tris–HCl pH 9.5/100 mM NaCl). The color reaction was then started by adding 1 ml of NBT/BCIP solution to each slide and incubating for 40 min in the dark at RT. The removal of the NBT/BCIP solution was done with two washes for 1 min each in distilled water. Hybridization sites were identified and photographed using 60× and 100× oil objectives (bright field), with reference to medfly salivary gland chromosome maps (Zacharopoulou 1990).

Results

The *Ceratitis capitata* putative *tsl* region

The *tsl* gene is located in the *wp–Zw* (*tsl*) genomic region, on the right arm of chromosome 5 (Kerremans and Franz 1994; Niyazi *et al.* 2005). According to the recently sequenced genome of the medfly EgII strain (*Ccap*3.2.1—accession GCA_905071925.1), both *wp* and *Zw* genes are located on scaffold 5, which represents chromosome 5 (Ward *et al.* 2021). The *wp* gene is at position 61,551,139, and the *Zw* gene (*glucose-6-phosphate 1-dehydrogenase I*) is at position 67,751,599. The *tsl* genomic region is 6,200,460 bp long and contains 561 putative genes (Funannotate genome annotation), averaging 45 genes/500 kb and 160 exons/500 kb.

RNA-Seq and DE genes

The number of RNA-Seq reads obtained for the different embryonic stages, and thermal treatments of the wild-type Benakeion and the mutant *wp tsl* strains are presented in Supplementary Table 2. The results show an average of 51,981,830 reads/sample with a percentage mapping rate of more than 75% on the Ccap 3.2.1 genome. Following 60 and 120 min of heat-shock at 34°C, 32 DE genes were observed comparing the wild-type Benakeion and the *wp tsl* mutant strains (Figs. 1 and 2).

Genomic analysis—detection of polymorphisms

The *wp tsl* (male and female) and VIENNA 7 (female) Illumina NGS whole genome sequencing read pairs (Supplementary Table 3) were mapped to the *tsl* genomic region extracted from Ccap3.2.1 resulting in a coverage of 98.4% and a pairwise identity of 92.6%. Using the mapped Illumina genome sequence data and the Geneious prime tool "*Find Variations/SNPs*", 2,782 SNPs, 220 insertions and 65 deletions in coding regions and 40,072 SNPs, 9,644 insertions and 2,352 deletions in noncoding (NC) regions were identified in the *tsl* genomic region. Most of the polymorphisms were SNPs and were detected in the NC sequences (72.7%), which included intronic sequences and sequences up to 1,000 bp upstream of the starting codon of the genes. It is worth noting that most detected indels were single nucleotides and were found in highly repetitive AT-rich regions.

Total polymorphism frequency distribution plots in coding (C) and NC regions showed (Supplementary Fig. 1) a nonuniform pattern along the *tsl* genomic region which was confirmed by three different ratios: (1) the number of polymorphisms found in noncoding regions (PNC) divided by the number of genes; (2) the number of polymorphisms found in coding regions (PC) divided by the number of genes; and (3) the number of PNC divided by the number of PC (Table 1).

The 3,067 polymorphisms detected in the coding sequences were distributed among 322 genes, representing 57.4% of the 561 genes in the *tsl* genomic region. The polymorphisms observed in the coding sequences were classified into transition and transversion point mutations, as well as synonymous and nonsynonymous mutations. Most of the SNPs, 1,806 or 64.9%, resulted in transitions, while only 976 (35.1%) were transversions. In addition, synonymous mutations were slightly higher than nonsynonymous mutations (1,555 vs 1,512 or 50.7% vs 49.3%), at a ratio of 1.03. Moreover, 1,229 out of the 3,067 polymorphisms detected in coding regions resulted in amino acid changes, while 248 in frameshift mutations.

Using the same procedure, a total of 320 SNPs, 3 insertions, and 1 deletion were detected in the 32 DE genes. Using MEGABLAST, all 324 polymorphisms were confirmed by the 10X genome sequence

	Ceratitis capitata gene	Ceratitis capitata Locus ID	Drosophila melanogaster ortholog	Position on scaffold_5 - Ccap3.2.1 (bp) (GCA_905071925.1)	SNP	25v25 a	60v60 b	120v120 c	Ben_25-60 d	Ben_25-120 e	tsl_25-60 f	tsl_25-120 g	Ben_25-tsl_60 h	Ben_25-tsl_120 i
1	zinc finger matrin-type protein	LOC101458006	CG9776	61,776,418	21		+		+					
2	uncharacterized protein	LOC101459618	-	61,822,952	1							+		+
3	thyroid adenoma-associated protein homolog	LOC101460518	THADA	61,831,192	32					+				
4	microtubule-associated protein futsch	LOC101462731	futsch	61,937,887	1			+		+		+		+
5	flocculation FLO11	LOC101449377	CG11409	62,749,878	52					+				+
6	small G protein signaling modulator 2	LOC101450993	CG1695	63,154,136	19					+			+	+
7	peroxiredoxin 1	LOC101452328	Prx1	63,171,064	4			+		+				
8	penguin	LOC101454189	peng	63,520,590	1						+	+		
9	b(0,+)-type amino acid transporter 1	LOC101456064	sbm	63,615,947	9					+				
10	coiled-coil domain-containing protein	LOC101454541	CG32809	64,785,569	17					+				
11	uncharacterized protein	LOC105665380	b6	64,821,433	5		+	+					+	+
12	mucin-5AC isoform X1, mucin-5AC	LOC101452564	CG2841	64,840,386	18									
13	uncharacterized protein	LOC101450909	PsGEF	64,857,733	13			+		+				
14	uncharacterized protein	LOC101451089	CG12496	64,910,055	16					+				
15	probable methylmalonate-semialdehyde dehydrogenase	LOC101455693	CG17896	64,936,622	1					+		+		
16	uncharacterized protein	LOC101451271	CG32815	64,945,302	5			+		+				
17	not found	not found	-	65,056,085	8				+		+	+	+	+
18	retinol dehydrogenase 12	LOC101452183	CG3842	65,074,990	18			+		+				
19	uncharacterized protein	LOC110117526	IP3K2	65,143,295	0									+
20	basement membrane-specific heparan sulfate proteoglycan	LOC101458763	trol	65,249,718	23			+	+	+	+		+	+
21	uncharacterized protein	LOC101460262	slpr	65,620,516	0			+		+				
22	transcription factor Adf-1	LOC101461280	RH45455p	65,670,967	0									+
23	amyloid-beta-like protein	LOC101459512	appl	65,968,645	10			+		+				+
24	arginase-1	LOC101461843	arg	66,139,728	0					+				+
25	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	LOC101448597	pis	66,228,387	0									+
26	uncharacterized protein	LOC101453429	CG43736	66,507,082	0	+								
27	inositol oxygenase	LOC101460467	CG6910	67,055,823	1						+	+	+	+
28	uncharacterized protein	LOC105664517	-	67,083,272	8									+
29	uncharacterized protein	LOC101457538	Fl23006p1	67,469,277	40							+		
30	alkaline phosphatase	LOC101450467	phu	67,477,142	1		+	+						
31	uncharacterized oxidoreductase SAR2567	LOC101451974	CG13377	67,669,192	0					+				
32	glucose-6-phosphate 1-dehydrogenase	LOC101453374	Zw	67,730,127	0			+						

Fig. 1. *Ceratitis capitata* differentially expressed genes located in the *tsl* genomic region. The *Ceratitis capitata* (*Cc*) gene name and locus, the *Drosophila melanogaster* (*Dmel*) ortholog name, the position in the reference genome sequence of medfly chromosome 5, the number of SNPs found in the coding region of the respective gene in the *wp tsl* mutant strain and the DE results for each gene are shown. All SNPs detected in the transcriptomic data were confirmed by the 10X genome sequence data of the *wp tsl* mutant strain.+ = differentially expressed; medfly strain names: "Benakeion" and "*wp tsl* mutant"; a, comparison of expression levels of Benakeion and *wp tsl* mutant kept at 25°C; b, comparison of expression levels of Benakeion and *wp tsl* mutant kept at 34°C for 60 min; c, comparison of expression levels of Benakeion and *wp tsl* mutant kept at 34°C for 120 min; d, comparison of expression levels of the Benakeion strain kept at 25°C and heat-shocked at 34°C for 60 min; e, comparison of expression levels of the Benakeion strain kept at 25°C and heat-shocked at 34°C for 120 min; f, comparison of expression levels of the *wp tsl* mutant strain kept at 25°C and heat-shocked at 34°C for 60 min; g, comparison of expression levels of the *wp tsl* mutant strain kept at 25°C and heat-shocked at 34°C for 120 min; h, expression levels of both strains kept at 25°C and the *wp tsl* mutant strain heat-shocked at 34°C for 60 min; i, expression levels of both strains kept at 25°C and the *wp tsl* mutant strain heat-shocked at 34°C for 120 min. The "SNP" column includes both synonymous and nonsynonymous substitutions.

data of the wild-type EgII and the *wp tsl* mutant strain (Supplementary Table 4).

Drosophila melanogaster heat-sensitive genes: medfly orthologs and detection of polymorphisms

Screening the FlyBase website with the keyword "heat sensitive genes", a total of 1,144 *D. melanogaster* heat-sensitive genes were found: 426 of them are located on chromosome 2, 480 on chromosome 3, 15 on chromosome 4, 219 on chromosome X, and 1 on chromosome Y. *Drosophila melanogaster* heat genes protein sequences were searched via tblastn, using Geneious Prime 2022.1.1, against *C. capitata tsl* genomic region resulting in the discovery of 33 orthologous genes: for 32 of them, the *D. melanogaster* gene are located on chromosome X (which is known for its synteny to the right arm of the medfly chromosome 5) and for just one, the gene is present on chromosome 2. Coding region polymorphism calling, using NGS data from the medfly *tsl* mutant strains, identified a total of 222 SNPs (37 of which resulted in amino acid changes and 13 in frameshift mutations), 12 insertions and 3 deletions in 19 genes of the medfly *tsl* genomic region in the mutant strain (Fig. 3). Using the 10X Genomics medfly genome sequence data, all 237 polymorphisms were confirmed.

In situ hybridization of *tsl* candidate genes

The results of the in situ hybridization analysis of 10 randomly selected *tsl* candidate genes are presented in Figs. 4 and 5 and Supplementary Fig. 2. All hybridizations provided a clear and unique signal confirming that these are single-copy genes (Fig. 4). In addition, the results of this analysis confirmed that the order of the genes tested largely agrees with the recently published assembly of the *tsl* genomic region, spanning the area from the *wp* gene to the *Zw* gene (6,200,460 bp long), except for one gene (*LOC101452328*; *peroxiredoxin 1*) (Figs. 4 and 5 and Supplementary Fig. 2) (Papanicolaou *et al.* 2016; Ward *et al.* 2021).

Discussion

The *tsl* gene has been used together with the *wp* gene as a selectable marker for the construction of the most successful GSS developed so far, the medfly VIENNA 8 GSS (Rössler 1979; Franz *et al.* 2021). This GSS is currently used in SIT applications against this major agricultural pest in almost all continents (Knipling 1955; Cayol *et al.* 2002; Millo *et al.* 2003; Porras-Reyes 2007; Zavala Lopez and Enkerlin 2017; Enkerlin 2021; Klassen and Vreysen 2021). It has been suggested that discovering the genes

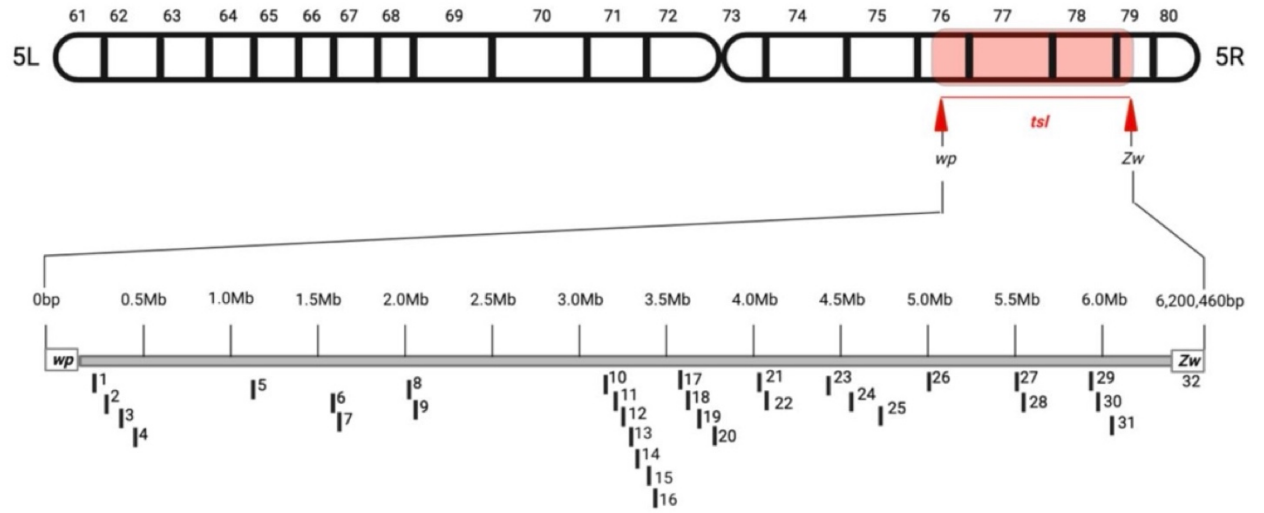

Fig. 2. Graphical representation of the *Ceratitis capitata tsl* genomic region including the in silico position of the 32 DE genes. Gene numbering is according to Fig. 1.

Table 1. Distribution of polymorphisms in the medfly *tsl* genomic region.

Position in *tsl* genomic region (Mb)	0–0.5	0.5–1.0	1.0–1.5	1.5–2.0	2.0–2.5	2.5–3.0	3.0–3.5	3.5–4.0	4.0–4.5	4.5–5.0	5.0–5.5	5.5–6.0	6.0–6.2	Total
Genes	46	22	43	34	33	29	54	49	58	48	55	70	20	561
Polymorphisms (NC)[a]	3,223	6,759	6,251	5,736	6,541	925	3,507	4,725	2,965	1,950	4,657	3,760	1,069	52,068
Polymorphisms (C)[b]	273	158	376	170	158	6	260	282	108	74	754	402	46	3,067
NC/genes	70.1	307.2	145.4	168.7	198.2	31.9	64.9	96.4	51.1	40.6	84.7	53.7	53.5	
C/genes	5.9	7.2	8.7	5.0	4.8	0.2	4.8	5.8	1.9	1.5	13.7	5.7	2.3	
NC/C	11.8	42.8	16.6	33.7	41.4	154.2	13.5	16.8	27.5	26.4	6.2	9.4	23.2	

[a] NC, polymorphisms (SNPs and indels) in the noncoding region.
[b] C, polymorphisms (SNPs and indels) in the coding region.

responsible for these two phenotypes could open the way for their generic use in constructing GSS against other insect pests and disease vectors (Caceres 2002; Franz *et al.* 2021). As the *wp* gene was recently discovered (Ward *et al.* 2021), the research efforts have focused on discovering the medfly *tsl* gene.

In the present study, we characterized the medfly *tsl* region using genomic, transcriptomic, bioinformatic, and cytogenetic analyses to identify candidate *tsl* genes for downstream functional analysis and potential linkage with the temperature-sensitive lethal phenotype reported in this species (Franz *et al.* 2021). Analysis of the available genetic and genomic information suggests that the *tsl* gene is in the *wp–Zw* (*tsl*) region which contains 561 genes. Earlier studies used deletion mapping and cytogenetic analysis to locate the *tsl* gene downstream of the *wp* locus, in the 59B–61C region of the trichogen polytene chromosome map. The most likely location of the gene is in the 60C–61B region, which corresponds to the 77–78 region of the polytene chromosome map, and is very close to the *Sergeant-2* gene (Kerremans and Franz 1994; Niyazi *et al.* 2005). However, since the insect lines used in the deletion mapping are no longer available, the comparative analysis of wild-type and *tsl* mutant strains was pursued.

Comparative transcriptomic analysis showed that 32 genes are DE between the wild-type and the *wp tsl* mutant strain upon a specific thermal treatment of embryos. The list of DE genes includes genes which are involved in diverse functions such as cell organization/biogenesis, development, response to stimulus, signaling as well as gene expression (Supplementary Table 5). Comparative genomic analysis of wild-type and mutant strains revealed the presence of 324 polymorphisms (320 SNPs, 3 insertions and 1 deletion) in the coding region of 24 DE genes. Interestingly, the orthologs of two of the 32 DE genes, *LOC101460262* and *LOC101448597*, have been associated with heat-sensitive phenotypes in *D. melanogaster* (Polaski *et al.* 2006; Wang and Montell 2006). The ortholog of *LOC101460262* is known as *slipper* in *D. melanogaster*. It has mainly been involved in functions such as system development, signal transduction, response to heat, transmembrane receptor protein tyrosine kinase signaling pathway, and regulation of the embryonic development (Harden 2002; Martin and Wood 2002; Sathyanarayana *et al.* 2002; Stronach and Perrimon 2002; Sathyanarayana *et al.* 2003; Ishimaru *et al.* 2004; Polaski *et al.* 2006; Baril *et al.* 2009; Gonda *et al.* 2012). The ortholog of *LOC101448597* is known as *phosphatidylinositol synthase* (*pis*) in *Drosophila*. It has been shown to participate in basement membrane organization, phototransduction, and phosphatidylinositol biosynthetic processes (Wang and Montell 2006; Gaudet *et al.* 2011; Devergne *et al.* 2014), as well as being responsible for lethality during the embryonic stage when mutated (Wang and Montell 2006; Liu *et al.* 2014).

Comparative genomic analysis between the wild-type and the *wp tsl* mutant strain identified many polymorphisms (SNPs and indels) in the coding region of 322 out of the 561 genes in the *tsl* region. Such polymorphisms, with or without transcriptomic data, can be exploited in genome-wide associated studies to assess genotype–phenotype associations (Rodrigues *et al.* 2016; Sim and

	Drosophila melanogaster gene	*Ceratitis capitata* ortholog	*Ceratitis capitata* Locus ID	Position on scaffold_5 · Ccap3.2.1 (bp) (GCA_905071925.1)	SNP	Gene involved in:
1	*pn*	*exopolyphosphatase PRUNE1*	LOC101460564	61,601,315	2	b, c
2	*su(sable)*	*protein suppressor of sable*	LOC101457344	61,757,672	27	d
3	*Slc25A46a*	*solute carrier family 25 member 46*	LOC101459433	61,820,012	3	a
4	*ras*	*inosine-5'-monophosphate dehydrogenase*	LOC101461588	62,129,554	0	a, b, e, f
5	*ct*	*homeobox protein cut*	LOC101462132	62,208,617	18	a,d, e, g, h
6	*CG32755*	*trypsin I-P1-like*	LOC101449736	62,776,135	0	i
7	*Dd*	*CTD nuclear envelope phosphatase 1 homolog*	LOC101450330	62,904,437	0	a, b, c, e, i
8	*Hecw*	*E3 ubiquitin-protein ligase HECW2*	LOC101451725	62,953,083	9	a, e, i, l
9	*Vap33*	*vesicle-associated membrane protein-associated protein B/C*	LOC101453359	63,268,157	0	a, b, c, e, l
10	*dor*	*vacuolar protein sorting-associated protein 18 homolog*	LOC101455883	63,563,525	36	a,b, c, e, g, i, l
11	*CG14414*	*probable RNA-binding protein 18 isoform X2*	LOC101456670	63,639,692	1	n/a
12	*Ucp4A*	*mitochondrial uncoupling protein 4*	LOC101449071	64,391,238	0	b, l
13	*para*	*sodium channel protein para (paralytic)*	LOC101449666	64,480,483	0	b, c, g, h, l
14	*hwt*	*uncharacterized*	LOC101454022	64,658,509	3	n/a
15	*exd*	*homeobox protein extradenticle*	LOC101451229	64,720,656	0	d, e
16	*CG14411*	*myotubularin-related protein 10-B*	LOC101456049	64,957,566	5	b
17	*wds*	*protein will die slowly*	LOC105664430	64,981,276	1	a, e, i
18	*sbr*	*nuclear RNA export factor 1*	LOC101458215	65,110,615	6	d, l
19	*z*	*regulatory protein zeste*	LOC101458939	65,416,004	16	a, d
20	*eag*	*potassium voltage-gated channel protein eag*	LOC101452546	65,510,114	4	b, e, g, h, l, m
21	*Tlk*	*uncharacterized protein isoform X1 (Slipper)*	LOC101460262	65,620,516	0	a, b, c, e, i, n
22	*elav*	*protein elav*	LOC101461306	66,144,096	0	d, e, g, i
23	*AMPKalpha*	*5'-AMP-activated protein kinase catalytic subunit alpha-2*	LOC101462970	66,215,860	0	a, b, c, e, i, l, n
24	*CG9240*	*mitochondrial inner membrane protease subunit 1*	LOC101463329	66,226,527	1	a, d, i, l
25	*pis*	*CDP-diacylglycerol--inositol 3-phosphatidyltransferase*	LOC101448597	66,228,387	0	a, b, c
26	*eIF5*	*eukaryotic translation initiation factor 5*	LOC101453609	66,551,774	0	a, d, i
27	*sno*	*protein strawberry notch*	LOC101455029	66,640,989	48	b, c, d, e
28	*su(w[a]*	*Ceratitis capitata protein suppressor of white apricot*	LOC101460656	67,058,480	5	n/a
29	*RpS27A*	*polyubiquitin – C*	LOC101461787	67,077,452	6	d, i
30	*Pi4KIIIalpha*	*phosphatidylinositol 4-kinase alpha*	LOC101462797	67,295,899	2	a, b, c, e, g
31	*sgg*	*protein kinase shaggy*	LOC101463278	67,386,322	0	a, b, c, e, g, h, i, l, m, n
32	*su(f)*	*protein suppressor of forked*	LOC101449978	67,464,312	21	d
33	*cin*	*molybdenum cofactor synthesis protein cinnamon*	LOC101451124	67,665,681	0	a, i, l, m

Fig. 3. *Drosophila melanogaster heat-sensitive* genes and their orthologs in the *Ceratitis capitata tsl* genomic region. *Drosophila melanogaster* gene name and locus, *C. capitata* ortholog gene name, locus, and position in the reference genome sequence, SNPs detected in the *wp tsl* mutant strain coding sequences, and gene function in *Drosophila* are shown. All SNPs were confirmed by the 10X Genomics medfly genome sequence data. a, cell organization/biogenesis; b, response to stimulus; c, signalin; d, gene expression; e, development; f, small molecule metabolism; g, reproduction; h, nervous system process; i, protein metabolism; l, transport/localization; m, behavior; n, cell cycle/proliferation; n/a, not available. The "SNP" column includes both synonymous and nonsynonymous substitutions.

