Institut für Insektenbiotechnologie
Professur für Insektenbiotechnologie im Pflanzenschutz
Justus-Liebig-Universität Gießen

Development of molecular approaches for Sterile Insect Technique involving RNAi in *Aedes aegypti* and functional validation of a *wp* rescue construct in *Ceratitis capitata*

INAUGURAL-DISSERTATION

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

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

vorgelegt von

Lucas Henrique Figueiredo Prates

aus Uberaba

Gießen, 2025

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1. Gutachter: **Prof. Dr. Marc F. Schetelig**
   Institut für Insektenbiotechnologie
   Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
   Justus-Liebig-Universität Gießen

2. Gutachter: **Prof. Dr. Nikola-Michael Prpic-Schäper**
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   Fachbereich Biologie und Chemie
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Fachbereich Veterinärmedizin
Justus-Liebig-Universität Gießen

Prüfer: **Prof. Dr. Miklós Bálint**
Institut für Insektenbiotechnologie
Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
Justus-Liebig-Universität Gießen

Prüfer: **Prof. Dr. Mathias Faßhauer**
Institut für Ernährungswissenschaft
Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
Justus-Liebig-Universität Gießen

Vorsitzender: **Prof. Dr. Gunter P. Eckert**
Institut für Ernährungswissenschaft
Fachbereich Agrarwissenschaften, Ökotrophologie und Umweltmanagement
Justus-Liebig-Universität Gießen

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1 Summary

Aedes mosquitoes, namely *Aedes albopictus* and *Ae. aegypti*, are competent vectors for several arboviruses with substantial public health importance, including dengue, chikungunya, Zika, and West Nile viruses, with no effective vaccines or drugs against most of the transmitted arboviruses. The control of mosquitoes is challenging and an effective system that could be safely and efficiently used against their increasing spread is not available. In addition, resistance development against pesticides traditionally used to control *Aedes* mosquitoes is observed worldwide. Modern molecular techniques have enabled the development of new environmentally-friendly systems for insect control. One such approach is the use of RNA interference (RNAi) triggered by double-stranded RNA (dsRNA). In this work, I focused on applying modern molecular techniques to develop efficient sustainable insect control systems.

In the **first study**, I optimized a scale-up ready protocol for extraction of bacterially produced dsRNA at low cost and good yields. The bacterial cells engineered to synthesize dsRNA were efficiently lysed in the early steps of the purification, ensuring no viable bacterial cells remaining in the purified dsRNA, which could then be used for several applications in insect control and plant protection. In the **second study**, I explored and discussed the challenges of establishing RNAi-mediated gene silencing in *Aedes* mosquitoes. Here, the versatility of the bacterial cells engineered to produce dsRNA and the extraction protocol established in the first study were fundamental for the readily production of different sequences of dsRNA on demand. The challenges identified in this study highlighted the need for additional research to ensure robust RNAi-based methodologies for *Aedes* control. In the **third study**, I applied modern molecular techniques on a generic approach to advance the concept of neo-classical genetic sexing strains (GSS) to support insect control applications based on the sterile insect technique (SIT). Specifically, I engineered and investigated the function of a minimal version of the *white pupae* gene in the non-model organism *Ceratitis capitata*. The pipeline for designing and testing the intronless version of the genetic marker described in this study could be extended to other species and genetic markers.

In general, while I developed a protocol for the cost-effective production of dsRNA, I also identified barriers to the application of RNAi in *Aedes* control. Additionally, the results of my third research project contribute to the further development of a generic approach for neo-classical GSS. This approach could improve the efficiency of existing GSS or be expanded to species for which GSS are not yet available.

1 Zusammenfassung

Aedes-Mücken, insbesondere *Aedes albopictus* und *Ae. aegypti*, sind kompetente Vektoren für mehrere Arboviren mit erheblicher Bedeutung für die öffentliche Gesundheit, darunter Dengue, Chikungunya-, Zika- und West-Nil-Viren. Gegen die meisten dieser übertragenen Arboviren gibt es keine wirksamen Impfstoffe oder Medikamente. Die Bekämpfung von Mücken stellt eine Herausforderung dar, da derzeit kein effektives System verfügbar ist, das sicher und effizient gegen ihre zunehmende Verbreitung eingesetzt werden könnte. Daneben wird weltweit die Entwicklung von Resistenzen gegen traditionell eingesetzte Pestizide zur Kontrolle von *Aedes*-Mücken beobachtet. Modernen molekularen Techniken haben die Entwicklung neuer umweltfreundlicher Systeme zur Insektenbekämpfung ermöglicht. Ein solcher Ansatz besteht im Einsatz von RNA-Interferenz (RNAi), die durch doppelsträngige RNA (dsRNA) ausgelöst wird. In dieser Arbeit habe ich mich darauf konzentriert, moderne molekulare Techniken anzuwenden, um effiziente und nachhaltige Systeme zur Insektenkontrolle zu entwickeln.

In der **ersten Studie** habe ich ein skalierbares Protokoll zur Isolierung von bakteriell produzierter dsRNA optimiert, das kostengünstig und ertragreich ist. Die bakteriellen Zellen, die zur Synthese von dsRNA veränderte wurden, wurden in den frühen Schritten der Isolierung effizient lysiert, sodass keine lebensfähigen bakteriellen Zellen in der isolierten dsRNA verbleiben konnten. Diese lässt sich anschließend für verschiedene Anwendungen in der Insektenbekämpfung und im Pflanzenschutz verwenden. In der **zweiten Studie** habe ich die Herausforderungen bei der Etablierung einer RNAi-vermittelten Genstilllegung in *Aedes*-Mücken untersucht und diskutiert. Dabei erwiesen sich sowohl die Vielseitigkeit der zur Produktion von dsRNA konstruierten Bakterienzellen, als auch das in der ersten Studie entwickelte Isolierungsprotokoll, als grundlegend für die schnelle und bedarfsorientierte Herstellung verschiedener dsRNA-Sequenzen. Die in dieser Studie identifizierten Herausforderungen verdeutlichen die Notwendigkeit weiterer Forschung, um robuste RNAi-basierte Methoden für die Kontrolle von *Aedes*-Mücken zu gewährleisten. In der **dritten Studie** habe ich moderne molekulare Techniken auf einen generischen Ansatz angewandt, um das Konzept der neoklassischen Genetischen Sexing Stämmen (GSS) weiterzuentwickeln und damit Anwendungen zur Insektenkontrolle auf Basis der Sterilen-Insekten-Technik (SIT) zu unterstützen. Konkret habe ich eine minimale Version des *white pupae*-Gens im Nicht-Modellorganismus *Ceratitis capitata* konstruiert und dessen Funktion untersucht. Der in dieser Studie beschriebene Ansatz zur Entwicklung und Testung einer intronlosen Version des genetischen Markers könnte auf andere Spezies und genetische Marker übertragen werden.

Zusammenfassend habe ich ein Protokoll für die kosteneffiziente Isolierung von dsRNA entwickelt und gleichzeitig Barrieren für die Anwendung von RNAi bei der Kontrolle von *Aedes*-Mücken identifiziert. Des Weiteren tragen die Ergebnisse meines dritten Forschungsprojekts zur Weiterentwicklung eines generischen Ansatzes für neoklassische GSS bei. Dieser Ansatz könnte die Effizienz bestehende GSS verbessern oder auf Spezies ausgeweitet werden, für die bisher keine GSS verfügbar sind.

2 Introduction

2.1 *Aedes* mosquitoes

Mosquitoes belong to the family *Culicidae*, within the order Diptera and the suborder Nematocera, and comprise over 3,500 species. While most of mosquito species would not attract much attention, some few species are intensively studied and monitored due to their worldwide spread beyond their original habitats and their role as vector of diseases, posing a serious threat to human and animal health (ECDC, 2024c; Hawkes & Hopkins, 2021; WHO, 2024a; b). Their biology and morphological traits have been reported in detail elsewhere (Becker et al, 2020; Das et al, 2024; Hawkes & Hopkins, 2021; Wilkerson et al, 2021).

Aedes mosquitoes, namely *Aedes (Stegomyia) albopictus* and *Aedes aegypti* (Diptera: Culicidae), are competent vector for at least 22 arboviruses, with substantial public health importance including dengue, chikungunya, and West Nile viruses or dirofilarial worms (Gratz, 2004; Mitchell, 1991; WHO, 2024a). Chikungunya and dengue infections through local transmission have been reported in Europe since 2007 and 2010, respectively. Concomitantly, it was shown that local *Ae. albopictus* populations from Italy, France, and Spain possess vector competence for chikungunya, yellow fever and Zika virus, respectively (Amraoui et al, 2016; Gutiérrez-López et al, 2019; Lindh et al, 2018; Rezza, 2018; Rezza et al, 2007), including the vertical viral transmission in mosquitoes. Data collected until September of 2024 showed local transmission of dengue virus in France, Italy and Spain (ECDC, 2024b; Fournet et al, 2023). In Brazil, one of the countries with higher rates of notifications, approximately 6.5 million cases and more than 5,500 death have been notified in 2024 until October (Saúde, 2024).

In their life cycle, *Aedes* mosquitoes go through four different morphological stages: from eggs, to aquatic larvae and pupae, and finally, to flying adults. The blood-feeding females lay eggs in water bodies or in wet areas in close proximity. The hatched larvae are aquatic and survive from filtering particles dispersed in water. After growing through four larval stages, the last aquatic stage is a pupa. At this stage, the most morphological changes are apparent with fully formed adults eclosing from the pupal cuticle (Figure 1). The development time is influenced by environmental conditions, as temperature and food availability, and can take as little a one-week from egg to adult. Despite a decrease in hatching rates, eggs of some Aedes species are reported to be desiccation resistant and survive for long periods, being viable when conditions are again favorable. This ability may specially contribute to the successful overwintering and further spread of *Aedes* mosquitoes in Europe (De Majo et al, 2016; Hawkes & Hopkins, 2021; Nicholson et al, 2014; Obholz et al, 2022).

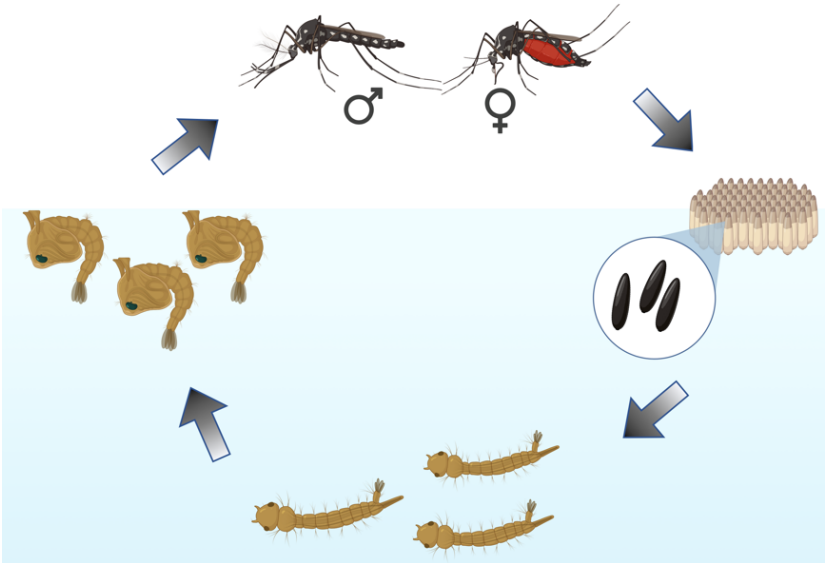


Figure 1. Life cycle of Aedes mosquitoes. Adapted from (Hawkes & Hopkins, 2021). Created with BioRender.com.

Also known as the Asian tiger mosquito, *Ae. albopictus* is native to tropical forests of South-East Asia but has been identified as an invasive pest worldwide. Similarly, the yellow fever mosquito, *Ae. aegypti*, originally found in tropical Africa, has found its way around the globe (Powell et al, 2018). The first record of *Ae. albopictus* in Europe occurred in Albania in 1979 (Adhami & Reiter, 1998) and it was found during the following years in Africa, North and South America (Aranda et al, 2006; Cornel & Hunt, 1991; Fontenille & Toto, 2001; Hahn et al, 2017; Ibáñez-Bernal et al, 1997; Moore & Mitchell, 1997; Schaffner et al, 2004). Currently, *Ae. albopictus* is considered established in several European countries including Austria, Belgium, Bulgaria, Croatia, France, Germany, Greece, Hungary, Italy, Malta, Portugal, Romania, Slovenia, and Spain. Furthermore, it is considered to have been introduced in Cyprus, Czechia, Liechtenstein, the Netherlands, Slovakia and Sweden (ECDC, 2024a). On the other hand, *Ae. aegy*pti has been historically reported in Europe in the Mediterranean countries and Portugal (ECDC, 2023). Invasion routes have been linked to increased international trade and public or private transport (Eritja et al, 2005; Reiter, 1998).

In Germany, a national program for monitoring the occurrence of mosquitoes is in place since 2011, involving also contributions of the population in a citizen science project to track the spread of mosquitoes (Pernat et al, 2021; Walther & Kampen, 2017; Werner et al, 2020). The first female of *Ae. albopictus* was detected in the Rhine Valley during the summer of 2011 (Werner et al, 2012). In 2015, the seasonal occurrence of *Ae. albopictus* had spread to North Rhine-Westphalia, Thuringia, Baden-Württemberg, and Bavaria (Walther et al, 2017), with the first mass development of *Ae. albopictus* in Germany reported in Freiburg (Becker et al, 2017). Only one year later, the first evidence of the successful overwintering of the Asian tiger

mosquito in Germany was observed in the region of Heidelberg (Pluskota et al, 2016). Last data by the end of 2023 indicates established populations in Baden-Württemberg, Rhineland-Palatinate, North Rhine-Westphalia, Hesse, Bavaria, in Jena (Thuringia), and Berlin (FLI, 2024). It is likely that *Ae. albopictus* will spread into all German states in the future, with the associated risk of spreading mosquito-borne diseases like dengue, chikungunya, and West Nile viruses or dirofilarial worms (Gratz, 2004; Paupy et al, 2009).

Following the first report of *Ae. albopictus* in Brazil in 1986, studies on the spatial distribution of Brazilian populations of *Ae*. *aegypti* and *albopictus* have indicated a preference of *Ae. albopictus* for areas with high to medium dense vegetation coverage, while *Ae. aegypti* is largely distributed in urban areas (Carvalho et al, 2014; Heinisch et al, 2019; Honório et al, 2009). Heinisch and colleagues highlighted the possibility of green areas in urban centers work as habitats for *Ae. albopictus.* In addition, contaminated *Ae*. *albopictus* could act as a bridge for the transmission of pathogens typically circulating in areas of dense vegetation, like the yellow fever virus (Carvalho et al, 2014; Couto-Lima et al, 2017; Gratz, 2004). In 2018, the Instituto Evandro Chagas (Brazil) reported the occurrence of *Ae. albopictus* infected with the yellow fever virus in Minas Gerais, Brazil (Damasceno-Caldeira et al, 2023).

The introduction and establishment of *Ae. albopictus* in several European countries, including Germany, associated with its potential to disseminate mosquito-borne diseases, highlight the importance of surveillance and control of this highly invasive mosquito. Moreover, there are no effective vaccines of broad application or drugs against most of the arboviruses transmitted by *Aedes* mosquitoes (Paupy et al, 2009).

2.2 The challenge of controlling mosquitoes

Despite the awareness of the threat by *Aedes* mosquitoes, their control is challenging, and an effective system that could be safely and efficiently used against the increasing spread of the mosquito is not available. Behavioral measures such as the use of repellents and insect screens in windows and doors can help to reduce the risk of infectious mosquito bites. However, such measures are not effective to control or reduce mosquito populations. Conventionally, mosquito control is based on the use of insecticides and on the reduction of breeding sites for egg laying and larval development (Paupy et al, 2009).

Permethrin, diflubenzuron, and lambda-cyhalothrin are classes of insecticides commonly used for mosquito control (Muzari et al, 2017; Wymann et al, 2008). As result of a global survey with 92 responding countries, the World Health Organization (WHO) estimated the use of nearly 6,000 metric tonnes of insecticides for disease vector control in the period 2010 – 2019 (WHO, 2021). On the other hand, there is still a notable expansion on the establishment of disease vectors, e.g., Aedes mosquitoes, into new geographic areas and an upward trend on cases of vector-borne diseases (ECDC, 2024a; b; WHO, 2024a; b).

Resistance development against these chemicals is being observed in field-collected *Aedes* mosquitoes worldwide, including in La Réunion Island (Tantely et al, 2010), Pakistan (Khan et al, 2011), Malaysia (Chen et al, 2013), USA (Estep et al, 2018), China (Li et al, 2021), and in Brazil (Rahman et al, 2021). In 2011, the Instituto Oswaldo Cruz and Fundação Oswaldo Cruz, both linked to the Brazilian Ministry of Health, recommended the interruption of using pyrethroids to control *Ae. aegypti* in Brazil given the kdr (knockdown resistance) mutation detected in mosquitoes across the country (FIOCRUZ, 2011). The genetic mutation known to confer resistance to permethrin was also reported in *Ae. albopictus* across Italy and Greece (Pichler et al, 2019).

Science projects involving the population for monitoring the presence of mosquitoes, like the Mückenatlas in Germany (Walther & Kampen, 2017) and ZanzaMapp in Italy (Caputo et al, 2020), are also relevant for mosquito control. Media presence and citizen science projects for mosquito surveillance can increase the community awareness and influence their behavior to positively take action to manage mosquitoes' population, e.g., by eliminating inhouse breeding sites for mosquitoes (Braz Sousa et al, 2024; Gubler & Clark, 1996; Pernat et al, 2022).

Considering the increase in mosquito-borne disease cases, together with the adaptability shown by *Aedes* mosquitoes and their potential for developing insecticide resistance, new vector control strategies are needed to enhance the 'toolbox' used in mosquito control programmes (Achee et al, 2019; Dusfour et al, 2019). Recently, the World Health Organization

launched the “Global Arbovirus Initiative: preparing for the next pandemic tackling mosquito-borne viruses with epidemic and pandemic potential” with focus on viruses transmitted by *Aedes* mosquitoes. In six pillars, strengthening vector control is highlighted to interrupt the mosquito-human-mosquito transmission cycle (WHO, 2024a). The vector control of *Ae. albopictus* and *Ae. aegypti* is also an ongoing concern present in the public health agenda of the European Union (EU, 2016).

2.3 Modern molecular techniques applied to insect control

In recent years, modern molecular techniques have been employed to boost and optimize insect control. This includes not only the discover of new active substances, but also completely new technologies applied to insect control. Among these innovations, RNA interference (RNAi) has gained attention for its potential applications in insect control (Christiaens et al, 2020; Fletcher et al, 2020; Koo & Palli, 2024; Nitnavare et al, 2021; Ortolá & Daròs, 2024). In parallel, advancements in gene editing also offer potential for developing more efficient sex-sorting systems for insects in mass-rearing facilities, supporting the cost-effective implementation of the sterile insect technique (SIT) (Häcker et al, 2021; Nguyen et al, 2021; Yan et al, 2023).

2.3.1 RNA interference for mosquito control

RNA interference (RNAi) is an antiviral defense mechanism first discovered in the nematode *Caenorhabditis elegans* that has been harnessed as a molecular technique for gene silencing triggered by double-stranded RNA (dsRNA). The post-transcriptional gene silencing can be triggered by naturally occurring or user-directed exposure to dsRNA, leading to degradation of specific (complementary) gene transcripts. RNAi can be designed to act specifically on target genes essential for life and development of the pest. After the dsRNA entered the cell, it is first processed into small interfering RNAs (siRNAs), approximately 21 to 23 nucleotides long, and one strand of siRNA is loaded into the RNA-induced silencing complex (RISC). The RISC localizes complementary messenger RNA (mRNA), promotes its degradation, and, thereof, the post-transcriptional gene silencing. While dsRNAs typically consist of longer sequences with hundreds of nucleotides, shorter RNA sequences can also be used to trigger RNAi. In this case, two complementary strands of approximately 19 to 22 nucleotides are linked by a loop, ranging from 4 to 11 nucleotides, to produce a short hairpin RNA (shRNA), which will also be processed into siRNAs upon entry in the cell (Fire et al, 1998).

Regardless of the architecture of the RNA molecule triggering RNAi, this mechanism can be used, in the context of pest control application, to specifically silence expression of genes essential to life of an insect, causing mortality of the target insect (Burand & Hunter, 2013; Joga et al, 2016; Murphy et al, 2016). The species-specific nature of RNAi for pest control highlights the environmentally-friendly aspect of this technology (Joga et al, 2016).

The potential of RNAi for insect pest control is, among other factors, depending on the insect species targeted. Multiple studies in different species over the past ten years indicate that a major challenge of establishing RNAi for insect control is the dsRNA stability and its uptake by the insect (Baum & Roberts, 2014; Bautista et al, 2009; Joga et al, 2016). dsRNA is

commonly applied to insects by injection, soaking, or feeding, with only the latter method being applicable to pest control. Successful oral application of dsRNA requires that the dsRNA is stable on its way to the target. The cellular dsRNA uptake mechanisms in insects have not been fully elucidated, but they seem to differ across insect orders and are probably involved in weak RNAi effects. While in some insect homologues to essential transmembrane transporters known from the RNAi model organism *C. elegans* seem to be involved, a large-scale study showed that the dsRNA uptake in *D. melanogaster* occurs via endocytosis (Saleh et al, 2006). This latter uptake mechanism faces the critical step of endosomal discharge of the dsRNA to reach the intracellular RNAi machinery (Varkouhi et al, 2011). The delivery and uptake of stable dsRNA into cells is, therefore, a threshold for the efficacy of RNAi (Joga et al, 2016).

The occurrence of RNases in the saliva and hemolymph as well as an alkaline pH in the gut may lead to dsRNA degradation, reducing the uptake by the insect, thus setting a barrier for RNAi efficiency (Allen & Walker, 2012; Christiaens et al, 2014). Recently, silencing genes expressing endonucleases together with targeting other genes has been investigated to potentialize initially low RNAi-based silencing effects observed in *Aedes aegypti* (Giesbrecht et al, 2020), *Ostrinia furnacalis* (Fan et al, 2021), *Ceratitis capitata (Volpe et al, 2024)*, *Zeugodacus cucurbitae* (Ahmad et al, 2024), *Nilaparvata lugens* (Gao et al, 2024), and *Papilio xuthus* (Shu et al, 2024). Similarly, the presence of viruses in the hemolymph of some species could constrain the RNAi efficiency (Swevers et al, 2013).

The effect of RNases in the gut and hemolymph of *Aedes* mosquitoes is not completely elucidated. While dsRNA could be partially extracted from mosquitoes after injection (Airs et al, 2023) and oral delivery (Coy et al, 2012; Romoli et al, 2024), it was not stable when exposed to the gut juice (Figueiredo Prates et al, 2024). Furthermore, the fate of the dsRNA after injection or oral delivery is not completely clear. While dsRNA could be traced to homocytes and ovaries of injected females, it could not be detected upon oral delivery (Airs et al, 2023). In another study, dsRNA could be detected in some gut epithelial cells upon oral delivery, but was not followed further (Romoli et al, 2024).

Nonetheless, several studies reported strong RNAi-induced effects on *Aedes* mosquitoes (Hapairai et al, 2017; Hapairai et al, 2021; Hapairai et al, 2020; Mysore et al, 2014a; Mysore et al, 2014b; Mysore et al, 2022; Mysore et al, 2017; Mysore et al, 2019a; Mysore et al, 2019b; Mysore et al, 2019c; Mysore et al, 2023; Mysore et al, 2015; Singh et al, 2013; Whyard et al, 2015). Small siRNAs and shRNAs, as well as long dsRNAs, targeting important genes have been reported to trigger RNAi-based responses. The application strategies included soaking of larvae in solutions containing the RNAi-trigger, feeding of adult mosquitoes with a mixture of

sugar and RNAi-trigger, feeding of larvae with microorganisms expressing the RNAi-trigger, and injection of RNAi-trigger into different life stages of *Aedes* mosquitoes (Munawar et al, 2020). Encapsulating of RNAi-trigger into nanoparticles has also been tested to increase RNAi-efficiency in mosquitoes (Das et al, 2015; Dhandapani et al, 2019; Kumar et al, 2016; Zhang et al, 2015; Zhang et al, 2010).

2.3.2 Double-stranded RNA production

Different approaches can be used to obtain dsRNA and these methods have been recently reviewed (Guan et al, 2021; Hough et al, 2022). DsRNA can be produced via *in vitro* transcription with different commercially available kits, mostly based on the bacteriophage T7 RNA polymerase, providing high quality transcribed dsRNA. However, such kits are costly, hampering future research and application of RNAi-based strategies. With the advances in gene editing, engineering bacterial and yeast cells to produce dsRNA became an alternative to produce dsRNA (Hough et al, 2022).

Since its manipulation to silence expression of RNase III and further insertion of an inducible T7-based system for production of dsRNA (Takiff et al, 1989; Timmons et al, 2001), *E. coli* bacterial cells HT115 (DE3) have been broadly used for dsRNA production (Lau et al, 2014; Taracena et al, 2019; Tenllado et al, 2003; Timmons et al, 2001). While the price for dsRNA synthesis has dropped strongly nowadays, with predicted production costs advertised as low as 1 USD/g using a proprietary cell-free methodology (www.greenlightbiosciences.com, accessed on 27 October 2024), the versality of engineered HT115 (DE3) *E. coli* cells for dsRNA production allows scientists to readily adjust dsRNA sequences according to experimental requirements or applications. Having a reliable and cost-effective protocol for extraction of dsRNA from large HT115 (DE3) culture volumes would further broaden the usability of this bacterially produced dsRNA in larger laboratory applications, such as high-throughput screening of potential target genes for RNAi in different species.

Although the application of bacterial crude extracts to trigger RNAi has been investigated (Lau et al, 2015; Lau et al, 2014; Tenllado et al, 2003; Timmons et al, 2001), a further purification process is required to obtain purer dsRNA, free of other bacterial nucleic acids and proteins. Besides avoiding potential immune responses due to the presence of bacterial cells (Li et al, 2019), the further purification of bacterially produced dsRNA is also relevant for legislation issues regulating the application of dsRNA-based products (De Schutter et al, 2022).

Several methods have been described for dsRNA extraction from bacterial cells (Ahn et al, 2019; Ongvarrasopone et al, 2007; Papić et al, 2018; Posiri et al, 2013; Solis et al, 2009; Verdonckt & Vanden Broeck, 2022). Despite the high yields and good quality of dsRNA extracted using phenol/guanidine-based protocol (Ongvarrasopone et al, 2007), the use of commercially available solutions of phenol/guanidine, like the costly TRIzol™ (Invitrogen) and QIAzol® (QIAGEN), drives up the costs for medium to large scale experiments. Undoubtedly, these costs pose an economic issue, especially for large laboratory set ups.

2.3.3 Neo-classical genetic sexing strains

Genetic sexing strains (GSS) are a proven valuable tool for sex sorting in insect mass-rearing facilities, allowing sex separation of insects for use in sterile insect technique (SIT)-based applications. The SIT is an environment-friendly technique used for species-specific and area-wide management of insects. The initial idea of the SIT is dated from the 1930s and 40s, and it has ever since benefited from scientific advances in different aspects of its concept and practical knowledge from field applications for several insect species (Bourtzis & Vreysen, 2021; Gilles et al, 2014; Klassen et al, 2021).

The SIT is based on mass rearing of the target insect, followed by its sterilization and release into a defined area. Sterile males mate with wild females, leading to no offspring, thus gradually reducing the insect population in the treated area. Successful SIT application can lead to a considerable suppression or even local eradication of an existing insect pest (Gilles et al, 2014; Klassen et al, 2021). In the case of disease vectors, like Aedes mosquitoes, it could effectively control outbreaks of diseases transmitted by the blood-feeding females.

A key element for SIT programmes is, among other factors, the so-called sexing, i.e., an effective sex separation in the mass rearing facilities to enable male-only releases. Although bisexual release has been conducted, e.g., to control lepidopteran pests (Marec & Vreysen, 2019), the SIT efficiency can be improved by male-only releases, as it avoids mating between the sterile males with the co-released sterile females instead of the wild females in the field. In addition, females of agricultural pests could still damage fruits by laying infertile eggs. Ruptured fruit tissues could facilitate the spread of phytopathogenic pests, leading to fungi and bacterial infections (Machota et al, 2016), besides the potential impact on fruit appearance and consumer perception. Furthermore, ensuring the biological safety of SIT programmes is crucial, especially when targeting disease-vectors of public health concern, like some mosquito species, as even sterile female mosquitoes remain capable of biting and transmitting diseases.

Nonetheless, sorting females out of the rearing process as early as possible reduces the rearing costs, therefore increasing the cost-effectiveness of SIT (Gilles et al, 2014; Parker et al, 2021).

Sex sorting of insects in mass rearing facilities can be achieved by using GSS. Classically, a GSS requires two components, i) a selectable mutated marker that allows sex separation or elimination of females at the earliest possible stage, and ii) linkage of the wild-type (WT) allele of this marker to the male sex. A well-known and successfully used example of a GSS for SIT is the VIENNA-8 strain, developed in *Ceratitis capitata*, the Mediterranean fruit fly (medfly) (Augustinos et al, 2017; Franz et al, 2021; Franz et al, 1994). This GSS carries two selectable markers, the *white pupae* (wp^-) and the *temperature-sensitive lethal* (tsl^-) mutations. These mutations were initially not sex-linked, but the wild-type alleles for both mutations were translocated onto the Y chromosome using irradiation. Thus, a separation system is possible where female flies, homozygous for both mutations, emerge from white pupae and are sensitive to high temperatures. In contrast, heterozygous male flies emerge from brown pupae and are not affected by the same high temperatures as females. Typically, female embryos do not survive a treatment at 34°C for 24 h, while males are unaffected (Franz et al, 2021).

The development of GSS strains has been highly time-consuming and luck-dependent. For instance, the wp^- phenotype, based on natural mutations, was found by chance in the medfly, in the melon fruit fly (*Zeugodacus cucurbitae*), and in the oriental fruit fly (*Bactrocera dorsalis*). In contrast, it could not be found in many other insect species of economic and public health importance. Furthermore, GSS based on irradiation-induced translocation of the WT alleles onto the Y chromosome are semi-sterile and mass rearing may produce recombinants, which could compromise the GSS in mass rearing facilities (Cáceres et al, 2023; Franz et al, 2021). Sex separation of *Ae. aegypti* based on naturally occurring size difference between male and female pupae is currently possible at large scale (Klassen et al, 2021). However, these size-based methods are not operationally effective for other mosquito species and prone to variations according to the diet used in the rearing (Gilles et al, 2014; Kavran et al, 2022). In sum, the current sex sorting of mosquitoes based on size difference is laborious and lacks efficiency (Gilles et al, 2014; Kavran et al, 2022; Klassen et al, 2021). The development of general tools for faster development of GSS remains a necessity for boosting and expanding SIT to other insect species (Bourtzis & Vreysen, 2021).

The discovery of selectable genetic markers, e.g., the genetic basis of the wp^- phenotype in medfly (Ward et al, 2021), together with modern molecular techniques, opened up new pathways to support and improve the efficiency of SIT as a sustainable control system for

insects (Bourtzis & Vreysen, 2021; Häcker et al, 2021; Ward et al, 2021; Yan et al, 2023). One of these possibilities is the use of modern molecular techniques to engineer new GSS, the so-called neo-classical GSS. In this approach, the WT allele of the genetic marker is inserted, e.g., via CRISPR-mediated gene editing, into the Y chromosome – or close to the male-determining factor - of a mutated strain (Figure 2). This insertion leads to sex separation as observed in the classical GSS, but avoids the issues observed from the irradiation-based translocations. Ideally, the genetic marker produces a phenotype suitable for efficient sorting in mass rearing facilities, either by morphological differences, e.g., body color (Ward et al, 2021), or by conditional response to conditions that can be included in the rearing process, e.g., a temperature-sensitive phenotype (Sollazzo et al, 2024).

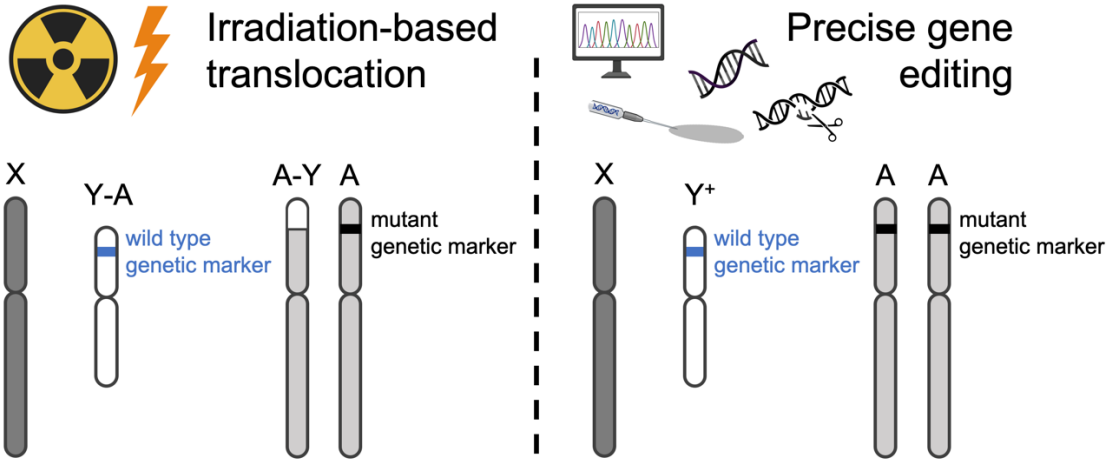


Figure 2. Schematic of male individuals of a GSS generated through classical methods with Y-autosome translocations induced by irradiation compared to the neo-classical GSS based on precise editing of the Y-chromosome. Y^+: Y-chromosome edited with insertion of the wild type allele of the genetic marker. Basic structure of GSS carrying Y-autosome translocation induced by irradiation adapted from (Franz et al, 2021). Partially created with BioRender.com.

A general pipeline could be established to develop neo-classical GSS. The first step involves identifying potential genetic markers. This may be accomplished through genetic analysis of naturally occurring mutations or by analysis of genetic markers already identified in similar species. Subsequently, in vivo validation of the identified marker is required. This step includes recreating the observed mutant phenotype through editing of the proposed genetic marker, as well as testing the capacity of the wild-type (WT) allele to restore, i.e., to rescue, the WT phenotype by introducing the WT allele of the genetic marker into the genome of a mutated strain of the species. Once the genetic marker's function and its rescue are confirmed, the next phase involves the precise insertion of the WT allele into the Y chromosome or near the male-

determining factor in a mutant strain. This strategy would achieve sex separation as males carry the WT allele, while females express only the mutant phenotype (Figure 3).

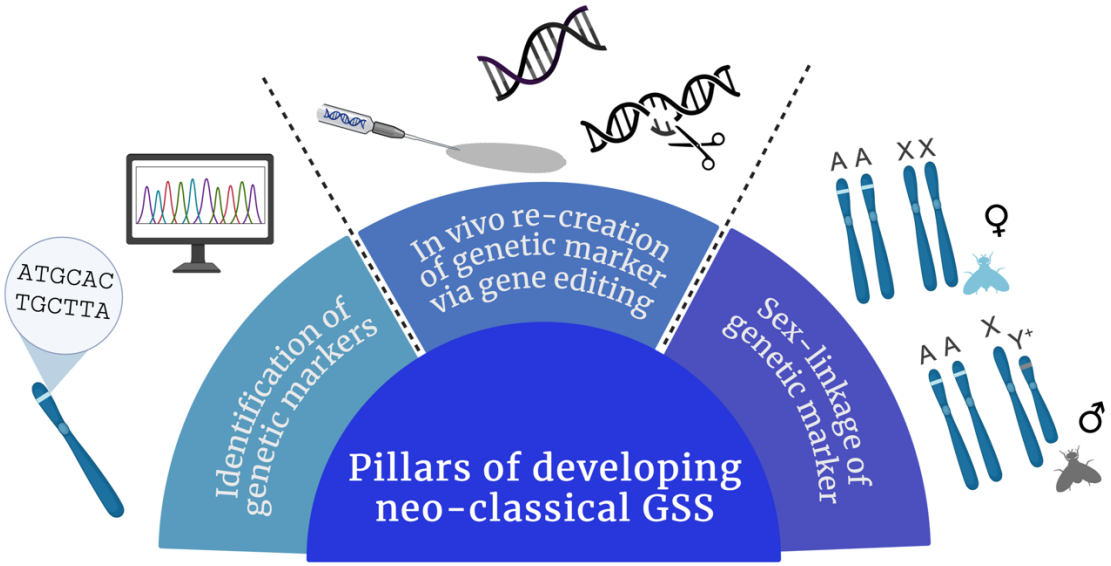


Figure 3. Main pillars to develop neo-classical GSS based on precise editing of the Y-chromosome or male-specific region. Y^{+}: Y-chromosome edited with insertion of the wild type allele of the genetic marker. Created with BioRender.com.

In medfly, the *maleness-on-the-Y* (*MoY*) has been identified in the Y chromosome (Meccariello et al, 2019) and there are ongoing efforts to assembly the Y chromosome in Tephritidae (Bayega et al, 2020; Congrains et al, 2024; Sim et al, 2024; Wang et al, 2023). Similarly, the male-determining factor (M-factor) has already been identified in *Ae. aegypti* (Hall et al, 2015) and *Ae. albopictus* (Gomulski et al, 2018). These male-specific genes – or male-specific regions in the genome - could be targeted for precise insertion of a genetic marker's WT allele**.** To enhance the efficiency of gene editing, particularly for CRISPR-mediated precision edits, the use of minimal gene constructs may be considered, as reducing cargo size can improve efficiency of CRISPR/Cas-mediated editing events (Li et al, 2014; Paix et al, 2017).

Taking the medfly as non-model organism with a successful case for GSS applied for SIT, it would be possible to directly compare a classical GSS with a neo-classical GSS. The genetic basis of the *wp*$^{-}$ phenotype has been recently identified and confirmed by generating several CRISPR-based strains expressing the *wp*$^{-}$ phenotype (Ward et al, 2021). Furthermore, the interspecies nature of this general pipeline has also been explored by the identification and validation of the genetic marker responsible for the *wp*$^{-}$ phenotype in other related Tephritidae species (Paulo et al, 2022).

2.4 Research objectives

Modern molecular techniques offer multiple tools and pathways to develop sustainable insect control systems. In my thesis, I focused on developing and optimizing such tools. First, I wanted to establish new RNAi-based larvicides in *Aedes* mosquitoes. This project required large quantities of dsRNA for the oral delivery to *Aedes* larvae. While the production of microgram to one-digit milligram amounts of dsRNA via in vitro transcription is typically not a problem for the budget, the production of higher milligram, or even gram amounts of clean dsRNA, can become financially challenging. Therefore, my **first objective** was to develop a protocol for **cost-effective extraction of bacterially produced dsRNA**. Here, the genetically modified *E. coli* bacterial strain HT115 (DE3) was used as platform for production of dsRNA. The developed protocol for extraction of dsRNA could then be used to produce large amounts of dsRNA for application in RNAi experiments.

The dsRNA produced with the protocol developed in my first objective was used in my **second objective** to **establish effective methods for dsRNA delivery to *Aedes* larvae**, and to further identify genes essential for the development and fitness of the insects, whose knockdown produces strong developmental or fitness defects.

Other useful sustainable systems for insect control are already available for some species, which, once fully understood and developed into a generic approach, could be transferred and optimized to mosquitoes. One of them is the successful use of genetic sexing strains (GSS) to support the sterile insect technique (SIT). In the final part of my thesis, I focused on using modern molecular techniques to advance the development of neo-classical GSS. My **third objective** was to **design and verify the function of a minimal version of the *white pupae* gene** as a genetic marker in the non-model organism *Ceratitis capitata*.

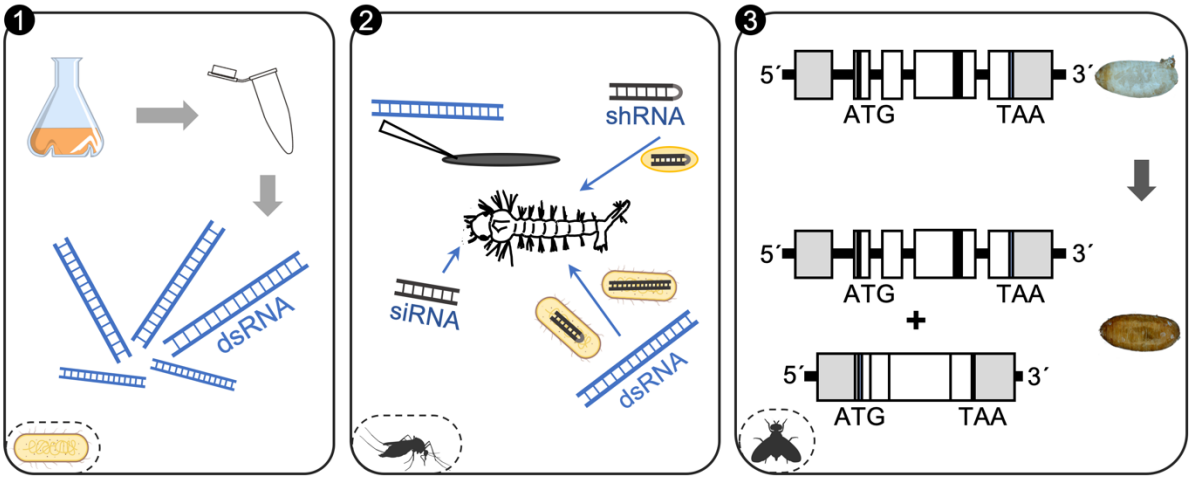


Figure 4. Schematic representation of the research objectives of this thesis. Partially created with BioRender.com.

3 Results

This section contains three manuscripts written with data that resulted from this thesis. Each chapter is introduced by a cover page with short information on the manuscript, main results achieved, list of authors and their contribution.

The manuscripts were published under open-access license. In accordance to the publisher (MDPI), copyright is retained by the authors. In addition, the article may be reused and quoted provided that the original published version is cited.

3.1 **Figueiredo Prates LH**, Merlau M, Rühl-Teichner J, Schetelig MF, Häcker I (**2023**) An optimized/scale up-ready protocol for extraction of bacterially produced dsRNA at good yield and low costs. ***International Journal of Molecular Sciences*****,** 24(11), 9266.
https://doi.org/10.3390/ijms24119266

3.2 **Figueiredo Prates LH**, Fiebig J, Schlosser H, Liapi E, Rehling T, Lutrat C, Bouyer J, Sun Q, Wen H, Xi Z, Schetelig MF, Häcker I (**2024**) Challenges of robust RNAi-mediated gene silencing in *Aedes* mosquitoes. ***International Journal of Molecular Sciences***, 25(10), 5218.
https://doi.org/10.3390/ijms25105218

3.3 **Figueiredo Prates LH**, Aumann RA, Sievers I, Rehling T, Schetelig MF. Towards neo-classical genetic sexing strains in *Ceratitis capitata* (Diptera: Tephritidae): engineering a functional intronless version of the *white pupae* gene in the Mediterranean fruit fly. ***Insect Science***.
(*to be submmited*)

3.1 An optimized/scale up-ready protocol for extraction of bacterially produced dsRNA at good yield and low costs

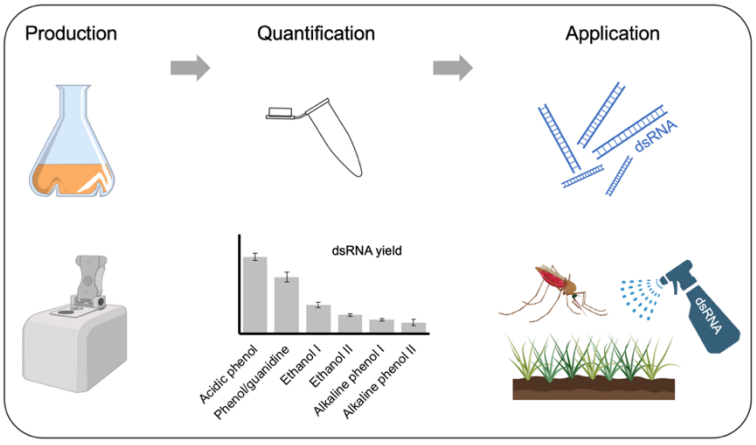
While the production of microgram to one-digit milligram amounts of dsRNA via in vitro transcription is typically not a problem for the budget, the production of higher milligram or even gram amounts of clean dsRNA can become financially challenging. I optimized a protocol for extraction of bacterially produced dsRNA at good yields and low costs using the genetically modified *E. coli* bacterial strain HT115 (DE3) as platform for production of dsRNA. In addition, I also assessed the influence of the induction time point for dsRNA expression on the dsRNA yield and performed a systematic comparative analysis of the dsRNA purity and yield achieved with the optimized protocol compared to five published protocols. Furthermore, I also showed that all bacterial cells are effectively lysed during the initial steps of the protocol, ensuring a GMO-free dsRNA product.

Title: An optimized/scale up-ready protocol for extraction of bacterially produced dsRNA at good yield and low costs.

Authors: **Lucas Henrique Figueiredo Prates**, Maximilian Merlau, Johanna Rühl-Teichner, Marc F. Schetelig, Irina Häcker.

Status: Published in the special issue on "RNA-Based Products as New Sustainable Strategies to Control Plant Diseases" in the International Journal of Molecular Sciences.

Contributions: all experiments reported in the manuscript were performed by Lucas Henrique Figueiredo Prates. Preliminary experiments to measure the amount of dsRNA extracted from bacterial cells grown in different media were performed by Johanna Rühl-Teichner and Maximilian Merlau.



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International Journal of
Molecular Sciences

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Article

An Optimized/Scale Up-Ready Protocol for Extraction of Bacterially Produced dsRNA at Good Yield and Low Costs

Lucas Henrique Figueiredo Prates *, Maximilian Merlau, Johanna Rühl-Teichner, Marc F. Schetelig and Irina Häcker *

Department of Insect Biotechnology in Plant Protection, Justus Liebig University Giessen, 35394 Giessen, Germany; maxmerlau@web.de (M.M.); johanna.ruehl@vetmed.uni-giessen.de (J.R.-T.); marc.schetelig@agrar.uni-giessen.de (M.F.S.)
* Correspondence: lucas.prates@agrar.uni-giessen.de (L.H.F.P.); irina.haecker@agrar.uni-giessen.de (I.H.)

Abstract: Double-stranded RNA (dsRNA) can trigger RNA interference (RNAi) and lead to directed silencing of specific genes. This natural defense mechanism and RNA-based products have been explored for their potential as a sustainable and ecofriendly alternative for pest control of species of agricultural importance and disease vectors. Yet, further research, development of new products and possible applications require a cost-efficient production of dsRNA. In vivo transcription of dsRNA in bacterial cells has been widely used as a versatile and inducible system for production of dsRNA combined with a purification step required to extract the dsRNA. Here, we optimized an acidic phenol-based protocol for extraction of bacterially produced dsRNA at low cost and good yield. In this protocol, bacterial cells are efficiently lysed, with no viable bacterial cells present in the downstream steps of the purification. Furthermore, we performed a comparative dsRNA quality and yield assessment of our optimized protocol and other protocols available in the literature and confirmed the cost-efficiency of our optimized protocol by comparing the cost of extraction and yields of each extraction method.

Keywords: double-stranded RNA; bacterial production system; RNA interference; RNAi-based pest control; dsRNA production costs; RNA-based biopesticides; organic purification; liquid–liquid extraction; selective extraction

Citation: Figueiredo Prates, L.H.; Merlau, M.; Rühl-Teichner, J.; Schetelig, M.F.; Häcker, I. An Optimized/Scale Up-Ready Protocol for Extraction of Bacterially Produced dsRNA at Good Yield and Low Costs. *Int. J. Mol. Sci.* **2023**, *24*, 9266. https://doi.org/10.3390/ijms24119266

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1. Introduction

RNA interference (RNAi) is a natural defense mechanism against foreign RNA, triggered by the presence of double-stranded RNA (dsRNA) and resulting in the silencing of specific genes via mRNA degradation or block of translation. Since its discovery in 1998 in the nematode *Caenorhabditis elegans* [1], RNAi has been transformed into a molecular technique to selectively silence gene expression and it has been extensively used in many different organisms. Besides basic and widely used gene function studies via RNAi-mediated gene knockdown and assessment of the corresponding phenotype, RNAi is also being studied for its application in gene therapy [2] or cancer therapy [3,4]. In addition, it can be used to screen lists of disease-causing gene candidates [5], just to name a few examples.

In recent years, RNAi has been applied to insect pest control, as it offers the possibility to species-specifically kill pest species by silencing essential genes. For instance, ingestion of bacterial cells expressing dsRNA targeting the heat shock protein and the shibire genes lead to gene silencing and lethality at the larval stage of the invasive emerald ash borer (*Agrilus planipennis* Fairmaire) [6]. Another possible strategy for insect pest control by RNAi is to target genes responsible for the (metabolic) resistance against commonly used insecticides [7]. Comprehensive reviews on this topic, including current achievements related to technical and insect-specific challenges, potential applications, regulatory considerations, and perspectives of RNAi for insect control have been recently published [8–11].

The potential of RNAi-based approaches for pest control has also gained attention in the industry. The first RNAi-based product for pest control to obtain approval from a regulatory agency was the maize "SmartStax® Pro" (Bayer, Leverkusen, Germany) approved in 2017 in the US [12]. According to its manufacturer, it expresses dsRNA that interferes in the natural production of a vital protein in the corn rootworm. Therefore, ingestion of this maize would be lethal for the insect. Other companies, such as Syngenta, also announced advances in the development of RNAi-based products (https://www.syngenta.com/en/innovation-agriculture/research-and-development/rna-based-biocontrols, accessed on 24 April 2023).

The application of RNAi for selective gene silencing experiments in the laboratory requires the production of gene-specific dsRNA, which can be obtained through different approaches, including in vitro transcription or in vivo production using genetically modified bacteria or yeast strains [13–15]. While the production of microgram to one digit milligram amounts of dsRNA via in vitro transcription is typically not a problem for the budget, the production of higher milligram or even gram amounts of clean dsRNA, for large scale laboratory experiments for insect pest control, can become financially challenging [13,16,17]. Here, the production of dsRNA via genetically modified bacterial cells can be an alternative.

Since its genetic modification to silence expression of RNase III and the insertion of a T7- inducible system for production of dsRNA [18,19], the bacterial strain HT115 (DE3) has been widely used for dsRNA production [6,19–23]. Although the application of bacterial crude extracts containing the produced dsRNA has been investigated in gene silencing experiments [19,20,22,24–26], purified dsRNA, free of other bacterial nucleic acids and proteins, is preferable or required in many cases, as it not only avoids potential immune responses due to the presence of bacterial cells [27], but also facilitates regulatory approvals [10,28].

Several methods have been described for dsRNA extraction from bacterial cells [29–34]. Phenol–guanidine-based protocols have been reported as efficient method for extraction of dsRNA at high yields (up to 30 µg/mL of bacterial cells) and good quality [29,34,35]. Other alternatives rely on fixing bacterial cells in ethanol and phosphate-buffered saline solution for extraction of dsRNA [32,33] or performing a phenol/chloroform/isoamyl alcohol (P/C/I) extraction and further DNase and RNase digestions [30,31].

The purification based on commercially available phenol–guanidine solutions inflates dsRNA production costs for medium- to large-scale laboratory experiments. Scaling up published protocols to extract dsRNA for example from a 5 L-bacterial culture, e.g., following the protocol from Verdonckt et al.'s work [34], would consume 500 mL of QIAzol® (QIAGEN, Venlo, The Netherlands), corresponding to 771.75 EUR just for the organic reagent. Processing the same amount of cells with a protocol using TRIzol™ (Invitrogen, Waltham, MA, USA) [29] would cost nearly EUR 1700 (794 mL of the organic reagent needed). While the required amounts of dsRNA for effective gene silencing vary with the target species, it is predicted that 2 to 10 g of dsRNA per hectare for crop protection would be needed in the field [36]. Based on the published yields, this would correspond to EUR 30,000 to 142,000 for TRIzol™, or EUR 38,000 to 192,000 for QIAzol®. However, for medium- to large-scale laboratory experiments the costs would also quickly be in the thousands to tens of thousands of Euros.

While the price for dsRNA synthesis has dropped strongly nowadays, with predicted production costs as low as 1 USD/g for a proprietary cell-free methodology (www.greenlightbiosciences.com, accessed on 24 April 2023), the versality of engineered HT115 (DE3) cells for dsRNA production allows scientists to readily adjust dsRNA sequences according to experimental requirements or for distinct applications. Having a reliable and cost-effective protocol for extraction of dsRNA from large HT115 (DE3) culture volumes would further broaden the usability of HT115 cultures for dsRNA production, e.g., for field trials or larger laboratory applications, such as high-throughput screening of potential targets for RNAi in different species. Therefore, we aimed to develop a low-cost protocol

for dsRNA extraction with high yield and quality, but at a fraction of the costs from the best currently available alternatives.

2. Results

2.1. dsRNA Can Be Successfully Extracted from HT115 DE3 Cells Using Either TRIzol™ or P/C/I

Several protocols for dsRNA extraction from bacterial cells have been published [29–34]. A protocol that reported good yield (30 µg per OD_{600} of cells) and purity of dsRNA uses boiling of the cells in sodium dodecyl sulfate (SDS), followed by RNase A digest of bacterial RNAs and TRIzol™ extraction of the dsRNA [29]. Applying TRIzol™ extraction to larger scale batch cultures or small fermenters, however, produces considerable costs, as described above. We therefore evaluated dsRNA extraction from HT115 DE3 cells induced to produce dsRNA at OD_{600} 0.8 following the general workflow of the phenol–guanidine-based protocol [29], but replacing the TRIzol™ with the cheaper chemical P/C/I (25:24:1, pH 4.5–5) and modifications to buffer to cell volume ratio and RNase A incubation time. The corresponding in vitro transcribed dsRNA was used as reference in gel electrophoresis. The obtained bands of the expected size indicate successful extraction of bacterially produced dsRNA using both TRIzol™ and P/C/I (25:24:1, pH 4.5–5) (Figure 1). Spectrophotometric quantification of the nucleic acids at 260 nm showed comparable mass yields per OD cells with both extraction reagents. However, the relative amount of a copurified high-molecular-weight band (presumably bacterial genomic DNA (gDNA)) and smaller bacterial RNAs relative to the dsRNA band was higher when using P/C/I for dsRNA extraction. We therefore started to systematically assess the nature of the copurified high-molecular-weight band, the influence of buffer pH, serial P/C/I extractions, and induction OD_{600} on the quality and yield of P/C/I-extracted dsRNA. Moreover, with regard to applying the protocol to dsRNA extractions from liters of culture, we assessed the efficiency of cell lysis in the reduced lysis buffer volumes, which reduces the amount of P/C/I needed for extraction.

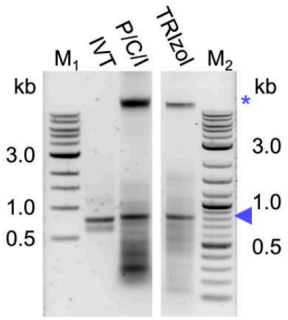

Figure 1. Comparison of yield and quality of bacterially produced dsRNA extracted with the protocol established by Ongvarrasopone et al. [29] using either P/C/I pH 4.5-5 or TRIzol™. In vitro transcribed (IVT) dsRNA (0.4 µg) is shown as a reference. Loaded were 2.8 µg and 1.5 µg of dsRNA extracted using P/C/I or TRIzol™, respectively. Blue arrowhead indicates the band of the dsRNA; the asterisk indicates a high-molecular-weight band, presumably genomic DNA, copurified from the bacterial cells. M1: 1 kb ladder. M2: 1 kb plus ladder (New England BioLabs Inc., Ipswich, MA, USA).

2.2. DNase Digestion Confirms Presence of Bacterial Genomic DNA in Extracted dsRNA

TRIzol™ as well as P/C/I extraction resulted in copurification of a high-molecular-weight nucleic acid. A similar high-molecular-weight band was observed in protocols without DNase treatment of the purified dsRNA [20,29,32], but was absent in protocols applying a DNase digestion [30,31]. We therefore included a DNase treatment step in the phenol–guanidine-based protocol [29]. This treatment completely removed the high-molecular-weight band compared to the extraction without DNase treatment (Figure 2, lane 2 compared to lane 1), showing that it consisted of bacterial (genomic) DNA.

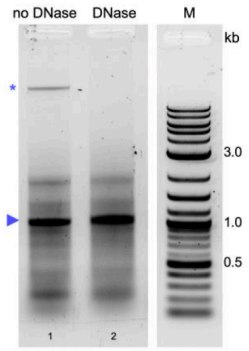

Figure 2. Effect of DNase treatment on dsRNA purity. Shown are the products of phenol–guanidine-based extraction following the protocol established by Ongvarrasopone et al. [29] (same conditions as in Figure 1) with mock DNase digestion (lane 1, no DNase) or with DNase digestion (lanes 2, DNase). Loadings were of 1.5 µg for each sample. Blue arrowhead indicates extracted dsRNA, blue asterisk indicates bacterial DNA. M: 1 kb plus ladder (New England BioLabs Inc., Ipswich, MA, USA).

2.3. Boiling in the Presence of Anionic Surfactant Efficiently Opens Bacterial Cells for dsRNA Extraction

Efficient cell lysis is critical for efficient dsRNA extraction, as dsRNA from unlysed cells would be lost in the pellet at the P/C/I extraction step. Moreover, the volume of buffers used in the initial steps of the protocol will eventually determine the volume of organic reagent (TRIzol™, QIAzol® or P/C/I) required for extraction of dsRNA, thereby directly impacting the costs and suitability of any extraction protocol for larger applications. We, therefore, investigated whether reducing the volume of SDS lysis buffer per OD_{600} of cells affects cell lysis. After boiling the bacterial cells at 95 °C for 2 min with a four-fold reduced volume of 0.1% (*w*/*v*) SDS per OD_{600} of cells compared to the original protocol [29], no viable cells were observed in a cell viability assay (Figure 3). Thus, boiling in the reduced volume of 0.1% SDS efficiently opens up bacterial cells for dsRNA extraction.

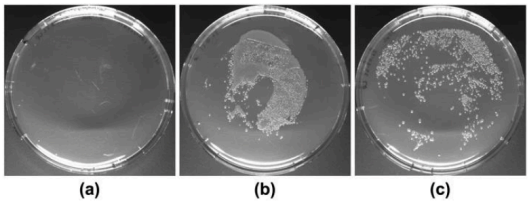

Figure 3. Cell viability assay with HT115 DE3 cells resuspended in reduced volume of 0.1% (*w*/*v*) SDS and boiled at 95 °C for 2 min (**a**) compared to cells resuspended in 2% (*w*/*v*) LB-medium without boiling and incubated at room temperature (**b**,**c**). Plated were 1 µL of undiluted cell suspension in (**a**,**b**), and 10 µL of 1:100 dilution in (**c**).

2.4. Low pH in a Second P/C/I Extraction Reduces Amount of Copurified DNA

DNase treatment of the extracted dsRNA might not be applicable to the processing of large culture batches. We therefore focused on the chemical approach of separating RNA and DNA to provide an alternative option of removing the contaminating DNA. At slightly alkaline conditions (pH 7.5–8.0), RNA and DNA partition into the aqueous phase of a phenol/chloroform extraction after phase separation, while at pH 4–6, the DNA should be retained in the lower, i.e., organic, phase and interphase [35]. Therefore, P/C/I pH 4.5–5.0 was chosen in our protocol for dsRNA extraction. Nevertheless, bacterial DNA was copurified (Figure 4a). To test if this contaminating DNA could be removed by further

acidifying the extraction conditions, we reduced the pH of the RNase A buffer or the SDS 0.1% (*w*/*v*) lysis buffer to pH 4.5 before performing the extraction with P/C/I pH 4.5–5.0.

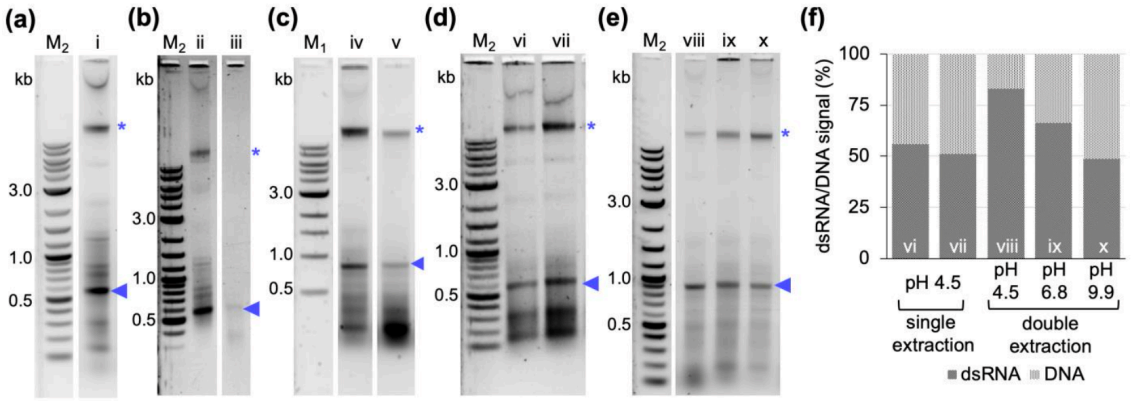

Figure 4. Comparison of different purification conditions on dsRNA purity and yield. (**a**) Product of single P/C/I extraction from cells lysed in 0.1% (*w*/*v*) SDS pH 7.1 and single-stranded RNA degradation performed in RNase A buffer at pH 7.5 (i) following the protocol established by Ongvarrasopone et al. [29] (same conditions as in Figure 1) (**b**) Cell lysis performed in 0.1% (*w*/*v*) SDS pH 7.1 (ii) or in 0.1% (*w*/*v*) SDS and 0.1 M sodium acetate buffer pH 4.5 (iii). (**c**) Single-stranded RNA degradation performed in RNase A buffer at pH 7.5 (iv) or pH 4.5 (v). (**d**) Reaction was acidified with 0.1 M sodium acetate buffer pH 4.5 before addition of P/C/I (vi and vii). (**e**) Product of the first P/C/I extraction was adjusted to pH 4.5, 6.8, or 9.9 (lanes (viii), (xi), and (x), respectively) by adding the respective buffers and a 2nd P/C/I extraction was performed. (**f**) Relative integrated densities of dsRNA and DNA bands in samples from (**d**,**e**) quantified with Adobe Photoshop (Adobe Inc., 2023, San José, CA, USA). Displayed is the percentage of the respective band intensities compared to the sum of dsRNA and DNA densities per lane. Loading amounts were 2.6 µg in (**a**), 3 µg in (**b**), 2.8 µg in (**c**), 2.0 µg in (**d**) and 1.1 µg in (**e**). Blue arrowheads indicate extracted dsRNAs of sizes 800 or 480 bp, blue asterisks indicate bacterial DNA copurified from the bacterial cells. M1: 1 kb ladder. M2: 1 kb plus ladder (both New England BioLabs Inc., Ipswich, MA, USA).

