

Recombinant venom proteins in insect seminal fluid reduce female lifespan

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The emergence of insecticide resistance has increased the need for alternative pest management tools. Numerous genetic biocontrol approaches, which involve the release of genetically modified organisms to control pest populations, are in various stages of development to provide highly targeted pest control. However, all current mating-based genetic biocontrol technologies function by releasing engineered males which skew sex-ratios or reduce offspring viability in subsequent generations which leaves mated females to continue to cause harm (e.g. transmit disease). Here, we demonstrate *intra*-generational genetic biocontrol, wherein mating with engineered males reduces female lifespan. The toxic male technique (TMT) involves the heterologous expression of insecticidal proteins within the male reproductive tract that are transferred to females via mating. In this study, we demonstrate TMT in *Drosophila melanogaster* males, which reduce the median lifespan of mated females by 37 – 64% compared to controls mated to wild type males. Agent-based models of *Aedes aegypti* predict that TMT could reduce rates of blood feeding by a further 40 – 60% during release periods compared to leading biocontrol technologies like fsRIDL. TMT is a promising approach for combatting outbreaks of disease vectors and agricultural pests.

Insect pests pose a major challenge to human and environmental health. Malaria, spread by several species of *Anopheles* mosquitoes, causes 608,000 deaths per year¹ and rates of arboviral diseases spread primarily by the *Aedes aegypti* mosquito, including dengue, Zika, chikungunya, and yellow fever, are reaching unprecedented levels due to increased global trade and warming climates². Dengue virus alone causes 390 million human infections each year and is now considered the most common vector-borne viral infection worldwide³. Recent studies have calculated that the cumulative cost of biological invasions is increasing four-fold every decade⁴, with a conservative estimate of US\$162.7 billion for damages caused in 2017⁵. Invertebrate pests, primarily insects, are responsible for ~40% of these damages^{5,6}, and estimates of annual losses for all major food crops ranges between 20 and 30% due to damages by pests and pathogens⁷. Arboviral diseases such as avian malaria also pose a significant ecological threat to many native species, and are believed to be a key driver of population decline in New Zealand and Hawaiian forest birds^{8,9}.

Pesticides are the first line of defence against many invasive species, particularly mosquitoes¹⁰. However, over-reliance on insecticides has resulted in the widespread emergence of resistance¹¹. Over-application of insecticides has also resulted in declining populations of non-target species, and are responsible for many other environmental and human-health concerns¹². Modern integrated pest management is trending towards reducing reliance on chemical insecticides in favour of other environmentally friendly management techniques to reduce the emergence of resistances^{10,13}.

Genetic biocontrol, defined as the release of organisms which have been genetically altered to reduce the spread and harm caused by a target species¹⁴, is one such alternative that's been gaining broader public acceptance¹⁵. Early and well established genetic biocontrol technologies (GBT) such as the sterile insect technique (SIT) involve the release of mass numbers of males which have been radiologically sterilised¹⁶, thereby reducing the reproductive potential of the females. More modern GBT, such as the release of insects carrying a

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dominant lethal gene (RIDL)¹⁷ and gene drives¹⁸ function by propagating transgenes which reduce the fitness of future generations as the transgenes are inherited. Cage and field trials of these second-generation GBT have begun to demonstrate their improved efficacy^{19–21}, requiring fewer released males compared to conventional approaches to achieve similar population control, albeit with a potential trade-off between enhanced efficacy and reduced control over the degree of transgene introgression¹⁴.

Currently, all GBT require a minimum of a generation to take effect on the target population and often much longer until the harm from a pest outbreak is mitigated¹⁹. While this generational lag period is less problematic for species where immature life stages are the primary cause of harm, it poses a significant challenge for many disease vectors and agricultural pests where adult females are the main concern. For example, wild female *Ae. aegypti* have a median adult lifespan of 2–3 weeks^{22,23}, will typically mate within 24–48 h of emerging²⁴, and on average will take 0.63–0.76 blood meals per day²⁵. Females that mate with GBT males may not produce viable offspring, but they can continue to spread disease or damage crops. Although current GBT are highly effective at intergenerational population control, a more rapid response to outbreaks of disease vectors is critical to mitigate the spread of arboviruses and avert the risk of epidemics, particularly in regions with seasonal population dynamics²⁶.

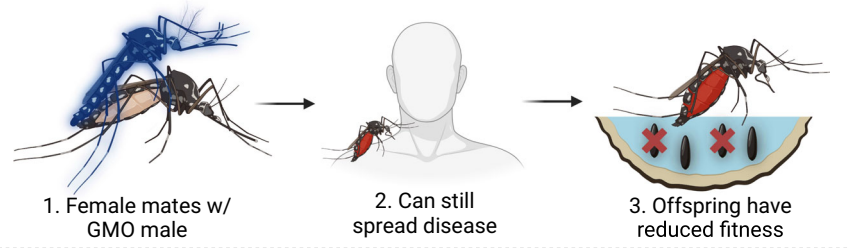
An alternative approach to GBT that has been speculated on is utilising seminal fluid proteins (SFP) as a target to affect the fitness or fertility of mated females^{27–30}. SFPs are produced within the male accessory glands (MAG) and stored within the lumen until they are transferred to females along with sperm and other compounds. SFPs such as ovulin (*Acp26Aa*) and sex peptides are known to generate various post-mating responses and physiological changes in females, such as reduced mating receptivity and median lifespan^{31,32}. It has been demonstrated in *Drosophila melanogaster* that several low molecular weight SFP can pass through the female reproductive tract and enter the circulatory system (haemolymph), and even to act on receptors in the central nervous system (CNS)^{33,34}. Evidence is mounting that mosquito SFP also act on receptors found within the CNS, with 30% of radiolabelled *Culex* SFP localising to the female head and thorax³⁵, and

intra-thoracic but not intra-abdominal SFP injections resulting in mating refractoriness in Anopheline and *Aedes* females^{36–39}.

Here we describe the toxic male technique (TMT), which is—to the best of our knowledge—the first example of an intragenerational GBT. By genetically engineering pest species to express toxic, low molecular weight compounds within the MAG, the survival of mated females can be reduced (Fig. 1). Using *D. melanogaster* as a proof of concept, we engineer males to express one of seven insect-specific venom proteins in a MAG-specific pattern. Of the >1600 characterised venom compounds, 60% of these are cysteine-knotted mini-proteins⁴⁰, which range from 2.6 to 14.8 kDa and are highly stable, taxonomically selective and less susceptible to pore region mutations associated with insecticide resistances⁴¹. Like endogenous SFP, the recombinant venom peptides should remain safely sequestered within the lumen of the MAG and not enter the male's haemolymph. If the recombinant venom peptides pass through the mated female's reproductive tract to affect the target ion channel receptors in her CNS, the neurotoxic effects are likely to be fast-acting and dose-dependent.

The median lifespans of wild-type females mated to two of the TMT strains are reduced by 37–64% after initial exposure compared to control females mated to wild-type males. Single-pair mating assays find that TMT males are able to court wild-type females as effectively as wild-type males. MAG-specific expression of a 5.2 kDa venom peptide has no significant effect on the lifespan of adult TMT males, but expression of a 2.9 kDa venom peptide reduces lifespan by 59% compared to wildtype and transgenic controls. To determine the theoretical capability of TMT to suppress a pest population in comparison to female-specific RIDL (fsRIDL), the current state-of-the-art GBT, we develop an agent-based model to simulate an idealised *Ae. aegypti* release programme⁴⁹. Sensitivity analysis of the model found that the improved performance of TMT compared to fsRIDL is consistent across a broad range of values of polyandry, density-dependent mortality, and release ratios, with TMT reducing blood feeding rates by a further 40–60% in most simulations. These results demonstrate the potential of TMT as the next generation of genetic biocontrol, which is especially suited to rapidly respond to outbreaks of disease vectors and agricultural pests.