Geib 2017; Sim *et al.* 2017; Mhoswa *et al.* 2020; Ward *et al.* 2021). It is also important to note that a significant number of polymorphisms was detected in NC regions because, as it has been shown in several studies, not only single point mutations (Aumann *et al.* 2020; Choo *et al.* 2020) or combination of different single point mutations (Lovato *et al.* 2009) in coding regions but also deletions (Kellis Jr *et al.* 1989; Sandberg and Terwilliger 1989; Lim *et al.* 1992; Varadarajan and Richards 1992; Matthews 1995; Shi *et al.* 2019) and insertions (Kellis Jr *et al.* 1989; Sandberg and Terwilliger 1989; Lim *et al.* 1992; Varadarajan and Richards 1992; Matthews 1995; Mela *et al.* 2009; Srinivas and Cronan 2017) located in intergenic and promoter regions can be responsible for temperature-sensitive lethal phenotypes. In addition, it was recently shown in the yeast *Saccharomyces cerevisiae* (Shen *et al.* 2022) that synonymous mutations, which do not alter protein sequences, may affect gene expression levels in a way similar to nonsynonymous mutations. This implies that each of the mutations identified in the *wp tsl* mutant strain could potentially be associated with the tsl phenotype.

Using a text mining approach, 1,144 known *D. melanogaster* heat-sensitive genes were retrieved from FlyBase, with 33 having orthologs in the medfly *tsl* genomic region. Polymorphisms, including SNPs and indels were found in 19 of the 33 medfly orthologs, expanding the list of candidate genes for a tsl phenotype. Among these, three *D. melanogaster* genes exhibit very interesting phenotypes: the gene *protein will die slowly* (*wds*; medfly ortholog *LOC105664430*), the gene *nuclear RNA export factor 1* (*sbr*; medfly ortholog *LOC101458215*) and the gene *deep orange* (*dor*; medfly ortholog *LOC101455883*).

They are involved in various cellular processes, including cell organization/biogenesis, development and protein metabolism, transport/localization, gene expression, cellular component organization, and response to a stimulus. Homozygous mutations in these genes lead to embryonic, larval, or pupal lethality at moderate or high temperatures (25°C for *wds* and 29°C for *sbr* and *dor*) (Lindsley and Grell 1968; Shannon *et al.* 1972; Belyaeva *et al.* 1982; Zhimulev *et al.* 1982; Sevrioukov *et al.* 1999; Wilkie *et al.* 2001; Sriram *et al.* 2003; Medioni and Noselli 2005; Suganuma *et al.* 2008; Wilkin *et al.* 2008; Gaudet *et al.* 2011; Pascual-Garcia *et al.* 2014; Takats *et al.* 2014; Guo and Jin 2015; Lorincz *et al.* 2016; Zhu *et al.* 2017).

Several *Dm* genes such as *shibire, notch, pale, transformer-2, downstream of Raf1* and *RNA polymerase II 215kD* subunit were considered potential *tsl* candidate genes as they exhibit temperature-sensitive lethal phenotype due to single point mutations (Nguyen *et al.* 2021). Still, none of them was found in the medfly *tsl* genomic region. It should be noted, however, that one of them, *shibire (shi)*, was localized downstream of the *Zw* gene by in situ hybridization (data not shown). Sequence analysis of *Cshi* in wild-type and *tsl* mutant strains showed no evidence of polymorphisms, thus allowing us to

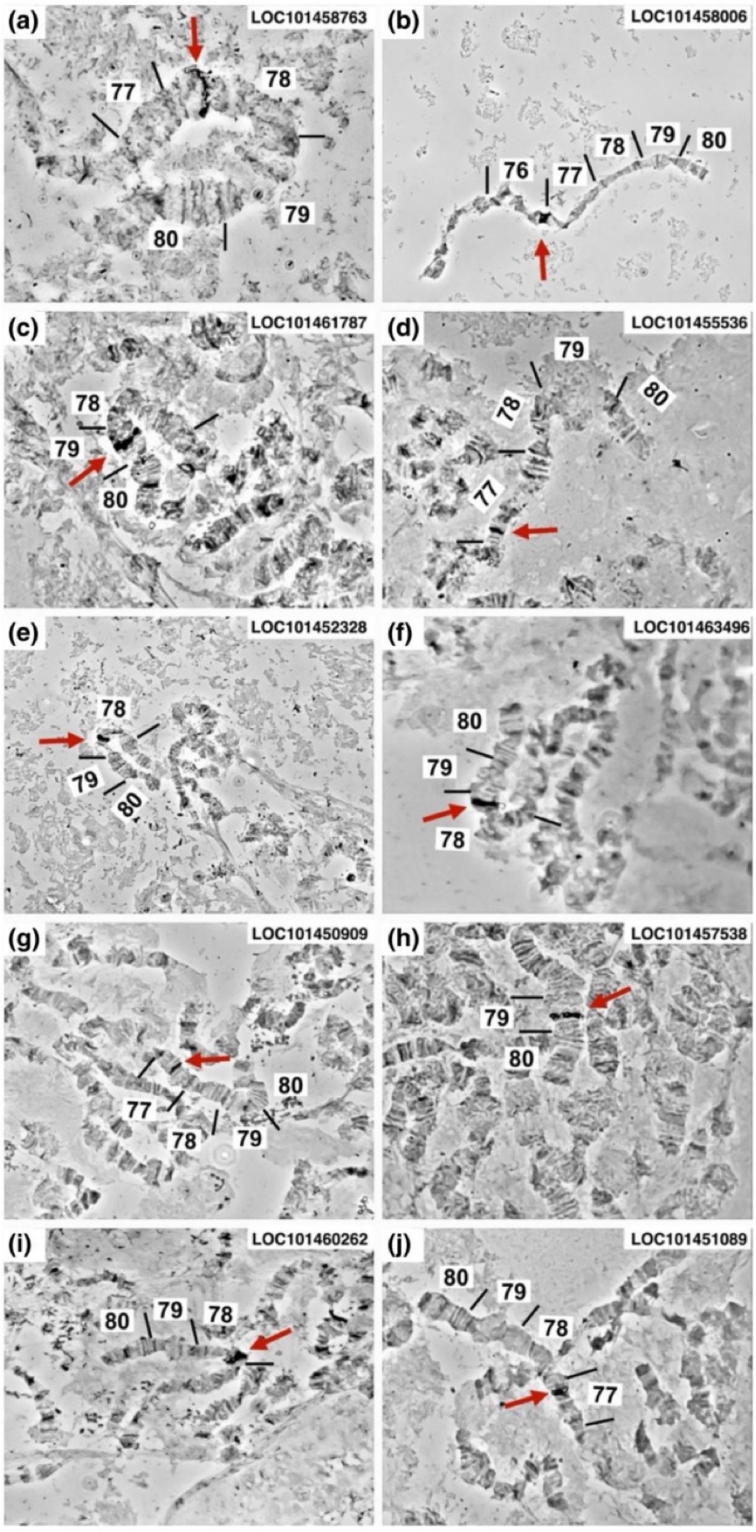

Fig. 4. In situ hybridization of *tsl* candidate genes on the right arm of the medfly polytene chromosome 5 (5R). Nuclei with the in situ hybridization signal (arrow) are presented for the following 10 genes: a) *LOC101458763*, b) *LOC101458006*, c) *LOC101461787*, d) *LOC101455536*, e) *LOC101452328*, f) *LOC101463496*, g) *LOC101450909*, h) *LOC101457538*, i) *LOC101460262*, and j) *LOC101451089*, respectively.

remove this gene from the list of *tsl* candidate genes (data not shown). However, the *shi* gene can be potentially exploited to induce a tsl-like temperature-sensitive lethal phenotype (Choo *et al.* 2020; Nguyen *et al.* 2021).

	Ceratitis capitata gene	*Ceratitis capitata* Locus ID	Position on scaffold_5 - Ccap3.2.1 (bp) (GCA_905071925.1)	Position on polytene chromosome map	Reference
1	*putative metabolite transport protein Hl_1104 (wp)*	LOC101451947	61,551,139	76B	(Ward et al. 2021)
2	*zinc finger matrin-type protein CG9776 isoform X2*	LOC101458006	61,796,353	76C/77A	This study
3	*protein halfway*	LOC101455536	63,564,784	77B	This study
4	*uncharacterized gene*	LOC101450909	64,877,668	77B	This study
5	*uncharacterized gene*	LOC101451089	64,929,990	77C	This study
6	*mitogen-activated protein kinase kinase kinase*	LOC101460262	65,636,787	78A	This study
7	*basement membrane-specific heparan sulfate proteoglycan core protein*	LOC101458763	65,269,744	78B	This study
8	*peroxiredoxin 1*	LOC101452328	63,190,999	78C	This study
9	*uncharacterized gene*	LOC101463496	66,252,484	78C	This study
10	*polyubiquitin-C*	LOC101461787	67,096,850	79A	This study
11	*uncharacterized gene*	LOC101457538	67,489,212	79B	This study
12	*glucose-6-phosphate 1-dehydrogenase (Zw)*	LOC101453374	67,751,598	79C	(Scott et al. 1993)

Fig. 5. In situ hybridization results of 10 *Ceratitis capitata tsl* candidate genes. In addition to the in situ localization results on the medfly polytene chromosome 5, the position of the genes on medfly chromosome 5 based on published assembly (Ccap3.2.1_scaffold_5) is indicated. The already-known localization of the *wp* and *Zw* genes is also presented.

As the entire in silico analysis of the present study was performed using the *C. capitata* 3.2.1 genome (Ccap3.2.1; accession GCA_905071925.1), it was important to confirm that the published assembly in the *tsl* genomic region is as accurate as possible. Using in situ hybridization analysis and a set of 10 genes spanning the *tsl* region, the localization of the genes tested was largely in agreement with the assembly of the *tsl* genomic region (Papanicolaou *et al.* 2016; Ward *et al.* 2021) except for one gene (*LOC101452328*, *peroxiredoxin 1*). According to earlier studies, the *tsl* gene is localized in the region 77–78 of the polytene chromosome map (Kerremans and Franz 1994; Niyazi *et al.* 2005). Our data show that the *peroxiredoxin 1* is mapped in 78C, which is close to the predicted localization of the *tsl* gene. This further emphasizes the importance of having a high-quality reference genome available.

Taken together, the present study unraveled the genomic and transcriptomic differences in the *tsl* genomic region of wild-type and *tsl* mutant strains. While it may not have produced a significant reduction in the target region, the study did identify several promising candidate genes that may be involved in the induction of temperature-sensitive lethal phenotypes. The genetic basis of the tsl phenotype remains unknown and can be due to one or more, protein or not, coding genes; the causal mutation(s) can be either in the coding or the regulatory region; the mutation(s) can be a single (or multiple) nucleotide (synonymous or nonsynonymous) substitution, indel(s) or chromosomal rearrangements (for example, a small size inversion). Clearly, the discovery of the *tsl* gene and its causal mutation(s) poses a significant challenge. However, this study's integrated approach has generated a list of potential candidate genes that could be linked to this phenotype. Downstream functional analysis based on genome editing tools is required to identify if any of these candidates is the *tsl* gene. If the causal mutation is an SNP, the functional verification will be based on inducing the same mutation in a wild-type strain and/or reconstructing the wild-type allele of the gene in a mutant strain followed by the necessary temperature sensitive lethal tests on the established genome edited lines to assess their temperature sensitivity. This process may also lead to the discovery of novel genes involved in the expression of temperature-sensitive lethal phenotypes.

Data availability

Sequencing files can be found at NCBI under the following project numbers: PRJEB57574.

Supplemental material available at G3 online.

Acknowledgements

This study benefitted from discussions at meetings for the Coordinated Research Project D44003, "Generic approach for the development of genetic sexing strains for SIT applications", funded by the International Atomic Energy Agency.

Funding

This study was supported by the Insect Pest Control Subprogramme of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture. Further support was provided by contract CAN 23358 in the frame of the CRP D44003 on "Generic approach for the development of genetic sexing strains for SIT applications", Genome Canada Platform grant and the Canada Foundation for Innovation to (JR) and the project was part of the "*Sex selection genes from fruit fly species for use in SITplus*" (FF17000) project funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and JLU Giessen and contributions from the Australian Government (MFS).

Conflicts of interest

The author(s) declare no conflict of interest.

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Editor: K. McKim

Supplementary Material

Genomic and cytogenetic analysis of the *Ceratitis capitata temperature-sensitive lethal* region

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Keywords:

Mediterranean fruit fly, sterile insect technique, genetic sexing strain, white pupae, Tephritidae

Table S1. Primers used for the preparation of probes for the *in situ* hybridization analysis.

***Ceratitis capitata* gene**	**Forward (F) and reverse (R) primers used for probes in *in situ* hybridization**
zinc finger matrin-type protein CG9776 isoform X2	F: ATTGGATCGGGCATAAGCTCT
	R: ACCTCCAGTGATTCCTCCTCA
protein halfway	F: TGCTCGTCAAATTATTCTTCGAGAT
	R: TCACTCCTTCTCTTCGGACTAAC
uncharacterized LOC101450909	F: CGATCCCGATGCCACACAG
	R: CGTCGCATACGTCACTCACT
uncharacterized LOC101451089	F: AGACATAAACCGACGGCAAC
	R: TGCTAAAGTGGACAGCGTCA
mitogen-activated protein kinase kinase kinase	F: GTTTGTCGGTTTTCAGGCGT
	R: GTGGCGCAAGTTCAGAAGAG
basement membrane-specific heparan sulfate proteoglycan core protein	F: AATCGCCAGCGGTATTGTGA
	R: GCACTGCCCGGTGGTATTAT
peroxiredoxin 1	F: TTGCAGCACATCCCACATTG
	R: TGGCGTGAAATGACGGTACA
uncharacterized LOC101463496	F: CACTCAGACACAAGCGACCT
	R: CGCGCCGCAGCTATAATAAC
polyubiquitin-C	F: GCACTGCCCGGTGGTATTAT
	R: ACGTTGTTGATCTGGGGGAA
uncharacterized LOC101457538	F: TTGCAGCACATCCCACATTG
	R: TGGCGTGAAATGACGGTACA

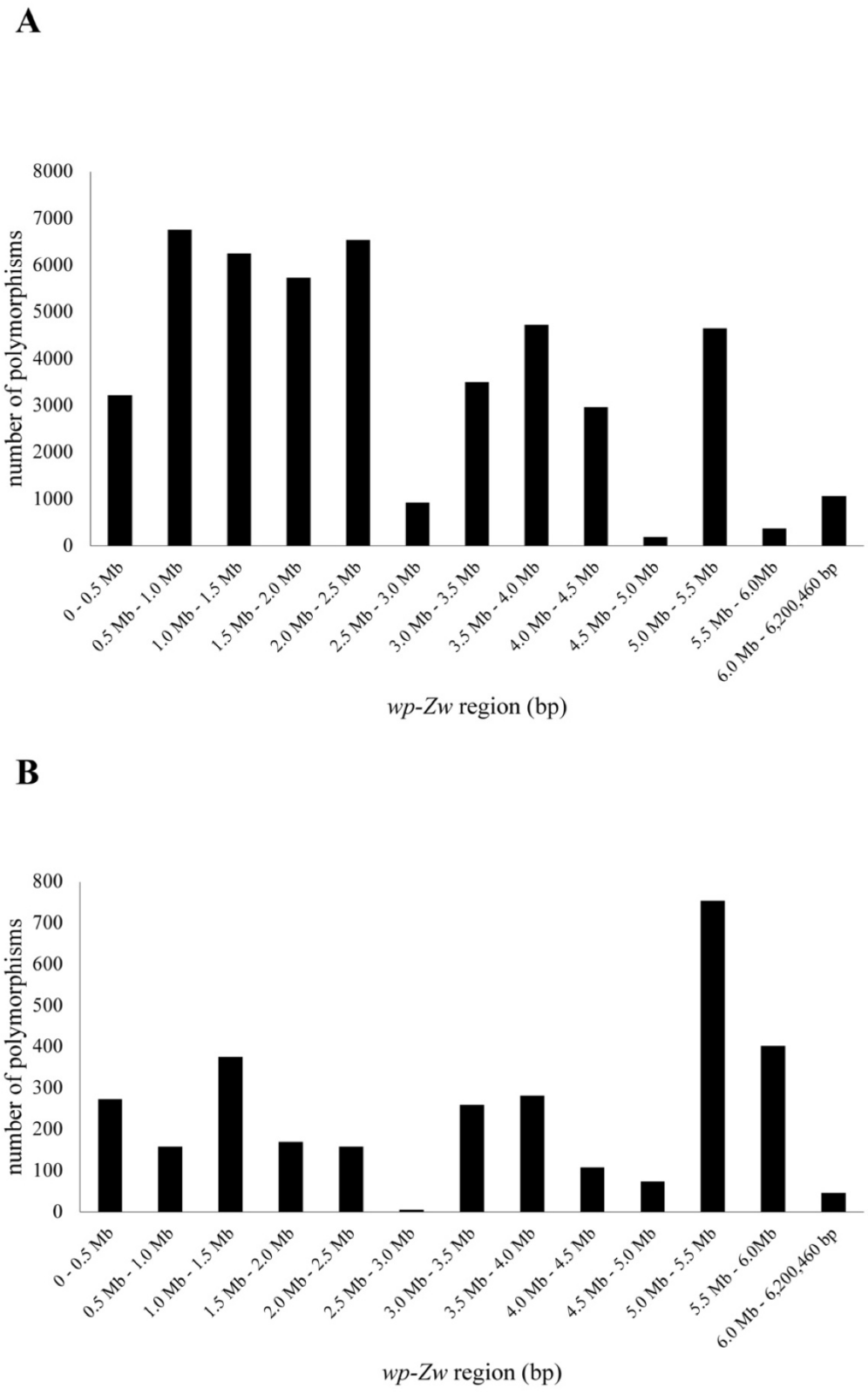
Table S2. RNA-seq results of the *Ceratitis capitata* wild-type Benakeion and *wp tsl* mutant strains (BioProject No PRJEB57574).

Strain	Name	Number of reads (bp)
Benakeion	Ben_25_R1	58,124,080
Benakeion	Ben_25_R2	37,604,618
Benakeion	Ben_25_R3	35,035,902
Benakeion	Ben_60_R1	99,190,658
Benakeion	Ben_60_R2	43,753,856
Benakeion	Ben_60_R3	34,902,306
Benakeion	Ben_120_R1	93,219,632
Benakeion	Ben_120_R2	40,344,904
Benakeion	Ben_120_R3	28,550,468
wp tsl	tsl_25_R1	79,382,592
wp tsl	tsl_25_R2	44,741,000
wp tsl	tsl_25_R3	37,458,356
wp tsl	tsl_60_R1	87,185,310
wp tsl	tsl_60_R2	47,286,386
wp tsl	tsl_60_R3	46,189,739
wp tsl	tsl_120_R1	48,851,950
wp tsl	tsl_120_R2	25,631,810
wp tsl	tsl_120_R3	48,219,365

Ben_25 and tsl_25 = Benakeion wild-type strain and *wp tsl* mutant strain kept at 25 °C.
Ben_60 and tsl_60 = Benakeion wild-type strain and *wp tsl* mutant strain kept at 34 °C for 60 minutes.
Ben_120 and tsl_120 = Benakeion wild-type strain and *wp tsl* mutant strain kept at 34 °C for 120 minutes.
R1, R2, R3 = three replicates were analyzed per strain and treatment.

Table S3. Illumina NGS results of the *Ceratitis capitata* VIENNA 7 GSS and *wp tsl* mutant strain (BioProject No PRJEB57574).

Strain	Total reads	Total reads bases (bp)
wp tsl (male)	58,541,784	8,839,809,384
wp tsl (female)	59,122,524	8,927,501,124
VIENNA 7 (female)	45,703,818	6,901,276,518



75 **Figure S1.** Non-coding (A) and coding (B) region polymorphisms frequency distribution plot
76 of the medfly *wp-Zw* genomic region. The horizontal axis shows the length of *wp-Zw* region
77 (bp) while the vertical axis indicates the number of polymorphisms.

Table S4. 10X Genomics sequencing results of the *Ceratitis capitata* wild-type EgII and *wp tsl* mutant strains (BioProject No PRJEB57574).

