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# Generation of a *Culex* Male Mosquito Sex-Separation RNAi Yeast Strain Using Cas-CLOVER and Super PiggyBac Engineering in *Saccharomyces cerevisiae*

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**Abstract:** Several emerging mosquito control technologies require mass releases of adult male mosquitoes. Previous studies resulted in the generation of a laboratory female-specific larvicidal yeast strain targeting the *GGT* gene, which facilitated the laboratory sex separation of male *Culex quinquefasciatus* mosquitoes. Global deployment of this yeast-based sex-separation technology requires engineering second generation yeast strains which can be used in industrial-scale fermentations to support global mosquito control programs. In this study, the RNA-guided Cas-CLOVER system was used in combination with piggyBac transposase to generate robust *Saccharomyces cerevisiae* strains with multiple integrated copies of the insecticidal GGT shRNA expression cassette. Top expressing Cas-CLOVER strains killed *Culex quinquefasciatus* female larvae which consumed the yeast, facilitating male sex separation. Scaled fermentation resulted in kilogram-scale production of the yeast, which can be heat-killed and dried for global deployment to mosquito mass-rearing facilities.

**Keywords:** *Culex quinquefasciatus*; female lethal; genetic engineering; larvicide; shRNA; synthetic biology



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## 1. Introduction

Mosquito-borne diseases result in hundreds of thousands of human deaths across the globe each year [1]. Mosquito control is currently the primary method of averting these diseases. However, insecticide resistance, concerns regarding the undesired effects of insecticides on human health and the environment, as well as the lack of support for control programs, hinder effective mosquito control efforts [1]. Such issues have led to renewed interest in alternative control methods, such as the sterile insect technique (SIT) [2]. The SIT, an environmentally friendly means of insect control, involves the release of mating-competitive sterile adult males that are unable to produce offspring [2]. Similarly, the incompatible insect technique (IIT), which can be paired with SIT, involves mass releases of *Wolbachia*-infected adult males for mosquito population suppression [3–5]. Likewise, population suppression approaches involving transgenic technology, including the release of insects which carry a dominant lethal (RIDL) gene [6,7] and emerging gene drive technologies that block pathogen transmission or suppress mosquito populations, involve male releases [8,9]. The release of only males is critical for limiting the health- and nuisance biting-risks posed by female contamination and is also thought to improve the efficacy of these interventions [10,11]. Unfortunately, the broad implementation of such mosquito

control programs has been hindered by a lack of inexpensive and scalable male mosquito sex-sorting strategies for various vector mosquito species that can be deployed globally for male mosquito production [10,11]. For some mosquitoes, including *Culex* spp., also referred to as common house mosquitoes [12], visual and mechanical separation methods, which are extremely labor intensive and insufficient for the large-scale implementation of SIT or IIT programs, are currently the only means of generating males [10]. Consequently, sex separation is a rate-limiting step that hampers the global deployment of population-based *Culex* mosquito control methodologies [10]. Such interventions would be useful, as these insects spread viruses that cause West Nile, St. Louis encephalitis, Japanese encephalitis, and viral diseases that infect horses and birds [12].

In previous studies, our RNAi screens led to the discovery of genes specifically required in female larvae. For example, the gamma-glutamyl transpeptidase (*GGT*) gene, which is well conserved in multiple species of mosquitoes, is required in female mosquito larvae. RNAi-mediated silencing of this gene in mosquitoes resulted in female-specific larval death but had no impact on male survival. The generation of a *Saccharomyces cerevisiae* strain GGT.566, which enabled inexpensive expression and oral delivery of shRNA corresponding to the mosquito *GGT* gene, resulted in female larval death and a 5:1 male/female adult mosquito ratio. GGT.566 yeast was incorporated into a larval mass rearing diet, permitting the generation of fit *C. quinquefasciatus* adult males. These studies suggested that yeast-based RNAi larvicides could help promote the global implementation of population-based control strategies that require the release of adult males. *Saccharomyces cerevisiae* is both genetically manipulable and inexpensive to grow in culture [13], facilitating economical production of the female-specific RNAi larvicides. Yeast RNAi strains effectively silence genes in mosquito larvae, generating relatively higher levels of larval mortality than other RNAi larvicide delivery methods [14]. Furthermore, the interfering RNA produced during yeast cultivation retains larvicidal activity when the yeast is heat-killed and dried [14], allowing female-specific insecticides to be packaged, shipped globally, and stored in an inactivated form.

The first generation of a GGT.566 yeast strain [15] was useful for demonstrating proof-of-concept that the yeast system enables male mosquito sex separation. However, the plasmid-based laboratory parent yeast strain used to generate this female-specific yeast larvicide is not suitable for commercial applications or global deployment of this sex-sorting technology. A stable transformant, rather than a plasmid-based strain, should be used for scaled production. Moreover, the growth of the lab strain is inadequate for scaled larvicide production, and the parent strain bears auxotrophic mutations that result in the need to use expensive complete media during culturing. Furthermore, the generation of yeast strains containing multiple copies of the GGT.566 shRNA expression cassette would likely decrease costs, as the amount of yeast required for male mass-rearing is expected to be less. In this investigation, we aimed to combine the use of the Cas-CLOVER and Super piggyBac (sPB) [16] systems to generate yeast strains bearing multiple copies of a high-expression GGT.566 expression cassette.