A. Traditional intergenerational genetic biocontrol



B. Intragenerational genetic biocontrol

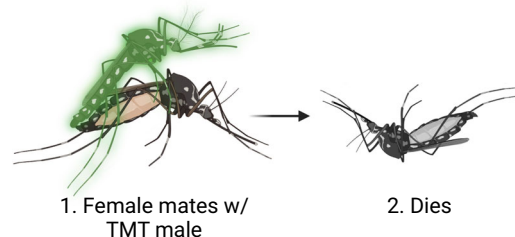


Fig. 1 | Intergenerational vs intragenerational genetic biocontrol of pest insects. Current mating-based methods of genetic biocontrol (A) function by affecting the viability or sex ratio of the offspring of subsequent generations. However, mated females persist in the target area and can continue to cause harm

(e.g. spread disease). Intragenerational biocontrol (B), such as TMT, directly affects the fitness of mated females, thereby rapidly reducing the harm caused by the target population. Created in BioRender. Maselko, M. (2024) <https://BioRender.com/r25q201>.

Table 1 | Candidate venom proteins investigated for accessory gland-specific recombinant expression

Venom protein	Native host	Target	kDA
μ -AGTX-Aa1d	<i>Agelenopsis aperta</i>	Na _v channel (site 4)	4.2
Γ -CNTX-Pn1a	<i>Phoneutria nigriventer</i>	NMDA glutamate receptors	5.2
δ -CNTX-Pn1a	<i>Phoneutria nigriventer</i>	Na _v channel (site 3)	5.3
κ -HXTX-Hv1c	<i>Hadronyche versuta</i>	BKCa channels	3.8
ω -HXTX-Hv1a	<i>Hadronyche versuta</i>	Ca _v 1 channels	4.1
ω -HXTX-Hv2a	<i>Hadronyche versuta</i>	Ca _v 2 channels	4.5
δ -AITX-Avd2a	<i>Anemonia sulcata</i>	Na _v channel (site 3)	2.9

Results

Heterologous expression of insecticidal venom proteins

We first identified a list of candidate venom proteins based on several criteria. Critically, the target receptor of the venom could not be present in the male reproductive tract, which was determined by tissue-specific gene enrichment values of the target ion channel given by FlyAtlas 2⁴². The venom proteins must also only interact with invertebrate ion channels, with no measurable LD50 found for mammals. Candidates were preferred if they had lower LD50 for *Drosophila* or another Dipteran. Candidates of lower molecular weight were also preferred, as we expect that to increase the likelihood of the venom proteins entering the haemolymph of mated females. To keep the number of candidate proteins manageable, we limited the list to the most promising candidate for a given ion channel target or class of neurotoxin (agatoxin, ctenitoxin, etc.) given our criteria (Table 1). Venom protein sequences were cloned into a UAS expression vector along with the signal peptide of an endogenous SFP (*Acp26Aa*) to generate transgenic *D. melanogaster* strains.

Testing functional expression of recombinant venoms

To assess the functionality of the recombinant venom proteins in a heterologous host, homozygous UAS:venom strains were crossed to hemizygous *Act5C-GAL4* flies⁴³. This GAL4 parental line will transmit one of two homologous chromosomes to its offspring: one containing the gene for the GAL4 transcriptional activator under regulation of the strong Actin-5C promoter; or the balancer chromosome containing a CyO marker resulting in a curled-wing phenotype. Progeny from this cross which inherit the *Act5C-GAL4* chromosome would be non-viable if the venom is functionally expressed by the flies, as ubiquitous expression of the venom peptide should be immediately lethal, so 100% of the viable offspring ought to have inherited the CyO chromosome and display curled-wing phenotype. Conversely, if the venom is non-functional, then inheritance of either the *Act5C-GAL4* or CyO chromosome would have no impact on the viability of the offspring, so 50% of the offspring would display the curled-wing phenotype. From these crosses ($N=3$), progeny from 5/7 of the UAS:venoms strains displayed 100% curled-wing phenotype, including μ -AGTX-Aa1d, Γ -CNTX-Pn1a, κ -HXTX-Hv1c, ω -HXTX-Hv1a and δ -AITX-Avd2a. Progeny from UAS:venom strains expressing δ -CNTX-Pn1a exhibited $46 \pm 2\%$ curled-wing phenotype, and ω -HXTX-Hv2a exhibited $44 \pm 10\%$, so these venom transgenes were presumed to be non-functional and not investigated further.

Accessory gland-specific expression of transgenes

The success of this approach hinges on our ability to get high levels of MAG-specific expression of the venom proteins, while reducing off-target expression as much as possible. We could not express the venom proteins directly by a MAG-specific promoter, as the strains would not be stable and would require backcrossing transgenic females to wild-type males each generation to maintain them. As such, a bipartite approach such as UAS:GAL4 was required to facilitate

separating the regulation of venom expression between the driver (GAL4) and responder (UAS) lines. To determine the most suitable expression pattern, we tested three different MAG-specific GAL4 drivers: *antr-GAL4*; *prd-GAL4*; and *Acp26Aa-GAL4*^{44–46}. These drivers were crossed to a UAS:*mCherry* reporter⁴⁷, and the degree of on and off-target expression for each cross was determined by fluorescence microscopy of dissected male progeny (Fig. 2). *prd-GAL4* produced the strongest accessory gland-specific RFP expression, but RFP was also observed in the digestive tracts of adult males (Fig. 2b, e). *Acp26Aa-GAL4* was highly specific to the accessory glands, but the expression was relatively weak (Fig. 2c, f) Only *antr-GAL4* produced a strong fluorescent signal in the accessory glands without also driving expression in earlier developmental stages or off-target tissue (Fig. 2a, d).

TMT males reduce the lifespan of mated females

Lifespan assays of mated females were performed to determine whether exposure to TMT males reduces the lifespan of wild-type females. TMT males were prepared by crossing UAS:venom strains (μ -AGTX-Aa1d, Γ -CNTX-Pn1a, κ -HXTX-Hv1c, ω -HXTX-Hv1a or δ -AITX-Avd2a) to the *antr-GAL4* driver, and transheterozygous male offspring were isolated soon after eclosion. As female *D. melanogaster* are poorly receptive to mating for 24–48 h post-eclosion⁴⁸, virgin wild-type females were isolated for 2–3 days to ensure that they had reached sexual maturity. Females were then housed in groups of eight with an equal number of TMT or wild-type males and were transferred to fresh media every 3 days. As female *D. melanogaster* are refractory to mating for at least 5 days after fertilisation⁴⁹, to ensure that females had access to mates with fully replenished SFP when they became receptive, as well as to maintain the 1:1 sex ratio, males were replaced during each transfer. Females lived for a median of 27 days post-initial exposure to wild-type males (95% CI: 25–31 days, $N=40$, Fig. 3a). Females mated to Γ -CNTX-Pn1a TMT males had a median post-exposure (MPE) lifespan of 17 days (95% CI: 12–19 days, $N=40$, Fig. 3a), 37% lower than controls (log-rank test, χ^2 (1, $N=80$) = 20.7, $P < 0.005$). Females mated to δ -AITX-Avd2a TMT males had an MPE lifespan of 15 days (95% CI: 11–18 days, $N=40$, Fig. 3a), 44% lower than controls (log-rank test, χ^2 (1, $N=80$) = 17.7, $P < 0.005$).