Strain	Total reads	Total reads bases (bp)
EgII (male)	188,183,315	56,831,361,130
EgII (female)	271,196,767	81,901,423,634
wp tsl (male)	207,972,616	62,807,730,032
wp tsl (female)	175,767,398	53,081,754,196

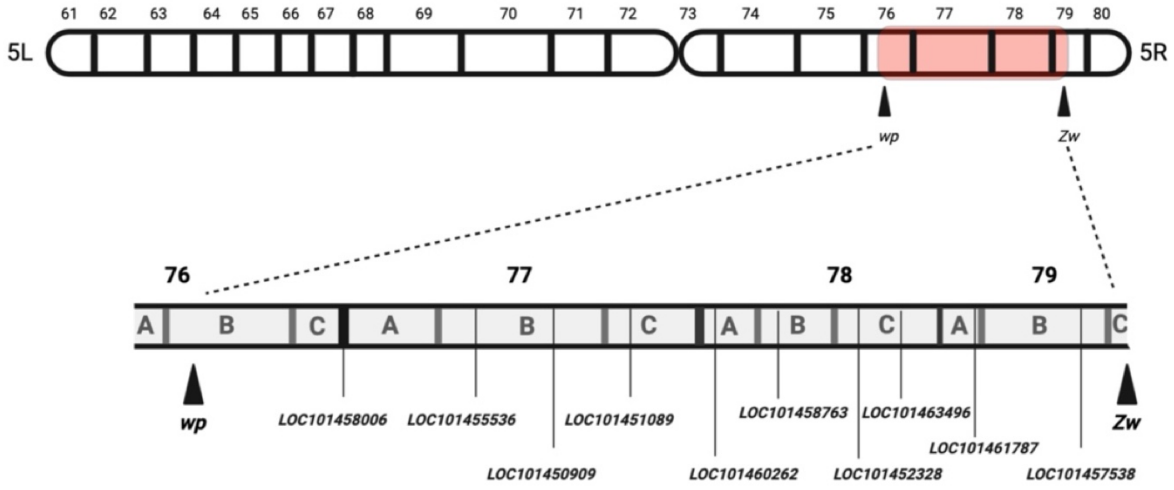


Figure S2. Schematic representation of *Ceratitis capitata* chromosome 5 showing the *tsl* genomic region [25, 27] and the localization of selected *tsl* genes by *in situ* hybridization on salivary gland polytene chromosomes.

Table S5. *Ceratitis capitata* differentially expressed genes located in the *tsl* region. The function of the *Drosophila melanogaster* orthologs is also shown.

Gene	Gene involved in:
zinc finger matrin-type protein CG9776	Gene expression
uncharacterized protein	n/a
thyroid adenoma-associated protein homolog	Transport/localization, gene expression
microtubule-associated protein futsch	Cell organization/biogenesis, transport/localization, development, nervus system process, behavior, response to stimulus
flocculation protein FLO11	n/a
small G protein signaling modulator 2	Transport/localization
peroxiredoxin 1	Development, reproduction, immune system, response to stimulus
protein penguin	Development, gene expression, protein metabolism
b(0,+)-type amino acid transporter 1	Transport/localization
coiled-coil domain-containing protein	n/a
uncharacterized protein	n/a
mucin-5AC isoform X1, mucin-5AC	n/a
uncharacterized protein	Cell organization/biogenesis, transport/localization, development, response to stimulus, signaling
uncharacterized protein	n/a
probable methylmalonate-semialdehyde dehydrogenase	Small molecule metabolism
uncharacterized protein	n/a
not found	n/a

retinol dehydrogenase 12	n/a
uncharacterized protein	Cell organization/biogenesis, transport/localization, development, nervus system process, behavior, response to stimulus, signaling
basement membrane-specific heparan sulfate proteoglycan core protein	Cell cycle proliferation, cell organization/biogenesis, development, response to stimulus, signaling
uncharacterized protein	Cell organization/biogenesis, development, reproduction, response to stimulus, signaling, protein metabolism
transcription factor Adf-1	Gene expression
amyloid-beta-like protein	Cell organization/biogenesis, development, nervus system process, behavior, response to stimulus
arginase-1	Small molecule metabolism
CDP-diacylglycerol--inositol 3-phosphatidyltransferase	Cell organization/biogenesis, response to stimulus, signaling
uncharacterized protein	n/a
inositol oxygenase	Small molecule metabolism
uncharacterized protein	n/a
uncharacterized protein	Cell organization/biogenesis, gene expression, protein metabolism
alkaline phosphatase	Response to stimulus
uncharacterized protein	n/a
glucose-6-phosphate 1-dehydrogenase	Small molecule metabolism

n/a = not available

3.3 Mutagenesis of a *tsl* candidate gene in *Ceratitis capitata*: the *deep orange* gene

The third goal was to use CRISPR/Cas9 technology to knock out *deep orange (Ccdor)* and to introduce one of the point mutations found in the *tsl* mutant strains into the wild-type background to determine whether one of these two strategies triggers a temperature-sensitive lethal phenotype. In this study, I successfully introduced the *Ccdor E839K* mutation by CRISPR HDR (*dor E839K*) and obtained two additional strains characterized by the presence of a 12 bp deletion (*dor 12del*) and a 51 bp duplication (*dor 51dup*). All three strains are temperature sensitive and show lethality after heat shock at embryonic, larval, and pupal stages.

Title: ***Deep orange* gene editing triggers temperature-sensitive lethal phenotypes in *Ceratitis capitata.***

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Status: **not published yet**

Contribution:

- Conceptualization: **Sollazzo G.,** Bourtzis K.
- Bioinformatic analysis: **Sollazzo G.,** Aumann RA.
- Data curation: **Sollazzo G.,** Nikolouli K.,
- Investigation: **Sollazzo G.**, Nikolouli K., Gouvi G., Aumann RA.
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- Supervision: Bourtzis K., Schetelig MF.
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- Writing-review and editing: **Sollazzo G.**, Nikolouli K., Gouvi G., Aumann RA., Schetelig MF., Bourtzis K.

Presentation: This work was presented at the 3rd Research Coordination Meeting on the "Generic approach for the development of genetic sexing strains for sterile insect technique applications" of the FAO/IAEA in Vienna, Austria (speaker; 2023 – Talk Title "Advancements in insect pest control through the identification and characterization of *wp* and *tsl* genes in the medfly for developing novel genetic sexing strains").

Deep orange gene editing triggers temperature-sensitive lethal phenotypes in *Ceratitis capitata*

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Abstract

Background. The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is a significant agricultural pest managed through area-wide integrated pest management (AW-IPM) including a sterile insect technique (SIT) component. Male-only releases increase the efficiency and cost-effectiveness of SIT programs, which can be achieved through the development of genetic sexing strains (GSS). The most successful GSS developed to date is the *C. capitata* VIENNA 8 GSS, constructed using classical genetic approaches and an irradiation-induced translocation with two selectable markers: the *white pupae* (*wp*) and *temperature-sensitive lethal* (*tsl*) genes. However, currently used methods for selecting suitable markers and inducing translocations are stochastic and non-specific, resulting in a laborious and time-consuming process. Recent efforts have focused on identifying the gene(s) and the causal mutation(s) for suitable phenotypes, such as wp and tsl, which could be used as selectable markers for developing a generic approach for constructing GSS. The *wp* gene was recently identified, and efforts have been initiated to identify the *tsl* gene. This study investigates *Ceratitis capitata deep orange* (*Ccdor*) as a *tsl* candidate gene and its potential to induce tsl phenotypes.

Results. An integrated approach based on (cyto)genetics, genomics, bioinformatics, and gene editing was used to characterize the *Ccdor*. Its location was confirmed on the right arm of chromosome 5 in the putative *tsl* genomic region. Knock-out of *Ccdor* using CRISPR/Cas9-NHEJ and targeting the fourth exon resulted in lethality at mid- and late-pupal stage, while the successful application of CRISPR HDR introducing a point mutation on the sixth exon resulted in the establishment of the desired strain and two additional strains (*dor 12del* and *dor 51dup*), all of them expressing tsl phenotypes and presenting no (or minimal) fitness cost when reared at 25°C. One of the strains exhibited complete lethality when embryos were exposed at 36°C.

Conclusions. Gene editing of the *deep orange* gene in *Ceratitis capitata* resulted in the establishment of temperature-sensitive lethal mutant strains. The induced mutations did not significantly affect the rearing efficiency of the strains. As *deep orange* is a highly conserved gene, these data suggest that it can be considered a target for the development of *tsl* mutations which could potentially be used to develop novel genetic sexing strains in insect pests and disease vectors.

Keywords. Mediterranean fruit fly, sterile insect technique, white pupae, Tephritidae, temperature sensitivity.

Background

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is one of the most important agricultural pests due to the damage it causes to many plant species of agronomic importance [1, 2]. The sterile insect technique (SIT), as part of area-wide integrated pest management (AW-IPM) programs, is a control tactic that has been developed to suppress, contain, and prevent the (re)introduction or locally eradicate populations of insect pests of agricultural, veterinary and human health importance [3–6].

One of the most critical aspects of SIT applications concerns the development of genetic sexing strains (GSS), which enable the mass production and separation of males and females. Male-only releases significantly improve the effectiveness and cost-efficiency of SIT applications [7–10]. Several GSSs have been developed using irradiation and classical genetic approaches for SIT applications against the Mediterranean fruit fly (medfly). The two strains used nowadays are VIENNA 7 and VIENNA 8 [10, 11].

The successful development and application of these GSS depend on the presence of (i) two selectable phenotypes, the *white pupae (wp)* gene and *temperature-sensitive lethal* (*tsl*), both being located on the right arm of chromosome 5, and (ii) a Y-autosome translocation, T(Y;A), which is required to link the wild-type alleles of these genes to the male sex chromosome [10]. Females of these GSS are homozygous for the recessive alleles, sensitive to high temperatures, and emerge from white puparia, while males are heterozygous at both loci and, since they carry a single copy of the wild-type alleles for both *wp* and *tsl* loci, they are resistant to high temperatures and emerge from brown puparia [8, 10, 11].

The development of these GSS was a rather lengthy process of over two decades, entirely based on the serendipitous discovery of the *wp* and *tsl* mutations and the stochastic induction of suitable translocations (T[Y;A]). The same approach was followed for all GSS constructed using classical genetic approaches [10]. Given recent advances in the field of genome editing, a generic (neoclassical) approach was proposed for the construction of non-transgenic GSS for

SIT applications [12, 13]. This approach requires the identification of gene(s) and the causal mutation(s) of suitable phenotypes, which could be used as selectable markers. The next step includes the induction of similar mutations in the orthologous gene(s) of SIT target species and the linkage of the wild-type allele of the gene marker(s) to the male sex using genome editing approaches [12, 13].

As the *wp* and *tsl* genes could be useful selectable markers for developing GSS in different SIT target species, initial efforts focused on identifying the genes responsible for these two phenotypes. The *wp* gene was recently mapped by *in-situ* hybridization to position 76B of the salivary gland polytene chromosomes and in earlier studies by deletion mapping to position 59B of the *C. capitata* trichogen polytene chromosome map [14, 15]. Based on similar transposition and deletion mapping experiments, the *tsl* gene was (cyto)genetically mapped at position 59B-61C [14, 16]. Based on these findings, efforts were initiated to identify the gene responsible for the tsl phenotype in *C. capitata* so that it could be used as a marker for the development of GSS in other SIT target species [12, 13]. As a first step, a tsl test (TSLT) was applied to several wild-type, GSS, and *tsl* mutant strains, and the results indicated that the lethality rates observed as a response to increasing temperatures depend on genetic and environmental factors [17]. This analysis also contributed to the identification of potential reference strains that could be used in functional tests of candidate genes [17].

C. capitata wild-type, GSS, and *tsl* mutant strains were recently used in genomic, transcriptomic, bioinformatic, and cytogenetic analyses to identify candidate genes in the so-called *C. capitata tsl* genomic region that may be involved in the tsl phenotype [18]. This region is defined by the *wp* gene at its left border and the *glucose-6-phosphate 1-dehydrogenase* gene (also known as *Zw*) at its right border, located at position 79C of the polytene chromosome map [18, 19]. It is 6,200,460 bp long and contains 561 genes [18]. The results of this integrated and comparative approach led to the identification of 33 *Drosophila melanogaster* temperature sensitive genes with orthologs in the *C. capitata tsl* genomic region. In addition, 214 polymorphisms were detected in 19 out of the 33 genes including locus LOC101455833 (*vacuolar protein sorting-associated protein 18 homolog, VPS18)* also known as the *deep orange* gene in *D. melanogaster* (*Dmdor*) [18].

The *deep orange* gene plays a major role in vesicle-mediated protein trafficking to lysosomal compartments and in membrane docking/fusion reactions of late endosomes/lysosomes

probably as part of the class C core vacuole/endosome tethering (CORVET) complex, as previously reported [20–23]. It is essential in larval neuromuscular junctions for endosomal sorting and trafficking old or dysfunctional synaptic vesicle proteins through a degradative endolysosomal route [22]. Moreover, it is essential for the biogenesis of eye pigment granules [21] and for maintaining normal levels of the protein Rush hour, which functions in endosome formation and trafficking [24].

Several *Dmdor* mutations have been associated with lethal phenotypes appearing at the third instar larval, pre-pupal, or pupal stage, with some mutations being temperature sensitive [19, 24, 25]. Exposure to elevated temperatures may not only result in lethality, but might also affect eyes, wings, late endosomes, thorax, and macrochaeta [20, 26].

The *C. capitata* orthologue of the *deep orange* gene (hereafter *Ccdor*) was selected as a potential *tsl* candidate gene and was targeted *via* Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) gene editing for further characterization. It is known that CRISPR/Cas9 genome editing can be used to target specific genes introducing a double-strand break (DSB), which can be repaired in two ways: by the non-homologous end-joining (NHEJ) or the homology-directed repair (HDR) [27, 28]. Both pathways can be exploited for gene editing. Performing NHEJ, mutations are induced, and the repair system implies the introduction of random INDELs (insertions or deletions), mainly used to knock out genes. Carrying out HDR, a DNA donor template is used to modify a specific region [29]. CRISPR/Cas9 has been successfully applied in many insect pest species targeted by SIT, such as *Ceratitis capitata* [15, 27, 28, 30, 31], *Bactrocera tryoni* [15, 32, 33], *Bactrocera dorsalis* [34, 35], *Anastrepha suspensa* [36] and *Zeugodacus cucurbitae* [37].

In the present study, we investigated whether the *C. capitata dor* gene is involved in a temperature-lethal phenotype in this species. We used CRISPR/Cas9-mediated NHEJ to knock out the *Ccdor* gene targeting the fourth exon and CRISPR/Cas9-mediated HDR to introduce a specific point mutation in the sixth exon and characterized the mutant strains with an emphasis on the expression of temperature-sensitive lethal phenotypes.

Results

Ceratitis capitata deep orange gene

The *Drosophila melanogaster deep orange* gene orthologue in *C. capitata (Ccdor)* is characterized by a length of 3,290 bp (973 aa) (RNAseq and genomic data), a total of 6 exons (Figure 1A), and 56.14% identity at the amino acid level with its *D. melanogaster* orthologue. Combined results from the NCBI Conserved Domain Database Server and SMART predicted the presence of four conserved domains for *Ccdor*: a *Pep3/Vps18/deep orange family* domain (300-452 aa), a Clathrin/VPS domain (627-772 aa), a Helo_like_N domain (804-865 aa) and a Ring finger/U-box domain (861-948 aa) (Figure 1A). The *C. capitata deep orange* gene is localized on the right arm of chromosome 5, in position 77B of the polytene chromosome map, as shown by *in-situ* hybridization analysis (Figure S1).

As reported in our previous study, polymorphism calling using the VIENNA 7 GSS (female) and *wp tsl* mutant strain (male and female) Illumina NGS data identified 36 SNPs in the respective *Ccdor* gene coding sequences [18]. The present study confirmed them *via* Sanger sequencing in the *wp tsl* strain. Six of them lead to amino acid changes (I83M, D100E, H313P, I458L, E839K, L940I). H313P is in the *Pep3/Vps18/deep orange family* domain, E839K in the Helo_like_N domain, and L940I in the Ring finger/U-box domain (Figure 1A; Table S1). These six positions were checked in several wild-type, GSS, and *tsl* mutant strains (Table 1) to confirm their homozygosity in wild-type and *tsl* mutant strains (including GSS females) and their heterozygosity in GSS males, a pattern which should be expected for a *tsl* mutation (Figure 1B). One of them (E839K) followed the proper pattern in all studied strains (Figure 1B). Further analyses were conducted to assess the conservation of DOR protein and the conservation of these positions across various species to evaluate *dor*'s eligibility as a selectable tsl marker (Figure S2). This assisted in identifying and prioritizing suitable target sites for CRISPR/Cas9 genome editing. The findings revealed that the DOR protein has a pairwise identity of 59.3% among insect species, which increases to 91.3% when only Tephritids are considered (Figure S2). Moreover, its secondary structure was evaluated and predicted by Phyre[2] and it contains 42% alpha helix, 19% beta-strand, and 22% disordered regions (Figure S3). Additionally, it was observed that three out of the six amino acids, in which polymorphisms were identified in *tsl* mutant strains, exhibit high conservation among insect species (Figure S2) and are located in the alpha helix (E839K & L940I) and disordered regions (D100E)

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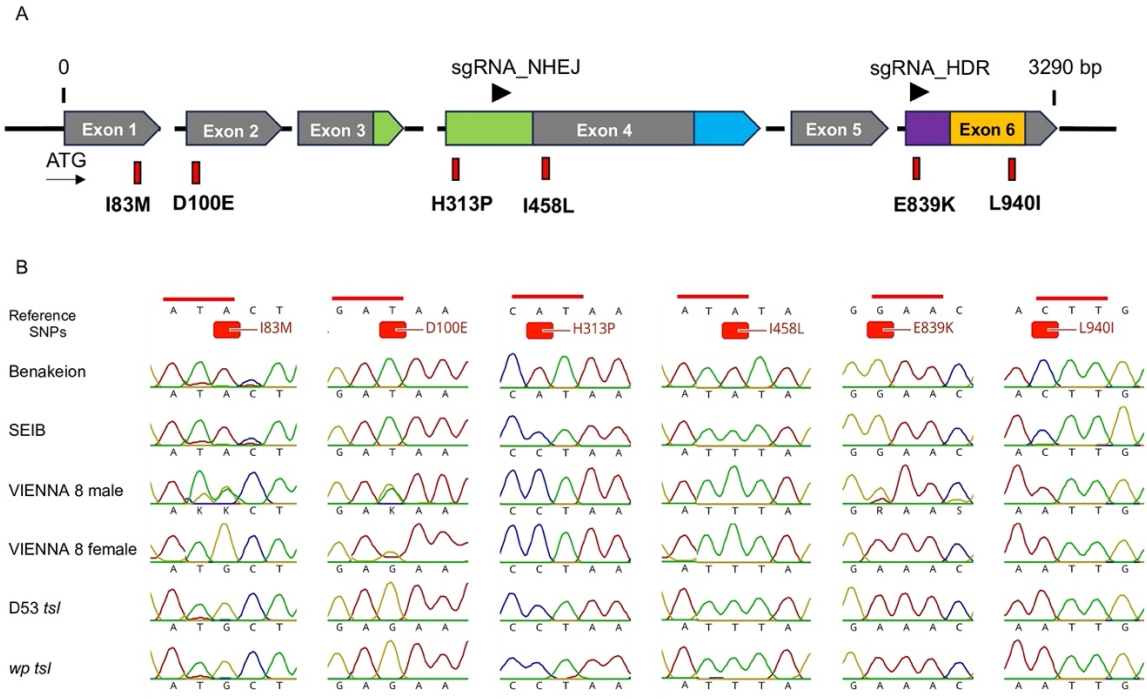

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199 **Figure 1. (A)** Schematic representation of the *Ccdor* gene spanning six exons. *Pep3/Vps18/deep orange family* domain, Clathrin/VPS domain,
200 Helo_like_N domain, Ring finger/U-box domain, and sgRNAs are shown in green, blue, purple, orange, and black, respectively. Red dashes
201 represent the six SNPs that lead to amino acid changes identified *via* wild type and *wp tsl* mutant strains' *deep orange* genomic sequence
202 comparison. **(B)** *Ccdor* gene SNPs Sanger sequenced in Benakeion Volos FF26 (hence Benakeion) (wild-type), Seibersdorf FF26 (hence SEIB)
203 (wild-type), VIENNA 8 2010 FF26 (hence VIENNA 8) (GSS), D53-3-28 FF21 (hence D53 *tsl*) (mutant) and *wp tsl* FF21 (hence *wp tsl)* (mutant)
204 strains using as reference *Ccap 3.2.1* (accession GCA_905071925.1) genome.

Knock-out of the *Ccdor* gene in exon 4 causes lethality at the pupal stage

The sgRNA_NHEJ (Table S2 targeting exon 4 and recombinant Cas9 protein were injected in 586 Egypt II FF26 (hence EgII) embryos to knock out the *Ccdor* gene. After injection, 145 embryos reached the larval stage, 70 reached the pupal stage, and 55 eclosed as adults (Table S3). Fifteen dead pupae were analyzed genotypically for CRISPR/Cas9-induced *dor* mutations; seven had NHEJ events (Figure S4).

Surviving adults were individually backcrossed to EgII wild-type virgin individuals and eggs from those crosses were collected three times in two-day intervals. G_1 adults were inbred and G_2 adults were subjected to non-lethal genotyping. 425 G_2 adults were screened for CRISPR-induced mutations, but none were detected. This suggested that all G_0 individuals with mosaic genotypes died at mid- or late-pupal stage (Figure 2), most likely due to the mutation that took place.

Figure 2. Lethal phenotype of *Ccdor* gene knock-out. Lethality was observed during mid- (A) and late-pupal stage (B) following *Ccdor* gene knock-out in exon 4 by CRISPR/Cas9 NHEJ targeted mutagenesis.

***Deep orange* E839K mutation introduced in EgII using CRISPR/Cas9 HDR**

Recombinant Cas9 protein, a sgRNA targeting the sixth exon (sgRNA_HDR) of *Ccdor,* close to the E839K (G2889A) mutation and a short single-stranded repair template (151 nt), designed in sense orientation of the gene (Table S2), were injected into 255 *Cc* EgII embryos (Table S3). In addition, 100 *Cc* EgII embryos were injected using the same mix but replacing the sense-oriented single-stranded repair template with the antisense-oriented one (Table S3). Twenty-eight adults survived the injections using the sense-oriented ssODN, and three survived using the antisense-oriented ssODN. Differently from what was done during the NHEJ experiment, G_0 adults were individually backcrossed to *wp tsl* FF21 (hence *wp tsl*) mutant strain virgin mates, trying to obtain complementation of the CRISPR allele with the *tsl* mutant one with the consequent manifestation of the desired phenotype. Using non-lethal genotyping on G_1 adults, we determined that at least one G_0 family produced HDR-positive offspring. Positive siblings were single-pair mated to EgII wild-type individuals of the opposite sex to remove the *wp tsl* background. Crosses and non-lethal genotyping were performed during the next generations to isolate flies carrying the E839K mutation. Once identified, they were inbred to obtain a homozygous mutant strain (Figure 3). No change in eye color or other visible mutant phenotype was detected.