Cas-CLOVER is an RNA-guided system which maintains the simplicity and high efficiency of the original CRISPR/Cas9 system but employs the high fidelity dimeric Clo051 genome editing nuclease [16] to prevent off-target editing and enable efficient knock-in generation. The Cas-CLOVER system seamlessly integrates with existing gRNA design and manufacturing platforms and is therefore applicable in a variety of synthetic applications [16]. Cas-CLOVER transgenesis, which is not limited by cargo size, is suitable for the integration of GGT.566 shRNA biopesticide cargo in conjunction with selectable nutritional markers [16]. Cas-CLOVER can be used in combination with the sPB transposase/transposon system, which recognizes transposon-specific inverted terminal repeats (ITRs), integrating the ITRs, intervening DNA at TTAA sites within the host genome, and enabling straightforward and consistent transgenesis [16]. In this study, it was hypothesized that multiple copies of the GGT.566 shRNA expression cassette could be successfully

integrated into the genome of a robust strain of *S. cerevisiae* using the Cas-CLOVER and sPB systems.

## 2. Materials and Methods

### *2.1. Yeast Strain Construction*

Generation of the *S. cerevisiae* FL100 bioproduction yeast strain was carried out as previously described in Brizzee et al. [17].

A *URA3* selection plasmid with a CEN/ARS origin of replication was used to express sPB transposase. A fragment containing the *LEU2* gene expressed under the control of the *LEU2d* promoter was incorporated between the piggyBac ITRs. The GGT.566 shRNA [15] expression cassette, which enables shRNA expression under the control of the *GAP* promoter [18], with termination via the *CYC1* terminator [19], was inserted into a multi-cloning site between the piggyBac ITRs and upstream of the *LEU2* marker. The *S. cerevisiae* strain FL100 was then transformed using the EZ-yeast Transformation Kit per the manufacturer's instructions (Zymo Research, Irvine, CA, USA). The desired transformants were selected according to their growth on SCD-Ura plates and then through a second round of selection on SCD-Leu plates. The selected colonies were subsequently transferred to 96-well plates and expanded for further analyses.

Following amplification of the region contained between 200 bp upstream and downstream of the *URA3* gene or the *LEU2* genes in the parent *S. cerevisiae* FL100 strain, auxotrophies were restored by transforming the products as described above, then growing the yeast on selective media (CM-URA or CM-LEU2). The resulting colonies were chosen for subsequent qPCR analysis, which enabled quantification of GGT.566 expression levels with respect to the control strains, DMT4-25.1R and DMT9-33.1, that had been engineered to express GGT.566 using transient expression from the pRS426_566 GPD shuttle vector [15] and piggyBac integration with a native *LEU2* promoter driving the expression of one copy of GGT.566, respectively.

A control strain, DMT4-347.1R, was generated in a similar manner in a previous study [17] and used for control studies in this investigation. This strain expresses an shRNA with no known target in mosquitoes [14] and has the following genotype: *MATa*, *PiggyBac* (*LEU2/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *2um* (*URA3/SPBase_Sc-CO*), *PiggyBac* (*HIS3/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) *PiggyBac* (*trp1d/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*).

### *2.2. Evaluation of GGT.566 Expression*

GGT.566 expression was assessed following RNA extraction from 200 µL of cells grown in a yeast extract peptone dextrose (YPD) culture using the YeaStar™ RNA Kit (Zymo Research, Irvine, CA, USA). cDNA was synthesized using the SuperScript™ IV VILO Master Mix with ezDNase (Thermo Fisher Scientific, Waltham, MA, USA). GGT.566 expression was quantified through cDNA amplification using the PowerUp™ SYBR™ Green Master Mix for qPCR kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems QuantStudio 6 Pro Real-Time PCR system. Primer sequences were as follows: *ALG9*, ALG9-Forward 5′-ATCGTGAAATTGCAGGCAGCTTGG-3′ and ALG9-Reverse 5′-CATGGCAACGGCAGAAGGCAATAA-3′; *GGT.566*, GGT.566-Forward 5′-TAGAACTAG TGGATCCAGACTTAC-3′ and GGT.566-Reverse 5′-TCCTTCCTTTTCGGTTAGAGC-3′. PCR reactions were performed in five replicate wells, and the results were standardized to *ALG9* levels and GGT.566 levels in DMT4-25.1R #1 using the ΔΔCt method [20]. The initial relative GGT.566 levels were compared to that of the strain DMT9-33.1, which was generated to express GGT.566 levels using piggyBac integration alone with a native *LEU2* promoter driving GGT.566 shRNA.

### *2.3. Whole Genome Sequencing (WGS) of Yeast Strains*

WGS performed by Oxford Nanopore Technology, Oxford, UK, as well as externally by NovaSeq PE150, Novogene, Sacramento, CA, USA, was used to identify the genomic

integration sites of DMT9-47.9R #2. Yeast gDNA was extracted using the NEB Monarch HMW gDNA Extraction Kit for Tissue (NEB #T3060S/L, Ipswich, MA, USA) and size selection to enrich for longer DNA fragments was performed as described by Maghini et al. [21]. The Flongle Flow Cell (FLO-FLG114) protocol [22] was used with the sequencing kit SQK-LSK114 for the sequencing of yeast gDNA, which was performed on a MinION Mk1B through MinKNOW 23.04.6 with a 20 h runtime. Live base calling was performed through internal guppy6.3.9 software in MinKNOW. Passed reads were mapped to the piggyBac transposon reference map with the Minimap2.24 plugin for Geneious Prime 2023.2.1 [23]. BLASTn [24] was used to identify the genomic integration sites of the sequences that mapped to the transposon.