Females mated to μ -AGTX-Aa1d or κ -HXTX-Hv1c TMT males did not have significantly reduced lifespans compared to wild-type controls, indicating that the reduction in lifespan observed in the Γ -CNTX-Pn1a and δ -AITX-Avd2a treatment groups is the result of specific venom expression rather than exposure to transgenic males. Females mated to ω -HXTX-Hv1a TMT males had an MPE lifespan of 20 days (95% CI: 17–23 days, $N=40$), 26% lower than controls (log-rank test, χ^2 (1, $N=80$) = 9.51, $P < 0.005$). However, most ω -HXTX-Hv1a TMT males were not surviving the 3 days until their replacement. Upon further investigation, we found that while expression of the primary subunit (*cac*) of the calcium channel targeted by ω -HXTX-Hv1a is not enriched in the MAG, the pore-forming subunit (*Ca- α 1D*) is. As such, expression of ω -HXTX-Hv1a was not investigated further.

Release programmes of currently available GBT such as SIT and RIDL utilise overflooding ratios of modified males to wild individuals^{19,50,51}. However, female *D. melanogaster* are known to have reduced fitness at higher rates of male exposure⁵². To determine if an increase in the sex ratio of TMT males results in a greater reduction in median lifespan than would be expected from a simple increase in male exposure, we performed another female lifespan assay with a 3:1 male-to-female sex ratio. In this assay, control females mated to wild-type males had an MPE lifespan of 22 days (95% CI: 15–30 days, $N=39$, Fig. 3b). Comparatively, females mated to Γ -CNTX-Pn1a TMT males had an MPE lifespan of 11 days (95% CI: 8–14 days, $N=40$, Fig. 3b), 50% lower than controls (log-rank test, χ^2 (1, $N=79$) = 24.4, $P < 0.005$), and females mated to δ -AITX-Avd2a TMT males had a MPE lifespan of

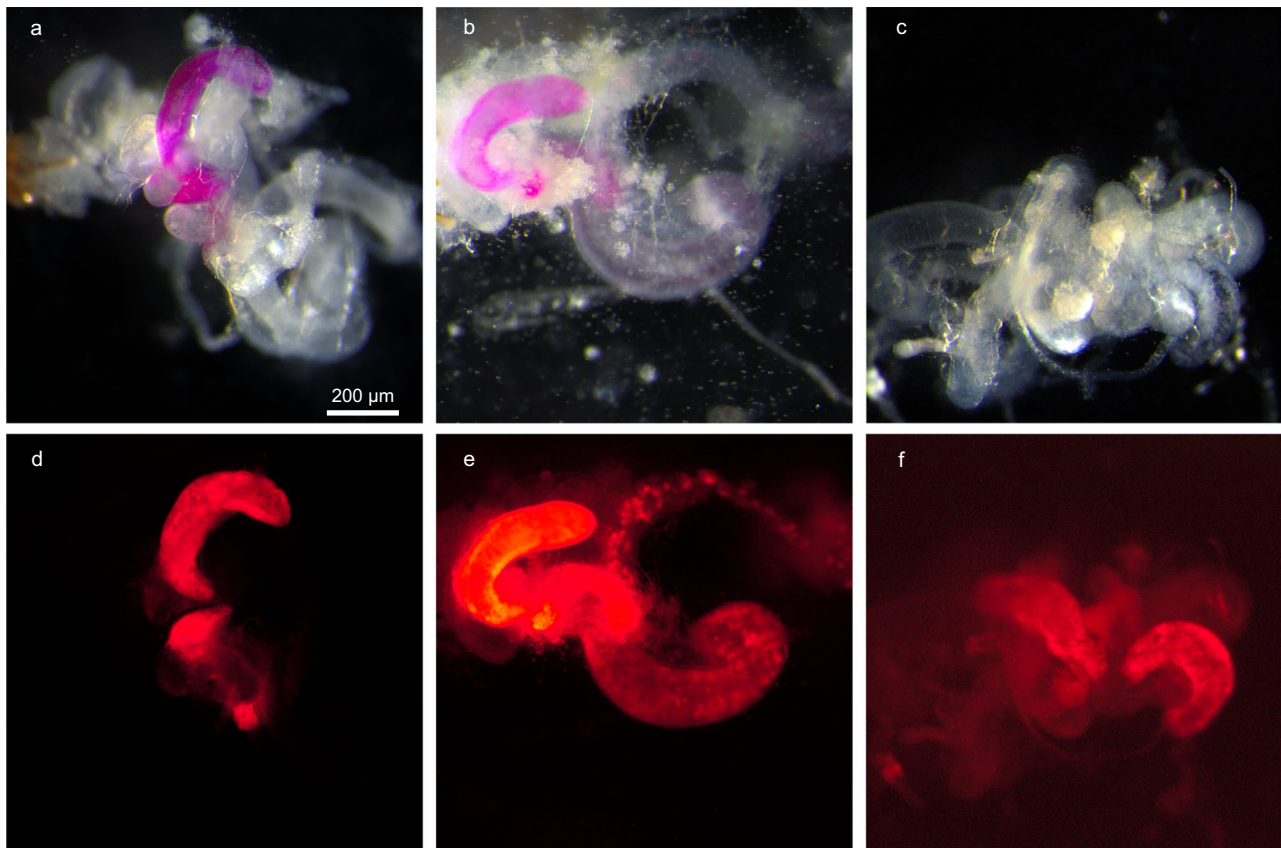


Fig. 2 | Accessory gland-specific expression of RFP in transgenic *D. melanogaster* males. Dissected reproductive and digestive tracts of adult male offspring from UAS:*mCherry* reporter and accessory gland-specific GAL4 drivers (*antr*-GAL4

(a, d), *prd*-GAL4 (b, e), and *Acp26Aa*-GAL4 (c, f) observed under bright-field (a–c) and red fluorescence (d–f) microscopy. Dissections were performed on ≥ 5 males per cross. Scale bar in a: 200 μm , applies to all panels.

8 days (95% CI: 6–13 days, $N = 40$, Fig. 3b), 64% lower than controls (log-rank test, χ^2 (1, $N = 79$) = 28.1, $P < 0.005$). The MPE lifespans of females mated 3:1 to wild-type males was not significantly reduced compared to females mated 1:1 to wild-type males (log-rank test, χ^2 (1, $N = 79$) = 0.2, $P = 0.6$), while females mated to 3:1 Γ -CNTX-Pn1a and δ -AITX-Avd2a TMT males had a 35% (log-rank test, χ^2 (1, $N = 80$) = 6.8, $P = 0.01$) and 47% (log-rank test, χ^2 (1, $N = 80$) = 7.4, $P = 0.01$) respectively reduced MPE lifespans relative to their equal ratio counterparts. Therefore, the negative effect of exposure to TMT males on the survival of mated females is greater than the negative effect of increased male exposure in general.

Single-pair courtship assay

To determine whether the expression of venom proteins in the accessory glands impaired the males' ability to court females, single-pair courtship assays were performed as previously described⁵³. A single wild-type virgin female was paired with a virgin male in a mating arena, which were observed for up to two hours to identify males which successfully courted a female. To control for off-target effects of accessory gland-specific expression of heterologous proteins, the competitiveness of negative control males expressing an unrelated low molecular weight protein (NbVHHO5⁵⁴, 13 kDa) in the same expression pattern (*antr*-GAL4/ UAS:*NbVHHO5*) was also measured. The percentage of wild-type males who successfully courted a female was 30% (95% CI: 19–44%, $N = 50$), Γ -CNTX-Pn1a TMT males was 30% (95% CI: 19–44%, $N = 50$), δ -AITX-Avd2a TMT males was 22% (95% CI: 13–35%, $N = 50$), and negative control males was 24% (95% CI: 14–37%, $N = 50$). No significant difference was observed in the ability of the males in any treatment group to court females (χ^2 (3, $N = 200$) = 1.3, $P = 0.73$).