***Deep orange* CRISPR NHEJ events obtained during the CRISPR HDR experiments**

In addition to the successful CRISPR HDR performed to introduce the E839K point mutation, the genotyping of G_1 embryo pools suggested the presence of at least three G_0 flies with editing events different from the expected one. Genotyping of G_1 adults showed flies with mosaic genotypes (Figure S4). However, unlike the latter ones, induced mutations were viable. We used non-lethal genotyping to determine that at least two G_1 families showed different NHEJ events. Crosses and non-lethal genotyping were performed during G_2 and G_3 to isolate the single events: a deletion of 12 bp (TGTGATAAACAA) and a duplication (which also included the E839K HDR event) of 51 bp (AAACGTGTTATGAAAGATTTACAAAATGTGCGTGAGAGAAGCATACAAGCG), both in frame (Figure 3), which were produced as a result of erroneous repair by the DNA polymerase. Once flies homozygous for the 12 bp deletion and the 51 bp duplication were identified, they were inbred at G_4 to establish the respective homozygous strains (*dor 12del* and *dor 51dup*) (Figure 3). These NHEJ events were unrelated to eye color change or other visible mutant phenotypes.

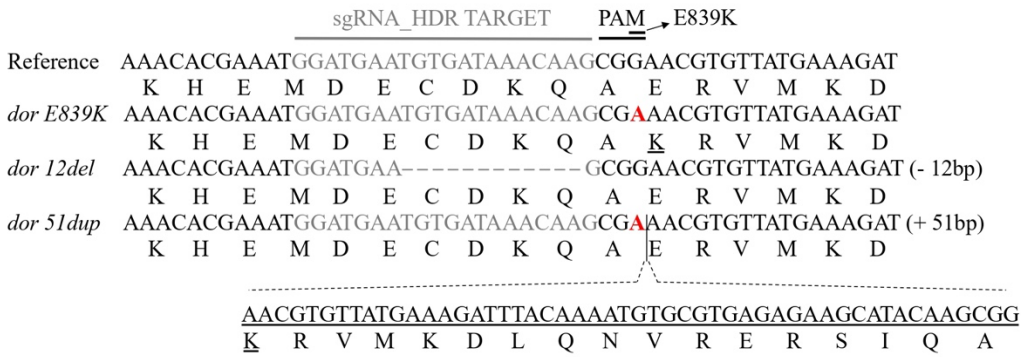

Figure 3. CRISPR strains obtained after targeting *Ccdor via* CRISPR HDR. The *Ccdor* reference sequence (*Ccap 3.2.1* (accession GCA_905071925.1)) with the sgRNA target sequence in grey and the PAM site is shown in the first row. The rows below represent the three mutant strains obtained *via* CRISPR HDR with the SNP responsible for the *E839K* mutation (*dor E839K*), the 12 bp deletion (*dor 12del)*, and the 51 bp duplication inserted via false integration of the HDR repair template (*dor 51dup)*, respectively. The nucleotide change responsible for the E839K mutation is shown in red, while the duplication is underlined.

Temperature-sensitive lethal tests of the three *dor* mutant strains

Egg hatching, pupal recovery, and adult emergence rates were assessed for all homozygous CRISPR-mutant (*dor 12del, dor 51dup,* and *dor E839K*) and control strain (wild type: EgII, SEIB, and mutant: *wp tsl*) (Table S4), based on the initial 100 embryos collected per each of the three replicates. Statistically significant differences were detected at the egg hatching rate among all the strains tested at 25°C ($F = 9.1437$, $df = 48$, $p = 3.674 \times 10^{-6}$), 31°C ($F = 54.733$, $df = 48$, $p < 2.2 \times 10^{-16}$), 32°C ($F = 18.745$, $df = 48$, $p = 2.746 \times 10^{-10}$), 33°C ($F = 260.49$, $df = 48$, $p < 2.2 \times 10^{-16}$), 34°C ($F = 686.77$, $df = 48$, $p < 2.2 \times 10^{-16}$), 35°C ($F = 79.944$, $df = 48$, $p < 2.2 \times 10^{-16}$) and 36°C ($F = 72.464$, $df = 48$, $p < 2.2 \times 10^{-16}$) (Figure 4, Table S4, Table S5).

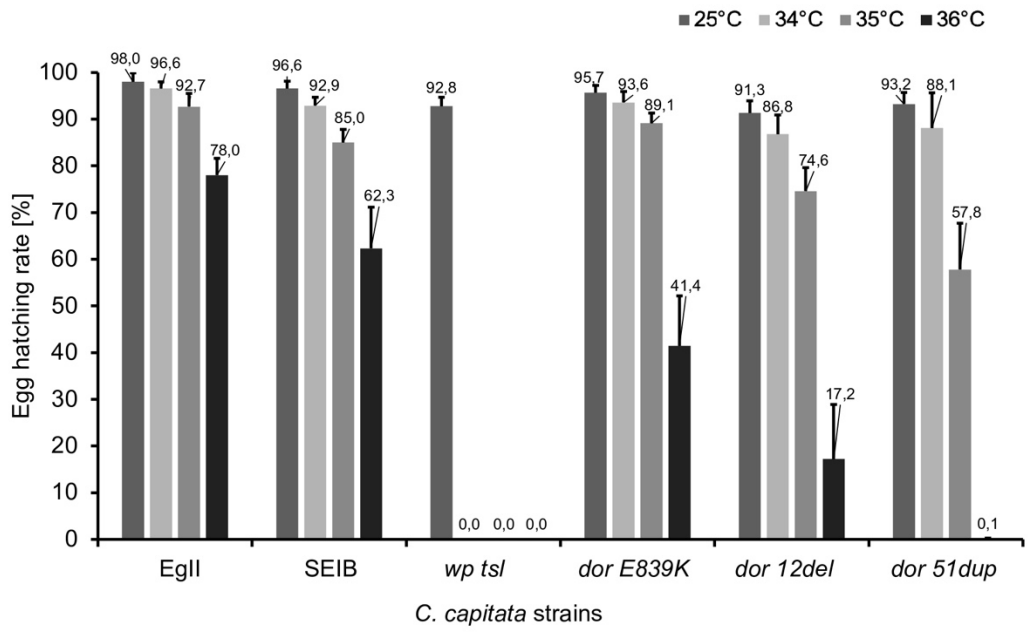

Figure 4. Egg hatching rates of wild-type control (EgII, SEIB), mutant (*wp tsl*), and CRISPR-mutant (*dor E839K, dor 12del,* and *dor 51dup*) strains. Egg hatching rates (shown as mean ± standard deviation) of strains reared without heat-shock treatment at 25°C and after 24 h heat-shock treatment at 34°C, 35°C or 36°C are shown. Values represent the mean of the three replicates for the three tested days.

291 At the embryonic stage, *dor 12del* and *dor 51dup* egg hatching rates at 25°C were significantly
292 different from those observed in the wild-type strains EgII and SEIB (Table S6), indicating
293 fitness cost, albeit minimal. On the contrary, no difference was detected among the *dor E839K*
294 and the two wild-type strains. Thermal sensitivity of wild-type and CRISPR strains started at
295 35°C (Table S5), and egg hatching rates ranged between 92.67 ± 2.81% (EgII) and 57.78 ±
296 9.94% (*dor 51dup*) (Table S4). At 36°C, the egg hatching rate of the EgII and SEIB strains was
297 78.00 ± 3.59% and 62.33 ± 8.81%, respectively, while that of the three CRISPR strains was
298 significantly decreased (*dor 12del* = 17.22 ± 11.70%, *dor 51dup* = 0.11 ± 0.19% and *dor E839K*
299 = 41.44 ± 10.75%; Table S4). Interestingly, at 36°C, *dor 51dup* and *wp tsl* were not statistically
300 different (Table S6), indicating a similarity between the behavior of the CRISPR strain and the
301 original *tsl* strain.
302
303 Statistically significant differences were also detected for pupal recovery among all the strains
304 tested at 25°C ($F = 2.5388$, $df = 48$, $p = 0.04064$), 31°C ($F = 27.071$, $df = 48$, $p = 6.635 \times 10^{-13}$), 32°C ($F = 18.848$, $df = 48$, $p = 2.524 \times 10^{-10}$), 33°C ($F = 10.445$, $df = 47$, $p = 8.825 \times 10^{-7}$),
306 34°C ($F = 28.269$, $df = 40$, $p = 3.475 \times 10^{-11}$) and 35°C ($F = 29.097$ $df = 40$, $p = 2.281 \times 10^{-11}$).
307 At 36°C, *wp tsl* and *dor 51dup* strains did not show any pupal recovery (Table S4) ($F = 2.518$,
308 $df = 28$, $p = 0.07845$) (Figure 5, Table S4, Table S7).
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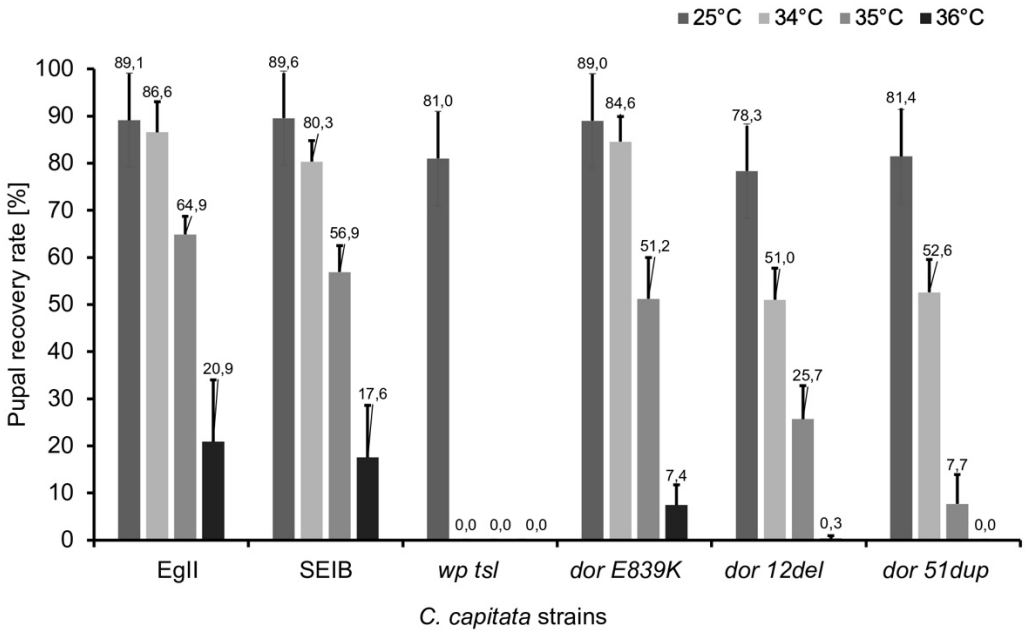

Figure 5. Pupal recovery rates of control (wild type: EgII, SEIB, and mutant: *wp tsl*) and CRISPR-mutant (*dor E839K, dor 12del,* and *dor 51dup*) strains. Pupal recovery rates (shown as mean ± standard deviation) of strains reared at 25°C without heat-shock treatment and after 24 h heat-shock treatment at 34°C, 35°C and 36°C are shown. Values represent the mean of the three replicates for the three tested days.

A significant difference was observed among the different strains concerning the temperature at which the pupal recovery rate started to decrease (Table S7). The *wp tsl* mutant strain was shown to be the most sensitive since the pupal recovery rate started to reduce at 31°C, while for *dor 12del* and *dor 51dup,* the reduction was initiated at 33°C, for *dor E839*K at 34°C (Table S7), and for the wild-type strains at 35°C, respectively (Table S7). In addition, the TSLT results provided clear evidence that the pupal recovery rate of the *dor 51dup* strain drastically decreases between 34°C (52.56 ± 6.98%) and 35°C (7.67 ± 6.24%) (Table S7), while for *dor 12del* (35°C = 25.67 ± 7.10; 36°C = 0.33 ± 0.58%) and *dor E839K* (35°C = 51.22 ± 8.74; 36°C = 7.44 ± 4.27%), an exposure at 36°C was required (Table S7).

Statistically significant differences were also detected for adult emergence among all the strains tested at 25°C ($F = 14.223$, $df = 48$, $p = 1.541 \times 10^{-8}$), 31°C ($F = 112.10$, $df = 48$, $p < 2.2 \times 10^{-16}$), 32°C ($F = 211.1$, $df = 48$, $p < 2.2 \times 10^{-16}$), 33°C ($F = 8.7947$, $df = 40$, $p = 3.424 \times 10^{-5}$),

34°C ($F = 434.56$, $df = 40$, $p < 2.2 \times 10^{-16}$) and 35°C ($F = 10.909$, $df = 40$, $p = 4.481 \times 10^{-6}$), while at 36°C ($F = 0.2303$, $df = 23$, $p = 0.7961$) no statistical differences were found (Figure 6, Table S4, Table S8).

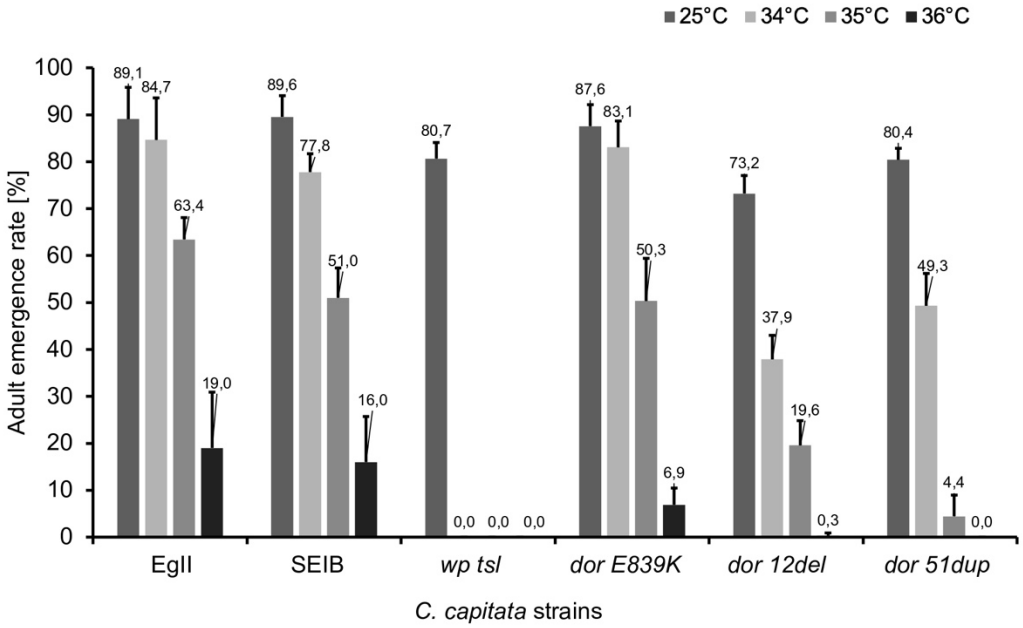

Figure 6. Adult recovery rates of control (wild type: EgII, SEIB, and mutant: *wp tsl*) and CRISPR-mutant (*dor E839K, dor 12del,* and *dor 51dup*) strains. Adult emergence rates (shown as mean ± standard deviation) of strains reared at 25°C without heat-shock treatment and after 24 h heat-shock treatment at 34°C, 35°C and 36°C are shown. Values represent the mean of the three replicates for the three tested days.

The impact of exposure to high temperatures on adult emergence differed among CRISPR and control strains. The adult emergence rate of the *wp tsl* mutant strain decreased at 31°C (Table S7) that of EgII and *dor 12del* at 34°C, while *dor 51dup* at 35°C, respectively (Tables S4 and S8). It is worth noting that when embryos were subjected to a 24-hour heat treatment at 36°C, the observed lethality was 100% for *dor 51dup* and almost 100% (99.67 ± 0.58%) for *dor 12del* (Tables S4 and S8).

Discussion

The temperature-sensitive lethal (tsl) phenotype has been used as a selectable marker in the most successful medfly GSS, VIENNA 7 and VIENNA 8, developed so far [10, 11]. More than two billion sterile GSS males are being produced in mass-rearing facilities every week and released to control populations of this major agricultural pest worldwide. Identifying the *tsl* gene and characterizing the mutation(s) responsible for the respective phenotype will pave the way for using it as a selectable marker for developing GSS in other SIT target species [10, 12, 13, 38–40]. In the present study, we characterized the *deep orange* locus of *C. capitata*. This gene is known to have temperature-sensitive lethal mutations in *D. melanogaster*, and we investigated whether a tsl phenotype, similar to the ones reported previously, can be reproduced by inducing CRISPR/Cas9 mutations in *Ccdor* [21, 25, 41].

The *Ccdor* gene was selected as a candidate *tsl* gene by thoroughly analyzing the *tsl* region on chromosome 5 [18]. Four sets of data were pointing towards that candidate: (a) the most recent genome assembly suggested the presence of *Ccdor* on the right arm of chromosome 5. Its position was confirmed by *in-situ* hybridization on polytene chromosomes, which localized *Ccdor* in 77B, in the area where the *tsl* gene is expected to be [14, 16]; (b) mutations in its *D. melanogaster* orthologue have resulted in a tsl phenotype [21, 25]; (c) the presence of point mutations resulting in amino acid substitutions when *Ccdor* gene sequences were compared between wild type and *tsl* mutant strains [18] and (d) its highly conserved amino acid sequence among insects, making it suitable for the construction of GSSs in other SIT target species.

CRISPR/Cas9-NHEJ targeting the *Ccdor* functional domain *Pep3/Vps18/deep orange,* present in exon 4, resulted in non-viable progeny with lethality being observed at the mid- and late-pupal stages (Figure 2A & 2B). This contrasts observations in *D. melanogaster*, where lethality occurred during larval and pre-pupal stages [20, 25, 26, 42, 43]. This difference in lethality stages observed in *Ccdor* after the knock-out may be influenced by the specific indel(s) introduced. However, the exact nature of the indel(s) has not been determined due to the genetic mosaicism encountered during G_0.

CRISPR/Cas9-HDR gene editing in the EgII wild-type strain introduced a point mutation detected on the sixth exon of the *Ccdor* gene of the *tsl* mutant strain. This resulted in the desired

correction and two additional mutations: a deletion and a duplication, both in frame, due to an error in the use of the repair template during the double-strand break sealing process. The efficiency of CRISPR HDR, including the appearance of errors, can be impacted by various factors, such as the activity of the endogenous repair systems, the cell cycle, and the length of the homology arms of the repair template [44]. The three strains obtained, *dor E839K*, *dor 12del*, and *dor 51dup*, are all temperature-sensitive lethal, but showed differences from the original *tsl* mutant strain. The embryonic lethality in the CRISPR *Ccdor* strains appears at higher temperatures than the original *tsl* mutant strain. Notably, only the *dor 51dup* strain exhibited almost complete embryonic lethality at 36°C, but all three strains reached high lethality rates at an early larval stage. The rearing efficiency of the new strains was satisfactory, and the *dor* gene is a valuable target for developing GSS in other species. However, more investigations are needed to employ *dor* as a selectable marker in *Ceratitis capitata*, and to explore the genetic background and possible involvement of other loci in the tsl phenotype.

Deep orange has several domains with diverse and essential functions. As the protein plays an important role in cellular activities, mutations in these domains may lead to loss/gain of function or significantly affect cellular activities, resulting in lethality [20, 67, 68]. The *Pep3_Vps18* domain, targeted by CRISPR NHEJ in the present study, is involved in endosomal sorting and vesicle trafficking [20, 67, 68]. Knock-out mutations in this domain have caused severe defects in endosomal sorting, vesicle trafficking, multivesicular body (MVB) formation, and increased levels of cellular stress and oxidative damage in yeast and mammalian cells [69, 70]. Thus, this might also be responsible for the lethal effect we experienced in the knock-out *via* NHEJ targeting in this study. The function of the *Helo_like_N* domain, which was targeted via CRISPR (HDR), is not fully understood. Previously, it was reported to play a role in RNA processing, DNA binding, transcriptional regulation, and cell death-inducing activity [71–75]. Moreover, mutations within the Helo_like_N domain, particularly in its transmembrane helix region, may compromise its function [76]. As shown in Figure S3, the E839K mutation, the deletion *dor 12del*, and the duplication *dor 51dup* are all located in an α-helix region. This could affect the regular gene expression pattern, leading to cellular function and development changes, including the gain of temperature sensitivity [73]. Although temperature sensitivity (ts) has been commonly associated with point mutations, deletions and duplications/insertions have also been reported, albeit less frequently, as the causal factor of tsl [31, 33, 46–66]. Such mutations may alter a functional domain, the overall native structure, or the specific activity of

the protein and these may be potential explanations for the tsl phenotype of *dor 12del* and *dor 51dup* strains [65, 66].

Mutations in the *ring finger/U-box* domain may replace highly conserved cysteine residues needed to form the "U-shaped" beta-sheet, destabilizing the protein and affecting its function [77–79]. This is the case of the *D. melanogaster* temperature-sensitive lethal mutation dor^{1} (C979Y) [21]. When insects carrying the *Dm* dor^{1} mutation are exposed to high temperatures, they die at the pupal stage [25] and present altered phenotypes in the eyes, thorax, and wings [20]. The *C. capitata tsl* mutant strain also has a non-synonymous point mutation in the same domain (L940I; Table S1). Whether this mutation has any effect on the tsl phenotype awaits investigation. It should be mentioned, however, that the expression of the typical tsl phenotype observed in the *C. capitata tsl* mutant strains may require the combination of point mutations in more than one domain of the *Ccdor*; for example, the E839K mutation in the *Helo_like_N* domain combined with the L940I in the *ring finger/U-box* domain or mutations in other domains or even other genes [80]. This was previously observed in *D. melanogaster* in genes involved in the control of body size [81], behavior [84], or tumor suppression [83]. Finally, none of the mutations in this study in the *Ccdor* resulted in eye colour alteration or any other visibly detectable phenotypes.