### *2.4. Pilot Fermentations*

Pilot fermentations were pursued at The Michigan State University (MSU) Bioeconomy Institute, Lansing, MI [25]. Two separate pilot fermentations were pursued at both the 5 L and 10 L scale using high cell density (HCD) [26] and a proprietary Demeetra fermentation media (DFM) recipe. The seed rounds required for the production of innocula for HCD cultivation were grown for 16 h in 50 mL of HCD seed media, as described by van Hoek et al. [26]. A total of 42.5 mL of seed 1 was added to 850 mL of HCD seed media, and seed 2 was subsequently grown for 24 h at 30 °C and 250 rpm. For DFM cultivation, the seed rounds were grown in YPD media at 28 °C and 225 rpm. For the HCD pilot, seed batch cultures were grown at 30 °C, pH 5.0, and 2 vessel volumes per minute (vvm) of air, while $O_2$ was supplied if dissolved oxygen fell below 20%. The same conditions were used for the DFM pilot, except that it was performed at 28 °C with 1 vvm of air. Optical density (OD) levels were recorded throughout the 72-h fermentation period and at 24-h intervals, beginning with the fermentation inoculation (timepoint 0). Cells were then pelleted and stored at −80 °C for subsequent qRT-PCR analyses in which the relative expression was compared to GGT.566 expression levels at the time of inoculation.

### *2.5. Mosquito Assays*

*Culex quinquefasciatus* JH (provided by the CDC to be distributed by BEI Resources, NIAID, NIH: Eggs, NR-43025, Manassas, VA, USA) were reared as described in [27] for use in larvicide assays. Adult females were given defibrinated sheep's blood (Hemostat) using a Hemotek membrane feeding system (Hemotek LLC, Blackburn, UK). The yeast for these assays was prepared as described in [28] and dried through lyophilization or prepared at the MSU facilities (see above) and subsequently lyophilized. Larvicide assays were performed as described in [28], using 20 mg of Koi food and 20 mg of either larvicidal yeast or control yeast fed to 20 first instar larvae in 500 mL plastic cups with 50 mL of distilled water. Mortality, adult emergence rates, and sex were recorded in three replicate trials per treatment. The Chi-squared test was used to identify statistically significant differences between the observed and expected 1:1 male/female ratios and male and female survival.

## 3. Results

### *3.1. Production and Evaluation of Culex Sex-Separation Yeast Strains*

#### 3.1.1. Generation of a Robust Yeast Strain with Multiple Integrations of the GGT.566 shRNA Expression Cassette

An auxotrophic yeast strain bearing *ura3* and *leu2* gene deletions was used in conjunction with the sPB transposase/transposon technology to facilitate positive selection and efficient detection of the integrated GGT.566 cargo marked by a nutritional rescue marker. Yeast strains displaying a range of GGT.566 expression levels were generated (Figure 1). Strains displaying the highest levels of GGT.566 shRNA, the expression of which was driven with a constitutive GAP promoter [18], were chosen for further characterization. Following the restoration of yeast nutritional gene auxotrophies (designated by 'R') in these strains (Table 1), the expression levels of GGT.566 shRNA were once again examined (Figure 2). These assays revealed that the expression levels of GGT.566 were ~40–80 times higher than

those observed in the plasmid-based yeast strain [15] (Figure 2, see DMT9-47.9R #1 to #6 clones). These results suggested that the new second-generation strains likely contained multiple copies of the GGT.566 shRNA expression cassette. Whole genome sequencing (WGS) of DMT9-47.9R #2, the strain with the highest and most consistent levels of GGT.566 shRNA expression (Figure 2), was used to further investigate this.