Competitive mating assay

To determine if the intrinsic competitiveness of the GAL4-UAS transgenic males was negatively affected by variations in genetic background, we performed a control competitive mating assay in which equal numbers of virgin wild-type females, wild-type males, and *antr*-GAL4/UAS:*NbVHHO5* males were housed together overnight and allowed to mate freely. As transgenic offspring lacked a distinct phenotypic marker, 10 offspring from each female were genotyped to determine paternity. Male competitiveness (C) was calculated as $C = PW/O(1 - P)^{19}$, where P is the proportion of females which produced transgenic offspring, W and O are the number of wild and transgenic males, respectively, with $C = 1$ indicating equal competitiveness to wild-type males. The competitiveness of the control males was calculated to be just 0.14 (95% CI: 0.05–0.25, $N = 42$), indicating that the GAL4-UAS genetic background of the transgenic males significantly affected their mating competitiveness.

TMT male longevity

Longevity assays were performed to determine what effect accessory gland-specific venom protein expression had on the longevity of TMT males relative to wild-type and negative control NbVHHO5 males. Males were isolated soon after eclosion and housed in groups of 8, being transferred onto fresh media every 3 days. The median lifespan of wild-type males was 46 days (95% CI: 38–50 days, $N = 40$, Fig. 4), negative control males was 43 days (95% CI: 41–46 days, $N = 40$), Γ -CNTX-Pn1a males was 46 days (95% CI: 38–47 days, $N = 40$), and δ -AITX-Avd2a males was 19 days (95% CI: 11–27 days, $N = 40$). Γ -CNTX-Pn1a TMT males had no significant difference in longevity compared to wild-type males (log-rank test, χ^2 (1, $N = 80$) = 2.8, $P = 0.09$) or negative control males (log-rank test, χ^2 (1, $N = 80$) = 0.8,

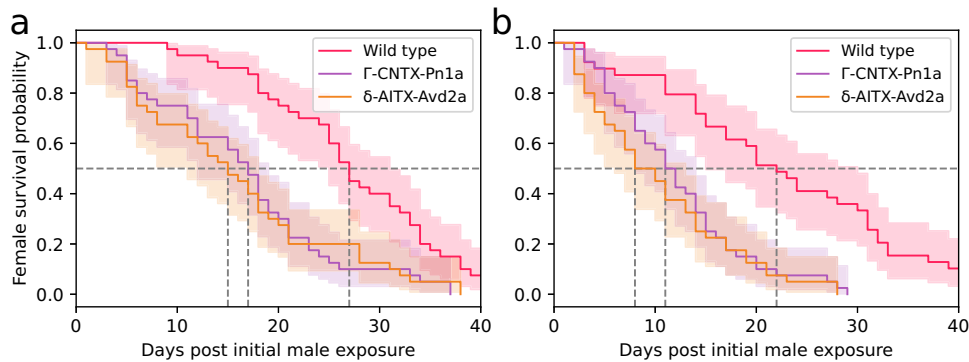


Fig. 3 | Female *D. melanogaster* exposed to TMT males have reduced lifespan. Females were exposed to males at an equal sex ratio (a), or with males at a three-fold excess (b). Data were presented as Kaplan–Meier survival probability estimates

with 95% Greenwood confidence intervals (indicated by the shaded area). The dashed lines indicate a median time of 50% survival. Source data are provided as a Source Data file.

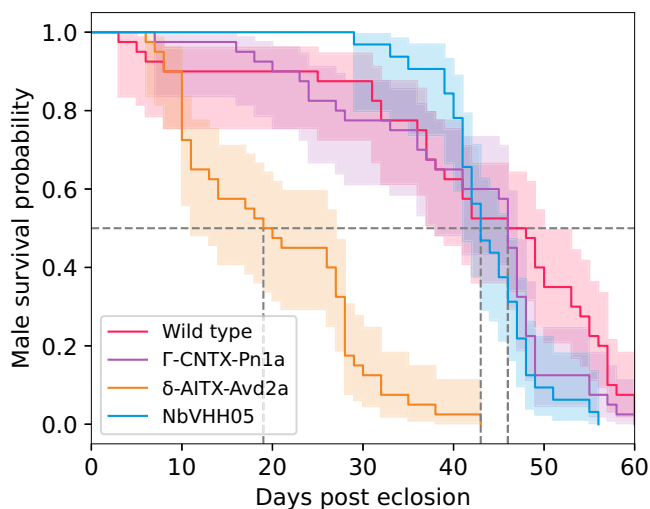


Fig. 4 | Longevity of male *D. melanogaster* expressing Γ -CNTX-Pn1a or δ -AITX-Avd2a venom proteins in their accessory glands compared to wild type and negative control (NbVHH05) strains. Data were presented as Kaplan–Meier survival probability estimates with 95% Greenwood confidence intervals (indicated by the shaded area). The dashed lines indicate time to 50% survival. Source data are provided as a Source Data file.

$P = 0.36$). However, δ -AITX-Avd2a TMT males had a 59% reduced median lifespan compared to wild-type males (log-rank test, χ^2 (1, $N = 80$) = 50.9, $P < 0.005$).

Modelling of *Ae. aegypti* population control

To evaluate how a TMT release programme might compare to currently deployed GBT in its ability to suppress a mosquito population, we developed an agent-based model simulating a hypothetical *Ae. aegypti* release programme of SIT, fsRIDL and TMT mosquitoes using GAMA 1.9 (<https://gama-platform.org>). This model simulates the full life cycle of each mosquito within the population in hourly intervals (Supplementary Fig. 1), with adult females having more complex behaviour in response to various internal and external factors such as age, time of day, mate choice, etc. (Supplementary Fig. 2). Models were instantiated with a seed population that is left for a year of simulation time to reach equilibrium, at which point adult transgenic males are released every 3 days until the end of the simulation at a release ratio of 12:1 males per wild adult. The frequency of polyandry was set to ~20%⁵⁵, and density-dependent mortality (DDM) of the immature stages was modelled as a logistic function (see Methods for further information). The fitness and lifespan of all transgenic

males were presumed to be equal to wild males, but the mating competitiveness estimated from Brazil field trials of RIDL mosquitoes⁹ (3.1%) was used. Each technology was likewise ‘idealised’, with all SIT offspring and female fsRIDL pupae being completely non-viable, and wild females that mated with TMT males dying within the hour (which functionally may also be acute incapacitation, eventually leading to their death). A negative control (no release) simulation was also performed for comparison.

The target female populations in SIT and fsRIDL simulations were reduced to 50% of their initial size (PR50) within 56.1 and 36.3 days, respectively, and reduced by 95% (PR95) within 109.6 and 87.8 days (Fig. 5a). Qualitatively, these results follow a similar trend to the rate of population reduction observed during the suppression phase of the Brazil field trials. The PR50 and PR95 for TMT simulations were 15 and 62.2 days, 58.7 and 29.2% faster than fsRIDL, respectively. The model also tracked key epidemiological factors such as cumulative blood feeding events, which on average were 8183 for SIT simulations, and 5659 for fsRIDL simulations (Fig. 5b). The average number of blood feeding events in TMT simulations was just 2804, 67% lower than SIT and 50% lower than fsRIDL simulations. The number of gonotrophic cycles undergone by a female mosquito correlates closely with two important factors, multiple feeding rates and the extrinsic incubation period (EIP) of arboviruses^{56,57}, with the earliest that a female is likely to transmit a virus being her second gonotrophic cycle. The median number of gonotrophic cycles per female was 5 for the negative control (IQR: 3–7), SIT (IQR: 3–7) and fsRIDL (IQR: 4–7) simulations, whereas the median for TMT simulations was just 1 (IQR: 1–5).