However, these treatments not only yielded weaker dsRNA bands, but also increased the contamination with small co-extracted RNAs, suggesting that low-pH conditions reduced RNase A activity (Figure 4b,c, compared to product of P/C/I extraction with unchanged cell lysis and RNase A buffers in Figure 4a). We subsequently assessed whether acidifying the reaction after RNase A digest and before P/C/I addition by adding 2.5 sample volumes of 0.1 M sodium acetate buffer, pH 4.5 would remove the DNA band (Figure 4d). However, this did not improve the dsRNA purity compared to the P/C/I extraction without additional acidification. It again increased the amount of copurified bacterial RNAs. In the next step we, therefore, included a 2nd acidic P/C/I extraction step into the protocol. For this, the dsRNA solution obtained after the first P/C/I extraction and nucleic acid precipitation was acidified by adding three sample volumes of 0.1 M sodium acetate buffer, pH 4.5, followed by a 2nd P/C/I extraction and nucleic acid precipitation. This treatment yielded strongly improved dsRNA purity compared to the result of a single P/C/I extraction (Figure 4e, lane viii, compared to Figure 4a, lane i). Two control experiments applying a 2nd P/C/I extraction at neutral (pH 6.8) or basic conditions (pH 9.9) (Figure 4e, lanes ix and x) showed that the combination of 2nd P/C/I extraction and low pH is needed for the effect. The improved dsRNA to DNA band ratio observed via gel electrophoretic analysis was confirmed by quantifying the integrated density of the bands with Adobe Photoshop (Adobe Inc., 2023, San José, CA, USA). The relative density of the dsRNA to DNA band is considerably higher when a second P/C/I extraction is performed with intermediate acidification of the sample (Figure 4f).

2.5. Inducing dsRNA Production at a Late Exponential Phase Reduces Yield and Quality of Extracted dsRNA

In vivo transcription of dsRNA in HT115 DE3 cells transformed with the vector L4440 can be induced with IPTG at any stage of bacterial growth. Considering different published manuscripts that induce dsRNA production in bacterial cells between OD_{600} of 0.4 and 0.8, we evaluated whether induction with IPTG during a late exponential phase of the bacterial growth would result in higher yields of dsRNA per bacterial cell. Thus, the dsRNA production was induced at an OD_{600} of either 0.4 or 0.8, and dsRNA purified with published protocols [29,32,33] and our own optimized protocol including the 2nd acidic P/C/I extraction. Nucleic acid yields determined via absorption measurement at 260 nm (A_{260}) of the purified nucleic acids did not significantly ($\alpha = 0.05$) increase when cultures were induced at a higher OD_{600} for three of the four protocols tested. For the ethanol-based protocol established by Posiri et al. [32], the induction at late growth stage even yielded significantly less nucleic acids per OD_{600} of cells ($p = 0.028$) (Figure 5a). Quality assessment of the purified nucleic acids via gel electrophoresis, however, revealed clear differences in the quality and quantity of the purified dsRNA at different induction ODs for all tested protocols. First, the band corresponding to the dsRNA was markedly stronger in relation to the background of copurified nucleic acids when induction of dsRNA production occurred at OD_{600} of 0.4. Second, the absolute dsRNA band intensity from cells induced at OD_{600} of 0.4 was stronger compared to the induction at OD_{600} of 0.8 (Figure 5b, same equivalent of cells was loaded). This was confirmed by quantifying the integrated densities of the dsRNA bands and of the complete lane by Adobe Photoshop to determine the fraction of dsRNA within each lane. Setting the fraction of dsRNA obtained from induction at OD_{600} 0.4 to 100 % for each purification protocol showed that induction at OD_{600} 0.8 results in 20.2 to 48.5 % less dsRNA per OD of cells (Figure 5c). Thus, induction at higher OD_{600} does not improve the amount of dsRNA produced per bacterial cell, nor the purity of the extracted dsRNA.

2.6. Comparative Assessment of Yield and Purity of the Optimized Low-Cost Protocol

We finally conducted a comparative dsRNA quality and yield assessment of our optimized P/C/I extraction protocol and the published protocols by performing dsRNA extractions from the same culture batches for all protocols in three biological replicates and two technical replicates each. To confirm that the remaining traces of the high-molecular-weight band copurified in our optimized protocol is DNA, as observed for the extraction with TRIzol^{TM} (Figure 2), we additionally included a facultative DNase I treatment step in the protocol before acidifying the sample for the second P/C/I extraction in one of the biological replicates (Figure 6a). In this comparative experiment, our protocol resulted in significantly higher nucleic acid amounts as determined by spectrophotometric analysis (17.7 ± 1.24 µg/OD_{600}) than even the best published protocol established by Ongvarrasopone et al. [29] (13.2 ± 1.00 µg/OD_{600}, p-value < 0.001), and two- to ten-fold higher nucleic acid amounts than both versions of the ethanol-based protocol [32,33] (8.2 ± 0.31 and 6.5 ± 0.22 µg/OD_{600}, respectively (p-value < 0.001)) and the alkaline phenol-based protocols [30,31] (1.4 ± 0.14 and 1.4 ± 0.42 µg/OD_{600}, respectively (p-value < 0.001)). DsRNA purity assessment via gel electrophoresis showed some background of different-size copurified bacterial nucleic acids in our protocol and the phenol–guanidine-based protocol established by Ongvarrasopone. et al. [29]. Both ethanol-based protocol presented a strong co-extraction of small bacterial RNAs; and both alkaline phenol-based protocols with RNase A and DNase I digestions produced overall the cleanest dsRNA, but at very low quantitative yields (Figure 6a). Considering that the absorbance measurements used to quantify the yields do not distinguish between the desired dsRNA and co-extracted bacterial nucleic acids in a sample, we determined the relative dsRNA amount per sample with Adobe Photoshop (integrated band densities, Figure 6b) and with these numbers calculated the absolute amount of dsRNA per sample (Figure 6c). This analysis showed that the highest absolute amount of dsRNA per OD cells can be extracted with our optimized protocol,

followed by the protocol of Ongvarrasopone et al. Three- to five-fold lower absolute yields of dsRNA were obtained using the ethanol-based and alkaline phenol-based protocols.

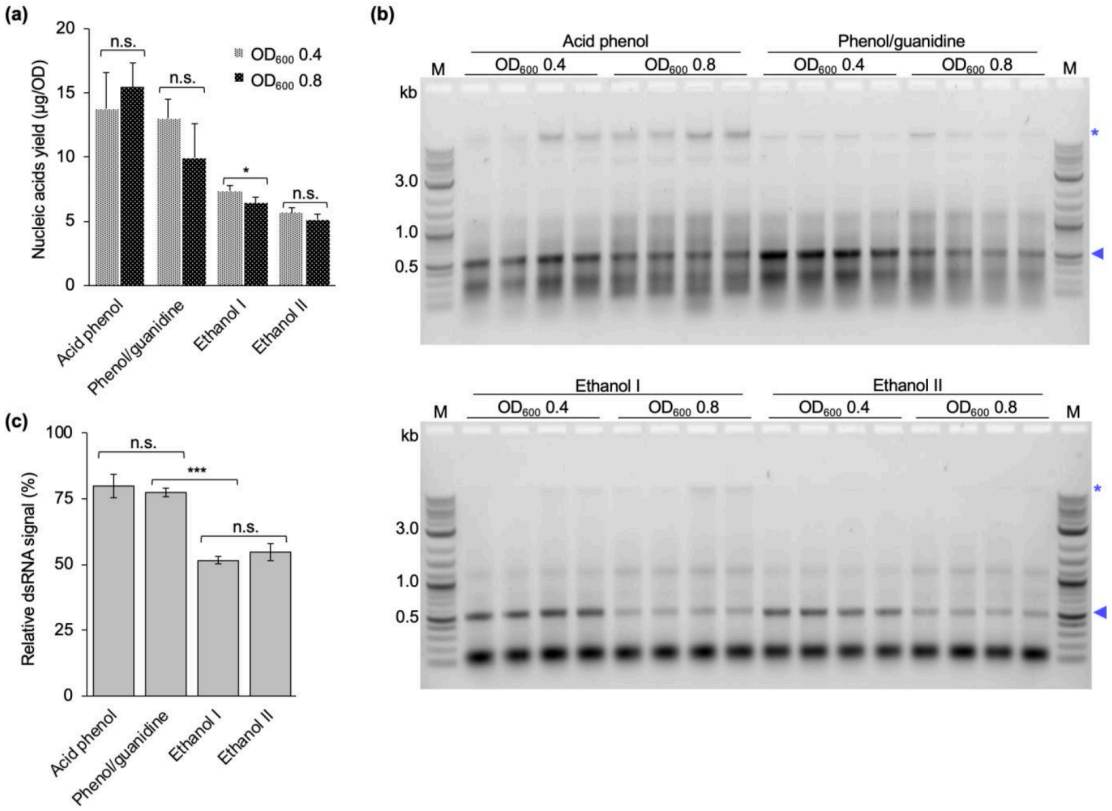

Figure 5. Yield and purity of dsRNA extracted from bacterial cells induced to produce dsRNA at OD_{600} of 0.4 or 0.8. Comparative extractions were performed with the acid phenol-based protocol developed in this study, the phenol–guanidine-based protocol established by Ongvarrasopone et al. [29] (Phenol/guanidine), or the ethanol-based protocols established by Posiri et al. [32] (Ethanol I) or Papić et al. [33] (Ethanol II). (**a**) Nucleic acid yield (µg per 1 OD_{600} of cells) extracted from bacterial cells induced to produce dsRNA at OD_{600} of 0.4 (grey) or 0.8 (black), measured by spectrophotometry at 260 nm. Data shown here are based on two technical replicates (extractions) from two biological replicates. n.s.: no statistically significant difference. *: statistically significant difference, p-value < 0.05. (**b**) Gel electrophoresis of dsRNA extracted from bacterial cells induced to produce dsRNA at OD_{600} of 0.4 or 0.8. Shown are the results of two technical replicates from two biological replicates for each condition (i.e., dsRNA was extracted from two samples per culture, for two different cultures). In all lanes, the equivalent of 0.2 OD_{600} of cells was loaded. Blue arrowheads indicate extracted dsRNA, blue asterisks indicate bacterial DNA. M: 1 kb plus ladder (New England BioLabs Inc., Ipswich, MA, USA). (**c**) Percentage of dsRNA obtained from cultures induced at OD_{600} of 0.8 compared to induction at OD_{600} of 0.4. Integrated density of dsRNA bands from the gels shown in (**b**) was quantified with Adobe Photoshop (Adobe Inc., 2023, San José, CA, USA), the intensity of the band obtained from the lower induction OD_{600} set to 100% and the intensity of the high induction OD_{600} put in relation; n.s. = no statistically significant difference. *** statistically significant difference, p-value < 0.001 (one-way ANOVA, Holm–Sidak method for all pairwise multiple comparison).

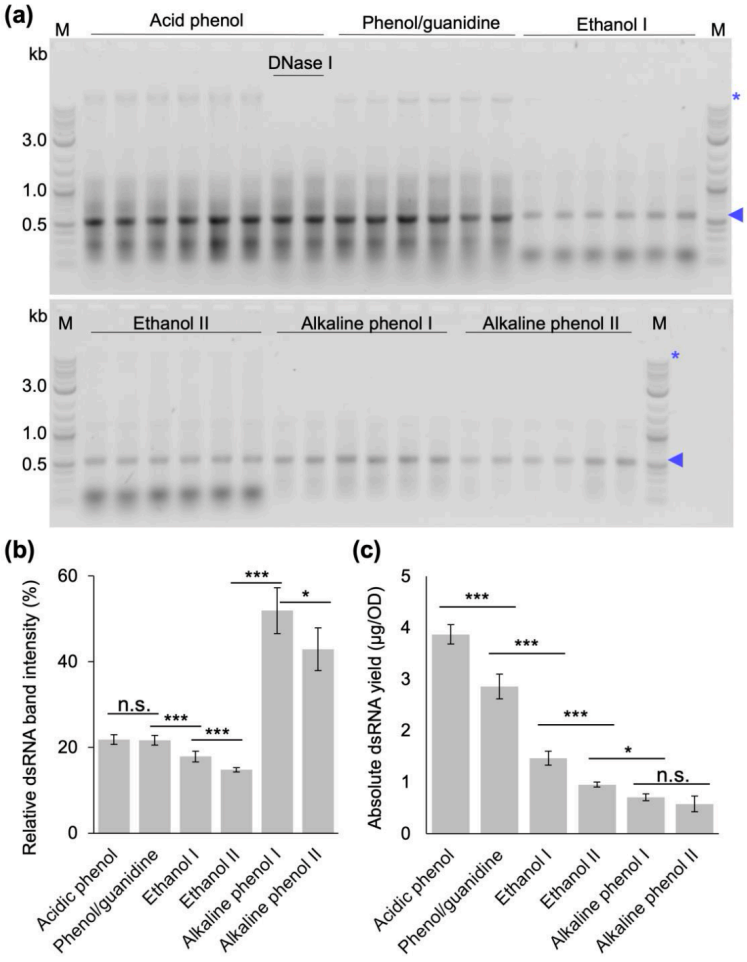

Figure 6. Comparative gel electrophoretic analysis, relative and absolute yield of dsRNA extracted from bacterial cells with different extraction protocols. Extractions were performed from cells induced at OD_{600} 0.4 with the acid phenol-based protocol developed in this study, the phenol–guanidine-based protocol established by Ongvarrasopone et al. [29] (Phenol/guanidine), the ethanol-based protocols established by Posiri et al. [32] (Ethanol I) or Papić et al. [33] (Ethanol II), the alkaline phenol-based protocols established by Ahn et al. [31] (Alkaline phenol I) or Solis et al. [30] (Alkaline phenol II). (**a**) Agarose gel electrophoresis of extracted dsRNA. Shown are the results of two technical replicates (i.e., extractions) from three biological replicates (i.e., bacterial cultures) for each extraction protocol. The product of the acid phenol-based protocol developed in this study with an additional DNase I digestion for one of the biological replicates is also shown. In all lanes, the equivalent of 0.4 OD of cells was loaded. (**b**) Relative dsRNA amount per lane (integrated density of the dsRNA band compared to the integrated density of the whole lane quantified by Adobe Photoshop (Adobe Inc., 2023, San José, CA, USA)). Data are based on gel shown in (**a**). (**c**) Absolute amount of dsRNA obtained from one OD of cells, calculated from the spectrophotometric analysis (absorbance at 260 nm) and the relative band intensities in (**b**) for each of the protocols tested. n.s. = no statistically significant difference. * statistically significant difference, p-value < 0.05, *** statistically significant difference, p-value < 0.001 (one-way ANOVA, Holm–Sidak method for all pairwise multiple comparison). Blue arrowheads indicate extracted dsRNA, blue asterisks indicate bacterial DNA. M: 1 kb plus ladder (New England BioLabs Inc., Ipswich, MA, USA).

2.7. P/C/I Extraction of Bacterially Produced dsRNA Is Cost-Effective

A cost analysis of the extraction protocols considering only the most expensive part, the organic reagent, resulted in estimated costs of EUR 3.58 to process 400 mL-bacterial culture with our optimized protocol. On the other hand, using either QIAzol® or TRIzol™ increases the dsRNA extraction costs to EUR 60 or even more than 100, respectively, when scaling the respective protocols up to 400 mL culture, while the ethanol-based protocols cost less than EUR 1 for this culture volume. A more realistic cost analysis considers the costs for obtaining a specific dsRNA amount. Based on the absolute dsRNA yields determined in our comparative experiment for each of the protocols (Figure 6c), we therefore calculated the costs to obtain one milligram of dsRNA. Compared to our optimized protocol, the ethanol-based protocol [32] is approximately 31% cheaper in producing the same amount of dsRNA. This calculation, however, does not yet include the costs for media preparation, which would be about four times higher for the ethanol-based protocols. On the other hand, our optimized protocol is approximately 90 to 98% cheaper than using the alkaline phenol-based protocols or the phenol–guanidine-based version that uses TRIzol™, respectively, to produce the same amount of purified dsRNA.

3. Discussion

Several methods have been developed for extraction of bacterially produced dsRNA for application of RNAi in crop protection, vector control, or functional genomic analysis, among other research fields [29–34,37]. In the initial experiments, we had tested most of these protocols for the dsRNA yield and purity and obtained the best results in yield with the protocol established by Ongvarrasopone et al., which is based on a TRIzol™ extraction. TRIzol™ (Invitrogen), TRI Reagent® (Merck, Darmstadt, Germany) and QIAzol® (QIAGEN) are examples of commercially available phenol–guanidine-based reagents that have been used for extraction of bacterially produced dsRNA for RNAi application [29,34]. They offer an all-in-one solution for cell lysis, protein denaturation and deactivation of nucleases by the chaotropic agent guanidinium thiocyanate in solution with phenol [35]. However, these commercially available monophasic solutions of phenol–guanidine are expensive. For example, in a protocol using QIAzol® the costs for the reagent contribute to up to 85% of the total costs of dsRNA extraction [34]. This becomes especially relevant when upscaling dsRNA purification from several liters of culture. Therefore, we exchanged the TRIzol™ for the about 10-fold cheaper chemical P/C/I (25:24:1), pH 4.5–5. This resulted in a higher background of copurified bacterial RNAs and also (genomic) DNA (Figure 1). The latter was unexpected, as the acidic conditions of the P/C/I extraction should partition the DNA into the interphase and organic phase [35]. However, the acidity of the P/C/I itself was not sufficient for this effect, and also acidifying the reaction before the P/C/I addition did not improve the partitioning. In a systematic optimization process we found that adding a 2nd acidic P/C/I extraction step reduces the copurified nucleic acids to a similar level as in the original TRIzol™-based protocol. Serial extractions to improve the quality of the dsRNA have previously been performed not only with phenol/chloroform-based protocols [30,31] but also with phenol–guanidine-based extraction methods [34]. Altogether, our findings suggest that the pH of the used P/C/I might not always be sufficient to result in effective separation of RNA and DNA in phenol-based extractions, and that stabilization of the acidic conditions in the sample by using a low pH-buffer might be needed to obtain better results. Moreover, the presence of certain chemicals in the aqueous phase might interfere with the proper separation of the nucleic acids in the respective phase during the P/C/I extraction, as in our case the additional acidification of the reaction before adding P/C/I did not improve the result. Only when the already one-step P/C/I-purified dsRNA dissolved in pure water was acidified, followed by a 2nd P/C/I extraction, could we achieve a better DNA and RNA separation. We suspect the SDS of the lysis buffer to be the interfering substance.

With regard to reducing the overall dsRNA production costs, we looked into possibilities to further increase the yield per culture volume and reduce extraction volumes to save on the most expensive component of the protocol, the organic reagent.

Cell lysis is a critical step in the extraction of bacterially produced dsRNA, as dsRNA would be lost with unlysed cells. Pre-treatment of cells, including sonication, heating, and enzymatic digestions, has been shown to increase the yield of dsRNA extraction [31,34]. However, quality analysis via gel electrophoresis has also shown that dsRNA extracted from sonicated samples yields less strong bands and smearing in the background, suggesting degradation of dsRNA [31,34]. We could show here that boiling in a reduced volume of the published buffer [29] efficiently lysed the bacterial cells while at the same time preserving the dsRNA quality (Figures 1 and 2), thereby abolishing the need for further treatments for cell lysis. Obtaining dsRNA free of bacterial cells is not only important to avoid possible unwanted immune responses in in vivo applications [27], but is also highly relevant for regulatory aspects. In Europe, for instance, if no genetically modified organism (GMO) has been used or it is proven to be inactivated, RNAi-based products intended for agricultural pest control would be regulated following the same regulations applied for classical synthetic chemical pesticides [28], which would apply to the dsRNA produced with our protocol.

Different studies in the literature have induced dsRNA production at cell densities ranging from 0.4 to 0.8 OD_{600}, and harvested cells after 4 to 5 h of dsRNA production or when the OD_{600} reached 1.0 [29–34]. Following the rationale that more cells per culture volume should yield more dsRNA per culture volume, we initially induced dsRNA production at a late exponential phase (OD_{600} = 0.8). To prove this point, we additionally performed dsRNA extractions from cultures induced closer to the mid-exponential phase (OD_{600} of 0.4). Regardless of the induction timepoint, the cells were allowed to produce dsRNA for 4 h before harvesting. In contrast to our expectations, the induction at the higher OD did not yield more dsRNA. On the contrary, the amount of dsRNA produced per cell was lower. In addition, a stronger background of copurified nucleic acids was observed. This was independent of the purification protocol used (Figure 5). We assume that at a late timepoint in the exponential phase the bacterial cells are less transcriptionally active due to nutrients starting to become limiting, and maybe also become less responsive to IPTG.

Finally, we compared the yield and quality of dsRNA extracted from bacterial cells induced at OD_{600} of 0.4 with our optimized protocol, the original protocol established by Ongvarrasopone et al. and the published extraction protocols that do not require expensive monophasic solutions of phenol–guanidine [29–33] in extensive side-by-side extractions. This experiment showed that our optimized protocol produces the highest absolute amount of dsRNA (Figure 6), and had the highest nucleic acid concentrations. The gel electrophoretic analysis also showed that the different protocols resulted in very different dsRNA qualities. For our protocol and the phenol–guanidine-based protocol, there are still remnants of bacterial RNAs and DNA (the latter can be removed via additional DNase digestion after the first P/C/I extraction if required for specific applications). The ethanol-based protocols showed a strong contamination with small bacterial RNAs in addition to the rather moderate dsRNA yield, while, in our hands, the alkaline phenol-based protocol performed best with regard to purity, but at the lowest quantitative yields (Figure 6a,b).

Interestingly, despite following the protocols as closely as the details reported in the respective methods sections allowed, we could not reproduce the reported dsRNA yields published in the literature. Possible reasons for the differences could be the different expression systems (strains and plasmids), culture media and types (batch or fed-batch), how the yields were determined, and the dsRNA constructs themselves. For instance, it has been shown that the dsRNA construct has a significative impact on the yield of the extracted dsRNA using a phenol–guanidine-based protocol [34]. Regarding the different plasmids for expression of dsRNA, the plasmid L4440 has been used in our experiments, while pET3a and pET17b were used in some of the other studies [29,32]. Moreover, the nutrient content of the medium used to grow the cells might influence the dsRNA yield. We have preliminary data confirming that the amount of extracted dsRNA (by absorption measurement) doubles when cells are grown in the nutrient-rich terrific broth (TB) medium

compared to LB medium. A very similar observation was made by Thammasorn et al. [38]. Ongvarrasopone et al., report obtaining 30 µg dsRNA per 1 OD of cells grown in 2xYT medium, while we obtained about 13 µg per OD from cells grown in LB medium with their protocol (both numbers based on absorption measurement at 260 nm, i.e., measuring the total nucleic acid content). Potentially, the combination of different expression plasmid and nutrient supply is responsible for the observed difference. Likewise, Ahn et al. obtained approximately 4.85 µg per OD from cell grown in 2xYT medium, while we obtained about 1.4 µg/OD in LB. Besides the nutrient content, the high salt present in some media, such as TB, could contribute to regulate the pH of the cultures, thereby optimizing cell growth and dsRNA production [38].

Taking into account only the costs for the organic reagent per microgram of produced dsRNA, our protocol is comparable to the two ethanol-based protocols (Table 1). All three are 15- to 75-fold cheaper than the other tested methods. When users have to decide between the three most cost-effective protocols, aspects to consider will be the use of ethanol versus P/C/I, the strong contamination with small bacterial RNAs in the ethanol-based protocols, and, last but not least, that for ethanol-based protocols about 4 times larger culture volumes are required to produce the same absolute amount of dsRNA as with our protocol. This might become a deciding factor especially for large-scale dsRNA productions.

Table 1. dsRNA purification costs based on the organic reagent costs for each protocol used in this study. Prices of the organic reagent used for calculations are list prices for the largest commercially available packing size at the time of writing this manuscript.

Protocol	Required Volume of Organic Reagent Per OD Cells (mL)	Price of Organic Reagent (EUR/mL)	Absolute dsRNA Yield [1] (µg/OD)	Organic Reagent Costs for 400 mL Culture (EUR)	Organic Reagent Costs Per mg of Purified dsRNA (EUR)
Acid phenol	0.0688	0.16	3.87	3.58	2.90
Phenol–guanidine	0.2000	2.14	2.86	135.93	149.57
Ethanol I	0.0375	0.08	1.47	0.93	1.99
Ethanol II	0.0350	0.08	095	0.87	2.86
Alkaline phenol I	0.0788	0.27	0.71	6.63	29.27
Alkaline phenol II	0.0627	0.27	0.58	5.28	28.32

[1] Absolute dsRNA yields were calculated based on the integrated density of the dsRNA band compared to the integrated density of the whole lane quantified by Adobe Photoshop (Adobe Inc., 2023, San José, CA, USA) and the nucleic acid yield as determined by spectrophotometric analysis (see Figure 6).

In summary, based on the results reported in the literature and our findings with the parallel purifications, we suggest that for a true comparison of the efficiency of a purification protocol, exactly the same dsRNA production system has to be used. Moreover, besides the extraction method, also the expression system, the culture conditions, and the induction time point for dsRNA production should be taken into account to further optimize the dsRNA yields from bacterial cultures. Finally, the combination of obtained dsRNA yields, dsRNA purity, and estimated production costs per milligram of purified dsRNA (based only on the organic reagent costs) suggests that when high purity dsRNA is required for the planned experiments, the alkaline phenol-based protocols perform best, but at considerable costs if high amounts are needed. In contrast, our optimized protocol has the best combined yield to cost and yield to culture volume ratios at only a moderate copurification of bacterial nucleic acids. This makes it the ideal protocol for dsRNA production for large scale laboratory or even field trial experiments not requiring the highest purity of dsRNA, meeting also potential regulatory demands for applications in the field.

4. Materials and Methods

4.1. In Vitro Production of dsRNA

Templates for in vitro transcription (IVT) of dsRNA were produced via polymerase chain reaction (PCR) using a fragment of the gene AAEL002851 from *Aedes aegypti* cloned into pCR4 TOPO vector and primers containing at T7 promoter overhang (marked in bold). PCR reactions contained 32.6 µL of nuclease-free water, 10 µL of 5x Q5 buffer, 5 µL of 2 mM dNTP mix, 0.5 µL of forward primer P884 (**CCCTTTAATACGACTCACTATAGGG** AGAAGGAAATCATCTCCGACGAAC) at 10 µM, 0.5 µL of reverse primer P885 (**CCCTTTA ATACGACTCACTATAGGG**AGAACACGGTACTGTTGCGATCC) at 10 µM, 1 ng of DNA template and 0.5 µL of Q5 Taq polymerase (New England Biolabs, Inc., Ipswich, MA, USA). The PCR reaction was performed as follows: 30 s at 98 °C; 30 cycles of 98 °C for 10 s, 65 °C for 20 s and 72 °C for 1 min; and final elongation at 72 °C for 2 min. The PCR product was analyzed and purified via gel electrophoresis using Zymo Research Gel DNA recovery kit (Zymo Research Europe GmbH, Freiburg, Germany) following the manufacturer's instructions. The purified template (200 ng) was used to generate dsRNA via in vitro transcription using the MegaScript Kit followed by purification of the in vitro transcription product using the MEGAclear™ kit according to the manufacturer's instructions (Ambion/Life Technologies, Thermo Fischer Scientific Inc., Waltham, MA, USA).

4.2. In Vivo Production of dsRNA

For in vivo production of dsRNAs, 275 mL of lysogeny broth (LB-Lennox) medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) supplemented with 100 µg/mL ampicillin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 12.5 µg/mL tetracycline (Fisher BioReagents, Geel, Belgium) were inoculated with 2.2 mL of an overnight culture of HT115 DE3 cells (F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase, from the Caenorhabditis Genetics Center) transformed with L4440 plasmid encoding either a dsRNA against *eGFP* (480 bp) or *Aae beta-tubulin* (AAEL002851) (800 bp). Cells were grown at 37 °C and 180 rpm until the optical density measured at 600 nm (OD_{600}) reached 0.4 or 0.8. dsRNA production was then induced via addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to a final concentration of 0.4 mM and cells grown for another 4 h. Cells were harvested at 4 °C for 15 min at 3214 rcf, resuspended in clean LB-Lennox medium, aliquoted in batches of 10 OD or 2 OD in 2 mL-tubes, collected via centrifugation and stored at −80 °C until further use.

4.3. dsRNA Extraction from HT115 DE3 Cells via Phenol/Chloroform/Isoamyl Alcohol Extraction

Extraction of dsRNA using P/C/I was performed with the following modifications from the published phenol–guanidine-based protocol [29]: bacterial cells were resuspended in 12.5 µL of 0.1% sodium dodecyl sulfate (SDS) per one OD of cells and incubated for 2 min at 95 °C. Then, 16.25 µL of neutral (pH 7.5) solution of 300 mM sodium acetate, 10 mM Tris-HCl, 5 mM EDTA, 1 µg RNase A per 1 OD cells were added to the lysed cells and incubated at 37 °C for 15 min. One volume of Roti® Aqua phenol/chloroform/isoamyl alcohol (P/C/I, 25:24:1, pH 4.5–5) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added to the mixture and vortexed at full speed for 15 s. After incubating at 65 °C for 15 min, the tubes were centrifugated at room temperature for 15 min at 17,949 rcf. The aqueous upper phase was transferred to a fresh 2 mL-tube without disturbing the protein interphase or the organic lower phase. Ethanol precipitation was performed by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol to the aqueous phase, followed by vortexing for 5 s. After precipitation for 1 h at −20 °C, dsRNA was pelleted at 17,949 rcf for 30 min at 4 °C. The dsRNA pellet was washed twice with 70% (*v*/*v*) cold ethanol, air dried, dissolved in 100 µL of nuclease-free water, incubated at 55 °C for 15 min and immediately placed on ice. For comparison with the original phenol–guanidine-based protocol, bacterial cells were also processed using TRIzol™ (Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) as published in the literature [29]. Concentration and quality of purified dsRNA was assessed via absorbance measurement and gel electrophoresis, respectively.

4.4. Removal of High-Molecular-Weight Nucleic Acid Contamination by DNase Digestion

Removal of high-molecular-weight nucleic acid contamination from dsRNA obtained via the phenol–guanidine-based extraction with TRIzol™ [29] was evaluated via DNase digestion. The final product from TRIzol™ extraction was resuspended in nuclease-free water and DNase digestion was performed at room temperature for 15 min. A mock digestion was performed for comparison. Following DNase digestion, the nucleic acids were extracted with P/C/I (25:24:1, pH 7.5–8) and precipitated with ethanol. Concentration measurement and quality assessment via gel electrophoresis were performed as described above.

4.5. Cell Lysis Efficiency Testing

Efficiency of cell lysis by boiling HT115 (DE3) cells at 95 °C for two minutes after resuspension in 12.5 µL of SDS 0.1% (*w*/*v*) per OD_{600} of cells was evaluated through a cell viability assay. After boiling, cells were homogenized and spread into LB-agar 1.5% (*w*/*v*) plates containing ampicillin (100 µg/mL). As control, cells were resuspended in the same volume of sterile LB-medium 2% (*w*/*v*) and incubated at room temperature for two minutes. Then, cells were homogenized and aliquots of the undiluted cells suspension and of 1:100 dilution were spread into LB-agar plates. All plates were incubated overnight at 37 °C before evaluation of colony development. The experiments were performed in biological duplicates.

4.6. Assessment of pH and Serial P/C/I Extractions on dsRNA Purity

The protocol described in Section 4.3 was tested in four different modifications to further improve dsRNA purity: (1) cell lysis was performed at 95 °C for 2 min in 0.1% (*w*/*v*) SDS, 0.1 M sodium acetate, pH 4.5. The rest of the protocol remained unchanged. (2) the RNase A digest was performed in 300 mM sodium acetate, 10 mM Tris-HCl, 5 mM EDTA, pH 4.5. The rest of the protocol remained unchanged. (3) The reaction was acidified by addition of 2.5 volumes of 0.1 M sodium acetate, pH 4.5 before the addition of one-volume equivalent (1000 µL) of P/C/I (25:24:1, pH 4.5–5.0). The rest of the protocol remained unchanged. (4) The product from the first P/C/I extraction, obtained as described in 4.3 and resuspended in 100 µL of nuclease-free water, was mixed with 300 µL of either 0.1 M sodium acetate at pH 4.5 or 0.2 M sodium phosphate dibasic at pH 6.8 or 0.1 M sodium bicarbonate/sodium carbonate at pH 9.9. Then, a second extraction was performed using 400 µL of P/C/I (25:24:1, pH 4.5–5). The downstream steps of dsRNA ethanol precipitation, washing, and resuspension in 100 µL of nuclease-free water, were performed as described in Section 4.3. Concentration measurement and quality assessment via gel electrophoresis of the obtained dsRNA from each experiment were performed as described above.

4.7. Optimization of the Induction OD_{600}

dsRNA was extracted from bacterial cells induced to produce dsRNA at OD_{600} of 0.4 or 0.8 using a phenol–guanidine-based protocol [29], two ethanol-based extraction protocols published in the literature [32,33], and the final version of the protocol developed in this study (two-step acidic P/C/I extraction, see Section 4.6). All extractions were performed as published with the exception of the phenol–guanidine-based protocol, where the RNase digestion was performed for 15 min instead of 5 min as originally published. Concentration measurement and quality assessment via gel electrophoresis were performed as described above. Extraction yields and dsRNA purity after induction at different cell densities was compared between all four protocols.

4.8. Systematic Comparison of dsRNA Extraction Protocol Efficiencies

To compare the yield and purity of phenol–guanidine-based [29], ethanol-based [32,33], and alkaline phenol-based [30,31] extraction protocols with the protocol developed in this study, dsRNA production in HT115 DE3 cells was induced at an OD_{600} of 0.4. Experiments were performed in three biological replicates and two technical replicates each. Importantly, the quantitative yields were calculated considering the initial amount of cells and volume

of nuclease-free water in the final resuspension step of each protocol. The method by Ongavarrasopone et al. [29] was followed as published, except for an RNase A digestion time of 15 min instead of 5 min. The methods established by Posiri et al. [32] and Papic et al. [33] were followed in their entirety as published. The protocol established by Ahn et al. [31] was adapted by opening the cells via homogenization in tubes containing ceramic beads in a tissue homogenizer (Precellys®, Bertin Instruments, Montigny-le-Bretonneux, France) for two runs at 6000 rpm for 20 s with an intermediate incubation on ice for 1 min. The method established by Solis et al. [30] was followed in its entirety, with exception of using Monarch DNase I (1 U/µL) and Monarch DNase I reaction buffer (New England Biolabs, Inc., Ipswich, MA, USA) for DNase digestion instead of Turbo DNase and DNase buffer (Ambion/Life Technologies, Thermo Fischer Scientific Inc., Waltham, MA, USA). These results were compared to the final version of the protocol developed in this study: Bacterial cells were resuspended in 12.5 µL of 0.1 % (*v*/*v*) SDS per OD of cells and incubated at 95 °C for 2 min. Then, 16.25 µL of 300 mM sodium acetate, 10 mM Tris-Cl, 5 mM EDTA, pH 7.5, 1 µg of PureLink RNase A per OD of cells were added and incubated at 37 °C for 15 min. The equivalent volume of P/C/I (25:24:1, pH 4.5–5) was added, thoroughly mixed for 15 s and incubated at 65 °C for 15 min. After centrifugation at 17,949 rcf for 15 min at 20 °C, the aqueous phase containing dsRNA was carefully transferred to a new tube without disturbing the protein interphase and the organic lower phase. Ethanol precipitation was performed by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol to the aqueous phase, followed by vortexing for 5 s and precipitation for 1 h at −20 °C. The dsRNA was pelleted at 17,949 rcf for 30 min at 4 °C and washed once with 70% (*v*/*v*) cold ethanol. The air-dried dsRNA pellet was dissolved in 100 µL of nuclease-free water and incubated at 55 °C for 10 min. Optional DNase digestion was performed with 25 µL DNase I mix and incubated at 30 °C for 15 min. Then, 375 µL of 0.1 M acetate buffer at pH 4.5 was added before repeating the P/C/I extraction and ethanol precipitation as described above, this time applying an additional ethanol wash step. Finally, the dsRNA pellet was dissolved in 100 µL of nuclease-free water, incubated at 55 °C for 10 min, and immediately placed on ice. DsRNA yields were calculated as total amount of nucleic acid quantified via spectrophotometry at 260 nm divided by the total OD of bacterial cells used for extraction to result in µg dsRNA/1 OD of cells. The quality of extracted dsRNA was inspected via gel electrophoresis analyzing the same volume of extracted dsRNA from each technical and biological replicates from each extraction method.

4.9. Analysis of Band Intensity from Gel Electrophoresis

Images acquired from the gel documentation system were analyzed using the software Adobe Photoshop 2023 (Adobe Inc., 2023, San José, CA, USA). In general, the bands or the region of interest or the complete lane were identified and their integrated density was quantified.

4.10. Statistical Analysis

Data analysis was carried out using the software SigmaPlot (Version 14.0, Systat Software Inc., San José, CA, USA) and MiniTab® (Minitab, LLC., State College, CA, USA). Yields of nucleic acids extracted from bacterial cells induced to produce dsRNA at OD_{600} of 0.4 or 0.8, and yield of extracted dsRNA using different published methods and the optimized version of our protocol were analyzed via one-way analysis of variance (ANOVA), with Shapiro–Wilk test for normality and Brown–Forsythe test for equal variance. Holm–Sidak method was used for multiple comparison of the data from each condition, when the differences in the mean values among the groups were greater than would be expected by chance ($\alpha = 0.05$). Data of the relative dsRNA band intensity, which failed Shapiro–Wilk test for normality, underwent a Box Cox transformation [39] prior to conducting the ANOVA. For the comparative analysis of the amounts of nucleic acids extracted using different published methods and the optimized version of our protocol, the yields of nucleic acid per OD_{600} for each extraction method were analyzed via Welch's test, which does not assume equal

variances for the analysis. The means were compared with the Games–Howell pairwise comparison at 95% confidence level.

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Supplementary information for "An optimized/scale up-ready protocol for extraction of bacterially produced dsRNA at good yield and low costs"

dsRNA extraction protocol

Notes: Reactions are exemplified for extraction from bacterial pellets 10xOD cells. Scale-up as needed,

1. Calculate the total buffer volumes needed for 0.1 % SDS and for the RNase A. Prepare the buffers accordingly:
   a. 12.5 µL of 0.1% SDS per 1 OD/mL cells.
      - 125 µL of 0.1% SDS
   b. 16.25 µL of 300 mM sodium acetate, 10 mM Tris-Cl pH 7.5, and 5 mM EDTA, 1 µg RNase A per 1 OD/ml cells. Mix very well (vortex for ~ 5 sec).
      - 162,5 µL of TRIS buffer containing 10 µg of RNase A

Prepare a 2 mL tube with 2 x 720 µL of TRIS + 4.46 µL of RNase

2. Set heat blocks to 95 °C and 37 °C
3. Resuspend the bacterial pellet using the 0.1% SDS buffer. Mix by pipetting up and down. Use 2 mL tubes.
4. Boil at 95 °C for 2 min to lyse the cells. Switch the heat block to 65 °C.
5. Add the TRIS/RNase A buffer to each tube according to the calculation made on step 1) b.
6. Incubate tubes at 37 °C for 15 min
7. Add 1 volume of PCI (25:24:1) for RNA extraction (pH: 4.5) and mix well by vortexing (15 sec).
8. Incubate at 65 °C for 15 min.
9. Centrifuge at room temperature (20°C) at 13,000 rpm for 15 min
10. Transfer the upper phase to fresh 2 mL tubes, avoid the interphase and lower phase. Use 200 µL pipette! Aim for 75%, not more than 80%, of the liquid phase volume.

Note: If the volume of the aqueous phase is higher than 550 µL, transfer half of the aqueous phase to a fresh 2 mL tube and proceed to ethanol precipitation.

11. Add to each tube:
    a. 1/10 volume of 3 M NaOAc pH 5.2. Mix well by vortexing (5 sec).
    b. 2.5 volumes of 100 % EtOH. Mix well by vortexing (5 sec).
12. Precipitate for at least 1 hour at – 20 °C.
13. Pellet the precipitated RNA at 13000 rpm for 30 min at 4 °C.
14. Wash pellet with 500 µL of ice-cold 70% ethanol, vortex briefly, centrifuge at 13,000 rpm for 15 min at 4°C.
15. Discard the supernatant. Make sure the pellet is well preserved.

Note: after removing the supernatant, spin down the tube and use a pipette to remove any leftover of ethanol

16. Resuspend pellet with 100 µL of nuclease-free water and incubate at 55 °C for 10 min

Note: Protocol can be interrupted here by storing samples at -80 °C to continue purification later

17. Prepare fresh 0.1 M acetate buffer pH 4.5
18. If stored at -80°C, thaw samples on ice.
19. Set heat block to 30°C

Steps 21 and 22 are optional, if DNAse digestion is desired.

20. Prepare DNase mix in a clean 1.5 mL tube by mixing 30 µL of Monarch DNase I + 30 µL of Monarch DNase I Reaction Buffer.
21. Incubate all tubes at 30°C for 15 min
22. Add 3 volumes of 0.1 M acetate buffer (pH ~4.5) and repeat P/C/I extraction
23. Add 1 volume of P/C/I (25:25:1) for RNA extraction (pH 4.5) and mix well by vortexing (15 s).
24. Incubate at 65°C for 15 min.
25. Centrifuge at 20°C for 15 min at 13000 rpm.
26. Transfer the upper phase to fresh 2 mL tubes. Avoid the inter- and lower phase.

Note: depending on the experience of the user, using a 200 µL pipette might be easier to avoid disturbing the interphase!

If the volume of the aqueous phase if higher than 550 µL, transfer half of the aqueous phase to a fresh 2 mL tube and proceed to ethanol precipitation.

Ethanol precipitation

27. Add to each tube:
    a. 1/10 volume of 3 M NaOAc pH 5.2. Mix well by vortexing (5 s)
    b. 2.5 volumes of 100 % EtOH. Mix well by vortexing (5 s).
28. Precipitate for at least 1 hour at – 20 °C.
29. Pellet the precipitated RNA at 13,000 rpm for 30 min at 4°C.
30. Wash the pellet with 500 µL of ice-cold 70% Ethanol, short vortexing to dislodge the pellet from the tube wall.
31. Centrifuge at 4°C for 15 min at 13000 rpm and remove the washing solution.
32. Repeat the wash step with centrifugation for 15 min.
33. Remove the washing solution as completely as possible by quick-spinning the tube after taking the majority of the supernatant out. Then, remove the rest of the liquid with a fine pipette tip.
34. Air dry the pellets and resuspend with 100 µL of nuclease-free water.
35. Gently pipet up and down to homogenize. Incubate at 55°C for 10 min, then place tubes immediately on ice.
36. Asses the quality and quantity of extracted RNA by established methods, as spectrophotometry and gel electrophoresis.

3.2 Challenges of robust RNAi gene silencing in *Aedes* mosquitoes

Following the optimization of dsRNA production, I investigated the efficacy of RNAi in controlling *Aedes* mosquitoes, with a particular focus on the reproducibility of previously reported RNAi effects achieved through various oral delivery methods. The research spanned various delivery methods and architecture of the RNA molecule triggering RNAi, including shRNA, dsRNA and siRNA, targeting genes implicated in mosquito viability. Additionally, I also explored the impact of different food formulations on RNAi effectiveness. Despite of following detailed published protocols and intensive troubleshooting, it was not possible to reproduce previously reported strong RNAi effects in *Aedes* mosquitoes. Similar results were also independently observed in other two research groups. Therefore, we decided to collectively publish a manuscript with data obtained independently in three laboratories. Our joint publication underscores the necessity for rigorous validation of RNAi-based strategies in vector control, highlighting potential challenges in their future practical implementation.

Title: Challenges of robust RNAi-mediated gene silencing in *Aedes* mosquitoes.

Authors: **Lucas Henrique Figueiredo Prates**, Jakob Fiebig, Henrik Schlosser, Eleni Liapi, Tanja Rehling, Célia Lutrat, Jeremy Bouyer, Qiang Sun, Han Wen, Zhiyong Xi, Marc F. Schetelig, Irina Häcker.

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Justus-Liebig University

- Production of dsRNA:	Lucas Henrique Figueiredo Prates
- Feeding assays, soaking and larval microinjections with dsRNA and siRNA:	Lucas Henrique Figueiredo Prates, Jakob Fiebig, Henrik Schlosser, Irina Häcker
- Analysis of gene expression:	Lucas Henrique Figueiredo Prates, Tanja Rehling, Irina Häcker

- Cloning of expression vectors for dsRNA expression in bacterial cells and shRNA in yeast:	Irina Häcker, Tanja Rehling, Lucas Henrique Figueiredo Prates
- Feeding assays with shRNA:	Eleni Liapi, Irina Häcker, Tanja Rehling
- Embryonic microinjections with dsRNA:	Tanja Rehling, Irina Häcker, Lucas Henrique Figueiredo Prates

ASTRE, CIRAD

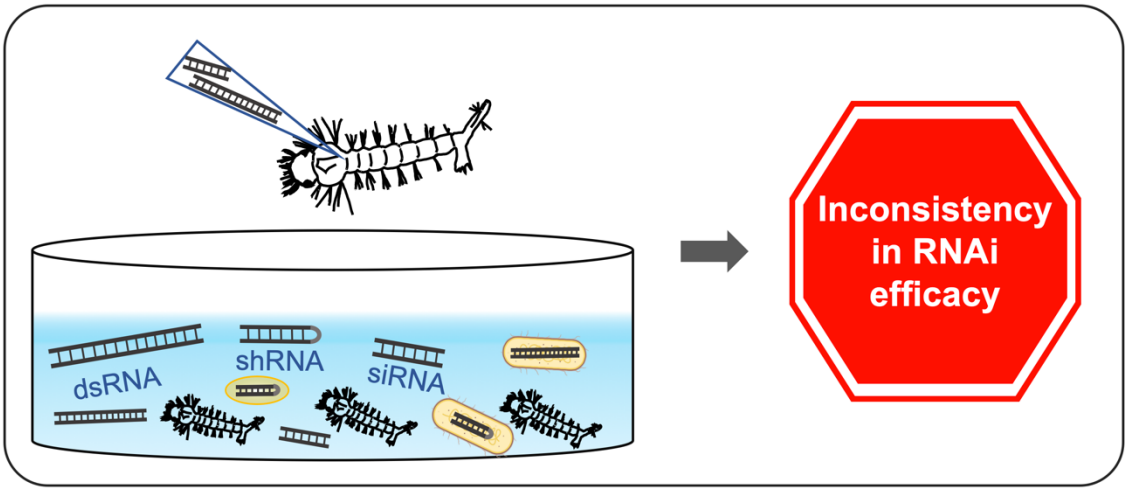
- Cloning of expression vectors for shRNA expression in bacterial cells and production of shRNA;	Célia Lutrat, Jeremy Bouyer
- Feeding assays with shRNA:	

Michigan State University

- Production of dsRNA;	Qiang Sun, Han Wen, Zhiyong Xi
- Larval soaking in dsRNA;	
- Analysis of gene expression:	

Presentations:

- Science flash talk on "Development of efficient sustainable control systems for disease transmitting mosquitoes" during the virtual DAAD Scholarship Holder Meeting (May, 2021).



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Challenges of Robust RNAi-Mediated Gene Silencing in *Aedes* Mosquitoes

Lucas Henrique Figueiredo Prates [1], Jakob Fiebig [1], Henrik Schlosser [1], Eleni Liapi [2], Tanja Rehling [1], Célia Lutrat [3], Jeremy Bouyer [3,4], Qiang Sun [5], Han Wen [5], Zhiyong Xi [5], Marc F. Schetelig [1,*] and Irina Häcker [1]

1 Department of Insect Biotechnology in Plant Protection, Justus Liebig University Giessen, 35394 Giessen, Germany; lucas.prates@agrar.uni-giessen.de (L.H.F.P.); jakob.fiebig@umwelt.uni-giessen.de (J.F.); h.schlosser@uq.edu.au (H.S.); tanja.rehling@agrar.uni-giessen.de (T.R.); irina.haecker@agrar.uni-giessen.de (I.H.)
2 Department of Biochemistry and Biotechnology, University of Thessaly, 41500 Larissa, Greece; eliapi@outlook.com
3 ASTRE, CIRAD, 34398 Montpellier, France; jeremy.bouyer@cirad.fr (J.B.)
4 ASTRE, CIRAD, INRAE, Univ. Montpellier, Plateforme Technologique CYROI, 97491 Sainte-Clotilde, La Réunion, France
5 Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA; sqiang617@gmail.com (Q.S.); wenhan@msu.edu (H.W.); xizy@msu.edu (Z.X.)
* Correspondence: marc.schetelig@agrar.uni-giessen.de

Abstract: In this study, we report the complexities and challenges associated with achieving robust RNA interference (RNAi)-mediated gene knockdown in the mosquitoes *Aedes aegypti* and *Aedes albopictus*, a pivotal approach for genetic analysis and vector control. Despite RNAi's potential for species-specific gene targeting, our independent efforts to establish oral delivery of RNAi for identifying genes critical for mosquito development and fitness encountered significant challenges, failing to reproduce previously reported potent RNAi effects. We independently evaluated a range of RNAi-inducing molecules (siRNAs, shRNAs, and dsRNAs) and administration methods (oral delivery, immersion, and microinjection) in three different laboratories. We also tested various mosquito strains and utilized microorganisms for RNA delivery. Our results reveal a pronounced inconsistency in RNAi efficacy, characterized by minimal effects on larval survival and gene expression levels in most instances despite strong published effects for the tested targets. One or multiple factors, including RNase activity in the gut, the cellular internalization and processing of RNA molecules, and the systemic dissemination of the RNAi signal, could be involved in this variability, all of which are barely understood in mosquitoes. The challenges identified in this study highlight the necessity for additional research into the underlying mechanisms of mosquito RNAi to develop more robust RNAi-based methodologies. Our findings emphasize the intricacies of RNAi application in mosquitoes, which present a substantial barrier to its utilization in genetic control strategies.

Keywords: RNA interference; *Aedes aegypti*; *Aedes albopictus*; RNAi-based pest control; RNAi delivery methods; reproducibility of RNAi protocols

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1. Introduction

RNA interference (RNAi) is a naturally occurring defense mechanism against foreign genetic material that has been exploited as a molecular technique primarily for reverse genetics studies by post-transcriptional gene silencing (PTGS) [1–5]. RNAi-based gene silencing can be triggered by double-stranded RNA (dsRNA), which can occur naturally or be supplied by the user, and that causes the degradation of the complementary gene transcript. Once incorporated, the dsRNA is first processed into smaller sequences of approximately 19 to 25 nucleotides, called small interfering RNAs (siRNAs). Then, one

strand of the siRNA is loaded into the RNA-induced silencing complex (RISC), which localizes the complementary messenger RNA (mRNA), leading to its degradation and gene silencing [3,5–10]. Instead of dsRNAs, which are usually hundreds of base pairs long, two complementary short RNA sequences of approximately 19 to 22 nucleotides linked by a short loop ranging from 4 to 11 nucleotides (short hairpin RNA, shRNA) could also be used to generate siRNA molecules to trigger RNAi [4,6,11]. Thus, different RNA architectures, such as dsRNAs, shRNAs, and siRNAs, can be used to induce RNAi. Herein, we refer to these molecules collectively as interfering RNAs (iRNAs).

RNAi has been successfully applied to reverse genetics studies on insects, including mosquitoes [12–14]. More recently, this technology has gained much attention as a promising new tool for insect pest control [4,15–18]. The potential of RNAi is based on the possibility of designing iRNA sequences to precisely target only the transcript of a particular species, avoiding off-targets in related species. Targeting genes essential for development or fundamental physiological or metabolic processes aims to kill pest species during developmental stages without affecting other species. Thus, iRNAs could act as a species-specific insecticides. A different approach targets genes involved in sex determination [19–22] or male or female fertility [23–25] to reduce the population size of the next generation by producing a sex bias, thereby reducing the availability of mating partners or the number of offspring per individual, respectively. An RNAi-based sexing approach could also be used for genetic control methods based on the release of males [26], as there is currently no perfect genetic sexing strain available [27], and most of the programs upscaling mosquito genetic control are based on automated sorters using the phenotypical differences between pupae [28,29] or adults [30].

For mosquitoes, several encouraging studies have been published over the past ten years presenting a strong sex bias or high sterility or larval mortality upon RNAi-mediated gene knockdown [19,31–36]. Generally, iRNA molecules that knocked down mosquito transcript levels were delivered as siRNAs [22,31,35,37], shRNAs [31,35,37–39], or >200 bp dsRNAs [19,31,36,40–45]. Application strategies for the iRNA molecules included soaking of larvae in iRNA solutions, feeding of adults with iRNA–sugar solutions, feeding of iRNA-expressing microorganisms (bacteria, yeast, and microalgae) to mosquito larvae, and injections of iRNA during different developmental stages [46]. Moreover, nanoparticles were used for protection and the better delivery of iRNA [32,45,47–52]. The oral delivery of iRNA molecules to larvae or adults would be the method of choice for mosquito control applications.

However, the level of iRNA-mediated gene silencing can vary strongly across insect orders, families, or species, and even within one species. Variance in RNAi effects has also been observed in *Aedes* mosquitoes [36,47,51,53–55]. While the reasons are not well understood in many cases, several factors have been associated with the success, failure, or variability of RNAi in insects and have been reviewed extensively [4,18,56]. Among these are the presence of gut RNases decreasing the amount of bioavailable iRNA molecules, the efficiency of iRNA uptake from the gut lumen during oral application, the accessibility of the iRNA to the intracellular RNAi machinery, and the amplification and spread of the RNAi signal from the cells that initially take up the molecules (systemic RNAi). Also, the conformation and length of the iRNA and the targeted region have been associated with RNAi efficiency [4,18,56]. Overall, the mechanisms involved in insect RNAi, especially mosquito RNAi, are poorly understood. The successful application of RNAi as a tool for mosquito control, however, will require a robust RNAi response, independent of external and internal variables like temperature or humidity, the availability of other food sources, the genetic background of the targeted mosquito populations, or factors like the nutritional condition, development stage, or overall fitness of the targeted individuals.

In three different laboratories, we initially and independently aimed to establish RNAi by oral delivery in the mosquitoes *Aedes aegypti* and *Ae. albopictus*. The goal was to identify genes that are essential for the development and fitness of the insects and that produce strong phenotypes upon knockdown. During this process, we collectively noticed that oral

delivery of iRNAs failed to reproduce previously reported strong RNAi effects, causing us to troubleshoot the possible reasons extensively and systematically. This included different iRNA delivery methods besides oral application, in combination with various iRNA architectures, such as siRNAs, shRNAs, and dsRNAs. Surprisingly, also with other delivery strategies the published results for the tested positive target genes could not be replicated. This raises doubts about the robustness of RNAi as a methodology in *Aedes* and implies that the complex underlying mechanisms are not yet understood well enough to make it a reliable method, which would also have implications for the use of RNAi for mosquito control.

2. Results

2.1. Variable RNAi Effects in Ae. aegypti upon Oral Delivery of iRNA-Expressing Microorganisms

2.1.1. Feeding Larvae with shRNA-Producing Yeast Strains

Based on several publications in recent years, the use of shRNA-expressing yeasts delivered orally to *Ae. aegypti* larvae seems to be one of the most reliable and effective ways to knock down gene expression via RNAi in this mosquito species [31,33,34,38]. To establish this oral delivery method in our lab, we set out to replicate the published results, following the detailed information provided in the literature for the design and cloning of shRNA sequences [35] and for the execution of yeast transformation, yeast culturing, and larval feeding assays [38].

Yeast shRNA feeding assays were performed with *semaphorin-1a* (*sem-1a*) [35], *fasciculation and elongation protein zeta2* (*fez2*), and *leukocyte receptor cluster member 8 homolog* (*lrc*) as target genes and with a scrambled shRNA as a negative control [31]. All three genes have been reported to cause up to 90% larval mortality until the L4 larval stage. The shRNA was provided to the larvae as dried yeast pellets, which were replaced with fresh ones as needed or, at the latest, every second day. *Sem-1a* and control shRNA assays were performed in eight biological replicates, and *lrc* and *fez2* assays were performed in four biological replicates, all distributed across two wild-type laboratory strains, namely, Orlando and Liverpool. The event of successful pupation was counted as survival. A significant reduction in larval survival or pupal transcript levels was observed in single experiments compared to the controls (Figure S1). Still, these reductions did not occur in the other replicates and were much lower than the published effects. Across all biological replicates, there was no significant difference in the survival rates between the control and the target genes for all three targets (p-values = 1.00 (*sem-1a*), 1.00 (*fez2*), 0.088 (*lrc*), one-way ANOVA, Bonferroni t-test) (Figure 1a).

In two large-scale feeding experiments with three technical replicates, performed once with Liverpool and once with Orlando larvae, we included sampling of batches of five larvae (on days 3 and 5 of the feeding) and five pupae for an RT-qPCR analysis. This analysis showed a moderate but significant reduction in *sem-1a* (p-value = 0.0141, one-sample t-test) and *fez2* (p-value = 0.0290, one-sample t-test) transcript levels in the 5-day larvae with the Orlando strain but not in the 3-day larvae or the pupae. The *lrc* transcript levels in Orlando did not differ from those in the controls. No effect was observed in the identical assay with the Liverpool lab strain (Figure 1b–g). The individual survival rates for these two experiments are shown in Figure S1, and the survival numbers and relative transcript ratios of all replicates performed are listed in Table S1.

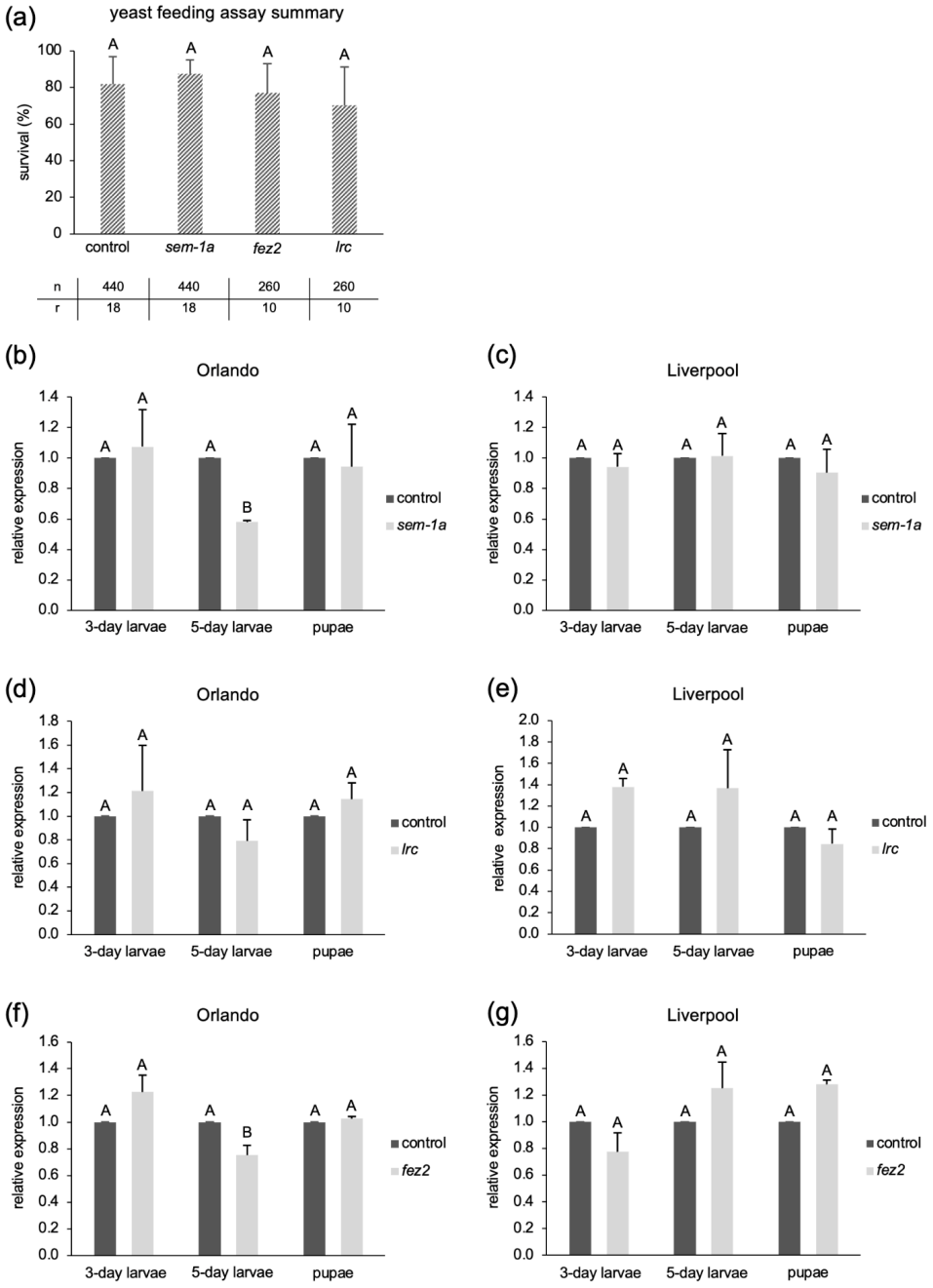

Figure 1. Results of oral delivery of shRNA-expressing yeast to *Ae. aegypti* larvae of the Orlando or Liverpool strains. Larvae were fed with the yeast 16–20 h after hatching until pupation. (**a**) Average survival rate to pupal stage (in percent) across all feeding assays performed per target gene, including eight biological replicates for *sem-1a* and the control shRNA, and four biological replicates for *lrc* and *fez2*. All biological replicates were performed with 2–3 technical replicates (20 to 30 individuals per replicate);

n = total number of individuals used in all combined replicates, r = total number of replicates performed. Panels (**b**–**g**) show target gene mRNA levels in larvae sampled after 3 or 5 days of yeast feeding or after pupation, determined by RT-qPCR, calculated by the Pfaffl method [57], using *rps17* as reference gene. Data shown are based on three technical replicates. Five larvae were pooled from each replicate. In panels (**a**–**g**), "control" is the feeding with the unspecific shRNA, *sem-1a* = *semaphorin-1a*, *fez2* = *fasciculation and elongation protein zeta2*, *lrc* = *leukocyte receptor cluster*. Error bars indicate standard deviation, and different letters above the bars indicate statistically significant differences between gene-specific shRNA treatments and the unspecific control with p-value < 0.05 (one-way ANOVA, Bonferroni t-test in (**a**), and one-sample t-test in (**b**–**g**)).

2.1.2. Feeding Larvae with Different Concentrations of dsRNA-Producing Bacteria

Other published oral delivery strategies that yielded sound RNAi effects are soaking early larvae in concentrated dsRNA or siRNA solutions [31,36] and feeding dsRNA-expressing bacterial strains to early larvae. In the latter assay, pelleted bacterial cells are mixed with LB agar and ground fish food to produce food pellets that are provided to the larvae daily until pupation [19]. The bacterial expression system is the widely used HT115 DE3 RNase III-deficient strain with the inducible L4440 expression vector [58–60].

One target gene, *beta-tubulin* (*βtub*, AAEL002851), was reported to yield high rates of *Ae. aegypti* late larval lethality by repeated soaking of L1 larvae in a 500 ng/µL solution of an 800 bp in vitro transcribed dsRNA [36]. We cloned the *βtub* dsRNA sequence from Singh et al. [36] into the L4440 expression vector and confirmed dsRNA expression by extraction from the bacterial cells [61].

Following the outline given in the bacterial feeding protocol [19], we produced food pellets by mixing bacterial cells from a 100 mL culture with 5 mL of LB-agar and ground fish food (food variant 1, 1X bacterial concentration) and fed them to *Ae. aegypti* larvae until pupation, starting with L1 larvae hatched overnight, and replacing food pellets as needed or, at the latest, after 48 h. This procedure did not yield significantly higher mortality than the bacteria expressing a 400 bp *eGFP* control dsRNA (Figure 2a). Therefore, we increased the amount of bacterial culture per food volume by 2.5- and 5-fold and used another version of the *βtub* gene (AAEL004939) [19], as well as the combination of both *βtub* versions at different bacterial concentrations. However, none of the experiments yielded a significantly higher larval death compared to the *eGFP* dsRNA control treatments (Figure 2a, p-values = 0.630, 0.882, 0.303, 0.568, 0.738, 0.597, 0.0107, Welch's t-test).