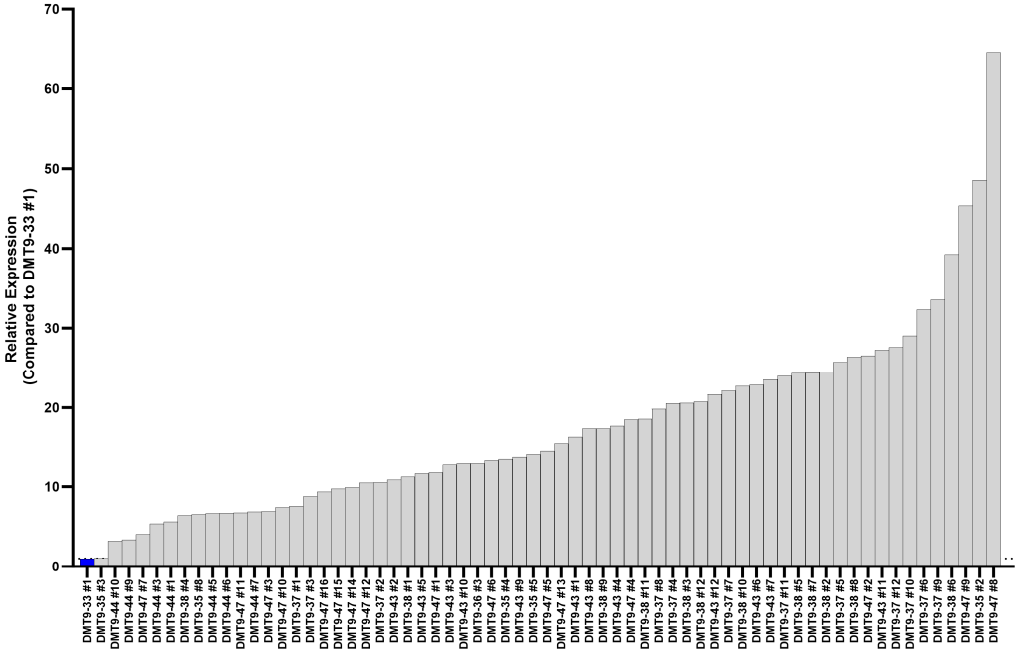


**Figure 1.** Yeast strains expressing varying levels of GGT.566 shRNA. Levels of GGT.566 shRNA expression in the Cas-CLOVER/piggyBac-engineered yeast strains were evaluated through qRT-PCR. The relative expression levels of GGT.566 shRNA with respect to the DMT4-251R #1 piggyBac integration strain (blue) are shown.

**Table 1.** Genotypes of *S. cerevisiae* strains.

| shRNA Type | Strain | Genotype | Original Auxotrophy | Restored Genotype |
|---|---|---|---|---|
| **GTT.566** | DMT9-47.9R #2 | *MATa*, *ura3Δ0*, *leu2Δ0*, *Piggybac* (*leu2d/*$P_{TDH3}$*-shRNA_566-*$T_{CYC1}$, $P_{TDH3}$*-shRNA_566-*$T_{CYC1}$, $P_{TDH3}$*-shRNA_566-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) | Uracil | *MATa*, *Piggybac* (*leu2d/*$P_{TDH3}$*-shRNA_566-*$T_{CYC1}$, $P_{TDH3}$*-shRNA_566-*$T_{CYC1}$, $P_{TDH3}$*-shRNA_566-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) |
| **Control** | DMT4-347.1R [a] | *MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ0*, *trp1Δ0*, *PiggyBac* (*LEU2/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *2um* (*URA3/SPBase_Sc-CO*), *PiggyBac* (*HIS3/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) *PiggyBac* (*trp1d/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) | Uracil | *MATa*, *PiggyBac* (*LEU2/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *2um* (*URA3/SPBase_Sc-CO*), PiggyBac (HIS3/$P_{TDH3}$-shRNA_Ctrl-$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) *PiggyBac* (*trp1d/*$P_{TDH3}$*-shRNA_Ctrl-*$T_{CYC1}$), *CEN/ARS* (*URA3/SPBase_Sc-CO*) |

[a] Described in reference [17].

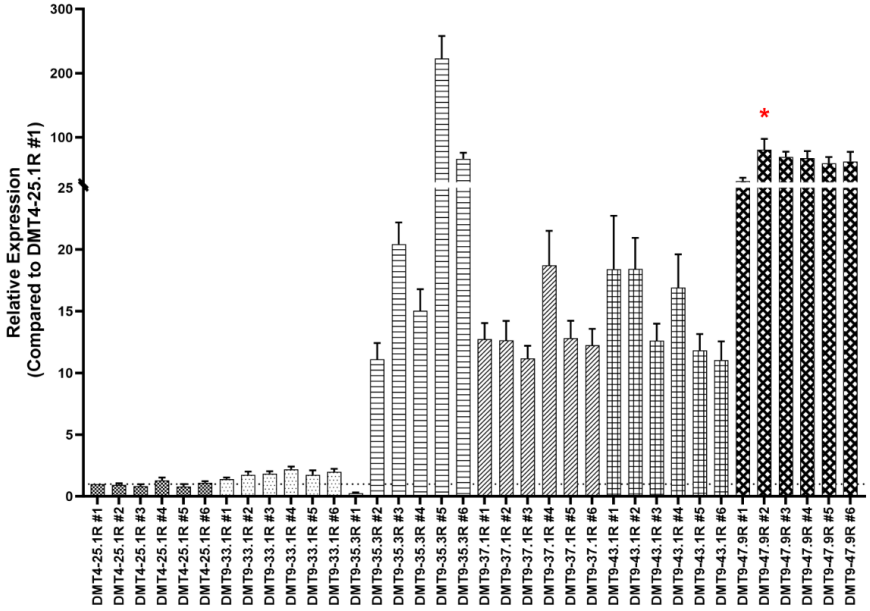


**Figure 2.** GGT.566 expression in down-selected yeast strains following auxotrophy restoration. Six clones (#1–#6) from each of the down-selected parental non-restored strains (each represented by a different histogram) were assayed for their GGT.566 expression. GGT.566 expression levels with respect to DMT4-25.1R #1, a transiently expressed GGT.566 strain, are shown. DMT9-47.9R #2 (red asterisk) was chosen for fermentation studies. Levels were assessed via qRT-PCR, and error bars correspond to SEM of five replicate wells.