Simulating reduced mating lethality

We then explored what performance could be expected if the mating lethality (i.e. likelihood that a female will die after mating with a TMT male) is less than 100%. We repeated the TMT simulations with mating lethality (hereafter ML) between 10 and 100% (in increments of 10%) and presumed that females that had survived the mating event had no other adverse effects and may even go on to produce offspring. Due to the non-linear response to venom dosage, and the uncertainty in the difference in venom production between homozygotes and heterozygotes, we decided that modelling heritability of the TMT construct was impractical at this stage. We, therefore, decided to model these simulations as a ‘worst-case’ scenario with TMT males producing fertile, wild-type offspring. However, it would be unlikely that no attempt to mitigate the risk of transgene escape would be made in the development of any TMT application. As such, we also modelled a scenario with TMT males that had reduced ML but would produce no viable offspring, which could be achieved through a combination with some form of SIT, IIT, or RIDL. We found

that the cumulative blood feeding rates for fertile TMT male scenarios were only significantly lower than fsRIDL simulations when the ML was $\geq 80\%$ ($P < 0.001$, Supplementary Fig. S3), but sterile males only required $\geq 40\%$ ML to outperform fsRIDL ($P < 0.001$, Supplementary Fig. S4). While the PR50 of fertile TMT males was lower than fsRIDL at ML $\geq 70\%$, the target population was not eliminated when ML was $< 80\%$ (Supplementary Fig. S3). However, sterile TMT males had a PR50 lower than fsRIDL at ML $\geq 50\%$ (Supplementary Fig. S4) and had a PR95 lower than fsRIDL at $\geq 80\%$.

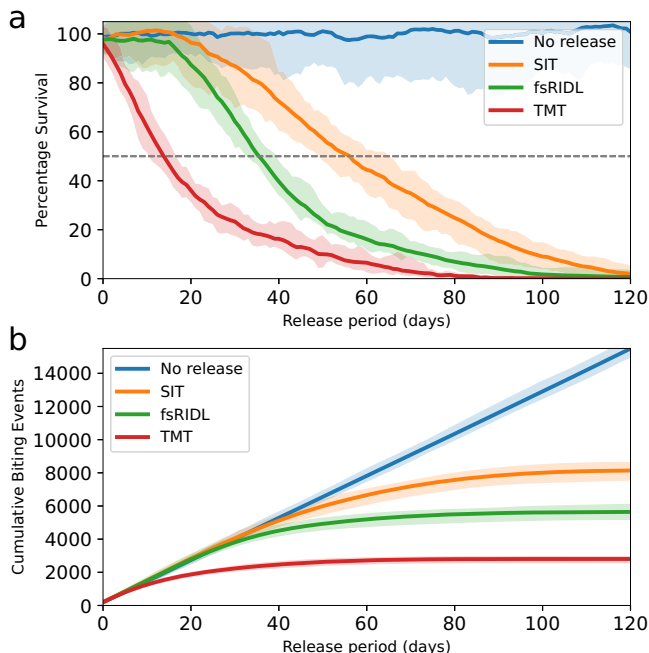


Fig. 5 | Agent-based models simulating *Ae. aegypti* population control programmes with SIT, fsRIDL, TMT, or no transgenic male releases. **a** The size of the female population during the release period relative to initial conditions. 50% reduction in the female population indicated by the dashed line. **b** The cumulative number of biting events which occurred throughout the releases. Data were presented as the mean of 10 simulations \pm the maximum and minimum values, respectively, observed at a given time point (indicated by the shaded area). Source data are available through Zenodo under accession code 13927099.

Sensitivity analysis

We conducted sensitivity analysis⁵⁸ to quantify the uncertainty in the model's outputs and to determine the relative influence of key parameters on the comparative efficacy of TMT and fsRIDL. Our analysis focused on three critical parameters: density-dependent mortality (DDM), polyandry, and release ratio. We systematically varied these parameters across defined ranges (see Methods) and assessed their impact on the percentage difference in cumulative blood-feeding events between TMT and fsRIDL simulations, serving as a proxy for their capacity to rapidly reduce the spread of disease (Fig. 6). We performed linear regression with polynomial and interaction terms by utilising a robust linear model (RLM) to account for non-normality and heteroscedasticity in the data, followed by global and local sensitivity analyses. We also calculated standardised regression coefficients (SRC) to compare the magnitude and direction of the parameters' effects, and variance inflation factors (VIF) to identify multicollinearity between effects.

No significant multicollinearity was observed (all VIF < 2.5), indicating that each predictor variable provides unique information to the model. The mean absolute error (MAE) of 15.7% reflects the average deviation in the model's predictions, indicating a reasonable level of uncertainty in its outputs. This, combined with the pseudo *R*-squared of 0.857, suggests that while the model captures a significant portion of the variability in the data, there remains some uncertainty in individual predictions. First-order Sobol indices indicate that the variance in the model's output can primarily be attributed to variation in polyandry ($45.0 \pm 6.2\%$) and release ratio ($38.2 \pm 4.8\%$), while DDM contributed less to output uncertainty ($14.8 \pm 3.5\%$). One-at-a-time sensitivity analysis revealed that polyandry and release ratio induced output variations of -45.0 to 60.1% and -46.0 to 46.8% , respectively, while DDM's impact ranged from -36.3 to 28.4% .

The regression model indicates that polyandry ($\beta = 1.51, P < 0.001$, SRC (95% CI) = $40.4-47.0$) and release ratio ($\beta = 17.4, P < 0.001$, SRC (95% CI) = $100.3-110.5$) have significant positive effects on the performance of TMT relative to fsRIDL, while DDM ($\beta = -89.0, P < 0.001$, SRC (95% CI) = -67.1 to -56.6) has significant negative effects. The SRC suggest that the release ratio has the strongest relative influence, followed by DDM and polyandry. However, the significant quadratic terms for all three parameters (all $P < 0.001$) indicate there may be non-linear effects, which may explain the discrepancy between Sobol indices and SRC rankings. Partial dependence plots (PDP) were used to further investigate these non-linear effects. While the effect of DDM on the relative performance of TMT and fsRIDL is negative, this trend

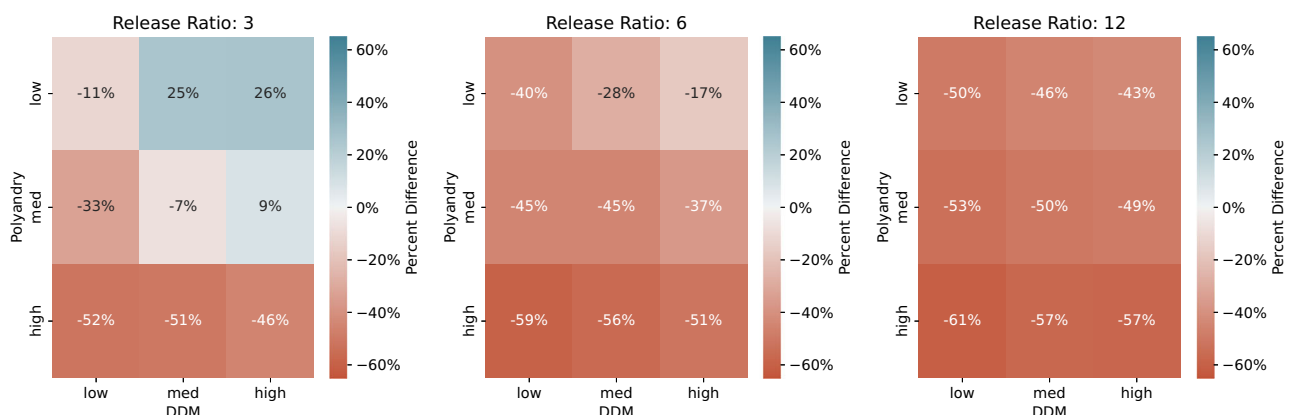


Fig. 6 | Heatmap of the percentage difference in cumulative bite rates between fsRIDL and TMT simulations under different input parameter combinations. The influence of key parameters (polyandry, release ratio, and density-dependent mortality) on the comparative ability of fsRIDL or TMT to reduce blood feeding rates was determined by sensitivity analysis. The data shown are the average of ten

simulations performed per technology and parameter value combination. Positive values (blue) indicate relatively lower bite rates for fsRIDL simulations, and negative values (red) indicate lower bite rates for TMT simulations. Source data are available through Zenodo under accession code 13927099.