Conclusions

The successful application of CRISPR/Cas9 genome editing targeting the *deep orange* gene of *Ceratitis capitata* resulted in three mutant strains that proved to be temperature sensitive. The presence of a 51 bp duplication together with the E839K mutation (*dor 51dup*) in the *Ccdor* coding region triggers total embryonic lethality following heat shock at 36°C. In addition, for all the CRISPR strains (*dor 51dup*, *dor 12del,* and *dor E839K),* a variable lethality was observed during the larval and pupal stages following heat shock at 35°C. Although two of the three CRISPR strains, *dor 51dup,* and *dor 12del*, exhibited minimal fitness cost at the embryonic stage when reared at 25°C, all gene-edited strains present a high productivity rate suggesting their suitability for breeding. Given this characteristic and the high conservation of the Deep orange protein sequence among insects, particularly Tephritids, the *dor* gene emerges as a promising selectable marker for creating new genetic sexing strains (GSS).

Methods

***Ceratitis capitata:* strains and fly rearing.**

In the frame of this study, seven *Ceratitis capitata* strains were used (Table 1) and reared under standard laboratory conditions (24 ± 2°C, 55 ± 10% RH, and 14/10 h light/dark cycle) as previously reported in Sollazzo et al, 2022 [17].

Table 1. *Ceratitis capitata* strains used in the present study.

	Strain	Group	Used for
1	Egypt II FF26	wild type	Illumina NGS, 10X Genomics, *tsl* test
2	Benakeion Volos FF26	wild type	Sanger sequencing, RNA-Seq
3	Seibersdorf FF26	wild type	Sanger sequencing
4	VIENNA 8 2010 FF26	GSS	Sanger sequencing
5	VIENNA 7	GSS	Illumina NGS, 10X Genomics
6	*wp tsl* FF21	mutant	Illumina NGS, 10X Genomics, Sanger sequencing, RNA-Seq, *tsl* test
7	D53-3-28 FF21	mutant	Sanger sequencing

Analysis of *Dm deep orange* gene orthologue in *Ceratitis capitata*

Using the *Cc deep orange* protein sequence (XP_004536447.1), a search for conserved domains was carried out through the NCBI Conserved Domain Database server (http://www.ncbi.nlm.nih.gov/cdd/cdd.shtml) [84] and the SMART online tool (http://smart.embl-heidelberg.de/) [85, 86]. The detected domains were annotated on *Ccdor* genomic sequence in Geneious Prime 2022.1.1 to check if any polymorphism found in its coding sequence (CDS) [18] was located inside a conserved domain. Secondary structure and

disorder predictions have been carried out using Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2) and the *Ccdor* wild-type protein sequence as input [87].

DNA extraction and Sanger sequencing of the *deep orange* gene

Genomic DNA was extracted from three males and three females of *C. capitata* Benakeion, SEIB, VIENNA 8, *wp tsl*, and D53 *tsl* strains using ExtractMe DNA tissue kit (Blirt, Poland) following the manufacturer's instructions. A NanoDrop spectrometer was used to assess the quantity and quality of the extracted DNA. Primers (Table S2) were designed using the Geneious Prime 2022.1.1 software. PCRs were performed in a 25 µL reaction volume using 12.5 µL Platinum™ Green Hot Start PCR Master Mix (2X) Kit (Thermo Fisher Scientific), 60–80 ng DNA, and the following PCR settings [94°C, 2 min; 35 cycles of (94°C, 30 s; 60°C, 30 s; 72°C, 120 s); 72°C, 5 min]. PCR products were analyzed by electrophoresis in 2% agarose gels and visualized under UV light. Amplicons were purified using the DNA Clean & Concentrator-25 kit according to the manufacturer's protocol (Zymo Research - Irvine, CA, USA). The purified products were adjusted to the concentration of 10 ng/µl while sequencing primers were diluted following the Eurofins Genomics instructions up to 100 nmol/µl. The sequencing mix was prepared in a final volume of 15 µl (13 µl of DNA and 2 µl of primer). Sequencing results were imported in Geneious Prime 2022.1.1 and aligned to the *Ccdor* gene wild-type sequence extracted from *Ccap 3.2.1* (accession GCA_905071925.1) using the Geneious Prime "Map to reference" tool with default parameters.

CRISPR/Cas9 genome editing

Lyophilized Cas9 protein from *Streptococcus pyogenes* (CP01 - PNA Bio, Newbury Park, California, USA) was resuspended in nuclease-free water to 1 µg/µl, separated in aliquots, and stored at −80°C until further use. Single guide RNAs: sgRNA_NHEJ (TCAAAATGCACCACGTGCCA) and sgRNA_HDR (GGATGAATGTGATAAACAAG) were designed and checked for off-targets using the "Find CRISPR site" tool in Geneious Prime 2022.1.1 [88] using the *Ceratitis capitata* 2.1 genome (accession GCF_000347755.2) from NCBI as the off-target database. sgRNAs were ordered from Sigma Aldrich, Germany, with the following specifications (Physical material: Synthetic RNA, Purification: HPLC, CRISPR species: SpCas9, Structure: sgRNA (crRNA+tracrRNA as one), Scale of synthesis: 3 nmol, modified, dry).

The two 151 bp single-stranded donor templates for CRISPR HDR, "ssODN_E839K_sense" and "ssODN_E839K_anti", designed in sense and antisense orientation (Table S2) to the double-strand break (DSB) [89] to re-build the mutation in position 839 (E839K), were synthesized by Eurofins Genomics (EXTREMer oligo, purified salt-free, quality control by CGE). They differ from the wild-type sequence by three bases (72A > G, 75G > C, 76G > A). The change in position 76 of the ssODN (G > A; Glu839 > Lys839) re-builds the mutation found in the *wp tsl* mutant strains while the second 75 (G > C; Ala838 > Ala838) and the third 72 (A > G; Gln837 > Gln837) are silent mutations to reduce the target sequence similarity after HDR and mutate the PAM site to prevent re-editing by the CRISPR/Cas9 machinery [90, 91].

Embryonic microinjections for CRISPR-Cas9 trageting

The injection mix for CRISPR NHEJ and HDR contained 360 ng/µl Cas9 protein, 200 ng/µl sgRNA, and a final concentration of 300 mM KCl in a 10 µl volume, as described in previous studies [27, 92, 93]. The mix was subjected to 10 min incubation at 37°C to complex the sgRNA and Cas9 protein. For CRISPR HDR, we added 200 ng/µl ssODN (sense or antisense) after the incubation step; also previously described [28, 30, 31, 92, 93].

Microinjections were carried out in 40-45 min old wild-type *C. capitata* EgII embryos which were previously chemically dechorionized (sodium hypochlorite, 3 min), fixed on double-sided sticky tape (Scotch 3 M), dehydrated (calcium chloride, 6 min) and covered with halocarbon oil 700 (Sigma-Aldrich) [94]. Microinjections were performed using siliconized quartz glass needles (Q100-70-7.5; Sutter Instruments, Novato, CA USA) drawn out on a laser-based micropipette puller (Sutter P-2000) with the following conditions (Heat = heat, Filament = Fil, Velocity = Vel, Delay = Del, Pull = Pull): Quartz (Q100-70-7.5): Heat 750, Fil 5, Vel 70, Del 130, Pull 175, a FemtoJet 4X micromanipulator/microinjector (Eppendorf, Hamburg, Germany) and a Leica DM IL LED inverted microscope (Leica Microsystems, Wetzlar, Germany). Once injected, embryos were kept at 25°C and 60% RH until larval hatching and transferred from the oil to the larval food using a brush.

Molecular detection of CRISPR/Cas9-induced *deep orange* mutations

Genomic DNA extraction from single G_0 flies and pupae, PCRs, DNA purification, and Sanger sequencing were performed as described above in "DNA extraction and Sanger sequencing of *deep orange* in *Ceratitis capitata* strains". G_2 and G_3 flies were analyzed *via* non-lethal genotyping using a single adult leg and Platinum™ Direct PCR Universal Master Mix kit

(Thermo Fisher Scientific). In more detail, PCRs were performed by cutting single legs from anesthetized flies using micro scissor (Hammacher Karl, Germany), placing each one of them into single PCR tubes containing 12.5 µL Platinum™ Direct PCR Universal Master Mix (Thermo Fisher Scientific), primers (100 nM) and water up to a final volume of 25 µL. The following pairs of primers were used: P58_NHEJ_geno_F / P58_NHEJ_geno_R for NHEJ and P58_HDR_geno_F / P58_HDR_geno_R for HDR, respectively (Table S2). PCR settings were the following: [94°C, 2 min; 35 cycles of (94°C, 15 s; 60°C, 15 s; 68°C, 20 s); 68°C, 5 min]. The 940 bp (CRISPR NHEJ) and the 829 bp (CRISPR HDR) PCR products were then verified by gel electrophoresis, purified using the DNA Clean & Concentrator-25 kit according to the manufacturer's protocol (Zymo Research - Irvine, CA, USA), Sanger-sequenced (NHEJ: P58_NHEJ_geno_F; HDR: P58_HDR_geno_F) using Eurofins Genomics Tube service and analyzed in Geneious Prime 2022.1.1.

Crossing and screening

G_0 adults that survived to sexual maturity were individually crossed to three EgII wild-type (CRISPR NHEJ) or *wp tsl* (CRISPR HDR) virgin mates. Eggs were collected three times, and G_1 adults were inter-crossed in mass, resulting in three potential G_2 genotypes: *dor* $^{+/+}$, *dor* $^{+/CRISPR,}$ and *dor* $^{CRISPR/CRISPR}$. Non-lethal genotyping allowed the screening of G_2 adults to isolate the *dor* $^{CRISPR/CRISPR}$ genotype and subsequent set up of single pair crosses according to the nature of the induced mutation found by sequencing. G_3 eggs from each single pair cross were collected three times, and adults were subjected to non-lethal genotyping to confirm the strain's stability. CRISPR strains with germline mutations of different natures were isolated and kept under laboratory conditions.

***In-situ* hybridization**

In-situ hybridization was performed as described in Gouvi et al. 2022 and Sollazzo et al. 2023 [18, 95]. Polytene chromosome preparations were prepared from third instar larvae salivary glands of *Ceratitis capitata* EgII strain, according to Mavragani-Tsipidou et al. 1990 [96]. The DNA labeling was performed using the “DIG-DNA Labeling and Detection” kit (Roche, Germany) following the manufacturer’s instructions. Well-spread nuclei were analyzed for the identification of the hybridization signals. Hybridization sites were photographed at a combined magnification of 60x and 100x using a phase contrast microscope DM2000 Led (Leica) and a camera DMC5400 (Leica). They were identified by using the salivary gland chromosome maps of *C. capitata* as reference [97].

Temperature-sensitive lethal test

Temperature-sensitive lethal tests (TSLT) were performed on CRISPR strains (two generations after the establishment of homozygous strains) and control strains (EgII, SEIB, and *wp tsl*) as previously described [11, 17, 45] to assess their temperature sensitivity. Briefly, three replicates of 100 eggs each were prepared, for each of the seven temperatures tested, resulting in the collection of 2100 eggs in total on a daily basis. This egg collection scheme was repeated for three consecutive days. For each replicate, the eggs were placed on black strips on top of 90 x 15 mm Petri dishes filled with larval carrot diet and kept at 25°C for 24 hours. Each set of three replicates was incubated at different temperatures (25, 31, 32, 33, 34, 35, and 36°C) for 24 hours. After the heat shock, Petri dishes were placed at 25°C to complete their development. 5-, 15-, and 23-days post egg collection, egg hatching, pupal recovery, and adult recovery rates were determined. Egg hatching, pupal recovery, and adult emergence rates were calculated for single replicate using the number of collected embryos (100) as a reference and the number of hatched eggs after five days, the number of puparia obtained after fifteen days, and the number of eclosed adults after twenty-three days, respectively.

Statistical analysis

All statistical analyses were performed using R version 4.2.0 [98]. All datasets of this study represent recovery rates (egg hatching, pupal recovery, and adult emergence) and were, therefore, analyzed using a GLM-binomial family or a GLM-quasi-binomial family, when overdispersion was detected [99]. The DHARMA package was used to check if the simulated dispersion is equal to the observed dispersion and identify overdispersion in the generalized linear models (GLM) [100]. In the case of overdispersion, a GLM-quasibinomial model using a logit link function was employed to address it [101]. The chi-square test for the GLM-binomial models and an F-test for the GLM-quasi-binomial models were used to analyze deviance. The goodness-of-fit of the models was inspected with simulation envelopes of half-normal plots [102]. The 'estimated marginal means' (emmeans) package was used for the pairwise comparisons of the fitted model estimates [103]. For all data, the significance level was set to $\alpha = 0.05$.

Availability of data and materials

All data generated or analyzed during this study are included in this published article, its supplementary information files, and publicly available repositories.

Abbreviations

AW-IPM: Area-Wide Integrated Pest Management.

SIT: Sterile insect technique.

GSS: Genetic sexing strain.

FAO/IAEA: Food and Agriculture Organization/International Atomic Energy Agency.

CRP: Coordinated research projects.

wp: *white pupae.*

tsl: temperature sensitive lethal.

EgII: Egypt II.

SEIB: Seibersdorf

dor: *deep orange.*

Ccdor: *Ceratitis capitata deep orange.*

Dmdor: *Drosophila melanogaster deep orange.*

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats.

NHEJ: Non-homologous end joining.

HDR: Homology-Directed Repair.

PAM: protospacer adjacent motif.

sgRNA: single guide RNA.

DSB: double-stranded DNA break.

ssODN: single-stranded oligodeoxynucleotide

SNP: single nucleotide polymorphism.

NGS: next-generation sequencing.

RH: relative humidity.

PCR: polymerase chain reaction.

aa: amino acid**.**

bp: base pair.

G_x***:*** generation.

Del: deletion.

Dup: duplication.

TSLT: Temperature-sensitive lethal test.

MVB: multivesicular body**.**

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Acknowledgments

This study benefitted from discussions at meetings for the Coordinated Research Project D44003, "Generic approach for the development of genetic sexing strains for SIT applications", funded by the International Atomic Energy Agency (IAEA).

Funding

This study was supported by the Insect Pest Control Subprogramme of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

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Ethics declarations

Ethics approval and consent to participate.

Not applicable.

Consent for publication

All authors of this manuscript consent to its publication as a research article in BMC Biology.

Competing interests

The authors declare that they have no competing interests.

Supplementary Material

Deep orange gene editing triggers temperature sensitive lethal phenotypes in *Ceratitis capitata*

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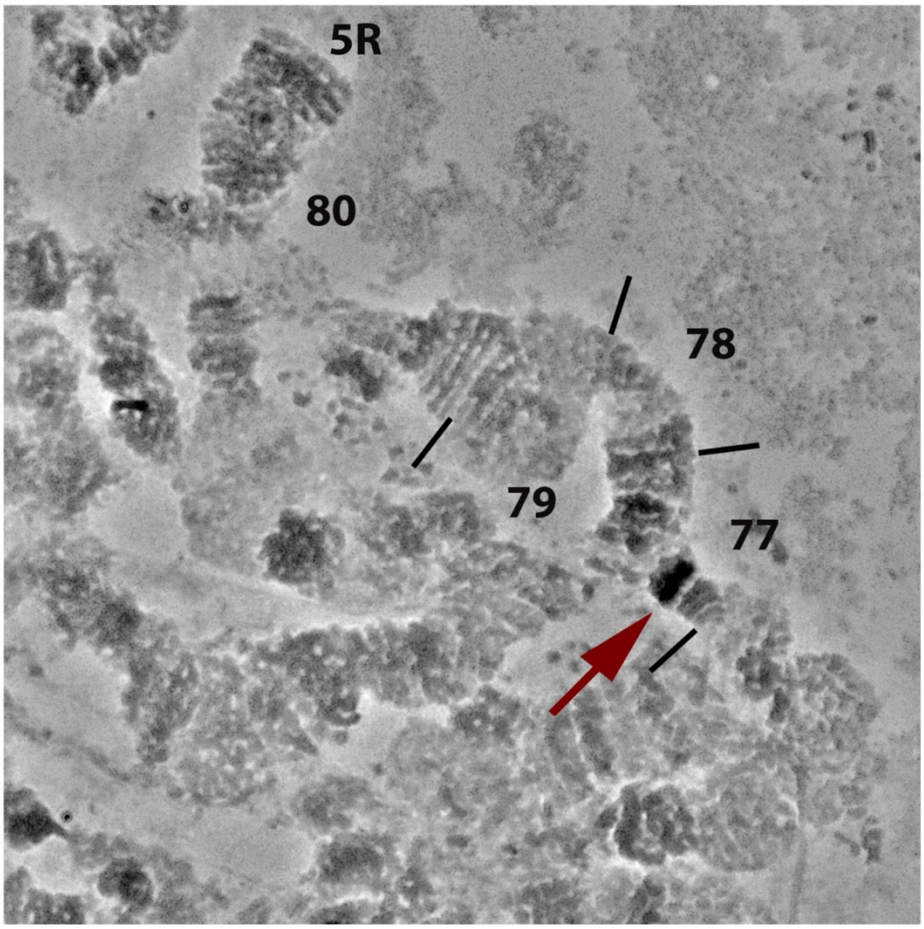

Figure S1. *In situ* hybridization of *Ccdor* in position 77B on the right arm of the medfly polytene chromosome 5.

Table S1. SNPs detected in the coding region of *deep orange* gene in the medfly *wp tsl* mutant strain. The position of SNPs in the *Ccdor* CDS, nucleotide change, type of polymorphism, and the amino acid change when it occurs are shown.

Position in *Ccdor* gene (bp)	Position in *Cc*DOR protein (aa)	Nucleotide change	Polymorphism type	Amino acid change
60		A -> T	SNP (transversion)	
87		T -> C	SNP (transition)	
249	83	A -> G	SNP (transition)	I -> M
362		T -> C	SNP (transition)	
367	100	T -> G	SNP (transversion)	D -> E
424		C -> T	SNP (transition)	
625		A -> G	SNP (transition)	
735		C -> T	SNP (transition)	
781		C -> T	SNP (transition)	
864		G -> T	SNP (transversion)	
1,032		A -> C	SNP (transversion)	
1,170		C -> G	SNP (transversion)	
1,184	313	A -> C	SNP (transversion)	H -> P
1,188		T -> C	SNP (transition)	
1,194		A -> C	SNP (transversion)	
1,212		C -> T	SNP (transition)	
1,227		A -> G	SNP (transition)	
1,240		T -> C	SNP (transition)	
1,431		A -> T	SNP (transversion)	
1,455		T -> C	SNP (transition)	
1,618	458	A -> T	SNP (transversion)	I -> L
1,650		G -> A	SNP (transition)	
1,932		A -> G	SNP (transition)	
2,016		C -> T	SNP (transition)	
2,028		T -> C	SNP (transition)	

2,070		C -> T	SNP (transition)	
2,238		T -> C	SNP (transition)	
2,346		G -> T	SNP (transversion)	
2,529		C -> T	SNP (transition)	
2,631		G -> A	SNP (transition)	
2,766		T -> C	SNP (transition)	
2,889	839	G -> A	SNP (transition)	E -> K
2,924		A -> G	SNP (transition)	
3,131		A -> G	SNP (transition)	
3,192	940	C -> A	SNP (transversion)	L -> I
3,248		T -> G	SNP (transversion)	

80 **Table S2.** List of primers and sgRNAs used in this study
81

Name	Sequence	Purpose
P58_2kb_F	GTTTCCGCATAGAAATCGGCT	Sanger sequencing
P58_2kb_R	CACTGAACTTTCCAGCAGCG	Sanger sequencing
P58_3kb_F	TCCTGAATGAAAAGTTAACCATTTGC	Sanger sequencing
P58_3kb_R	GAAACTGAGTTAAACTATTCACTGT	Sanger sequencing
P58_F1	GGTTCATAAATATCATCATTCC	Sanger sequencing
P58_F2	AATAGTAACAGTCAGCCGCAA	Sanger sequencing
P58_F3	TCAAAATCGGAGAAAAATGGCA	Sanger sequencing
P58_F4	TATAGCATATTCCAAATAA	Sanger sequencing
P58_F5	CCGTCGGGGATAATTGATTTGTG	Sanger sequencing
P58_F6	TGTTCATGGAAATACTCCTCGT	Sanger sequencing
P58_F7	TCTTCGCCGTGCATTTGTTG	Sanger sequencing
P58_F8	TGCTGATAATCCACTGGTTTGTG	Sanger sequencing
P58_F9	CGAACGACGACATTTTACTCGT	Sanger sequencing
P58_NHEJ_geno_F	TGTTCATGGAAATACTCCTCGT	No-lethal genotyping
P58_NHEJ_geno_R	TAACAAAGTCATCTTAGAAA	No-lethal genotyping
P58_HDR_geno_F	CGTTCACTTGATCCCAATCGC	No-lethal genotyping
P58_HDR_geno_R	TGCGGCTGTTGAATTAGCAC	No-lethal genotyping
P58_probe_F	CTAATTCAACAGCCGCAGCC	*In situ* hybridization
P58_probe_R	TGCACCTAACGTCCTCCTTG	*In situ* hybridization
sgRNA_NHEJ	TCAAAATGCACCACGTGCCATGG	CRISPR NHEJ
sgRNA_HDR	GGATGAATGTGATAAACAAGCGG	CRISPR HDR

ssODN_E839K_sense	AAAGATTACAATTTTCCTGTGCGCTA ATGCGTATGCTTCTCTCACGCACATT TTGTAAATCTTTCATAACACGTTTGG CCTGTTTATCACATTCATCCATTTCGT GTTTTAATTCTTGAATTTTTTGATTGT AATCCTGAATGAAAAGGTG	CRISPR HDR
ssODN_E839K_anti	CACCTTTTCATTCAGGATTACAATCA AAAAATTCAAGAATTAAAACACGAA ATGGATGAATGTGATAAACAGGCCA AACGTGTTATGAAAGATTTACAAAAT GTGCGTGAGAGAAGCATACGCATTA GCGCACAGGAAAATTGTAATCTTT	CRISPR HDR

82


C.capitata
I83M
D100E
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
H313P
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
I458L
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
E839K
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes
C.capitata
L940I
B.dorsalis
B.oleae
B.tryoni
Z.cucurbitae
D.melanogaster
M.domestica
A.aegypti
A.albopictus
G.fuscipes

Figure S2. Amino acid sequence alignment of different insect species' *deep orange* homologs using MUSCLE. Identical residues are shaded in black, and conserved residues are shaded grey. Red dashes represent the six SNPs (that lead to amino acid changes) found in *tsl* mutant strains.