### 3.1.2. WGS Reveals the GGT.566 Expression Cassette Integration Sites

The genomic integration sites of GGT.566 were revealed through WGS of DMT9-47.9R #2 using Illumina paired short reads and Oxford Nanopore Technologies long reads. Illumina sequencing yielded 10,835,186 reads, of which 69,272 reads mapped to the piggyBac transposon cargo. A total of 277 reads mapped to the flanking regions of the 3′ ITR, while 447 reads mapped to the flanking regions of the 5′ ITR, revealing the three genomic integration sites of DMT9-47.9R #2 (Figure 3, Table 2). Four copies of GGT.566 shRNA were integrated at each site, for a total of twelve copies (Figure 3, Table 2). The first integration was in Chromosome 7 (NC_001139) between the *TDH3* and *PDX1* genes. The second integration was in Chromosome 8 (NC_001140) between the *OSH3* and *QNS1* genes. Lastly, the third integration was found in Chromosome 10 between the *CYC1* and *FMP46* genes. All integrations by sPB were intergenic.

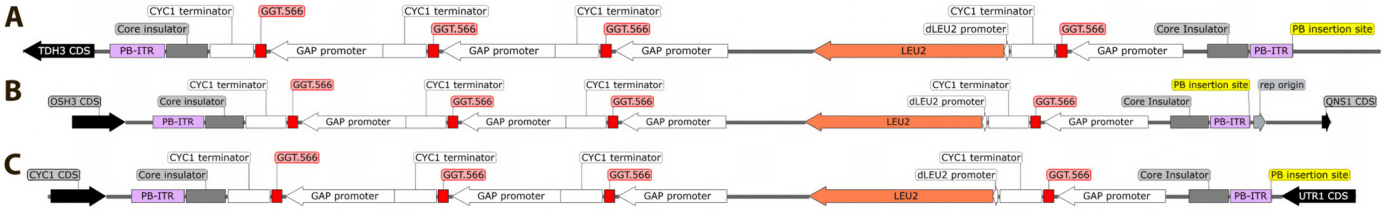


**Figure 3.** Genomic integration sites of the GGT.566 expression cassette in the DMT9-47.9R #2 strain. A GGT.566 shRNA expression cassette bearing four copies of the GGT.566 shRNA expression construct (purple) is stably integrated between the *TDH3* and *PDX1* genes in Chromosome 7 (**A**), between the *OSH3* and *QNS1* genes in Chromosome 8 (**B**), and between the *CYC1* and *UTR1* genes in Chromosome 10 (**C**). Black arrows = flanking genes' coding sequence (CDS) and transcriptional direction. Light purple boxes = piggyBac ITRs; gray boxes = ITR core insulators; White arrows = constitutive GAP promoters driving expression of GGT.566 shRNA (red) and terminated by the *CYC1* terminators (white boxes); Small white arrows = defective/truncated *LEU2* promoters for expression of the *LEU2* gene (orange arrows); piggyBac integration sites are highlighted in yellow.

**Table 2.** Summary of genomic integration sites revealed by WGS of strain DMT47.9R #2.

| Strain | Integrations | GGT.566 Copies | Total Copies | Genomic Integration Site | 5′ Flanking Sequence (60 bp) | 3′ Flanking Sequence (60 bp) |
|---|---|---|---|---|---|---|
| DMT9-47.9R #2 | 3 | 3 | 9 | Chromosome VII (883,906) | AAGTTCTTGGTGTTT-TAAAACTAAAAA-AAAGACTAACTA-TAAAAGTAGAAT-TTAAGAAGT | GAAATAGATTTACAG-AATTACAATCAATAC-CTACCGTCTTATATA-CTTATTAGTCAAGT |
| | | | | Chromosome VIII (245,747) | ACTATATACAACATG-AAGGACTTTATAAAT-TCTTGATCAATCTTT-CACACTAATTTTATA | CAGTTCTACACTATT-TTTGTTTGCTTGACT-GTAAAAACATTCTAT-TTTATGATTAGTTGC |
| | | | | Chromosome X (526,821) | AGGAGTTAGACAACC-TGAAGTCTAGGTCCC-TATTTATTTTTTTTA-ATAGTTATGTTAGTA | GAACGTTATTTATAT-TTCAAATTTTTCTTT-TTTTTCTGTACAAAC-GCGTGTACGCATGTA |

### 3.2. Assessment of Female-Specific Yeast Larvicide Activity

The impact of rearing larvae on DMT9-47.9R #2 yeast was examined in *C. quinquefasciatus* larvae. No significant male or female death was observed in larvae reared on the DMT4-347.1R (Table 1) control RNAi yeast or on a normal laboratory diet (Figure 4). However, larval consumption of DMT9-47.9R #2 yeast resulted in significantly higher than expected male/female ratios (Figure 4, Table 3, $p < 0.001$). Although treatments of larvae with DMT47.9R #2 had no significant impact on the survival of males to adulthood (Figure 4, $p > 0.05$), only 23 ± 6% of expected *C. quinquefasciatus* female mosquitoes survived to adulthood (Figure 4, $p < 0.001$).