reverses as the influence of DDM increases (SRC (95% CI) = 37.4–47.5, Supplementary Fig. S5). The positive effect of increased release ratios also appears to plateau around the highest value observed in the model (SRC (95% CI) = –74.9 to –65.0, Supplementary Fig. S6). However, the positive effect of polyandry on TMT's relative performance continues to increase up to the highest value observed in the model (SRC (95% CI) = 5.3–11.1, Supplementary Fig. S7). The interaction term between release ratio and polyandry ($\beta = -0.09$, $P < 0.001$, SRC (95% CI) = –24.5 to –21.2) suggests that the positive effect of release ratios may be mitigated in populations with high polyandry. Conversely, the negative effect of DDM may be mitigated by the interaction of increased release ratios ($\beta = 1.58$, $P < 0.001$, SRC (95% CI) = 7.1–10.5).

Discussion

Here, we describe a genetic biocontrol technology which can reduce the lifespan of mated females, called the toxic male technique, or TMT. The heterologous expression of insect-specific venom proteins, Γ -CNTX-Pn1a from the *P. nigriventer* spider and δ -AITX-Avd2a from the *A. sulcata* sea anemone, within the seminal fluid of male *D. melanogaster* resulted in a 37–64% reduction in the median lifespan of mated wild-type females after initial exposure to TMT males. The development of intragenerational genetic biocontrol technologies such as TMT represents a paradigmatic shift in pest management. Relative to current biocontrol technologies, the speed of population suppression achievable by intragenerational biocontrol may make these techniques a viable alternative to chemical pesticides as a first-line response to outbreaks while retaining taxonomic specificity and reducing off-target effects on local ecologies.

Over 1000 unique spider venom proteins have been characterised⁴¹, and many more from hosts such as scorpions and centipedes⁵⁹. Many insecticidal venom proteins are highly taxonomically selective, with toxicities that vary by orders of magnitude between different classes of insect⁶⁰, which allows for targeted application of particular use cases. Other insecticidal proteins from plants or bacteria may also make good candidates for TMT, though further investigation is required to determine their suitability. Co-expression of multiple toxins could help to mitigate the emergence of resistance alleles, as well as potentially resulting in synergistic toxicity from affecting multiple ion channel targets. It needs to be determined whether heterologous venom protein expression may result in TMT males becoming toxic to natural predators. However, the oral toxicity of venom proteins is typically between 1 and 2 orders of magnitude lower than when they are directly injected⁶¹, and venom proteins can be selected which have greater toxicity for the target species relative to natural predators.

To determine the theoretical capabilities of TMT, we developed an agent-based model of an *Ae. aegypti* release programme to compare its performance to fsRIDL under different assumptions of density-dependent mortality, polyandry, and release ratios of transgenic males. These results suggest that compared to fsRIDL, TMT may reduce the incidence of blood feeding during the release period by a further 40–60% under most scenarios, which has significant epidemiological implications. Our model indicates for TMT to outperform fsRIDL in this regard, it would require ~80% of the females to die during mating. However, this presumes that the TMT males are fertile, and any application of TMT will likely also require sterilisation of the males to mitigate the risk of transgene escape. If the TMT males cannot produce viable offspring, then only 40% mating lethality is required to outperform fsRIDL.

Sensitivity analysis revealed that the model has a mean absolute error of 17%, with most of the variation being explained by the direct effects of these key parameters. Release ratio had the most significant effect on the relative performance of TMT simulations, with TMT benefitting from higher ratios more so than fsRIDL, up to a point of diminishing returns where the wild population is fully saturated by

transgenic males. As expected, DDM had a negative effect on TMT's performance relative to fsRIDL, though increases in release ratio may help to overcome this limitation. Higher rates of polyandry increased TMT's performance, and the positive effect continues up to the highest rates tested, suggesting that TMT would likely be even more effective in disease vectors with higher polyandry, such as Brown Planthoppers^{62,63}. As *Ae. aegypti* females typically mate and attempt to blood feed within 24–48 h of eclosion²⁴, and arboviruses such as Dengue require a 3–10-day incubation period⁵⁷, most females killed by TMT males are not likely to transmit these illnesses. However, further studies will be required to understand the effects of epidemiology and other extrinsic factors (e.g. migration) on TMT's ability to mitigate epidemics.

The ideal phenotype for TMT would be immediate and irreversible incapacitation of the mated female. While the observed reduction in median female lifespan of 37–64% demonstrates the concept of TMT, mating lethality will inevitably need to be increased for TMT to approach the performance observed in the modelling. Anecdotally, we observed that mated females appeared lethargic and perhaps even mildly paralysed prior to their death, which might suggest that even if the TMT males do not kill the females directly, they may increase their mortality indirectly by making them more susceptible to predation. The expression of Γ -CNTX-Pn1a in male accessory glands did not affect the lifespan of TMT males, but expression of δ -AITX-Avd2a reduced median lifespan by 59% compared to wild-type males. We believe that this discrepancy is likely due to the difference in molecular weights of these venoms (5.2 and 2.9 kDa, respectively). The smaller size of δ -AITX-Avd2a may allow it to pass through the accessory gland epithelium into the haemolymph via paracellular routes, as has been described for some orally toxic insecticidal proteins⁶⁴. The performance of TMT can, therefore, potentially be improved by identifying venoms or other insecticidal proteins within some optimal molecular weight range, and by characterising accessory gland-specific regulatory elements to increase their expression.

The expression of either Γ -CNTX-Pn1a or δ -AITX-Avd2a did not reduce TMT male's ability to court females in single-pair settings, though due to differences in genetic background, we were unable to perform a meaningful assay to determine their ability to compete with wild-type males for mates. Competitive mating assays, which assess the ability of genetically engineered males to compete with wild-type males for mates, predominately utilise transgenic males prepared from wild-type genetic background^{65–67}. This is because males prepared using the GAL4-UAS system⁶⁸, as well as ectopic expression of the *white (mini-w+)* marker, can confound the results of competitive mating assays due to differences in body size and colour⁶⁹ or by increased rates of male-male courtship⁷⁰. Due to the stringent spatio-temporal regulation necessary to characterise TMT, the use of *antr*-GAL4 drivers were required to regulate the MAG-specific expression of the venom peptides. While it may have been possible to isogenize the *antr*-GAL4 and UAS strains by multigenerational backcrossing, characteristics unique to TMT (i.e. increased female mortality) would skew the paternity share to wild males regardless, further confounding indirect estimations of male competitiveness.