We finally increased the amount of bacterial cells by 15-fold compared to the starting amount (food variant 2). To exclude RNAi inefficiency due to the refractoriness of the *βtub* target gene in our strain, we also included more positive control target genes, *fez2*, *lrc*, and *sem-1a*, from the yeast shRNA feeding publications [31,35]. The corresponding dsRNA sequences were designed to include the published siRNA target regions for these genes (see Table S2H). Moreover, additional targets, *acetylcholine esterase 1* (*ache1*) and *vacuolar-type ATPase* (*V-ATPase*), which showed potent lethal effects in other insects when knocked down by RNAi [62], were included. Despite the increased amount of bacteria per food volume, there was no significant difference in the larval survival rates between the *eGFP* control and any of the target genes (p-value = 0.324, one-way ANOVA, Welch's test, Figure 2b).

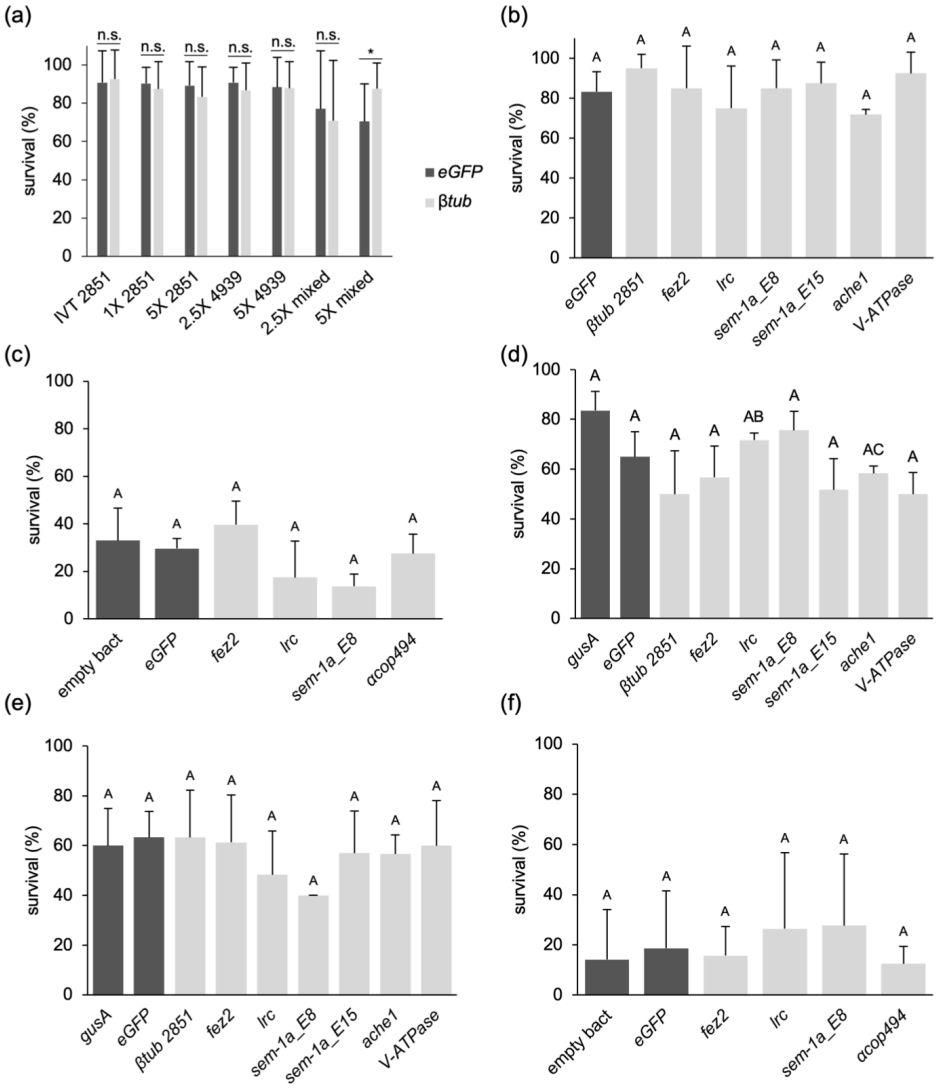

Figure 2. Survival rates of *Ae. aegypti* larvae fed with dsRNA-expressing bacteria. Panels (**a**,**b**): bacterial pellets with high nutrient content. (**a**) Food variant 1, containing different bacterial doses (1X, 2.5X, 5X). All results represent one biological replicate with 12 technical replicates each (except for *βtub 4939* 5X, which has two biological replicates). IVT represents the result of larval soaking in 500 ng/µL of in vitro transcribed AAEL002851 dsRNA (one biological replicate with 12 technical replicates) based on the protocol in [36]. "mixed" means that larvae were fed with a mixture of bacteria expressing dsRNAs against AAEL002851 and AAEL004939. (**b**) Food variant 2, data presented are based on two biological replicates with 20 larvae each. The bacteria used per food volume was 15X, i.e., 3-fold higher than the highest in (**a**). Panels (**c**–**f**) show feeding with reduced nutrient content. (**c**) Food variant 3, two biological replicates with 30 and 40 larvae each. (**d**) Food variant 4, addition of fish food on day 11; three biological replicates with 20 larvae each. (**e**) Food variant 4, addition of fish food on days 11, 13, 15; three biological replicates with 20 larvae each. (**f**) Food variant 5, two biological replicates with

40 larvae each. Average survival rates are shown (in percent), and error bars represent standard deviation. n.s. indicates no statistical significance, * indicates a significant difference with p-value < 0.05 (Welch's t-test in (**a**)). Different letters above the bars indicate statistically significant differences with p-value < 0.05 (one-way ANOVA, Welch's test in (**b**,**c**,**d**,**f**); one-way ANOVA in (**e**)). Additionally, 2851 = *βtub* gene AAEL002851, 4939 = *βtub* gene AAEL004939, *ache1* = *acetylcholine esterase 1*, *acop494* = *coat protein alpha* (494 bp dsRNA), *eGFP* = *enhanced green fluorescent protein*, empty bact = bacteria not expressing any dsRNA, *fez2* = *fasciculation and elongation protein zeta2*, *gusA* = *E. coli beta-glucuronidase*, *lrc* = *leukocyte receptor cluster member 8 homolog*, *sem-1a* = *semaphorin-1a*; *sem-1a-E8* and *sem-1a-E15* are two different dsRNAs, targeting *sem-1a* exon 8 and exon 15, respectively. In exon 8, the siRNA target sequence is from [63]; in exon 15, the siRNA target sequence is from [35]. *V-ATPase* = *vacuolar-type ATPase*.

We extracted dsRNAs from the bacterial cells to exclude the lack of RNAi-induced mortality due to the lack of bacterial expression of the corresponding dsRNA. Gel electrophoresis revealed strong bands corresponding to the expected sizes for each dsRNA that was unique to the respective bacterial strain, indicating that all strains correctly expressed the dsRNAs (Figure 3).

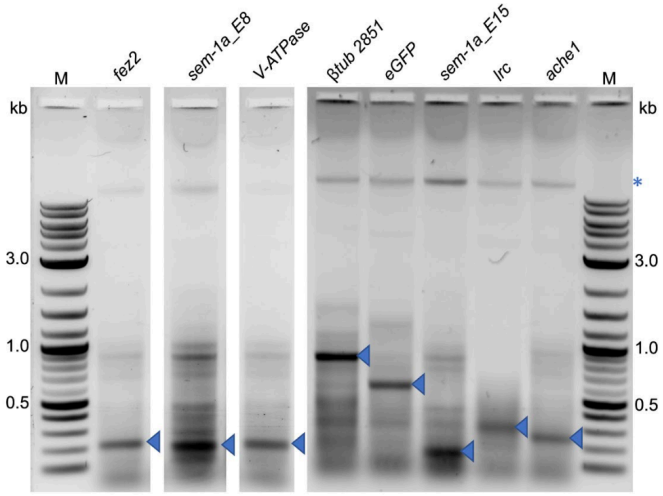

Figure 3. Gel electrophoresis of bacterially produced dsRNAs after phenol/chloroform/isoamyl alcohol extraction from bacterial cells [61]. Blue arrows indicate the expected size of the dsRNA. The blue asterisk indicates bacterial chromosomal DNA. M = 1 kb plus ladder (New England Biolabs Inc., Ipswich, MA, USA); kb = kilobases; for target gene name abbreviations, see Figure 2.

2.1.3. Further Enhancement of Bacteria Ingestion Does Not Improve the RNAi Effect

We hypothesized that fast larval growth, promoted by the high nutrient content in the bacterial food pellets used so far, limits the volume of food (i.e., bacteria) intake, thereby keeping the amount of ingested dsRNA below a biologically relevant threshold. If this is correct, a lower nutrient content in the bacterial feeding pellets could enhance the consumption of the bacterial cells, i.e., dsRNA. Moreover, a slower larval development would increase the dsRNA action time. We therefore decided to evaluate food formulations with a reduced nutrient content for RNAi efficiency. These experiments included an additional target gene, *coat protein (coatomer) alpha* (*αcop*), and bacteria transformed with the empty expression plasmid L4440 as an additional negative control.

Food variant 3 consisted of bacteria mixed with LB-agar, corresponding to a 5X bacterial dose per food volume. To support larval development towards the end of the experiment, the larvae were supplied with baker's yeast after the end of the bacterial pellet

feeding. We also performed a parallel feeding assay with fish food to obtain a standard for development time and survival rate under normal rearing conditions. The reduced nutrients resulted in a slower development time—from 13 days for the first pupation to up to 33 days until the last pupation or the death of the larvae. In contrast, the larvae fed with fish food had all pupated between 5 and 8 days and showed a >90% pupation rate. While an overall high lethality was observed in all feedings with the bacteria–LB-agar pellets, it could not be assigned to the dsRNA treatment (p-value = 0.355, one-way ANOVA, Welch's test, Figure 2c).

With food variant 4, the last extra nutrients besides the bacteria, i.e., the salts from the LB medium, were omitted. Thus, the food consisted only of bacteria and agar. Moreover, the relative amount of bacteria per food volume was increased again to 15X. Two experiments with small differences in the feeding regime were performed (Table S3). In experiment 1, the larval age at feeding start was ~20 h; in experiment 2, the larvae were less than one hour old to test for the effect of very early exposure to the dsRNA.

In both experiments, we again observed delayed development (pupation onset 13–15 days after feeding start) and overall increased mortality, which was not different between the targets and the control dsRNAs *gusA* or *eGFP* (experiment 1: p-value = 0.017, one-way ANOVA, Welch's test, Figure 2d; experiment 2: p-value = 0.612, one-way ANOVA, Figure 2e). The observed p-value < 0.05 for experiment 1 was due to differences between two of the treatment conditions (*lrc* and *ache1*, p-value = 0.042) and not to the control dsRNAs *gusA* and *eGFP* (Table S4, p. 31).

In a final iteration, we decided to follow the yeast feeding protocol, which uses dried tablets of pure yeast [38], and correspondingly produced pure bacterial tablets by drying *E. coli* pellets at 30 °C for 48 h. As these experimental conditions were prone to low water quality, the larvae were counted and transferred to clean water with a fresh bacterial tablet every 24 h for eight consecutive days, starting with approximately 20 h old L1 larvae. While 97.3 ± 2.1% (n = 200) of the larvae treated under standard rearing conditions pupated by the end of the eighth day, all larvae treated with the bacterial tablets were still in the larval stage. Starting from day 9, we added baking yeast to each container to support development. Under these conditions, pupation typically started after 14 days, and no significant difference was observed in the survival rates between the transcript-specific dsRNAs and the controls (p-value = 0.985, one-way ANOVA, Welch's test, Figure 2f).

Thus, none of the dsRNA feeding strategies yielded larval mortality significantly different from that of the treatments with control RNAs. To exclude that the lack of the RNAi effect was caused by dsRNA degradation under the experimental conditions, we extracted dsRNA from the food pellets after 24, 48, or 72 h of incubation with mosquito larvae in water at 27 °C. Undegraded dsRNA could be recovered from the formulations with agar for up to 72 h of incubation with the larvae. In the feeding assays with the dried bacteria tablets, the dsRNA was stable for up to 48 h (Figure 4). Therefore, dsRNA degradation under the experimental conditions could be excluded as a probable cause for the absence of target gene-specific dsRNA-induced lethality.

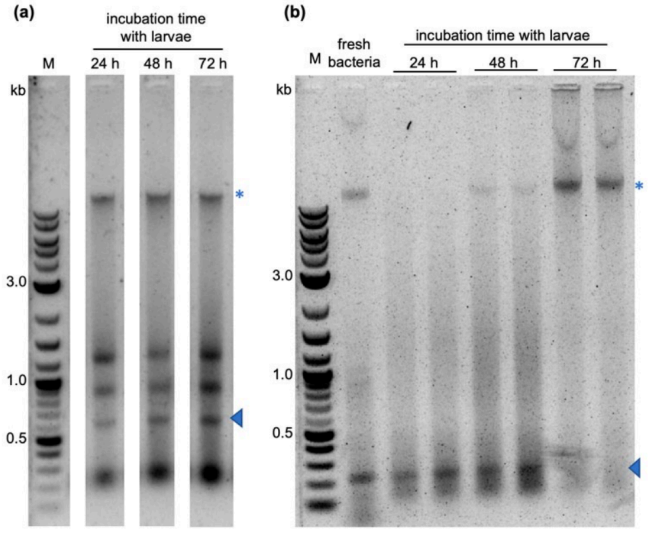

Figure 4. Gel electrophoresis of dsRNA recovered from bacteria–agar pellets (**a**) and pure dried bacteria tablets (**b**) after incubation in water with *Ae. aegypti* larvae. Each lane represents RNA extracted from one food pellet after incubation with 40 (**a**) or 20 (**b**) L1 to L2 larvae for the indicated periods; "fresh bacteria" represents dsRNA extracted from bacteria without larval incubation; blue arrows indicate the expected size of the dsRNA (*eGFP* in (**a**) and *fez2* in (**b**)). The blue asterisk indicates bacterial chromosomal DNA. M = 1 kb plus ladder (New England Biolabs Inc., Ipswich, MA, USA); kb = kilobases; for target gene name abbreviations, see Figure 2.

2.2. Co-Delivery of shRNAs against Gut RNases Does Not Enhance Target Gene-Specific RNAi Effects

A major obstacle to the oral delivery of iRNAs in insects can be the presence of RNases in the gut, which degrade the biologically active RNA molecules before they can be taken up into the gut epithelial cells. One possible solution is the co-delivery of iRNAs targeting the RNase transcripts [64–66]. We therefore performed feeding assays with bacteria expressing shRNAs against *fez2* or *lrc* with or without gut RNase-specific shRNAs in two *Aedes* species, *Ae. aegypti* and *Ae. albopictus*. These experiments were conducted independently from the above feeding experiments in a different laboratory.

In *Ae. aegypti*, one nuclease gene was targeted, XM_001653429.2, while three putative gut nucleases were targeted in *Ae. albopictus*, XM_019679594.1 (referred to as "nuclease 1"), XM_019701402.1 ("nuclease 2"), and XM_019683641.1 ("nuclease 3"; for more detailed information, see Table S2). shRNA sequences against the target genes were the ones published previously (*Aae fez2* v2 and *Aae lrc* [31]) or designed de novo (*Aae fez2* v1, *Aae* nuclease, *Aal fez2*, and *Aal nucleases 1-3*). The shRNA design followed the information and loop sequence published by Mysore et al. [35]. The experiments were performed with the *Ae. aegypti* Liverpool and La Réunion strains and an *Ae. albopictus* strain collected in Montpellier, France. Control experiments were performed with bacteria that did not express shRNA ("none") or that expressed an unspecific shRNA ("scramble").

While not all conditions were tested in multiple replicates, these tests also lacked the previously reported strong lethal effect of *fez2* or *lrc* knockdown [31,35], and the presence of RNase-specific shRNAs did not improve the RNAi effect. Moreover, as also observed in the bacterial feeding assays described above, the treatment resulted in overall increased development times, and control survival numbers varied within and between experiments, partially due to limiting larvae feeding on the pellets (Table S5). For statistical analysis, the controls or the assays for the same target gene with or without RNase-targeting shRNA

were combined (Figure 5). No significant lethal effects were observed in the feeding assays with the Liverpool strain (Figure 5a, p-value = 0.844, one-way ANOVA, Welch's test), the La Réunion strain when targeting *fez2* v1 (Figure 5b, p-value = 0.828, Student's t-test), or the *Ae. albopictus* Montpellier strain (Figure 5c, p-value = 0.272, Student's t-test). Only when targeting *fez2* v2 in the La Réunion strain was a significant reduction in survival observed (Figure 5b, p-value = 0.0489, Welch's t-test), but it was about five times lower than previously reported [31].

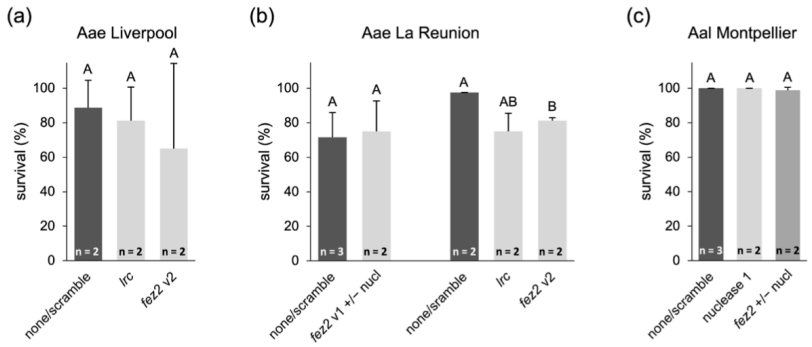

Figure 5. Survival rates of *Ae. albopictus* and *Ae. aegypti* following larval feeding with bacteria producing shRNAs against *fasciculation and elongation protein zeta2 (fez2)*, *leukocyte receptor cluster member 8 homolog* (*lrc)*, or nuclease-encoding transcripts (nucl). Each replicate contained 40 neonate larvae. Two types of negative controls were used: "none" means that no bacteria-expressed shRNA was added to the agar pellet; "scramble" means that bacteria expressing an unspecific shRNA were added to the agar pellet. Shown are average survival rates (in percent); error bars indicate standard deviation; different letters above the bars indicate statistically significant differences between gene-specific shRNA treatment and the unspecific control with p-value < 0.05 (one-way ANOVA, Welch's test in (**a**), Student's t-test and Welch's t-test in (**b**), Student's t-test in (**c**)); the numbers inside each bar indicate the number of replicates for each condition. Aae = *Ae. aegypti*, Aal = *Ae. albopictus; fez2* v1 is a newly designed shRNA sequence against *fez2*; *fez2* v2 and *lrc* correspond to the published siRNA sequences [31].

In another series of feeding experiments with the parallel knockdown of gut RNases, a study in *Ae. aegypti*, in which the knockdown of the female-specific isoform of *doublesex* (*dsx*) by RNAi caused a sex ratio distortion resulting in more than 90% male development [19], was replicated in *Ae. albopictus*. Here, two female-specific isoforms of the *dsx* transcript were targeted.

Ae. albopictus larvae were fed a mixture of bacteria expressing shRNAs against the female-specific isoforms of *dsx* and all three RNase genes in multiple replicates. In some replicates (treatment and control), the agar pellets used for dsRNA feeding were barely consumed over time. Since larval development was much slower in such replicates, counting was stopped on day 30, meaning that it was not possible to determine the sex of the remaining underdeveloped larvae (Table S6), and the male–female ratio was evaluated based on the obtained pupae. The combined feeding of female *dsx*- and nuclease-targeting shRNAs did not result in any significant sex bias (p-value = 0.415, one-way ANOVA) in adult mosquitoes (Figure 6 and Table S6).

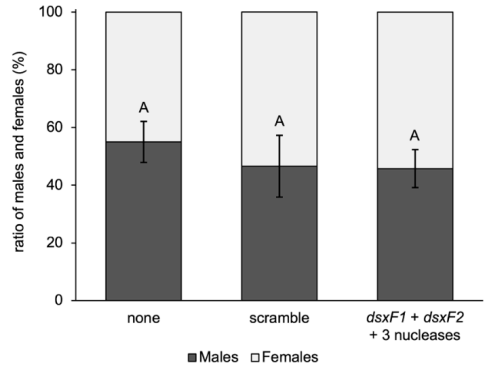

Figure 6. The sex ratio of *Ae. albopictus* adults (Montpellier strain) fed with bacteria producing shRNAs targeted against the two female-specific isoforms of *doublesex* (*dsxF1* and *dsxF2*). Two rounds of experiments were carried out, with 2–3 replicates for each condition. Each replicate contained 50 neonate larvae at the start of feeding. Two types of negative controls were used: "none" means that no bacteria-expressed shRNA was added to the agar pellet, and "scramble" means that bacteria expressing an unspecific shRNA were added to the agar pellet. The average male and female ratios (in percent) of both experiments combined are shown. Error bars indicate standard deviation; bars with common letters are not significantly different at 95% confidence level (one-way ANOVA).

In summary, these feeding experiments independently replicated the above results and did not show an increased RNAi effect when co-feeding with shRNAs targeting gut RNases. Possibly, insufficient amounts of RNase-targeting shRNAs reached the gut epithelial cells, either because gut RNases also degraded them before cellular uptake, or because the uptake of the RNA into the gut epithelial cells or the release from the bacterial cells in the gut are limiting steps.

2.3. Soaking of Ae. aegypti Larvae in Concentrated dsRNA or siRNA Solutions Does Not Improve RNAi Efficiency

If the release of dsRNA molecules from the producing microbial cells was a limiting factor, the exposure of the larvae to concentrated dsRNA solutions could result in the desired effect. The so-called soaking, i.e., the incubation of mosquito larvae in concentrated dsRNA or siRNA solutions, has often been reported [19,34–36,41,43] and used, for example, to screen for potential RNAi targets [31]. We tested soaking with freshly hatched Liverpool L1 larvae (not older than 90 min) using concentrations from 500 to 1500 ng/µL of bacterially produced and extracted dsRNA targeting *fez2*, *lrc*, and *sem-1a_E8* for 4 h, and we subsequently monitored larval and pupal survival under standard rearing conditions. Control experiments used either only water, *gusA*, or *eGFP* dsRNA. All treatments, on average, yielded survival rates to pupal stage higher than 80% (Figure 7a), with no significant difference between treatments and the controls (p-value = 0.154, one-way ANOVA, Welch's test).

The soaking experiment was repeated at the highest dsRNA concentration with an *Ae. aegypti* wild-type strain from Brazil to exclude a strain-specific lack of the RNAi effect (e.g., insensitivity to RNAi). Again, the survival rates for all treatments were at least 90% (Figure 7b), with no significant difference between the target gene treatments and the control targeting *gusA* (p-value = 0.857, one-way ANOVA, Welch's test).

To test whether the absence of dsRNA-induced lethality was due to the failed processing of the dsRNA by the RNAi machinery after uptake into the gut cells, we next tried soaking the larvae in in vitro-synthesized siRNAs (Integrated DNA Technologies Inc., Coralville, IA, USA). The target and control siRNA sequences were taken from the literature [31,35], and an additional control with water was performed. Despite increasing the siRNA concentration by two- to four-fold compared to published assays, we did not

observe any statistically significant differences in survival rates (Figure 7c, *p*-value = 0.223, one-way ANOVA).

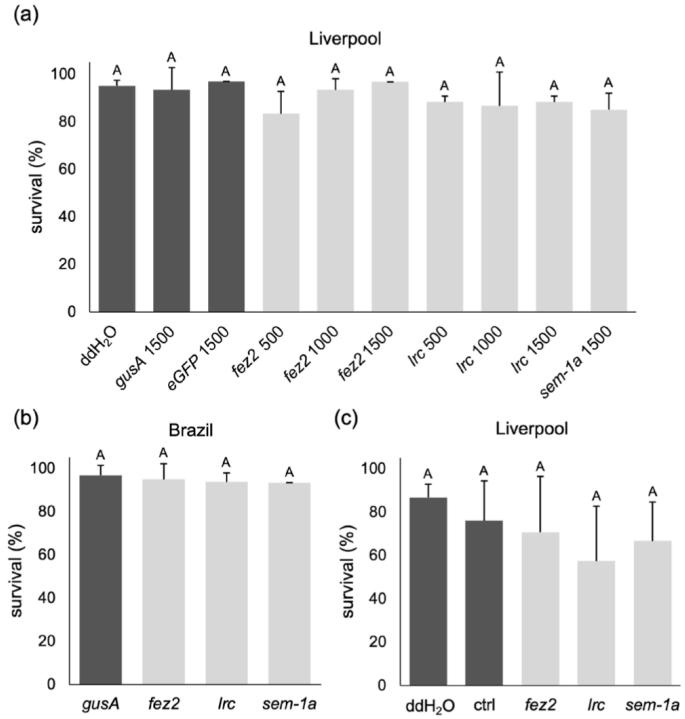

Figure 7. Survival rates after soaking *Ae. aegypti* early L1 larvae in concentrated dsRNA or siRNA solutions. (**a**) Liverpool wild-type strain larvae soaked in three different dsRNA concentrations, 500, 1000, and 1500 ng/µL; (**b**) Brazil wild-type strain larvae soaked in 1500 ng/µL dsRNA; (**c**) Liverpool larvae soaked in 1000 ng/µL siRNA solutions, with sequences from the literature [31,35]. Data in (**a**,**b**) are based on two biological replicates with 30 neonate L1 larvae each. Data in (**c**) are based on three biological replicates with 25 neonate L1 larvae each. Bars represent average survival rates in percent, error bars represent standard deviation, and bars with common letters are not significantly different at 95% confidence level (one-way ANOVA, Welch's test in (**a**,**b**), one-way ANOVA in (**c**)). For abbreviations of target gene names, see Figure 2; ddH_2O = double-distilled water; ctrl = unspecific siRNA sequence [31].

Independent dsRNA soaking experiments were performed in another laboratory, again targeting the female-specific transcript of *dsx*. While exposure to the *dsx* dsRNA resulted in about a 50% reduction in the levels of the female-specific *dsx* transcript (Figure 8a, *p*-value = 0.0000334, Welch's *t*-test), there was no difference in the male-to-female ratio between the control and *dsx* dsRNA treatments (Figure 8b, *p*-value = 0.815, Student's *t*-test), which is in contrast to the published sex bias [19]. Overall, the lack of an RNAi response in the soaking experiments indicates that releasing the RNA molecules from the producing cells is not the limiting step. This still leaves multiple reasons for the failure to produce an RNAi response, including degradation by gut RNases, failed uptake into the gut epithelial cells, or the inability of the RNAi machinery to process the dsRNAs or shRNAs.

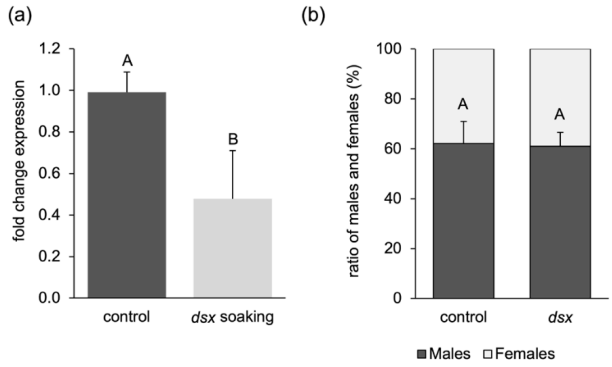

Figure 8. Targeting the female-specific transcript of the *doublesex* gene (*dsx*) by soaking *Ae. aegypti* larvae in *dsx*-dsRNA. Data are based on five to seven replicates with 50 to 100 neonate larvae each. Shown is the average fold change in *dsx* transcript levels after repeated soaking of larvae in *dsx*-dsRNA compared to the control (=*GFP*-dsRNA) in (**a**), as determined by RT-qPCR and calculated with the ΔΔCt method [67]; 8 × 3 larvae were pooled and analyzed for the control, and 11 × 3 larvae for the *dsx*-dsRNA. Panel (**b**) shows the male-to-female ratio (in percent) of adult mosquitoes after larval soaking in *dsx* or control (=*GFP*) dsRNA (**b**). Error bars represent standard deviation, and different letters above the bars indicate statistically significant differences with p-value < 0.05 (Welch's t-test in (**a**), Student's t-test in (**b**)).

2.4. siRNA or dsRNA Injections of Ae. aegypti Larvae Do Not Lead to Gene Knockdown

Delivering dsRNA by injection into larvae circumvents the gut RNases and a possible gut epithelial barrier. Injection of siRNAs would avoid issues with the intracellular processing of the dsRNAs. We started with injections of the bacterially produced and extracted anti-*fez2*, -*lrc*, and -*sem-1a* dsRNAs into L2 larvae. dsRNA solutions were mixed with a food dye to track the injection and ensure the uniformity of the injected volume. The *E. coli*-specific *gusA* dsRNA, total RNA extract from bacteria transformed with the empty L4440 expression plasmid ("bact ctrl"), and water with food dye served as negative controls. One replicate without dye was also included to verify the non-toxicity of the food dye. After injection, the larvae were allowed to recover, moved to normal rearing conditions, and monitored for pupation rate. No significant lethality was observed in the injections with target dsRNAs compared to the controls (p-value = 0.499, one-way ANOVA, Figure 9a). To assess the mRNA levels of the targeted genes, pools of three to five larvae from each injection were sampled 24 h after the injection and analyzed by RT-qPCR. However, also at the transcript level, no effect of the dsRNA injections could be observed (*fez2*: p-value = 0.824, *lrc*: p-value = 0.917, and *sem-1a_E8*: p-value = 0.337, one-way ANOVA, Welch's test, Figure 9d).

To assess the possible bottleneck of dsRNA processing, we injected siRNAs into L2 and L4 larvae, using the same siRNA sequences as for the soaking experiments. Water, water plus food dye, and an unspecific siRNA were used as controls. Neither in L2 nor in L4 larvae did we observe significant lethality with the target gene siRNAs (Figure 9b,c; L2 larvae: p-value = 0.127, L4 larvae: p-value = 0.363, one-way ANOVA). The general lower survival rates of the L2 larvae compared to L4 larvae are likely due to increased sensitivity to the injection procedure at a younger age. As the expression of *fez2* and *lrc* was detected in the fourth larval instar brain [31], we assessed target gene transcript levels by RT-qPCR in L4 as described above. We did not detect a significant reduction compared to the control injections at the transcript level (Figure 9e; *fez2*: p-value = 0.719, *lrc:* p-value = 0.999, *sem-1a*: p-value = 0.690, one-way ANOVA, Welch's test).

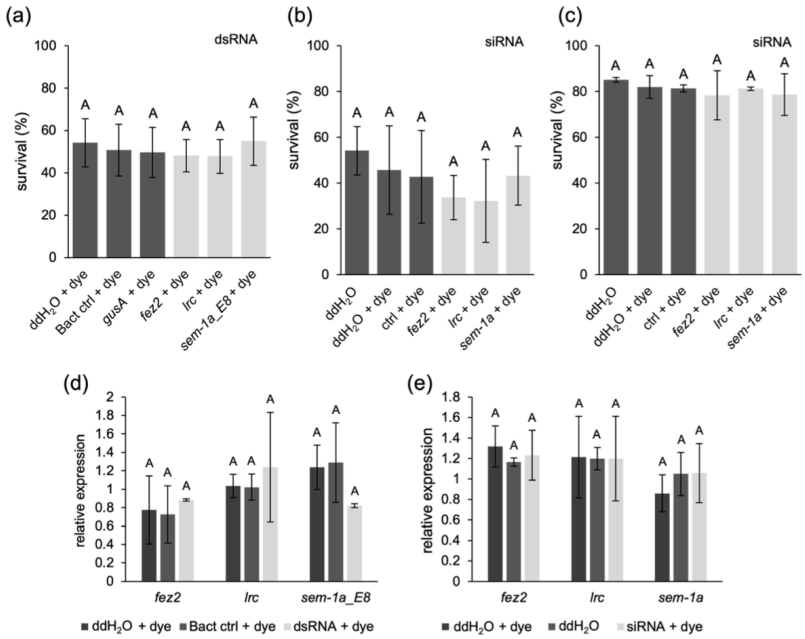

Figure 9. Effects of siRNA and dsRNA injections into *Ae. aegypti* L2 or L4 larvae. Average survival rates (in percent) to pupal stage (**a**–**c**) and average target gene transcript levels 24 h after injection (**d**,**e**) are shown. (**a**) dsRNA injections into L2 larvae; (**b**) siRNA injections into L2 larvae and (**c**) into L4 larvae; the *sem-1a* target sequence corresponds to the one published in [35]; data shown in (**a**–**c**) are based on three biological replicates, with 100 individuals per replicate. Three larvae in (**d**) and five larvae in (**e**) were pooled for RNA extraction and RT-qPCR 24 h post-injection. Relative expression was calculated following the Pfaffl method [57], using *rps17* as a reference gene. Data shown are based on two technical replicates from two biological replicates. For abbreviations of target gene names, see Figure 2; "dye" is food color mixed with ddH_2O or with siRNA or dsRNA solutions to visualize the success of injection, and "bact ctrl" is total RNA extracted from bacteria transformed only with the empty expression vector L4440. Error bars indicate standard deviation, and bars with common letters are not significantly different at a 95% confidence level (one-way ANOVA in (**a**–**c**), and one-way ANOVA and Welch's test in (**d**,**e**)).

2.5. Embryonic Injection with eGFP dsRNA Reduces eGFP mRNA and Protein Levels in a Transgenic Line

We finally evaluated the RNAi sensitivity in very early *Ae. aegypti* embryos. For this, preblastodermal embryos of an *eGFP*-expressing transgenic line were injected with bacterially produced and extracted *eGFP* dsRNA (same sequence as used in the experiments described above as a negative control) in two independent experiments (Table S7). Injection survivors were screened in the late L2 to L4 larval stages for *eGFP* fluorescence intensity compared to individuals injected with water or total RNA extract from wild-type bacterial cells. While all injection survivors from the control injections showed bright *eGFP* expression in the eyes, most *eGFP*-injected individuals showed a clearly reduced, sometimes very weak *eGFP* fluorescence intensity (Figure 10a). After fluorescence screening, each individual was quickly frozen at −80 °C for total RNA extraction and RT-qPCR analysis of *eGFP* transcript levels. RT-qPCR confirmed a significant downregulation of *eGFP* transcript levels in the individuals with weak eGFP expression, but also in the individuals with a stronger eGFP phenotype (Figure 10b, p-value = 0.002, one-way ANOVA, Welch's test). These results show that the bacterially produced dsRNA is biologically active.

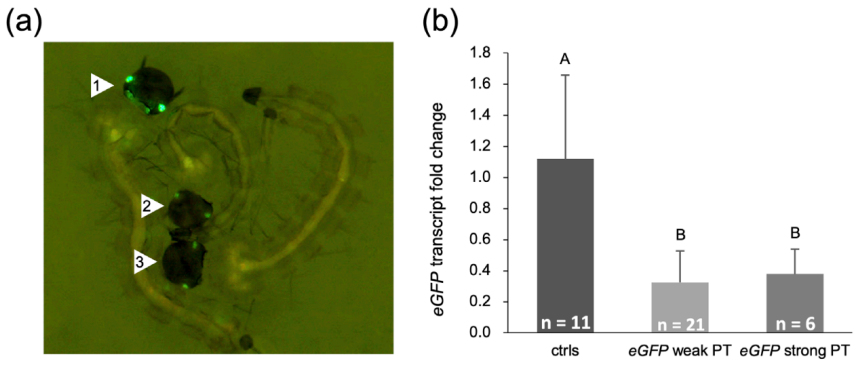

Figure 10. Targeting of *eGFP* transcript in early *Ae. aegypti* transgenic embryos via *eGFP* dsRNA injection. (**a**) Exemplary fluorescence image of larvae injected with bacterial extract as control (animal 1) or dsRNA targeting the *eGFP* transcript (animals 2, 3). (**b**) Relative mRNA levels were assessed in larvae injected with an extract from bacteria transformed with the empty expression plasmid or with ddH_2O (ctrls), or with bacterially produced *eGFP* dsRNA (*eGFP* weak PT = weak phenotype and *eGFP* strong PT = stronger phenotype). Shown is the average fold change in transcript levels for the respective groups measured by RT-qPCR and calculated with the ΔΔCt method [67]. For the bacterial extract control, five individuals from a total of 20 injection survivors from two independent experiments were analyzed; for the ddH_2O control, six out of seven injection survivors; and for the *eGFP* dsRNA, 24 out of 32 survivors from two independent injections; n = number of analyzed individuals (also see Table S7). Error bars indicate standard deviation, and different letters above the bars indicate statistically significant differences with p-value < 0.05 (one-way ANOVA, Welch's test).

3. Discussion

Gene knockdown by RNAi has been used to study gene function in mosquitoes and has gained a lot of attention regarding the development of RNAi-based vector control. For the success of this approach, the RNAi response should be robust and insensitive to potential external and internal variables. When we, the authors, independently of each other, started to set up RNAi as a tool in our laboratories, we chose protocols and target sequences reported to result in a high larval lethality or sex bias [19,31,35,36] for assay establishment. Surprisingly, however, we collectively failed to reproduce the published effects, although these well-designed studies provided exact information on the iRNA structure and sequences and detailed protocols for producing and delivering the iRNAs.

Several factors are critical for the successful induction of an RNAi response, including the stability of the RNA molecules in the environment and during delivery, the uptake from the gut lumen and intracellular release, the processing of the iRNA by the RNAi machinery, and the induction of systemic RNAi [4,17,18,56,62,68]. To troubleshoot the reasons for our failure to reproduce the published results, we independently started to test different RNAi-triggering molecules (siRNA, shRNA, and dsRNA) in combination with different delivery pathways (oral delivery via iRNA-producing microorganisms provided to the larvae in various formulations, soaking in pure iRNA solutions, and microinjections). None of the different combined strategies resulted in strong or consistent phenotypic effects. This also applied to the respective target gene transcript levels. Only in two assays could a consistent effect be observed: (1) *eGFP* knockdown in embryos of a transgenic line by *eGFP*-dsRNA injections, resulting in significant transcript level reductions combined with a clearly reduced fluorescence phenotype, and (2) knockdown of the female-specific *dsx* transcript in *Ae. aegypti* (approximately 50% across all replicates), but without the published sex bias phenotype [19]. Otherwise, only moderate reductions occurred randomly in single experiments or at a specific sampled stage.

Five different *Ae. aegypti* laboratory strains were used in total for all the assays performed in the three labs, which excludes the possibility of RNAi resistance in a specific strain.

An often-described bottleneck in oral RNAi is the presence of RNases in the insect gut that potentially degrade the iRNAs before they can be taken up by the gut epithelial cells [18,56]. One solution is the co-delivery of iRNA(s) that target the RNase transcripts [64–66]. In our experiments, however, the oral delivery of RNase-targeting shRNAs, together with shRNAs against *fez2*, *lrc*, or *dsx*, did not result in a target-specific phenotype in *Ae. aegypti* or *Ae. albopictus*.

The delivery of the iRNA molecules by microorganisms would also protect the RNAs from the nucleases during passage through the gut. However, if RNAi is to be triggered via the ingestion of iRNA-producing microorganisms, these need to be lysed in the gut to set the iRNA free. Coon et al. [69] reported that bacterial cells are required for normal larval development and can be later found dead in the gut of the mosquito larvae. We therefore assume that at least part of the bacterially produced dsRNA was released into the gut lumen upon death of the microorganisms. However, the amount could have been below a biologically meaningful threshold. This would be supported by observations made by Romoli et al. [68], who could not detect siRNA enrichment in the tissues of *Ae. aegypti* females whose gut had been colonized by dsRNA-producing bacteria. Attempts to detect siRNA enrichment following oral administration of heat-killed *E. coli* also yielded no positive results. However, upon oral administration of naked dsRNA or dsRNA injection into adult females, siRNAs were enriched, indicating the delivery and uptake of sufficient amounts with these strategies, but not with the microorganisms.

To address the possibility that the microorganisms' iRNA molecules were not expressed or degraded in the formulated food pellets during the assay, we purified dsRNAs from fresh cells or from food pellets after extended exposure to larval feeding, showing that dsRNAs were produced and stable. However, it is impossible to compare our RNA yield to that of other studies, as, overall, the amount of iRNA produced by and released from microorganisms in the published assays is a black box. Therefore, there could be a strong inherent variance between the used iRNA-producing strains, which could be a major factor in the observed lack of reproducibility.

Another possibility for the lack of RNAi effects is the failed processing of the delivered dsRNA into siRNAs by the cellular RNAi machinery. While we did not experimentally assess this step, the assays described above from Romoli et al. [68] show the processing of dsRNAs by the RNAi machinery. Based on this, we would have expected better RNAi efficiency when delivering naked dsRNA or chemically synthesized siRNAs against *fez2*, *lrc*, and *sem-1a*, via larval soaking or larval injections, using the published highly successful target sequences. However, except for the approximately 50% *dsx* transcript level knockdown after the soaking of larvae in dsRNA (see above), none of the experiments resulted in a measurable phenotypic or transcript level effect. Conversely, McFarlane et al. [70] showed that, despite successful in vitro siRNA-mediated gene silencing in *Ae. aegypti*-derived Aag2 cells, the same effect was not observed upon injection of *Ae. aegypti* female adults with siRNAs. However, strong knockdown was observed when the mosquitoes were injected with dsRNA targeting the same gene.

So far, the mechanisms involved in RNAi in mosquitoes have been barely studied and need to be better understood to be able to explain the observed variances. The effect of RNases in the gut and hemolymph remains a major mystery in mosquito RNAi. While dsRNA could be reextracted from mosquitoes at least in part up to two days post-oral delivery [25,68] and up to 7 days post-injection [71], the incubation of dsRNA with serial dilutions of gut juice led to the fast degradation of the dsRNA (Figure S2). Also, the pathway of dsRNA upon ingestion or injection is barely known. Fluorescently labeled dsRNA could be detected in some gut epithelial cells after oral delivery, but was not followed further [68]. Labeled dsRNA injected into females was found enriched mainly in hemocytes and ovaries [71], but could not be detected after oral administration. On the

contrary, many solid studies show high RNAi efficiency upon the oral administration of naked iRNAs [31,33,43,72,73].

Other studies have tested different nanomaterials for the packaging of dsRNA and the effect on the extent of gene silencing upon oral administration of such nanoparticles. Nanoparticles provide protection from gut RNases and are also used for the delivery of therapeutics to target cells in medical research. In several studies in *Aedes*, the RNAi effect was stronger when using packaged compared to naked dsRNA. Interestingly, the extent of gene silencing was dependent on the nanomaterial used [45,47,51]. But also here, the results are not consistent, and the effects can be gene-specific [47]. The potential of nanoparticles for RNAi-based insect management, including different nanomaterials, nanoparticle formulations, and their effect on different insect species, has recently been reviewed elsewhere [74–76]. One should consider that RNAi technology, when incorporating additional components formulated with the RNA, might be subject to additional regulatory frameworks akin to those for chemicals, potentially losing the regulatory advantages typically associated with RNA-only biologicals.

The apparent limitations of RNAi are not unique to mosquitoes and have been observed in several species over the past decade. The research performed so far clearly shows that not all insects are equally susceptible to dsRNA and/or RNAi in general. While Coleopterans are considered very RNAi-susceptible, Hemipterans show varying effects, and Dipterans and Lepidopterans show overall low RNAi efficiencies. The Coleopteran *Tribolium castaneum* is the model insect for RNAi, in which a comprehensive gene function study by systematic RNAi-mediated gene knockdown has been performed [77]. Even parental RNAi works very well in these beetles. In contrast, lepidopteran species are often considered RNA recalcitrant. One of the main reasons for that seems to be the presence of dsRNases in the saliva or the midgut, which quickly digest the ingested dsRNA in recalcitrant but not in susceptible species [78–83].

Overall, the studies performed over the past 10–15 years have also helped to uncover several barriers to RNAi in insects besides gut RNases, such as dsRNA uptake into cells, intracellular release, or the spread of the RNAi signal in the insect body. A lot of our understanding of the RNAi mechanism and the involved genes and processes comes from model organisms such as *C. elegans* or from human studies. Core RNAi enzymes like Dicer and Ago2 have been found in many insects. However, other mechanisms and factors involved in the RNAi process are less conserved and seem to differ between orders, families, species, and even tissues or stages. Thus, there is not "one mechanism of insect RNAi" but many varying factors, which probably contribute to the high variability in RNAi effects across and within insect species. It is beyond the scope of this manuscript to go into further mechanistic details, but current knowledge has been summarized comprehensively before [4,5,18,56]. However, it is essential to further investigate the molecular mechanisms of RNAi across different species.

For *Aedes* mosquitoes, the inconsistent and sometimes contradictory RNAi effects in the literature, in combination with our failed attempts to reproduce published data independently in three independent laboratories, strongly suggest a high complexity of the RNAi mechanisms in *Aedes* mosquitoes and of the factors influencing these mechanisms, which are both insufficiently understood and produce unexpected and currently unexplainable variability in experiments. It also shows that RNAi application in *Aedes* mosquitoes is less robust and straightforward than the successful studies imply. This is important information that needs to be shared to raise awareness and open the discussion about the challenges involved, with the goal to further study and improve RNAi in *Aedes* species.

4. Materials and Methods

The data presented in this study were generated in three different laboratories independently and without knowledge of each other; therefore, the methods presented in this section are subdivided by laboratory. All primer and interfering RNA sequences used in this study are provided in the Supplementary Material (Table S2).

4.1. Protocols from Department of Insect Biotechnology in Plant Protection, Justus Liebig University Giessen, Germany

4.1.1. Mosquito Rearing

Ae. aegypti wild-type strains and the transgenic line V19 were reared in an insectary at 27 °C with 70% relative humidity (RH) and a 12:12 h light–dark cycle. Larvae were fed on Tetra TabiMin fish food pellets (Tetra GmbH, Melle, Germany), and adults were fed on sterile-filtered 10% (w/v) sucrose solution. Adult females were fed once per week with pig blood purchased from a butcher shop.

The *Ae. aegypti* laboratory strains used in the following experiments were the Orlando (collected from Orlando, FL, USA, in 1952) [84], Liverpool (the Liverpool reference strain, originating from West Africa in 1936) [85–88], and Brazil (collected in Juazeiro, Carnaiba do Sertão, Brazil, between 2012 and 2014) wild-type strains.

The transgenic *Ae. aegypti* line V19 contains one copy of the *piggyBac* construct pB_*attp_3xP3-eGFP_attPrev.* When homozygous, it shows strong *eGFP* expression in the eyes and in the ventral nerve cord, which has been published previously [89].

4.1.2. Cloning of dsRNA Expression Vectors for Bacterial Expression

The dsRNA sequences for the *beta-tubulin* (*βtub*) genes AAEL002851 and AAEL004939, as well as for the *E. coli beta-glucuronidase* (*gusA*) control, were identical to the ones published [19,36], using the primer sequences provided in these publications, with overhangs for ApaI-NotI cloning. PCRs were performed on *Ae. aegypti* complementary DNA (cDNA) and *E. coli* XL1-Blue genomic DNA (gDNA) with Platinum Tag polymerase (Invitrogen, Waltham, MA, USA). A 20 µL reaction contained 1X Platinum Taq buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 500 nM forward and reverse primers, and the DNA template. The cycling conditions were 1× 95 °C 90 s, 30× 94 °C 30 s, 56 °C 20 s, 72 °C 1 min/1 kb, and 1× 72 °C 5 min. Products were digested with ApaI and NotI (25 µL PCR reaction, 4 µL CutSmart, 10–20 U enzyme, double-distilled water (ddH_2O) to 40 µL; 1.5 h, 37 °C), and gel was purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research Europe GmbH, Freiburg, Germany) following the manufacturer's instructions, but elution was performed with a pre-heated buffer (60 °C), and T4 DNase-ligated (New England BioLabs, Inc., Ipswich, MA, USA) into the ApaI-NotI-digested and gel-purified L4440 expression plasmid (Addgene plasmid # 1654) at 16 °C overnight.

The dsRNA sequences for *acetylcholine esterase 1* (*ache1*, LOC5578456), *vacuolar-type ATPase* (*V-ATPase*, LOC5575718, AAEL012035), *fasciculation and elongation protein zeta-2* (*fez2*, LOC5569012, AAEL007292), *leukocyte receptor cluster member 8 homolog* (*lrc*, LOC5569340, AAEL007548), *semaphorin-1a* (*sem-1a*, LOC5575438, AAEL002653) exon 8 and exon 15, and *coat protein (coatomer) alpha* (αcop) (LOC5577214, AAEL015001) were designed with the online tool eRNAi [90] using the following parameters: |siRNA length for specificity prediction: 17 bp, exclude low complexity regions, exclude > 5× CA[ACGT] repeats, siRNA seed length: 6, efficiency scoring: weighted, minimal siRNA efficiency score: 40, homology e-value cut-off: 1×10^{-5}, amplicon size range: 150–500|. Only sequences producing zero off-target siRNAs in the *Ae. aegypti* genome were used. For *fez2*, *lrc*, and *sem-1a*, the dsRNA sequences suggested by eRNAi, which contained the published siRNA sequences [31,35,63], were chosen. The primers for *ache1*, *V-ATPase*, *fez2*, *lrc*, and *sem-1a* were designed with overhang for KpnI-SacI restriction cloning into the L4440 plasmid. The primers for αcop were designed with overhang for Gibson Assembly cloning (New England Biolabs, Inc., Ipswich, MA, USA) into the KpnI-EcoRI-digested L4440 plasmid. All PCRs were performed on genomic DNA (gDNA) with Phusion Flash High-Fidelity polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). A 20 µL reaction contained 100 ng gDNA, 250 nM forward and reverse primers, and 1X Phusion Flash Master mix. Reactions were performed as 1× 98 °C 10 s, 30× 98 °C 1s, 54 °C 5 s, 72 °C 15 s, 1× 72 °C 1 min. PCR products were gel-purified and extracted with a Zymoclean Gel DNA Recovery Kit (Zymo Research). *ache1*, *V-ATPase*, *fez2*, *lrc*, and *sem-1a* PCR products were digested with KpnI and SacI (50 µL rxn with 20 U each enzyme, 1X CutSmart buffer and the complete purified PCR product) and ligated

with the KpnI-SacI-digested plasmid L4440 (2 µg plasmid, 20 U each enzyme, 1X CutSmart buffer in 50 µL) using T4 DNA ligase overnight at 16 °C. The αcop PCR product was cloned into the KpnI-EcoRI-digested L4440 plasmid following the manufacturer's Gibson Assembly cloning protocol. All ligated plasmids were first transformed into XL1-Blue cells (*E. coli* [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]]; Agilent Technologies, Santa Clara, CA, USA) for sequence verification and, after that, into HT115 DE3 (*E. coli* [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lacUV5 promoter -T7 polymerase]; Caenorhabditis Genetics Center) cells, followed again by plasmid extraction and sequencing.

4.1.3. In Vitro Transcription (IVT) of *βtub* and *gusA* dsRNA

IVT templates were produced by PCR on the respective L4440 plasmid using Q5 High-Fidelity polymerase (New England BioLabs Inc., Ipswich, MA, USA) and the gene-specific primers with a 5′ extension for the T7 promoter adapter. A 50 µL reaction contained 1X Q5 buffer, 100 nM forward and reverse primers, 200 µM dNTPs, and 0.5 µL Q5 polymerase. The cycling conditions were 1× 98 °C 30 s, 30× 98 °C 10 s, 65 °C 20 s, 72 °C 60 s, and 1× 72 °C 2 min. The PCR product was purified by gel electrophoresis and extraction with the Zymoclean Gel DNA Recovery Kit (Zymo Research Europe GmbH, Freiburg, Germany).

IVT was performed using the MEGAscript kit (Ambion/Life Technologies, Thermo Fischer Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions, using 500 ng of the PCR product as the template. Reactions were incubated for 4 h at 37 °C. Then, an annealing step was performed: 75 °C for 5 min and cooling down to room temperature, followed by DNase digest at 37 °C for 15 min. The dsRNA was purified using the MEGAclear Transcription clean-up kit (Ambion/Life Technologies, Thermo Fischer Scientific) according to the manufacturer's instructions. The dsRNA was eluted with 50 µL elution solution incubated on a column at 65 °C for 5 min. The elution was performed twice. The dsRNA quality was analyzed by agarose gel electrophoresis.

4.1.4. Cloning of shRNA Expression Vectors for Expression in the Yeast Strain BY4742

shRNA sequences were deducted from the literature for *fez2* and *lrc* [31] and for *sem-1a* [35]. Sequences were ordered as DNA oligo templates from IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA) containing a 5′ overhang with a BamHI restriction site and a 3′ overhang with an XhoI restriction site for cloning into the pRS426 YE shuttle vector (plasmid #77107, American Type Culture Collection, ATCC).

shRNA templates were amplified using the Phi29 primer extension method [91]. A 20 µL reaction contained 20 pmol oligo template, 20 pmol 3′ primer ATCTCCATGCAGCTC-GAG, 1X Phi29 reaction buffer, 8 µg BSA, 50 mM dNTPs, and 10 U Phi29 DNA polymerase (New England BioLabs Inc., Ipswich, MA, USA). The reaction was incubated for 10 min at 30 °C, followed by heat inactivation of the enzyme for 10 min at 65 °C. The gel-purified PCR products (Zymoclean Gel DNA Recovery Kit, Zymo Research Europe GmbH, Freiburg, Germany) were subcloned into the Zero Blunt TOPO vector (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions and transformed into XL1-Blue cells. Colonies with the correct cloning product were identified by plasmid preparation (NucleoSpin Plasmid Mini kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sequencing. The shRNA sequence was then cut out of the correct plasmids by restriction digest (100 ng plasmid DNA, 20 U XhoI and BamHI (New England BioLabs), 1X CutSmart buffer in 25 µL total volume, 1 h at 37 °C), purified via gel electrophoresis, and ligated into the BamHI- and XhoI-digested and gel-purified pRS426 vector using T4 DNA ligase (New England BioLabs) at 16 °C overnight, followed by transformation of the ligated products into XL1-Blue cells. Colonies with the correct cloning product were again identified by plasmid preparation (NucleoSpin Plasmid Mini kit, Macherey-Nagel) and sequencing and then transformed into the BY4742 *S. cerevisiae* strain following the protocol from Mysore et al. [38] step by step. To confirm correct plasmids, single colonies were grown in 3 mL YPD medium for 24 h at 30 °C, and DNA was extracted using a NucleoSpin Plasmid Mini

kit (Macherey-Nagel) with one adaptation of the protocol: cells from 2 mL yeast culture were pelleted and resuspended in 250 µL Buffer A1, transferred to a tube with ceramic beads, and homogenized (Precellys®, Bertin Instruments, Montigny-le-Bretonneux, France) at 6000 rpm for 20 s. Then, the protocol was continued according to the manufacturer's instructions. Plasmid sequence was verified by sequencing.

4.1.5. Culturing of dsRNA-Expressing Bacteria

Bacteria were grown at 37 °C and 150 rpm in LB medium containing 12.5 ng/µL tetracycline (Fisher BioReagents, Geel, Belgium) and 100 ng/µL ampicillin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to an optical density per mL measured at a wavelength of 600 nm (OD_{600}/mL) of 0.4 to 0.8. dsRNA expression was induced by the addition of 0.4 mM IPTG. Cells were grown for another 4 h at 37 °C and 150 rpm, harvested, and further treated according to the different food preparation protocols.

4.1.6. Feeding of *Ae. aegypti* L1 Larvae with dsRNA-Expressing Bacteria

The dsRNA-expressing HT115 DE3 cells used for these feeding experiments were grown and induced for dsRNA production as described above.

Variant 1: The composition of food pellets was based on the information from Whyard et al. [19]. As no exact amounts were indicated, the following amounts were mixed as starting conditions: bacterial pellet from 100 mL culture grown to an OD_{600}/mL of 0.7–1.0, 5 mL of LB-agar cooled to 45–50 °C, and 1.25 g of ground TabiMin fish food (Tetra GmbH, Melle, Germany). After mixing well by pipetting, the solution was aliquoted in ca. 100 µL droplets in a Petri dish, cooled down, and stored at 4 °C in a sealed dish if not used immediately. This food composition contained the equivalent of 20 mL bacterial culture per 1 mL of food = 1X. The amount of bacterial cells was further increased to 50 mL and 100 mL bacterial culture per 1 mL of food (= 2.5X and 5X, respectively).

L1 larvae of the Orlando wild-type strain (ca. 15 h old) were counted in 12 groups of 10 larvae per target, incubated in 6-well plates in ddH_2O, and fed ad libitum with the food pellets. Larvae were supplied with ca. 50 µL food pellet in the first four to five days of the experiment. Then, feeding increased, with 100 µL food pellet per feeding. Water was exchanged as needed. If food or water needed to be replaced in one well, then it was also replaced in all other wells. The feeding amount across all wells was always consistent. Every individuum that pupated was counted as a survivor.

Variant 2: Bacterial cells from 50 mL culture harvested at OD_{600} of 1.4–1.6 were mixed with 12 mg of finely ground Tetra TabiMin fish food. Then, 55 µL of 6% (w/v) pre-melted agarose solution was added, and the mix was thoroughly homogenized using a pipette tip. Bacterial pellets were allowed to solidify at room temperature for at least 10 min and transferred to 4 °C for at least 20 min before use for larvae feeding, or they were stored at −20 °C for later use within one week. Each pellet was used as one feeding portion to 20 L1 larvae in 50 mL of autoclaved double-distilled water. Fresh feeding portions were provided as needed or, at the latest, every 48 h. This food composition provided the equivalent of 300 mL culture/mL of food (=15X).

Variant 3: Bacterial cells from 400 mL culture were resuspended in 4 mL of pre-melted LB-agar, together with ampicillin (final concentration 100 µg/mL) and tetracycline (final concentration 12.5 µg/mL). This corresponded to the equivalent of 100 mL culture/1 mL of food pellet (=5X). The solution was transferred to a 5 mL syringe with a cut-off tip, allowed to solidify, and slices of 0.5 mL were used to feed 40 neonate larvae in 50 mL autoclaved ddH_2O. Fresh feeding portions were provided as needed or, at the latest, every 48 h. To support larval development after the end of the bacterial feeding, baker's yeast was added to the water to a final concentration of 0.08 mg/mL.

Variant 4: Bacterial cells from 50 mL of culture harvested at OD_{600}/mL of 1.6–2.2 were mixed with 55–110 µL of 2–6% (w/v) of pre-melted agarose (see Table S3 for specific amounts) in 1.5 mL microfuge tubes. Bacterial pellets were left to cool down and solidify at room temperature for at least 10 min and transferred to 4 °C for at least 20 min before use

for larvae feeding, or they were stored at −20 °C for later use within one week. To support larval development towards the end of the bacterial feeding, 12 mg of finely ground fish food was added to the food pellets (Table S3). Each pellet was used as one feeding portion to 20 neonate or 20 h old L1 larvae in 50 mL of autoclaved ddH_2O. Pellets were replaced when consumed or, at the latest, after 3 days.

Variant 5: Similar to the production of dried tablets of shRNA-expressing yeast [38], 50 mL of bacterial cells cultured and harvested as above was collected in 1.5 mL microfuge tubes and dried at 30 °C and 50% RH for 48 h. Dried pellets were used immediately for feeding assays or stored for up to one week at −20 °C. Each pellet was used as one feeding portion for 40–60 L1 larvae in 50 mL of autoclaved ddH_2O and replaced daily, including water change. After 8 days, baking yeast was provided at 0.08 mg/mL as only source of nutrition.

4.1.7. Feeding of *Ae. aegypti* L1 Larvae with shRNA-Expressing Yeast

For the preparation of the yeast cultures and dried yeast pellets, as well as the feeding procedure of the *Ae. aegypti* larvae, we closely followed the protocol by Mysore et al. [38] with some small changes: larvae were supplied with yeast on the first day of the experiment (pellet corresponding to 50 mL culture grown to OD_{600}/mL of 2.5–3) and again on the fourth day of the experiment, including a water change. In cases where transcript levels were assessed by RT-qPCR, the experiment started with 50 L1 larvae in 50 mL ddH_2O, and 2 × 5 larvae were sampled from each cup on day 3 and day 5 of the experiment, as well as 2 × 5 pupae, and stored at −80 °C immediately. The amount of yeast added per mL of ddH_2O was not adjusted, as the water remained cloudy from the yeast, so there was never a shortage in yeast cells, even with the increased number of larvae. Each biological replicate was performed with 2–3 technical replicates.

4.1.8. Soaking of Neonate *Ae. aegypti* Larvae with dsRNA or siRNA

The dsRNAs used in these experiments were extracted from HT115 DE3 cells as previously described [61]. siRNAs were purchased from IDT (Integrated DNA Technologies, Inc., Coralville, IA USA). dsRNAs or siRNAs were diluted in ddH_2O to 500 ng/µL, 1000 ng/µL, or 1500 ng/µL. *Ae. aegypti* larvae either less than 90 min old or between 20 and 24 h old were gently transferred to 1.5 mL microfuge tubes containing 25 to 100 µL of either the dsRNA or siRNA solutions. Soaked larvae were checked under a microscope to ensure complete immersion. After 4 h of soaking, larvae were gently transferred to autoclaved ddH_2O and further reared under regular rearing conditions. Development was monitored daily until pupation. Soaking assays were performed with 20 to 30 larvae per replicate.

4.1.9. RNA Extraction and RT-qPCR of Larvae/Pupae Sampled from Yeast shRNA Feeding Assays

Total RNA was extracted from samples stored at −80 °C with a Monarch total RNA Miniprep kit (New England Biolabs Inc., Ipswich, MA, USA) or TRIzol™ Reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Then, 0.5–1 µg of RNA was reverse-transcribed using a QuantiNova Reverse Transcription Kit (QIAGEN, Venlo, The Netherlands) according to the kit's protocol. The kit uses an integrated gDNA removal step and a mix of oligo-dT and random primers. cDNA was diluted either 1:5 or 1:6 in ddH_2O, depending on the expected level of gene expression, and 2 µL was used per qPCR reaction. qPCR was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a 10 µL reaction volume with 2 technical replicates for each condition. qPCR primers were evaluated for primer efficiency and primer dimer formation. The reference gene was the ribosomal protein S17 (*rps17*, AAEL004175) [92]. Changes in transcript levels relative to those of the control treatments were calculated using

Pfaffl's mathematical model [57], which incorporates an efficiency correction to account for the real-time PCR efficiency of the individual transcripts with the following formula:

$$ratio = \frac{\left(E_{target}\right)^{\Delta CP_{target}(control-sample)}}{\left(E_{ref}\right)^{\Delta CP_{ref}(control-sample)}}$$

Here, "*ratio*" is the relative expression ratio, E_{target} is the real-time PCR efficiency of the target gene transcript, $E_{ref}$is the real-time PCR efficiency of the reference gene transcript, ΔCP_{target} is the CP difference of the control to the sample of the target gene transcript, and ΔCP_{ref} is the CP difference of the control to the sample of the reference gene transcript. The ratio was first calculated separately for each experimental replicate by normalizing the target gene CP for each replicate to the reference gene CP averaged across all replicates per treatment or control. Then, the ratios of all experimental replicates of one target gene or control treatment were averaged, and the standard deviation was calculated.