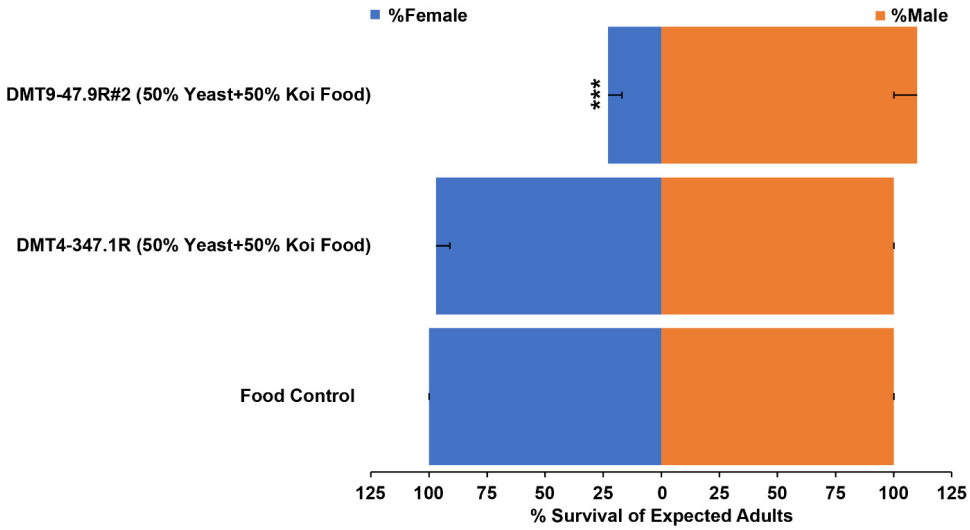


**Figure 4.** Female-specific larvicide activity of the DMT9-47.9R #2 Cas-CLOVER yeast strain. Larval consumption of yeast-interfering RNA larvicide DMT9-47.9R #2 resulted in significant female *C. quinquefasciatus* larval mortality (*** = $p < 0.001$) but did not impact male survival ($p > 0.05$) with respect to larvae reared on DMT4-347.1R control RNAi yeast or on a normal laboratory diet (food control). The percentage of adult emergence is shown, and error bars correspond to SDs.

**Table 3.** DMT9-47.9R #2 induces significantly higher male/female ratios.

| Treatment | Male:Female [a] | *p*-Value |
|---|---|---|
| Food Control | 1:1 | 1 |
| DMT4-347.1R (50% Yeast + 50% Koi Food) | 1:1 | 0.86 |
| DMT9-47.9R #2 (50% Yeast + 50% Koi Food) | 5:1 | $2.1 \times 10^{-9}$ |

[a] The male/female ratios shown correspond to the data displayed in Figure 4.

### *3.3. Performance of Strains in Pilot Fermentations*

The potential for culturing the DMT9-47.9R #2 yeast strain in industrial-sized fermentations was evaluated in pilot studies (Figure 5). At a 5–10 L fermentation scale, DMT9-47.9R #2 yielded 31.7 and 88.6 g/L in dry cell weight (DCW) when cultured using two different fermentation media, respectively (Figure 5). The optical densities at 600 nm ($OD_{600}$) reached an astonishing 93.45 and 156.9 in Demeetra proprietary fermentation media (DFM) and in high cell density (HCD) media, respectively.

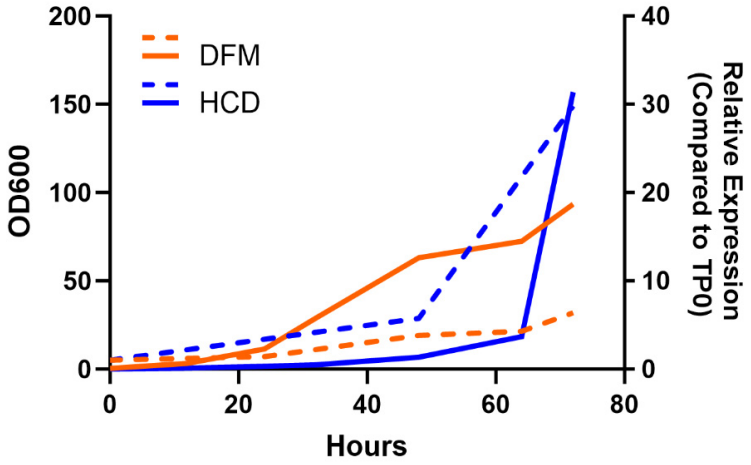


**Figure 5.** Growth curves and corresponding GGT.566 shRNA expression observed in pilot fermentations of strain DMT9-47.9R #2 using two fermentation media, high cell density (HCD) and Demeetra proprietary fermentation media (DFM). The growth curves obtained during the 72 h pilot-scale fermentations in HCD (blue) and DFM (red) media are shown. The solid lines correspond to OD600 readings obtained for cultures grown in each type of media. The expression levels of GGT.566 shRNA (dashed line) were quantified via qRT-PCR.

### *3.4. Evaluation of the Larvicidal Activity of Yeast Produced during the Scaled Fermentation*

The activity of DMT9-47.9R #2 yeast prepared during the pilot fermentation was confirmed in *C. quinquefasciatus* larvae (Figure 6). As expected, these assays demonstrated that the yeast produced during pilot fermentations induced significant female larval death ($p < 0.001$). Only 23 ± 6% of expected female larvae survived to adulthood. Female larval death resulted in significantly increased male/female ratios in adults (Table 4, $p < 0.001$), even at dosages that were one-half of those utilized in the first-generation GTT.566 yeast strain [15].