Figure S3. Secondary structure and disorders prediction of *CcDOR* wild type protein done with the Phyre[2] online tool (http://www.sbg.bio.ic.ac.uk/phyre2).

Table S3. Summary of the microinjections performed using sgRNA_NHEJ and sgRNA_HDR + ssODN to induce *Ccdor* gene knock-out and introduce the E839K point mutation, respectively.

Injection	sgRNA	ssODN	# embryos injected	# G_0 pupae (% survival) [a]	# G_0 adults (% survival) [b]	#G_0 germline mutants [c]	Detected mutation	
							G_1 mutant families	Mutation
NHEJ	sgRNA_NHEJ	no	586	70 (11.9 %)	55 (9.4 %)	7 (12.7 %) [d]	0	mosaicism
HDR	sgRNA_HDR	ssODN_E839K_sense	255	28 (11.0 %)	28 (11.0 %)	1 (4.5 %)	1	Knock-in E839K
		ssODN_E839K_antisense	100	4 (4.0 %)	3 (3.0 %)	3 (100.0 %)	2	Deletion (12 bp) Knock-in E839K + Duplication (51 bp)

[a] Pupal recovery rate is presented in parentheses as the percentage of G0 pupae out of the total number of injected embryos.

[b] Adult recovery rate is presented in parentheses as the percentage of G0 adults out of the total number of injected embryos.

[c] Mutagenesis efficiency is presented in parentheses as the percentage of G0 individuals with a CRISPR-induced mutation out of the total number of G0 adults obtained.

[d] All died at mid- or late-pupal stage.

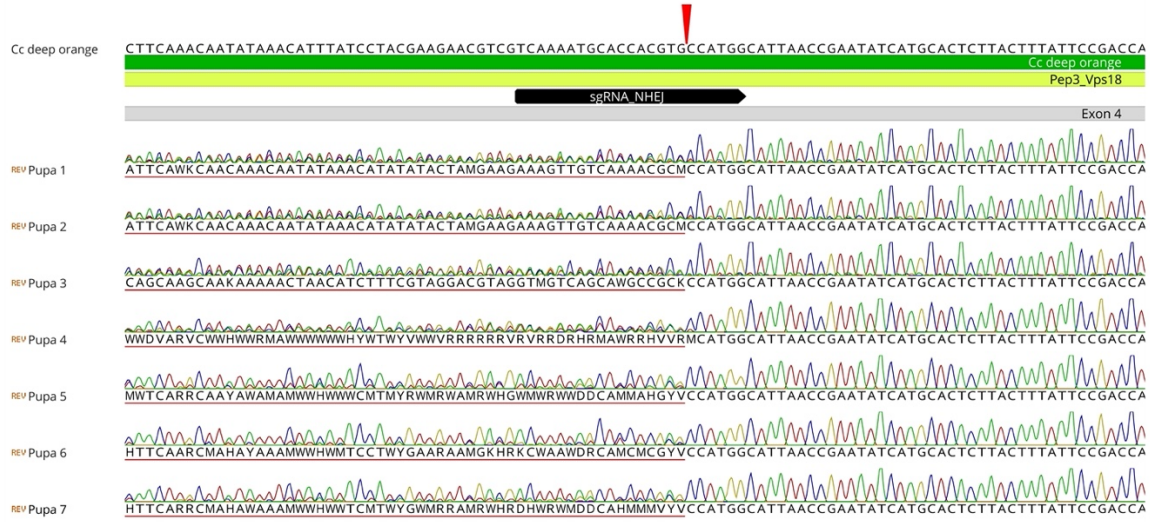

Figure S4. CRISPR/Cas9 NHEJ induced mutation in G_0. Sanger sequencing of seven dead pupae recovered from sgRNA_NHEJ injections targeting *Ccdor* gene exon 4. The red arrow indicates the Cas9 cutting site. Poor sequencing results upstream of the Cas9 cutting site could be due to mosaicism induced by CRISPR NHEJ.

111 **Table S4**: Egg hatching, pupal recovery, and adult emergence rates (shown as mean and
112 standard deviation) of *Ceratitis capitata* control and CRISPR strains at different temperatures.

Strain	Temperature (°C)	Egg hatching (%)	Pupal recovery (%)	Adult emergence (%)
EgII	25	98.00 ± 1.91	89.11 ± 6.75	89.11 ± 6.75
	31	95.89 ± 0.96	87.67 ± 5.78	87.44 ± 5.75
	32	97.22 ± 0.96	90.33 ± 6.14	91.11 ± 4.11
	33	96.89 ± 1.47	87.00 ± 6.36	86.44 ± 7.27
	34	96.56 ± 1.40	77.11 ± 6.46	84.67 ± 8.95
	35	92.67 ± 2.81	64.89 ± 3.81	63.44 ± 4.63
	36	78.00 ± 3.59	20.89 ± 13.10	19.00 ± 11.93
SEIB	25	96.56 ± 1.54	89.56 ± 4.52	89.56 ± 4.52
	31	94.89 ± 1.28	86.22 ± 9.10	86.22 ± 9.10
	32	95.56 ± 2.04	88.00 ± 4.24	88.00 ± 4.24
	33	94.11 ± 2.94	78.00 ± 4.08	76.56 ± 4.13
	34	92.89 ± 1.79	80.33 ± 4.42	77.78 ± 3.93
	35	85.00 ± 2.81	56.89 ± 5.61	51.00 ± 6.35
	36	62.33 ± 8.81	17.56 ± 11.07	16.00 ± 9.74
wp tsl	25	92.78 ± 1.93	81.00 ± 3.41	80.67 ± 3.41
	31	79.00 ± 2.40	38.22 ± 4.12	29.11 ± 5.39
	32	85.78 ± 2.37	52.11 ± 6.23	23.11 ± 3.42
	33	9.11 ± 1.92	2.00 ± 0.58	0.33
	34	0.00	0.00	0.00
	35	0.00	0.00	0.00
	36	0.00	0.00	0.00
dor E839K	25	95.67 ± 1.58	89.00 ± 5.30	87.56 ± 4.64
	31	96.22 ± 0.38	88.00 ± 3.79	87.44 ± 3.99
	32	94.56 ± 1.46	84.89 ± 5.09	84.11 ± 5.46
	33	95.44 ± 2.59	87.89 ± 2.04	86.89 ± 2.10
	34	93.56 ± 2.35	84.56 ± 5.37	83.11 ± 5.55
	35	89.11 ± 2.19	51.22 ± 8.74	50.33 ± 9.11
	36	41.44 ± 10.75	7.44 ± 4.27	6.89 ± 3.60
dor 12del	25	91.33 ± 2.63	78.33 ± 3.94	73.22 ± 3.87
	31	91.00 ± 1.49	78.67 ± 6.16	78.11 ± 5.63
	32	89.44 ± 1.46	77.56 ± 4.93	74.89 ± 5.12
	33	89.22 ± 3.99	70.89 ± 8.33	67.22 ± 8.13
	34	86.78 ± 4.14	51.00 ± 6.70	37.89 ± 5.13
	35	74.56 ± 5.06	25.67 ± 7.10	19.56 ± 5.28
	36	17.22 ± 11.70	0.33 ± 0.58	0.33 ± 0.58

	25	93.22 ± 2.52	81.44 ± 2.11	80.44 ± 2.41
	31	92.56 ± 2.49	80.33 ± 5.58	80.11 ± 5.73
	32	90.78 ± 2.78	79.22 ± 5.63	78.44 ± 5.72
dor 51dup	33	90.89 ± 1.28	75.22 ± 4.13	74.11 ± 4.40
	34	88.11 ± 7.54	52.56 ± 6.98	49.33 ± 6.89
	35	57.78 ± 9.94	7.67 ± 6.24	4.44 ± 4.50
	36	0.11 ± 0.19	0.00	0.00

Table S5: Pairwise comparisons of egg hatching rates of *Ceratitis capitata* control and CRISPR strains at different temperatures.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII	25 - 31	0.7843	0.585	Inf	1.340	0.1804
	31 - 32	-0.5733	0.548	Inf	-1.047	0.2951
	32 – 33	0.1752	0.592	Inf	0.296	0.7671
	33 – 34	0.1500	0.547	Inf	0.274	0.7840
	34 – 35	0.9927	0.441	Inf	2.249	0.0245
	35 - 36	13.690	0.275	Inf	4.971	<.0001
SEIB	25 - 31	0.5371	0.472	Inf	1.137	0.2555
	31 - 32	-0.1839	0.429	Inf	-0.429	0.6680
	32 – 33	0.3653	0.413	Inf	0.883	0.3770
	33 – 34	0.2361	0.359	Inf	0.657	0.5111
	34 – 35	0.9167	0.292	Inf	3.139	0.0017
	35 - 36	12.687	0.204	Inf	6.205	<.0001
wp tsl	25 - 31	13.265	0.278	Inf	4.764	<.0001
	31 - 32	-0.4952	0.223	Inf	-2.223	0.0262
	32 – 33	40.664	0.256	Inf	15.906	<.0001
	33 – 34	sanp	-	-	sanp	sanp
	34 – 35	sanp	-	-	sanp	sanp
	35 - 36	sanp	-	-	sanp	sanp
dor E839K	25 - 31	-0.1881	0.475	Inf	-0.396	0.6922
	31 - 32	0.4873	0.448	Inf	1.087	0.2768
	32 – 33	-0.2324	0.418	Inf	-0.556	0.5782
	33 – 34	0.4459	0.401	Inf	1.111	0.2666
	34 – 35	0.6450	0.319	Inf	2.024	0.0430
	35 - 36	25.142	0.225	Inf	11.159	<.0001
dor 12del	25 - 31	0.0462	0.303	Inf	0.152	0.8789
	31 - 32	0.1947	0.289	Inf	0.674	0.5001
	32 – 33	0.0254	0.276	Inf	0.092	0.9264
	33 – 34	0.2506	0.262	Inf	0.958	0.3382
	34 – 35	0.8431	0.221	Inf	3.815	0.0001
	35 - 36	26.298	0.200	Inf	13.118	<.0001
dor 51dup	25 - 31	0.1163	0.340	Inf	0.342	0.7326
	31 - 32	0.2628	0.314	Inf	0.836	0.4033
	32 – 33	-0.0148	0.297	Inf	-0.050	0.9603
	33 – 34	0.3252	0.280	Inf	1.160	0.2462
	34 – 35	17.474	0.219	Inf	7.989	<.0001
	35 - 36	48.434	0.562	Inf	8.625	<.0001

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.
sanp = statistical analysis not possible due to values of 0 (zero) in the calculations.

Table S6: Pairwise comparisons of egg hatching rates between *Ceratitis capitata* control and CRISPR strains egg at different temperatures.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
dor E839K vs EgII	25	-0.8556	0.579	Inf	-1.477	0.1398
	31	0.1168	0.482	Inf	0.242	0.8087
	32	-0.9438	0.518	Inf	-1.824	0.0682
	33	-0.5362	0.507	Inf	-1.057	0.2903
	34	-0.8320	0.451	Inf	-1.845	0.0651
	35	-0.4843	0.305	Inf	-1.587	0.1124
	36	-16.295	0.183	Inf	-8.896	<.0001
dor E839K vs SEIB	25	-0.3193	0.492	Inf	-0.649	0.5162
	31	0.4059	0.455	Inf	0.892	0.3722
	32	-0.2653	0.422	Inf	-0.629	0.5291
	33	0.3324	0.410	Inf	0.811	0.4173
	34	0.1226	0.350	Inf	0.351	0.7258
	35	0.3943	0.254	Inf	1.550	0.1211
	36	-0.8511	0.167	Inf	-5.101	<.0001
dor E839K vs *wp tsl*	25	0.6545	0.400	Inf	1.638	0.1014
	31	21.691	0.379	Inf	5.727	<.0001
	32	11.866	0.327	Inf	3.629	0.0003
	33	54.854	0.365	Inf	15.034	<.0001
	34	74.497	0.632	Inf	11.791	<.0001
	35	68.048	0.610	Inf	11.157	<.0001
	36	42.906	0.590	Inf	7.269	<.0001
dor 12del vs EgII	25	-17.344	0.529	Inf	-3.280	0.0010
	31	-0.9962	0.394	Inf	-2.530	0.0114
	32	-17.643	0.478	Inf	-3.693	0.0002
	33	-16.145	0.445	Inf	-3.628	0.0003
	34	-17.151	0.412	Inf	-4.161	<.0001
	35	-15.655	0.272	Inf	-5.762	<.0001
	36	-28.263	0.205	Inf	-13.754	<.0001
dor 12del vs SEIB	25	-11.981	0.431	Inf	-2.779	0.0054
	31	-0.7071	0.359	Inf	-1.968	0.0491
	32	-10.857	0.372	Inf	-2.922	0.0035
	33	-0.7459	0.330	Inf	-2.261	0.0238
	34	-0.7605	0.298	Inf	-2.554	0.0107
	35	-0.6868	0.213	Inf	-3.221	0.0013
	36	-20.479	0.191	Inf	-10.717	<.0001
dor 12del vs *wp tsl*	25	-0.2243	0.322	Inf	-0.697	0.4860
	31	10.561	0.256	Inf	4.118	<.0001
	32	0.3662	0.259	Inf	1.412	0.1579
	33	44.071	0.272	Inf	16.196	<.0001
	34	65.667	0.605	Inf	10.859	<.0001
	35	57.236	0.594	Inf	9.637	<.0001

	36	30.938	0.598	Inf	5.177	<.0001
dor 51dup vs EgII	25	-14.313	0.542	Inf	-2.641	0.0083
	31	-0.7632	0.406	Inf	-1.879	0.0602
	32	-15.993	0.483	Inf	-3.308	0.0009
	33	-14.093	0.453	Inf	-3.114	0.0018
	34	-15.845	0.416	Inf	-3.808	0.0001
	35	-23.391	0.264	Inf	-8.869	<.0001
	36	-58.136	0.567	Inf	-10.250	<.0001
dor 51dup vs SEIB	25	-0.8950	0.447	Inf	-2.002	0.0453
	31	-0.4742	0.373	Inf	-1.272	0.2035
	32	-0.9208	0.379	Inf	-2.430	0.0151
	33	-0.5407	0.340	Inf	-1.589	0.1120
	34	-0.6299	0.303	Inf	-2.077	0.0378
	35	-14.605	0.203	Inf	-7.194	<.0001
	36	-50.352	0.562	Inf	-8.958	<.0001
dor 51dup vs *wp tsl*	25	0.0788	0.343	Inf	0.230	0.8182
	31	12.890	0.275	Inf	4.685	<.0001
	32	0.5311	0.270	Inf	1.968	0.0491
	33	46.123	0.285	Inf	16.211	<.0001
	34	66.973	0.607	Inf	11.025	<.0001
	35	49.499	0.590	Inf	8.385	<.0001
	36	0.1065	0.798	Inf	0.133	0.8938

Table S7: Pairwise comparisons of pupal recovery rates of *Ceratitis capitata* control and CRISPR strains at different temperatures

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII	25 - 31	-0.06162	0.378	Inf	-0.163	0.8705
	31 - 32	-0.45807	0.427	Inf	-1.073	0.2834
	32 – 33	0.62500	0.415	Inf	1.506	0.1321
	33 – 34	0.04157	0.355	Inf	0.117	0.9068
	34 – 35	130.976	0.301	Inf	4.349	<.0001
	35 - 36	185.422	0.255	Inf	7.272	<.0001
SEIB	25 - 31	0.25150	0.395	Inf	0.636	0.5248
	31 - 32	-0.15761	0.388	Inf	-0.406	0.6847
	32 – 33	0.87785	0.349	Inf	2.517	0.0119
	33 – 34	-0.27882	0.305	Inf	-0.915	0.3604
	34 – 35	164.128	0.272	Inf	6.045	<.0001
	35 - 36	sanp	-	-	sanp	sanp
wp tsl	25 - 31	199.296	0.287	Inf	6.945	<.0001
	31 - 32	-0.50159	0.235	Inf	-2.131	0.0331
	32 – 33	156.827	0.599	Inf	2.619	0.0088
	33 – 34	sanp	-	-	sanp	sanp
	34 – 35	sanp	-	-	sanp	sanp
	35 - 36	sanp	-	-	sanp	sanp
dor E839K	25 - 31	0.22102	0.405	Inf	0.546	0.5850
	31 - 32	0.19784	0.372	Inf	0.532	0.5946
	32 – 33	-0.28113	0.380	Inf	-0.740	0.4590
	33 – 34	0.21361	0.385	Inf	0.554	0.5793
	34 – 35	193.867	0.307	Inf	6.324	<.0001
	35 - 36	242.078	0.407	Inf	5.944	<.0001
dor 12del	25 - 31	-0.05689	0.320	Inf	-0.178	0.8587
	31 - 32	-0.02248	0.326	Inf	-0.069	0.9450
	32 – 33	0.52300	0.304	Inf	1.722	0.0850
	33 – 34	0.99789	0.254	Inf	3.924	0.0001
	34 – 35	0.99886	0.244	Inf	4.093	<.0001
	35 - 36	240.694	0.885	Inf	2.721	0.0065
dor 51dup	25 - 31	0.05078	0.326	Inf	0.156	0.8764
	31 - 32	-0.04216	0.328	Inf	-0.128	0.8978
	32 – 33	0.35618	0.313	Inf	1.137	0.2555
	33 – 34	117.814	0.263	Inf	4.479	<.0001
	34 – 35	198.186	0.308	Inf	6.438	<.0001
	35 - 36	sanp	-	-	sanp	sanp

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.
sanp = statistical analysis not possible due to values of 0 (zero) in the calculations.

Table S8: Pairwise comparisons of adult recovery rates of *Ceratitis capitata* control and CRISPR strains at different temperatures.

Strain	Temperature [°C] pairwise comparisons	Estimate	Standard Error	df	z ratio	*p* value
EgII	25 - 31	15.2199	1.752	Inf	0.009	0.9931
	31 - 32	105.754	1.672	Inf	0.632	0.5271
	32 – 33	0.20673	1.142	Inf	0.181	0.8563
	33 – 34	248.619	0.814	Inf	3.053	0.0023
	34 – 35	-155.782	0.624	Inf	-2.496	0.0126
	35 - 36	147.399	0.774	Inf	1.905	0.0568
SEIB	25 - 31	0.2477	Inf	0.345	0.313	0.2477
	31 - 32	0.2477	Inf	0.221	0.292	0.2477
	32 – 33	0.1753	Inf	0.010	0.9922	0.1753
	33 – 34	0.718	Inf	0.773	0.4396	0.718
	34 – 35	0.525	Inf	2.394	0.0167	0.525
	35 - 36	0.644	Inf	-0.267	0.7896	0.644
wp tsl	25 - 31	432.731	1.210	Inf	3.575	0.0004
	31 - 32	138.861	0.321	Inf	4.327	<.0001
	32 – 33	sanp	-	-	sanp	sanp
	33 – 34	sanp	-	-	sanp	sanp
	34 – 35	sanp	-	-	sanp	sanp
	35 - 36	sanp	-	-	sanp	sanp
dor E839K	25 - 31	-0.95424	1.081	Inf	-0.883	0.3772
	31 - 32	0.37534	1.201	Inf	0.312	0.7547
	32 – 33	0.21882	1.035	Inf	0.211	0.8326
	33 – 34	0.41218	0.892	Inf	0.462	0.6442
	34 – 35	0.01600	0.926	Inf	0.017	0.9862
	35 - 36	211.953	1.221	Inf	1.736	0.0825
dor 12del	25 - 31	-228.384	0.969	Inf	-2.357	0.0184
	31 - 32	161.074	1.011	Inf	1.593	0.1111
	32 – 33	0.42645	0.560	Inf	0.761	0.4465
	33 – 34	184.752	0.426	Inf	4.340	<.0001
	34 – 35	-0.10195	0.384	Inf	-0.266	0.7906
	35 - 36	sanp	-	-	sanp	sanp
dor 51dup	25 - 31	-149.992	1.601	Inf	-0.937	0.3490
	31 - 32	127.379	1.643	Inf	0.776	0.4380
	32 – 33	0.41350	1.013	Inf	0.408	0.6832
	33 – 34	147.168	0.760	Inf	1.936	0.0528
	34 – 35	291.085	0.587	Inf	4.956	<.0001
	35 - 36	sanp	-	-	sanp	sanp

* Inf = Infinite degrees of freedom. Z test used for pairwise comparison is not affected by the number of observations.

sanp = statistical analysis not possible due to values of 0 (zero) in the calculations.

4. Discussion

For over 60 years, the sterile insect technique (SIT) has been extensively utilized as an environmentally friendly and species-specific approach in integrated pest management programs (AW-IPM) to control insect pests and disease vectors (Enkerlin, 2003; Dyck *et al*, 2021; Klassen & Vreysen, 2021). As SIT involves the release of both bisexual and/or sexed sterile individuals (Hendrichs *et al*, 1995; Rendón *et al*, 2004; Augustinos *et al*, 2017; Franz *et al*, 2021), the development of genetic sexing strains (GSS) has been crucial. GSS enables the production of exclusively male individuals by eliminating females during the early stages of development. The *Ceratitis capitata* VIENNA-8 and VIENNA-7 GSSs (Augustinos *et al*, 2017; Franz *et al*, 2021), which are currently employed in large-scale AW-IPM programs (Augustinos *et al*, 2017; Franz *et al*, 2021), utilize Y-autosome translocation and selectable markers such as *white pupae (wp)* (Ward *et al*, 2021; Rössler, 1979) and *temperature-sensitive lethal (tsl)* (Franz *et al*, 2021). By subjecting GSS embryos to elevated temperatures, female individuals can be eliminated at the embryonic stage while allowing the survival of males (Augustinos *et al*, 2017). Given thet successful identification of the mutation responsible for the wp phenotype (Ward *et al*, 2021) in *Ceratitis capitata,* once the *tsl* gene will be identified, this will open up the possibility of using both markers in the construction of GSS for targeting other insect pests and disease vectors (FAO/IAEA, 2019, 2021). The development of a genetic sexing system in the Mediterranean fruit fly has demonstrated enhanced cost-effectiveness and efficiency in the field, indicating the potential benefits of similar systems for other species.