4.1.10. Larval Injections with siRNA or dsRNA Solutions

Following standard rearing conditions, larvae of *Ae. aegypti* at two different development stages (L2 or early L4) were randomly selected to undergo microinjections. Animals were injected in the section between the thorax and abdomen, taking care to avoid the gut. Needles for injection were prepared using siliconized quartz glass capillaries (Science Products for Research in Life Science GmbH, Product Number Q100-70-7.5, Hofheim, Germany) pulled in a P-2000 laser puller (Sutter Instruments, Novato, CA, USA). The injection setup consisted of an MN-151 micromanipulator (Narishige, Tokyo, Japan), a FemtoJet 4i (Eppendorf, Hamburg, Germany), and a SZX16 stereo microscope (Olympus, Tokyo, Japan). The dsRNAs used in these experiments were extracted from the HT115 DE3 cells as previously described [61]. siRNAs were purchased from IDT (Integrated DNA Technologies, Inc., Coralville, IA USA). Larvae were injected with solutions of siRNA or dsRNA at 1 µg/µL in three biological replicates with 100 individuals each. Food dye was mixed into the injection solution to allow for the tracking of the injected volume and ensure the uniformity of the injections throughout the experiments. The food dye was verified to not degrade siRNA or dsRNA during the time of the injection. After the injection, larvae were allowed to recover shortly, gently transferred to normal rearing conditions, and monitored daily. RNA extraction and RT-qPCR of larvae sampled 24 h after injection was performed as described above for larvae and pupae sampled from yeast shRNA feeding assays.

4.1.11. Embryonic Microinjections with *eGFP* dsRNA

Preblastodermal embryos of the V19 *eGFP*-expressing transgenic line (pB_attp_3xP3-eGFP_attprev) of *Ae. aegypti* [89] were collected by allowing females homozygous for the single-copy transgene construct to oviposit for 30 min. Eggs were then rowed on a wet filter paper to ensure that all were in the same anterior–posterior orientation, transferred to double-sided sticky tape on a cover slide, and covered with halocarbon oil 27 (Sigma Aldrich/Merck KG, Darmstadt, Germany). Embryos were injected between 60 and 120 min post-oviposition start into the posterior pole with *eGFP* dsRNA produced by and extracted from *E. coli* HT115 DE3 cells [61] and dissolved in ddH_2O at 1 µg/µL. Control injections were performed with ddH_2O or with total RNA extracted from wild-type HT115 DE3 cells not expressing any dsRNA. Surviving G_0 individuals were reared to the L2 to L4 larval stage under standard rearing conditions and assessed for *eGFP* expression level by fluorescence microscopy. Larvae were then quick-frozen individually at −80 °C for later RNA and DNA extraction using the TRIzol™ method (see above). *eGFP* mRNA levels were measured by RT-qPCR for each larva individually as described above, and the fold change in transcript levels was calculated by the ΔΔCt method [67]. The fold change was first calculated separately for each experimental replicate by normalizing the target gene CP of each replicate to the reference gene (= *rps17*) CP averaged across all replicates per treatment or control. Then, the fold changes in all experimental replicates of one target gene

or control treatment were averaged, and the standard deviation was calculated. Moreover, the transgene copy number of each larva was confirmed to be two by digital droplet PCR according to the protocol in [89].

4.2. Protocols from ASTRE, CIRAD, Montpellier, France

4.2.1. Mosquito Rearing

The mosquito strains used for the following experiments were the *Ae. aegypti* La Réunion wild-type strain, collected from La Réunion Island, France, in or before 2018; the Liverpool strain; and an *Ae. albopictus* wild-type strain collected in Montpellier, France. The *Ae. albopictus* strain was reared in a climatic chamber at 25 °C and 70% RH, and *Ae. aegypti* strains were reared at 27 °C and 70% RH. Both light cycles were 14 h light–10 h dark with dawn and dusk. Other rearing conditions were essentially as described above.

4.2.2. shRNA Cloning for Bacterial Expression

Novel shRNA sequences were designed with the RNA interference tool from IDT: https://www.idtdna.com/site/order/designtool/index/DSIRNA_PREDESIGN (accessed on 25 February 2019). Obtained sequence suggestions were checked with Blast for off-targets. Every sequence that produced more than 17 bp off-target hits was excluded. Oligo primers were designed to represent the shRNA target sequence and the reverse complement linked by the loop sequence (5′AAGTTCTCT3′). An 'AATG' overhang was added to the 5′ end and 'TGAG' to the 3′ end of the top strand. The forward oligo started with the 5′ overhang (AATG) and ended at the end of the reverse complement. The reverse oligo started with the 3′ overhang (=CTCA, i.e., the reverse complement of TGAG) and ended at the start of the shRNA target sequence. The oligos were phosphorylated with polynucleotide kinase (New England BioLabs Inc., Ipswich, MA, USA) in a 10 µL reaction containing 1X PNK buffer, 1 µL PNK, and 100 µmol oligo for 1 h at 37 °C. Oligo pairs were mixed, the volume was increased to 200 µL, and oligos were annealed in a thermal cycler: 96 °C 6 min, 96 °C–1 °C per cycle down to 23 °C. Annealed oligos were blunt-cloned into the PJet1.2/blunt cloning vector (Thermo Fisher Scientific Inc., Waltham, MA, USA) and transformed into *E. coli* DH5alpha cells. Colonies with the correct sequence plasmid were identified by colony PCR. Plasmids were purified (E.Z.N.A. Plasmid DNA Mini Kit I, Omega Bio-tek, Inc., Norcross, GA, USA) and transformed into HT115 DE3 cells, and resulting colonies screened again for correct plasmid sequence by colony PCR. Positive plasmids were confirmed by sequencing.

4.2.3. Culturing of shRNA-Expressing Bacteria and Preparation of Food Pellets

HT115 DE3 cells were grown as described above, and shRNA expression was induced at 0.8 OD_{600}/mL. Food pellets were prepared by pelleting cells from 40 mL culture, resuspending the cells in 4 mL of 1% (w/v) agar at 80 °C, and adding 1 mL brewer's yeast slurry. The mixture was incubated for 15 min at 80 °C to heat-kill the bacteria and filled into a 5 mL syringe with a cut-off tip. Upon solidifying, the food was cut into 0.5 mL sections.

4.2.4. Feeding of *Ae. aegypti* and *Ae. albopictus* Larvae with shRNA-Expressing Bacteria

Forty first-instar larvae of *Ae. aegypti* or *Ae. albopictus* were kept in a Petri dish with 25 mL of ddH_2O, supplied with two 0.5 mL food pellets, and incubated at 28 °C for 4–6 days until the fourth instar.

4.3. Protocols from Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, USA

4.3.1. Mosquito Rearing

Mosquito rearing conditions were essentially as described above. The *Ae. aegypti* wild-type strain Waco (originally collected from the field in Waco Texas and has been colonized in the lab for >20 years [93]) was used for the following soaking experiments.

4.3.2. *Ae. aegypti Doublesex* and *GFP* dsRNA Synthesis by IVT

For dsRNA synthesis by IVT, total RNA was extracted from fourth-instar larvae and reverse-transcribed to cDNA as described below. cDNA was used as a template for PCR. Primers with a T7 promoter sequence added to their 5′ end were employed for amplification. The PCR product was purified using a NucleoSpin Gel and PCR Clean-up kit (Takara Bio Inc., San Jose, CA, USA) after cutting the PCR product bands from gel analysis. Subsequently, dsRNAs were synthesized using the T7 RiboMAX™ Express RNAi System (Promega Corp., Madison, WI, USA). As a control, ds*GFP* was also synthesized for larval RNAi experiments.

4.3.3. *Ae. aegypti* Larval Soaking in *dsx* dsRNA

Purified dsRNA was dissolved to a concentration of 1000 ng/µL. Approximately 50 to 100 newly hatched larvae were transferred into a 50 mL conical tube, with its bottom replaced with a sieve to enable soaking. Subsequently, all the larvae were soaked in dsRNA at a concentration of 1000 ng/µL in soaking buffer for 2 h per day over 6 days. *dsGFP* was used as the control, with at least five replicates for each treatment. After soaking, the larvae were returned to a cup containing fresh water and food and allowed to recover for 24 h at 27 °C and 80% RH. Mosquito sexes were determined based on the morphological character of the adults developed after treatment. For RNAi efficiency determination via target gene transcript levels, larvae were collected in TRIzol™ solution after the last soaking with 3 larvae/pool.

4.3.4. Total RNA Extraction and RT-qPCR of *dsx* dsRNA-Treated *Ae. aegypti* Larvae

Insect tissues were collected in 1.5 mL tubes and immediately flash-frozen in liquid nitrogen. Total RNA was then extracted using TRIzol™ reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. cDNA was synthesized from purified total RNA using a QuantiTect Reverse Transcription Kit (QIAGEN, Venlo, The Netherlands; integrated gDNA removal step and mix of oligo-dT and random primers). Subsequently, real-time quantitative PCR (RT-qPCR) was performed for 40 cycles on the QuantStudio Real-Time PCR System (Applied Biosystems/Thermo Fischer Scientific Inc., Waltham, MA, USA) using SYBR™ Green PCR Master Mix (Thermo Fischer Scientific Inc., Waltham, MA, USA). *rps6* was used as a reference gene. Transcript levels of the target mRNA were normalized to reference gene levels within the same samples. Each experiment involved the collection of four separate samples, with duplicate measurements conducted for each. The relative gene expression was calculated as $rq = 2^{-\Delta\Delta Ct}$ [67].

4.4. Statistical Analysis

Data analysis was carried out using the software SigmaPlot (Version 14.0, Systat Software Inc., San José, CA, USA) and MiniTab® (Minitab, LLC., State College, CA, USA). Data were analyzed via a one-way analysis of variance (ANOVA), with the Shapiro–Wilk test for normality and the Brown–Forsythe test for equal variance. The Holm–Sidak method was used for multiple comparisons of the data from each condition, or the Bonferroni test was performed for comparison with a control group when the differences in the mean values among the groups were significant ($\alpha = 0.05$). Data that failed the Shapiro–Wilk test for normality underwent an arcsin or Box-Cox transformation [94] prior to conducting the ANOVA. When equal variance could not be assumed, the data were analyzed via Welch's one-way ANOVA. The means were then compared with the Games–Howell pairwise comparison at a 95% confidence level. Student's *t*-test or Welch's *t*-test was used to compare the means between two groups when equal variances were or were not assumed, respectively. One sample *t*-test was performed to compare observed means to a hypothesized value. The Wilcoxon signed rank test was performed for non-parametric data. All statistical analyses are provided in Table S4.

5. Conclusions

In summary, the results of this study, produced independently in different laboratories, in combination with other RNAi results published to date, demonstrate inconsistent effects, indicating a high variability in RNAi efficiency in *Aedes* mosquitoes, for which the reasons are currently not understood. For the reproducible and reliable application of RNAi in (*Aedes*) mosquitoes, we need to better understand the involved molecular mechanisms and contributing factors, which will require further basic research on the different steps of the RNAi process and the fate of RNAi-triggering molecules inside the mosquito body. Only then will we be able to develop RNAi protocols that are robust, not only under laboratory conditions but also in the field, where varying climatic conditions and other variables such as the larval development stage, nutrition status, or availability of alternative nutrition sources influence the system. Only if the system is stable enough to be unaffected by these variables can the technology reliably be used for mosquito control or sexing within genetic control programs.

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Supplementary information for "Challenges of robust RNAi-mediated gene silencing in *Aedes* mosquitoes"

Supplementary information includes the list of primer sequences; the list of siRNA, shRNA, and dsRNA sequences used in this study; experimental setup of feeding assays; raw data of survival rates and phenotypical observations.

Figure S1

Figure S2

Tables S1-S3, and Tables S5-S7

Table S4 with statistical analysis is provided in the Appendix of this thesis.

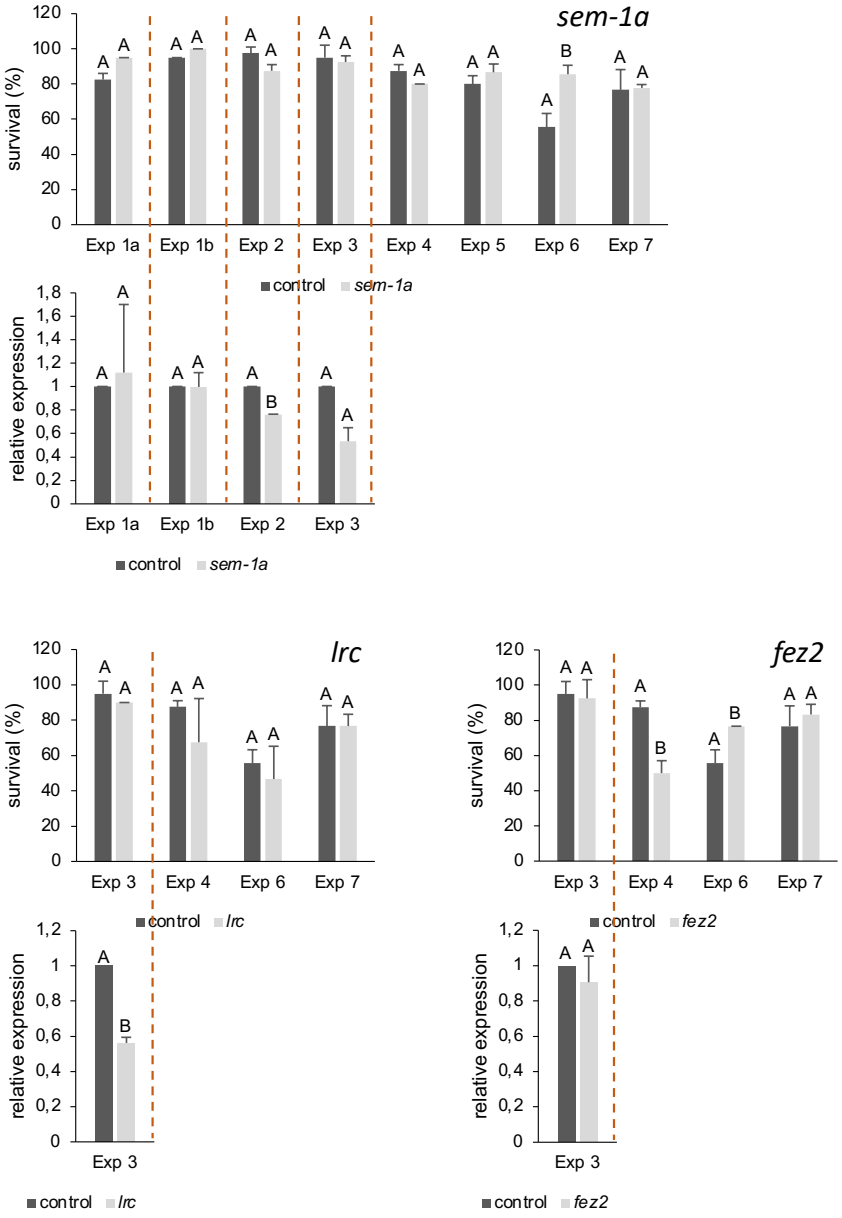

Figure S1: Percent survival until pupation of *Ae. aegypti* larvae fed with shRNA-expressing yeast, and corresponding transcript levels of targeted genes in the surviving pupae. Shown are the average survival rates of the different feeding experiments with shRNA-producing yeasts that are summrized in Figure 1 of the study. Insects were monitored until pupation and all viable pupae were counted as survivors. In case transcript levels were assessed by RT-qPCR, five pupae were pooled. Relative expression was calculated with the Pfaffl method. Bars and error bars represent the average survival and standard deviation, respectively, of two technical replicates for experiments 1 to 5, and three replicates for experiments 6 and 7. Each replicate contained 20 (exp. 1-4) to 30 (exp. 5-7) individuals. Exp. 1-4 and 6 were performed with larvae of the Orlando wild type strain, exp. 5 and 7 with the Liverpool wild type strain. Exp. 1a and 1b were performed with yeast from the same culture batch, but on different days. Relative transcript levels of experiments 6 and 7 are shown in Figure 1. "Control" is the feeding with yeasts expressing an unspecific shRNA); "Exp" = independent experiment (i.e. biological replicate); *sem-1a = semaphorin-1a*, *lcr = leukocyte receptor cluster*, *fez2 = fasciculation and elongation protein zeta2;* Raw data are provided in Table S1

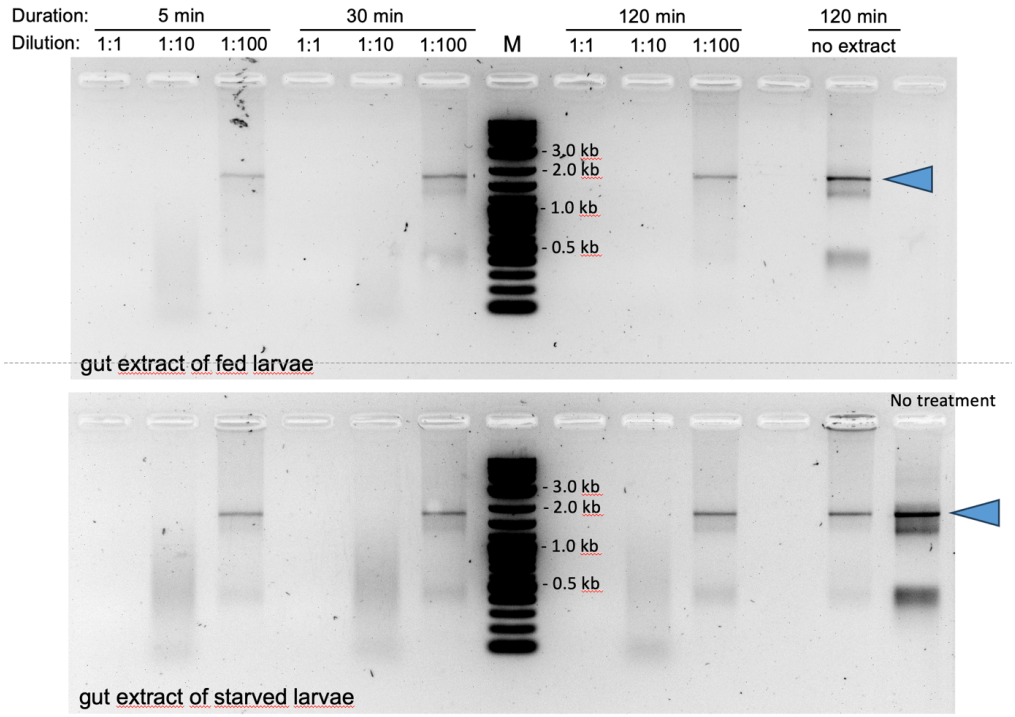

Figure S2. Gel electrophoretic analysis of dsRNA incubated with a serial dilution of *Ae. aegypti* gut extract. Guts of ten L3/L4 larvae (fed on fish food or starved for 24 h) were pooled in RNase-free 1x TE buffer and homogenized with a pistil. The supernatant was serially diluted and incubated as indicated with 200 ng of in vitro transcribed *gusA* dsRNA. The digestion was stopped by proteinase K digest before gel analysis. Arrows indicate the position of the undigested *gusA* dsRNA (size: ~1.8 kb). M = 1 kb plus ladder (NEB).

Table S1. General summary of experiments and raw data

Approach/delivery method	"source" of RNAi molecule	Developmental stage	RNAi molecule	Targets	total replicates (biological + technical)	Total number of animals	Main results	Statistics	Reference
Oral delivery	Yeast	L1	shRNA	*fez2*, *lrc*, *sem1a*, control shRNA	10 to 18	1400	Lethality not higher than controls	*p*-values = 1.00 (*sem-1a*), 1.00 (fez2), 0.088 (*lrc*)	Figure 1a
	In vitro transcription	L1	dsRNA	*beta-tubulin*, *eGFP*	24	240	Lethality not higher than controls	*p*-values = 0.630, 0.882, 0.303, 0.568, 0.738, 0.597, 0.0107	Figure 2a
	Bacteria	L1	dsRNA	*fez2*, *lrc*, *sem1a_E8*, sem1a_E15, beta-tubulin, ache1, V-ATPase, αcop, *gusA*, *eGFP*, bact ctrl	12 to 24	3980	Lethality not higher than controls	*p*-value = 0.324 (Figure 2b) *p*-value = 0.355 (Figure 2c) *p*-value = 0.017 (Figure 2d)[1] *p*-value = 0.612 (Figure 2e) *p*-value = 0.985 (Figure 2f)	Figure 2a-f
	Bacteria	L1	shRNA	*fez2*, *lrc*, *dsx*, *endonucleases*	2 to 3	1460	Weak lethal effect when targeting *fez2* v2 in the La Réunion strain	*p*-value = 0.844 (Figure 5a) *p*-value = 0.828 (Figure 5b) *p*-value = 0.0489 (Figure 5b) *p*-value = 0.272 (Figure 5c) *p*-value = 0.415 (Figure 6)	Figure 5, Figure 6
Soaking	Bacterially produced and purified	L1 nenonate larvae	dsRNA	*fez2*, *lrc*, *sem1a_E8*, *gusA*, *eGFP*, bact ctrl, H_2O	4	840	Lethality not higher than controls	*p*-value = 0.154 (Strain Liverpool), *p*-value = 0.857 (Strain Brazi)	Figure 7a, 7b
	Chemically synthesized, purchased from IDT	L1 nenonate larvae	siRNA	*fez2*, *lrc*, *sem1a_E8*, *unspecific siRNA*, H_2O	3	375	Lethality not higher than controls	*p*-value = 0.223	Figure 7c
	In vitro transcription	L1 nenonate larvae	dsRNA	*dsx*, *eGFP (control)*	3	600	Reduced levels of the female-specific *dsx* transcript, no difference in the male-to-female ratio between control and *dsx* dsRNA treatments	*p*-value = 0.0000334 (Figure 8a) *p*-value = 0.815 (Figure 8b)	Figure 8
Injection	Bacterially produced and purified	L2 larvae	dsRNA	*fez2*+dye, *lrc*+dye, *sem1a_E8*+dye, *gusA*+dye, H_2O+dye, bact ctrl+dye	3	1800	Lethality not higher than controls	*p*-value = 0.499	Figure 9a
	Chemically synthesized, purchased from IDT	L2, L4 Larvae	siRNA	*fez2*+dye, *lrc*+dye, *sem1a* +dye, *unspecific siRNA*+dye, H_2O+dye, H_2O	6	3600	Lethality not higher than controls	*p*-value = 0.127 (L2 larvae), *p*-value = 0.363 (L4 larvae)	Figures 9b, 9c
					total number of animals	*14295*			

[1] The observed p-value < 0,05 for this experiment was due to differences between two of the treatment conditions (lrc and ache1) and not to the control dsRNAs *gusA* or *eGFP*

"bact ctrl" is total RNA extracted from bacteria transformed only with the empty expression vector L4440

Supplementary material - Figueiredo Prates et al. (2024) IJMS

Figure 1A
Yeast feeding assay summary

Control		
control total	starting number	final # alive
C-1	20	17
C-2	20	16
C-1	20	19
C-2	20	19
C-1	20	19
C-2	20	20
C-1	20	18
C-2	20	20
C-1	20	17
C-2	20	18
C-1	30	25
C-2	30	23
C-1	30	14
C-2	30	18
C-3	30	18
C-1	30	21
C-2	30	21
C-3	30	27
n	440	
reps	18	

Sem1a		
Sem1a total	starting number	final # alive
S-1	20	19
S-2	20	19
S-1	20	20
S-2	20	20
S-1	20	17
S-2	20	18
S-1	20	18
S-2	20	19
S-1	20	16
S-2	20	16
S-1	30	27
S-2	30	25
S-1	30	24
S-2	30	27
S-3	30	26
S-1	30	24
S-2	30	23
S-3	30	23
n	440	
reps	18	

Fez2		
fez2 total	starting number	final # alive
F-1	20	20
F-2	20	17
F-1	20	11
F-2	20	9
F-1	30	23
F-2	30	23
F-3	30	23
F-1	30	23
F-2	30	26
F-3	30	26
n	260	
reps	10	

Lrc		
lrc total	starting number	final # alive
L-1	20	18
L-2	20	18
L-1	20	17
L-2	20	10
L-1	30	8
L-2	30	15
L-3	30	19
L-1	30	25
L-2	30	23
L-3	30	21
n	260	
reps	10	

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Figure 1B relative expression data

Control, day 3	Sem1a, day 3
1	1,128863867
	0,810532194
	1,283704058

Control, day 5	Sem1a, day 5
1	0,57288702
	0,59140946

Control, pupae	Sem1a, pupae
1	1,26405353
	0,767413261
	0,799633637

Figure 1C relative expression data

Control, day 3	Sem1a, day 3
1	1,002571451
	0,841435249
	0,982877873

Control, day 5	Sem1a, day 5
1	1,162348625
	1,010494305
	0,865882198

Control, pupae	Sem1a, pupae
1	0,907193656
	1,0543007
	0,748771909

Figure 1D relative expression data

Control, day 3	Irc, day 3
1	0,801533758
	1,282367402
	1,557145661

Control, day 5	Irc, day 5
1	0,663156174
	0,919453457

Control, pupae	Irc, pupae
1	1,082308505
	1,298959893
	1,04915697

Figure 1E

Control, day 3	Irc, day 3
1	1,432085699
	1,325369338

Control, day 5	Irc, day 5
1	1,681262032
	1,44720348
	0,969209546

Control, pupae	Irc, pupae
1	1,00647434
	0,749632535
	0,777034657

Figure 1F relative expression data

Control, day 3	fez2, day 3
1	1,176094999
	1,132116769
	1,368799731

Control, day 5	fez2, day 5
1	0,832362953
	0,742311815
	0,684625401

Control, pupae	fez2, pupae
1	1,042965329
	1,013381281
	1,022788857

Figure 1G relative expression data

Control, day 3	fez2, day 3
1	0,617411017
	0,811603557
	0,892663919

Control, day 5	fez2, day 5
1	1,083803082
	1,197327784
	1,470543557

Control, pupae	fez2, pupae
1	1,316735404
	1,253608746
	1,272232264

Figure 2A

target: EGFP IVT (1X)			
	Number of animals		
	day1	day10	survival (%)
well 1	5	5	100
well 2	5	5	100
well 3	5	4	80
well 4	5	4	80
well 5	5	5	100
well 6	5	5	100
well 7	5	5	100
well 8	5	3	60
well 9	5	5	100
well 10	5	5	100
well 11	5	5	100
well 12	5	5	100
well 13	5	5	100
well 14	5	3	60
well 15	5	4	80
well 16	5	5	100
well 17	5	5	100
well 18	5	5	100
well 19	5	4	80
well 20	5	5	100
well 21	5	5	100
well 22	5	5	100
well 23	5	2	40
well 24	5	5	100

target: β-tub 2851 IVT (1X)			
	Number of animals		
	day1	day10	survival (%)
well 1	5	3	60
well 2	5	3	60
well 3	5	5	100
well 4	5	5	100
well 5	5	5	100
well 6	5	5	100
well 7	6	6	100
well 8	5	5	100
well 9	5	5	100
well 10	5	5	100
well 11	6	5	83,3333333
well 12	5	5	100
well 13	5	5	100
well 14	5	5	100
well 15	5	5	100
well 16	5	3	60
well 17	5	5	100
well 18	5	5	100
well 19	5	5	100
well 20	5	3	60
well 21	5	5	100
well 22	5	5	100
well 23	5	5	100
well 24	5	5	100

target: EGFP (1X)			
	Number of animals		
	day1	day12	survival (%)
well 1	10	10	100
well 2	10	9	90
well 3	10	8	80
well 4	10	7	70
well 5	10	9	90
well 6	11	10	90,9090909
well 7	11	10	90,9090909
well 8	12	11	91,6666667
well 9	10	9	90
well 10	10	9	90
well 11	10	10	100
well 12	10	10	100

target: βtub 2851 (1X)			
	Number of animals		
	day1	day12	survival (%)
well 1	10	7	70
well 2	10	9	90
well 3	10	10	100
well 4	10	9	90
well 5	10	10	100
well 6	10	9	90
well 7	10	10	100
well 8	10	10	100
well 9	10	7	70
well 10	10	8	80
well 11	10	6	60
well 12	10	10	100

target: EGFP (5X)			
	Number of animals		survival (%)
	day1	day10	
well 1	10	7	70
well 2	10	7	70
well 3	10	10	100
well 4	10	10	100
well 5	10	10	100
well 6	10	9	90
well 7	10	9	90
well 8	10	9	90
well 9	10	10	100
well 10	10	7	70
well 11	10	10	100
well 12	11	10	90,9090909

target: βtub 2851 (5X)			
	Number of animals		
	day1	day10	survival (%)
well 1	10	9	90
well 2	10	6	60
well 3	10	9	90
well 4	10	8	80
well 5	10	7	70
well 6	10	9	90
well 7	10	9	90
well 8	10	10	100
well 9	10	9	90
well 10	10	9	90
well 11	10	5	50
well 12	10	10	100

target: EGFP (2.5X)			
	Number of animals		
	day1	day11	survival (%)
well 1	10	9	90
well 2	10	8	80
well 3	10	10	100
well 4	10	10	100
well 5	10	9	90
well 6	10	8	80
well 7	10	10	100
well 8	10	9	90
well 9	10	10	100
well 10	10	8	80
well 11	10	9	90
well 12	10	9	90

target: β-tub 4939 (2.5X)			
	Number of animals		
	day1	day11	survival (%)
well 1	10	8	80
well 2	10	10	100
well 3	10	10	100
well 4	10	10	100
well 5	10	9	90
well 6	10	9	90
well 7	10	6	60
well 8	10	10	100
well 9	10	9	90
well 10	10	8	80
well 11	10	9	90
well 12	10	6	60

Figure 2A (continuation)

target: EGFP (5X)

	Number of animals		
	day1	day10	survival (%)
well 1	10	9	90
well 2	10	10	100
well 3	11	10	90,9090909
well 4	10	10	100
well 5	10	7	70
well 6	10	5	50
well 7	10	5	50
well 8	10	6	60
well 9	10	10	100
well 10	10	8	80
well 11	10	9	90
well 12	10	9	90
well 1	10	10	100
well 2	10	9	90
well 3	10	9	90
well 4	10	9	90
well 5	10	10	100
well 6	10	10	100
well 7	10	10	100
well 8	10	9	90
well 9	10	10	100
well 10	10	10	100
well 11	10	10	100
well 12	10	9	90

target: βtub 4939 (5X)

	Number of animals		
	day1	day7	survival (%)
well 1	10	9	90
well 2	10	9	90
well 3	10	10	100
well 4	10	10	100
well 5	10	10	100
well 6	10	10	100
well 7	10	8	80
well 8	10	9	90
well 9	10	9	90
well 10	10	9	90
well 11	10	9	90
well 12	10	10	100
well 1	10	5	50
well 2	11	9	81,8181818
well 3	10	10	100
well 4	10	8	80
well 5	10	9	90
well 6	10	10	100
well 7	10	10	100
well 8	10	6	60
well 9	10	9	90
well 10	10	10	100
well 11	10	7	70
well 12	10	7	70

target: EGFP (2.5X)

	Number of animals		
	day1	day13	survival (%)
well 1	10	10	100
well 2	10	10	100
well 3	10	3	30
well 4	10	10	100
well 5	10	10	100
well 6	10	10	100
well 7	11	5	45,4545455
well 8	10	2	20
well 9	10	10	100
well 10	10	8	80
well 11	10	9	90
well 12	10	6	60

target: βtub 2851 + βtub 4939 (2.5x)

	Number of animals		
	day1	day13	survival (%)
well 1	10	9	90
well 2	10	5	50
well 3	10	1	10
well 4	10	10	100
well 5	10	5	50
well 6	10	3	30
well 7	10	5	50
well 8	10	10	100
well 9	10	9	90
well 10	10	10	100
well 11	10	10	100
well 12	10	8	80

target: EGFP (5X)

	Number of animals		
	day1	day10	survival (%)
well 1	10	9	90
well 2	10	10	100
well 3	10	4	40
well 4	10	8	80
well 5	10	6	60
well 6	10	8	80
well 7	11	10	90,9090909
well 8	10	4	40
well 9	10	6	60
well 10	10	8	80
well 11	10	7	70
well 12	11	6	54,5454545

target: βtub 2851, 4393 (5X)

	Number of animals		
	day1	day10	survival (%)
well 1	10	8	80
well 2	10	10	100
well 3	10	9	90
well 4	10	9	90
well 5	10	10	100
well 6	10	8	80
well 7	11	10	90,9090909
well 8	10	9	90
well 9	10	5	50
well 10	10	10	100
well 11	10	9	90
well 12	10	9	90

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Figure 2B

Orlando			
	Initial	Pupae	Pupation
eGFP	21	19	90,4761905
βtub2851	20	20	100
fez2	20	20	100
lrc	20	18	90
Sem1a_E8	20	19	95
Sem1a_E15	20	19	95
Ache1	20	14	70
VATPase	20	20	100

Liverpool			
	Initial	Pupae	Pupation
eGFP	21	16	76,1904762
βtub2851	20	18	90
fez2	20	14	70
lrc	20	12	60
Sem1a_E8	20	15	75
Sem1a_E15	20	16	80
Ache1	19	14	73,6842105
VATPase	20	17	85

Figure 2C

Replicate 1			
	initial	Final living	Survival
Empty bact	30	7	23,3333333
eGFP	30	8	26,6666667
fez2	30	14	46,6666667
lrc	30	2	6,66666667
sem1a_E8	30	3	10
αCOP494	30	10	33,3333333

Replicate 2			
	initial	Final living	Survival
Empty bact	47	20	42,5531915
eGFP	46	15	32,6086957
fez2	46	15	32,6086957
lrc	46	13	28,2608696
sem1a_E8	46	8	17,3913043
αCOP494	46	10	21,7391304

Figure 2D

Replicate 1			
	Initial	Pupae alive	Pupation
GusA	20	18	90
eGFP	20	13	65
ßtub	20	12	60
Fez2	20	11	55
Lrc	20	15	75
Sem1a_E8	20	16	80
Sem1a_E15	20	13	65
Ache1	20	12	60
VATPase	20	12	60

Replicate 2			
	Initial	Pupae alive	Pupation
GusA	20	15	75
eGFP	20	15	75
ßtub	20	12	60
Fez2	20	9	45
Lrc	20	14	70
Sem1a_E8	20	16	80
Sem1a_E15	20	10	50
Ache1	20	11	55
VATPase	20	9	45

Replicate 3			
	Initial	Pupae alive	Pupation
GusA	21	18	85,7142857
eGFP	20	11	55
ßtub	20	6	30
fez2	20	14	70
lrc	20	14	70
sem1a_E8	21	14	66,6666667
sem1a_E15	20	8	40
Ache1	20	12	60
VATPase	20	9	45

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Figure 2E

	Replicate 1				Replicate 2				Replicate 3			
	initial	Pupae	Pupation			initial	Pupae	Pupation		initial	Pupae	Pupation
Gus A	20	12	60		Gus A	20	9	45	Gus A	20	15	75
eGFP	20	12	60		eGFP	20	11	55	eGFP	20	15	75
βtub	20	10	50		βtub	20	11	55	βtub	20	17	85
Fez2	21	17	80,952381		Fez2	21	9	42,8571429	Fez2	20	12	60
lrc	20	6	30		lrc	20	13	65	lrc	20	10	50
sem1a_E8	20	8	40		sem1a_E8	20	8	40	sem1a_E8	20	8	40
sem1a_E15	21	16	76,1904762		sem1a_E15	20	9	45	sem1a_E15	20	10	50
Ache1	20	11	55		Ache1	20	10	50	Ache1	20	13	65
VATPase	20	15	75		VATPase	20	8	40	VATPase	20	13	65

Figure 2F

	Replicate 1				Replicate 2			
	Initial	Final living	survival (%)			Initial	Final living	survival (%)
Empty bact	40	0	0		Empty bact	46	13	28,2608696
eGFP	40	1	2,5		eGFP	46	16	34,7826087
fez2	40	3	7,5		fez2	46	11	23,9130435
lrc	40	2	5		lrc	46	22	47,826087
sem1a_E8	40	3	7,5		sem1a_E8	46	22	47,826087
αCOP494	40	3	7,5		αCOP494	46	8	17,3913043

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Figure 5

Species	Strain	Treatment	Replicate no.	Total L1	Pupae + adults	Larvae	Dead	Total alive (% survival)
Ae. albopictus	Montpellier	**nuclease 1**	1	40	22	18	0	40 (100%)
		nuclease 1	2	40	18	22	0	40 (100%)
		fez2	1	40	6	34	0	40 (100%)
		***fez2* + nuclease 1**	1	40	18	21	1	39 (97.5%)
		none	1	40	0	40	0	40 (100%)
		scramble	1	40	20	20	0	40 (100%)
		scramble	2	40	18	22	0	40 (100%)
Ae. aegypti	La Réunion	***fez2* v1**	1	40	23	2	15	25 (62.5%)
		***fez2* v1 + nuclease**	1	40	33	2	5	35 (87.5%)
		none	1	40	30	5	5	35 (87.5%)
		scramble	1	40	23	1	16	24 (60%)
		scramble	2	40	24	3	13	27 (67.5%)
	La Réunion	***Irc***	1	40	14	19	7	33 (82.5%)
		Irc	2	40	19	8	13	27 (67.5%)
		***fez2* v2**	1	40	12	21	7	33 (82.5%)
		***fez2* v2**	2	40	14	18	8	32 (80%)
		none	1	40	19	20	1	39 (97.5%)
		scramble	1	40	20	19	1	39 (97.5%)
	Liverpool	***Irc***	1	40	6	32	2	38 (95%)
		Irc	2	40	0	27	13	27 (67.5%)
		***fez2* v2**	1	45	1	44	0	45 (100%)
		***fez2* v2**	2	40	0	12	28	12 (30%)
		none	1	44	3	41	0	44 (100%)
		scramble	1	40	1	30	9	31 (77.5%)

Figure 6

Treatment	Replicate	No. (%) males	No. (%) females	Could not be sexed	Reached adulthood (%)	Remaining larvae/ pupae after 1 month
***dsxF1* + *dsxF2* + 3 nucleases**	1	17 (50.0 %)	17 (50.0 %)	0	34 (68.0 %)	5
***dsxF1* + *dsxF2* + 3 nucleases**	2	19 (54.3 %)	16 (45.7 %)	0	35 (70.0 %)	1
***dsxF1* + *dsxF2* + 3 nucleases**	3*	12 (38.7 %)	19 (61.3 %)	0	31 (62 %)	0
***dsxF1* + *dsxF2* + 3 nucleases**	4*	11 (45.7%)	19 (54.3%)	5	35 (70%)	0
***dsxF1* + *dsxF2* + 3 nucleases**	5*	13 (40.0%)	21 (60.0%)	1	35 (70%)	0
none	1	12 (60.0 %)	8 (40.0%)	0	20 (40.0 %)	15
none	2*	14 (50.0%)	14 (50.0%)	2	30 (60%)	0
scramble	1	18 (56.3 %)	14 (43.8%)	0	32 (64.0 %)	2
scramble	2	14 (48.3 %)	15 (51.7%)	0	29 (58.0 %)	2
scramble	3*	13 (35.1%)	24 (64.9 %)	0	37 (74.0 %)	0

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Figure 7A
Soaking L1 4h dsRNA, Liverpool strain

Replicate 1				Replicate 2			
	Initial	Total (pupae) alive	survival (%)		Initial	Total (pupae) alive	survival (%)
H2O	30	29	96,6666667	H2O	30	28	93,33333333
GusA 1500	30	26	86,6666667	GusA 1500	30	30	100
eGFP 1500	32	31	96,875	eGFP 1500	31	30	96,77419355
fez 500	30	27	90	fez 500	30	23	76,66666667
fez 1000	30	27	90	fez 1000	30	29	96,66666667
fez 1500	30	29	96,6666667	fez 1500	30	29	96,66666667
lrc 500	30	26	86,6666667	lrc 500	30	27	90
lrc 1000	30	29	96,6666667	lrc 1000	30	23	76,66666667
lrc 1500	30	27	90	lrc 1500	30	26	86,66666667
Sem 1500	30	27	90	Sem 1500	30	24	80

Figure 7B
Soaking L1 4h dsRNA, Brazil strain

Replicate 1				Replicate 2			
	Initial	Total (pupae) alive	survival (%)		Initial	Total (pupae) alive	survival (%)
gusA dsRNA 1500	25	25	100	gusA dsRNA 1500	30	28	93,3333333
fez2 dsRNA 1500	29	26	89,6551724	fez2 dsRNA 1500	31	31	100
lrc dsRNA 1500	30	29	96,6666667	lrc dsRNA 1500	32	29	90,625
sem1a dsRNA 1500	30	28	93,3333333	sem1a dsRNA 1500	30	28	93,3333333

Figure 7C
Soaking L1 4h siRNA, Liverpool strain

Replicate 1				Replicate 2				Replicate 3			
	initial	Total (pupae) alive	survival (%)		initial	Total (pupae) alive	survival (%)		initial	Total (pupae) alive	survival (%)
ddH2O	25	23	92	ddH2O	25	22	88	ddH2O	25	20	80
ctrl siRNA	25	24	96	ctrl siRNA	25	18	72	ctrl siRNA	25	15	60
fez2 siRNA	26	26	100	fez2 siRNA	25	15	60	fez2 siRNA	25	13	52
lrc siRNA	25	7	28	lrc siRNA	25	18	72	lrc siRNA	25	18	72
sem1a siRNA	25	21	84	sem1a siRNA	25	12	48	sem1a siRNA	25	17	68

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Figure 8A

Fold change expression

Control	dsx soaking
1,0777	0,5822
0,8491	0,5761
1,0867	0,9116
0,9469	0,7027
1,1281	0,6861
0,9852	0,4363
0,9361	0,2279
0,9225	0,2596
	0,3847
	0,3131
	0,1805
Average control	**Average dsx soaking**
0,9916	0,4783

Figure 8B - male to female ratio data

Control soaking result					Dsx soaking result			
replicate	number of females	number of males	male ratio		replicate	number of females	number of males	male ratio
control-1	38	49	56,32%		dsx-1	18	33	64,71%
control-2	24	29	54,72%		dsx-2	42	68	61,82%
control-3	22	38	63,33%		dsx-3	29	41	58,57%
control-4	11	39	78,00%		dsx-4	25	51	67,11%
control-5	17	39	69,64%		dsx-5	65	73	52,90%
control-6	24	33	57,89%					
control-7	47	57	54,81%					

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Figure 9A
Injections L2 dsRNA

Replicate 1				Replicate 2				Replicate 3			
	Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)
ddH2O + color dye	88	44	50,0	ddH2O + color dye	88	40	45,5	ddH2O + color dye	88	59	67,0
fez2 dsRNA + color dye	88	39	44,3	fez2 dsRNA + color dye	88	38	43,2	fez2 dsRNA + color dye	88	50	56,8
lrc dsRNA + color dye	88	39	44,3	lrc dsRNA + color dye	88	50	56,8	lrc dsRNA + color dye	88	37	42,0
sem1a dsRNA + color dye	88	49	55,7	sem1a dsRNA + color dye	88	58	65,9	sem1a dsRNA + color dye	88	38	43,2
Gus A dsRNA (ctrl) + color dye	88	47	53,4	Gus A dsRNA (ctrl) + color dye	88	52	59,1	Gus A dsRNA (ctrl) + color dye	88	32	36,4
Coextract empty bact + dye	88	40	45,5	Coextract empty bact + dye	88	57	64,8	Coextract empty bact + dye	88	37	42,0

Figure 9B
Injections of L2 Liverpool with siRNA at 1000 ng/µL

Replicate 1				Replicate 2				Replicate 3			
	Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)
ddH2O + color dye	88	43	48,9	ddH2O + color dye	98	62	63,3	ddH2O + color dye	88	22	25,0
fez2 siRNA + color dye	88	33	37,5	fez2 siRNA + color dye	88	36	40,9	fez2 siRNA + color dye	88	20	22,7
lrc siRNA + color dye	88	24	27,3	lrc siRNA + color dye	88	46	52,3	lrc siRNA + color dye	88	15	17,0
sem1a siRNA + color dye	88	43	48,9	sem1a siRNA + color dye	88	46	52,3	sem1a siRNA + color dye	88	25	28,4
ctrl siRNA + color dye	88	30	34,1	ctrl siRNA + color dye	88	58	65,9	control siRNA + color dye	88	25	28,4
ddH2O	88	52	59,1	ddH2O	88	54	61,4	ddH2O	88	37	42,0

Figure 9C
injections L4 siRNA 1000 ng/µL

Replicate 1				Replicate 2				Replicate 3			
	Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)		Initial	Total pupae alive	survival (%)
ddH2O + color dye	100	77	77,0	ddH2O + color dye	100	82	82,0	ddH2O + color dye	100	87	87,0
fez2 siRNA + color dye	100	66	66,0	fez2 siRNA + color dye	100	85	85,0	fez2 siRNA + color dye	100	84	84,0
lrc siRNA + color dye	93	75	80,6	lrc siRNA + color dye	100	81	81,0	lrc siRNA + color dye	100	82	82,0
sem1a siRNA + color dye	100	80	80,0	sem1a siRNA + color dye	100	87	87,0	sem1a siRNA + color dye	90	62	68,9
ctrl siRNA + color dye	100	81	81,0	ctrl siRNA + color dye	100	80	80,0	ctrl siRNA + color dye	100	83	83,0
ddH2O	100	84	84,0	ddH2O	100	86	86,0	ddH2O	110	94	85,5

Figure 9D - relative expression data
qPCR L2 Ae aegypti Liverppol injected with dsRNA, collected 24h after injection

Replicate 1				Replicate 2			
	fez2	lrc	sem1a_E8		fez2	lrc	sem1a_E8
control (Gus A dsRNA) + dye	1	1	1	control (Gus A dsRNA) + dye	1	1	1
dsRNA + dye	0,874117623	0,81786592	0,837453705	dsRNA + dye	0,8925208	1,660127047	0,80702
ddH2O + dye	1,035464259	1,12460263	1,407912342	ddH2O + dye	0,51421146	0,945320615	1,06575119
Coextract empty bact + dye	0,9469824	0,92366885	1,594883155	Coextract empty bact + dye	0,50862485	1,122507194	0,98379157

Figure 9E - relative expression data
qPCR L4 Ae aegypti Liverppol injected with siRNA, collected 24h after injection

Replicate 1				Replicate 2			
	fez2	lrc	sem1a		fez2	lrc	sem1a
control (siRNA control sequence) + dye	1	1	1	control (siRNA control sequence)	1	1	1
siRNA + dye	1,402656985	0,90681831	1,261929078	siRNA + dye	1,05928275	1,490449432	0,85347562
ddH2O + dye	1,458675232	0,93159529	0,98613623	ddH2O + dye	1,17590347	1,494621468	0,73218448
ddH2O	1,135550494	1,27640081	1,198676897	ddH2O	1,19399648	1,11995812	0,89985403

Table S2

General notes:

Sequences used in this study. All sequences listed here are shown in 5' --> 3' direction.

Primer melting temperatures were determined with Geneious software, which uses a modified version of Primer3 2.3.7 (Formula: SantaLucia 1998)

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Table S2A. Primers for producing templates for dsRNA synthesis by in vitro transcription (IVT). Gene-specific parts of the primers are shown; a T7 promoter and linker sequence (ccctttaatacgactcactatagggagaa) was added to the 5' end of each primer for IVT. Capital "F" and "R" denote forward and reverse primers, respectively. βtub and and gusA primers were used for the projects at Justus Liebig University Giessen, dsx and GFP primers at Michigan State University.

Species	purpose	Target / Gene symbol	mRNA	Primer Name	Primer sequence	Primer length (bp)	%GC	Hairpin Tm (°C)	Tm (°C)	Amplicon length
Escherichia coli	IVT of dsRNA	beta-glucuronidase / uidA [36]	NP_416134.1	886_Ecoli-gusA-dsF for IVT	TGGTCCGTCCTGTAGAAACC	20	55.0	37.1	59.0	1866
				887_Ecoli-gusA-dsR for IVT	CCCCACCGAGGCTGTAGC	18	72.2	41.1	62.2	
Aedes aegypti		tubulin beta-1 chain / LOC5576244 (AAEL002851) [36]	XM_001655975.2	884_Aae-btub-dsF for IVT (AAEL002851)	GGAAATCATCTCCGACGAAC	20	50.0	None	56.3	786
				885_Aae-btub-dsR for IVT (AAEL002851)	CACGGTACTGTTGCGATCC	19	57.9	None	58.6	
		doublesex / dsx (AAEL009114) [19]	XM_021837269.1	dsDSX2-F	GCAAATGCTGTTTAACGATAATAG	24	33.3	38.2	55.3	462
				dsDSX2-R	CGGAGCCGTTTGGCAACGG	19	68.4	65.5	64.8	
Aequorea victoria		green fluorescent protein		dsGFP-F	GGAGAAGAACTTTTCACTGGAGTTG	25	44.0	34.0	60.0	411
				dsGFP-R	TTTGTGTCCGAGAATGTTTCCATCT	25	40.0	None	61.0	

Table S2B. Primers for producing L4440 dsRNA expression plasmids, for dsRNA production by the bacterial strain HT115 DE3. Gene-specific parts are shown in uppercase letters, sequences added to the 5' end for cloning into L4440 plasmid are shown in lowercase letters. Sequences used for projects at Justus Liebig University Giessen.

Species	purpose	Target / Gene symbol	mRNA	Primer Name	Primer sequence (with extension)	Primer length (bp)	%GC	Hairpin Tm (°C)	Tm (°C)	Amplicon length
Aequorea victoria	dsRNA L4440 bacterial expression vector cloning	enhanced green fluorescent protein		P1022_EGFP_dsRNA_for	tacaagcttACCACATGAAGCAGCACGAC	29	48.3	48.5	67.3	476
				p1023_egfp_dsrna_rev	atagagctcgtccatgccgagagtgatc	28	53.6	51.5	67.4	
Escherichia coli		beta-glucuronidase / uidA [36]	NP_416134.1	P1267_Ecoli-gusA F2	agatgggcccTGGTCCGTCCTGTAGAAACC	30	56.7	55.7	71.2	1866
				P1268_Ecoli-gusA R2	agatgcggccgcCCCCACCGAGGCTGTAGC	31	64.5	51.6	76.3	
Aedes aegypti		tubulin beta chain / LOC5565681 (AAEL004939) [19]	XM_001650004.2	P1297_Aae-btub-F AAEL004939	agatgggcccTATCCTGGGCAGCTGAACTC	30	60.0	52.3	72.1	308
				P1298_Aae-btub-R AAEL004939	agatgcggccgcCAATGCGAGAAGGTACCGTG	30	73.3	51.1	79.3	
		tubulin beta-1 chain / LOC5576244 (AAEL002851) [36]	XM_001655975.2	P1299_Aae-btub-dsRNA-F3 AAEL002851	agatgggcccGGAAATCATCTCCGACGAAC	30	60.0	65.9	72.2	786
				P1300_Aae-btub-dsRNA-R3 AAEL002851	agatgcggccgcCACGGTACTGTTGCGATCC	32	62.5	64.5	76.0	
		coatomer subunit alpha / LOC5577214 (AAEL015001)	XM_001663259.2	P1569_ aCOP-E6-for1	atagggcgaattgggtacCAAGGGCTTCTACGCTG TTC	38	52.6	67.5	67.5	494
				p1570_ aCOP-E6-rev1	agatctgatatcatcgatgTTTCCATCTGAAGTCC GACC	39	43.6	40.2	63.1	
		Semaphorin-1a / LOC5575438 (AAEL002653) [35]	XM_021844665.1, XM_021844666.1	P1693_sem1a-E15-dsfor1	atagggtaccGGTGATAATTATCGTCGCGGT	31	48.4	64.8	68.4	51
				P1694_sem1a-E15-dsrev1	ataggagctcTTTCACGCTTCTCGTTGTTGAG	32	46.9	52.3	68.0	
		Semaphorin-1a / LOC5575438 (AAEL002653) [35]	XM_021844665.1, XM_021844666.1 , XM_021844668.1, XM_021844667.1, XM_021844669.1	P1695_sem1a-E8-dsfor1	atagggtacCCATCAAAACGGTGAATGCAGA	31	45.2	62.4	67.7	131
				P1696_sem1a-E8-dsrev2	ataggagctcCGTATTGCATGGTTCTAACGA	31	45.2	42.1	67.1	
		fasciculation and elongation protein zeta-2 / LOC5569012 (AAEL007292) [31]	XM_021849792.1	P1697_fez2-E1-dsfor1c	atagggtaccATTTCGGAAACATTTTACCAATCG	34	38.2	63.0	65.6	171
				P1698_fez2-E1-dsrev	ataggagctcGAATCAATGCGTGCATATCCA	31	45.2	45.9	67.8	
		leukocyte receptor cluster member 8 homolog / LOC5569340 (AAEL007548; AAEL007550) [31]	XM_001658387.2	P1699_lrc-E1-dsfor1	atagggtaccCGGGAATTGTCGGAAGTATC	30	50.0	62.4	67.0	166
				P1700_lrc-E1-dsrev1c	ataggagctcTCGAACCGGACTTATTGCC	29	51.7	None	67.6	
		acetylcholinesterase / LOC5578456 (AAEL000511)	all predicted transcript variants	P1737_ache1-E3-dsfor1	atagggtaccACCTACCACTGGCTAGCGAAC	31	54.8	63.0	69.9	171
				P1738_ache1-E3-dsrev1	ataggagctcACGATGCGACAAAGCTCACT	30	50.0	45.4	69.3	

Table S2C. Sequences of the forward (F) and reverse (R) oligos used for cloning PJet-shRNA expression vectors for shRNA production by HT115 DE3 bacteria. The shRNA and reverse complement sequences are shown in uppercase letters, the hairpin sequence is shown in lowercase and underlined letters. Four base pair overhangs used for cloning are shown in lowercase letters. Sequences used for projects at ASTRE, CIRAD, Montpellier.

Species	purpose	Target / Gene symbol	mRNA	Primer Name	Primer sequence (with extension)	Primer length (bp)	%GC	Hairpin Tm (°C)	Tm (°C)	Amplicon length
Aedes albopictus	shRNA PJet bacterial expression vector cloning	Fez2	(XM_019709180.1)	Aal Fez2 F	aatgCATGATGACTAAAGCAGAAATCAaagttctctTGATTTCTGCTTTAGTCATCATG	59	39.0	30.2	67.2	63
				Aal Fez2 R	ctcaCATGATGACTAAAGCAGAAATCAagagaacttTGATTTCTGCTTTAGTCATCATG	59	40.7	42.9	67.1	
		Nuclease 1	(XM_019679594.1)	Aal nuclease 1 F	aatgGGTAGGATTTCTGACTTACATTGaagttctctTCGTCAATTTCCTTGATGACCTC	59	39.0	40.0	67.0	63
				Aal nuclease 1 R	ctcaGGTAGGATTTCTGACTTACATTGagagaacttTCGTCAATTTCCTTGATGACCTC	59	40.7	35.9	67.0	
		Nuclease 2	(XM_019701402.1)	Aal nuclease 2 F	aatgGGGTAAAGAATTCAACACCAAGCaagttctctTTCACTCCTAGCTCCGAGCAAT	58	43.1	39.6	69.8	62
				Aal nuclease 2 R	ctcaGGGTAAAGAATTCAACACCAAGCagagaacttTTCACTCCTAGCTCCGAGCAAT	58	44.8	41.1	69.7	
		Nuclease 3	(XM_019683641.1)	Aal nuclease 3 F	aatgCGAGTATCAGCTGAAATTGAATGaagttctctCGCCACCGCAAGAGGTACTTGT	58	44.8	38.4	70.7	62
				Aal nuclease 3 R	ctcaCGAGTATCAGCTGAAATTGAATGagagaacttCGCCACCGCAAGAGGTACTTGT	58	46.6	41.4	70.5	
		dsxF1	(MF682531.1)	Aal dsxF1 F	aatgCAAGCCGTGGTAAATGAATACTCaagttctctCAATGTAAGTCAGAAATCCTACC	59	39.0	30.2	67.2	63
				Aal dsxF1 R	ctcaCAAGCCGTGGTAAATGAATACTCagagaacttCAATGTAAGTCAGAAATCCTACC	59	40.7	42.9	67.1	
		dsxF2	(MF682532.1)	Aal dsxF2 F	aatgGAAGTGCAAATGCTGTTCAACAAaagttctctGCTTGGTGTTGAATTCTTTACCC	59	39.0	42.5	68.6	63
				Aal dsxF2 R	ctcaGAAGTGCAAATGCTGTTCAACAAagagaacttGCTTGGTGTTGAATTCTTTACCC	59	40.7	41.4	68.6	
Aedes aegypti		Fez2 v1	(XM_021849792.1)	Aae Fez2 v1 F	aatgGAGGTCATCAAGGAAATTGACGAaagttctctCATTCAATTTCAGCTGATACTCG	59	39.0	45.3	67.4	63
				Aae Fez2 v1 R	ctcaGAGGTCATCAAGGAAATTGACGAagagaacttCATTCAATTTCAGCTGATACTCG	59	40.7	50.4	67.4	
		Nuclease	(XM_001653429.2)	Aae Nuclease F	aatgACAAGTACCTCTTGCGGTGGCGaagttctctATATTTATCTGACTTGCTTCTCC	58	43.1	48.6	68.6	62
				Aae Nuclease R	ctcaACAAGTACCTCTTGCGGTGGCGagagaacttATATTTATCTGACTTGCTTCTCC	58	44.8	57.5	68.8	
		Fez2 v2 [31]	(XM_021849792.1)	Aae Fez2 v2 F	aatgCTAGCATCATCTTCCGACCGAACCAaagttctctTAATATTTATCTGACTTGCTTCT	61	37.7	74.1	74.1	65
				Aae Fez2 v2 R	ctcaCTAGCATCATCTTCCGACCGAACCAagaggaacttTAATATTTATCTGACTTGCTTCT	61	39.3	74.8	74.8	

Table S2D. qPCR primers. *dsx* and *rps6* primers were used for the projects at Michigan State University, all others for projects at Justus Liebig University Giessen.

Species	purpose	Target / Gene symbol	Primer Name	Primer sequence (with extension)	Primer length (bp)	%GC	Hairpin Tm (°C)	Tm (°C)	Amplicon length
Aequorea victoria	qPCR	enhanced green fluorescent protein	P1712_EGFP_qPCR_F	AAGTTCATCTGCACCACCGG	20	55.0	None	60.6	81
			P1713_EGFP_qPCR_R	GAAGCACTGCACGCCGTAG	19	63.2	None	61.7	
Aedes aegypti		ribosomal protein S17 / LOC110680939 (AAEL004175)	P1512_RPS17_FW	AAGAAGTGGCCATCATTCCA	20	45.0	None	57.1	200
			P1513_RPS17_REV	GGTCTCCGGGTCGACTTC	18	66.7	None	59.1	
		sem1a / LOC5575438	P1759_Sem1a_qPCR_F1	GAGATCACGCTGCCTGTCC	19	63.2	None	60.5	131
			P1760_Sem1a_qPCR_R1	GCTGATCATGTCGTTGCCG	19	57.9	None	59.7	
		fez2 / LOC5569012 (AAEL007292)	P1761_fez2_qPCR_F1	TGCAGTGTCCAGGTTATACGG	21	52.4	None	59.8	76
			P1762_fez2_qPCR_R1	CCAGTGGCGGTTACAAGC	18	61.1	None	58.7	
		lrc / LOC5569340 (AAEL007548)	P1763_lrc_qPCR_R1	CGTTCGGTCGGAGTGAGAG	19	63.2	None	59.9	110
			P1764_lrc_qPCR_F1	GGAGGCGAGAACATATTGGAG	21	52.4	None	58.2	
		doublesex / dsx (AAEL009114)	qPCR-Aae.DSX2-F	CTCTTCAATCGTTTCCGACA	20	45.0	47.6	55.8	140
			qPCR-Aae.DSX2-R	CAGTGTCTGTGTCTGCTGGA	20	55.0	None	59.6	
		ribosomal protein S6 / LOC5563590 (AAEL000032)	qPCR-Aae.RPS6-F	GAAGTTGAACGTATCGTTTC	20	40.0	50.3	52.7	118
			qPCR-Aae.RPS6-R	GAGATGGTCAGCGGTGATTT	20	50.0	34.4	58.0	

Table S2E. Sequences of shRNAs used for feeding *Ae. aegypti* with shRNA-expressing yeasts; siRNA sequence and complement are underlined; hairpin loop sequence is denoted by **bold** letters; lowercase sequences were used for PCR amplification and cloning into the pRS426 shuttle vector; projects at Justus Liebig University Giessen.

Target	shRNA sequence
Irc shRNA LOC5569340 (AAEL007548) [31]	ctgcatggagatgggatccGTATCAGTCAGTATCAGAACCAGAA**TCAAGAG**TTCTGGTTCTGATACTGACTGATACTT TTTTctcgagctgcatggagat
sem1a shRNA LOC5575438 [35]	ctgcatggagatgggatccATTATCGTCGCGGTGACGGATT**TCAAGAG**AATCCGTCACCGCGACGATAATTTTTTTct cgagctgcatggagat
fez2 shRNA LOC5569012 (AAEL007292) [31]	ctgcatggagatgggatccCTAGCATCATCTTCCGACCGAACCA**TCAAGAG**TGGTTCGGTCGGAAGATGATGCTAGTT TTTTctcgagctgcatggagat
Universal control [35]	ctgcatggagatgggatccGAAGAGCACUGAUAGAUGUUAGCGU**TCAAGAG**ACGCTAACATCTATCAGTGCTCTTCTT TTTTctcgagctgcatggagat

Table S2F. shRNA target sequences used for feeding of *Ae. aegypti* and *Ae. albopictus* L1 larvae with shRNA-expressing bacteria. Consistency of target sequences in all tested strains were controlled by Sanger sequencing; projects at ASTRE, CIRAD, Montpellier.

Species	Target (NCBI identifier or literature reference)	Target sequence
Aedes albopictus	fez2 (XM_019709180.1)	CATGATGACTAAAGCAGAAATCA
	nuclease 1 (XM_019679594.1)	GGTAGGATTTCTGACTTACATTG
	nuclease 2 (XM_019701402.1)	GGGTAAAGAATTCAACACCAAGC
	nuclease 3 (XM_019683641.1)	CGAGTATCAGCTGAAATTGAATG
	dsxF1 (MF682531.1)	CAAGCCGTGGTAAATGAATACTC
	dsxF2 (MF682532.1)	GAAGTGCAAATGCTGTTCAACAA
Aedes aegypti	fez2 v1 (XM_021849792.1)	GAGGTCATCAAGGAAATTGACGA
	fez2 v2 [31]	CTAGCATCATCTTCCGACCGAACCA
	lrc [31]	GTATCAGTCAGTATCAGAACCAGAA
	nuclease (XM_001653429.2)	ACAAGTACCTCTTGCGGTGGCG
-	No target (unspecific sequence « scramble »)	GTATAGTATAGTATACCGTATAA

Table S2G. siRNA sequences used for *Ae. aegypti* injection and soaking experiments; siRNAs were purchased from Integrated DNA Technologies Ltd.; projects at Justus Liebig University Giessen.

Target	siRNA target sequence
fez2; LOC5569012 (AAEL007292) [31]	CTAGCATCATCTTCCGACCGAACCA
lrc; LOC5569340 (AAEL007548) [31]	GTATCAGTCAGTATCAGAACCAGAA
sem1a; LOC5575438 (AAEL007548; AAEL007550) [35]	ATTATCGTCGCGGTGACGGATT

Table S2H. dsRNA sequences designed to contain the published potent siRNA sequences [31, 35, 63] shown in **bold;** projects at Justus Liebig University Giessen.