**Table 4.** DMT9-47.9R #2 yeast produced during a pilot fermentation induces significantly higher male/female ratios.

| Treatment | Male/Female [a] | *p*-Value |
|---|---|---|
| Food Control | 1:1 | 0.72 |
| DMT9-47.9R #2 (50% Yeast + 50% Koi Food) | 3.5:1 | $8.4 \times 10^{-10}$ |

[a] The male/female ratios shown correspond to the results displayed in Figure 6.

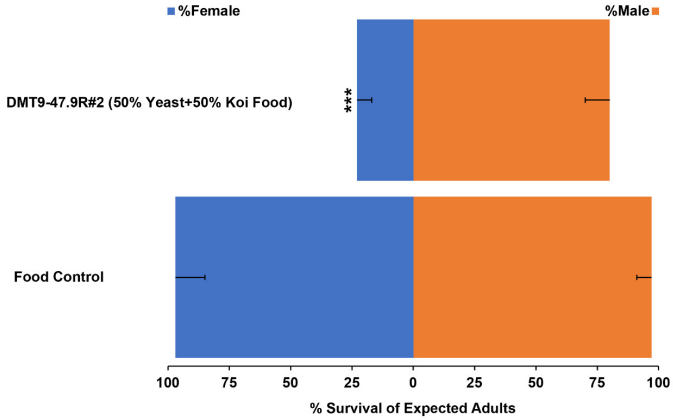


**Figure 6.** Female-specific larvicide activity is maintained following pilot fermentations. DMT9-47.9R #2 yeast prepared in pilot fermentations resulted in significant mortality of *C. quinquefasciatus* female larvae ($p < 0.001$, denoted by ***) yet did not significantly impact male survival with respect to larvae reared on a regular diet ($p > 0.05$). The percentage of adult emergence is shown, and error bars correspond to SDs.

## 4. Discussion

Although several emerging mosquito control technologies, such as SIT and IIT, involve mass releases of adult males, methods of sex sorting which can be deployed globally have not yet been established [10]. Our previous screens, combined with genetic engineering in *S. cerevisiae*, resulted in the production of first-generation laboratory-based RNAi yeast strains that permitted proof-of-concept male sex-sorting experiments on mosquito larvae [15,29]. We predicted that *S. cerevisiae* would be an excellent system for enabling the broad application of RNAi technologies, which are dependent upon the production of interfering RNA molecules in an economically feasible, scalable, and sustainable fashion [13]. *S. cerevisiae* is a genetic model organism that lacks its own functional RNAi system [30,31], suggesting that it may be an excellent choice for expressing and accumulating high levels of recombinant shRNA [13]. In this study, we have succeeded in generating robust commercial-ready yeast strains that can withstand scaled fermentations on non-specialized yeast media. Moreover, we have demonstrated that the production of shRNA targeting the *C. quinquefasciatus GGT* gene in these strains greatly exceeds those of laboratory strains. These yeast strains, like the first-generation laboratory predecessor, GGT.566 [15], can result in significantly higher male/female ratios in mosquito larvae that are fed with the yeast during larval development (Figures 4 and 6; Tables 3 and 4). These findings demonstrate that the combined use of Cas-CLOVER and sPB represent an excellent method of integrating several copies of shRNA expression constructs into the yeast genome. This strategy, which is helpful for increasing the production of shRNA levels in a yeast strain, would likely be useful for increasing the production of many other RNAs or proteins in *S. cerevisiae.*

The Cas-CLOVER yeast strains generated in the current study performed well during pilot fermentations. The expression of shRNA GGT.566 in the DMT9-47.9R #2 strain consistently increased, reaching over five-fold higher expression levels during the 72-h fermentation period (Figure 5). These observations, combined with another recent study in which expression of a separate shRNA molecule was maintained uniformly in a pilot fermentation trial [17], are notable given that yeast has rarely been used as a system for RNA production [13]. These results suggest that yeast, as an excellent system for scaled shRNA production, could potentially be used for expressing other RNA molecules of interest. Although lyophilization was sufficient for drying the yeast generated in these pilot fermentation studies, commercial production will likely involve spray drying, which is more cost-effective [13]. Moreover, heat-inactivation of the yeast, which was pursued just prior to lyophilization in the current study, could potentially be achieved during the spray drying process, which can be pursued at temperatures that will inactivate yeast [13]. At the commercial stage, it will be important to institute quality control measures to ensure

that heat-inactivation during spray drying is complete prior to shipment of the yeast to mass-rearing facilities. In the laboratory, we have confirmed that heat-killing is sufficient by attempting to culture the yeast after heating, and no yeast growth has been observed in such experiments to date.

The levels of shRNA production achieved during the scaled fermentations were successfully monitored using a qRT-PCR approach (Figure 5). It should be noted that although amplification of cDNA generated from shRNA can be challenging due to the secondary structures of the hairpin, the strategy applied herein, which is based upon the one utilized to study first-generation *GGT*-silencing yeast strains [15], enabled successful amplification of the GGT.566 shRNA (Figures 2 and 4). While qRT-PCR reactions can be performed quickly, the development of faster methods of monitoring shRNA expression could nevertheless prove useful, particularly as optimization of larger industry-scale fermentations are pursued [13]. For example, Crook et al. [32] used a yellow fluorescence protein reporter system to monitor shRNA production during fermentation. This system, which can be monitored in even more "real time" than qRT-PCR, allowed the researchers to optimize shRNA expression during fermentation. Regardless, the levels of shRNA production achieved herein were more than sufficient for successful mosquito sex-separation applications (Figures 5 and 6).