Determining the mating competitiveness of TMT males in target species will, therefore, require these strains to be engineered in wild-type genetic backgrounds, as well as utilising phenotypic markers such as fluorescent sperm so that female preference can be determined directly rather than through offspring. *D. melanogaster* is useful as a model for characterising systems like TMT, but limitations inherent in model systems hinder our ability to conclude how effective TMT may be if it can be implemented in other species, even closely related Dipterans like *Ae. aegypti*. However, current evidence indicates that successful translation of TMT into mosquitoes appears probable, as SFP are known to act on targets in the CNS of both *Drosophila* and mosquito females^{33–39}, males of both species can mate three to five

times before depleting their SFP^{27,71}, and accessory gland-specific promoters have been characterised in *Ae. aegypti*²⁷. However, female *Ae. aegypti* are more monandrous than *D. melanogaster* females, and it is unclear whether this difference in mating strategies may affect the quantity or proportion of SFP transferred by males.

Optimisations in the expression patterns of insecticidal proteins, such as changes to regulatory elements or co-expression of multiple venoms, will likely result in further decreases in mated female lifespan. Dose-dependent responses to venoms tend to follow a sigmoidal curve, so depending on the current levels of expression, it's possible that minor improvements could result in a significant increase in female mortality. In order to develop a stable TMT strain for mass rearing that does not produce venom peptides until the males are released, it will be necessary to engineer conditional expression or post-translational activation of venom proteins through mechanisms such as tetracycline-repression. As UAS:venom/*antr*-GAL4 crosses produced viable male and female offspring, it may also be beneficial to engineer TMT strains with genetic sorting of females to increase the efficiency of large-scale production of male-only cohorts.

Intrigenerational biocontrol techniques such as TMT present an approach to pest management which addresses many limitations in current insecticide and intergenerational biocontrol practices. TMT is inherently self-limiting due to strong selection pressure against venom expression, which may be advantageous from a regulatory perspective as, unlike GBT such as gene drives, there is little risk of substantial transgene introgression into non-target populations, and TMT transgenes are predicted to be rapidly lost from all populations in the absence of ongoing releases. TMT could theoretically be implemented in any species with internal fertilisation, provided that heterologous expression of the target protein can be constrained to the seminal fluid. Compared to current GBT such as SIT and fSRIDL, TMT will likely be most effective in species where the primary harm-causing life stage is the adult female, particularly anautogenous disease vectors like Black Flies and Horse Flies. TMT may also be highly effective for species which are already candidates for SIT that have relatively long lived and highly polyandrous females, including many fruit flies and Lepidoptera. In species where the adults are short lived, have low rates of female remating, and the immature stages are the primary concern, TMT will likely function comparably to SIT in its ability to suppress the population. As an alternative to chemical insecticides, TMT may be uniquely well suited for rapidly responding to outbreaks of human, animal, and agricultural disease vectors.

Methods

Ethical statement

Biosafety approval was granted by the Macquarie University Institutional Biosafety Committee (#5202).

Design and construction of UAS expression plasmids

The coding sequences for mature venom proteins (μ -AGTX-Aa1d, δ -CNTX-Pn1a, Γ -CNTX-Pn1a, κ -HXTX-Hv1c, ω -HXTX-Hv1a, ω -HXTX-Hv2a and δ -AITX-Avd2a), NbVHH05, as well as the *Acp26Aa* signal peptide, were codon optimised for expression in *D. melanogaster* using Geneious Prime 2023.2.1. pJFRC81-10XUAS:IVS-Syn21-GFP-p10 (hereafter pJFRC81) was a gift from Gerald Rubin (Addgene plasmid # 36432; <http://n2t.net/addgene:36432>; RRID:Addgene_36432). The region 67 bp upstream of the start codon of the GFP CDS of pJFRC81 was used as an extended upstream homology arm to re-introduce the Syn21 5'UTR during assembly. About 30 bp of the p10 3' UTR was used as the downstream homology arm, with a SapI recognition site introduced between the stop codon and terminator for future mutagenesis. Sequences comprised of the *Acp26Aa* signal peptide, a coding sequence, and both homology arms were ordered as gBlocks from Integrated DNA Technologies (IDT) and assembled (NEBuilder® HiFi

DNA Assembly Master Mix (New England Biolabs (NEB))) into pJFRC81 after linearisation with NotI and XbaI. All plasmids were sequence verified by Plasmidsaurus (Eugene, OR) whole plasmid sequencing. Annotated plasmid DNA sequences used in this study have been deposited in Zenodo under accession code 13910640 (<https://doi.org/10.5281/zenodo.13910639>)⁷².

Fly strains, transgenesis, and husbandry

Canton S wild-type flies were sourced from the Bloomington Drosophila Stock Centre (RRID: BDSC #64349). *Act5C-GAL4/CyO* flies were sourced from the Bloomington Drosophila Stock Centre (RRID: BDSC #4414). UAS:*mCherry* flies were sourced from the Bloomington Drosophila Stock Centre (RRID: BDSC #52268). *prd-GAL4*, *antr-GAL4* and *Acp26Aa-GAL4* flies were a kind gift from Mariana F. Wolfner from Cornell University.

UAS expression plasmids were sent to BestGene Inc (Chino Hills, CA) for plasmid DNA midpreps, microinjections into a strain with a 2nd chromosome attP docking site for ϕ C31 mediated genomic integration (RRID: BDSC #9752) and outcrossing of transgenic lines to balancer strains. Homozygous UAS stocks were prepared by selecting against the CyO marker on the balancer chromosome.

Flies were maintained on a cornmeal diet based on the Bloomington Drosophila Stock Centre (BDSC) standard Nutri-Fly formulation (catalogue number 66-113; Genesee Scientific). Flies were reared in a controlled environment room at 25 °C, 75% humidity, and a 12 h light-dark cycle with a 30 min transition period.

Recombinant venom functionality assay

To test the functionality of recombinant venom expression, hemizygous *Act5C-GAL4/CyO* virgin females were crossed to homozygous UAS:(μ -AGTX-Aa1d, δ -CNTX-Pn1a, Γ -CNTX-Pn1a, κ -HXTX-Hv1c, ω -HXTX-Hv1a, ω -HXTX-Hv2a and δ -AITX-Avd2a) males. Three crosses were prepared for each of the venoms, with each cross comprising ten females and ten males, which mated freely for 7 days before the parents were discarded. Offspring were collected for up to 10 days after the parents were discarded, and the presence or absence of the CyO marker was scored for each offspring.

Characterising accessory gland-specific GAL4 drivers

The strength of on and off-target heterologous gene regulation by various accessory gland GAL4 drivers was determined by crossing UAS:*mCherry* reporter strains to *prd-GAL4*, *antr-GAL4* and *Acp26Aa-GAL4* driver strains. Offspring were observed throughout their development, and five to ten adult male offspring from each cross were dissected to isolate their reproductive tracts. The strength of off-target and accessory gland-specific expression of mCherry was identified by fluorescence microscopy (Olympus SZX16 stereomicroscope, 6.3x magnification, Excitation: 530–550 nm, Emission: 575 nm longpass).

Mated female lifespan assays

Wild-type females were isolated soon after eclosion and housed together in groups of eight for 2–3 days prior to male exposure to ensure that they would be receptive to male courtship. Transhemizygous *antr-GAL4/UAS*:(μ -AGTX-Aa1d, Γ -CNTX-Pn1a, κ -HXTX-Hv1c, ω -HXTX-Hv1a and δ -AITX-Avd2a) males were prepared continuously throughout the experiment by crossing homozygous UAS males to homozygous *antr-GAL4* virgin females. Male offspring, including wild-type males, were isolated soon after eclosion and housed in groups of eight for 3–5 days prior to female exposure. Five groups of eight females were allocated to each treatment group. Females were briefly anaesthetised with CO₂ to be transferred to fresh vials every 3 days, and deaths were scored every 1–2 days. Males were initially added at this first transfer (2–3 days post-female eclosion) and were replaced during subsequent transfers to maintain the desired sex ratio, as well

as to ensure that females had constant access to robust males with full reserves of SFP.