Species	Target	dsRNA sequence	dsRNA length
Aedes aegypti	lrc [31]	CGGGAATTGTCGGAA**GTATCAGTCAGTATCAGAACCAGAA**CCTCCGAAGCAGCAGTCCTCCCAACG GCTTCGGTCGAAACAGTCAAAATTTCACTCGCAAAAGCGGTAATTCCGTGGGGACTCCGCATTCGC TTGGATCTCGCCTAGGCAATAAGTCCGGTTCGA	165
	sem1a exon 8 [63]	CATCAAAACGGTGAATGCAGAATCAGCCGACTCCAACAAGAAGGTCACCTCGGTTGTCATCGAGGA GATCGATGCTCTTCCAACTAGTGAACCGGTGCGGAGCTTGGAA**ATCGTTAGAACCATGCAAT**ACG	131
	sem1a exon 15 [35]	GGTGATA**ATTATCGTCGCGGTGACGGATT**CTCAACAACGAGAAGCGTGAAA	51
	fez2 [31]	ATTTCGGAAACATTTTACCAATCGATTGGTCAAAAACGTACGCGAGACAAATGCATGTACCGGCAC TGAAA**CTAGCATCATCTTCCGACCGAACCA**ACTACAATGACATTCAAGATCTTAGCTCCGAAGATG AGGCCGTTGCTAACGATTTGGATATGCACGCATTGATTC	171

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Table S3. Feeding scheme for bacteria-agar pellets in Orlando, food variant 4. As the first day pellet in experiment 1 was not consumed at all, the day 4 pellet was produced with less agar to facilitate the young larvae feeding. In experiment 2, the agar concentration of the first food pellet was reduced to 2% to account for the putatively low strength in the mouth parts of the very young larvae. “Agar content” specifies the concentration (w/v) and the volume of the agar solution used to prepare the food pellets.

Experiment 1			Experiment 2		
Feeding day	Agar content	Fish food	Feeding day	Agar content	Fish food
1	6% (w/v), 110 µL	-	1	2 % (w/v), 110 µL	-
4	4% (w/v), 110 µL	-	4	6 % (w/v), 110 µL	-
6	6% (w/v), 55 µL	-	6	6 % (w/v), 110 µL	-
8	6% (w/v), 55 µL	-	8	6 % (w/v), 110 µL	-
10	6% (w/v), 55 µL	-	10	6 % (w/v), 110 µL	-
11	6% (w/v), 55 µL	yes	11	6 % (w/v), 110 µL	yes
			13	6 % (w/v), 110 µL	yes
			15	6 % (w/v), 110 µL	yes

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Table S5. Survival rates of *Ae. albopictus* and *Ae. aegypti* following feeding with bacterially produced shRNAs targeting *fez2, lrc* and nuclease-encoding genes. Each replicate contained n = 40 neonate larvae at the start. In some of the replicates (treatment and control), the agar pellets used for dsRNA feeding were hardly consumed over time and larvae died from starvation. In some replicates, the target-specific shRNA was combined with an shRNA against a gut-specific nuclease. Two types of negative controls were used: « none » means that no bacterially expressed shRNA was added to the agar pellet, « scramble » means that unspecific bacterial-expressed shRNA was added to the agar pellet.

Species	Strain	Treatment	Replicate no.	Total L1	Pupae + adults	Larvae	Dead	Total alive (% survival)
Ae. albopictus	Montpellier	**nuclease 1**	1	40	22	18	0	40 (100%)
		nuclease 1	2	40	18	22	0	40 (100%)
		fez2	1	40	6	34	0	40 (100%)
		***fez2* + nuclease 1**	1	40	18	21	1	39 (97.5%)
		none	1	40	0	40	0	40 (100%)
		scramble	1	40	20	20	0	40 (100%)
		scramble	2	40	18	22	0	40 (100%)
Ae. aegypti	La Réunion	***fez2* v1**	1	40	23	2	15	25 (62.5%)
		***fez2* v1 + nuclease**	1	40	33	2	5	35 (87.5%)
		none	1	40	30	5	5	35 (87.5%)
		scramble	1	40	23	1	16	24 (55%)
		scramble	2	40	24	3	13	27 (67.5%)
	La Réunion	***lrc***	1	40	14	19	7	33 (82.5%)
		lrc	2	40	19	8	13	27 (67.5%)
		***fez2* v2**	1	40	12	21	7	33 (82.5%)
		***fez2* v2**	2	40	14	18	8	32 (80%)
		none	1	40	19	20	1	39 (97.5%)
		scramble	1	40	20	19	1	39 (97.5%)
	Liverpool	***lrc***	1	40	6	32	2	38 (95%)
		lrc	2	40	0	27	13	27 (67.5%)
		***fez2* v2**	1	45	1	44	0	45 (100%)
		***fez2* v2**	2	40	0	12	28	12 (30%)
		none	1	44	3	41	0	44 (100%)
		scramble	1	40	1	30	9	31 (77.5%)

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Table S6. Sex-ratio and survival rates in *Ae. albopictus* (Montpellier strain) fed with bacterially produced shRNAs targeted against two female-specific isoforms of *doublesex (dsxF1 and dsxF2)*. Two rounds of experiments were carried out. Replicates annotated with an asterisk were performed on a different day than replicates without. Each replicate contained n = 50 neonate *Ae. albopictus* larvae at the start. In some of the replicates (treatment and control), the agar pellets used for dsRNA feeding were hardly consumed over time. Larval development being much slower in such replicates, counting was stopped at day 30 even though there remained young larvae whose sex could not be determined. *dsx*-shRNAs were combined with shRNAs against three different gut nucleases of *Ae. albopictus*. Two types of negative controls were used: « none » means that no bacterially expressed shRNA was added to the agar pellet, « scramble » means that an unspecific bacterially expressed shRNA was added to the agar pellet.

Treatment	Replicate	No. (%) males	No. (%) females	Could not be sexed	Reached adulthood (%)	Remaining larvae/ pupae after 1 month
***dsxF1* + *dsxF2* + 3 nucleases**	1	17 (50.0 %)	17 (50.0 %)	0	34 (68.0 %)	5
***dsxF1* + *dsxF2* + 3 nucleases**	2	19 (54.3 %)	16 (45.7 %)	0	35 (70.0 %)	1
***dsxF1* + *dsxF2* + 3 nucleases**	3*	12 (38.7 %)	19 (61.3 %)	0	31 (62 %)	0
***dsxF1* + *dsxF2* + 3 nucleases**	4*	11 (45.7%)	19 (54.3%)	5	35 (70%)	0
***dsxF1* + *dsxF2* + 3 nucleases**	5*	13 (40.0%)	21 (60.0%)	1	35 (70%)	0
none	1	12 (60.0 %)	8 (40.0%)	0	20 (40.0 %)	15
none	2*	14 (50.0%)	14 (50.0%)	2	30 (60%)	0
scramble	1	18 (56.3 %)	14 (43.8%)	0	32 (64.0 %)	2
scramble	2	14 (48.3 %)	15 (51.7%)	0	29 (58.0 %)	2
scramble	3*	13 (35.1%)	24 (64.9 %)	0	37 (74.0 %)	0

Supplemental Table S7A: Overview of injection and screening data of bacterially expressed and purified eGFP dsRNA into embryos of the V19 transgenic line carrying a GFP fluorescent marker. Listed are the number of injected embryos, the number of larvae hatched from the injected embryos, the number of larvae showing weak or strong GFP fluorescence in the L2 to L4 larval stage (weak / strong GFP PT; PT = phenotype), the number of individuals frozen for qPCR analysis and the number of individuals analyzed for eGFP expression level by RT-qPCR; the results of 2 independent injection experiments are shown. Control injections were double-distilled water (ddH2O) and bacterial extract obtained from bacteria transformed with the empty dsRNA expression plasmid.

injection 1

	embryos	larvae hatched	weak GFP PT	strong GFP PT	frozen weak	frozen strong	qPCR weak	qPCR strong
ddH2O	215	19	0	19	0	16	0	6
bacterial extract	552	16	0	16	0	13	0	5
eGFP dsRNA	556	17	9	6	8	5	8	5

injection 2

	embryos	larvae hatched	weak GFP PT	strong GFP PT	frozen weak	frozen strong	qPCR weak	qPCR strong
bacterial extract	76	4	0	4	0	1	0	1
eGFP dsRNA	513	15	12	0	12	0	12	0

Supplemental Table S7B: Results of the RT-qPCR analysis of the embryonic injections with eGFP dsRNA into the eGFP-expressing transgenic line V19. Shown are the fold changes of eGFP transript levels in eGFP-dsRNA-injected indivial larvae compared to the bacterial extract and water controls. Fold changes were calculated with the ddCt method (according to Livak et al. 2001). For this, dCt values were averaged for all the control individuals (ddH2O plus bact. extract) and the average dCT(control) subtracted from the dCt value of each eGFP-dsRNA-injected individual to obtain the ddCt value. Fold change was calculated for each individual as 2^-ddCt. Shown here are the fold changes in eGFP transcript levels for each analyzed individual, the average fold changes and standard deviations for all eGFP-dsRNA-injected individuals showing a weak GFP phenotype, a strong GFP phenotype, and for the control injected individuals.

eGFP dsRNA weak GFP fluoresence phenotype

sample name	fold change eGFP expr	avg	stdev
eGFP 1	0,15	0,32	0,20
eGFP 2	0,26		
eGFP 3	0,44		
eGFP 4	0,43		
eGFP 5	0,30		
eGFP 6	0,24		
eGFP 7	0,52		
eGFP 8	0,62		
eGFP 9	0,80		
eGFP 10	0,32		
eGFP 11	0,42		
eGFP 12	0,21		
eGFP 13	0,68		
eGFP 14	0,23		
eGFP 15	0,11		
eGFP 16	0,03		
eGFP 17	0,10		
eGFP 18	0,21		
eGFP 19	0,34		
eGFP 20	0,23		
eGFP 21	0,16		

eGFP dsRNA strong GFP fluoresence phenotype

sample name	fold change eGFP expr	avg	stdev
eGFP 22	0,34	0,38	0,16
eGFP 23	0,42		
eGFP 24	0,36		
eGFP 25	0,24		
eGFP 26	0,24		
eGFP 27	0,68		

ddH2O and bacterila extract control strong GFP fluorescence phenotype

sample name	fold change eGFP expr	avg	stdev
H2O ctrl 1	0,94	1,12	0,5395706
H2O ctrl 2	0,49		
H2O ctrl 3	0,42		
H2O ctrl 4	0,81		
H2O ctrl 5	0,84		
Bact. ctrl 1	0,99		
Bact. ctrl 2	1,86		
Bact. ctrl 3	0,81		
Bact. ctrl 4	1,78		
Bact. ctrl 5	1,90		
Bact. ctrl 6	1,46		

3.3 Towards neo-classical genetic sexing strains in *Ceratitis capitata* (Diptera: Tephritidae): engineering a functional intronless version of the *white pupae* gene in the Mediterranean fruit fly

In the next step of my research on developing efficient sustainable control systems for invasive insects, I focused on using modern molecular techniques to advance the development of neo-classical genetic sexing strains (GSS). My main goal was to engineer an intronless version of the recently discovered *white pupae* gene to be used in the concept of neo-classical GSS. The function of the minimal version of the *white pupae* gene was proven in vivo, as one copy of the intronless gene successfully rescued the wild type phenotype in homozygous mutant individuals. The molecular techniques and work-pipeline applied for this project could be further used as a toolbox to expand or generate novel neo-classical GSS.

Title: Towards neo-classical genetic sexing strains in *Ceratitis capitata* (Diptera: Tephritidae): engineering a functional intronless version of the white pupae gene in the Mediterranean fruit fly.

Authors: **Lucas Henrique Figueiredo Prates**, Roswitha A. Aumann, Inga Sievers, Tanja Rehling, Marc F. Schetelig.

Status: To be submitted to the special issue on "Generic Approach for the Development of Genetic Sexing Strains for Sterile Insect Technique (SIT) Applications" in the Insect Science (Wiley).

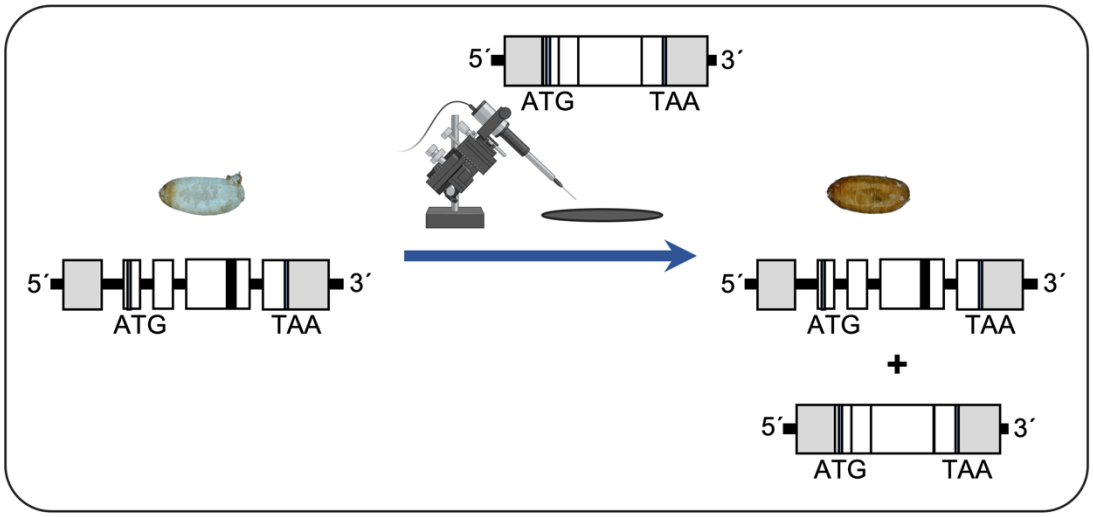
Contributions: Organization, compilation, statistical analysis and writing of manuscript: Lucas Henrique Figueiredo Prates.

- Design and cloning of intronless version of the *wp* gene:	Lucas Henrique Figueiredo Prates, Roswitha A. Aumann, Tanja Rehling, Marc F. Schetelig
- Germline transformation:	Lucas Henrique Figueiredo Prates, Tanja Rehling,
- Screening and molecular analysis of transformed flies:	Lucas Henrique Figueiredo Prates
- Identification of integration sites:	Lucas Henrique Figueiredo Prates, Inga Sievers, Tanja Rehling
- Droplet digital PCR:	Tanja Rehling, Lucas Henrique Figueiredo Prates
- Remobilization of *piggyBac* integration and screening:	Lucas Henrique Figueiredo Prates

- Functional analysis of rescue capability: Lucas Henrique Figueiredo Prates, Inga Sievers

Presentations: "Developing novel Genetic Sexing Strains for SIT" at Chinese Academy of Agricultural Sciences in Shenzhen, China (27.02.2024).

"Advancements in insect pest control through the identification and characterization of *wp* and *tsl* genes in the medfly for developing novel genetic sexing strains" at the 3rd Research Coordination Meeting on "Generic approach for the development of genetic sexing strains for SIT application" at the International Atomic Energy Agency (IAEA) (25.04.2023).



Partially created with BioRender.com.

Title: "Towards neo-classical genetic sexing strains in *Ceratitis capitata* (Diptera: Tephritidae): engineering a functional intronless version of the *white pupae* gene in the Mediterranean fruit fly"

Short running title: Rescue of the white pupae in medfly

Lucas Henrique Figueiredo Prates, Roswitha A. Aumann, Inga Sievers, Tanja Rehling, Marc F. Schetelig*

Department of Insect Biotechnology in Plant Protection, Justus Liebig University Giessen, 35394 Giessen, Germany (LHFP, lucas.prates@agrar.uni-giessen.de; RAA, roswitha.aumann@agrar.uni-giessen.de; IS, inga.sievers@ag.uni-giessen.de; TR, tanja.rehling@agrar.uni-giessen.de; MFS, marc.schetelig@agrar.uni-giessen.de)

*Correspondence: Prof. Dr. Marc F. Schetelig

Department of Insect Biotechnology in Plant Protection, Justus Liebig University Giessen

Winchester Str. 2, 35394 Giessen, Germany

Tel.: +49 641/99-35900

marc.schetelig@agrar.uni-giessen.de

Abstract: Genetic sexing strains (GSS) are important tools for the sterile insect technique (SIT), an environmentally friendly and species-specific insect pest control method. GSS feature sex-specific phenotypes, enabling sex sorting in mass-rearing facilities and male-only releases, which significantly improve the cost-effectiveness and efficiency of SIT programs. In classical GSS, sex linkage of marker gene(s), such as *white pupae* (*wp*), is achieved through an irradiation-induced translocation between the marker-carrying autosome and the Y chromosome. However, this approach renders GSS males semi-sterile. The recently proposed neo-classical GSS concept suggests using genome editing to achieve sex linkage by directly inserting the wild-type marker allele onto the Y chromosome, potentially yielding GSS males with higher fertility. In this study, we examined the *wp* gene as a genetic marker for the neo-classical GSS concept and developed a minimal, intronless version of this gene, termed mini-*wp*. We demonstrate that a single copy of mini-*wp* is sufficient to restore the wild-type brown puparium phenotype and is functional when integrated at various positions within the medfly genome, including the X chromosome. Due to its smaller size (4,689 bp, including 2,000 bp of putative promoter region) relative to the full WT allele (20,868 bp), mini-*wp* may facilitate its precise insertion into the Y chromosome, representing an important step towards realizing neo-classical GSS. Furthermore, the methodology developed for designing and testing mini-*wp* in medfly may be adapted to other Tephritidae species with an identified *wp* gene.

Keywords: gene editing, Mediterranean fruit fly, minimal gene, *piggyBac*, selectable marker, SIT.

Introduction

Genetic sexing strains (GSS) are a proven valuable tool for sex-sorting in insect mass-rearing facilities and a key element for cost-effective application of the sterile insect technique (SIT) for pest control (Franz et al., 2021, Klassen et al., 2021, Mumford, 2021). The SIT is based on mass rearing of the target insect, followed by its sterilization and release into the infested area. Sterile males mate with wild females, leading to no offspring, thus gradually reducing the insect population in the treated area. A GSS classically requires two components: a selectable mutated marker that allows sex separation or elimination of females at the earliest possible stage, and linkage of the wild-type (WT) allele of this marker to the male sex. A well-known and successful example of a GSS is the VIENNA-8, developed in *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), commonly known as the Mediterranean fruit fly, or medfly (Franz et al., 1994, Augustinos et al., 2017, Franz et al., 2021).This GSS carries two selectable markers, the *white pupae* (wp^-) and the *temperature-sensitive lethal* (tsl^-) mutations. These mutations were initially not sex-linked, but the WT alleles for both mutations were translocated onto the Y chromosome using irradiation. This made sex separation possible: female flies, homozygous for both mutations, emerge from white puparia and are sensitive to high temperatures. In contrast, heterozygous male flies emerge from brown puparia and are not affected by the same high temperatures. Typically, during operation in the mass rearing facilities, female embryos do not survive a treatment at 34°C for 24 h, while males are unaffected (Franz et al., 2021).

However, developing GSS strains has been time-consuming and reliant on chance. For example, the wp^- phenotype in Tephritidae, arising from natural mutations, was discovered by chance only in the medfly (Rössler, 1979), the melon fly (*Zeugodacus cucurbitae*) (McInnis et al., 2004), and the oriental fruit fly (*Bactrocera dorsalis*) (McCombs & Saul, 1992, McCombs & Saul, 1995). Furthermore, GSS based on irradiation-induced translocation of the autosomal WT alleles onto the Y chromosome are semi-sterile and mass rearing may produce recombinants, which could compromise the GSS in mass-rearing facilities (Franz et al., 2021, Cáceres et al., 2023). Therefore, the development of general tools for faster development of GSS remains a necessity for boosting and expanding SIT to other insect species (Bourtzis & Vreysen, 2021).

Although the wp^- phenotype has been used in GSS since decades, the genetic basis for this phenotype in Tephritids has only recently been revealed, enabling the creation of multiple CRISPR/Cas-based lines with the wp^- phenotype in medfly and in the Queensland fruit fly, *B. tryoni* (Ward et al., 2021). Shortly after, the identified domain was also shown to cause the wp^- phenotype in the melon fly (Paulo et al., 2022). The identification of this and additional suitable

marker genes (Robinson, 2002, Chen et al., 2022, Sollazzo et al., 2024), combined with advancements in genetic editing in insects using modern molecular techniques (Aumann et al., 2018, Buchman & Akbari, 2019, Meccariello et al., 2019, Aumann et al., 2020, Gamez et al., 2021, Häcker et al., 2021, Yan et al., 2023), enables the concept of 'neo-classical' GSS. In this approach, sex linkage could be achieved by targeted insertion of the WT allele of selectable marker(s) into the Y chromosome or near the male determining factor in a strain with mutated markers (Nguyen et al., 2021, Yan et al., 2023, Yan et al., 2024). Since the insertion efficiency of precise genetic editing with CRISPR/Cas is sensitive to the size of the insert and may improve with smaller cargos (Li et al., 2014, Paix et al., 2017), using engineered minimal gene constructs instead of the full endogenous allele could enhance editing success. This might be particularly relevant for the development of neo-classical GSS, where targeting the gene-poor and highly repetitive Y-chromosome is required.

Considering the identification of the gene responsible for the *wp*$^{-}$ phenotype in Tephritids, we examined the *wp* gene as a genetic marker for the neo-classical GSS concept in the non-model organism *C. capitata*. Specifically, we report the successful rescue of the WT brown pupae phenotype in the medfly by *piggyBac* integration of an intronless version of the *white pupae* gene into a *wp*$^{-}$ strain.

Materials and Methods

All primers used in this work are listed in Table S1.

Insect strains and rearing

Ceratitis capitata (Wiedemann) strains wild-type *Egypt-II* (*EgII*), white eye (we^-), and white pupae (wp^-) were obtained from the Insect Pest Control Laboratory, joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (IPCL/IAEA, Seibersdorf, Austria). The CRISPR-modified wp^- strain (wp^-_{CRISPR}) was previously established in the laboratory (strain *Cc_D*) (Ward et al., 2021). The strain double homozygous for the white eye and white pupae natural mutations ($we^-;we^-/wp^-;wp^-$) was produced by inbreeding the naturally mutated white eye and the naturally mutated white pupae strains. All insect strains were reared under standard laboratory conditions at 25.2 °C, 48 % relative humidity (RH) and 14/10 h light/dark cycle. Larvae were reared on carrot-based larval food prepared with 1.4 kg of cooked frozen carrots, 16 g of sodium benzoate (VWR International GmbH, Darmstadt, Germany), 500 g of carrot powder (Van Drunen Farms, Momence, IL, USA), 168 g of yeast hydrolysate enzymatic (MP Biomedicals, Solon, OH, USA), 20 mL of hydrochloric acid solution 25 % (v/v) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), blended with approximately 2 L of distilled water to adjust to a smooth consistency. Adults were fed ad libitum with a mixture of sugar and yeast hydrolysate enzymatic (MP Biomedicals) (3:1, v:v), and water.

Rapid amplification of cDNA ends

Total RNA was extracted from single pre-pupae of *EgII* strain using Monarch total RNA Miniprep kit (New England Biolabs Inc., Ipswich, MA, USA) following the manufacturer's instructions. Then, 5 µg of DNA-free total RNA was used to isolate mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490, New England Biolabs Inc., Ipswich, MA, USA). Both 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed using SMARTer® RACE 5'/3' Kit (Takara Bio USA, Inc., Mountain View, CA, USA) following the manufacturer's instructions with primers P2125 and P2126, designed to include the sequence GATTACGCCAAGCTT in the 5' end of both primers. Cycler conditions were as follows: 5 cycles 94 °C for 30 s, 72 °C for 3 min, 5 cycles 94 °C for 30 s, 70 °C for 30 s, 72°C for 3 min, 25 cycles 94 °C for 30 s, 65 °C for 30 s, 72 °C for 3 min. PCR products were analyzed via gel electrophoresis, extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research Europe GmbH, Freiburg, Germany), cloned into the linearized pRACE vector (Takara Bio USA, Inc.), and transformed into XL1-Blue MR Supercompetent cells (*E. coli* [Δ(mcrA)183 Δ(mcrCB-

hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tetr)]]; Agilent Technologies, Santa Clara, CA, USA). Individual colonies were grown in LB medium containing 100 ng/µL ampicillin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and plasmids were extracted using NucleoSpin Plasmid Mini kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Finally, plasmids were pre-selected by restriction digestion with EcoRI-HF and HindIII-HF (New England Biolabs Inc., Ipswich, MA, USA) and Sanger-sequenced (Macrogen). Sequencing results were aligned to the medfly reference genome version 2.1 (GCF_000347755.3) (Papanicolaou et al., 2016).

Intronless version of the white pupae gene

Construction of the intronless version of the *white pupae* gene (mini-*wp*) was performed by amplifying a 2 kb region upstream of the 5' UTR, assumed to contain the promoter, along with the 5' UTR itself (521 bp) using 200 ng of genomic DNA (gDNA) extracted from a virgin female adult of the strain *EgII* and primers P2237 and P2238. The coding sequence, together with part of the 5' UTR and the 3' UTR, in total 2,168 bp, were amplified using 3 µL of 1:5 diluted cDNA from a single pre-pupae of the strain *EgII*, prepared as above for RACE PCR, using primers P2239 and P2240 spanning from the originally annotated 5' UTR to the end of the 3' UTR. Phusion Flash High-Fidelity PCR Mastermix was used for both amplifications and cycler conditions were as follows: 98 °C for 10 s, 35 cycles 98 °C for 1 s, 50 °C for 5 s, 72°C for 1 min, 72 °C for 3 min.

All primers were designed with Geneious Prime (version 2021.2.2, (Kearse et al., 2012)) and contained overhangs for Gibson cloning (underlined). These PCR products were analyzed via gel electrophoresis, extracted using Zymoclean Gel DNA Recovery Kit (Zymo Research Europe GmbH, Freiburg, Germany), and assembled into the *piggyBac* transformation vector *AH465 (pXLBacII_IE1hr5-DsRed.T3-SV40*) (Li & Handler, 2017), previously digested with SacII and XhoI, using the Gibson Assembly Cloning Kit (New England Biolabs Inc., Ipswich, MA, USA). The resulting plasmid *M6620* (*pXLBacII_mini-wp_IE1hr5-DsRed.T3-SV40*) was used to transform XL1-Blue MR Supercompetent cells (Agilent Technologies, Santa Clara, CA, USA). Correct assembly and sequence of the mini-*wp* insert (in total 4,689 bp, including 2,000 bp of putative promoter region) was verified by restriction digestion with NgoMIV and AfiII and Sanger sequencing. Finally, endotoxin-free plasmid DNA was prepared using the purification kit NucleoBond® Xtra Maxi EF (Macherey-Nagel GmbH & Co. KG, Düren, Germany), following manufacturer's instructions.

Germline transformation

Germline transformation was achieved through microinjection into embryos of the *C. capitata* CRISPR-modified white-pupae strain (wp^-_{CRISPR}, Ward et al., 2021), using a mixture of KCl (5 mM) and $NaPO_4$ (0.1 mM) buffer at pH 6.8, *piggyBac* donor plasmid *M6620* (500 ng/µL), and the standard *phsp-piggyBac* helper plasmid (200 ng/µL) (Handler & Harrell Ii, 1999). Microinjection was performed following previously described standard procedures (Handler et al., 1998, Rong & Golic, 2000, Aumann et al., 2018). Briefly, embryos of the wp^-_{CRISPR} strain were collected for up to 40 min, dechorionized in 1.4 % (w/w) solution of sodium hypochlorite for 3 min, rowed on double-sided sticky tape and covered with halocarbon oil 700 (Sigma Aldrich/Merck KG, Darmstadt, Germany). Needles for injection were made out of siliconized quartz glass capillaries (Science Products for Research in Life Science GmbH, Product Number Q100-70-7.5, Hofheim, Germany) crafted in a P-2000 laser puller (Sutter Instruments, Novato, CA, USA). The injection setup consisted of a MN-151 micromanipulator (Narishige, Tokyo, Japan), a FemtoJet 4i (Eppendorf, Hamburg, Germany), and a SZX16 stereo microscope (Olympus, Tokyo, Japan). After injection, embryos were placed into an oxygen chamber with moistened filter paper at 21 °C. Hatched larvae were carefully transferred into larval food and reared under standard conditions at 25.2 °C. Eclosed flies were backcrossed to the parental strain, and their progeny were inbred to establish the rescued strain. Screening for transformed progeny, i.e., rescued flies, was performed under Leica M205FC stereo microscope for expression of DsRed fluorescence and, at pupal stage, for WT color of the puparia.

Screening and image acquisition

Flies anesthetized with CO_2 were screened under Leica M205 FCA coupled to camera DMC6200. The microscope was equipped with following fluorescence filters: DsRed filter: excitation 530–560 nm, emission 590–650 nm; YFP filter: excitation 490–510 nm, emission 520–550 nm; GFP filter: excitation 460–500 nm, emission 510 nm (longpass). To facilitate image acquisition, flies were cooled down on ice. Pictures were taken using Leica Application Suit X software (version 3.8.1.26810, Leica, Wetzlar, Germany).

Non-lethal molecular characterization of mini-wp rescue

For molecular confirmation of the observed rescued phenotype, Platinum™ Direct PCR Universal Master Mix (Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was used for non-lethal genotyping using gDNA extracted from single legs of adult flies. One middle leg was carefully excised from CO_2-anesthetized fly, immediately transferred into 20

µL of lysis solution in a microcentrifuge tube and shortly spun down to ensure complete immersion in the solution. The lysis solution was prepared following manufacturer's instruction and contains 0.6 µL of proteinase K to 20 µL of lysis buffer, all components from the Platinum™ Direct PCR Universal Master Mix. Each fly was kept separately until genotyping was concluded and allowed to recover at standard rearing conditions. The lysis supernatant containing genomic DNA was used for PCR amplification with primers P1634 and P1936, following manufacturer's instructions. The primers amplify part of the coding sequence from exon 1 to exon 3 of the *white pupae* gene, spanning 135 bp of introns. Therefore, the expected size of the amplicon for the naturally occurring *wp* gene is 786 bp, while the amplicon from the mini-*wp* is expected to be 651 bp. The PCR products were analyzed by agarose gel electrophoresis.

Droplet digital PCR

To confirm the number of integration events in the first generation of positively transformed flies and their heterozygous offspring, droplet digital PCR (ddPCR) was performed with the BIO-RAD QX200 and Auto-DG System as previously described (Häcker et al., 2023). DsRed in the mini-*wp* cassette was used as target gene. Primers and probe were: P49, P50, and DsRed-probe (5'FAM-TCGTTGTGGGAGGTGATGTC-BHQ1).

Inverse PCR and sequence confirmation of integration site

Inverse PCR (iPCR) was performed to determine the genomic location of the *piggyBac* insertions. 600 ng of gDNA was extracted from single virgin flies and digested with 4 Units of MspI (New England Biolabs Inc., Ipswich, MA, USA) in a 20 µL-reaction at 37 °C for 1 h. Digested DNA was precipitated in 3 M NaOAc and ethanol, and recovered in 50 µL of TE buffer. The resuspended DNA was allowed to re-ligate overnight (approximately 18 h) at 16 °C with 800 Units of T4 DNA ligase (New England Biolabs Inc., Ipswich, MA, USA) in a 350 µL-reaction. Ligated DNA was again precipitated in NaOAC and ethanol, and recovered in 50 µL TE buffer. Inverse PCR reactions were performed using Phusion Flash High-Fidelity PCR Mastermix and 3 µL of the ligated DNA in a total volume of 20 µL. Primers to amplify the region flanking the 5' *piggyBac* insertion site were mfs11 and mfs10. Primers to amplify the region flanking the 3' *piggyBac* insertion site were mfs34 and P815. PCR conditions were as follows: 1 x 98 °C 10 s; 5x 98 °C 1 s, 66-56 °C (5'*piggyBac)/64-54 °C (3'piggyBac) (-2 °C per cycle)* 5 s, 72 °C 60 s; 35x 98 °C 1 s, 56 °C (5'*piggyBac*)/54 °C (3'*piggyBac*) 5 s, 72 °C 60 s, 1x 72 °C 60 s, 12 °C hold. PCR products were purified by gel electrophoresis and extracted

with the Zymoclean Gel DNA Recovery Kit (Zymo Research Europe GmbH, Freiburg, Germany). Finally, PCR products were Sanger-sequenced and the results were analyzed for fragments of the *piggyBac* vector and restriction sites of MspI. The sequences were used for BLAST search against the reference genome of *C. capitata* (GCF_000347755.3-Genome assembly Ccap_2.1) (Papanicolaou et al., 2016) and the assembly version EGII-3.2.1 (GCA_905071925.1-Genome assembly EGII-3.2.1) (Ward et al., 2021) using Geneious Prime. In case of weak or no bands observed in the agarose gel, 1 µL of the iPCR reaction was reserved for a semi-nested PCR. In this case, iPCR product was diluted 1:100 and 1 µL was used for semi-nested PCR with primers mfs10 and mfs31 for the 5' *piggyBac* insertion, or primers mfs34 and P139 for the 3' *piggyBac* integration. Cycling conditions were: 1x 98 °C 10 s; 35x 98 °C 1 s, 54 °C (5'*piggyBac*)/52 °C (3'*piggyBac*) 5 s, 72 °C 60 s, 1x 72 °C 3 min s, 12 °C hold. In addition, the integration sites were confirmed by PCR with primers binding in the genomic regions flanking the integration site paired to another primer within the transgene, followed by Sanger sequencing of the produced amplicon. PCR reactions were performed using Phusion Flash High-Fidelity PCR Mastermix and 40 ng of gDNA in a 20 µL-reactions.

Remobilization of piggyBac integration

To generate strains with new integration sites of the mini-*wp*, the strain *piggyBac*-Jumpstarter 3 containing the pMi|Ccwhite+; hspBac| (AH_370) (Schetelig et al., 2009) was used as source of transposase to remobilize the *piggyBac* cassette containing the mini-*wp* rescue. Since the cassette for expression of the *piggyBac* transposase in the Jumpstarter 3 strain is linked to the cDNA of the *white* gene, thereby rescuing the white-eye phenotype, and to easily identify whether this cassette would still be present in the flies after remobilization, it was necessary to first obtain the mini-*wp* rescue in the white eye/white pupae background. As preliminary work for the remobilization, the mini-*wp* strain with the original integration site was outcrossed to a strain double homozygous for natural mutations of the white eye and white pupae (we^-;we^-/wp^-;wp^-). After establishing a mini-*wp* rescued strain with we^-;we^-/wp^-;wp^- background, males and females of this strain were mated with their counterparts of the Jumpstarter 3 strain. The offspring was screened for DsRed fluorescence, color of the eyes and non-lethally genotyped with primers binding to the genomic DNA flaking the original integration site and primers inside the mini-*wp* cassette to determine whether the cassette remained in the original integration site or possibly remobilized. Primers P2357, P2358 and P2273 were used on the 5' *piggyBac* end, while mfs34 and P2359 were used on the 3' *piggyBac* end. To facilitate identification of the flies during genotyping and crosses, all flies were consecutively numbered

and the families established thereof were named after their numbers. The family established from the fly with the original integration site was named after the plasmid number (M6620). Flies with potentially new integration sites were individually outcrossed with counterparts of the strain *we*$^{-}$;*we*$^{-}$/*wp*$^{-}$;*wp*$^{-}$. The offspring was again screened and rescued flies with white eye phenotype were individually outcrossed with the *we*$^{-}$;*we*$^{-}$/*wp*$^{-}$;*wp*$^{-}$ strain. The white eye phenotype indicates the absence of *piggyBac* transposase expression, therefore, indicating that the new integration sites have been successfully stabilized. The offspring was then screened again and rescued flies were inbred to establish strains of mini-*wp* rescued flies in the *we*$^{-}$;*we*$^{-}$/*wp*$^{-}$;*wp*$^{-}$ background with different integration sites of the *piggyBac* cassette (Figure 1). Integration sites of the new strains were identified through iPCR and compared to the available genomic sequences of the medfly, as described above. Flanking sequences of the integration sites for all mini-*wp* strains are given Table S2.

Functional rescue capability from mini-wp integrated in difference genomic positions

To verify the functionality of the mini-*wp* construct to rescue the white pupae phenotype when integrated in different genomic positions, homozygous flies expressing the mini-*wp* rescue were outcrossed to their homozygous counterparts of a strain carrying the natural mutation of the *white pupae* gene. Eggs were collected on two consecutive days between oviposition days 5 to 8, in two to six batches, depending on the total number of laid eggs. Ideally, one hundred eggs were collected from each cage per batch. After collection, each batch of eggs was reared separately. Pupae were screened for DsRed fluorescence and puparium color and counted as "rescued" (brown puparium) or "white pupae" (white puparium). Heterozygous adults eclosing from rescued pupae were screened for DsRed fluorescence and again outcrossed to their homozygous counterparts of a strain with the natural mutation of the *wp* gene. Again, pupae were screened and counted as rescued or white pupae. Rescue capability is given as percentage of pupae with rescued phenotype in relation to the total amount of pupae obtained. Numbers of collected eggs, pupae and adults for each experiment are given in Tables S3, S4, and S5.

Statistical analysis

Data analysis was carried out using the software MiniTab® (Minitab, LLC., State College, CA, USA). Data were analyzed via one-way analysis of variance (ANOVA), with Ryan-Joiner test for normality and Levene's test for equal variance. The means were then compared with the Tukey simultaneous test for differences of means at 95% confidence level. All statistical analyses are provided in Table S6.

Results

Analysis of the white pupae *gene structure and cloning of an intronless minimal gene fragment into a piggyBac vector*

The structure of the *white pupae* gene (LOC101451947) (Ward et al., 2021), *in silico* annotated with a total length of 20,868 bp and coding sequence (CDS) of 1,596 bp over four exons, was successfully verified via Rapid Amplification of cDNA Ends (RACE) PCR. Mapping the obtained sequences to the medfly reference genome (GCF_000347755.3) (Papanicolaou et al., 2016) placed the start of the 5'UTR at 18,489 bp upstream the start of the predicted CDS, signaled by the starting codon. As shorter cargo sequences might facilitate insect transgenesis, a minimal gene version of the *white pupae* gene (mini-*wp, mwp*) was designed to facilitate subsequent insect transgenesis efforts. A putative endogenous promoter region (2,000 bp), along with the 5'UTR, the CDS and the 3'UTR of the *white pupae* gene, in total 4,689 bp, were cloned into a *piggyBac* vector containing a DsRed fluorescent marker (Figure 2A).

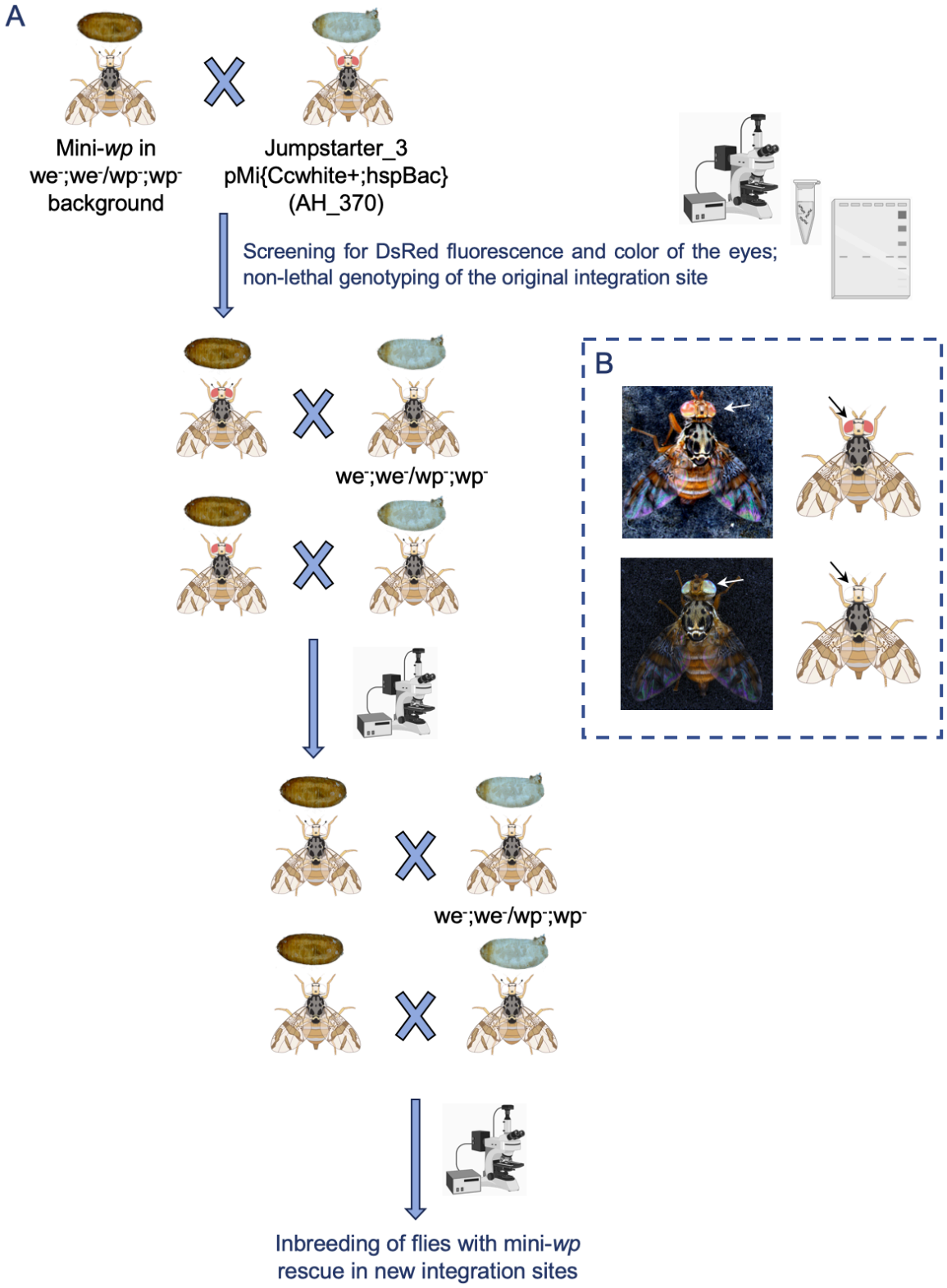


Figure 1. Schematic of crosses for remobilization of the *piggyBac* integrated mini-*wp* rescue cassette using the Jumpstarter 3 strain as source of transposase (A). The phenotypical differences in expression of the Jumpstarter 3 strain with peach-colored eyes, indicating expression of transposase (top), and the white-eye phenotype (bottom), are highlighted in (B) and respectively indicated in the schematic of the flies. Partially created with BioRender.com.

Positive germline transformation with mini-wp restores wild-type pupal phenotype

Germline transformation was performed through microinjection into 849 embryos of a CRISPR-modified *wp*$^{-}$ strain (Ward et al., 2021) (Figure 2C). All G_0 adults, eight males and eight females, were individually backcrossed to the parental *wp*$^{-}_{CRISPR}$ strain. Offspring was screened for DsRed fluorescence and color of the puparium at pupal stage and one out of 124 pupae from the female #6 showed the rescued phenotype, i.e., wild-type brown puparium color and expression of DsRed fluorescence. The transgene copy number in the rescued individual and its heterozygous progeny was confirmed to be one by ddPCR, indicating a unique *piggyBac* integration event. The rescued male fly (M6620_M1F6) was backcrossed to females of the *wp*$^{-}_{CRISPR}$ strain and their progeny was inbred to establish the rescued strain (Figure 2D). PCR amplification with primers spanning two introns between exons 1 and 3 of the *wp* gene performed on gDNA successfully allowed to molecularly distinguish between flies with WT color of the puparium due to the engineered mini-*wp* in comparison to flies with the WT allele of the *wp* gene (Figure 2B-D).

Transposase-mediated remobilization integrates mini-wp in different genomic positions

To verify that the mini-*wp* rescue construct could be integrated and functional in other genomic positions within the medfly genome, the Jumpstarter 3 strain (Schetelig et al., 2009) was used as source of *piggyBac* transposase to remobilize the integrated mini-*wp* cassette from its original integration site. Following the crossing scheme to mobilize the *piggyBac* integration and to stabilize the newly integrated transgene sites (Figure 1), eight new strains with the rescued phenotype have been established. The integration sites for all rescued strains were determined and the genomic region flanking the integration site confirmed by Sanger sequencing (Table S2). Notably, integration site of strain #160 was identified in a scaffold region predicted to be the chromosome X. The integration sites of all other strains were identified in autosomal positions, and based on *in silico* analysis of annotated genes and microsatellite sequences (Papanicolaou et al., 2016), likely to be on chromosomes 2 (scaffold 1), 4 (scaffold 6), 5 (scaffold 5), and 6 (scaffold 4) (Figure 2E).

Mini-wp successfully rescues WT pupal phenotype from different integration sites

To functionally evaluate the mini-*wp* rescue integrated in different genomic positions, flies from the rescued strains were crossed to flies carrying the natural mutation of the *white pupae* gene, reported as an insertion of ~8150 bp in exon 3 (Ward et al., 2021). The offspring was screened for the rescued WT phenotype and DsRed fluorescence at pupal stage and the adults

were outcrossed again to evaluate the rescue capability in heterozygosity. The rescue from all evaluated positions was proven to be effectively functional, with 100% of the offspring from homozygous crosses showing the restored WT phenotype at pupal stage. The rescue was also proven to be inheritable and functional in heterozygosity, i.e., a single mini-*wp* copy can rescue a homozygous *wp*- mutant. Averaging across all strains with different integration sites of the mini-*wp,* 47.43 ± 4.5 % of the offspring expressed the rescued pupal phenotype in the second generation of outcrosses, with no significant statistical difference between the strains (*p*-value = 0.297, one-way ANOVA). To functionally validate the integration predicted to be on the X chromosome, we outcrossed males of strain #160 to their counterparts carrying the natural mutation on the *wp* gene. As expected for a marker located on the X chromosome, all rescued offspring was female, while all flies that eclosed from the white puparia were males (Table 1). Interestingly, in a subsequent round of rearing for maintenance of the line #160, when outcrossing the rescued male offspring to females of the *wp*- strain, we observed two males that eclosed from rescued pupae. In this occasion, a total of 600 eggs were collected. Upon the hypothesis that a recombination event might have occurred, these males were outcrossed to females of the *wp*- strain. Despite several egg collections, no adult offspring was obtained and it was not possible to establish new families from these male flies. As these males did not survive or produced offspring, no further analysis was carried out as part of this work. Lines #104 and #183 were not viable after approximately five generations of inbreeding, suggesting that the integration of the rescue cassette in the different lines might have fitness costs, impacting their maintenance.

Table 1. Inheritance distribution of rescued phenotype in the offspring of male medflies with the mini-*wp* integrated into the X chromosome crossed to homozygous *wp*- females

Phenotype	Number of pupae	Number of eclosed males	Number of eclosed females
Rescued	208	0	200
White pupae	263	247	0

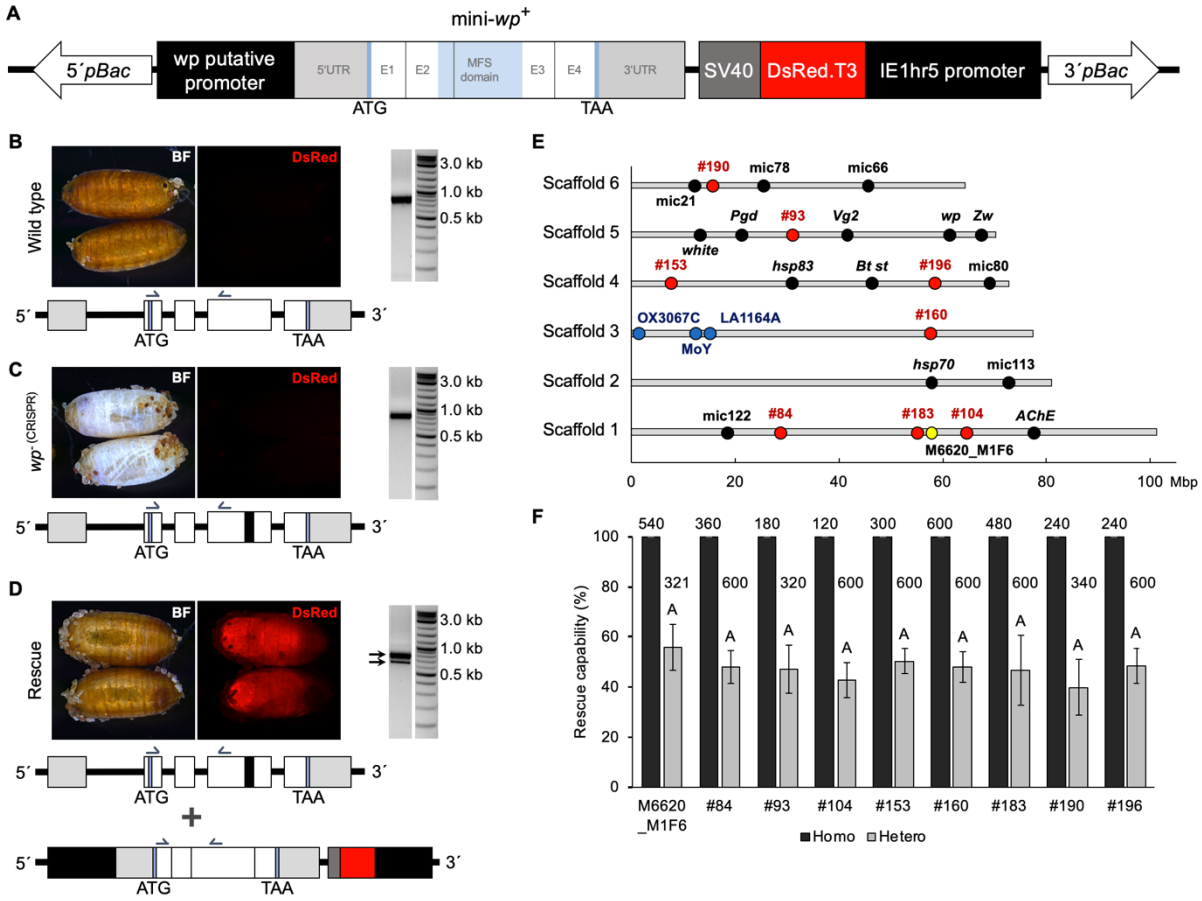


Figure 2. Functional rescue of the wild-type brown pupae phenotype through *piggyBac* integration of *white pupae* minimal gene (4,689 bp) in medfly. (**A**) Schematic of the mini-*white-pupae* construct in a *piggyBac* vector. The total size of the *piggyBac* cargo was 6,856 bp. ATG and TAA represent the position of the *wp* start and stop codons, respectively. E1, E2, E3, E4 represent the exon count. Phenotypical expression of the wild-type version of the *white pupae* gene (EgII strain) is show in (**B**), compared to a CRISPR-mediated *wp*$^{-}$ strain (**C**), and the phenotype observed after germline transformation of the intronless version of the *wp* gene, i.e., the phenotype of the rescued strain in (**D**). Each figure with the phenotypical expression is followed by a schematic of their respective *wp* gene version, plus the intronless version in (D). Semi-arrows in the schematic of the genes represent primer binding sites used for genotyping each version of the different phenotypes. Black stripe in the third exon of the *wp* gene in (C) and (D) represents the CRISPR-mediated deletion reported to cause the white pupae phenotype (Ward et al., 2021). 1 kb plus ladder (New England Biolabs Inc., Ipswich, MA, USA) was used as marker in gel electrophoresis; kb = kilobases. BF - bright field. DsRed – fluorescence filter. (**E**) displays the integration sites mapped to the medfly genome assembly version 3.2.1 (GCA_905071925.1). Yellow dot indicates the original *piggyBac* integration site obtained through germline transformation; red dots indicate integration sites following remobilization of the original integration site; black dots denote reference genes, known genomic regions, or microsatellite (*mic*) sequences previously mapped to autosomal chromosomes or to the Y chromosome (blue dots) (Condon et al., 2007, Papanicolaou et al., 2016, Meccariello et al., 2021, Ward et al., 2021). *AChE – Acetylcholinesterase, hsp70 - heat shock 70, MoY – Maleness-on-Y, hsp83 - partial gene for heat shock protein 83, Bt st - Bactrocera tryoni scarlet gene, Pgd*

- *6-phosphogluconate dehydrogenase, Vg2 - Vitellogenin 2, wp - white pupae*, *Zw - glucose-6-phosphate 1 dehydrogenase*; OX3067C and LA1164A are Y-specific *piggyBac* transposon 5'-flanking sequence from transgenic lines reported in the literature (Condon et al., 2007). (**F)** Rescue capability of mini-*wp* integrated in different genomic positions. Shown are the mean values in percentage of offspring that expressed the rescued WT phenotype at pupal stage (rescue capability). Data shown is based on 2 to 6 batches of egg collections. Total number of eggs collected are given above the bars. Error bars indicate the standard deviation and bars with common letter are not significantly different at 95% confidence level (one-way ANOVA).

Unexpected phenotypes derived from remobilization of the piggyBac integration

Among the flies obtained from the remobilization experiment, flies #171 and #193 stood out with unexpected phenotypes. Fly #171 showed no rescued phenotype of the puparium color, but positive expression of the fluorescent marker (Figure 3A, 3C). PCR amplification flanking the genomic position of the original integration site showed partial amplification of the cassette on the 3' end of the integrated cassette in the original position. However, amplification spanning the 5' end of the cassette was not possible, suggesting that part of the construct is missing (Figure 3G). It was also not possible to amplify parts of the mini-*wp* cassette upstream from the fluorescent marker (Figure S1), suggesting that part of the cassette might have been excised out. Fly #193 presented a segmented WT phenotype of the puparium (Figure 3B), and no expression of the fluorescent marker (Figure 3B, 3D). PCR amplification on both 3' and 5' ends of the rescue cassette confirmed the integration site of the original strain (M6620_M1F6), suggesting that there was no remobilization (Figure 3G).

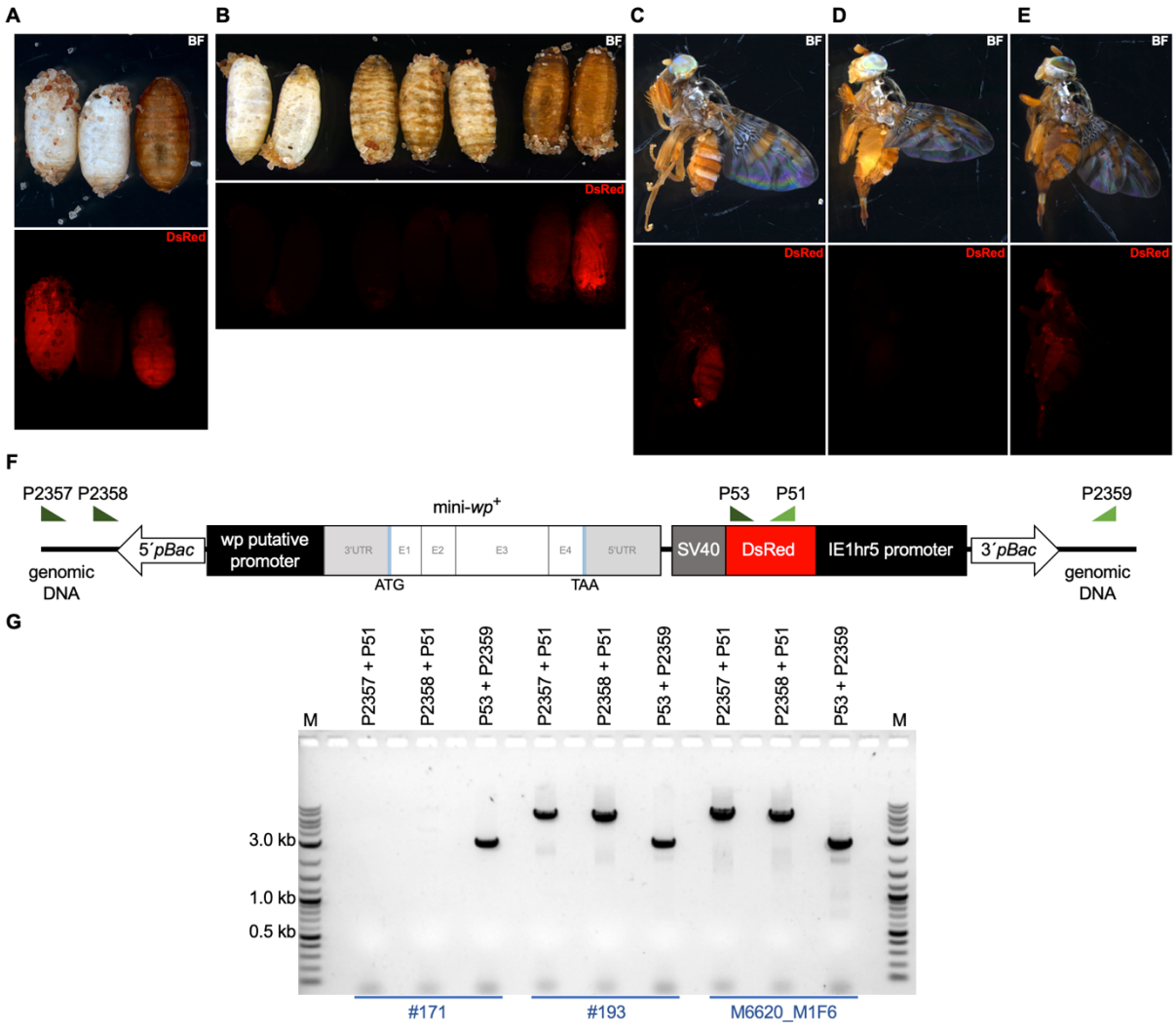


Figure 3. Unexpected phenotypes obtained from remobilization of the *mwp*-DsRed *piggyBac* cassette. (**A**) Phenotypical expression of strain #171 at pupal stage (left), natural mutated white-pupae strain (middle), and rescued strain M6620_M1F6 (right). (**B**) Two pupae from the natural mutated white-pupae strain (left), three pupae with segmented expression of WT puparium color (#193) (middle), two pupae from the rescued strain M6620_M1F6 (right). Adults from strains #171 in (**C**), #193 in (**D**) and M6620_M1F6 in (**E**). Position of forward (P2357, P2358, P53) and reverse (P51, P2359) primers used to check integration of the rescue cassette are schematically shown in (**F**). The original integration site was analyzed in genomic DNA of all three strains with the following primer combinations: P2357 and P51 (expected amplicon size: 6,455 bp), P2358 and P51 (expected amplicon size: 6080 bp), P53 and P2359 (expected amplicon size: 2839 bp). PCR products were analyzed via gel electrophoresis (**G**). 1 kb plus ladder (New England Biolabs Inc., Ipswich, MA, USA) was used as marker in gel electrophoresis; kb = kilobases. BF - bright field. DsRed – fluorescence filter.

Discussion

The concept of neo-classical GSS provides a generic approach for engineering GSS across various insect pest species in a targeted and time-efficient manner (Häcker et al., 2021, Nguyen et al., 2021, Yan et al., 2024, Yan et al., 2023). Unlike classical GSS, which depend on the chance identification of naturally occurring or chemically induced mutations, the proposed neo-classical approach employs molecular techniques and targeted genome editing, making the process more predictable and efficient. Methods such as CRISPR/Cas can be used to induce mutations in suitable marker genes of SIT target species (Ward et al., 2021, Chen et al., 2022, Sollazzo et al., 2024), thereby facilitating the establishment of phenotypic mutant strains. Neo-classical GSS may also overcome the issues of semi-sterility and potential recombination commonly associated with classical GSS, which are generated via irradiation-induced translocation between an autosome carrying the WT allele and the Y chromosome (Franz et al., 2021, Cáceres et al., 2023). In the neo-classical GSS concept, genome editing is intended to precisely insert a copy of the WT allele of the genetic marker into the Y chromosome or close to a male-determining factor in the mutant strain. Here, we investigated the *white pupae* gene as a potential marker for the generic approach to construct neo-classical GSS and demonstrated the functional rescue of the wild-type brown pupal color phenotype through *piggyBac*-mediated insertion of an intronless version of the *wp* gene (mini-*wp)* into the genome of a white pupae mutant strain.

Previous studies indicate that knock-in efficiency decreases as cargo size increases (Li et al., 2014, Paix et al., 2017), and that targeted insertions may be particularly challenging in repetitive and heterochromatic regions, such as the Y chromosome (Bernardini et al., 2014, Buchman & Akbari, 2019). Consequently, using the complete *white pupae* gene to establish sex-linkage in neo-classical GSS might present a challenge, as the cargo would reach approximately 20 kb due to an 18 kb intron located between the 5' UTR and the start codon of the *wp* gene. To address this challenge, we designed a minimal, intronless version of the *wp* gene with only 4,689 bp, including a 2,000 bp-genomic sequence upstream the 5'UTR, that we used as the putative promoter region. Although not investigated in this study, further reductions could be achieved by testing a minimal endogenous promoter or alternative promoters. The successful *piggyBac*-mediated integration of the mini-*wp* and the resulting restoration of the WT pupal phenotype in a wp^{-}_{CRISPR} strain demonstrates the feasibility of obtaining complete phenotypical rescue using an intronless *wp* gene. In addition, genotyping with primers spanning the original introns of the *wp* gene enables rapid differentiation between flies carrying the naturally occurring WT alleles (wp^{+}/wp^{+}) and those with brown pupae phenotype due to at least

one copy of the engineered mini-*wp* allele (*wp*$^{-}$/ *wp*$^{-}$; *mwp*$^{+}$/*mwp*$^{+}$) (Figure 2B, 2D), eliminating the need for a linked fluorescent marker. This feature might be important for quality control in mass rearing facilities and for identifying recaptured flies in SIT programs.

During maintenance of the strains generated in the remobilization experiment, we observed variations in overall performance (Table S3), with two out of the nine strains (#104 and #183) unable to be maintained. This variability likely results from the random nature of *piggyBac* integration, as integrations into essential genes or regulatory regions may disrupt gene expression and compromise fly fitness. For instance, in strain #104, the *mwp* integration site was located within the locus of a predicted protein-coding gene, identified as cyclic nucleotide-binding domain-containing protein 2, which disruption may have impacted its viability. The white pupal phenotype accompanied by DsRed expression observed in *mwp* strain #171 (Fig. 3A, 3G) may also be attributed to remobilization mechanism. It appears that part of the *mwp* cassette was excised, leaving only the section containing the fluorescent marker and the 3' end of the *piggyBac* vector stably retained at the original integration site. A possible hypothesis for the phenotype with WT stripes in the puparium of the strain #193 could involve mutations in the regulatory region of the *wp* gene, in another gene or regulatory element. Zhang et al. (2019) examined the role of tyrosine hydroxylase (TH) in *Zeugodacus tau* pigmentation and found that feeding larvae with 3-IT, a specific inhibitor of TH, led to white scattered areas in the pupal surface. The predicted protein sequence of *Z. tau* TH is highly conserved within the Tephritidae family, showing over 90% similarity with the medfly homolog (Gene ID 101450123) (Zhang et al., 2019). However, such mutations would not account for the lack of fluorescent marker expression observed. The potential connection between this phenotype and the remobilization experiment remains unsolved, and further investigation into its underlying cause was beyond the scope of this study.

For the next steps in engineering neo-classical GSS, CRISPR/Cas could be used to integrate mini-*wp* into unique regions of the Y chromosome in white pupae phenotype strains. The toolkit developed to design and validate the intronless version of the *wp* gene could be extended to related species where this gene has been identified and CRISPR/Cas-mediated *wp*$^{-}$ strains have been generated (Ward et al., 2021, Paulo et al., 2022), but also to other genetic markers and insect species with potential target genes that could serve as selectable markers (Robinson, 2002, Chen et al., 2022, Sollazzo et al., 2024).

Overall, our findings mark an important advance toward developing neo-classical GSS for use in SIT. To facilitate future gene editing efforts, we have demonstrated that the WT phenotype can be restored by integrating an intronless version of the *wp* gene into various

genomic sites of medfly. This work supports future gene-editing efforts aimed at engineering GSS in various SIT-target species using modern molecular techniques to create sex-specific selectable makers.

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Disclosure

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Supplementary material

Figure S1. PCR amplification of different parts within the mini-*wp* rescue in strains #171 and #193.

Table S1. Primer sequences used in this study.