We had previously demonstrated that yeast targeting *Culex GGT* genes can be incorporated into mosquito larval mass-rearing diets, permitting the generation of fit adult male *Culex* mosquitoes [15]. The generation of the Cas-CLOVER strains will permit scaled yeast production to support mosquito mass-rearing facilities worldwide. This could facilitate the development and expansion of eco-friendly SIT and IIT programs. Such efforts will be beneficial in many areas of the world which are impacted by *Culex* mosquitoes, nuisance biters which can also vector several arboviral diseases [12]. Moreover, *Culex* mosquitoes are a major threat to birds located on islands throughout the tropics, including the Hawaiian Islands, where avifauna are susceptible to significant population declines and future extinctions as a result of the introduction of non-native pathogens by *Culex* mosquitoes [33].

The *GGT* gene is well conserved in multiple species of mosquitoes, and our past efforts have demonstrated that silencing the *Aedes aegypti*, *Aedes albopictus*, and *Anopheles gambiae GGT* orthologs results in female larval death, with no significant impact on male survival [15]. Moreover, yeast has also been successfully incorporated into larval diets in support of the scaled rearing of these species [15,29]. The *Culex* results described herein indicate that the combined use of Cas-CLOVER and sPB would enable the preparation of robust second-generation yeast strains to support mass rearing of *A. aegypti*, *A. albopictus*, and *A. gambiae* mosquitoes. It would also be useful to design and characterize yeast strains targeting additional species of *Anopheles* mosquitoes. For example, the World Health Organization (WHO) recently issued a vector alert for *Anopheles stephensi* invasion and spread in Africa and Sri Lanka. *A. stephensi*, a highly competent malaria parasite vector, is an efficient vector of urban malaria [34]. The expansion of this invasive mosquito is a major threat to malaria control and elimination in Africa and southern Asia [34].

Notably, the successful separation of male mosquitoes observed with the new yeast strains was accomplished with only half of the amount of dried yeast that had been used in the original studies pursued with first-generation laboratory strains of yeast [15]. This is likely due to the significantly higher level of gene expression observed in the Cas-CLOVER strains of yeast (Figure 1) and the high levels of shRNA produced in the yeast during scaled fermentations (Figure 5), findings which demonstrate the cost-saving benefits of using Cas-CLOVER combined with sPB for strain optimization. Although female-specific yeast larvicides will be beneficial to *Culex* mosquito control programs, as discussed previously [15], the use of the yeast larvicides is not likely to be a stand-alone technique for programs that require >99% male mosquito purity, as ~20% of female mosquitoes do not succumb to yeast treatments, irrespective of which female-specific lethal genes have been targeted ([15,29], this study). The high levels of shRNA produced in the new Cas-CLOVER strains did not further increase female morality rates, perhaps because

some larvae may be eating the remains of dead larvae in the rearing trays rather than the yeast. Despite this, the male/female ratios achieved in this investigation (Tables 3 and 4) may nevertheless be sufficient for SIT releases in which adult mosquitoes are irradiated prior to releases [10]. Moreover, in instances in which even minor female contamination is problematic, it would still be possible to pair female-specific yeast feedings with other sex-separation methodologies [15]. For example, yeast feedings would greatly reduce the labor required for hand separation, and it might also allow automated sorting systems to act more efficiently [10,15].

The production of bioprocessing platforms using targeted gene-editing technology such as Cas-CLOVER has infinite potential in a variety of biotechnology sectors. Whether specifically knocking in RNAi cargo or knocking out genes essential for non-auxotrophic growth, Cas-CLOVER can be utilized in microorganisms to manufacture high quantities of RNAi-based biopesticides [16]. piggyBac has been shown to prefer highly accessible chromatin structures and highly transcribed genomic areas [35]. Thus, in combination with Cas-CLOVER, piggyBac transposase technology can identify genomic hotspots as well as enabling selection marker recycling in certain cases in which the knowledge of the genomic landscape of the microorganism is little to unknown. Another benefit of piggyBac is that piggyBac transposition does not require DNA synthesis, which is a characteristic of other transposases. Therefore, transposition by piggyBac requires no DNA repair due to the precise excision and integration of the transposable element, allowing for more streamlined strain verification strategies [36].

Given the growing interest in the use of RNAi applications in agriculture [37,38] and medicine [39,40], the results of this study indicate that the combined use of Cas-CLOVER and piggyBac technology could facilitate the generation of a variety of different RNA-expressing yeast strains. The use of yeast as an efficient, effective, and inexpensive system for RNA production could likely advance the development of new crop protection tools and RNAi-based therapeutics. The approval of RNAi-based agricultural interventions by the U.S. Environmental Protection Agency [41] and several RNAi-based therapeutics by the U.S. Food and Drug Administration [42–46] has paved the way for the development and potential registry of additional RNAi-based agricultural [47] and medical technologies. Thus, in addition to helping to minimize the global burden of mosquito-borne illnesses, the results of this study could have broader implications in the agricultural and medical communities.

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