4.1 Temperature sensitivity of *Ceratitis capitata* strains

The *temperature-sensitive lethal (tsl)* gene plays a crucial role in sterile insect technique (SIT) application against the Mediterranean fruit fly, *Ceratitis capitata*, as it acts as a selectable marker for sex separation in the genetic sexing strains VIENNA 7 and VIENNA 8 (Augustinos *et al*, 2017; Franz *et al*, 2021). To ensure the overall stability and robustness of the developed GSSs, regular monitoring of the genetic stability of the *tsl* gene is necessary (Augustinos *et al*, 2017). First, we conducted an essential test to evaluate the sensitivity to temperature of several wild-type, genetic sexing, and *tsl* mutant strains in the laboratory (see 3.1). The results showed that the GSSs used in our study exhibited varying sensitivities to temperature. This variability can be attributed to various factors, including different genomic backgrounds of the strains and the different translocations they carry (VIENNA 7 and VIENNA 8) (Fisher & Caceres, 2000; Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017). Due to the genomic background of GSSs, where males possess the *tsl* mutation in heterozygosity and females in homozygosity, lethality

following heat treatment has been found mostly in the latter. Moreover, it is highly recommended to regularly employ "TSLTs" along with the "filter rearing system" to mitigate the risk of accumulating recombinants (Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017). These recombinants, characterized by white puparia and resistance to high temperatures, have the potential to accumulate in mass-rearing conditions. This accumulation poses a significant threat, as it can result in the loss of sexing properties and the collapse of the GSS (Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017). During our experiments, and as also observed in previous studies (Augustinos *et al*, 2017), we ran temperature-sensitive lethal tests (hence TSLT) at temperatures of 25°C, 31°C, 32°C, 33°C, 34°C, and 35°C. This was done for two reasons: first, to evaluate the exact temperature at which the *tsl* mutation induces lethality, and second, to evaluate the stress response and consequently the fitness cost to which the wild-type embryos are subjected following heat shock. Subjecting embryos to a thermal shock of 35°C rather than 34°C for 24 hours could imply a reduction in the survival of the male embryos, making them less suitable for mass rearing. Therefore, using a lower temperature, it is possible to preserve the quality of male embryos by avoiding high-temperature treatments. SIT facilities might consider changing the insect strain by opting for a variant that performs better at 25°C, thus enhancing the overall performance by enriching it with a local genetic background. Furthermore, achieving complete female lethality at a lower temperature would mean that the recovered males have experienced less stress and potentially possess higher biological quality. Several studies have indicated that prolonged mass rearing can lead to laboratory adaptation, resulting in a loss of genetic diversity and significant changes in alleles (Hoffmann *et al*, 2001; Zygouridis *et al*, 2014; Raphael *et al*, 2014; Hoffmann & Ross, 2018). These changes can impact various phenotypes and traits related to the insects' life cycle. Therefore, it is crucial to continuously monitor these alterations to ensure the maintenance of genetic diversity, biological quality, competitiveness, and the production of high-quality sterile insects for SIT applications (Reynolds *et al*, 2012; Gaire *et al*, 2022). To achieve this objective, it has been demonstrated that periodic introductions of wild or partially inbred individuals into mass-reared colonies can greatly benefit mass-rearing facilities (Hoffmann *et al*, 2001; Zygouridis *et al*, 2014; Ward *et al*, 2021). By infusing the mass-reared colony with "fresh" flies, genetic bottlenecks can be minimized, and the genetic diversity and biological quality of the mass-reared insects can be preserved.

Moreover, the recombination phenomena should be taken into consideration as a cause of the variation in the temperature sensitivity among the tested strains. To overcome this problem, the D53 inversion, located between the *wp* and *tsl* loci, was introduced (Fisher & Caceres, 2000;

Augustinos *et al*, 2017; Zacharopoulou *et al*, 2017). Among the inversion strains studied, one behaved like a typical *tsl* mutant strain, while the other two showed a significant number of survivors at high temperatures. All three strains contained the D53 inversion, which suppresses genetic recombination. Recombination outside the boundaries of the D53 inversion could explain the presence of survivors. Revertant mutations in the *tsl* gene and mutations in secondary genetic loci may also affect the tsl phenotype. Increasing the heat-shock temperature up to 36°C could overcome this issue, as has already been performed in other studies (Augustinos *et al*, 2017; Porras *et al*, 2020). Similarly, as observed in GSS and *tsl* mutant strains, for wild-type strains, the values relating to egg hatching, pupal recovery, and adult emergence vary greatly depending on the strain. The wild-type strains used in this study come from different parts of the world, and some have undergone laboratory rearing for decades. When subjected to heat shock at high temperatures (34°C and 35°C), a few of them (Benakeion Volos FF26, Benakeion TR 34 S FF26, Benakeion TR 35 S FF26) show a clear reduction in survival already at the embryonic stage, while others adapt in a better way (EgII FF21 and SEIB). This behavior can be traced back to the presence of a hidden *tsl* allele, not necessarily "the original *tsl*," but also to other gene(s) implicated in a temperature-sensitive phenotype activated at high temperatures. TSLTs, which were used to assess the thermal tolerance of different strains such as wild-type, GSS, and *tsl* mutants, yield valuable data. These results provide insights into the survivability of each strain under varying temperature stress levels. Understanding the variability within strains is crucial in comprehending the range of temperatures at which wild-type strains can effectively survive and reproduce. In the context of the SIT, where sterilized males are released into the wild, the lethality and survival values of males and females belonging to GSSs become significant factors. TSLT data enable the identification of GSSs with higher thermal tolerance, thereby increasing the competitiveness and survival rates of sterilized males upon release. This knowledge is invaluable when selecting strains for mass-rearing facilities worldwide, ensuring the success of SIT applications. It should also be mentioned that in a historical period in which climate change is an imminent threat, the latter's assessment should not be underestimated (Malacrida *et al*, 2007). Climate change has significant implications for the management of *Ceratitis capitata*. The species has expanded into new areas due to climate change (Weldon *et al*, 2018; Lemic *et al*, 2021), but rising temperatures pose challenges for area-wide integrated pest management programs using the sterile insect technique (SIT). Increased temperatures can lead to the mortality of released sterile males, although *Ceratitis capitata* has shown the ability to adapt (Weldon *et al*, 2018; Pörtner *et al*, 2022). The temperature increases in the coming decades may further impact the

species, potentially altering its life cycle, generation numbers, and geographic range. Additionally, climate change brings unpredictable weather events that can affect IPM programs with SIT components, affecting the performance of sterile males during release. Furthermore, the results obtained from TSLT have played a crucial role in selecting the appropriate strains, including wild-type, GSS, and tsl mutants, for subsequent genomics, transcriptomics, cytogenetics, and genome editing experiments. The TSLT results have influenced the decision-making process (based on strains' fitness costs and survival rates at different temperatures) and have guided us in choosing the most suitable strains to further investigate and manipulate through the genome editing technique CRISPR/Cas9.

4.2 *Wp-Zw* region: the putative location for the *tsl* gene

In testing the temperature sensitivity of twenty-seven *Ceratitis capitata* strains, we obtained a general overview of their behavior, which can be different according to the origin, genetic composition, and rearing conditions. Moreover, we were able to establish a solid temperature-response baseline for a wild-type (wt) and a natural *tsl* mutant strain, enabling us to choose three strains, one for each category (wild-type, genetic sexing, and *tsl* mutant strain), to be used in subsequent experiments.

Given the project's main aim, "the search for the *Ceratitis capitata tsl* gene", it was necessary to decide the strains on which sequencing, cytogenetic and transcriptomic experiments were to be carried out. Evaluating the results obtained (see 3.1), the choice fell on the Egypt II FF26 (hence EgII), VIENNA 8 2010 FF26 (hence VIENNA 8) and *wp tsl* FF21 (hence *wp tsl*) strains. Several years of TSLTs performed on these strains (data not shown) have demonstrated their stability regarding the hatching rate at the rearing temperature of 25°C and recombination phenomena (VIENNA 8). The next step was investigating the putative region where the *tsl* gene should be located on the *Ceratitis capitata* chromosome 5 (Kerremans & Franz, 1994; Niyazi *et al*, 2005). Several criteria have been considered for the selection of *tsl* candidate genes: (1) the *in silico* position on the *Ceratitis capitata* genome, (2) gene expression in wild-type and *tsl* mutant strains, (3) the presence of polymorphisms that alter the amino acid sequence in the mutant strain, and (4) the function, location, and presence of heat-sensitive phenotypes of orthologous genes in *Drosophila melanogaster*. The analysis was divided into several steps, each allowing us to obtain a list of candidate genes (see 3.2). Subsequently, the results were compared to identify common genes across all the lists. For example, a gene displaying differential expression but lacking mutations in coding regions was not regarded as a strong candidate. Conversely, some genes, although not differentially expressed, were

considered strong candidates due to mutations leading to an amino acid change and/or heat-sensitive phenotypes in their *Drosophila melanogaster* orthologs. Cytogenetic studies based on deletion and transposition mappings allowed us to identify the *tsl* gene on the right arm of chromosome 5 (Kerremans & Franz, 1994; Niyazi *et al*, 2005). It is closely linked to the *wp* gene, which has been identified and mapped in position 59B of the trichogen polytene chromosome map, which corresponds to position 76B of the salivary gland polytene chromosome map (Kerremans & Franz, 1994; Ward *et al*, 2021). Furthermore, these analyses also allowed the establishment of a right border for the region of interest represented by the *glucose-6-phosphate 1-dehydrogenase* gene (also known as *Zw*), which is located at positions 71C and 78C of the trichogen polytene chromosome and salivary gland polytene chromosome map (Scott *et al*, 1993), respectively. Our results reduced the area of interest to a 6,200,460 bp-long region containing 561 genes (see 3.2). Transcriptomic data were generated using a wild-type (Benakeion) and a *tsl* mutant (*wp tsl*) strain, both subjected to heat shock. This was done because the gene(s) responsible for the tsl phenotype could exhibit differential expression in wild-type strains compared to *tsl* mutant strains and in heat-treated embryos compared to untreated embryos. To achieve this, we used untreated embryos kept at 25°C and heat-treated embryos kept for 60 and 120 minutes at 34°C. The RNA Seq data were compared using the Degust platform, revealing significant differences in gene expression levels in 32 genes. Furthermore, using genomic data, it was possible to evaluate the presence of 324 polymorphisms, some of which are located in genes that are particularly intriguing due to their association with recognized phenotypes in *Drosophila melanogaster*. However, as previously mentioned, the absence of mutations that lead to an amino acid change makes a candidate less likely to be chosen. This concerns the genes *slipper* and *pis*. Although they are expressed differently in wild-type and tsl mutant strains and can cause a temperature-sensitive phenotype in *Drosophila melanogaster* when mutated (Wang & Montell, 2006; Gonda *et al*, 2012), their *Ceratitis capitata* orthologs do not display any mutations in their coding regions. Therefore, they have not been considered. The analysis aimed to identify SNPs or INDELs within the coding regions of these genes because, as observed in previous studies in *Drosophila melanogaster* and other tephritids, point mutations (Aumann *et al*, 2020; Choo *et al*, 2020; Lovato *et al*, 2009), deletions, and insertions (Lim & Snyder, 1968; Kellis Jr *et al*, 1989; Sandberg & Terwilliger, 1989; Varadarajan & Richards, 1992; Lim *et al*, 1992; Matthews, 1995; Mela *et al*, 2009; Srinivas & Cronan, 2017; Shi *et al*, 2019) altering the amino acid sequence of the protein product of the gene can cause a change in the proteins' tertiary structure at restrictive temperatures, leading to the expected (tsl) phenotype. Moreover, it has been

demonstrated that when subjected to gamma-ray and EMS-induced mutagenesis, a certain proportion of induced mutations (ranging from 6.3% to 3.2%) are characterized as temperature-sensitive lethal and semi-lethal mutations (Suzuki *et al*, 1967). Further confirmation of the identified polymorphisms was performed via Sanger sequencing. In fact, through this method, it was possible to confirm the homozygosity/heterozygosity condition in the various strains used. In detail, a putative *tsl* mutation must be homozygous for the wild-type allele in the wild-type strain, homozygous for the mutated allele in the *tsl* mutant strain and GSS females, while it must be heterozygous for the mutated and wild-type allele in males belonging to the GSS strains. Subsequently, using our transcriptomic and genomic data, we extended the analysis to the entire *tsl* putative region. The *wp-Zw* region was screened using transcriptomics data to then confirm the detected mutations using Illumina NGS data. This led to the identification of 322 genes characterized by the presence of polymorphisms in coding regions. This result has greatly extended the list of putative *tsl* genes (see 3.2). Simultaneously, the results obtained were integrated with a comparative analysis with the model organism *Drosophila melanogaster* to evaluate the position of their orthologous genes in the genome of the latter. Cytogenetic studies carried out over the years have shown that ortholog-characterized genes located on the X chromosome of *Drosophila melanogaster* were then identified on chromosome 5 of *Ceratitis capitata* (Brown *et al*, 1989; Konsolaki *et al*, 1990; Tolias *et al*, 1990; Rina & Savakis, 1991; Zacharopoulou *et al*, 1992; Scott *et al*, 1993: Zwiebel *et al*, 1995; Saccone *et al*, 1998; Lindsley & Zimm, 2012), supporting the hypothesis of strong homology between the *Drosophila melanogaster* X chromosome and *Ceratitis capitata* chromosome 5 (Gariou-Papalexiou *et al*, 2002). All *in silico* analyses were performed using the *Ceratitis capitata* 3.2.1 genome (Ccap3.2.1; accession GCA_905071925.1) (Ward *et al*, 2021), and given that the *tsl* region was narrowed down before, we used *in situ* hybridization to confirm the *in silico* data (Papanicolaou *et al*, 2016; Ward *et al*, 2021). Probes were designed for ten genes as widespread as possible within the region of interest for two reasons: first, to confirm their position, and second, to narrow down the region and detect any errors in the genome assembly, as has been observed for *peroxiredoxin 1 (LOC101452328)* which was supposed to be mapped approximately 2 million bp upstream; however, its actual position indicates that misassemblies may occur, and additional probes are needed to confirm the position of genes along this region. In other situations, such as *LOC101458006*, interpreting the results of *in situ* hybridization is difficult due to chromosome condensation (see 3.2). Another analysis carried out was a cross-search using the *Drosophila melanogaster* database on FlyBase.org, searching for the term "heat-sensitive". This allowed the identification of 1144 *Drosophila melanogaster* heat-sensitive

genes, of which 33 orthologs were mapped via *in silico* analysis in the region of interest on the right arm of *Ceratitis capitata* chromosome 5. Moreover, following a comparative analysis using wild-type and *tsl* mutant strain genomic data, 19 out of these 33 genes showed the presence of mutations in coding regions, resulting in an amino acid change. Through literature research, we examined the functions of these genes in the model organism *Drosophila melanogaster*, their expression patterns, and the presence of known mutations that lead to temperature-sensitive phenotypes. These genes participate in diverse cellular activities, including cell formation, growth and regulation of proteins, transportation within the cell, gene expression, cellular structure, and response to external stimuli. It is worth noting that, except for one gene, all of them are located on the X chromosome of *Drosophila melanogaster* (see 3.2). In parallel, the selected candidate genes underwent a final analysis focusing on evaluating their protein structure and sequence. Specifically, (1) the research of conserved domains to see if any of the point mutations is located inside a functional domain, (2) 3D structure prediction of both the wild-type and TSL proteins, comparing them to identify any structural changes resulting from the point mutations, (3) prediction of the effects of the amino acid changes, and (4) assessment of the conservation of the protein sequence among tephritids and other insect species to determine if it is conserved, as well as identifying if any of the point mutations are located in highly conserved regions that could be targeted as the primary candidate for CRISPR/Cas9 genome editing. It should also be noted that the literature contains numerous *Drosophila melanogaster* genes associated with "temperature-sensitive lethal" phenotypes when point mutations occur. Some of these genes, such as *shibire*, *notch*, *pale, transformer-2*, *downstream of Raf1,* and *RNA polymerase II 215 kD* subunit (Nguyen *et al*, 2021), were analyzed to evaluate the presence of mutations in their *tsl* mutant strain orthologs in *Ceratitis capitata* (data not shown). For instance, the *shibire* gene, which has been subject to further studies in other insect species with the aim of introducing *Drosophila melanogaster* temperature-sensitive (ts) mutations (Choo *et al*, 2020), did not exhibit any polymorphisms in coding regions or intragenic and regulatory regions. However, these genes hold the potential to induce a temperature-sensitive lethal phenotype similar to *tsl* (Choo *et al*, 2020; Nguyen *et al*, 2021). However, before choosing a candidate for genome editing, it was necessary to consider the possible mutation(s) responsible for the tsl phenotype in *Ceratitis capitata*. No evidence allowed us to state that the sensitivity to temperature in the mutated strain is due to the presence of a single gene. It could be two or more genes that contribute to the appearance of this phenotype. Furthermore, the polymorphisms involved in this process must also be evaluated. It may be possible that the tsl phenotype is caused by multiple SNPs or INDELs acting together.

This would make identifying the original *tsl* gene and mutation(s) rather difficult, as it would be necessary to test various combinations of genes and mutations to identify the one causing the phenotype. However, to date, there is no evidence in the literature of research based on the identification of ts phenotypes caused by mutations localized on multiple genes linked to each other or combinations of mutations. Moreover, the putative causal *tsl* mutation(s) may be in an intragenic or promoter region. Thus, there could be no alteration in the amino acid sequence, but the mutation could still affect gene expression levels in a way similar to synonymous mutations, as observed in the yeast *Saccharomyces cerevisiae* (Shen *et al*, 2022).

4.3 *Ceratitis capitata deep orange* gene

Considering the aforementioned information and the conducted analyses, I have chosen to target a gene identified through the comparative analysis with the heat-sensitive genes of *Drosophila melanogaster*: the *deep orange* gene. It is also necessary to mention the other parameters that led to this choice. The *deep orange* gene of *Drosophila melanogaster* (*Dmdor*) is located on the X chromosome (Sevrioukov *et al*, 1999). As expected, its ortholog in *Ceratitis capitata* (*Ccdor*) was mapped in our study on chromosome 5, specifically at position 77B of the salivary gland polytene chromosome map (see 3.3). This position perfectly aligns with the *in silico* analyses and our expectations, given that the *tsl* region ranges from 76B to 78C (Kerremans and Franz 1994; Niyazi et al. 2005). As previously mentioned, *Ccdor* was selected following an analysis based on the heat-sensitive genes of *Drosophila melanogaster* (see 3.2). Studies carried out over the last 25 years showed that *Dmdor* is involved in various cellular processes, including vesicle-mediated protein transport to lysosomes, membrane docking and fusion reactions of endosomes/lysosomes, endosomal sorting in neuromuscular junctions, biogenesis of eye pigment granules, and maintenance of normal levels of a RUSH protein involved in endosome formation and trafficking (Sevrioukov *et al*, 1999; Gailite *et al*, 2012; Fernandes *et al*, 2014). Moreover, it plays a significant role in processes such as border follicle cell migration, and given its location in the endosome, lysosome, and synapse, it contributes to syntaxin binding activity (Sevrioukov *et al*, 1999; Gailite *et al*, 2012; Fernandes *et al*, 2014). The gene is part of the CORVET and HOPS complexes; its transcript is detected in all developmental stages, from the embryo to the adult, and it is expressed in various structures, including the embryonic brain, embryonic/larval crystal cell, extended germ band embryo, larval ventral nerve cord, and ovary (Shestopal *et al*, 1997; Sevrioukov *et al*, 1999; Gailite *et al*, 2012; Fernandes *et al*, 2014; Lőrincz *et al*, 2016). Known lethal phenotypes in *Dmdor* occur in the presence of SNPs and insertions of long pieces of DNA (Shestopal *et al*, 1997; Sevrioukov

et al, 1999) and induce lethality during the larval and pre-pupal stages (Belyaeva, E.S *et al*, 1982; Georgel *et al*, 1991; Shestopal *et al*, 1997; Schwartz Y, 1999; Sriram *et al*, 2003; Chi *et al*, 2010). The analysis of the *Ccdor* protein showed the presence of four conserved domains (Pep3/Vps18/deep orange family, Clathrin/VPS, a Helo_like_N, Ring finger/U-box), while the comparative analysis using wild-type and *tsl* mutant genomic data resulted in 36 identified SNPs, six of which resulted in an amino acid change, and three of these are located in the aforementioned conserved domains (see 3.3). The Sanger sequencing in several *Ceratitis capitata* wild-type, genetic sexing, and *tsl* mutant strains was necessary to confirm its alleles in strains with different genomic backgrounds. The condition of homozygosity for the wild-type alleles, the mutated alleles, and the condition of heterozygosity in wild-type, *tsl* mutant strains, and GSS males, respectively, is a fundamental requirement for a mutation to potentially be attributed to a temperature-sensitive lethal phenotype. Furthermore, *Ccdor* does not show any difference in expression between untreated and treated embryos belonging to wild-type and *tsl* mutant strains. CRISPR/Cas9 NHEJ and HDR were used to target *dor*'s fourth and sixth exons, respectively. This resulted in lethality of injected individuals at mid- and late-pupal stages when *Ccdor* was targeted via CRISPR NHEJ. Additionally, three strains (*dor E839K, dor 12del,* and *dor 51dup*) were obtained as a result of CRISPR HDR, with different characteristics when subjected to TSLTs (see 3.3). The three strains obtained through CRISPR HDR, *dor E839K, dor 12del*, and *dor 51dup*, are temperature-sensitive lethal and show differences from the original *tsl* mutant strain. The *tsl* strain showed significant lethality at the embryonic stage following heat shock at 33°C (90.89% ± 1.92), which increased during the pupal (98.00% ± 0.58) and adult (99.67%) stages. Starting from a temperature of 34°C, 100% lethality was observed at the embryonic stage. In contrast, for the CRISPR strains, lethality starting from a temperature of 35°C was observed, which became even more marked at 36°C during the embryonic and larval stages and continued into the pupal stage. Specifically, after heat shock at 36°C, while the EgII and SEIB wild-type strains' embryonic lethality rates were 22.00% ± 3.59 and 37.67% ± 8.81, respectively, the *dor E839K* (58.56% ± 10.75), *dor 12del* (82.78% ± 11.70), and *dor 51dup* (99.89% ± 0.19) strains had significantly higher lethality rates. This behavior continues during the larval stage, where lethality values reached 92.56% ± 4.27 in *dor E839K*, 99.67% ± 0.58 in *dor 12del* and 100.00% in *dor 51dup*. All CRISPR strain egg hatching and pupal recovery rates at 36°C were significantly different from wild-type (EgII and SEIB) values. In comparison to point mutations, which are often linked to temperature sensitivity, deletions and duplications/insertions are less commonly associated with gaining this

trait (Lim & Snyder, 1968; Kellis Jr *et al*, 1989; Sandberg & Terwilliger, 1989; Varadarajan & Richards, 1992; Lim *et al*, 1992; Matthews, 1995; Mela *et al*, 2009; Srinivas & Cronan, 2017; Shi *et al*, 2019). However, the cause of this may be attributed to the hindrance of proper DNA binding activity development or alteration of a functional domain in the *dor 12del* line. In contrast, for the *dor 51dup* line, duplications may result in a deviation from the protein's native structure, leading to denaturing behavior (Xu *et al*, 1990; Lim *et al*, 1992). The results of the TSLTs conducted on the strains obtained following CRISPR HDR allowed us to highlight the promising behavior of the *dor 51dup* strain following heat shock. It is important to note that the manifestation of the typical tsl phenotype observed in *Ceratitis capitata tsl* mutant strains may necessitate the presence of multiple point mutations in different domains of the *Ccdor* gene. Similar phenomena have been observed in *D. melanogaster* for genes involved in body size regulation (Vonesch *et al*, 2016), behavior (Sambandan *et al*, 2006), or tumor suppression (Cerrato *et al*, 2006).