Table S2. Flanking sequences of the integration site for all mini-*wp* strains.

Table S3. Rescue capability in homozygosity.

Table S4. Rescue capability in heterozygosity.

Table S5. Rescue of strain #160 with mini-*wp* on the X chromosome.

Table S6. Statistical analysis - provided in the Appendix of this thesis.

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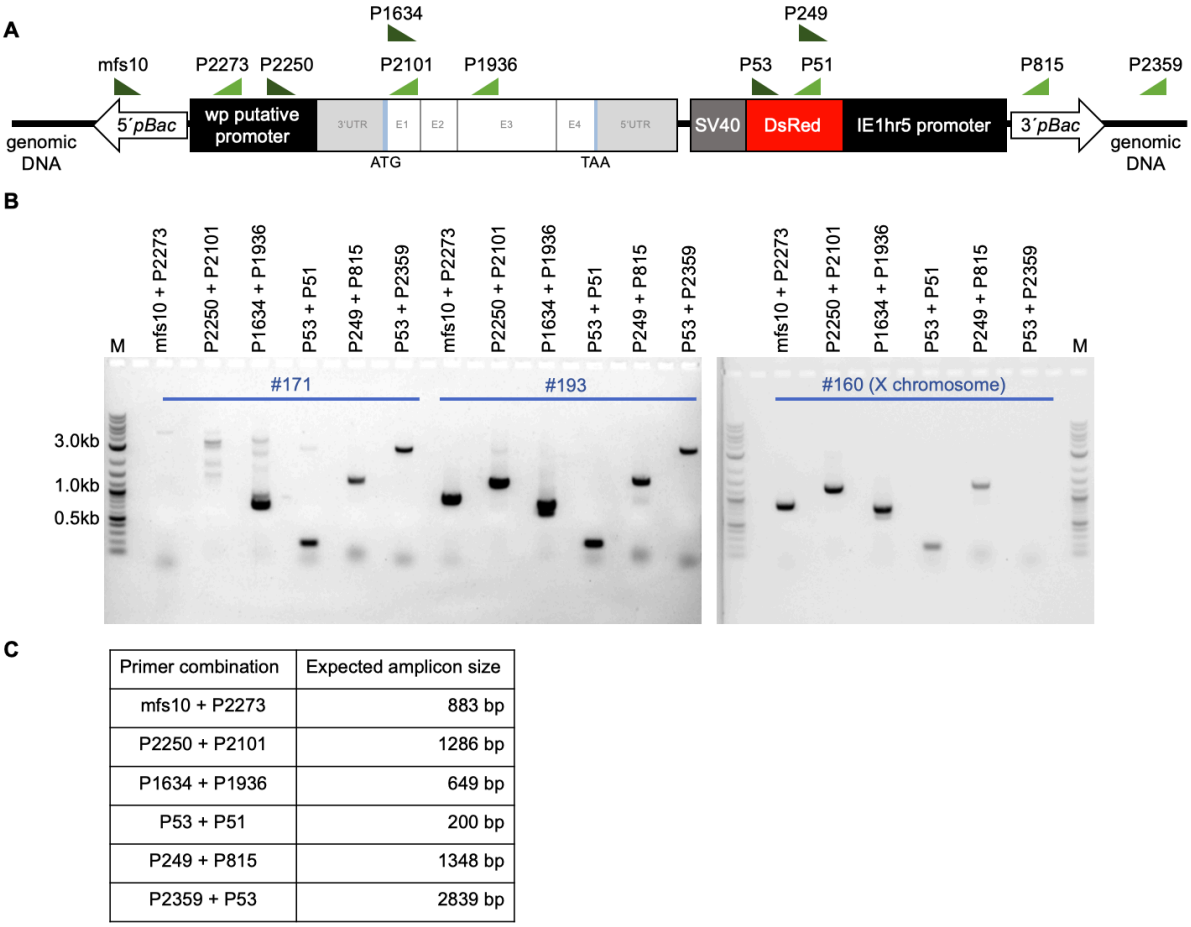
Supplementary information



Primer combination	Expected amplicon size
mfs10 + P2273	883 bp
P2250 + P2101	1286 bp
P1634 + P1936	649 bp
P53 + P51	200 bp
P249 + P815	1348 bp
P2359 + P53	2839 bp

Figure S1. Position of primers used to verify different parts within the rescue cassette are schematically shown in (**A**). PCR products amplified from genomic DNA of strains #171, #193, and #160 were analyzed via gel electrophoresis (**B**). Primer combinations and expected sizes of the amplicons are shown in (**C**). P2359 is specific to the genomic position in the original integration site (M6620_M1F6), so amplification with gDNA from strain #160 is not expected. M: Marker, 1 kb plus ladder (New England Biolabs Inc., Ipswich, MA, USA); kb = kilobases.

Table S1. Primer sequences used in this study. All sequences are shown in 5' --> 3' direction

Primer number	Primer sequence
mfs10	ACGACCGCGTGAGTCAAAATGACG
mfs11	ATCAGTGACACTTACCGCATTGACA
mfs31	CGACTGAGATGTCCTAAATGCACAG
mfs34	CGTACGTCACAATATGATTATCTTTCTAGG
P49	GATCCACAAGGCCCTGAAGC
P50	GCTCCACGATGGTGTAGTCC
P51	GTAATGCAGAAGAAGACTATGGGCTGGGAG
P53	ATGTCCAGCTTGGAGTCCACGTAGTAGTAG
P139	CTTTTATCGAATTCCTGCAGC
P815	ATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATT
P1633	TCCAGTGCAGTTCGGCTTAA
P1634	CGGCTTTTACAACGCTTATGTTC
P1643	TTGAAGAGCGCACTTGCAAC
P1936	CAAATAGCACATACGCCAGCC
P2048	GGTACCGTAGAAGCGTACTGG
P2100	AACGGTGTTTCGAGTTTGCG
P2102	TCATTGGTGTAAAAATTCACATCTTTG
P2125	GATTACGCCAAGCTTGCCTGTAGCCAGTGGGTTGCAACCG
P2126	GATTACGCCAAGCTTACGCCCGTTACACGTGAGAGCAGCAG
P2237	TTATCGATACCGTCGACCTCGAGGAGTGTTAATAGATGAAAGAAAAGGA
P2238	AAATGTGTGCTTTGGTTGTTGTTTAATATATTTGT
P2239	CAACCAAAGCACACATTTAAGTCACAAAATGCT
P2240	TAGAGCGGCCGCCACCGCGGTGTTGGTTAAATTGCTTATAAAAC
P2241	ACGTGCAACACAAGCAACGAAACC
P2250	CACGAACAACAACAACAACTGCG
P2273	AGCTGACGTTGTTCTGCCC
P2278	CTGACCGCTCCAAATTACGC
P2357	GTAAGCCATGGATTCAGTGTGC
P2358	GAGTTGGTCCAGCAGTACACG
P2359	CAGTCATTGAATGTCGTGTGCC
P2585	ATTTAAGGTTCGATCGATAATTGCGC
P2586	GTGGTCACTTTCTTCCAGAGTC
P2587	CACAACTGCGGTAAGGCTAAAC
P2590	TCACGACACACAAGAATTCTGAAAATTATT
P2593	GTACCCTGTGAGCAAACAAACG
P2612	CATTGCCGACTGAGAGTCCCA
P2614	TTGTGATGCAGTACCAGTCGG
P2618	ATTCCATTATAGTTCTGGTATACAAGGTG
P2621	ATGGAATGCATCGAGTTAGACCG
P2622	TAATATCAATAAATGGGTGAGCATTTGTGG
P2625	TCAGACTCGTTGCTTGCATACTAC
P2670	GTCCTGCCACTAAAGCTCTTGC

Table S1. Primer sequences used in this study – continuation

Primer number	Primer sequence
P2671	TGAGCCGGCACATATGATACC
P2672	ATTTAGCCTTCTCGTCGTCTTCG
P2673	CAAAGCGATTCTGTGTGACTGC
P2674	TGTTGTGCTGTTGTAGACGGG
P2675	TTCAGTGACTTCGCTTGGTATGC

Underlined are overhangs for Gibson cloning.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping. Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
M1F6_M6620 (original integration)	**GTAAGCCATGGATTCAGTGTGC**AGTGAAGACGGAAGACGCCTTG CAACTTTTTTTTACAGAATCCACGCTGACGAAGGATGAACAAAG CTATATTCTCCAACTTTAAAAGTAGACAGAAACCTTATTGTACC CCGAGCATGGCTAAATATCATGAGGTATGGGTAGTTTATTCTTT CACCTGGAGAAAAGCGACCAAAAAGGCGCAGGAATTCACATGAA TGACATCAAAGTAGCGAAGAAAACTACACAGTGTGGCTCTGTCA TAACAAATGTAGGTGACGGTTGGGGAAATATAGCCTAAAAATGC TGAAAGTCTTCCAAATAAACCTTTGGTCTTATAAAATGGCTTCA AGTGCAAGGATTTATTCAACGTGGAGTTGGTCCAGCAGTACACG GTGTTGGCCAAATGTAGTGTTTCTGGTAGCACGCGATCAGATAT CGTTTACGAGGAACATACGAAACACTCTTGATTAGTCAGTATAT TAGATGGTACACGTATAGCTATCCAAAATAAGTTGGTAGTGGAT TGGATGGGCGAAAGAAGATTTGAAATAGGTTTGAAGCGCGAATT CTTAAGCAATCAACTTTATTATGGACATTATCAAATAAAGCTGA CCCTATCAGATCAGTGCAAATTTAATTAAAAAAAAAAAATTAGT AAAAAATAACTTCTTAGTTTTGAAAACTTGCCAAATATTTTACT TGAATAGTACAAAGAGATCTCCCTTTAGTGAAGATTTGGAGATT TTCTGATACCAAATTGTACGAAGGACAAAAGCAGCAGGAAGAAA TGAAAGTTCAACACGAGAATAACACCTGCAACATATGTAAGGCA ATTCTCTCTATAGAAATAAAAAATGTC**TTAA**…	*insert*	…**TTAA**TTATGATTTAACCCTATGCATCAATCCTTGTTTAATATGG ATTATTATAGTTTATGAATGGGATAAAAAACTATTTATATGGAAT ATTAATTGTATCAGGCGACATTCTGCTGCTCAAAATAGGGTTTGG AGTTTTTCCAATGCGCTCTACAGAAATCATAAACAAAATTAGCAA ACATAGAAGTAATCATCCAACAGCAACTTGCAGAAACATTCTCCA GAACTAGTGATGATCTGTAAACTACTACAGTTTTAGTCCCGTATA TGGTCTGGCGAGGTTTTTCGTATAGGAAAGGCGGATCAGTAATGT GTTTAGGCCTATTAATATATATACAAAGCTCGATCTATTTTGGAA TTTAAAATATATTTCAGCTGAAAGTGTAATCGAAAACCATATGCT GCCACTTAATTTATTAATACGATTAGTAGTAGGCTATAGATACAT ATAATAAATACTATTTGCTACTAAAATAAATTCATCAAACATCTC ATATTTAGTAAAAATTTAACGCCGAAAAACAAAAATATCAATCTA CCTAATAATATAAATGGTAAACTAGTTCAATATTCCGTTTCACGC GTAAATTCAAAAAATTAGCATTCAAACTACACCTGCTTAAAGTAC ATACTCTGCTCCAAATTTTTGGAGGCAACACCAAGTCTAATTAAG TGCCACTGCAGTGACTTCACATTCCACGCGCACACACTTAATGCG ATCGTGTAACATAAATCACTATTGTATAAAATAAATTTAAAAAAA TTATGTGCAAATTTTCAACTTTTGCACAAATCGCAAACAGAAAAT TCGCAAAAATAAAAATTTGAATTTTAATCTCACAAAAGTCACTGC TCCACTTCAATAGATATTTTT**GGCACACGACATTCAATGACTG**	Genome 3.2.1: Scaffold 1. Genome 2.1: Scaffold 16.
#84	**TTGTGATGCAGTACCAGTCGG**TGGAAGGAAGACCTCCACTCCAT TGGATAGACCAGGTAGAGAAGGATCTGGCCGCACATGGTATTTC CAATAGGCGCCAAACAGCAAAAAGAAGAAATGAGCACCGCTGCA GCAAAGATATTCGTTACTCAGATCTACGGATAGTGGAATGGCGG ACGTGCCGTTGTTTTTGCTTAAAATGCTTAAACAGAAAGCTGTG ATAATTCGGTCCAGCTTTTCTGGATTTTTAAACATGTGCGGTAC TCAGCGAAAACTTTTTGTTATTATACAACCTCTCTTTCTCTCTG CGTACGAGACAAAGGTCGTAGACTCGCGAGAACTATGGGCAAAA CTTACGAAGTTTTTACAATCGGTGCCGAGACGGGATTCCAGGCT GCTAGTACTTGTA**TTAA**…	*insert*	…**TTAA**AGAGGCATTGCCCTTTCACGTCGCAGTCTTTTAATTTATA CCCGATAAGGGACCACAAATGAAAGTAAATGCAAGAAATATCTGG AAACAGTGGATCATGGGTGAGTGTCCAGCACTCGGCAGGCAAAAG CTATGCTATAC**TGGGACTCTCAGTCGGCAATG**	Genome 3.2.1: Scaffold 1. Genome 2.1: Scaffold 104, intron of LOC101453768, LOC101453768 putative uncharacterized protein DDB_G0282133, involved in protein phosphorylation, enables protein serine/threonine kinase activity.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping. Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
#93	**TCAGACTCGTTGCTTGCATACTAC**ATATTATACACATGCACATA AGTATGTACGCAGATACATATCTTCAGATCTGCATGTTTGTAGT TGAACATTTTCATGTGGCAAGACATAAAGTGTTTTTTCCTTCGT CGTATGAACATTGAATGGTAAATATGAAAAACAATTCTGAAGCT TAAACATCACAACCTCGCACCACAACGAGTCAATGGACTGTGAA ACAAAATGCTAAACAAAAACAATAGCAATAGCAGCAAAGGCGCC TAGCGAAAATATGCAATAATATGCTGCAAGCTTACATGCATGTA TATATATATATACATATATATATATATATAAGTATGTACGTGGA AGATTGCTGCAGTAAAAGGCATCAAGCCAGCGTGCGACCAGCTC AAGACAATTATCCATTCAACGGCAGGAGCAGCAGCGATTCAGCA GTCATCAGCATATGAACATGATAGTTTTGTGTTGCATTTATTGA GCAATATTGTTGTTGTTGTTGTTGTTGCTGCTGCTGCTGTT GCTGTTGTTTATATGCTCTGATTCTTATTGTTTTAC**TTAA**…	*insert*	…**TTAA**ACTATGCAAGTATGCACACACACACACCAAACTCTCAC ACCCATATTGATTGTTGTTGTTTCGTTGTTGTTTTTGTTGTTGTT AATGTATTGTCTTGTCGTAAAAACAGCGTCTTGAGTCGCTGGCGA AATGTGGTTTTCAACACGTAACATACGCGACTTTTACGAGCGGTA CTTATGGTCAAGTTCA**CCACAAATGCTCACCCATTTATTGATATT A**	Genome 3.2.1: Scaffold 5. Genome 2.1: Scaffold 68.
#104	**ATTCCATTATAGTTCTGGTATACAAGGTG**TTTAAACTAACATTA AGAAAGAACAAAAAGCAGATACATAGGGCAAAAATGTGTTTCGT AGTTTTGTTGTGGAAAGTGTTATTCATCCTTGTTTTGCATTTGG TTTTATCAAATACGTAGAATCTTGACGCCCTGTCTTATTAAATA AACATATACGATAAGGAAGCGGATGTGCCTCGATTCGCGAGTTA AGACTGTTAGCGCCTTCCAATTTTGGGCAGGAATTGTAAGCCT**T TAA**…	*insert*	…**TTAA**TATAAGGTTGTCAAATATCTCCCTTCCGCTTTTTTGCTCT TCATTCAATGCTTTATAAAAAGTGTTACAGTGATCGGATTCAGTT AAAATATGCGCCGTTTTGTTCGATGATCTGTTTCCATCTAGACGG CAACTTCATAATACCTTCCTCGTAGAAGCCCCCCTCCTTATTTGC GAAGAATTCGGACAGCCACTTTTCACAAGCCTCTTTTGAGTTCAA CTTCACACCACCAAGGGCATTCGCCATGGACAGGAACAGGTGGTA ATCACTTGGCGCTACGTCCGGGCTATATGGTGGATGCGATAAAAC CTCCCATCCGAGCTCCCGTAGCTTCTGACGAGTCATCAACGAAGT GTGTGGTCTGGCGTTGTCCTGGTGGAACACTACACCCTTCCTGTT GGCCAATTCTGGACGCTTCTGGTCGATCGCCTGCTTCAAGCGGTC CAGTTGTTCGCAGTAGATGGTAGAATTAAGCGTCTGGCCATATGG GAGCAGCTCATAGTGGATGATTCCCTTCCAATCCCACCAAACACA CAGCAAAACCGTCCGGCATGGCCACTATTTGGGACGATTCACCGG CCTTCGACCACGACCGTTTTCGCTTGATATTGTCGTATGTAATCC ATTTTTCGTCGCCAGTCACCATCCGCTTCAAGAATGGGTCGAGTT CGTTACGTCTCAGCAGCATATCGCAGGCACCCAAACATCAAACTT TTTTTTTTGTATCCAGCCTTCTGCAGATGGTTCAAAATGGTTTGG TGACTAACTCCCATCTCCTGGGCAATGTCACGAGATGCCATATGC **CGGTCTAACTCGATGCATTCCAT**	Genome 3.2.1: Scaffold 1. Genome 2.1: Scaffold 44, uncharacterized LOC101458382 (intron), also known as CT152.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping.
Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
#153	**TCACGACACACAAGAATTCTGAAAATTATT**CTTGGGCCACGCTG ATGCAGCGATTGCGTTAAAGAAATAAAAAAGCAAAAAAAAAAAA TATTGTTGATAGAGAGAACTAACTCCGCGACATTAATTGATATA ATTGATAGAACAGTAAAGGACCCAAATGAATTCGC**TTAA**…	*insert*	…**TTAA**GGGGTGAACCGACCACAGTCGCCGAGTGGTTATCATTACG TACTGCTATTCGGAAGGCCCGGTGGTGCGACCAACATCCAAAATT TATAGAAAATTAATTTAAACAAAAATTTAAAATAAATTTTCTAAA TGCGGTCGCCCCTCGGCAGGGAATGTCAACACATCCGAGTGTAGT TCTGACATGAAATAAAGGGTCCTTATTGAAAACAGTATCAGCCTT TCGTAGGCGGCTTAGTATTAATCGTAGGTCCCTCCATTTCTTGGA AAAAACATCACGACGAAAAACCACAAATTGGAGGAGGAGCTCGGC CGTAAATCGCTTAAGCGTTTACAAAATACCAATTATATATATATA AGGGGTAAACCCGTTCTAGGATTTTGAAAACGCAATTT**CGTTTGT** **TTGCTCACAGGGTAC**	Genome 3.2.1: Scaffold 4. Genome 2.1: Scaffold 49 LOC101457311 (intron) Gene description: talin-1, Tln1.
#160	**GTGGTCACTTTCTTCCAGAGTC**TTTTTTTCTTTTTTTATATTTT GTGTGTGTCCTTTAAATTTTTAAAGCATCCACAATCTTATGAAT TCTAACAGTTTTACACTTTAATTTGTTAAGGATTCGTTCTTCAA TCATATGAAGTTCAAATGTCACATAAGTTTCACAAAAATTTTAC ACTTTAATTTTTTAAAGGACTCGTTCTTCAATCTTATGAAGTTC AAATGTCACAAAAATTTTGTACGTCTTTTAATCTTTTAAAGGAT CCGTTCTTATTGTATTTTACCCGTTACGAATATTAAAATATCCA ATAGCCTTTCTCATGTAACTCGAATTTAAATGATTTACCCATAA GGATATACGGGTATTCGACCGACTGCGCCACTTTTTTCCTCGTA ACGAGAAACAACTAAAAATAAAACAACTCC**ATTTAAGGTTCGAT** **CGATAATTGCGC**TTTATTCTTTATTTTTCACTTTCTTATATACT ATTATTTTGTTAATTTTGCATTTTCGTTAGCGGAAATGCACCTG AAAATAAAGTTA**TTAA**…	*insert*	…**TTAA**AAGTATTTGGAATTAATGATACATTTTTGTTAACCATTGC ATTTTCGTTTGTTCGTTAGCGGAAATGCACTTGGACAACAATTAT TTGTAAAACACAATTTATAAATATATTTTGAAATTCTTAGCGAAA ATACAATTGACAAAGTTATAAATATAAATATTAAAATTCTTAGGA ATTAGTATACTTTGCAGTGAATAGAAAAATAAATAAAAAAAACAA TTGGTAATATTTTTAATGAGTTAGAATAGCGTGTGCATGTGTGTA TTTTCAAAATAACGTTAAGGGAGTAGTGGGAGACACTATAACACC GCCACCGTTAGTTTGAGGCTTCGACGAAATTATATTATTTATGCC GGGTTGTTTATAGTAGTTCCGTTTTTTAATTTCACGTTCGGAATA TATGTTTTTTTATTCCTGAAGAAGTATATAAAAATAATTAAGACA CATATTGATATAAGAATTGAGATATCTATGCTAAAACTAAGTACT TTCGAGTTCTTATCTGTATATAGTACTTGTTTCAATGTTTTTTCG TATTCTACTTGTTTTAGATATAAAGATTCAATTTATATGTCTGCA AGCGTTATATTACTGTTGTCTTTGATTTTCGTTATGAAATTAGGT AGGATAAACTTGTCTTTTGTTTTAAGTTTAATATTTGTAAAAGTT TGGTTTAACGTGTTTATCTCACAAGTTTTAAATTTTATCAAGTAA TTTCCTGAAATTTTTA**GTTTAGCCTTACCGCAGTTGTG**	Genome 3.2.1: Scaffold 3. Genome 2.1: Scaffold 308.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping.
Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
#183	**GTCCTGCCACTAAAGCTCTTGC**TAAGCCTGCTATATAAAATTAA ATTTCAATTTCGTTTCGTTCCTATAATGATTTGGTAAGAAATAG TGAATATGTCGTAGTCTTAAGAAAATTTCAGTTTAATTTTTATA TTTCCTTTTGAATCTTTTGCTAAATAAACCTTTTTGACTAAAAC TTTTTTCCACACCCAACACCTCTGTGATTTAAAAAAATCGGTAT CGGCCATTCATTTGGAGTCGGTCGTACTGTTCTTTGTACGGGAA GAGATGAGAGCTAAGGAAGTGTAGGAAATTCCAGGGTAGCCGTT TTACGAGTAGATCTTCCGATAGTCAACACTTTATGAATTCGAAA TGCCTCAATAGTTTAAGCTGGACGATGCACGACTTTTATTAGCT TTCTTGGTCTTTGCAGATTTAGGAATTCTCAAATGGAGCTATCA CAACGATGGTAAAGTAGTCGAACAAAATTATTCCGTGAGGTAAT ATATCAGCAGAAAATCTGATTTTCTTACTGGAAATTCACCTCGG C**TTAA**…	*insert*	…**TTAA**ATGAGATCTCGAGTAGTGGTAATTGAAGGTAGTTTCCGTC TCAAGAGTGAGATAGTTATTGAGAGAGCATTATTTTTTTATTAGT TCCTGTTCTAGAAATATTCACAGCTTTGATTTGAAATGAGTTTGT TTGCGAGATTGAAATCACGTTTTGTTATTCTCCCTTAACTTTGAC GAATCGTAGAGTTAGAGAATTTAATTTTGGACGACAACCCCACGT ATATTGCTCTTAATGGACTATAGATGAATGTATCTATATATTTAT ATTAATAAAAGTTTTATAAATTTATTTTTTATGCAAACTTTAGTA TTTACTTATTTTAATAAGATTTCTTTAAAAACTTAAATAATTTAG AAAATGAAAATATTTGAAACTTTAAATTCAGTAACATAAAATCTG AAAAATTTAAAATAACAAGTTATAAATGGTAAAGTAGATTCCTAC ATAGACTACAGCAGTAATTTATTAATTATAAAGATTCTAAAACTT AGGCTTATGGATAGGCTTAAATTAAGCGGACTCAATAAATAAATT TCTTTGGTCTGAAAAAACATCTTAGACGAAGGCCATCGTACCGGG TCGAATTCTCGTATCCGGGCGATGAACAGGAGGAGATTTTGACTA ATATGGAAGATATTTTACAGTTTCGGCTATAAGGATAGTGGATGC CATACTTTATTCTACAATATTATTGGGCCGCCTTTTAACCACCCA AAGATAAAGTTTTGGGATATTAAACTGCCACAGTGCCTCTCTCAT AGAATCCCTATTGGCAAAATATTTGAATAATCGAATTTCACAGTC TTCTTTGAGGCTAACTTTTCGCCAGCCAAATCATTTGTCATATGC AGTAACGGATGGTAATCACTTGATGCGAGGTCCGAACTATAATGA GGATCCATAAGAACTTCTGAACAAAGCTCCTGGAGCTTTTGCACA GTCATTATCGATGTCATTACCCTAGTTGTGCGCTTGTGGGTGATT GCTTCCTTCAGATGGTCAAATTACTGAGAGTAAAGGTCCAAAATA ATAATTTGGCCTCAGGGTAGCGGCTCATAATAGATGAATCCTTGG CGATCTCACCAAGCACACAAGAAAACTTTCCTGGTTAGCAATTCT GCCTTG**GGTATCATATGTGCCGGCTCA**	Genome 3.2.1: Scaffold 1. Genome 2.1: Scaffold 45.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping.
Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
#190	**ATTTAGCCTTCTCGTCGTCTTCG**TCGTTCTTTAGACGCCTTAAA ACTTTTTCAATTCATTTATTTACGTTTACGTTTACGTATATGTA TTTTTCATTTATTTTTGCATTTATTTATTTACCAAACTTTCCTC GTTTTACCTAATCTCTCACACTTATATATAAGAAAATTCTTATG TATGCATTATGTATTATATTTAATTGTCACCGTTGTATGTGCCT TCGCCTTCACCTAAATTTCAAGAAAAAGTTTCACTTAGCAACGT CAAAATCATTTTATCTTTATTTTTATACATATTTTTCCTTCTTT TTTTATGTTTTTTTTTATTATTTTTTCCTTAATTTCTTTTGGTA GTTTTTTGATTTACTTTTATATATTTTTTATTTTTTTTTCCTTA TTTTTTTTTAATTTTTTCTTTATTTTATATTTATATTTATTTGT ATCTCTTTTCTTTCTTTTTTACAAAGTTTCCATCATGAGTTTCC GCATCTACCTCTACTTTTTCTATTTTGTTGTTTTTATTTTGAAC ATTTATTTCTTCGGACATTTCTAAAATGTTCTCGGAATAAAGCA AAAATATTTTTCGTTAGAAGAGTTGAACTCATTTCAACAATAAC TAAATTTAATTTATTTCAAAGAACGAAGAGATGTCAAAAAGCCC TAAGATTGCCTC**TTAA**…	*insert*	…**TTAA**AACTGTTAAATTACCAGTCCATTTATTCAATTCAGCTTTT TTAAGTTCCCGCTTTGCTTTGCTTACGGTTGTTATGCCGAAAGCC GGCACAATTTTTTTTAATAAGTAAAAGTGGCCTTCTGTCAATGTT ACTATTCGTTCTCTTTTTTGTTTTTTTTTTTTTGTTTAACTTGTA CGTGTTTTATAGTCCAATTTTTATTACATTTGCATTAAAGCAGGA AATAAAGCGCAAAAAATCCGAAAATAATGTAAGACGGTAATAAAA AGCTTTTGACTCTGACGTCTTCATATTCTTGTTTTCGTTCTTTTG TTACAACGCGACCCATCCTTATTTATGCCTATCAGCATATTTACA TATATATATGTATATATTTTCCCACCTTTGTTTGTCGAACGTTTT TTGAATGTCAAGTGCGCAGTATTTTCAATATCTGTAGTACATACC TACATACATACATATGTACATACGTATATTTTTTTTTTGCATATT TGGGCTCTTCAATTACTACCACATACAGTTTACTAAATTTTCAAT CATGTTTGGCTGTGTCATTTATTTTTTCCTGTTTGTTCATATATG TACATATGTATGTATGCCTATTCTTACATTTGCCTTAAATAATAA AAAATGTGTTGAATTTGCGCATTACGTCAGCGTTTGTTAATAAAT GCATACAAACAACTATGTAGAGTTTTTTTTCCGCTTTCATTTGTT TTTTGTTTGTATGTACTATGTATTCATAATTTTGTTGACGCATCG CAATTGTTTGTTTCTCATTAGTTTCGTTAATCAAAGTTGATTTTG AAAGTAGACTGTTTTTTTTTTAGGTAGAACACATTTTTTTTGTTT TTTTTTTTAGGTGTGGCGCATTCT**GCAGTCACACAGAATCGCTTT G**	Genome 3.2.1: Scaffold 6. Genome 2.1: Scaffold 4, LOC101462599, 14-3-3 protein zeta, also known as 1433z.

Table S2. Flanking sequences of piggyBac insertions of the mini-wp rescue. In bold are primer-binding sites used to confirm sequence/genotyping.
Genome 3.2.1 refers to genome assembly GCA_905071925.1-Genome assembly EGII-3.2.1.
Genome 2.1. refers to the current reference genome assembly GCF_000347755.3-Genome assembly Ccap_2.1.

Strain	Flanking sequence 5' *piggyBac* integration		Flanking sequence 3' *piggyBac* integration	Identified position in genome assembly
#196	**TGTTGTGCTGTTGTAGACGGG**CACGAGATAGTTTTTTTTTTGTT CTCTTACGGTATTTTATTAAATTTATATATATATATATATATTT TTTTTTTATATTAATTTGTATTGCTTTAATTGAATTGGCTTACA AACAAATGATTTAGTTTTATGGTCATTGATGATATCGCGCTCAT AATGATGTCTCTATTAACTTTTTGGTTTAACTATATGACAAGGC CAAACATACAGAGATTTTATTATATACACAGAGTTTTCGTGACA GCTTTACGGTTACAGTTTTGCGTTTGAAGACACCCAATAAGTAG AGTAAACAGCGTTTTGAGTCTAACCTAACCTCAATATGTGTTTC AAAATTAGTCTCTGGAACATAGTATATCGGTAGTTGTTGGCAGG TTGTAGTGTGGCGAGTTGAGAATATTAAAATCTTAGTGCCAGAA TAGAGTTTGAGGTTGTGTTGCTTCGGATATTCTCTGGTTGAAGT CCCTAACTACTTTGCTCGTTCTTACGTTTCCTTTTCTTCTCATT CACTATAGTTCAAATCCATCTTAAACTGCTCATCACTGAGTAGT GCGTACATCAGGACGGAAACAAGC**TTAA**…	*insert*	…**TTAA**AGAGCTTGTACATGGTTAGTCAAGAGAATACTTTACTTTT CAATTGCCTAATCTCCGTTGCCTGCTGTGTTGGATATCCGGGCTG ACAAAGTTGTTGGTGTGGTCATTGCTTTGCATATAGACGATAGAC GTACACAGCTCTTCCGAGTTATGACTGCCAATTTTGACGCGGTAG CCAAGGCACGAATATGCTGTCTCTTTTGTCGGACGACAGGAGGAG GGATAATATTTCTTCGAGCTCTTGTTTAGTACCAGAACCATTCTA TTAGATAGACCAGCCTGGATGTGCATAATTTGAGGTTGGGAACCT TGGGTTCCACCGCAGTGGCAAAGCCTTTTTGCTAACTACTACCAG CACCTGCTGAATATTGGATTTACTGGTTGGAAGAACCTCTCTCTG GTACTCTCTATTACTCGGTCATTTGTATAAGTGACAGAGCTGCTG GAATACGTTGCTCTGGCTTAGTGAGAGGTTTTATTCGGGAAAAAC AAGTATAAGCTCTTTATTCGTCGCTTCCATTGACGGAGCCTGTAC AAAGTCAAGATAGTTCGTTTGTCAATACGTCTTCCTCGTCGCACA GCAGAGTATAGTACTTTATTTTTCCAAGAGATTTAGCACAGTTTG AATGTCTTCGTTCCAAACTCCTAGAGTTCCTTGGATATGTGTTAC CTGACGTTACTTTAAACGCTGCTTTGCTCTTAACAACACTTTGTT CGGTTAACCAAAAATTCGTTTTTACACTTCTTCCTGTCTTTTCCC ACTGCTCCTACCCACTCTTCAATGGAATTTAGAACAAAACCATTA AAAGTTGCATAGTGAATTTCGCGGCACTCCCTTTTCTCATCTCAA TGAAATTGCTTACAAGACCAAGACGAAGAGTTGCTGAGCCACAGC AAGACAAGAATACGAAAACGTTGATACAAAATACTTCCCAAGCTA AAACCAAGAATATTTTGGCCCTTCATTTTCTTTATTTATTTTTGT TTTTGCAAATATTCTACTGCTTTTTGTGTATTTCGCTTGTAAGCT TTCCAATTTGCTTAACGAACGCTTTTGTTTTATAAGAGTAACCAG AAACCGGTTGTGTAGATTGCAGAAAAGGAAAAAAAAATACATTTA AATTATGTGAAGAGTCAAGGAGAAACATGCATTTCTGTGAGAAGG AAAGACGAGCAGTTGAAA**GCATACCAAGCGAAGTCACTGAA**	Genome 3.2.1: Scaffold 4. Genome 2.1: Scaffold 1, LOC111591518, bromodomain-containing protein DDB_G0271118-like.

Table S3. Rescue capability in homozygosity. Homozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
M6620_M1F6	day 5	100	59		0	
			29	28	0	0
		100	69		0	
			26	42	0	0
		100	70		0	
			37	31	0	0
	day 6	80	54		0	
			26	27	0	0
		80	62		0	
			26	37	0	0
		80	61		0	
			23	38	0	0
#84	day 5	60	23		0	
			11	12	0	0
		60	27		0	
			10	16	0	0
		60	23		0	
			12	11	0	0
	day 6	60	27		0	
			14	13	0	0
		60	20		0	
			9	11	0	0
		60	29		0	
			13	16	0	0
#93	day 7	80	25		0	
			13	12	0	0
		/	/		0	
			/	/	0	0
		/	/		0	
			/	/	0	0
	day 8	100	37		0	
			21	16	0	0
		/	/		0	

Table S3. Rescue capability in homozygosity. Homozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
			/	/	0	0
		/	/		0	
			/	/	0	0
#104	day 5	60	13		0	
			3	10	0	0
		/	/		0	
			/	/	0	0
		/	/		0	
			/	/	0	0
	day 6	60	33		0	
			19	12	0	0
		/	/		0	
			/	/	0	0
		/	/		0	
			/	/	0	0
#153	day 5	60	32		0	
			18	12	0	0
		60	41		0	
			22	19	0	0
		/	/		0	
			/	/	0	0
	day 6	60	41		0	
			15	23	0	0
		60	47		0	
			24	21	0	0
		60	54		0	
			20	27	0	0
#160	day 5	100	86		0	
			37	45	0	0
		100	81		0	
			35	37	0	0
		100	67		0	
			36	29	0	0

Table S3. Rescue capability in homozygosity. Homozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
#183	day 6	100	78		0	
			46	35	0	0
		100	84		0	
			39	41	0	0
		100	78		0	
			29	49	0	0
	day 7	80	56		0	
			35	20	0	0
		80	46		0	
			28	17	0	0
		80	42		0	
			18	23	0	0
	day 8	80	25		0	
			16	9	0	0
		80	27		0	
			14	11	0	0
		80	37		0	
			20	17	0	0
#190	day 7	60	26		0	
			12	13	0	0
		60	33		0	
			19	13	0	0
		/	/		0	
			/	/	0	0
	day 8	60	30		0	
			14	16	0	0
		60	19		0	
			4	13	0	0
		/	/		0	
			/	/	0	0
#196	day 6	60	24		0	
			12	12	0	
		60	28		0	

Table S3. Rescue capability in homozygosity. Homozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
			14	13	0	0
		/	/		0	
			/	/	0	0
	day 7	60	10		0	
			6	3	0	0
		60	13		0	
			9	4	0	0
		/	/		0	
			/	/	0	0

"/" indicates that no more eggs were oviposited during the day of egg collection

Table S4. Rescue capability in heterozygosity. Heterozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
M6620_M1F6	day 7	21	11		5	
			3	8	0	2
		/	/		/	
			/	/	/	/
		/	/		/	
			/	/	/	/
	day 8	100	34		27	
			8	25	9	15
		100	30		34	
			9	19	10	18
		100	40		37	
			19	19	14	18
#84	day 5	100	43		41	
			19	17	16	24
		100	32		42	
			13	19	19	23
		100	35		36	
			17	14	19	15
	day 6	100	38		28	
			16	16	14	11
		100	30		33	
			15	12	17	10
		100	20		32	
			6	9	10	16
#93	day 6	100	34		32	
			15	18	12	19
		100	37		30	
			16	19	13	16
		/	/		/	
			/	/	/	/
	day 7	40	11		9	
			3	6	6	3
		40	6		12	
			1	5	6	5

Table S4. Rescue capability in heterozygosity. Heterozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
		40	7		10	
			1	5	8	2
#104	day 5	100	30		30	
			14	15	9	20
		100	32		36	
			16	14	19	15
		100	31		36	
			18	11	20	15
	day 6	100	23		43	
			8	13	17	24
		100	20		41	
			10	5	13	21
		100	26		31	
			10	13	14	13
#153	day 5	100	45		38	
			21	23	18	18
		100	42		44	
			23	18	20	23
		100	33		48	
			22	11	22	26
	day 6	100	43		37	
			15	27	22	13
		100	42		38	
			17	24	21	16
		100	44		42	
			23	20	23	19
#160	day 7	100	30		38	
			15	14	18	18
		100	41		39	
			17	21	19	18
		100	33		34	
			18	13	14	18
	day 8	100	23		38	
			11	11	15	20

Table S4. Rescue capability in heterozygosity. Heterozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
		100	39		33	
			18	18	17	14
		100	35		33	
			18	11	19	13
#183	day 7	100	2		4	
			2	0	1	2
		100	6		7	
			3	3	2	5
		100	2		5	
			2	0	3	2
	day 8	100	19		10	
			10	8	7	1
		100	18		17	
			10	8	8	7
		100	22		18	
			11	10	8	9
#190	day 7	80	7		6	
			2	3	2	4
		80	3		6	
			0	3	3	3
		/	/		/	
			/	/	/	/
	day 8	60	1		3	
			1	0	1	2
		60	9		11	
			4	4	5	6
		60	8		11	
			5	3	6	4
#196	day 6	100	33		42	
			16	17	21	21
		100	42		31	
			19	21	22	9
		100	39		32	
			15	22	16	16

Table S4. Rescue capability in heterozygosity. Heterozygous rescued flies were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
	day 7	100	29		39	
			13	15	17	22
		100	39		37	
			14	25	19	18
		100	29		43	
			10	19	20	23

"/" indicates that no more eggs were oviposited during the day of egg collection

Table S5. Rescue of strain #160 with mini-*wp* on the X chromosome. Male flies carrying the mini-*wp* rescue on X chromosome were crossed to their homozygous counterparts carrying the white pupae natural mutation.

Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
#160	day 6	60	13		22	
			13	0	0	20
		60	21		18	
			19	0	0	16
		60	13		20	
			12	0	0	19
	day 7	50	5		15	
			5	0	0	13
		50	10		13	
			9	0	0	13
		50	11		21	
			11	0	0	20
Cage	Oviposition day	Number of eggs taken	Number of brown pupae		Number of white pupae	
			Female adults	Male adults	Female adults	Male adults
#160	day 7	60	24		29	
			24	0	0	25
		60	27		25	
			27	0	0	24
		60	21		23	
			21	0	0	22
	day 8	60	17		24	
			16	0	0	23
		60	20		24	
			19	0	0	23
		60	26		29	
			24	0	0	29

4 Discussion

4.1 Scale-up ready protocol for extraction of dsRNA

Several methods have been developed for extraction of bacterially produced dsRNA for application of RNAi in crop protection, vector control, functional genomic analysis, among other research fields (Ahn et al, 2019; Ongvarrasopone et al, 2007; Papić et al, 2018; Posiri et al, 2013; Shen, 2019; Solis et al, 2009; Verdonckt & Vanden Broeck, 2022). Combined with the flexibility of designing and cloning different dsRNA sequences into an expression vector, having a cost-effective protocol for extraction of the bacterially produced dsRNA expands the potential of research on RNAi in different application fields. This could be particularly interesting in research projects screening for different target genes, where changes or optimization on the dsRNA sequence might be required, or in establishing different delivery or application methods, which often demands larger amounts of dsRNA.

Considering the different methods and promising protocols available in the literature, the yield, quality and production costs of phenol-based (Ahn et al, 2019; Solis et al, 2009), phenol/guanidine-based (Ongvarrasopone et al, 2007), and ethanol-based (Papić et al, 2018; Posiri et al, 2013) extraction methods for a small laboratory-scale application were evaluated. Another phenol/guanidine-based protocol for dsRNA extraction using QIAzol® has also been recently published (Verdonckt & Vanden Broeck, 2022). Based on the published yields and to reduce the costs with the commercially available phenol/guanidine-based reagents for nucleic acids extraction, experimental comparisons only with the protocol by Ongvarrasopone et al. using TRIzol™ was performed, while still keeping at least one protocol from each class of extraction method. Due to mixed results from the published protocols, some producing high quality RNA at low yields or high yields at high costs, further optimization to obtain an extraction protocol with high yields at reduced costs was necessary. Finally, a comparative assessment of yield, purity and costs of the optimized version and other available protocols was performed (Ahn et al, 2019; Ongvarrasopone et al, 2007; Papić et al, 2018; Posiri et al, 2013; Solis et al, 2009).

Guanidinium thiocyanate-based methods are commonly used for extraction of nucleic acids. Here, cell lysis, protein denaturation and deactivation of nucleases are enhanced by the chaotropic agent guanidinium thiocyanate in solution with phenol (Shen, 2019). TRIzol™ (Invitrogen), TRI Reagent® (Merck) and QIAzol® (QIAGEN) are examples of commercially available phenol/guanidine-based reagents for nucleic acids extraction, that have been used for extraction of bacterially produced dsRNA for RNAi application (Ongvarrasopone et al, 2007; Verdonckt & Vanden Broeck, 2022). Despite of efficiently extracting dsRNA, these

commercially available monophasic solutions of phenol/guanidine are costly, thus driving up the costs for extraction of bacterially produced dsRNA**.** As discussed in the literature, the costs with organic extraction, represented by the use of QIAzol®, corresponded to 85 % of the total costs of dsRNA extraction (Verdonckt & Vanden Broeck, 2022). Based on list prices of these chemicals at the time of performing this study, the costs of these monophasic solutions of phenol/guanidine are at least 10-fold more expensive when compared to the alternative phenol/chloroform/isoamyl alcohol (P/C/I) solution (25:24:1) at pH 4.5-5.0. The economic pressure for a more cost-efficient method for extraction of bacterially produced dsRNA for RNAi application and studies in laboratory larger scale has also been pointed out in recent publications (Guan et al, 2021; Silver et al, 2021).

Considering the promising high yields of the phenol/guanidine-based protocol, TRIzol^{TM} was replaced with the cheaper chemical P/C/I for extraction of dsRNA in that protocol. Despite of the successful isolation of dsRNA, the direct replacement of TRIzol^{TM} by acidic P/C/I (25:24:1, pH 4.5-5) led to a stronger co-extraction of bacterial genomic DNA (gDNA) together with noise background.

The co-extraction of gDNA was unexpected, as the DNA should be partitioned into the interphase and organic phase under acidic conditions (Shen, 2019). However, the acidity of the P/C/I itself was not sufficient for this effect, and also acidifying the reaction before the P/C/I addition didn't improve the partitioning. In a systematic optimization process it was observed that acidifying the product of a first P/C/I extraction before performing a 2nd acidic P/C/I extraction considerably increases the quality of the extracted dsRNA. These results suggest that the pH of the used P/C/I might not always be sufficient to result in effective separation of RNA and DNA in phenol-based extractions, and that a pre-acidification of the samples is required to obtain better results.

Pre-treatments to open up bacterial cells, including sonication, heating, and enzymatic digestions, in the upstream process have been shown to increase the yields of dsRNA extraction (Ahn et al, 2019; Verdonckt & Vanden Broeck, 2022). Despite of the increased yield, quality analysis via gel electrophoresis has also indicated that dsRNA extracted from sonicated samples presented the weakest bands and smearing in the background, suggesting degradation of dsRNA (Ahn et al, 2019; Verdonckt & Vanden Broeck, 2022). From the protocol optimized in this study, it is shown that boiling in a reduced volume of anionic surfactant efficiently opens up bacterial cells, therefore consisting of a quick and inexpensive approach in the upstream process for dsRNA extraction with no further treatments required for cell lysis.

Obtaining dsRNA free of viable bacterial cells is important not only to avoid possible unwanted immune responses (Li et al, 2019), but also relevant for regulatory issues. In Europe, for instance, if no genetically modified organism (GMO) has been used or if it is proven to be inactivated, RNAi-based products intended for agricultural pest control would be regulated following the same regulations applied for classical synthetic chemical pesticides (De Schutter et al, 2022). It is noteworthy that no viable cells were observed in the upstream process of our protocol, supporting not only the safety of the next steps of the extraction, but also guaranteeing a GMO-free dsRNA as final product.

Different studies in the literature have induced cells with OD_{600} ranging from 0.4 to 0.8, and harvested after 4 to 5 h of dsRNA production or when OD_{600} reached 1.0 (Ahn et al, 2019; Ongvarrasopone et al, 2007; Papić et al, 2018; Posiri et al, 2013; Solis et al, 2009; Verdonckt & Vanden Broeck, 2022). As the bacterial growth consists of an initial lag-phase, followed by exponential phase and stationary phase, inducing dsRNA production with IPTG in a late exponential phase ($OD_{600} = 0.8$) was evaluated. At this point, the culture would have more cells and could produce more dsRNA. To avoid any possible limitations from the different extraction methods, dsRNA extraction was performed with published ethanol-based, phenol/guanidine-based methods and with the protocol optimized in this study (Ongvarrasopone et al, 2007; Papić et al, 2018; Posiri et al, 2013). Regardless of the extraction method, it was observed that induction at later growth stage yielded weaker dsRNA bands and up to two-thirds of dsRNA extracted with induction at OD_{600} 0.4 is not recovered when the induction occurred at the later stage.

Based on the quality analysis via gel electrophoresis, the different protocols produced dsRNA with different purities. There is still some contamination with gDNA when no DNase A digestions is performed in the dsRNA extracted using the protocol optimized in this study and the phenol/guanidine-based protocol; for the ethanol-based protocols there is a strong contamination with small bacterial RNAs, and the alkaline phenol-based protocol presents the best quality, but the lowest quantitative yields. Despite of using ethanol, which is cheap and readily available, the ethanol-based protocol presented a strong contamination with bacterial RNAs, which drives up the costs of purification once the yield and the estimated amount of dsRNA are considered. On the other hand, the phenol/guanidine presented higher yields, with less co-extraction of other nucleic acids, but increased costs given the use of the costly TRIzol™. The alternative optimized protocol produced dsRNA at comparable quality and yields as the phenol/guanidine-based protocol, but at much lower costs, as the cheaper P/C/I was used for extraction. Moreover, the optional DNase I digestion is not expected to

significantly increase the costs of the protocol. The alkaline phenol-based protocols did produce dsRNA at a considerably good quality, but at low yields, driving up the costs when a scale-up in dsRNA production is expected.

The optimized protocol for extraction of bacterially produced dsRNA combines good qualitative and quantitative yields of extraction at a considerable low costs. Together with the flexibility of engineering bacterial cells to express dsRNA, it offers a solution for 'on demand' scalable production of dsRNA in laboratory conditions for further applications.

4.2 Challenges of robust RNAi gene silencing in *Aedes* mosquitoes

Several approaches to harness RNAi technology for gene silencing and *Aedes* control have been reported in the last years. The present study sought to replicate and build upon previously published results demonstrating the efficacy of RNAi effects in *Aedes* mosquitoes through oral delivery of RNAi-triggering molecules (Hapairai et al, 2017; Mysore et al, 2019c; Singh et al, 2013; Whyard et al, 2015). Specifically, the experiments first focused on the oral delivery of shRNA-expressing yeasts. Despite promising results described in the literature (Hapairai et al, 2017; Mysore et al, 2019c), it was not possible to successfully reproduce them. Induction of RNAi response could be affected by several factors, such as the stability of RNA molecules in the environment or during delivery, uptake from the gut lumen, intracellular release, processing of the dsRNA by the RNAi machinery, induction of systemic RNAi, among others (Baum & Roberts, 2014; Christiaens et al, 2022; Christiaens et al, 2020; Cooper et al, 2019; Joga et al, 2016; Romoli et al, 2024). To troubleshoot some of these possible factors influencing the success of RNAi in *Aedes,* different RNAi-triggering molecules (si-, sh-, dsRNA) and different delivery pathways (oral delivery of naked molecules and of microorganisms producing shRNA or dsRNA, soaking into naked molecules and microinjection) were systematically tested. Despite of following methods and protocols from publication which presented a strong RNAi effect, none of the different combined strategies resulted in robust phenotypic effects.

If RNAi is to be triggered via ingestion of shRNA- or dsRNA-producing microorganisms, these need to be lysed in the gut to set the RNA free. Coon and colleagues observed that bacterial cells are needed for normal development of the larvae and can be later found dead in the gut of mosquito larvae (Coon et al, 2017). Thus, bacterially produced dsRNA could have been set free in the gut of the larvae after feeding. However, since no RNAi effect has been observed, dsRNA could have still not been correctly processed into siRNA by the RNAi machinery. Recently, Romoli and colleagues investigated the use of RNAi in mosquitoes as a potential mechanism to block virus transmission and observed no RNAi effect upon

colonization of *Ae*. *aegypti* with *E. coli* bacterial cells producing dsRNA. Additionally, they investigated the use of in vitro transcribed dsRNA, but did not observe a RNAi-induced effect upon oral delivery. On the other hand, viral replication was completely blocked upon injection of the in vitro transcribed dsRNA (Romoli et al, 2024). Similarly, McFarlane and colleagues reported successful knockdown of targeted genes upon injection of female *Ae*. *aegypti* adults with dsRNA. Interestingly, however, no RNAi effect was observed when siRNA was used in vivo, although successful knockdown was observed in in vitro experiments with the *Ae. aegypti*-derived Aag2 cells and siRNA (McFarlane et al, 2021).

Upon successful bacterial expression of dsRNA and its stability in the food, yet with no RNAi effect, it is possible that degradation upon uptake by the larvae or lack of correct processing of the RNAi-triggers might have undermined the attempts to trigger RNAi. To circumvent this issue, soaking and microinjection assays were used to trigger RNAi using siRNA. Although these delivery strategies for triggering RNAi might circumvent possible barriers from the oral delivery, no improvement in triggering RNAi was observed, except by the proof-of-principle embryonic microinjection of the transgenic line expressing eGFP. Here, we have additionally confirmed the bioactivity of the bacterially produced dsRNA. Additionally, the hypothesis of strains being recalcitrant to RNAi has been tested by using different strains, yet with no success in triggering robust RNAi-based effects.

Despite extensive troubleshooting and experimental adjustments, it was not possible to observe the RNAi-based effects in *Aedes* mosquitoes as published in the literature. Together with other studies showing the limitations on RNAi for *Aedes* mosquitoes, this study suggests that establishing RNAi in *Aedes* mosquitoes might be more complex than previously reported or assumed. There is also an indication of several knowledge gaps or inconsistencies in the mechanisms underlying RNAi in *Aedes* mosquitoes. Further basic research into the mechanisms and fate of RNAi-triggering molecules inside the mosquito body will be needed for a better understanding of the process and the factors influencing it. Currently, the results indicate a clear limitation of RNAi as an applicable tool for mosquito control. Furthermore, considering a possible practical application under environmental conditions for *Aedes* control, the RNAi-based effects should be robust and reproducible enough to withstand environmental variations, such as temperature changes, food availability and interaction with other organisms and natural conditions.

4.3 Minimal version of selectable genetic marker for neo-classical GSS

The sterile insect technique (SIT) is a well-established strategy for environmentally friendly and species-specific control of pest insects. It relies on introducing sterility in a wild insect population, causing suppression, containment or local eradication of the targeted insect species (Klassen et al, 2021). Genetic sexing strains (GSS) can be used for sex separation in the rearing facilities, enabling male-only releases, contributing for the cost-effectiveness of SIT programmes (Franz et al, 2021; Mumford, 2021). Classically, a GSS requires a phenotypic marker linked to the male sex (Franz et al, 2021). Despite the white pupae phenotype being used as a selectable marker for sex separation in *C. capitata* for several years, its genetic basis was only recently elucidated (Ward et al, 2021). Following the logic to use modern molecular techniques to create new generation of GSS (Häcker et al, 2021; Yan et al, 2023), the *white pupae* (*wp*) gene was further explored in this study as a selectable marker to facilitate the creation of the so-called 'neo-classical' GSS. Specifically, the robust and functional rescue of the WT pupal color phenotype through *piggyBac* insertion of a minimal, intronless, version of the *wp* gene (mini-*wp*), is reported.

The identification of the 5' untranslated region (5'UTR) mapped approximately 18 kb upstream the starting codon of the *wp* gene might pose a challenge for site-specific knock-in of the WT allele, e.g., through CRISPR/HDR-mediated knock-in of the *wp* gene in the Y chromosome. Considering the whole gene, the cargo would be about 20 kb long. As observed in the literature, the knock-in efficiency is likely to decrease with an increase in the size of the cargo (Li et al, 2014; Paix et al, 2017) and a successful targeted insertion might be even more challenging in repetitive and heterochromatic regions, as the Y chromosome. A minimal version of the gene could help overcome this barrier. At this step and to first evaluate the functionality of such construct, the well-established *piggyBac* transposon system was used for random transposon-mediated integration of the mini-*wp* rescue into the genome of a CRISPR-modified wp^{-} medfly strain.

The successful *piggyBac* integration of the mini-*wp* and the expression of the rescued WT phenotype indicates the feasibility of obtaining fully functional phenotypical rescue using an intronless version of the *wp* gene. The preserved functionality of the mini-*wp* rescue integrated into different genomic positions, predicted to correspond to different chromosomes, confirms its robust function. Despite of not being investigated in this study, a smaller endogenous putative promoter region or other (shorter) exogeneous promoters could also be evaluated, further reducing the overall size of the rescue.

In the next steps of creating neo-classical GSS, the mini-*wp* designed in this study could be inserted into the Y chromosome of a *white pupae* mutated medfly strain, thereof promoting sex sorting based on the pupal phenotype. When applying this pipeline to create neo-classical GSS in other insects, the insertion of the WT allele of a genetic marker could be introduced into the Y chromosome or close to the male-determining factor (M factor) already identified, for example, in *Ae. aegpyti* (Hall et al, 2015) and *Ae. albopictus* (Gomulski et al, 2018).

Despite of being luck-dependent, random *piggyBac* integrations into the Y chromosome, or close to the male-determining factor, could also be used to achieve sex linkage of the WT allele. The *piggyBac* integration into the Y chromosome of the mosquito *Anopheles gambiae* (Bernardini et al, 2014), and as well for several *Tephritidae* flies have been reported: the Mexican fruit fly, *Anastrepha ludens* (Meza et al, 2011), the Caribean fruit fly, *Anastrepha suspensa* (Schetelig & Handler, 2013), and for the medfly (Condon et al, 2007). However, these transposon-mediated integrations on the Y chromosome are reported as rare events and might be subjected to position effects, or might be suppressed due to the heterochromatic nature of the Y chromosome (Bernardini et al, 2014; Condon et al, 2007; Elgin & Reuter, 2013). In the reported *piggyBac* integration of a fluorescent marker into the Y chromosome of medfly, the authors speculated that the observed patchy fluorescence was due to position effect (Condon et al, 2007). In case of silent integrations, where expression of an integrated construct is completely suppressed due to the heterochromatin or other regulatory elements in the region of integration, only the molecular analysis of the integrated cassette and integration site could shed light to this matter. Similarly, in a CRISPR/Cas-mediated editing of the Y chromosome, offspring with no expression of the expected phenotype might also require genotyping to verify for the intended integration event or a possible silent integration. On the other hand, a larger experimental setup with mobilization of *piggyBac* integration might also be sufficient to produce Y-specific expression in a useful level (Condon et al, 2007). This approach would, however, not be applicable for *Aedes* mosquitoes, as the *piggyBac* transposase, and other transposable elements, are reported to lack mobility in the mosquito genome (Häcker & Schetelig, 2018; Palavesam et al, 2013; Sethuraman et al, 2007). While luck-dependent, if successful, such remobilization screening would have the advantage of identifying integration sites that are effectively expressed exclusively in males, bypassing the search for unique regions to be targeted or possible position effect suppression.

The toolkit here evaluated could also be extrapolated not only to other related species, where the *wp* gene has already been identified and CRISPR/Cas-mediated mutated strains have been generated (Paulo et al, 2022; Ward et al, 2021), but it could also be used in other insect

species with potential target genes to be tested as selectable markers (Chen et al, 2022; Paulo et al, 2022; Robinson, 2002; Sollazzo et al, 2024; Ward et al, 2021). Altogether, the findings reported represent an important advancement toward developing neo-classical GSS for SIT. It was demonstrated that the *wp* gene can be used as selectable marker and that the WT phenotype can be rescued by a minimal, intronless, version of the gene integrated into different genomic positions. This will facilitate further gene editing approaches for generating neo-classical GSS.

4.4 General regulatory framework

The advances in modern molecular techniques has enabled new strategies for insect pest control, like RNAi-based insecticides, or improvement and expansion of strategies already well established, like the SIT (Christiaens et al, 2020; Fletcher et al, 2020; Häcker et al, 2021; Joga et al, 2016; Nitnavare et al, 2021; Ortolá & Daròs, 2024; Yan et al, 2023). This occurs in times of increased insecticide resistance across several species around the world and search for new environmentally friendly approaches for pest control.

The RNAi mechanism could be used for insect control essentially in two ways: either by engineering organisms that produce RNAi-triggers or by external application of such RNAi-triggers. While the first is more likely in the context of plant protection, e.g., with plants engineered to produce dsRNA targeting genes specific of a certain insect pest (EFSA et al, 2024; EFSA et al, 2018), the last could be applied not only for plant protection, e.g., through sprayable dsRNA-based biopesticides (Chakraborty & Ghosh, 2022; Rodrigues et al, 2021; Yan et al, 2020), but also for vector control. This external, i.e., topical, application will likely also involve some system for packaging or stabilizing the RNAi-triggers in the environment.

Recently, the genetically modified maize DP23211 was authorized for placing in the market, except for cultivation, by the European Commission (EFSA et al, 2024; EU, 2024). Among other features, this genetically modified maize expresses *DvSSJ1* dsRNA, which confers resistance to certain coleopteran pests. Besides the Official Journal of the European Union, the European GMO initiative for a unified database system provides a useful platform to those who seek accurate information on genetically modified organisms (EUginius, 2014).

In case of external application of dsRNA to trigger RNAi, it is possible that microbial systems will be used for large scale production. In this case, it will be important to guarantee the inactivation of the microorganisms used for dsRNA production, safeguarding the application of the produced dsRNA. If this requirement is fulfilled and the dsRNA is used, for instance, as a topically applied plant protection product, it will likely not be regulated as a genetically modified organisms (GMO) within the European Union (EU) (De Schutter et al,

2022; Schenkel & Gathmann, 2021). In this sense, the protocol for extraction of bacterially produced dsRNA developed in this thesis represents an important step to provide dsRNA at low cost and good yields, with efficient lysis of the bacterial cells during the extraction and no viable microorganism present in the purified dsRNA (Figueiredo Prates et al, 2023). Yet, dsRNA as active substance for pest control would have to be approved under the EU Regulation (EC) No 1107/2009 (EC, 2022).

There is an ongoing discussion to change the regulation on plants produced using new genomic techniques (NGT) in the EU (EC, 2024a). In the future, the changes in the regulation for plants may eventually be extended to insects and regulate the insects produced in mass rearing facilities for SIT. The current proposal for the new regulation on plants produced by NGT states that, under certain conditions, these engineered plants might be considered equivalent to conventional plants (EC, 2024b). Extending these conditions to insects, it is possible to hypothesize that insects engineered under the neo-classical GSS concept and carrying a WT allele of a genetic marker on the Y chromosome - or close to a male-determining factor – could be considered equivalent to conventional insects.

The simplest scenario would be the targeted insertion of the complete WT allele of a genetic marker into a male-specific genomic region. Here, the targeted insertion of a contiguous DNA sequence existing in the insect's gene pool, as long as the genetic modification does not interrupt an endogenous gene, would result in engineered insects considered equivalent to conventional insects. This would represent the scenario of inserting the WT allele of a genetic marker without any further modification. Although possible for short-sequence genetic markers, it might become technically challenging to insert genetic markers with longer sequences, as the *white pupae* gene. In the case of a minimal version of a gene, as explored in this thesis (Figueiredo Prates et al, 2024), the engineered insects could still be considered equivalent to conventional insects according to the point (5) "any other targeted modification of any size, on the condition that the resulting DNA sequences already occur (possibly with modifications as accepted under points (1) and/or (2)) in a species from the breeders' gene pool". Since the point (2) indicates that "deletion of any number of nucleotides" would be accepted, it is possible to speculate that insects engineered with such minimal version of a gene could still be considered as equivalent to conventional insects, and therefore not regulated as GMO.

5 Appendix

5.1 Statistical analysis of data in manuscript "An optimized/scale up-ready protocol for extraction of bacterially produced dsRNA at good yield and low costs"

Figure 5a

Descriptive Statistics

Data source: Data

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
Papic 04	4	0	5,659	0,364	0,182	0,579
Papic 08	4	0	5,116	0,470	0,235	0,748
Prates 04	4	0	13,757	2,812	1,406	4,474
Prates 08	4	0	15,463	1,905	0,953	3,032
Ong 04	4	0	12,995	1,546	0,773	2,461
Ong 08	4	0	9,945	2,636	1,318	4,194
Posiri 04	4	0	7,335	0,420	0,210	0,669
Posiri 08	4	0	6,388	0,502	0,251	0,798
Papic 04	4	0	5,659	0,364	0,182	0,579
Papic 08	4	0	5,116	0,470	0,235	0,748

Column	Range	Max	Min	Median	25%	75%
Papic 04	0,796	6,084	5,288	5,631	5,325	6,020
Papic 08	1,024	5,660	4,636	5,084	4,683	5,581
Prates 04	5,609	16,911	11,302	13,407	11,344	16,519
Prates 08	4,168	18,286	14,118	14,725	14,245	17,421
Ong 04	3,374	14,124	10,750	13,554	11,365	14,067
Ong 08	5,625	13,822	8,198	8,880	8,245	12,710
Posiri 04	0,969	7,840	6,870	7,315	6,939	7,751
Posiri 08	1,061	7,135	6,074	6,171	6,086	6,905
Papic 04	0,796	6,084	5,288	5,631	5,325	6,020
Papic 08	1,024	5,660	4,636	5,084	4,683	5,581

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
Papic 04	0,273	-3,040	0,231	0,516	0,945	0,684
Papic 08	0,239	-3,232	0,232	0,509	0,941	0,663
Prates 04	0,259	-4,488	0,292	0,256	0,853	0,238
Prates 08	1,852	3,560	0,382	0,042	0,771	0,059
Ong 04	-1,648	2,649	0,306	0,207	0,825	0,155
Ong 08	1,783	3,165	0,336	0,121	0,779	0,070
Posiri 04	0,222	-1,399	0,176	0,692	0,987	0,939
Posiri 08	1,917	3,709	0,383	0,041	0,735	0,028
Papic 04	0,273	-3,040	0,231	0,516	0,945	0,684
Papic 08	0,239	-3,232	0,232	0,509	0,941	0,663

Column	Sum	Sum of Squares
Papic 04	22,634	128,474
Papic 08	20,464	105,357
Prates 04	55,027	780,704
Prates 08	61,854	967,363
Ong 04	51,981	682,686
Ong 08	39,781	416,468
Posiri 04	29,340	215,731
Posiri 08	25,551	163,964
Papic 04	22,634	128,474
Papic 08	20,464	105,357

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,210)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,212)

Group Name	N	Missing	Mean	Std Dev	SEM
Prates 04	4	0	13,757	2,812	1,406
Prates 08	4	0	15,463	1,905	0,953

Source of Variation	DF	SS	MS	F	P
Between Groups	1	5,826	5,826	1,010	0,354
Residual	6	34,607	5,768		
Total	7	40,433			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,354).

Power of performed test with alpha = 0,050: 0,051

The power of the performed test (0,051) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,468)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,628)

Group Name	N	Missing	Mean	Std Dev	SEM
Ong 04	4	0	12,995	1,546	0,773
Ong 08	4	0	9,945	2,636	1,318

Source of Variation	DF	SS	MS	F	P
Between Groups	1	18,607	18,607	3,985	0,093
Residual	6	28,014	4,669		
Total	7	46,621			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,093).

Power of performed test with alpha = 0,050: 0,308

The power of the performed test (0,308) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,206)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,877)

Group Name	N	Missing	Mean	Std Dev	SEM
Posiri 04	4	0	7,335	0,420	0,210
Posiri 08	4	0	6,388	0,502	0,251

Source of Variation	DF	SS	MS	F	P
Between Groups	1	1,794	1,794	8,382	0,028
Residual	6	1,284	0,214		
Total	7	3,079			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,028).

Power of performed test with alpha = 0,050: 0,623

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
Posiri 04 vs. Posiri 08	0,947	2,895	0,028	Yes

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,384)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,407)

Group Name	N	Missing	Mean	Std Dev	SEM
Papic 04	4	0	5,659	0,364	0,182
Papic 08	4	0	5,116	0,470	0,235

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0,589	0,589	3,331	0,118
Residual	6	1,060	0,177		
Total	7	1,649			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,118).

Power of performed test with alpha = 0,050: 0,252

The power of the performed test (0,252) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 5C

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,392)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,381)

Group Name	N	Missing	Mean	Std Dev	SEM
Acid phenol	4	0	79,784	4,550	2,275
Phenol/guanidine	4	0	77,430	1,643	0,822
Et I	4	0	51,546	1,510	0,755
Et II	4	0	54,592	3,119	1,560

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2638,373	879,458	99,346	<0,001
Residual	12	106,230	8,853		
Total	15	2744,603			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
Acid phenol vs. Et I	28,238	13,422	<0,001	Yes
Phenol/guanidine vs. Et I	25,884	12,303	<0,001	Yes
Acid phenol vs. Et II	25,192	11,974	<0,001	Yes
Phenol/guanidine vs. Et II	22,838	10,855	<0,001	Yes
Et II vs. Et I	3,046	1,448	0,316	No
Acid phenol vs. Phenol/guani	2,354	1,119	0,285	No

Figure 6B

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
AcidPhen	6	0	0,215	0,210	0,227
PhenGuan	6	0	0,217	0,206	0,228
EthI	6	0	0,173	0,171	0,191
EthII	6	0	0,148	0,143	0,153
AlkPheI	6	0	0,504	0,487	0,569
AlkPheII	6	0	0,421	0,384	0,487

H = 32,889 with 5 degrees of freedom. (P = <0,001)

> The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P	P<0,050
AlkPheI vs EthII	178,000	6,897	<0,001	Yes
AlkPheI vs EthI	142,000	5,502	0,002	Yes
AlkPheI vs PhenGuan	90,000	3,487	0,134	No
AlkPheI vs AcidPhen	86,000	3,332	0,172	Do Not Test
AlkPheI vs AlkPheII	32,000	1,240	0,952	Do Not Test
AlkPheII vs EthII	146,000	5,657	<0,001	Yes
AlkPheII vs EthI	110,000	4,262	0,031	Yes
AlkPheII vs PhenGuan	58,000	2,247	0,606	Do Not Test
AlkPheII vs AcidPhen	54,000	2,092	0,678	Do Not Test
AcidPhen vs EthII	92,000	3,565	0,118	No
AcidPhen vs EthI	56,000	2,170	0,642	Do Not Test
AcidPhen vs PhenGuan	4,000	0,155	1,000	Do Not Test
PhenGuan vs EthII	88,000	3,410	0,152	Do Not Test
PhenGuan vs EthI	52,000	2,015	0,712	Do Not Test
EthI vs EthII	36,000	1,395	0,922	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

> A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance

Data source: Data in dsRNA band intensities transformations

Normality Test (Shapiro-Wilk): Passed (P = 0,166)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,866)

Group Name	N	Missing	Mean	Std Dev	SEM
AcidPhen	6	0	4,582	0,223	0,0910
PhenGuan	6	0	4,623	0,241	0,0985
EthI	6	0	5,607	0,376	0,153
EthII	6	0	6,770	0,238	0,0970
AlkPheI	6	0	1,943	0,197	0,0804
AlkPheII	6	0	2,356	0,268	0,110

Source of Variation	DF	SS	MS	F	P
Between Groups	5	103,975	20,795	299,493	<0,001
Residual	30	2,083	0,0694		
Total	35	106,058			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
EthII vs. AlkPheI	4,827	31,729	<0,001	Yes
EthII vs. AlkPheII	4,414	29,016	<0,001	Yes
EthI vs. AlkPheI	3,664	24,086	<0,001	Yes
EthI vs. AlkPheII	3,252	21,374	<0,001	Yes
PhenGuan vs. AlkPheI	2,680	17,618	<0,001	Yes
AcidPhen vs. AlkPheI	2,639	17,350	<0,001	Yes
PhenGuan vs. AlkPheII	2,268	14,905	<0,001	Yes
AcidPhen vs. AlkPheII	2,227	14,637	<0,001	Yes
EthII vs. AcidPhen	2,188	14,379	<0,001	Yes
EthII vs. PhenGuan	2,147	14,111	<0,001	Yes
EthII vs. EthI	1,163	7,642	<0,001	Yes
EthI vs. AcidPhen	1,025	6,737	<0,001	Yes
EthI vs. PhenGuan	0,984	6,468	<0,001	Yes
AlkPheII vs. AlkPheI	0,413	2,713	0,022	Yes
PhenGuan vs. AcidPhen	0,0408	0,268	0,790	No

Figure 6C

One Way Analysis of Variance

Data source: Data

Normality Test (Shapiro-Wilk): Passed (P = 0,065)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,496)

Group Name	N	Missing	Mean	Std Dev	SEM
Acid phenol	6	0	3,872	0,190	0,0774
PhenGua	6	0	2,861	0,241	0,0985
Et I	6	0	1,468	0,135	0,0552
Et II	6	0	0,954	0,0500	0,0204
AlkPhe I	6	0	0,710	0,0653	0,0267
AlkPhe II	6	0	0,579	0,152	0,0622

Source of Variation	DF	SS	MS	F	P
Between Groups	5	53,430	10,686	450,236	<0,001
Residual	30	0,712	0,0237		
Total	35	54,142			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
Acid phenol vs. AlkPhe II	3,294	37,031	<0,001	Yes
Acid phenol vs. AlkPhe I	3,163	35,555	<0,001	Yes
Acid phenol vs. Et II	2,919	32,812	<0,001	Yes
Acid phenol vs. Et I	2,405	27,034	<0,001	Yes
PhenGua vs. AlkPhe II	2,282	25,656	<0,001	Yes
PhenGua vs. AlkPhe I	2,151	24,181	<0,001	Yes
PhenGua vs. Et II	1,907	21,438	<0,001	Yes
PhenGua vs. Et I	1,393	15,660	<0,001	Yes
Acid phenol vs. PhenGua	1,012	11,374	<0,001	Yes
Et I vs. AlkPhe II	0,889	9,996	<0,001	Yes
Et I vs. AlkPhe I	0,758	8,521	<0,001	Yes
Et I vs. Et II	0,514	5,778	<0,001	Yes
Et II vs. AlkPhe II	0,375	4,219	<0,001	Yes
Et II vs. AlkPhe I	0,244	2,743	0,020	Yes
AlkPhe I vs. AlkPhe II	0,131	1,475	0,151	No

5.2 Statistical analysis of data in manuscript "Challenges of robust RNAi-mediated gene silencing in *Aedes* mosquitoes"

Figure 1a

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,066)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	18	0	81,759	15,151	3,571
sem1a	18	0	87,407	7,632	1,799
fez2	10	0	76,500	15,937	5,040
lrc	10	0	68,500	20,942	6,622

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2484,346	828,115	3,871	0,014
Residual	52	11125,525	213,952		
Total	55	13609,871			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,014).

Power of performed test with alpha = 0,050: 0,653

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,806)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,480)

Group Name	N	Missing	Mean	Std Dev	SEM
asinsqrt(control)	18	0	1,172	0,223	0,0526
asinsqrt(sem1a)	18	0	1,236	0,153	0,0360
asinsqrt(fez2)	10	0	1,098	0,224	0,0710
asinsqrt(lrc)	10	0	0,991	0,233	0,0736

Source of Variation	DF	SS	MS	F	P
Between Groups	3	0,422	0,141	3,350	0,026
Residual	52	2,185	0,0420		
Total	55	2,608			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,026).

Power of performed test with alpha = 0,050: 0,558

Multiple Comparisons versus Control Group (Bonferroni t-test):

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0,050
asinsqrt(con vs. asinsqrt(lrc	0,181	2,238	0,088	No
asinsqrt(con vs. asinsqrt(fez	0,0748	0,925	1,000	Do Not Test
asinsqrt(con vs. asinsqrt(sem	0,0641	0,939	1,000	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Figure 1B

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,624)

Group Name	N	Missing	Mean	Std Dev	SEM
4_Sem1a_1, day 3	3	0	1,074	0,241	0,139

Hypothesized population mean 1,000

t = 0,534 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,475 to 1,674

Two-tailed P-value = 0,647

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,647).

One-tailed P-value = 0,323

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,323).

One-Sample t-test

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
16_Sem1a_1, day 5	2	0	0,582	0,0131	0,00926

Hypothesized population mean 1,000

t = -45,118 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,464 to 0,700

Two-tailed P-value = 0,0141

There is a statistically significant difference between the mean of the sampled population and the hypothesized population mean (P = 0,014).

One-tailed P-value = 0,00705

The hypothesized mean exceeds the sample mean of the group by an amount that is greater than would be expected by chance, rejecting the hypothesis that the true mean of group is greater than or equal to the hypothesized mean. (P = 0,007).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,111)

Group Name	N	Missing	Mean	Std Dev	SEM
28_Sem1a_1,, pupae	3	0	0,944	0,278	0,160

Hypothesized population mean 1,000

t = -0,351 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,253 to 1,634

Two-tailed P-value = 0,759

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,759).

One-tailed P-value = 0,380

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,380).

Figure 1C

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,214)

Group Name	N	Missing	Mean	Std Dev	SEM
4_Sem1a_1, day 3	3	0	0,942	0,0879	0,0507

Hypothesized population mean 1,000

t = -1,137 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,724 to 1,161

Two-tailed P-value = 0,373

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. **There is not a significant difference between the two means (P = 0,373).**

One-tailed P-value = 0,187

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,187).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,973)

Group Name	N	Missing	Mean	Std Dev	SEM
16_Sem1a_1, day 5	3	0	1,013	0,148	0,0856

Hypothesized population mean 1,000

t = 0,151 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,645 to 1,381

Two-tailed P-value = 0,894

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,894).

One-tailed P-value = 0,447

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,447).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,959)

Group Name	N	Missing	Mean	Std Dev	SEM
28_Sem1a_1,, pupae	3	0	0,903	0,153	0,0882

Hypothesized population mean 1,000

t = -1,095 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,524 to 1,283

Two-tailed P-value = 0,388

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,388).

One-tailed P-value = 0,194

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,194).

Figure 1D

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,702)

Group Name	N	Missing	Mean	Std Dev	SEM
7_lrc_1, day 3	3	0	1,214	0,382	0,221

Hypothesized population mean 1,000

t = 0,968 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,264 to 2,164

Two-tailed P-value = 0,435

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,435).

One-tailed P-value = 0,218

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,218).

One-Sample t-test

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
20_lrc_2, day 5	2	0	0,791	0,181	0,128

Hypothesized population mean 1,000

t = -1,629 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: -0,837 to 2,420

Two-tailed P-value = 0,351

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,351).

One-tailed P-value = 0,175

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,175).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,234)

Group Name	N	Missing	Mean	Std Dev	SEM
31_lrc_1, pupae	3	0	1,143	0,136	0,0783

Hypothesized population mean 1,000

t = 1,832 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,806 to 1,480

Two-tailed P-value = 0,208

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,208).

One-tailed P-value = 0,104

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,104).

Figure 1E

One-Sample t-test

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
7_lrc_1, day 3	2	0	1,379	0,0755	0,0534

Hypothesized population mean 1,000

t = 7,098 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,701 to 2,057

Two-tailed P-value = 0,0891

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,089).

One-tailed P-value = 0,0446

The sample mean of the group exceeds the hypothesized mean by an amount that is greater than would be expected by chance, rejecting the hypothesis that the hypothesized mean is greater than or equal to the true mean. (P = 0,045).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,627)

Group Name	N	Missing	Mean	Std Dev	SEM
19_lrc_1, day 5	3	0	1,366	0,363	0,210

Hypothesized population mean 1,000

t = 1,746 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,464 to 2,267

Two-tailed P-value = 0,223

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,223).

One-tailed P-value = 0,111

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,111).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,186)

Group Name	N	Missing	Mean	Std Dev	SEM
31_lrc_1, pupae	3	0	0,844	0,141	0,0814

Hypothesized population mean 1,000

t = -1,911 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,494 to 1,195

Two-tailed P-value = 0,196

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,196).

One-tailed P-value = 0,0981

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,098).

Figure 1F

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,335)

Group Name	N	Missing	Mean	Std Dev	SEM
10_fez2_1, day 3	3	0	1,226	0,126	0,0727

Hypothesized population mean 1,000

t = 3,105 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,913 to 1,538

Two-tailed P-value = 0,0900

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,090).

One-tailed P-value = 0,0450

The sample mean of the group exceeds the hypothesized mean by an amount that is greater than would be expected by chance, rejecting the hypothesis that the hypothesized mean is greater than or equal to the true mean. (P = 0,045).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,760)

Group Name	N	Missing	Mean	Std Dev	SEM
22_fez2_1, day 5	3	0	0,753	0,0745	0,0430

Hypothesized population mean 1,000

t = -5,743 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,568 to 0,938

Two-tailed P-value = 0,0290

There is a statistically significant difference between the mean of the sampled population and the hypothesized population mean (P = 0,029).

One-tailed P-value = 0,0145

The hypothesized mean exceeds the sample mean of the group by an amount that is greater than would be expected by chance, rejecting the hypothesis that the true mean of group is greater than or equal to the hypothesized mean. (P = 0,015).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,604)

Group Name	N	Missing	Mean	Std Dev	SEM
34_fez2_1, pupae	3	0	1,026	0,0151	0,00873

Hypothesized population mean 1,000

t = 3,023 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,989 to 1,064

Two-tailed P-value = 0,0942

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,094).

One-tailed P-value = 0,0471

The sample mean of the group exceeds the hypothesized mean by an amount that is greater than would be expected by chance, rejecting the hypothesis that the hypothesized mean is greater than or equal to the true mean. (P = 0,047).

Figure 1G

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,555)

Group Name	N	Missing	Mean	Std Dev	SEM
10_fez2_1, day 3	3	0	0,774	0,141	0,0817

Hypothesized population mean 1,000

t = -2,769 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,423 to 1,125

Two-tailed P-value = 0,109

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,109).

One-tailed P-value = 0,0547

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,055).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,553)

Group Name	N	Missing	Mean	Std Dev	SEM
22_fez2_1, day 5	3	0	1,251	0,199	0,115

Hypothesized population mean 1,000

t = 2,183 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,757 to 1,744

Two-tailed P-value = 0,161

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,161).

One-tailed P-value = 0,0804

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,080).

One-Sample t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,556)

Group Name	N	Missing	Mean	Std Dev	SEM
34_fez2_1, pupae	3	0	1,281	0,0324	0,0187

Hypothesized population mean 1,000

t = 14,998 with 2 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 1,200 to 1,361

Two-tailed P-value = 0,00442

There is a statistically significant difference between the mean of the sampled population and the hypothesized population mean (P = 0,004).

One-tailed P-value = 0,00221

The sample mean of the group exceeds the hypothesized mean by an amount that is greater than would be expected by chance, rejecting the hypothesis that the hypothesized mean is greater than or equal to the true mean. (P = 0,002).

Figure 2a

t-test

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,697)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
EGFP IVT	24	0	0,908	0,167	0,0340
β-tub 2851 IVT	24	0	0,926	0,153	0,0312

Difference of means -0,0181

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,391 with 46 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,111 to 0,0749

Two-tailed P-value = 0,697

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,697).

One-tailed P-value = 0,349

The sample mean of group β-tub 2851 IVT does not exceed the sample mean of the group EGFP IVT by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group EGFP IVT is greater than or equal to the population mean of group β-tub 2851 IVT cannot be rejected. (P = 0,349).

Equal Variances Not Assumed (Welch's t-test):

t = -0,391 with 45,666 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,111 to 0,0749

Two-tailed P-value = 0,697

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,697).

One-tailed P-value = 0,349

The sample mean of group β-tub 2851 IVT does not exceed the sample mean of the group EGFP IVT by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group EGFP IVT is greater than or equal to the population mean of group β-tub 2851 IVT cannot be rejected. (P = 0,349).

Box-Cox Transformation

Two-Sample T-Test and CI: C3, C2

Method

μ_1: population mean of C3 when C2 = EGFP IVT

μ_2: population mean of C3 when C2 = β-tub 2851 IVT

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C3

C2	N	Mean	StDev	SE Mean
EGFP IVT	24	7698533333	3740213378	763467859
β-tub 2851 IVT	24	8213715622	3607999659	736479846

Estimation for Difference

Difference	95% CI for Difference
-515182288	(-2651732811, 1621368235)

Test

Null hypothesis H_0: $\mu_1 - \mu_2 = 0$

Alternative hypothesis H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-0.49	45	0.630

t-test

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,098)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
βtub 2851 einfach	12	0	0,875	0,142	0,0411
EGFP einfach	12	0	0,903	0,0855	0,0247

Difference of means -0,0279

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,583 with 22 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,127 to 0,0714

Two-tailed P-value = 0,566

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,566).

One-tailed P-value = 0,283

The sample mean of group EGFP einfach does not exceed the sample mean of the group βtub 2851 einfach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 einfach is greater than or equal to the population mean of group EGFP einfach cannot be rejected. (P = 0,283).

Equal Variances Not Assumed (Welch's t-test):

t = -0,583 with 18,028 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,129 to 0,0727

Two-tailed P-value = 0,567

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,567).

One-tailed P-value = 0,284

The sample mean of group EGFP einfach does not exceed the sample mean of the group βtub 2851 einfach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 einfach is greater than or equal to the population mean of group EGFP einfach cannot be rejected. (P = 0,284).

Box-Cox Transformation

Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = EGFP einfach

μ_2: population mean of C4 when C1 = βtub 2851 einfach

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
EGFP einfach	12	6455650412	2557120655	738177149
βtub 2851 einfach	12	6260875000	3707638894	1070303157

Estimation for Difference

Difference	95% CI for Difference
194775412	(-2526521591, 2916072415)

Test

Null hypothesis	H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis	H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.15	19	0.882

t-test

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,868)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
βtub 2851 6-fach	12	0	0,833	0,156	0,0449
EGFP 6-fach	12	0	0,892	0,124	0,0358

Difference of means -0,0591

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -1,028 with 22 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,178 to 0,0601

Two-tailed P-value = 0,315

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,315).

One-tailed P-value = 0,158

The sample mean of group EGFP 6-fach does not exceed the sample mean of the group βtub 2851 6-fach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 6-fach is greater than or equal to the population mean of group EGFP 6-fach cannot be rejected. (P = 0,158).

Equal Variances Not Assumed (Welch's t-test):

t = -1,028 with 20,957 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,179 to 0,0605

Two-tailed P-value = 0,316

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,316).

One-tailed P-value = 0,158

The sample mean of group EGFP 6-fach does not exceed the sample mean of the group βtub 2851 6-fach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 6-fach is greater than or equal to the population mean of group EGFP 6-fach cannot be rejected. (P = 0,158).

Box-Cox Transformation

Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = EGFP 6-fach

μ_2: population mean of C4 when C1 = βtub 2851 6-fach

Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
EGFP 6-fach	12	69763445	31486318	9089317
βtub 2851 6-fach	12	56486667	30151641	8704029

Estimation for Difference

Difference	95% CI for Difference
13276779	(-12894631, 39448189)

Test

Null hypothesis H_0: $\mu_1 - \mu_2 = 0$

Alternative hypothesis H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
1.05	21	0.303

t-test

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,228)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
β-tub 4939 dreifach	12	0	0,867	0,144	0,0414
EGFP dreifach	12	0	0,908	0,0793	0,0229

Difference of means -0,0417

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,880 with 22 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,140 to 0,0565

Two-tailed P-value = 0,388

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,388).

One-tailed P-value = 0,194

The sample mean of group EGFP dreifach does not exceed the sample mean of the group β-tub 4939 dreifach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group β-tub 4939 dreifach is greater than or equal to the population mean of group EGFP dreifach cannot be rejected. (P = 0,194).

Equal Variances Not Assumed (Welch's t-test):

t = -0,880 with 17,141 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,141 to 0,0582

Two-tailed P-value = 0,391

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,391).

One-tailed P-value = 0,195

The sample mean of group EGFP dreifach does not exceed the sample mean of the group β-tub 4939 dreifach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group β-tub 4939 dreifach is greater than or equal to the population mean of group EGFP dreifach cannot be rejected. (P = 0,195).

Box-Cox Transformation
Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = EGFP dreifach
μ_2: population mean of C4 when C1 = β-tub 4939 dreifach
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
EGFP dreifach	12	70910833	23772348	6862486
β-tub 4939 dreifach	12	64190000	32285349	9319978

Estimation for Difference

Difference	95% CI for Difference
6720833	(-17421947, 30863614)

Test

Null hypothesis H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.58	20	0.568

Box-Cox Transformation
Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = eGFP 6-fach
μ_2: population mean of C4 when C1 = βtub 4939 6-fach
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
eGFP 6-fach	24	6658688051	3411898544	696450874
βtub 4939 6-fach	24	6329615763	3353619834	684554782

Estimation for Difference

Difference	95% CI for Difference
329072288	(-1637809829, 2295954405)

Test

Null hypothesis H_0: $\mu_1 - \mu_2 = 0$
Alternative hypothesis H_1: $\mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.34	45	0.738

t-test

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,768)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
βtub 2851 + βtub 4939 einfach	12	0	0,708	0,315	0,0908
EGFP einfach doppelt	12	0	0,771	0,303	0,0874

Difference of means -0,0629

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,499 with 22 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,324 to 0,199

Two-tailed P-value = 0,623

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,623).

One-tailed P-value = 0,311

The sample mean of group EGFP einfach doppelt does not exceed the sample mean of the group βtub 2851 + βtub 4939 einfach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 + βtub 4939 einfach is greater than or equal to the population mean of group EGFP einfach doppelt cannot be rejected. (P = 0,311).

Equal Variances Not Assumed (Welch's t-test):

t = -0,499 with 21,968 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,324 to 0,199

Two-tailed P-value = 0,623

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,623).

One-tailed P-value = 0,311

The sample mean of group EGFP einfach doppelt does not exceed the sample mean of the group βtub 2851 + βtub 4939 einfach by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group βtub 2851 + βtub 4939 einfach is greater than or equal to the population mean of group EGFP einfach doppelt cannot be rejected. (P = 0,311).

Box-Cox Transformation
Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = EGFP einfach doppelt
μ_2: population mean of C4 when C1 = βtub 2851 + βtub 4939 einfach
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
EGFP einfach doppelt	12	6789	3951	1141
βtub 2851 + βtub 4939 einfach	12	5925	3937	1136

Estimation for Difference

Difference	95% CI for Difference
864	(-2484, 4212)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
0.54	21	0.597

Box-Cox Transformation
Two-Sample T-Test and CI: C4, C1

Method

μ_1: population mean of C4 when C1 = eGFP 6-fach
μ_2: population mean of C4 when C1 = βtub 2851, 4393 6-fach (dreifach, 2 targets)
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics: C4

C1	N	Mean	StDev	SE Mean
eGFP 6-fach	12	423467	297863	85986
βtub 2851, 4393 6-fach (dreifach, 2 targets)	12	712110	247972	71583

Estimation for Difference

Difference	95% CI for Difference
-288643	(-521316, -55970)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-2.58	21	0.017

Figure 2B

Descriptive Statistics:

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
Ache1	2	0	0,718	0,0261	0,0184	0,234
fez2	2	0	0,850	0,212	0,150	1,906
lrcE1	2	0	0,750	0,212	0,150	1,906
Sem1aE8	2	0	0,850	0,141	0,1000	1,271
Sem1aE15	2	0	0,875	0,106	0,0750	0,953
VATPase	2	0	0,925	0,106	0,0750	0,953
eGFP	2	0	0,833	0,101	0,0714	0,908
ßtub2851	2	0	0,950	0,0707	0,0500	0,635

Column	Range	Max	Min	Median	25%	75%
Ache1	0,0368	0,737	0,700	0,718	0,700	0,737
fez2	0,300	1,000	0,700	0,850	0,700	1,000
lrcE1	0,300	0,900	0,600	0,750	0,600	0,900
Sem1aE8	0,200	0,950	0,750	0,850	0,750	0,950
Sem1aE15	0,150	0,950	0,800	0,875	0,800	0,950
VATPase	0,150	1,000	0,850	0,925	0,850	1,000
eGFP	0,143	0,905	0,762	0,833	0,762	0,905
ßtub2851	0,1000	1,000	0,900	0,950	0,900	1,000

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
Ache1	--	--	0,260	0,481	--	--
fez2	--	--	0,260	0,481	--	--
lrcE1	--	--	0,260	0,481	--	--
Sem1aE8	--	--	0,260	0,481	--	--
Sem1aE15	--	--	0,260	0,481	--	--
VATPase	--	--	0,260	0,481	--	--
eGFP	--	--	0,260	0,481	--	--
ßtub2851	--	--	0,260	0,481	--	--

Column	Sum	Sum of Squares
Ache1	1,437	1,033
fez2	1,700	1,490
lrcE1	1,500	1,170
Sem1aE8	1,700	1,465
Sem1aE15	1,750	1,543
VATPase	1,850	1,722
eGFP	1,667	1,399
ßtub2851	1,900	1,810

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,302)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Ache1	2	0	0,718	0,700	0,737
fez2	2	0	0,850	0,700	1,000
lrcE1	2	0	0,750	0,600	0,900
Sem1aE8	2	0	0,850	0,750	0,950
Sem1aE15	2	0	0,875	0,800	0,950
VATPase	2	0	0,925	0,850	1,000
eGFP	2	0	0,833	0,762	0,905
ßtub2851	2	0	0,950	0,900	1,000

H = 5,605 with 7 degrees of freedom. (P = 0,586)

> The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,586)

One-way ANOVA: Ache1, fez2, lrc_E1, Sem1a_E8, Sem1a_E15, VATPase, eGFP, βtub2851

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	8	Ache1, fez2, lrc_E1, Sem1a_E8, Sem1a_E15, VATPase, eGFP, βtub2851

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	7	3.21282	1.83	0.324

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
36.99%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Ache1	2	0.7184	0.0261	(0.4844, 0.9525)
fez2	2	0.850	0.212	(-1.056, 2.756)
lrc_E1	2	0.750	0.212	(-1.156, 2.656)
Sem1a_E8	2	0.850	0.141	(-0.421, 2.121)
Sem1a_E15	2	0.8750	0.1061	(-0.0780, 1.8280)
VATPase	2	0.9250	0.1061	(-0.0280, 1.8780)
eGFP	2	0.8333	0.1010	(-0.0743, 1.7409)
βtub2851	2	0.9500	0.0707	(0.3147, 1.5853)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
βtub2851	2	0.9500	A
VATPase	2	0.9250	A
Sem1a_E15	2	0.8750	A
Sem1a_E8	2	0.850	A
fez2	2	0.850	A
eGFP	2	0.8333	A
lrc_E1	2	0.750	A
Ache1	2	0.7184	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
fez2 - Ache1	0.132	0.151	(-4.367, 4.631)	0.87	0.950
lrc_E1 - Ache1	0.032	0.151	(-4.467, 4.531)	0.21	1.000
Sem1a_E8 - Ache1	0.132	0.102	(-2.639, 2.903)	1.29	0.846
Sem1a_E15 - Ache1	0.1566	0.0772	(-1.7231, 2.0362)	2.03	0.650
VATPase - Ache1	0.2066	0.0772	(-1.6731, 2.0862)	2.67	0.520
eGFP - Ache1	0.1149	0.0738	(-1.6371, 1.8670)	1.56	0.771
βtub2851 - Ache1	0.2316	0.0533	(-0.7665, 1.2297)	4.35	0.299
lrc_E1 - fez2	-0.100	0.212	(-2.054, 1.854)	-0.47	0.998
Sem1a_E8 - fez2	0.000	0.180	(-1.982, 1.982)	0.00	1.000
Sem1a_E15 - fez2	0.025	0.168	(-2.364, 2.414)	0.15	1.000
VATPase - fez2	0.075	0.168	(-2.314, 2.464)	0.45	0.998
eGFP - fez2	-0.017	0.166	(-2.495, 2.462)	-0.10	1.000
βtub2851 - fez2	0.100	0.158	(-3.099, 3.299)	0.63	0.988
Sem1a_E8 - lrc_E1	0.100	0.180	(-1.882, 2.082)	0.55	0.995
Sem1a_E15 - lrc_E1	0.125	0.168	(-2.264, 2.514)	0.75	0.978
VATPase - lrc_E1	0.175	0.168	(-2.214, 2.564)	1.04	0.919
eGFP - lrc_E1	0.083	0.166	(-2.395, 2.562)	0.50	0.997
βtub2851 - lrc_E1	0.200	0.158	(-2.999, 3.399)	1.26	0.855
Sem1a_E15 - Sem1a_E8	0.025	0.125	(-1.239, 1.289)	0.20	1.000
VATPase - Sem1a_E8	0.075	0.125	(-1.189, 1.339)	0.60	0.993
eGFP - Sem1a_E8	-0.017	0.123	(-1.300, 1.267)	-0.14	1.000
βtub2851 - Sem1a_E8	0.100	0.112	(-1.493, 1.693)	0.89	0.953
VATPase - Sem1a_E15	0.050	0.106	(-0.927, 1.027)	0.47	0.998
eGFP - Sem1a_E15	-0.042	0.104	(-0.999, 0.916)	-0.40	0.999
βtub2851 - Sem1a_E15	0.0750	0.0901	(-0.9161, 1.0661)	0.83	0.968
eGFP - VATPase	-0.092	0.104	(-1.049, 0.866)	-0.89	0.961
βtub2851 - VATPase	0.0250	0.0901	(-0.9661, 1.0161)	0.28	1.000
βtub2851 - eGFP	0.1167	0.0872	(-0.8075, 1.0408)	1.34	0.836

Figure 2C

Descriptive Statistics - data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
empty bact	2	0	0,671	0,136	0,0961	1,221
eGFP	2	0	0,704	0,0420	0,0297	0,378
Fez2	2	0	0,604	0,0994	0,0703	0,893
Sem1aE8	2	0	0,863	0,0523	0,0370	0,470
aCOP494	2	0	0,725	0,0820	0,0580	0,737
lrcE1	2	0	0,825	0,153	0,108	1,372

Column	Range	Max	Min	Median	25%	75%
empty bact	0,192	0,767	0,574	0,671	0,574	0,767
eGFP	0,0594	0,733	0,674	0,704	0,674	0,733
Fez2	0,141	0,674	0,533	0,604	0,533	0,674
Sem1aE8	0,0739	0,900	0,826	0,863	0,826	0,900
aCOP494	0,116	0,783	0,667	0,725	0,667	0,783
lrcE1	0,216	0,933	0,717	0,825	0,717	0,933

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
empty bact	--	--	0,260	0,481	--	--
eGFP	--	--	0,260	0,481	--	--
Fez2	--	--	0,260	0,481	--	--
Sem1aE8	--	--	0,260	0,481	--	--
aCOP494	--	--	0,260	0,481	--	--
lrcE1	--	--	0,260	0,481	--	--

Column	Sum	Sum of Squares
empty bact	1,341	0,918
eGFP	1,407	0,992
Fez2	1,207	0,739
Sem1aE8	1,726	1,492
aCOP494	1,449	1,057
lrcE1	1,651	1,386

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,408)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
empty bact	2	0	0,671	0,574	0,767
eGFP	2	0	0,704	0,674	0,733
Fez2	2	0	0,604	0,533	0,674
Sem1aE8	2	0	0,863	0,826	0,900
aCOP494	2	0	0,725	0,667	0,783
lrcE1	2	0	0,825	0,717	0,933

H = 6,079 with 5 degrees of freedom. (P = 0,299)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,299)

One-way ANOVA: Empty bact, eGFP, fez2, sem1a_E8, αCOP494_1B, lrc_E1

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	Empty bact, eGFP, fez2, sem1a_E8, αCOP494_1B, lrc_E1

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	5	2.71285	1.76	0.355

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
59.92%	26.52%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Empty bact	2	0.6706	0.1359	(-0.5505, 1.8916)
eGFP	2	0.7036	0.0420	(0.3261, 1.0811)
fez2	2	0.6036	0.0994	(-0.2895, 1.4967)
sem1a_E8	2	0.8630	0.0523	(0.3935, 1.3326)
αCOP494_1B	2	0.7246	0.0820	(-0.0120, 1.4612)
lrc_E1	2	0.825	0.153	(-0.547, 2.197)

Games-Howell Pairwise Comparisons - Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
sem1a_E8	2	0.8630	A
lrc_E1	2	0.825	A
αCOP494_1B	2	0.7246	A
eGFP	2	0.7036	A
Empty bact	2	0.6706	A
fez2	2	0.6036	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
eGFP - Empty bact	0.033	0.101	(-1.881, 1.947)	0.33	0.998
fez2 - Empty bact	-0.067	0.119	(-1.167, 1.033)	-0.56	0.984
sem1a_E8 - Empty bact	0.192	0.103	(-1.471, 1.856)	1.87	0.613
αCOP494_1B - Empty bact	0.054	0.112	(-1.151, 1.259)	0.48	0.991
lrc_E1 - Empty bact	0.155	0.145	(-1.064, 1.373)	1.07	0.863
fez2 - eGFP	-0.1000	0.0763	(-1.2364, 1.0364)	-1.31	0.780
sem1a_E8 - eGFP	0.1594	0.0474	(-0.2560, 0.5749)	3.36	0.265
αCOP494_1B - eGFP	0.0210	0.0651	(-0.7911, 0.8331)	0.32	0.998
lrc_E1 - eGFP	0.122	0.112	(-2.167, 2.411)	1.09	0.854
sem1a_E8 - fez2	0.2594	0.0794	(-0.7070, 1.2258)	3.27	0.323
αCOP494_1B - fez2	0.1210	0.0911	(-0.6676, 0.9096)	1.33	0.768
lrc_E1 - fez2	0.222	0.129	(-1.076, 1.519)	1.72	0.632
αCOP494_1B - sem1a_E8	-0.1384	0.0687	(-0.8423, 0.5655)	-2.01	0.544
lrc_E1 - sem1a_E8	-0.038	0.114	(-2.058, 1.983)	-0.33	0.998
lrc_E1 - αCOP494_1B	0.101	0.123	(-1.362, 1.563)	0.82	0.934

Figure 2D

One-way ANOVA: GusA, ßtub, Fez2, Lrc_E1, Sem1a_E8, Sem1a_E15, Ache1, VATPase, eGFP

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	GusA, ßtub, Fez2, Lrc_E1, Sem1a_E8, Sem1a_E15, Ache1, VATPase, eGFP

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	8	7.30229	5.40	**0.017**

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
65.74%	50.51%	22.90%

Means

Factor	N	Mean	StDev	95% CI
GusA	3	83.57	7.73	(64.38, 102.76)
ßtub	3	50.0	17.3	(7.0, 93.0)
Fez2	3	56.67	12.58	(25.41, 87.92)
Lrc_E1	3	71.67	2.89	(64.50, 78.84)
Sem1a_E8	3	75.56	7.70	(56.43, 94.68)
Sem1a_E15	3	51.67	12.58	(20.41, 82.92)
Ache1	3	58.33	2.89	(51.16, 65.50)
VATPase	3	50.00	8.66	(28.49, 71.51)
eGFP	3	65.00	10.00	(40.16, 89.84)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
GusA	3	83.57	A
Sem1a_E8	3	75.56	A
Lrc_E1	3	71.67	A
eGFP	3	65.00	A
Ache1	3	58.33	A
Fez2	3	56.67	A
Sem1a_E15	3	51.67	A
VATPase	3	50.00	A
ßtub	3	50.0	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
ßtub - GusA	-33.6	10.9	(-109.4, 42.2)	-3.07	0.321
Fez2 - GusA	-26.90	8.53	(-78.32, 24.51)	-3.16	0.274
Lrc_E1 - GusA	-11.90	4.76	(-47.33, 23.52)	-2.50	0.466
Sem1a_E8 - GusA	-8.02	6.30	(-41.86, 25.82)	-1.27	0.899
Sem1a_E15 - GusA	-31.90	8.53	(-83.32, 19.51)	-3.74	0.184
Ache1 - GusA	-25.24	4.76	(-60.66, 10.18)	-5.30	0.110

VATPase - GusA	-33.57	6.70	(-69.86, 2.72)	-5.01	0.065
eGFP - GusA	-18.57	7.30	(-59.22, 22.08)	-2.55	0.407
Fez2 - ßtub	6.7	12.4	(-63.4, 76.8)	0.54	0.999
Lrc_E1 - ßtub	21.7	10.1	(-69.5, 112.9)	2.14	0.592
Sem1a_E8 - ßtub	25.6	10.9	(-50.4, 101.5)	2.34	0.506
Sem1a_E15 - ßtub	1.7	12.4	(-68.4, 71.8)	0.13	1.000
Ache1 - ßtub	8.3	10.1	(-82.9, 99.5)	0.82	0.981
VATPase - ßtub	0.0	11.2	(-73.7, 73.7)	0.00	1.000
eGFP - ßtub	15.0	11.5	(-56.4, 86.4)	1.30	0.885
Lrc_E1 - Fez2	15.00	7.45	(-48.77, 78.77)	2.01	0.629
Sem1a_E8 - Fez2	18.89	8.52	(-32.54, 70.32)	2.22	0.530
Sem1a_E15 - Fez2	-5.0	10.3	(-60.2, 50.2)	-0.49	1.000
Ache1 - Fez2	1.67	7.45	(-62.11, 65.44)	0.22	1.000
VATPase - Fez2	-6.67	8.82	(-57.62, 44.28)	-0.76	0.992
eGFP - Fez2	8.33	9.28	(-42.98, 59.65)	0.90	0.980
Sem1a_E8 - Lrc_E1	3.89	4.75	(-31.35, 39.13)	0.82	0.983
Sem1a_E15 - Lrc_E1	-20.00	7.45	(-83.77, 43.77)	-2.68	0.438
Ache1 - Lrc_E1	**-13.33**	**2.36**	**(-26.00, -0.67)**	**-5.66**	**0.042**
VATPase - Lrc_E1	-21.67	5.27	(-62.47, 19.14)	-4.11	0.198
eGFP - Lrc_E1	-6.67	6.01	(-55.32, 41.99)	-1.11	0.931
Sem1a_E15 - Sem1a_E8	-23.89	8.52	(-75.32, 27.54)	-2.81	0.351
Ache1 - Sem1a_E8	-17.22	4.75	(-52.46, 18.02)	-3.63	0.244
VATPase - Sem1a_E8	-25.56	6.69	(-61.79, 10.68)	-3.82	0.149
eGFP - Sem1a_E8	-10.56	7.29	(-51.20, 30.09)	-1.45	0.836
Ache1 - Sem1a_E15	6.67	7.45	(-57.11, 70.44)	0.89	0.972
VATPase - Sem1a_E15	-1.67	8.82	(-52.62, 49.28)	-0.19	1.000
eGFP - Sem1a_E15	13.33	9.28	(-37.98, 64.65)	1.44	0.841
VATPase - Ache1	-8.33	5.27	(-49.14, 32.47)	-1.58	0.780
eGFP - Ache1	6.67	6.01	(-41.99, 55.32)	1.11	0.931
eGFP - VATPase	15.00	7.64	(-26.53, 56.53)	1.96	0.620

Figure 2E

One Way Analysis of Variance - data given in lethality

Normality Test (Shapiro-Wilk): Passed (P = 0,230)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,899)

Group Name	N	Missing	Mean	Std Dev	SEM
Ache1	3	0	0,433	0,0764	0,0441
Fez2	3	0	0,387	0,191	0,110
lrcE1	3	0	0,517	0,176	0,101
sem1aE8	3	0	0,600	0,000	0,000
Sem1aE15	3	0	0,429	0,168	0,0967
VATPase	3	0	0,400	0,180	0,104
eGFP	3	0	0,367	0,104	0,0601
ßtub	3	0	0,367	0,189	0,109
GusA	3	0	0,400	0,150	0,0866

Source of Variation	DF	SS	MS	F	P
Between Groups	8	0,144	0,0180	0,798	0,612
Residual	18	0,406	0,0225		
Total	26	0,550			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,612).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 2F

One Way Analysis of Variance – data given in lethality

Normality Test (Shapiro-Wilk): Passed (P = 0,202)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
empty bact	2	0	0,859	0,717	1,000
eGFP	2	0	0,814	0,652	0,975
fez2	2	0	0,843	0,761	0,925
sem1E8	2	0	0,723	0,522	0,925
aCOP494	2	0	0,876	0,826	0,925
lrcE1	2	0	0,736	0,522	0,950

H = 0,998 with 5 degrees of freedom. (P = 0,963)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,963)

One-way ANOVA: Empty bact, eGFP, fez2, sem1a_E8, αCOP494_1B, lrc_E1

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	Empty bact, eGFP, fez2, sem1a_E8, αCOP494_1B, lrc_E1

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	5	2.64824	0.10	0.985

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
12.77%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Empty bact	2	0.859	0.200	(-0.937, 2.654)
eGFP	2	0.814	0.228	(-1.237, 2.865)
fez2	2	0.8429	0.1161	(-0.1998, 1.8857)
sem1a_E8	2	0.723	0.285	(-1.839, 3.285)
αCOP494_1B	2	0.8755	0.0699	(0.2471, 1.5039)
lrc_E1	2	0.736	0.303	(-1.985, 3.457)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
αCOP494_1B	2	0.8755	A
Empty bact	2	0.859	A
fez2	2	0.8429	A
eGFP	2	0.814	A
lrc_E1	2	0.736	A
sem1a_E8	2	0.723	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
eGFP - Empty bact	-0.045	0.215	(-1.862, 1.772)	-0.21	1.000
fez2 - Empty bact	-0.016	0.163	(-1.830, 1.798)	-0.10	1.000
sem1a_E8 - Empty bact	-0.135	0.246	(-2.479, 2.208)	-0.55	0.985
αCOP494_1B - Empty bact	0.017	0.150	(-2.590, 2.624)	0.11	1.000
lrc_E1 - Empty bact	-0.123	0.257	(-2.677, 2.431)	-0.48	0.992
fez2 - eGFP	0.029	0.181	(-2.245, 2.303)	0.16	1.000
sem1a_E8 - eGFP	-0.090	0.258	(-2.357, 2.176)	-0.35	0.998
αCOP494_1B - eGFP	0.062	0.169	(-3.169, 3.293)	0.37	0.997
lrc_E1 - eGFP	-0.078	0.268	(-2.510, 2.355)	-0.29	0.999
sem1a_E8 - fez2	-0.120	0.218	(-3.471, 3.232)	-0.55	0.983
αCOP494_1B - fez2	0.0326	0.0958	(-0.9972, 1.0624)	0.34	0.998
lrc_E1 - fez2	-0.107	0.229	(-3.822, 3.608)	-0.47	0.991
αCOP494_1B - sem1a_E8	0.152	0.208	(-4.356, 4.660)	0.73	0.950
lrc_E1 - sem1a_E8	0.013	0.294	(-2.437, 2.462)	0.04	1.000
lrc_E1 - αCOP494_1B	-0.140	0.220	(-5.046, 4.766)	-0.64	0.968

Figure 5A

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,903)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
lrc	2	0	81,250	19,445	13,750
fez2	2	0	65,000	49,497	35,000
none scr	2	0	88,750	15,910	11,250

Source of Variation	DF	SS	MS	F	P
Between Groups	2	589,583	294,792	0,287	0,769
Residual	3	3081,250	1027,083		
Total	5	3670,833			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,769).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Statistics

Variable	N	Mean	SE Mean	StDev	Var	CoefVar	Min	Median	Max
lrc	2	81.3	13.8	19.4	378.1	23.93	67.5	81.3	95.0
fez2	2	65.0	35.0	49.5	2450.0	76.15	30.0	65.0	100.0
none scr	2	88.8	11.3	15.9	253.1	17.93	77.5	88.8	100.0

One-way ANOVA: lrc, fez2, none scr

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	lrc, fez2, none scr

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.82124	0.19	0.844

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
16.06%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
lrc	2	81.3	19.4	(-93.5, 256.0)
fez2	2	65.0	49.5	(-379.7, 509.7)
none scr	2	88.8	15.9	(-54.2, 231.7)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
none scr	2	88.8	A
lrc	2	81.3	A
fez2	2	65.0	A

Means that do not share a letter are significantly different.

Figure 5B

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,191)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,860)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
fez2 v1	2	0	75,000	17,678	12,500
none scr	3	0	71,667	14,216	8,207

Difference of means 3,333

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 0,236 with 3 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -41,569 to 48,235

Two-tailed P-value = 0,828

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,828).

One-tailed P-value = 0,414

The sample mean of group fez2 v1 does not exceed the sample mean of the group none scr by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group none scr is greater than or equal to the population mean of group fez2 v1 cannot be rejected. (P = 0,414).

Equal Variances Not Assumed (Welch's t-test):

t = 0,223 with 1,874 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -186,671 to 193,337

Two-tailed P-value = 0,846

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,846).

One-tailed P-value = 0,423

The sample mean of group fez2 v1 does not exceed the sample mean of the group none scr by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group none scr is greater than or equal to the population mean of group fez2 v1 cannot be rejected. (P = 0,423).

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,683)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
fez2v2	2	0	81,250	1,768	1,250
non scr	2	0	97,500	0,000	0,000
Difference of means		-16,250			

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -13,000 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -21,628 to -10,872

Two-tailed P-value = 0,00587

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,006).

One-tailed P-value = 0,00293

The sample mean of group non scr exceeds the sample mean of group fez2v2 by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group fez2v2 is greater than or equal to the population mean of group non scr. (P = 0,003).

Equal Variances Not Assumed (Welch's t-test):

t = -13,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -32,133 to -0,367

Two-tailed P-value = 0,0489

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,049).

One-tailed P-value = 0,0244

The sample mean of group non scr exceeds the sample mean of group fez2v2 by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group fez2v2 is greater than or equal to the population mean of group non scr. (P = 0,024).

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,683)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
lrc	2	0	75,000	10,607	7,500
non scr	2	0	97,500	0,000	0,000

Difference of means -22,500

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -3,000 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -54,770 to 9,770

Two-tailed P-value = 0,0955

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,095).

One-tailed P-value = 0,0477

The sample mean of group non scr exceeds the sample mean of group lrc by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group lrc is greater than or equal to the population mean of group non scr. (P = 0,048).

Equal Variances Not Assumed (Welch's t-test):

t = -3,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -117,797 to 72,797

Two-tailed P-value = 0,205

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,205).

One-tailed P-value = 0,102

The sample mean of group non scr does not exceed the sample mean of the group lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group lrc is greater than or equal to the population mean of group non scr cannot be rejected. (P = 0,102).

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,980)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
lrc	2	0	75,000	10,607	7,500
fez2v2	2	0	81,250	1,768	1,250

Difference of means -6,250

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,822 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -38,965 to 26,465

Two-tailed P-value = 0,497

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,497).

One-tailed P-value = 0,249

The sample mean of group fez2v2 does not exceed the sample mean of the group lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group lrc is greater than or equal to the population mean of group fez2v2 cannot be rejected. (P = 0,249).

Equal Variances Not Assumed (Welch's t-test):

t = -0,822 with 1,056 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -102,861 to 90,361

Two-tailed P-value = 0,556

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,556).

One-tailed P-value = 0,278

The sample mean of group fez2v2 does not exceed the sample mean of the group lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group lrc is greater than or equal to the population mean of group fez2v2 cannot be rejected. (P = 0,278).

Figure 5C

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,325)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
fez2 nuc	2	0	98,750	1,768	1,250
non scr	3	0	100,000	0,000	0,000

Difference of means -1,250

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -1,342 with 3 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -4,215 to 1,715

Two-tailed P-value = 0,272

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,272).

One-tailed P-value = 0,136

The sample mean of group non scr does not exceed the sample mean of the group fez2 nuc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group fez2 nuc is greater than or equal to the population mean of group non scr cannot be rejected. (P = 0,136).

Equal Variances Not Assumed (Welch's t-test):

t = -1,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -17,133 to 14,633

Two-tailed P-value = 0,500

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,500).

One-tailed P-value = 0,250

The sample mean of group non scr does not exceed the sample mean of the group fez2 nuc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group fez2 nuc is greater than or equal to the population mean of group non scr cannot be rejected. (P = 0,250).

Figure 6

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,730)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,102)

Group Name	N	Missing	Mean	Std Dev	SEM
Males dsx	5	0	45,740	6,594	2,949
Males scramble	3	0	46,567	10,706	6,181
Males none	2	0	55,000	7,071	5,000

Source of Variation	DF	SS	MS	F	P
Between Groups	2	129,445	64,723	1,000	0,415
Residual	7	453,159	64,737		
Total	9	582,604			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,415).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7A

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
H2O	2	0	0,0500	0,0236	0,0167	0,212
Gus 1500	2	0	0,0667	0,0943	0,0667	0,847
eGFP 1500	2	0	0,0318	0,000713	0,000504	0,00640
fez 500	2	0	0,167	0,0943	0,0667	0,847
fez 1000	2	0	0,0667	0,0471	0,0333	0,424
fez 1500	2	0	0,0333	0,000	0,000	0,000
lrc 500	2	0	0,117	0,0236	0,0167	0,212
lrc 1000	2	0	0,133	0,141	0,1000	1,271
lrc 1500	2	0	0,117	0,0236	0,0167	0,212
sem 1500	2	0	0,150	0,0707	0,0500	0,635

Column	Range	Max	Min	Median	25%	75%
H2O	0,0333	0,0667	0,0333	0,0500	0,0333	0,0667
Gus 1500	0,133	0,133	0,000	0,0667	0,000	0,133
eGFP 1500	0,00101	0,0323	0,0313	0,0318	0,0313	0,0323
fez 500	0,133	0,233	0,1000	0,167	0,1000	0,233
fez 1000	0,0667	0,1000	0,0333	0,0667	0,0333	0,1000
fez 1500	0,000	0,0333	0,0333	0,0333	0,0333	0,0333
lrc 500	0,0333	0,133	0,1000	0,117	0,1000	0,133
lrc 1000	0,200	0,233	0,0333	0,133	0,0333	0,233
lrc 1500	0,0333	0,133	0,1000	0,117	0,1000	0,133
sem 1500	0,1000	0,200	0,1000	0,150	0,1000	0,200

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
H2O	--	--	0,260	0,481	--	--
Gus 1500	--	--	0,260	0,481	--	--
eGFP 1500	--	--	0,260	0,481	--	--
fez 500	--	--	0,260	0,481	--	--
fez 1000	--	--	0,260	0,481	--	--
fez 1500	--	--	0,000	<0,001	--	--
lrc 500	--	--	0,260	0,481	--	--
lrc 1000	--	--	0,260	0,481	--	--
lrc 1500	--	--	0,260	0,481	--	--
sem 1500	--	--	0,260	0,481	--	--

Column	Sum	Sum of Squares
H2O	0,1000	0,00556
Gus 1500	0,133	0,0178
eGFP 1500	0,0635	0,00202
fez 500	0,333	0,0644
fez 1000	0,133	0,0111
fez 1500	0,0667	0,00222
lrc 500	0,233	0,0278
lrc 1000	0,267	0,0556
lrc 1500	0,233	0,0278
sem 1500	0,300	0,0500

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,949)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
H2O	2	0	0,0500	0,0333	0,0667
Gus 1500	2	0	0,0667	0,000	0,133
eGFP 1500	2	0	0,0318	0,0313	0,0323
fez 500	2	0	0,167	0,1000	0,233
fez 1000	2	0	0,0667	0,0333	0,1000
fez 1500	2	0	0,0333	0,0333	0,0333
lrc 500	2	0	0,117	0,1000	0,133
lrc 1000	2	0	0,133	0,0333	0,233
lrc 1500	2	0	0,117	0,1000	0,133
sem 1500	2	0	0,150	0,1000	0,200

H = 10,461 with 9 degrees of freedom. (P = 0,314)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,314)

One-way ANOVA: H2O, Gus 1500, eGFP 1500, fez 500, fez 1000, lrc 500, lrc 1000, lrc 1500, Sem 1500 – data in lethality

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	H2O, Gus 1500, eGFP 1500, fez 500, fez 1000, lrc 500, lrc 1000, lrc 1500, Sem 1500

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	8	3.33600	3.39	0.154

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
43.54%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
H2O	2	0.0500	0.0236	(-0.1618, 0.2618)
Gus 1500	2	0.0667	0.0943	(-0.7804, 0.9137)
eGFP 1500	2	0.031754	0.000713	(0.025350, 0.038158)
fez 500	2	0.1667	0.0943	(-0.6804, 1.0137)
fez 1000	2	0.0667	0.0471	(-0.3569, 0.4902)
lrc 500	2	0.1167	0.0236	(-0.0951, 0.3284)
lrc 1000	2	0.133	0.141	(-1.137, 1.404)
lrc 1500	2	0.1167	0.0236	(-0.0951, 0.3284)
Sem 1500	2	0.1500	0.0707	(-0.4853, 0.7853)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
fez 500	2	0.1667	A
Sem 1500	2	0.1500	A
lrc 1000	2	0.133	A
lrc 1500	2	0.1167	A
lrc 500	2	0.1167	A
fez 1000	2	0.0667	A
Gus 1500	2	0.0667	A
H2O	2	0.0500	A
eGFP 1500	2	0.031754	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
Gus 1500 - H2O	0.0167	0.0687	(-1.7122, 1.7455)	0.24	1.000
eGFP 1500 - H2O	-0.0182	0.0167	(-0.5738, 0.5373)	-1.09	0.914
fez 500 - H2O	0.1167	0.0687	(-1.6122, 1.8455)	1.70	0.755
fez 1000 - H2O	0.0167	0.0373	(-0.5362, 0.5695)	0.45	0.999

lrc 500 - H2O	0.0667	0.0236	(-0.1590, 0.2923)	2.83	0.421
lrc 1000 - H2O	0.083	0.101	(-2.882, 3.048)	0.82	0.969
lrc 1500 - H2O	0.0667	0.0236	(-0.1590, 0.2923)	2.83	0.421
Sem 1500 - H2O	0.1000	0.0527	(-1.0113, 1.2113)	1.90	0.698
eGFP 1500 - Gus 1500	-0.0349	0.0667	(-2.2661, 2.1963)	-0.52	0.996
fez 500 - Gus 1500	0.1000	0.0943	(-0.8027, 1.0027)	1.06	0.938
fez 1000 - Gus 1500	0.0000	0.0745	(-1.1057, 1.1057)	0.00	1.000
lrc 500 - Gus 1500	0.0500	0.0687	(-1.6789, 1.7789)	0.73	0.982
lrc 1000 - Gus 1500	0.067	0.120	(-1.308, 1.442)	0.55	0.997
lrc 1500 - Gus 1500	0.0500	0.0687	(-1.6789, 1.7789)	0.73	0.982
Sem 1500 - Gus 1500	0.0833	0.0833	(-0.7929, 0.9596)	1.00	0.949
fez 500 - eGFP 1500	0.1349	0.0667	(-2.0963, 2.3661)	2.02	0.682
fez 1000 - eGFP 1500	0.0349	0.0333	(-1.0799, 1.1497)	1.05	0.924
lrc 500 - eGFP 1500	0.0849	0.0167	(-0.4707, 0.6405)	5.09	0.318
lrc 1000 - eGFP 1500	0.102	0.100	(-3.246, 3.449)	1.02	0.931
lrc 1500 - eGFP 1500	0.0849	0.0167	(-0.4707, 0.6405)	5.09	0.318
Sem 1500 - eGFP 1500	0.1182	0.0500	(-1.5549, 1.7913)	2.36	0.612
fez 1000 - fez 500	-0.1000	0.0745	(-1.2057, 1.0057)	-1.34	0.855
lrc 500 - fez 500	-0.0500	0.0687	(-1.7789, 1.6789)	-0.73	0.982
lrc 1000 - fez 500	-0.033	0.120	(-1.408, 1.342)	-0.28	1.000
lrc 1500 - fez 500	-0.0500	0.0687	(-1.7789, 1.6789)	-0.73	0.982
Sem 1500 - fez 500	-0.0167	0.0833	(-0.8929, 0.8596)	-0.20	1.000
lrc 500 - fez 1000	0.0500	0.0373	(-0.5029, 0.6029)	1.34	0.855
lrc 1000 - fez 1000	0.067	0.105	(-2.156, 2.289)	0.63	0.992
lrc 1500 - fez 1000	0.0500	0.0373	(-0.5029, 0.6029)	1.34	0.855
Sem 1500 - fez 1000	0.0833	0.0601	(-0.6042, 0.7709)	1.39	0.843
lrc 1000 - lrc 500	0.017	0.101	(-2.948, 2.982)	0.16	1.000
lrc 1500 - lrc 500	0.0000	0.0236	(-0.2257, 0.2257)	0.00	1.000
Sem 1500 - lrc 500	0.0333	0.0527	(-1.0780, 1.1447)	0.63	0.992
lrc 1500 - lrc 1000	-0.017	0.101	(-2.982, 2.948)	-0.16	1.000
Sem 1500 - lrc 1000	0.017	0.112	(-1.642, 1.675)	0.15	1.000
Sem 1500 - lrc 1500	0.0333	0.0527	(-1.0780, 1.1447)	0.63	0.992

One-Sample T: fez 500, fez 1000, Gus 1500, eGFP 1500, lrc 1500, Sem 1500, H2O

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean	95% CI for μ
fez 500	2	0.1667	0.0943	0.0667	(-0.6804, 1.0137)
fez 1000	2	0.0667	0.0471	0.0333	(-0.3569, 0.4902)
Gus 1500	2	0.0667	0.0943	0.0667	(-0.7804, 0.9137)
eGFP 1500	2	0.031754	0.000713	0.000504	(0.025350, 0.038158)
lrc 1500	2	0.1167	0.0236	0.0167	(-0.0951, 0.3284)
Sem 1500	2	0.1500	0.0707	0.0500	(-0.4853, 0.7853)
H2O	2	0.0500	0.0236	0.0167	(-0.1618, 0.2618)

μ: population mean of fez 500, fez 1000, Gus 1500, eGFP 1500, lrc 1500, Sem 1500, H2O

Test

Null hypothesis	H_0: $\mu = 0.0333333$
Alternative hypothesis	H_1: $\mu \neq 0.0333333$

Sample	T-Value	P-Value
fez 500	2.00	0.295
fez 1000	1.00	0.500
Gus 1500	0.50	0.705
eGFP 1500	-3.13	0.197
lrc 1500	5.00	0.126
Sem 1500	2.33	0.258
H2O	1.00	0.500

Figure 7B

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
Gus 1500	2	0	0,0333	0,0471	0,0333	0,424
fez2 1500	2	0	0,0517	0,0731	0,0517	0,657
sem1a 1500	2	0	0,0667	0,000	0,000	0,000
lrcE1 1500	2	0	0,0635	0,0427	0,0302	0,384

Column	Range	Max	Min	Median	25%	75%
Gus 1500	0,0667	0,0667	0,000	0,0333	0,000	0,0667
fez2 1500	0,103	0,103	0,000	0,0517	0,000	0,103
sem1a 1500	0,000	0,0667	0,0667	0,0667	0,0667	0,0667
lrcE1 1500	0,0604	0,0938	0,0333	0,0635	0,0333	0,0938

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
Gus 1500	--	--	0,260	0,481	--	--
fez2 1500	--	--	0,260	0,481	--	--
sem1a 1500	--	--	0,000	<0,001	--	--
lrcE1 1500	--	--	0,260	0,481	--	--

Column	Sum	Sum of Squares
Gus 1500	0,0667	0,00444
fez2 1500	0,103	0,0107
sem1a 1500	0,133	0,00889
lrcE1 1500	0,127	0,00990

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,665)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Gus 1500	2	0	0,0333	0,000	0,0667
fez2 1500	2	0	0,0517	0,000	0,103
sem1a 1500	2	0	0,0667	0,0667	0,0667
lrcE1 1500	2	0	0,0635	0,0333	0,0938

H = 0,753 with 3 degrees of freedom. P(est.)= 0,861 P(exact)= 0,886

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,886)

One-way ANOVA: Gus A dsRNA 1500, fez2 dsRNA 1500, lrc_E1 dsRNA 1500

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	Gus A dsRNA 1500, fez2 dsRNA 1500, lrc_E1 dsRNA 1500

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.92818	0.17	0.857

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
8.98%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Gus A dsRNA 1500	2	0.0333	0.0471	(-0.3902, 0.4569)
fez2 dsRNA 1500	2	0.0517	0.0731	(-0.6055, 0.7089)
lrc_E1 dsRNA 1500	2	0.0635	0.0427	(-0.3203, 0.4474)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
lrc_E1 dsRNA 1500	2	0.0635	A
fez2 dsRNA 1500	2	0.0517	A
Gus A dsRNA 1500	2	0.0333	A

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
fez2 dsRNA 1 - Gus A dsRNA	0.0184	0.0615	(-0.4189, 0.4557)	0.30	0.953
lrc_E1 dsRNA - Gus A dsRNA	0.0302	0.0450	(-0.2376, 0.2980)	0.67	0.801
lrc_E1 dsRNA - fez2 dsRNA 1	0.0118	0.0599	(-0.4490, 0.4726)	0.20	0.979

One-Sample T: Gus A dsRNA 1500, fez2 dsRNA 1500, lrc_E1 dsRNA 1500

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean	95% CI for μ
Gus A dsRNA 1500	2	0.0333	0.0471	0.0333	(-0.3902, 0.4569)
fez2 dsRNA 1500	2	0.0517	0.0731	0.0517	(-0.6055, 0.7089)
lrc_E1 dsRNA 1500	2	0.0635	0.0427	0.0302	(-0.3203, 0.4474)

μ: population mean of Gus A dsRNA 1500, fez2 dsRNA 1500, lrc_E1 dsRNA 1500

Test

Null hypothesis $H_0: \mu = 0.0666667$
Alternative hypothesis $H_1: \mu \neq 0.0666667$

Sample	T-Value	P-Value
Gus A dsRNA 1500	-1.00	0.500
fez2 dsRNA 1500	-0.29	0.821
lrc_E1 dsRNA 1500	-0.10	0.934

One-Sample T: fez2 dsRNA 1500, sem1a_E8 dsRNA 1500, lrc_E1 dsRNA 1500

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean	95% CI for μ
fez2 dsRNA 1500	2	0.0517	0.0731	0.0517	(-0.6055, 0.7089)
sem1a_E8 dsRNA 1500	2	0.06667	0.00000	0.00000	(0.06667, 0.06667)
lrc_E1 dsRNA 1500	2	0.0635	0.0427	0.0302	(-0.3203, 0.4474)

μ: population mean of fez2 dsRNA 1500, sem1a_E8 dsRNA 1500, lrc_E1 dsRNA 1500

Test

Null hypothesis $H_0: \mu = 0.03333$
Alternative hypothesis $H_1: \mu \neq 0.03333$

Sample	T-Value	P-Value
fez2 dsRNA 1500	0.36	0.782
sem1a_E8 dsRNA 1500	*	*
lrc_E1 dsRNA 1500	1.00	0.500

*** NOTE * All values in column are identical.**

Figure 7C

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
H2O dye	3	0	0,0533	0,0462	0,0267	0,115
H2O	3	0	0,133	0,0611	0,0353	0,152
ctl siRNA	3	0	0,240	0,183	0,106	0,455
fez2 siRNA	3	0	0,293	0,257	0,148	0,639
lrc siRNA	3	0	0,427	0,254	0,147	0,631
sem1a siRNA	3	0	0,333	0,180	0,104	0,448

Column	Range	Max	Min	Median	25%	75%
H2O dye	0,0800	0,0800	0,000	0,0800	0,000	0,0800
H2O	0,120	0,200	0,0800	0,120	0,0800	0,200
ctl siRNA	0,360	0,400	0,0400	0,280	0,0400	0,400
fez2 siRNA	0,480	0,480	0,000	0,400	0,000	0,480
lrc siRNA	0,440	0,720	0,280	0,280	0,280	0,720
sem1a siRNA	0,360	0,520	0,160	0,320	0,160	0,520

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
H2O dye	-1,732	--	0,385	0,089	0,750	<0,001
H2O	0,935	--	0,253	0,487	0,964	0,637
ctl siRNA	-0,935	--	0,253	0,487	0,964	0,637
fez2 siRNA	-1,545	--	0,328	0,225	0,871	0,298
lrc siRNA	1,732	--	0,385	0,089	0,750	<0,001
sem1a siRNA	0,331	--	0,196	0,636	0,996	0,878

Column	Sum	Sum of Squares
H2O dye	0,160	0,0128
H2O	0,400	0,0608
ctl siRNA	0,720	0,240
fez2 siRNA	0,880	0,390
lrc siRNA	1,280	0,675
sem1a siRNA	1,000	0,398

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,980)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,197)

Group Name	N	Missing	Mean	Std Dev	SEM
H2O dye	3	0	0,0533	0,0462	0,0267
H2O	3	0	0,133	0,0611	0,0353
ctl siRNA	3	0	0,240	0,183	0,106
fez2 siRNA	3	0	0,293	0,257	0,148
lrc siRNA	3	0	0,427	0,254	0,147
sem1a siRNA	3	0	0,333	0,180	0,104

Source of Variation	DF	SS	MS	F	P
Between Groups	5	0,277	0,0554	1,641	0,223
Residual	12	0,405	0,0338		
Total	17	0,682			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,223).