4.4 SIT application via edited genetic sexing strains using *Ccdor*

To establish effective large-scale operational SIT programs, a range of common issues must be resolved, irrespective of the targeted species. The primary challenge lies in developing effective methods, including GSS, that can facilitate the production of substantial quantities of male insects in mass-rearing establishments (Dyck *et al*, 2021; Franz *et al*, 2021; Häcker *et al*, 2021). Despite the evident advantages, there is currently no "one-size-fits-all" method for developing GSSs that can be readily applied to various insect species. Therefore, the primary goal of research endeavours should be to explore the feasibility of developing such an approach. Presently, several strategies are being considered for devising GSSs as alternatives to mechanical separation of the sexes, including developing strains that feature female-specific lethal phenotypes that are conditional and strains in which the sex determination pathway can be manipulated conditionally to achieve sex conversion from female to male (Hendrichs *et al*, 1995; Thomas *et al*, 2001; Leftwich *et al*, 2014; Kandul *et al*, 2019; Li *et al*, 2021; Franz *et al*, 2021). However, it is essential to determine the most widely applicable approaches with minimal research and development requirements across various target species. Moreover, it is critical to assess the degree of transferability of each system between species since gene functions may not be conserved across species.

Deep orange mutation-based GSSs.

Given the highly conserved sequence of the *deep orange* gene in tephritids and in many other insect species, it emerges as a potential candidate for generating new GSSs. However, to effectively employ *dor* as a selectable marker in *Ceratitis capitata*, further investigations are necessary. This includes investigating other SNPs identified in the *tsl* mutant strain, either individually or in combination with the E839 mutation. Additionally, it is crucial to investigate the genetic context and potential contributions of other loci to the tsl phenotype. In the next step, the wild-type rescue alleles can be linked directly to Y chromosomes or M-loci (Meccariello *et al*, 2019; Lutrat *et al*, 2023) using CRISPR to induce homologous recombination or large chromosomal rearrangements. This strategy, which we call 'neo-classical', essentially replicates the 'classical' genetic efforts and does not include the introduction of foreign DNA, which may be a benefit given current legislation. This will allow sexing to take place during the embryonic stage or, at the latest, induce total lethality before the pupal stage is reached. The proven acquisition of temperature sensitivity through induced mutation in *Ccdor* (*dor 51dup*) during the embryonic stage, along with the minimal fitness cost observed during its rearing at 25°C, provides a solid basis for replicating this mutation in other species. Regardless of the phylogenetic distance, the conservation of the *Ccdor* sequence should allow the observation of the same tsl phenotypes in other species of insects.

4.5 Future of the fight against insect pests and regulatory status of CRISPR-edited organisms

Introducing advanced molecular technologies has brought about a revolutionary transformation in the battle against insect pests and disease vectors. These technologies offer many possibilities to overcome the challenges and hurdles faced in current population suppression programs. Significant breakthroughs include developing techniques such as insect sexing for male-only releases, sterilization methods, and marking techniques (Häcker *et al*, 2021; Hendrichs & Robinson, 2021). These biotechnologies also allow the tailoring of strains to meet the specific requirements of control programs while facilitating the transfer of successful strategies to new pest species. However, the successful targeting of these technologies is highly dependent on the availability of genome sequence data and molecular tools for insect transformation in the species of interest. As such, further advancements in molecular technologies hold great promise for improving the efficiency and effectiveness of insect pest and disease-vector control programs (International Glossina Genome, 2014; Papanicolaou *et al*,

2016; Matthews *et al*, 2018; Turner *et al*, 2018; Häcker *et al*, 2021). SIT application through GSSs is one of the main suppression strategies suitable for controlling agricultural pests and disease vectors. Molecular technologies have been used to create sexing and sterility strains and introduce stable markers in insect species such as the Mediterranean fruit fly (Ogaugwu *et al*, 2013), Mexican fruit fly (Schetelig *et al*, 2016) and Australian sheep blowfly (Yan & Scott, 2020). Although these strains have undergone initial evaluations under mass-rearing scenarios or in open-field trials, their adoption has been slow due to low public acceptance of transgenic methods, as well as the regulatory requirements and approvals necessary for their implementation in most countries (Reeves *et al*, 2012). It should be noted that the regulatory framework for transgenic insects varies among different countries and continents and is not expected to be harmonized soon. Therefore, scientists and managers planning to use transgenic insects in the field must address questions and concerns raised by the public and stakeholders in advance (FAO/IPPC, 2004, 2017). The decision-making process for using transgenic insects in the field is based on scientific evidence, evaluations, risk assessment studies, and large-scale experiments in the laboratory, as well as in contained and open-field release trials. Due to the different control scenarios, a clear case-by-case evaluation is needed, and a generalized treatment of different systems and/or insect strains should be avoided. Alternatively, non-transgenic approaches, including classical genetic approaches, RNAi strategies, CRISPR/Cas, and, therefore, the strains produced in this study, could be pursued. However, the European Court of Justice ruled in 2021 that all gene-edited organisms fall under the European Union's GMO regulations (European Commission, 2021), sparking a debate over regulatory reform at the EU level. Many argue that the current regulations are disproportionate and should be amended to allow for exceptions or simplifications, as some of these organisms carry genetic changes that could also result from conventional breeding techniques and do not pose significant health or environmental risks (EFSA GMO Panel *et al*, 2020, 2022). The *tsl* gene is a prime example of this scenario. It was obtained through an EMS experiment (Busch-Petersen, 1990), but it cannot be confirmed with certainty that mutagenesis induced this mutation deliberately. It is plausible that the *tsl* gene arose as a spontaneous mutation during the breeding process, occurring naturally and randomly in the strain. Moreover, the EU's regulation of gene-edited plants is not consistent with that of major trade partners, including the US Department of Agriculture, the Government of India (Government of India *et al*, 2022), the Government of Japan (Nature, 2018) and the United Kingdom, where a new regulation has recently been approved, effectively allowing the use of edited organisms (UK Parliament, 2023). To address this issue, the European Commission published a study on new genomic techniques in April

2021 and plans to propose a legal framework for gene-edited plants and their products in the second quarter of 2023, based on the findings of the study (European Commission, 2021, 2022). In the future, if regulations for edited organisms are implemented, there is a possibility that strains generated through CRISPR mutagenesis might be exempted from regulations applicable to transgenic organisms. In addition, RNAi methods (Fire *et al*, 1998; Sharp, 1999) also offer a way to work without transgenic organisms, but only if the dsRNA is not conditionally or constitutively expressed in the insect and does not persist in the environment. Overall, the deep search performed in the putative region where the *tsl* gene should be localized has helped highlight numerous genes that could be targeted to evaluate their phenotype and the possibility of manifesting temperature-sensitive lethality when mutated. The findings of this thesis contribute to the understanding of the *deep orange* gene (*Ccdor*) and its potential applications in the development of GSSs and the sterile insect technique for controlling *Ceratitis capitata* and other insect pests. The findings provide insights into the feasibility of utilizing *Ccdor* as a selectable marker and highlight the importance of further research and development to optimize its implementation in SIT programs.

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6. Appendix.

6.1 Material and dataset used for this study.

6.1.1 *Ceratitis capitata* strains.

6.1.2 Oligonucleotides, sgRNA, and CRISPR HDR templates.

6.2 Methods used and established during these studies.

6.2.1 *Ceratitis capitata* temperature sensitive lethal test.

6.2.2 CRISPR/Cas9 gene editing.

6.2.3 Non-lethal genotyping of *Ceratitis capitata* using single-leg DNA.

6.2.4 Polymorphism detection using Illumina NGS genomic data.

6.1 Material and dataset used for this study.

6.1.1 *Ceratitis capitata* strains.

Strain	Group	Used for project
EgII FF21	wild-type	3.1
EgII FF26	wild-type	3.1, 3.2, 3.3
Benakeion Volos FF26	wild-type	3.1, 3.2, 3.3
Seibersdorf (SEIB) FF26	wild-type	3.1, 3.3
Argentina FF26	wild-type	3.1
Benakeion TR 34 FF26	wild-type	3.1
Benakeion TR 35 FF26	wild-type	3.1
Benakeion TR 34S FF26	wild-type	3.1
Benakeion TR 35S FF26	wild-type	3.1
VIENNA 8 2010 FF26	GSS	3.1, 3.3
VIENNA 8 2018 FF26	GSS	3.1
VIENNA 8 2019 FF26	GSS	3.1
VIENNA 8 Sr^2 FF26	GSS	3.1
VIENNA 8 “El Pino” FF26	GSS	3.1
VIENNA 8 Israel FF26	GSS	3.1
VIENNA 8 Argentina FF26	GSS	3.1
VIENNA 7 2017 FF26	GSS	3.1, 3.2
VIENNA 7 2018 FF26	GSS	3.1
VIENNA 7 2019 FF26	GSS	3.1
VIENNA 7 2020 FF26	GSS	3.1
wp tsl FF21	mutant	3.1
wp tsl FF26	mutant	3.1
wp tsl (EgII) FF21	mutant	3.1, 3.2, 3.3
wp tsl (EgII) FF26	mutant	3.1
D53-3-28 FF21	mutant	3.1, 3.3
D53-3-28 FF26	mutant	3.1
D53-1 FF26	mutant	3.1
dor E839K	CRISPR mutant	3.3
dor 12del	CRISPR mutant	3.3
dor 51dup	CRISPR mutant	3.3

6.1.2 Oligonucleotides, sgRNA, and CRISPR HDR templates.

Name	Sequence	Purpose
P58_2kb_F	GTTTCCGCATAGAAATCGGCT	Sanger sequencing
P58_2kb_R	CACTGAACTTTCCAGCAGCG	Sanger sequencing
P58_3kb_F	TCCTGAATGAAAAGTTAACCATTTGC	Sanger sequencing
P58_3kb_R	GAAACTGAGTTAAACTATTCACTGT	Sanger sequencing
P58_F1	GGTTCATAAATATCATCATTCC	Sanger sequencing
P58_F2	AATAGTAACAGTCAGCCGCAA	Sanger sequencing
P58_F3	TCAAAATCGGAGAAAAATGGCA	Sanger sequencing
P58_F4	TATAGCATATTCCAAATAA	Sanger sequencing
P58_F5	CCGTCGGGGATAATTGATTTGTG	Sanger sequencing
P58_F6	TGTTCATGGAAATACTCCTCGT	Sanger sequencing
P58_F7	TCTTCGCCGTGCATTTGTTG	Sanger sequencing
P58_F8	TGCTGATAATCCACTGGTTTGTG	Sanger sequencing
P58_F9	CGAACGACGACATTTTACTCGT	Sanger sequencing
P58_NHEJ_geno_F	TGTTCATGGAAATACTCCTCGT	No-lethal genotyping
P58_NHEJ_geno_R	TAACAAAGTCATCTTAGAAA	No-lethal genotyping
P58_HDR_geno_F	CGTTCACTTGATCCCAATCGC	No-lethal genotyping
P58_HDR_geno_R	TGCGGCTGTTGAATTAGCAC	No-lethal genotyping
P58_probe_F	CTAATTCAACAGCCGCAGCC	*In situ* hybridization
P58_probe_R	TGCACCTAACGTCCTCCTTG	*In situ* hybridization
sgRNA_NHEJ	TCAAAATGCACCACGTGCCA	CRISPR NHEJ
sgRNA_HDR	GGATGAATGTGATAAACAAG	CRISPR HDR
ssODN_E839K_sense	AAAGATTACAATTTTCCTGTGCGCTA ATGCGTATGCTTCTCTCACGCACATT TTGTAAATCTTTCATAACACGTTTGG CCTGTTTATCACATTCATCCATTTCGT GTTTTAATTCTTGAATTTTTTGATTGT AATCCTGAATGAAAAGGTG	CRISPR HDR

ssODN_E839K_anti	CACCTTTTCATTCAGGATTACAATCA AAAAATTCAAGAATTAAAACACGAA ATGGATGAATGTGATAAACAGGCCA AACGTGTTATGAAAGATTTACAAAAT GTGCGTGAGAGAAGCATACGCATTA GCGCACAGGAAAATTGTAATCTTT	CRISPR HDR

6.2 Methods used and established during these studies.

6.2.1 *Ceratitis capitata* temperature-sensitive lethal test

This protocol is based on a protocol designed by Augustinos et *al*., 2017.

Material

- Cages containing 5- to 7-day-old *Ceratitis capitata* adults.
- Petri dishes filled with carrot diet.
- Black filter paper (e.g., Whatman® - WHA10300012)
- • Spring steel tweezers (blunt), brushes, and plastic Pasteur pipettes.
- Incubators set at different temperatures (25-31-32-33-34-35-36°C)
- Petri boxes with sand.
- Sieve
- Manual counter

Protocol

- Collect 5-hour-old eggs.
- Place 300 eggs (3 x 100) on black filter paper and place it on a Petri dish filled with carrot diet.
- Repeat the previous step for each temperature to assess for three days in a row.
- Place the Petri dishes for 24 hours at 25°C.
- Place the Petri dishes in the incubator (at the temperature to be evaluated) for 24 hours.
- Remove the Petri dishes from the incubators and place them at 25°C.
- Determinate egg hatching rates after 5 days.
- Determinate pupal recovery rates after 15 days.
- Determinate adult emergence rates after 23 days.

6.2.2 CRISPR/Cas9 gene editing.

This protocol is based on a protocol designed for *Ceratitis capitata* by Aumann *et al*, 2018.

Cas9 protein

All experiments were performed with recombinant Cas9 protein from *Streptococcus pyogenes* obtained from PNA Bio Inc. (catalog number CP01, Lot number PC16912). The protein was shipped in lyophilized form and subsequently reconstituted to a stock concentration of 1 µg/µl in 20 mM HEPES, 150 mM KCl, 2% sucrose, and 1 mM DTT (pH 7.5) by adding nuclease-free ddH_2O to the protein pellet. Cas9 protein lyophilizate and ddH_2O were incubated at room temperature for 10 min and gently mixed by tapping to ensure complete dissolution while avoiding foam formation. The dissolved protein was stored at -80°C in single-use aliquots (3.6 µl). A concentration of 360 ng/µl Cas9 protein was used for microinjections of *Ceratitis capitata* embryos.

gRNA design and usage

gRNAs were designed using the Geneious software package (Kearse *et al*., 2012) and subsequently ordered from Sigma Aldrich, Germany with the following specifications (Physical material: Synthetic RNA, Purification: HPLC, CRISPR species: SpCas9, Structure: sgRNA (crRNA+tracrRNA as one), Scale of synthesis: 3 nmol, modified, dry). gRNAs for gene knockout via NHEJ were positioned either in an early exon or a conserved domain to ensure that an editing event causes a nonfunctional protein. For ssODN knock-in (HDR), gRNAs were designed to bind as close as possible to the target site, as the likelihood of incorporation decreases rapidly with increasing distance to the target site (0 bp cut-to-mutation distance equals ~ 90-100% mutation incorporation, 10 bp cut- to-mutation distance equals ~ 35-50% (Paquet *et al*, 2016; Kwart *et al*, 2017)). The Geneious tool ‘find CRISPR sites’ was used with the following prerequisites for the search: Target: N(20); PAM: NGG; Activity scoring: Doench 2014; Specificity scoring against an off-target database. For specificity scoring and off-target analysis, the most recent genome version and an algorithm proposed by the Zhang lab (Hsu *et al*, 2013) were used to score CRISPR sites. Denoted scores are between 0 and 100, with 100 being the highest specificity and lowest off-target activity. gRNAs without an off-target effect were chosen for experiments. The on-target activity was scored using the method from Doench et al. 2014. Scores are between 0 and 1, with a higher score representing higher expected activity (Doench *et al*, 2014).

ssODN repair template

Single-stranded DNA was used as a repair template. Short templates (151 bp, ssODN) were synthesized by Eurofins Genomics (EXTREMer oligo, purified salt-free, quality control by CGE).

Microinjections and post-injection treatment

Embryos for microinjections were collected for 30-60 min, dechorionated by immersion in a 1:1 solution of Chlorix (DanKlorix Hygiene Reiniger mit Aktiv Chlor) and demineralized H2O (3 min, freshly prepared), and then washed with demineralized H2O. Cas9 protein (360 ng/µl), gRNA (200 ng/µl), KCl (final concentration: 300 mM; including 150 mM KCl in the Cas9 protein stock solution in the calculation), ddH2O, and, for HDR experiments, ssODN repair template (200 ng/µl) were mixed (final volume: 10 µl) and incubated for 10 min at 37°C to enable RNP formation. The injection mix was freshly prepared for each injection day and stored on ice until use. Injections were performed using siliconized quartz needles (Q100-70-7.5; O.D.: 1.0 mm, I.d.: 0.70; 7.5 cm length; Science Products, Hofheim). Needles were drawn out on a Sutter P-2000 laser-based micropipette puller with the following conditions (Heat = heat, Filament = Fil, Velocity = Vel, Delay = Del, Pull = Pull): Quartz (Q100-70-7.5): Heat 750, Fil 5, Vel 70, Del 130, Pull 175.

6.2.3 Non-lethal genotyping of *Ceratitis capitata* using single-leg DNA.

This protocol is based on a protocol designed for *Drosophila* (Carvalho *et al*, 2009) and was adapted and modified to facilitate genotyping of *Ceratitis capitata* based on genomic DNA extraction of a single leg (non-lethal).

Material

- Micro scissors (e.g., Hammacher (Solingen), article nr. HSB 530-08, KG115).
- Spring steel tweezers (blunt).
- Cages for keeping single flies while waiting for the genotyping results.
- Genotyping must be performed on virgin flies (either freshly eclosed or sexed).

Procedure

- Cut off a single middle leg of a CO_2-anesthetized fly using micro scissors while holding the leg with spring steel tweezers.

- Place the leg in the reaction tube inside 12.5 µL Platinum™ Direct PCR Universal Master Mix (Thermo Fisher Scientific), primers (100 nM) according to the mutation to assess, and water up to a final volume of 25 µL. The leg was on the bottom and not attached to the side of the reaction tube. Store it on ice.
- Place sampled flies inside the single cage until PCR results are ready.
- Cross the single fly.

6.2.4 Polymorphism detection using Illumina NGS genomic data.

- *DNA extraction*
  - gDNA from males and females of the *wp tsl* mutant strain was extracted using an ExtractMe DNA tissue kit (Blirt, Poland).
  - gDNAs from VIENNA 7 GSS strain males and females were extracted using phenol/chloroform (Saccheri & Bruford, 1993).

- *Library preparation and sequencing*
  - Libraries for the *wp tsl* strain were prepared using an Illumina TruSeq Nano DNA Kit (Illumina, USA) and sequenced on an Illumina HiSeq X platform, resulting in 150-bp paired-end reads by Macrogen, Korea.
  - Libraries for the VIENNA 7 GSS were prepared using an Illumina TruSeq PCR-free kit (Illumina, USA) and sequenced on an Illumina HiSeq 400 platform, resulting in 150-bp paired-end reads (Centre for Genomic Research, Liverpool).

- Import Raw fastaq NGS reads in Geneious Prime 2022.1.1.
- Trim the reads using BBDuk Adapter/Quality Trimming Version 38.37 with default settings (Adapters: AllTruseq, Nextera, and PhiX adapters; Trim: Right End, Kmer Length: 27; Maximum Substitutions: 1)
- Map the trimmed reads to the reference sequence using the *"Bowtie2" (v. 2.4.5 - © Ben Langmead)* Geneious Prime 2022.1.1 plug-in with default parameters (*alignment type: end to end; high sensitivity*)
- The tool "*Find Variations/SNPs*" was used for polymorphism calling, customizing it according to the variant P value, minimum strand-bias P value, and location of variants.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

Erklärung gemäß der Promotionsordnung des Fachbereichs 09 vom 07. Juli 2004 § 17 (2)

“Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

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London, den 17.09.2023

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