Power of performed test with alpha = 0,050: 0,165

The power of the performed test (0,165) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 8A

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,720)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
control	8	0	0,955	0,0530	0,0187
dsx2 soaking	11	0	0,478	0,232	0,0700

Difference of means 0,477

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 5,661 with 17 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,299 to 0,654

Two-tailed P-value = 0,0000282

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0,001).

One-tailed P-value = 0,0000141

The sample mean of group control exceeds the sample mean of group dsx2 soaking by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group dsx2 soaking is greater than or equal to the population mean of group control. (P = <0,001).

Equal Variances Not Assumed (Welch's t-test):

t = 6,580 with 11,403 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,318 to 0,635

Two-tailed P-value = 0,0000334

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0,001).

One-tailed P-value = 0,0000167

The sample mean of group control exceeds the sample mean of group dsx2 soaking by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group dsx2 soaking is greater than or equal to the population mean of group control. (P = <0,001).

Figure 8B

t-test

Normality Test (Shapiro-Wilk): Passed (P = 0,282)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,540)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
male control	7	0	62,102	8,851	3,345
male dsx	5	0	61,021	5,551	2,482

Difference of means 1,081

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 0,240 with 10 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -8,968 to 11,130

Two-tailed P-value = 0,815

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,815).

One-tailed P-value = 0,408

The sample mean of group male control does not exceed the sample mean of the group male dsx by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group male dsx is greater than or equal to the population mean of group male control cannot be rejected. (P = 0,408).

Equal Variances Not Assumed (Welch's t-test):

t = 0,260 with 9,917 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -8,211 to 10,373

Two-tailed P-value = 0,801

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,801).

One-tailed P-value = 0,400

The sample mean of group male control does not exceed the sample mean of the group male dsx by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group male dsx is greater than or equal to the population mean of group male control cannot be rejected. (P = 0,400).

Figure 9A

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
H2O dye	3	0	0,458	0,114	0,0657	0,283
fez dsRNA	3	0	0,519	0,0757	0,0437	0,188
lrc dsRNA	3	0	0,523	0,0795	0,0459	0,198
sem1a dsRNA	3	0	0,451	0,114	0,0657	0,283
GusA dsRNA	3	0	0,504	0,118	0,0683	0,294
Coextract	3	0	0,492	0,123	0,0708	0,304
Handling	3	0	0,360	0,0739	0,0427	0,184

Column	Range	Max	Min	Median	25%	75%
H2O dye	0,216	0,545	0,330	0,500	0,330	0,545
fez dsRNA	0,136	0,568	0,432	0,557	0,432	0,568
lrc dsRNA	0,148	0,580	0,432	0,557	0,432	0,580
sem1a dsRNA	0,227	0,568	0,341	0,443	0,341	0,568
GusA dsRNA	0,227	0,636	0,409	0,466	0,409	0,636
Coextract	0,227	0,580	0,352	0,545	0,352	0,580
Handling	0,148	0,432	0,284	0,364	0,284	0,432

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
H2O dye	-1,427	--	0,310	0,283	0,900	0,384
fez dsRNA	-1,688	--	0,358	0,142	0,812	0,144
lrc dsRNA	-1,574	--	0,333	0,210	0,862	0,274
sem1a dsRNA	0,298	--	0,193	0,640	0,997	0,890
GusA dsRNA	1,293	--	0,292	0,344	0,923	0,463
Coextract	-1,583	--	0,334	0,205	0,860	0,266
Handling	-0,230	--	0,187	0,646	0,998	0,915

Column	Sum	Sum of Squares
H2O dye	1,375	0,656
fez dsRNA	1,557	0,819
lrc dsRNA	1,568	0,832
sem1a dsRNA	1,352	0,635
GusA dsRNA	1,511	0,789
Coextract	1,477	0,757
Handling	1,080	0,399

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,136)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,977)

Group Name	N	Missing	Mean	Std Dev	SEM
H2O dye	3	0	0,458	0,114	0,0657
fez dsRNA	3	0	0,519	0,0757	0,0437
lrc dsRNA	3	0	0,523	0,0795	0,0459
sem1a dsRNA	3	0	0,451	0,114	0,0657
GusA dsRNA	3	0	0,504	0,118	0,0683
Coextract	3	0	0,492	0,123	0,0708
Handling	3	0	0,360	0,0739	0,0427

Source of Variation	DF	SS	MS	F	P
Between Groups	6	0,0583	0,00971	0,938	0,499
Residual	14	0,145	0,0103		
Total	20	0,203			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,499).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 9B

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
H2O dye	3	0	0,543	0,193	0,112	0,480
fez2 siRNA	3	0	0,663	0,0966	0,0558	0,240
lrc siRNA	3	0	0,678	0,181	0,105	0,450
sem1a siRNA	3	0	0,568	0,129	0,0745	0,321
ctl siRNA	3	0	0,572	0,202	0,117	0,502
H2O	3	0	0,458	0,106	0,0610	0,262
Handling	3	0	0,330	0,0227	0,0131	0,0565

Column	Range	Max	Min	Median	25%	75%
H2O dye	0,383	0,750	0,367	0,511	0,367	0,750
fez2 siRNA	0,182	0,773	0,591	0,625	0,591	0,773
lrc siRNA	0,352	0,830	0,477	0,727	0,477	0,830
sem1a siRNA	0,239	0,716	0,477	0,511	0,477	0,716
ctl siRNA	0,375	0,716	0,341	0,659	0,341	0,716
H2O	0,193	0,580	0,386	0,409	0,386	0,580
Handling	0,0455	0,352	0,307	0,330	0,307	0,352

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
H2O dye	0,715	--	0,231	0,557	0,980	0,729
fez2 siRNA	1,493	--	0,319	0,251	0,885	0,339
lrc siRNA	-1,132	--	0,274	0,412	0,945	0,546
sem1a siRNA	1,597	--	0,337	0,197	0,855	0,253
ctl siRNA	-1,579	--	0,333	0,207	0,861	0,269
H2O	1,642	--	0,346	0,172	0,837	0,206
Handling	-0,0000000660	--	0,175	0,654	1,000	1,000

Column	Sum	Sum of Squares
H2O dye	1,629	0,959
fez2 siRNA	1,989	1,337
lrc siRNA	2,034	1,445
sem1a siRNA	1,705	1,002
ctl siRNA	1,716	1,063
H2O	1,375	0,653
Handling	0,989	0,327

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,574)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,679)

Group Name	N	Missing	Mean	Std Dev	SEM
H2O dye	3	0	0,543	0,193	0,112
fez2 siRNA	3	0	0,663	0,0966	0,0558
lrc siRNA	3	0	0,678	0,181	0,105
sem1a siRNA	3	0	0,568	0,129	0,0745
ctl siRNA	3	0	0,572	0,202	0,117
H2O	3	0	0,458	0,106	0,0610
Handling	3	0	0,330	0,0227	0,0131

Source of Variation	DF	SS	MS	F	P
Between Groups	6	0,260	0,0434	2,043	0,127
Residual	14	0,297	0,0212		
Total	20	0,558			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,127).

Power of performed test with alpha = 0,050: 0,283

The power of the performed test (0,283) is below the desired power of 0,800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 9C

Descriptive Statistics – data in lethality

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
H2O dye	3	0	0,180	0,0500	0,0289	0,124
fez2 siRNA	3	0	0,217	0,107	0,0617	0,266
lrc siRNA	3	0	0,188	0,00703	0,00406	0,0175
sem1a siRNA	3	0	0,214	0,0913	0,0527	0,227
ctl siRNA	3	0	0,187	0,0153	0,00882	0,0379
H2O	3	0	0,148	0,0103	0,00597	0,0257
Handling	3	0	0,121	0,0186	0,0107	0,0462

Column	Range	Max	Min	Median	25%	75%
H2O dye	0,1000	0,230	0,130	0,180	0,130	0,230
fez2 siRNA	0,190	0,340	0,150	0,160	0,150	0,340
lrc siRNA	0,0135	0,194	0,180	0,190	0,180	0,194
sem1a siRNA	0,181	0,311	0,130	0,200	0,130	0,311
ctl siRNA	0,0300	0,200	0,170	0,190	0,170	0,200
H2O	0,0200	0,160	0,140	0,145	0,140	0,160
Handling	0,0372	0,140	0,103	0,120	0,103	0,140

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
H2O dye	-2,498E-015	--	0,175	0,654	1,000	1,000
fez2 siRNA	1,715	--	0,369	0,120	0,789	0,089
lrc siRNA	-1,248	--	0,287	0,364	0,930	0,488
sem1a siRNA	0,660	--	0,226	0,571	0,983	0,751
ctl siRNA	-0,935	--	0,253	0,487	0,964	0,637
H2O	1,206	--	0,282	0,382	0,936	0,510
Handling	0,225	--	0,187	0,647	0,998	0,917

Column	Sum	Sum of Squares
H2O dye	0,540	0,102
fez2 siRNA	0,650	0,164
lrc siRNA	0,564	0,106
sem1a siRNA	0,641	0,154
ctl siRNA	0,560	0,105
H2O	0,445	0,0664
Handling	0,363	0,0446

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
H2O dye	3	0	0,180	0,130	0,230
fez2 siRNA	3	0	0,160	0,150	0,340
lrc siRNA	3	0	0,190	0,180	0,194
sem1a siRNA	3	0	0,200	0,130	0,311
ctl siRNA	3	0	0,190	0,170	0,200
H2O	3	0	0,145	0,140	0,160
Handling	3	0	0,120	0,103	0,140

H = 8,861 with 6 degrees of freedom. (P = 0,182)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,182)

Descriptive Statistics, transformed (arcsin transformation) data

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
asinsqrt(col(1))	3	0	0,436	0,0657	0,0379	0,163
asinsqrt(col(2))	3	0	0,477	0,126	0,0728	0,313
asinsqrt(col(3))	3	0	0,448	0,00902	0,00521	0,0224
asinsqrt(col(4))	3	0	0,475	0,112	0,0646	0,278
asinsqrt(col(5))	3	0	0,447	0,0197	0,0114	0,0490
asinsqrt(col(6))	3	0	0,395	0,0145	0,00835	0,0359
asinsqrt(col(7))	3	0	0,355	0,0286	0,0165	0,0709

Column	Range	Max	Min	Median	25%	75%
asinsqrt(col(1))	0,131	0,500	0,369	0,438	0,369	0,500
asinsqrt(col(2))	0,225	0,623	0,398	0,412	0,398	0,623
asinsqrt(col(3))	0,0174	0,456	0,438	0,451	0,438	0,456
asinsqrt(col(4))	0,223	0,592	0,369	0,464	0,369	0,592
asinsqrt(col(5))	0,0387	0,464	0,425	0,451	0,425	0,464
asinsqrt(col(6))	0,0280	0,412	0,383	0,391	0,383	0,412
asinsqrt(col(7))	0,0571	0,383	0,326	0,354	0,326	0,383

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.	SWilk W	SWilk Prob
asinsqrt(col(1))	-0,165	--	0,181	0,651	0,999	0,939
asinsqrt(col(2))	1,709	--	0,366	0,126	0,796	0,105
asinsqrt(col(3))	-1,259	--	0,288	0,359	0,928	0,482
asinsqrt(col(4))	0,442	--	0,206	0,619	0,993	0,836
asinsqrt(col(5))	-0,968	--	0,256	0,475	0,961	0,622
asinsqrt(col(6))	1,183	--	0,279	0,391	0,938	0,521
asinsqrt(col(7))	0,126	--	0,178	0,653	0,999	0,953

Column	Sum	Sum of Squares
asinsqrt(col(1))	1,307	0,578
asinsqrt(col(2))	1,432	0,715
asinsqrt(col(3))	1,345	0,603
asinsqrt(col(4))	1,424	0,701
asinsqrt(col(5))	1,340	0,599
asinsqrt(col(6))	1,186	0,470
asinsqrt(col(7))	1,064	0,379

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,082)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,327)

Group Name	N	Missing	Mean	Std Dev	SEM
asinsqrt(col(1))	3	0	0,436	0,0657	0,0379
asinsqrt(col(2))	3	0	0,477	0,126	0,0728
asinsqrt(col(3))	3	0	0,448	0,00902	0,00521
asinsqrt(col(4))	3	0	0,475	0,112	0,0646
asinsqrt(col(5))	3	0	0,447	0,0197	0,0114
asinsqrt(col(6))	3	0	0,395	0,0145	0,00835
asinsqrt(col(7))	3	0	0,355	0,0286	0,0165

Source of Variation	DF	SS	MS	F	P
Between Groups	6	0,0351	0,00584	1,196	0,363
Residual	14	0,0684	0,00488		
Total	20	0,103			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,363).

Power of performed test with alpha = 0,050: 0,084

The power of the performed test (0,084) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 9D

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,443)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
fez2 dsRNA	2	0	0,883	0,0130	0,00920
fez2 h2o+dye	2	0	0,775	0,369	0,261
fez2 coextract	2	0	0,728	0,310	0,219

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,0254	0,0127	0,164	0,856
Residual	3	0,232	0,0774		
Total	5	0,258			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,856).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: dsRNA fez2, ddH2O + dye fez2, Coextract empty bact fez2

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	dsRNA fez2, ddH2O + dye fez2, Coextract empty bact fez2

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.33734	0.23	0.824

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
9.88%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
dsRNA fez2	2	0.88332	0.01301	(0.76640, 1.00024)
ddH2O + dye fez2	2	0.775	0.369	(-2.537, 4.086)
Coextract empty bact fez2	2	0.728	0.310	(-2.057, 3.513)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
dsRNA fez2	2	0.88332	A
ddH2O + dye fez2	2	0.775	A
Coextract empty bact fez2	2	0.728	A

Means that do not share a letter are significantly different.

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,841)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
lrc dsRNA	2	0	1,239	0,596	0,421
lrc h2o+dye	2	0	1,035	0,127	0,0896
lrc coextrct	2	0	1,023	0,141	0,0994

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,0589	0,0295	0,226	0,810
Residual	3	0,391	0,130		
Total	5	0,449			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,810).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: dsRNA lrc, ddH2O + dye lrc, Coextract empty bact lrc

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	dsRNA lrc, ddH2O + dye lrc, Coextract empty bact lrc

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.79989	0.09	0.917

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
13.11%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
dsRNA lrc	2	1.239	0.596	(-4.112, 6.590)
ddH2O + dye lrc	2	1.0350	0.1268	(-0.1040, 2.1740)
Coextract empty bact lrc	2	1.0231	0.1406	(-0.2402, 2.2863)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
dsRNA lrc	2	1.239	A
ddH2O + dye lrc	2	1.0350	A
Coextract empty bact lrc	2	1.0231	A

Means that do not share a letter are significantly different.

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,969)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
sem1a dsRNA	2	0	0,822	0,0215	0,0152
sem1a h2o+dye	2	0	1,237	0,242	0,171
sem1a coextract	2	0	1,289	0,432	0,306

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,262	0,131	1,599	0,337
Residual	3	0,246	0,0819		
Total	5	0,508			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,337).

Power of performed test with alpha = 0,050: 0,090

The power of the performed test (0,090) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: dsRNA sem1a, ddH2O + dye sem1a, Coextract empty bact sem1a

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	dsRNA sem1a, ddH2O + dye sem1a, Coextract empty bact sem1a

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.34707	2.72	0.337

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
51.59%	19.32%	0.00%

Means

Factor	N	Mean	StDev	95% CI
dsRNA sem1a	2	0.8222	0.0215	(0.6289, 1.0156)
ddH2O + dye sem1a	2	1.237	0.242	(-0.937, 3.411)
Coextract empty bact sem1a	2	1.289	0.432	(-2.593, 5.172)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
Coextract empty bact sem1a	2	1.289	A
ddH2O + dye sem1a	2	1.237	A
dsRNA sem1a	2	0.8222	A

Means that do not share a letter are significantly different.

Figure 9E

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,551)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
fez2 siRNA	2	0	1,231	0,243	0,172
fez2 h2o+dye	2	0	1,317	0,200	0,141
fez2 h2o	2	0	1,165	0,0413	0,0292

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,0234	0,0117	0,349	0,731
Residual	3	0,101	0,0335		
Total	5	0,124			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,731).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: siRNA fez2, ddH2O + dye fez2, ddH2O fez2

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	siRNA fez2, ddH2O + dye fez2, ddH2O fez2

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.42374	0.42	0.719

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
18.86%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
siRNA fez2	2	1.231	0.243	(-0.951, 3.412)
ddH2O + dye fez2	2	1.317	0.200	(-0.479, 3.114)
ddH2O fez2	2	1.1648	0.0413	(0.7935, 1.5361)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
ddH2O + dye fez2	2	1.317	A
siRNA fez2	2	1.231	A
ddH2O fez2	2	1.1648	A

Means that do not share a letter are significantly different.

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,259)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
lrc siRNA	2	0	1,199	0,413	0,292
lrc h2o+dye	2	0	1,213	0,398	0,282
lrc h2o	2	0	1,198	0,111	0,0782

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,000288	0,000144	0,00127	0,999
Residual	3	0,341	0,114		
Total	5	0,341			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,999).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: siRNA lrc, ddH2O + dye lrc, ddH2O lrc

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	siRNA lrc, ddH2O + dye lrc, ddH2O lrc

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.51017	0.00	0.999

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
0.08%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
siRNA lrc	2	1.199	0.413	(-2.509, 4.907)
ddH2O + dye lrc	2	1.213	0.398	(-2.364, 4.790)
ddH2O lrc	2	1.1982	0.1106	(0.2043, 2.1921)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
ddH2O + dye lrc	2	1.213	A
siRNA lrc	2	1.199	A
ddH2O lrc	2	1.1982	A

Means that do not share a letter are significantly different.

One Way Analysis of Variance

Normality Test (Shapiro-Wilk): Passed (P = 0,154)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
sem1a siRNA	2	0	1,058	0,289	0,204
sem1 H2O+dye	2	0	0,859	0,180	0,127
sem1a H2O	2	0	1,049	0,211	0,149

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0,0504	0,0252	0,472	0,664
Residual	3	0,160	0,0534		
Total	5	0,211			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,664).

Power of performed test with alpha = 0,050: 0,050

The power of the performed test (0,050) is below the desired power of 0,800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One-way ANOVA: siRNA sem1a, ddH2O + dye sem1a, ddH2O sem1a

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	siRNA sem1a, ddH2O + dye sem1a, ddH2O sem1a

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	1.93831	0.45	0.690

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
23.93%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
siRNA sem1a	2	1.058	0.289	(-1.537, 3.653)
ddH2O + dye sem1a	2	0.859	0.180	(-0.754, 2.473)
ddH2O sem1a	2	1.049	0.211	(-0.849, 2.948)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
siRNA sem1a	2	1.058	A
ddH2O sem1a	2	1.049	A
ddH2O + dye sem1a	2	0.859	A

Means that do not share a letter are significantly different.

Figure 10

One Way Analysis of Variance

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,064)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

Group Name	N	Missing	Mean	Std Dev	SEM
h2o+bact ctrl	11	0	1,118	0,540	0,163
weak dsRNA	21	0	0,324	0,201	0,0439
strong dsRNA	6	0	0,380	0,163	0,0665

Source of Variation	DF	SS	MS	F	P
Between Groups	2	4,793	2,396	21,756	<0,001
Residual	35	3,855	0,110		
Total	37	8,648			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

Statistics

Variable	Total Count	Mean	SE Mean	StDev	Variance	CoefVar	Median
dsRNA weak GFP	21	0.324324	0.0442844	0.202937	0.0411833	62.57	0.257507
dsRNA strong GFP	21	0.379385	0.0655798	0.160637	0.0258043	42.34	0.350258
ddH2O and bact ctrl	21	1.11952	0.162687	0.539571	0.291136	48.20	0.943620

One-way ANOVA: dsRNA weak GFP, dsRNA strong GFP, ddH2O and bact ctrl

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Rows unused	25

Equal variances were not assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	dsRNA weak GFP, dsRNA strong GFP, ddH2O and bact ctrl

Welch's Test

Source	DF Num	DF Den	F-Value	P-Value
Factor	2	13.6678	10.60	0.002

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
55.43%	52.88%	46.75%

Means

Factor	N	Mean	StDev	95% CI
dsRNA weak GFP	21	0.3243	0.2029	(0.2319, 0.4167)
dsRNA strong GFP	6	0.3794	0.1606	(0.2108, 0.5480)
ddH2O and bact ctrl	11	1.120	0.540	(0.757, 1.482)

Games-Howell Pairwise Comparisons

Grouping Information Using the Games-Howell Method and 95% Confidence

Factor	N	Mean	Grouping
ddH2O and bact ctrl	11	1.120	A
dsRNA strong GFP	6	0.3794	B
dsRNA weak GFP	21	0.3243	B

Means that do not share a letter are significantly different.

Games-Howell Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
dsRNA strong - dsRNA weak G	0.0551	0.0791	(-0.1615, 0.2716)	0.70	0.771
ddH2O and ba - dsRNA weak G	0.795	0.169	(0.342, 1.248)	4.72	0.002
ddH2O and ba - dsRNA strong	0.740	0.175	(0.276, 1.204)	4.22	0.003

Figure S1

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 1a_sem1a	2	0	1,120	0,581	0,411

Hypothesized population mean 1,000

t = 0,293 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: -4,097 to 6,337

Two-tailed P-value = 0,818

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,818).

One-tailed P-value = 0,409

The sample mean of the group does not exceed the hypothesized mean by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the hypothesized mean is greater than or equal to the true mean cannot be rejected. (P = 0,409).

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 1b_sem1a	2	0	0,998	0,122	0,0863

Hypothesized population mean 1,000

t = -0,0276 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: -0,0987 to 2,094

Two-tailed P-value = 0,982

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,982).

One-tailed P-value = 0,491

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,491).

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 2_sem1a	2	0	0,761	0,00378	0,00267

Hypothesized population mean 1,000

t = -89,267 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,727 to 0,795

Two-tailed P-value = 0,00713

There is a statistically significant difference between the mean of the sampled population and the hypothesized population mean (P = 0,007).

One-tailed P-value = 0,00357

The hypothesized mean exceeds the sample mean of the group by an amount that is greater than would be expected by chance, rejecting the hypothesis that the true mean of group is greater than or equal to the hypothesized mean. (P = 0,004).

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3_sem1a	2	0	0,534	0,116	0,0817

Hypothesized population mean 1,000

t = -5,697 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: -0,504 to 1,573

Two-tailed P-value = 0,111

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,111).

One-tailed P-value = 0,0553

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,055).

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3 fez2	2	0	0,906	0,148	0,104

Hypothesized population mean 1,000

t = -0,899 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: -0,420 to 2,233

Two-tailed P-value = 0,534

The difference between the mean of the sampled population and the hypothesized population mean is not great enough to reject the hypothesis that the difference is only due to random sample variability. There is not a significant difference between the two means (P = 0,534).

One-tailed P-value = 0,267

The hypothesized mean does not exceed the sample mean of the group by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the true mean is greater than or equal to the hypothesized mean cannot be rejected. (P = 0,267).

One-Sample t-test_qPCR yeast feeding assays, relative expression ratio

Normality Test (Kolmogorov-Smirnov): Passed (P = 0,481)

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3 lrc	2	0	0,562	0,0320	0,0226

Hypothesized population mean 1,000

t = -19,345 with 1 degrees of freedom.

95 percent two-tailed confidence interval for the population mean: 0,275 to 0,850

Two-tailed P-value = 0,0329

There is a statistically significant difference between the mean of the sampled population and the hypothesized population mean (P = 0,033).

One-tailed P-value = 0,0164

The hypothesized mean exceeds the sample mean of the group by an amount that is greater than would be expected by chance, rejecting the hypothesis that the true mean of group is greater than or equal to the hypothesized mean. (P = 0,016).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,683)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 1a_Sem-1a	2	0	0,950	0,000	0,000
Exp 1a_control	2	0	0,825	0,0354	0,0250

Difference of means 0,125

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 5,000 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,0174 to 0,233

Two-tailed P-value = 0,0377

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,038).

One-tailed P-value = 0,0189

The sample mean of group Exp 1a_Sem-1a exceeds the sample mean of group Exp 1a_control by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 1a_control is greater than or equal to the population mean of group Exp 1a_Sem-1a. (P = 0,019).

Equal Variances Not Assumed (Welch's t-test):

t = 5,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,193 to 0,443

Two-tailed P-value = 0,126

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,126).

One-tailed P-value = 0,0628

The sample mean of group Exp 1a_Sem-1a does not exceed the sample mean of the group Exp 1a_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 1a_control is greater than or equal to the population mean of group Exp 1a_Sem-1a cannot be rejected. (P = 0,063).

One-Sample t-test_ Survival numbers yeast feeding assays

Normality Test (Kolmogorov-Smirnov): Failed (P < 0,050)

Test execution ended by user request, One-Sample Signed Rank Test begun

One-Sample Signed Rank Test

Group	N	Missing	Median	25%	75%
Exp 1b_Sem-1a	2,000	0,000	1,000	1,000	1,000

Hypothesized population median 1,000

W= 0,000 T+ = 0,000 T-= 0,000
Z-Statistic (based on positive ranks) = 6,790E-313

Yates continuity correction option applied to calculations.

P(est.)= 1,000 P(exact)= 1,000

95 percent confidence interval for the population median: 1,000 to 1,000

The difference between the median of the group and the hypothesized population median is not great enough to reject the possibility that the difference is due to random sampling variability.There is not a significant difference between the two medians (P = 1,000).

One-Sample t-test

Normality Test (Kolmogorov-Smirnov): Failed (P < 0,050)

Test execution ended by user request, One-Sample Signed Rank Test begun

One-Sample Signed Rank Test

Group	N	Missing	Median	25%	75%
Exp 1b_control	2,000	0,000	0,950	0,950	0,950

Hypothesized population median 1,000

W= -3,000 T+ = 0,000 T-= 3,000
Z-Statistic (based on positive ranks) = -1,414

Yates continuity correction option applied to calculations.

P(est.)= 0,346 P(exact)= 0,500

95 percent confidence interval for the population median: 0,950 to 0,950

The difference between the median of the group and the hypothesized population median is not great enough to reject the possibility that the difference is due to random sampling variability.There is not a significant difference between the two medians (P = 0,500).

Wilcoxon Signed Rank Test: Exp 1b_Sem-1a

Method

η: median of Exp 1b_Sem-1a

Descriptive Statistics

Sample	N	Median
Exp 1b_Sem-1a	2	1

Test

Null hypothesis	H_0: η = 0.95
Alternative hypothesis	H_1: η ≠ 0.95

Sample	N for Test	Wilcoxon Statistic	P-Value
Exp 1b_Sem-1a	2	3.00	0.371

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 2_Sem-1a	2	0	0,875	0,0354	0,0250
Exp 2_control	2	0	0,975	0,0354	0,0250

Difference of means -0,1000

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -2,828 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,252 to 0,0521

Two-tailed P-value = 0,106

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,106).

One-tailed P-value = 0,0528

The sample mean of group Exp 2_control does not exceed the sample mean of the group Exp 2_Sem-1a by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 2_Sem-1a is greater than or equal to the population mean of group Exp 2_control cannot be rejected. (P = 0,053).

Equal Variances Not Assumed (Welch's t-test):

t = -2,828 with 2,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,252 to 0,0521

Two-tailed P-value = 0,106

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,106).

One-tailed P-value = 0,0528

The sample mean of group Exp 2_control does not exceed the sample mean of the group Exp 2_Sem-1a by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 2_Sem-1a is greater than or equal to the population mean of group Exp 2_control cannot be rejected. (P = 0,053).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,969)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
asinsqrt(col(9))	2	0	1,211	0,0537	0,0380
asinsqrt(col(10))	2	0	1,458	0,159	0,113

Difference of means -0,247

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -2,076 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,759 to 0,265

Two-tailed P-value = 0,174

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,174).

One-tailed P-value = 0,0868

The sample mean of group asinsqrt(col(10)) does not exceed the sample mean of the group asinsqrt(col(9)) by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group asinsqrt(col(9)) is greater than or equal to the population mean of group asinsqrt(col(10)) cannot be rejected. (P = 0,087).

Equal Variances Not Assumed (Welch's t-test):

t = -2,076 with 1,224 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -1,759 to 1,265

Two-tailed P-value = 0,248

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,248).

One-tailed P-value = 0,124

The sample mean of group asinsqrt(col(10)) does not exceed the sample mean of the group asinsqrt(col(9)) by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group asinsqrt(col(9)) is greater than or equal to the population mean of group asinsqrt(col(10)) cannot be rejected. (P = 0,124).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,714)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3_sem-1a	2	0	0,925	0,0354	0,0250
Exp 3_control	2	0	0,950	0,0707	0,0500

Difference of means -0,0250

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,447 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,266 to 0,216

Two-tailed P-value = 0,698

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,698).

One-tailed P-value = 0,349

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_sem-1a by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_sem-1a is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,349).

Equal Variances Not Assumed (Welch's t-test):

t = -0,447 with 1,471 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,735 to 0,685

Two-tailed P-value = 0,712

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,712).

One-tailed P-value = 0,356

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_sem-1a by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_sem-1a is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,356).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,683)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 4_Sem-1a	2	0	0,800	0,000	0,000
Exp 4_control	2	0	0,875	0,0354	0,0250

Difference of means -0,0750

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -3,000 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,183 to 0,0326

Two-tailed P-value = 0,0955

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,095).

One-tailed P-value = 0,0477

The sample mean of group Exp 4_control exceeds the sample mean of group Exp 4_Sem-1a by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 4_Sem-1a is greater than or equal to the population mean of group Exp 4_control. (P = 0,048).

Equal Variances Not Assumed (Welch's t-test):

t = -3,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,393 to 0,243

Two-tailed P-value = 0,205

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,205).

One-tailed P-value = 0,102

The sample mean of group Exp 4_control does not exceed the sample mean of the group Exp 4_Sem-1a by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 4_Sem-1a is greater than or equal to the population mean of group Exp 4_control cannot be rejected. (P = 0,102).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 5_Sem-1a	2	0	0,867	0,0471	0,0333
Exp 5_control	2	0	0,800	0,0471	0,0333

Difference of means 0,0667

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 1,414 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,136 to 0,269

Two-tailed P-value = 0,293

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,293).

One-tailed P-value = 0,146

The sample mean of group Exp 5_Sem-1a does not exceed the sample mean of the group Exp 5_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 5_control is greater than or equal to the population mean of group Exp 5_Sem-1a cannot be rejected. (P = 0,146).

Equal Variances Not Assumed (Welch's t-test):

t = 1,414 with 2,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,136 to 0,269

Two-tailed P-value = 0,293

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,293).

One-tailed P-value = 0,146

The sample mean of group Exp 5_Sem-1a does not exceed the sample mean of the group Exp 5_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 5_control is greater than or equal to the population mean of group Exp 5_Sem-1a cannot be rejected. (P = 0,146).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,101)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
asinsqrt(col(20))	2	0	1,200	0,0699	0,0494
asinsqrt(col(21))	2	0	1,108	0,0591	0,0418

Difference of means 0,0912

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 1,409 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,187 to 0,370

Two-tailed P-value = 0,294

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,294).

One-tailed P-value = 0,147

The sample mean of group asinsqrt(col(20)) does not exceed the sample mean of the group asinsqrt(col(21)) by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group asinsqrt(col(21)) is greater than or equal to the population mean of group asinsqrt(col(20)) cannot be rejected. (P = 0,147).

Equal Variances Not Assumed (Welch's t-test):

t = 1,409 with 1,947 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,731 to 0,913

Two-tailed P-value = 0,297

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,297).

One-tailed P-value = 0,149

The sample mean of group asinsqrt(col(20)) does not exceed the sample mean of the group asinsqrt(col(21)) by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group asinsqrt(col(21)) is greater than or equal to the population mean of group asinsqrt(col(20)) cannot be rejected. (P = 0,149).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,059)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 6_Sem-1a	3	0	0,856	0,0509	0,0294
Exp 6_control	3	0	0,556	0,0770	0,0444

Difference of means 0,300

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 5,630 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,152 to 0,448

Two-tailed P-value = 0,00490

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,005).

One-tailed P-value = 0,00245

The sample mean of group Exp 6_Sem-1a exceeds the sample mean of group Exp 6_control by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 6_control is greater than or equal to the population mean of group Exp 6_Sem-1a. (P = 0,002).

Equal Variances Not Assumed (Welch's t-test):

t = 5,630 with 3,469 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,143 to 0,457

Two-tailed P-value = 0,00742

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,007).

One-tailed P-value = 0,00371

The sample mean of group Exp 6_Sem-1a exceeds the sample mean of group Exp 6_control by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 6_control is greater than or equal to the population mean of group Exp 6_Sem-1a. (P = 0,004).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,190)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 7_Sem-1a	3	0	0,778	0,0192	0,0111
Exp 7_control	3	0	0,767	0,115	0,0667

Difference of means 0,0111

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 0,164 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,177 to 0,199

Two-tailed P-value = 0,877

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,877).

One-tailed P-value = 0,439

The sample mean of group Exp 7_Sem-1a does not exceed the sample mean of the group Exp 7_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_control is greater than or equal to the population mean of group Exp 7_Sem-1a cannot be rejected. (P = 0,439).

Equal Variances Not Assumed (Welch's t-test):

t = 0,164 with 2,111 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,280 to 0,302

Two-tailed P-value = 0,884

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,884).

One-tailed P-value = 0,442

The sample mean of group Exp 7_Sem-1a does not exceed the sample mean of the group Exp 7_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_control is greater than or equal to the population mean of group Exp 7_Sem-1a cannot be rejected. (P = 0,442).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,683)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3_lrc	2	0	0,900	0,000	0,000
Exp 3_control	2	0	0,950	0,0707	0,0500

Difference of means -0,0500

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -1,000 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,265 to 0,165

Two-tailed P-value = 0,423

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,423).

One-tailed P-value = 0,211

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_lrc is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,211).

Equal Variances Not Assumed (Welch's t-test):

t = -1,000 with 1,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,685 to 0,585

Two-tailed P-value = 0,500

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,500).

One-tailed P-value = 0,250

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_lrc is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,250).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,962)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 4_lrc	2	0	0,675	0,247	0,175
Exp 4_control	2	0	0,875	0,0354	0,0250

Difference of means -0,200

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -1,131 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,961 to 0,561

Two-tailed P-value = 0,375

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,375).

One-tailed P-value = 0,188

The sample mean of group Exp 4_control does not exceed the sample mean of the group Exp 4_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 4_lrc is greater than or equal to the population mean of group Exp 4_control cannot be rejected. (P = 0,188).

Equal Variances Not Assumed (Welch's t-test):

t = -1,131 with 1,041 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -2,446 to 2,046

Two-tailed P-value = 0,455

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,455).

One-tailed P-value = 0,228

The sample mean of group Exp 4_control does not exceed the sample mean of the group Exp 4_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 4_lrc is greater than or equal to the population mean of group Exp 4_control cannot be rejected. (P = 0,228).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,588)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 6_lrc	3	0	0,467	0,186	0,107
Exp 6_control	3	0	0,556	0,0770	0,0444

Difference of means -0,0889

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,766 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,411 to 0,233

Two-tailed P-value = 0,486

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,486).

One-tailed P-value = 0,243

The sample mean of group Exp 6_control does not exceed the sample mean of the group Exp 6_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 6_lrc is greater than or equal to the population mean of group Exp 6_control cannot be rejected. (P = 0,243).

Equal Variances Not Assumed (Welch's t-test):

t = -0,766 with 2,668 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,588 to 0,410

Two-tailed P-value = 0,506

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,506).

One-tailed P-value = 0,253

The sample mean of group Exp 6_control does not exceed the sample mean of the group Exp 6_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 6_lrc is greater than or equal to the population mean of group Exp 6_control cannot be rejected. (P = 0,253).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,110)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 7_lrc	3	0	0,767	0,0667	0,0385
Exp 7_control	3	0	0,767	0,115	0,0667

Difference of means 0,000

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 0,000 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,214 to 0,214

Two-tailed P-value = 1,000

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 1,000).

One-tailed P-value = 1,000

The sample mean of group Exp 7_control does not exceed the sample mean of the group Exp 7_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_lrc is greater than or equal to the population mean of group Exp 7_control cannot be rejected. (P = 1,000).

Equal Variances Not Assumed (Welch's t-test):

t = 0,000 with 3,200 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,237 to 0,237

Two-tailed P-value = 1,000

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 1,000).

One-tailed P-value = 1,000

The sample mean of group Exp 7_control does not exceed the sample mean of the group Exp 7_lrc by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_lrc is greater than or equal to the population mean of group Exp 7_control cannot be rejected. (P = 1,000).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,348)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 3_fez2	2	0	0,925	0,106	0,0750
Exp 3_control	2	0	0,950	0,0707	0,0500

Difference of means -0,0250

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -0,277 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,413 to 0,363

Two-tailed P-value = 0,808

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,808).

One-tailed P-value = 0,404

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_fez2 by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_fez2 is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,404).

Equal Variances Not Assumed (Welch's t-test):

t = -0,277 with 1,742 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -1,170 to 1,120

Two-tailed P-value = 0,811

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,811).

One-tailed P-value = 0,405

The sample mean of group Exp 3_control does not exceed the sample mean of the group Exp 3_fez2 by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 3_fez2 is greater than or equal to the population mean of group Exp 3_control cannot be rejected. (P = 0,405).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,714)

Equal Variance Test (Brown-Forsythe): Failed (P < 0,050)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 4_fez2	2	0	0,500	0,0707	0,0500
Exp 4_control	2	0	0,875	0,0354	0,0250

Difference of means -0,375

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = -6,708 with 2 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,616 to -0,134

Two-tailed P-value = 0,0215

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,022).

One-tailed P-value = 0,0108

The sample mean of group Exp 4_control exceeds the sample mean of group Exp 4_fez2 by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 4_fez2 is greater than or equal to the population mean of group Exp 4_control. (P = 0,011).

Equal Variances Not Assumed (Welch's t-test):

t = -6,708 with 1,471 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -1,085 to 0,335

Two-tailed P-value = 0,0445

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,045).

One-tailed P-value = 0,0223

The sample mean of group Exp 4_control exceeds the sample mean of group Exp 4_fez2 by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 4_fez2 is greater than or equal to the population mean of group Exp 4_control. (P = 0,022).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,078)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 6_fez2	3	0	0,767	0,000	0,000
Exp 6_control	3	0	0,556	0,0770	0,0444

Difference of means 0,211

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 4,750 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,0877 to 0,335

Two-tailed P-value = 0,00897

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,009).

One-tailed P-value = 0,00449

The sample mean of group Exp 6_fez2 exceeds the sample mean of group Exp 6_control by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 6_control is greater than or equal to the population mean of group Exp 6_fez2. (P = 0,004).

Equal Variances Not Assumed (Welch's t-test):

t = 4,750 with 2,000 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 0,0199 to 0,402

Two-tailed P-value = 0,0416

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,042).

One-tailed P-value = 0,0208

The sample mean of group Exp 6_fez2 exceeds the sample mean of group Exp 6_control by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group Exp 6_control is greater than or equal to the population mean of group Exp 6_fez2. (P = 0,021).

t-test_ Survival numbers yeast feeding assays

Normality Test (Shapiro-Wilk): Passed (P = 0,091)

Equal Variance Test (Brown-Forsythe): Passed (P = 1,000)

The result of the equal variance test indicates the likelihood that the two groups are sampled from populations with equal variances, but does not guarantee the equality or inequality of the two variances.

Group Name	N	Missing	Mean	Std Dev	SEM
Exp 7_fez2	3	0	0,833	0,0577	0,0333
Exp 7_control	3	0	0,767	0,115	0,0667

Difference of means 0,0667

Use the results of Welch's test, where equal variances are not assumed, if the equality of the population variances of the two groups is in doubt.

Equal Variances Assumed (Student's t-test):

t = 0,894 with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,140 to 0,274

Two-tailed P-value = 0,422

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,422).

One-tailed P-value = 0,211

The sample mean of group Exp 7_fez2 does not exceed the sample mean of the group Exp 7_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_control is greater than or equal to the population mean of group Exp 7_fez2 cannot be rejected. (P = 0,211).

Equal Variances Not Assumed (Welch's t-test):

t = 0,894 with 2,941 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: -0,254 to 0,387

Two-tailed P-value = 0,438

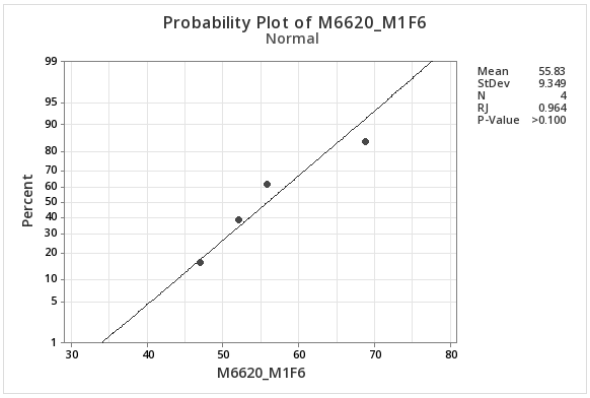
The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0,438).

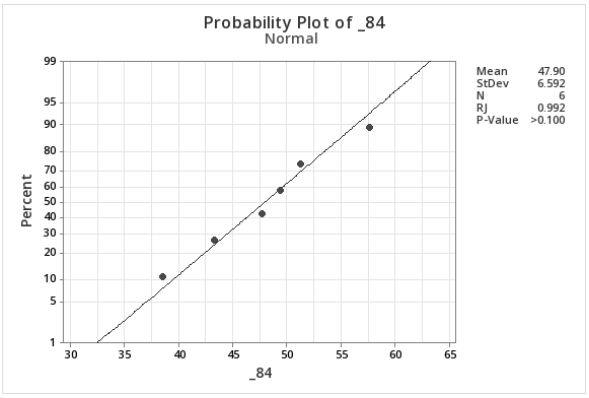
One-tailed P-value = 0,219

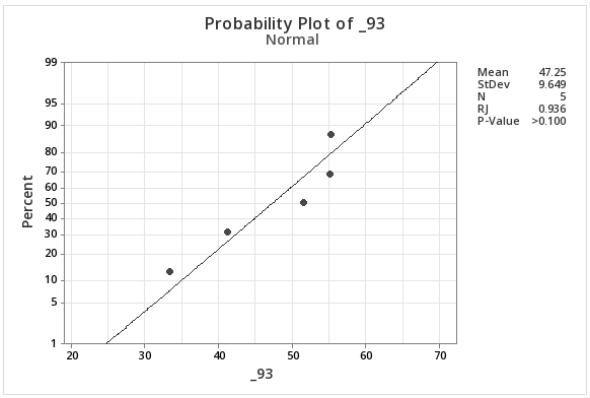
The sample mean of group Exp 7_fez2 does not exceed the sample mean of the group Exp 7_control by an amount great enough to exclude the possibility that the difference is due to random sampling variability. The hypothesis that the population mean of group Exp 7_control is greater than or equal to the population mean of group Exp 7_fez2 cannot be rejected. (P = 0,219).

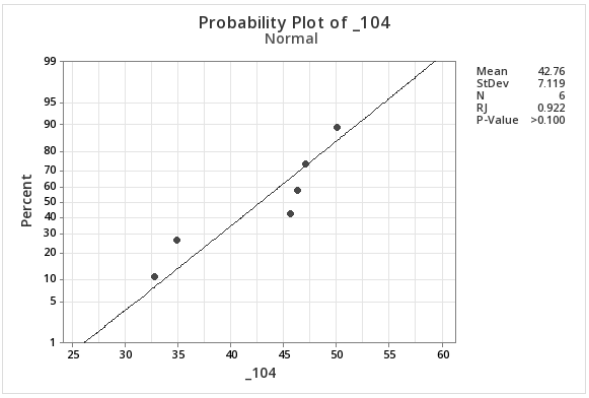
5.3 Statistical analysis of data in manuscript "Towards neo-classical genetic sexing strains in *Ceratitis capitata* (Diptera: Tephritidae): engineering a functional intronless version of the white pupae gene in the Mediterranean fruit fly"

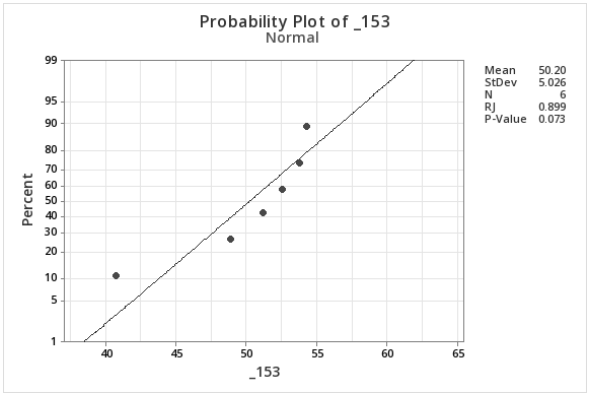
Figure 2F
Ryan-Joiner normality test:

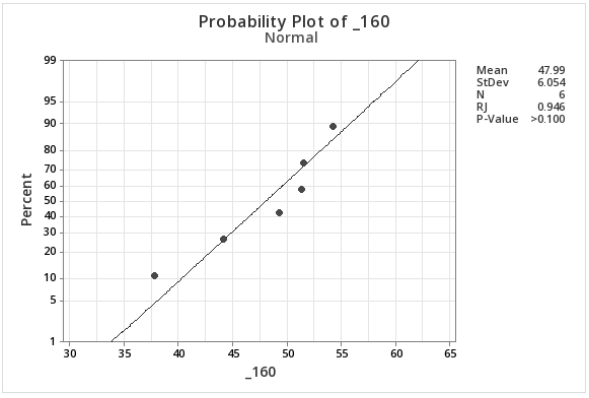




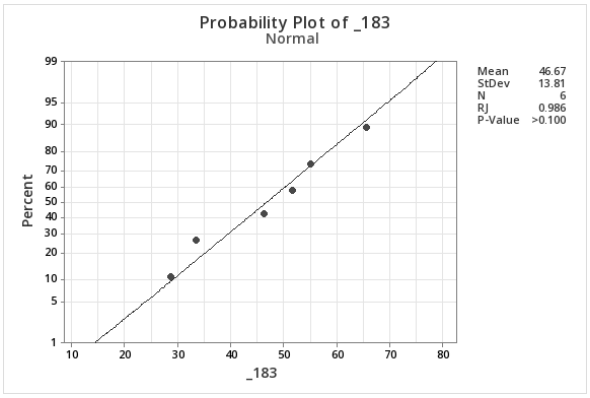


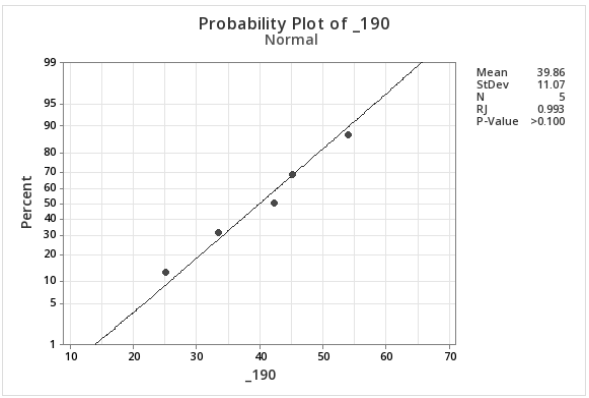


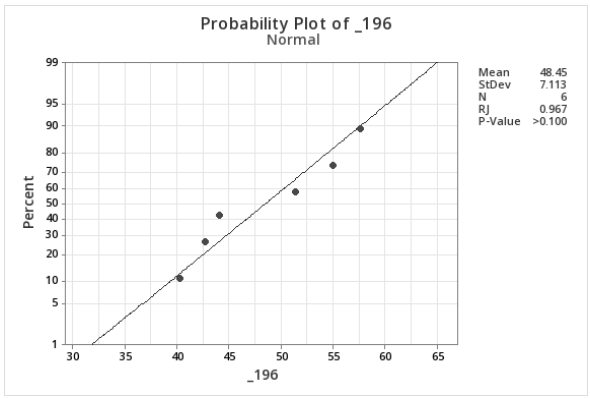




Ryan-Joiner normality test (continuation):







Test for Equal Variances
Samples: M6620_M1F6, _153, _104, _84, _93, _183, _190, _196, _160

Method

Null hypothesis	All variances are equal
Alternative hypothesis	At least one variance is different
Significance level	α = 0.05

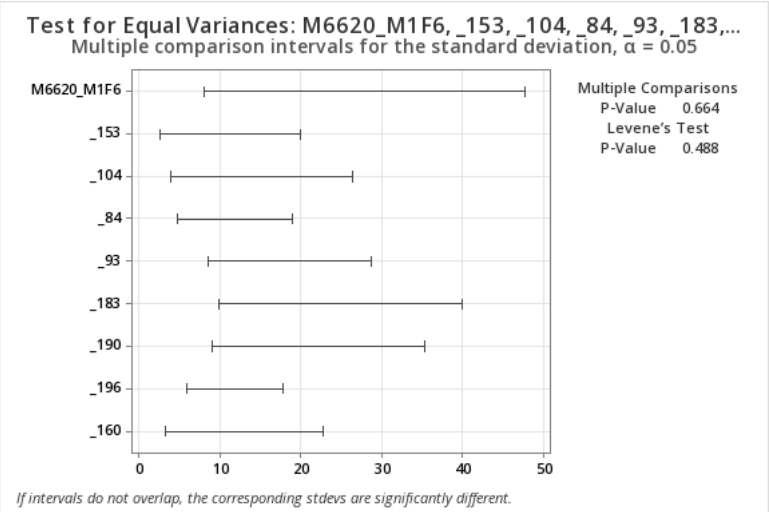
95% Bonferroni Confidence Intervals for Standard Deviations

Sample	N	StDev	CI	
M6620_M1F6	4	9.3487	(0.52991,	537.641)
_153	6	5.0257	(0.48962,	95.914)
_104	6	7.1191	(1.04501,	90.173)
_84	6	6.5916	(1.95650,	41.290)
_93	5	9.6488	(2.46975,	84.631)
_183	6	13.8054	(4.56722,	77.587)
_190	5	11.0735	(2.52836,	108.884)
_196	6	7.1131	(3.01907,	31.159)
_160	6	6.0542	(0.77247,	88.222)

Individual confidence level = 99.4444%

Tests

Method	Test Statistic	P-Value
Multiple comparisons	—	0.664
Levene	0.95	0.488



One-way ANOVA

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Rows unused	4

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	9	M6620_M1F6, _153, _104, _84, _93, _183, _190, _196, _160

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	757.2	94.65	1.25	0.297
Error	41	3111.2	75.88		
Total	49	3868.4			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.71111	19.57%	3.88%	0.00%

Means

Factor	N	Mean	StDev	95% CI	
M6620_M1F6	4	55.83	9.35	(47.03,	64.62)
_153	6	50.20	5.03	(43.02,	57.38)
_104	6	42.76	7.12	(35.58,	49.94)
_84	6	47.90	6.59	(40.72,	55.08)
_93	5	47.25	9.65	(39.38,	55.12)
_183	6	46.67	13.81	(39.49,	53.85)
_190	5	39.86	11.07	(31.99,	47.72)
_196	6	48.45	7.11	(41.27,	55.63)
_160	6	47.99	6.05	(40.81,	55.18)

Pooled StDev = 8.71111

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
M6620_M1F6	4	55.83	A
_153	6	50.20	A
_196	6	48.45	A
_160	6	47.99	A
_84	6	47.90	A
_93	5	47.25	A
_183	6	46.67	A
_104	6	42.76	A
_190	5	39.86	A

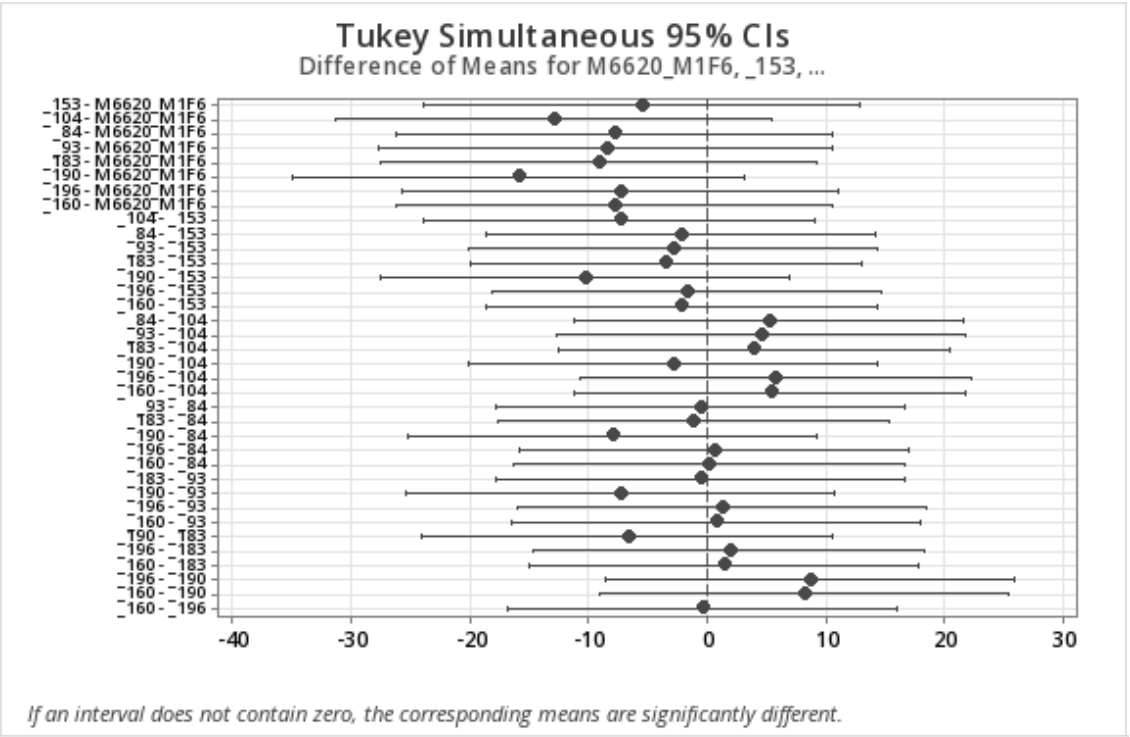
Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

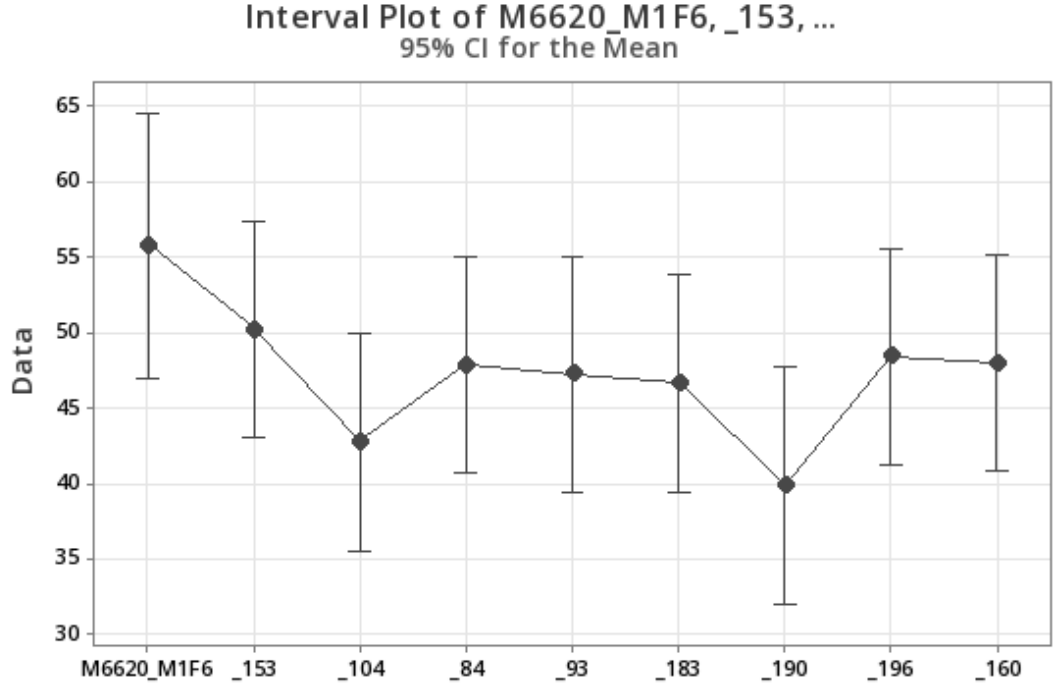
Difference of Levels	Difference of Means	SE of Difference	95% CI		T-Value	Adjusted P-Value
_153 - M6620_M1F6	-5.63	5.62	(-24.04,	12.78)	-1.00	0.984
_104 - M6620_M1F6	-13.06	5.62	(-31.47,	5.34)	-2.32	0.352
_84 - M6620_M1F6	-7.93	5.62	(-26.34,	10.48)	-1.41	0.887
_93 - M6620_M1F6	-8.58	5.84	(-27.71,	10.55)	-1.47	0.864
_183 - M6620_M1F6	-9.16	5.62	(-27.57,	9.25)	-1.63	0.784
_190 - M6620_M1F6	-15.97	5.84	(-35.10,	3.16)	-2.73	0.168
_196 - M6620_M1F6	-7.38	5.62	(-25.79,	11.03)	-1.31	0.922
_160 - M6620_M1F6	-7.83	5.62	(-26.24,	10.58)	-1.39	0.894
_104 - _153	-7.44	5.03	(-23.90,	9.03)	-1.48	0.859
_84 - _153	-2.30	5.03	(-18.77,	14.16)	-0.46	1.000
_93 - _153	-2.95	5.27	(-20.22,	14.32)	-0.56	1.000
_183 - _153	-3.53	5.03	(-20.00,	12.93)	-0.70	0.998
_190 - _153	-10.34	5.27	(-27.61,	6.93)	-1.96	0.578
_196 - _153	-1.75	5.03	(-18.22,	14.72)	-0.35	1.000
_160 - _153	-2.21	5.03	(-18.67,	14.26)	-0.44	1.000
_84 - _104	5.13	5.03	(-11.33,	21.60)	1.02	0.982

Difference of Levels	Difference of Means	SE of Difference	95% CI		T-Value	Adjusted P-Value
_93 - _104	4.49	5.27	(-12.78,	21.76)	0.85	0.994
_183 - _104	3.90	5.03	(-12.56,	20.37)	0.78	0.997
_190 - _104	-2.91	5.27	(-20.18,	14.36)	-0.55	1.000
_196 - _104	5.69	5.03	(-10.78,	22.15)	1.13	0.966
_160 - _104	5.23	5.03	(-11.23,	21.70)	1.04	0.979
_93 - _84	-0.65	5.27	(-17.92,	16.62)	-0.12	1.000
_183 - _84	-1.23	5.03	(-17.70,	15.24)	-0.24	1.000
_190 - _84	-8.04	5.27	(-25.31,	9.23)	-1.52	0.838
_196 - _84	0.55	5.03	(-15.91,	17.02)	0.11	1.000
_160 - _84	0.10	5.03	(-16.37,	16.56)	0.02	1.000
_183 - _93	-0.58	5.27	(-17.85,	16.69)	-0.11	1.000
_190 - _93	-7.39	5.51	(-25.43,	10.64)	-1.34	0.912
_196 - _93	1.20	5.27	(-16.07,	18.47)	0.23	1.000
_160 - _93	0.74	5.27	(-16.53,	18.01)	0.14	1.000
_190 - _183	-6.81	5.27	(-24.08,	10.46)	-1.29	0.928
_196 - _183	1.78	5.03	(-14.68,	18.25)	0.35	1.000
_160 - _183	1.33	5.03	(-15.14,	17.79)	0.26	1.000
_196 - _190	8.59	5.27	(-8.68,	25.86)	1.63	0.783
_160 - _190	8.14	5.27	(-9.13,	25.41)	1.54	0.829
_160 - _196	-0.46	5.03	(-16.92,	16.01)	-0.09	1.000

Individual confidence level = 99.78%



If an interval does not contain zero, the corresponding means are significantly different.



The pooled standard deviation is used to calculate the intervals.

6 Statement on data usage in this thesis

Part of the results shown and discussed in this thesis have been peer-reviewed and published under open access license in the following special issues:

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8 Erklärung gemäß der Promotionsordnung des Fachbereichs 09

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.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Osnabrück, den 08.01.2025

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Lucas Henrique Figueiredo Prates

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