Male lifespan assays

Transhemizygous *antr-GAL4/UAS:(Γ -CNTX-Pn1a, δ -AITX-Avd2a and NbVHH05)* males were prepared by crossing homozygous UAS males to homozygous *antr-GAL4* virgin females. Forty male offspring, including wild-type males, were isolated soon after eclosion and housed in five groups of eight. Males were briefly anaesthetised with CO₂ to be transferred to fresh vials every 3 days, and deaths were scored every 1–2 days.

Single-pair courtship assays

About 200 virgin wild-type females, 50 virgin wild-type males and 50 virgin transhemizygous *antr-GAL4/UAS:(Γ -CNTX-Pn1a, δ -AITX-Avd2a and NbVHH05)* males were isolated soon after eclosion and housed in groups of 10–15. Males were aged for 3–5 days, and females for 2–3 days prior to the experiment. Mating arenas were prepared by adding 6.2 g of sucrose diet (5% sucrose, 2% agar) to 35-mm petri dishes, leaving enough headroom for the flies to comfortably move around. Experiments were performed at the same time of day, starting 30 min after dawn. Flies were briefly anaesthetised on ice, and single pairs were transferred to mating arenas. The pairs were observed for up to 2 h, with courtship considered to be successful if the male remained firmly mounted on the female for >1–2 min, and the fraction of successful courtship was calculated to be the percentage courtship of a given treatment group.

Competitive mating assay

About 50 virgin wild-type females, 50 virgin wild-type males and 50 virgin *antr-GAL4/UAS:NbVHH05* males were isolated soon after eclosion and housed in groups of 5. Males were aged for 3–5 days, and females for 2–3 days prior to the experiment. Assays were prepared just before dusk to reduce visual courtship stimuli and increase reliance on chemosensory cues⁷³. Flies were briefly anaesthetised, and ten groups of five wild-type females, five wild-type males and five transgenic males were transferred to fresh media. The flies were immediately placed into darkened environment chambers and were allowed to mate freely until the next morning, at which point the males were discarded, and the females were isolated on fresh media. Ten offspring from each female were collected, and genomic DNA was extracted from pooled offspring as previously described⁷⁴. The flies were homogenised in 20 μ L PBS, after which 20 μ L extraction buffer (10 Mm Tris HCl, 10 Mm KCl, 10 Mm MgCl₂, 2 mM EDTA and 0.4 M NaCl) and 10 μ L SDS (10%) were added. The homogenate was vortexed for 1 min, briefly centrifuged, incubated for 25 min at 60 °C, and 10 μ L of NaCl (5 M) added. Finally, the samples were cooled to 4 °C, centrifuged for 30 min at 4 °C, 16,000 \times g. The clarified supernatant containing the genomic DNA was extracted, diluted 1:10 to reduce the salt concentration, then used as a template for 20 μ L PCR reactions with OneTaq[®] DNA Polymerase (New England Biolabs (NEB)). The quality of the extracted genomic DNA was determined by using primers against the endogenous *white* locus using the following primers: white-F (GCCAACTAGCCGAGACCTC), and white-R (GCCGTTAGGGAGCCGATAAA). Paternity of the offspring was determined by the presence or absence of the UAS:NbVHH05 locus using the following primers: UAS-F (TCAAC-CACTGATGCCTAGGC), and UAS-R (CACGTTCTGACTGCGTACT).

Agent-based modelling

To model the expected performance of TMT relative to existing GBT such as fsRIDL, we developed an agent-based model of *Ae. aegypti* using GAMA 1.9 (<https://gama-platform.org>). The model is not spatially explicit, but instead assumes a homogenous, randomly mixing population of a size for which the major limiting factor (in the absence of

extrinsic effects such as predation or fluctuations in temperature and precipitation) is density-dependent mortality (DDM) of the early life stages (eggs and first and second instar larvae)⁷⁵. We incorporated empirically derived parameters such as density-independent survivorship, durations of immature stages, and polyandry (model functionality and parameters detailed in Supplementary Figs. S1, S2 and Supplementary Data S1A–E)^{76–79}. DDM was modelled as a non-linear logistic (sigmoidal) function of the larval population⁷⁵, which was calibrated so that the population equilibrium and proportion of life stages fit observations from field studies²². DDM was calibrated such that the midpoint of the sigmoidal curve was centred on the hourly survival rate (0.97) and number of larvae (300) which produced a stable population of 500–600 adults, manually determining reasonable constants which produced the strongest and weakest response to larval density while maintaining the population equilibrium, then using the midpoint between these extremes for the analysis (see Supplementary Data S1A). Female remating was modelled as a logarithmic decay function which was calibrated to result in the desired rate of polyandry while producing a similar pattern of refractoriness as described in the literature⁵⁵. Females that mate with fsRIDL males will produce carrier offspring, with all female carriers dying during pupation and male carriers passing the fsRIDL transgene on to 50% of their offspring. Females that mate with SIT males will produce no viable offspring, and females that mate with TMT males will die immediately after mating.

Simulating reduced mating lethality

GAMA's factorial sampling method was used to run batches of 10 simulations for each value for mating lethality to be tested (i.e. 0.1–1.0), in combination with either fertile TMT males (surviving females produced offspring of a 'wild' genotype) or sterile males (surviving females produced non-viable 'SIT' genotype offspring). The differences between the cumulative biting rates between fsRIDL simulations and each of the reduced mating lethality simulations were assessed by performing a two-sample independent *t*-test.

Sensitivity analysis

We performed a sensitivity analysis to assess the robustness of our results and the model's sensitivity to changes in three key parameters: density-dependent mortality (DDM) of the immature stages, polyandry, and release ratio. Female *Ae. aegypti* become receptive to mating within 24–48 h of eclosion²⁴, and upon mating for the first time, will become increasingly refractory to remating over the course of 24 h⁵⁵, after which she will never remate. The fraction of *Ae. aegypti* females which mate with more than a single male is estimated to be between 14 and 24%^{38,55}, so the medium value for polyandry was set as 20%, with 0% remating as low, and the highest rates of polyandry reported for *Ae. aegypti* (23% of females remating two or more times, 53% remating at least once)⁸⁰ as high. The values for release ratio were set at 3, 6 and 12, respectively, roughly corresponding to the per-hectare release ratios in the range-finder, scale-up, and suppression phases of the Brazil field trials¹⁹. The high and low values for DDM were derivations of the logistic function, which produced the strongest and weakest response to larval density, respectively while maintaining the population equilibrium, with the medium value set as the midpoint between these (the same function as used in the initial analysis). GAMA's factorial sampling method was used to run batches of 10 simulations for each parameter and technology combination, with the number of successful blood meals taken between the start of the release period and the final cycle of the simulation used as the cumulative bite rate.

The *statsmodels* (v0.14.1)⁸¹ package for Python 3.12 was used to perform the regression analysis using a robust linear model (RLM) with polynomial and interaction terms, as well as to evaluate multicollinearity using variance inflation factors (VIF). To perform global

sensitivity analysis, the *SALib* (v1.4.5)⁸² package was used to calculate first-order, total-order, and second-order Sobol indices with 95% confidence intervals. One-at-a-time (OAT) sensitivity analysis was also used to examine the individual effects of each parameter. The *Scikit-learn* (v1.5.1)⁸³ package was used to calculate standardised regression coefficients (SRC) with 95% confidence intervals and assess model fit using pseudo *R*-squared and mean absolute error (MAE).

Statistics and reproducibility

Lifespans of mated females and transheterozygous males were analysed with the *lifelines* (v0.28.0)⁸⁴ Python package. Survival functions were calculated using the Kaplan–Meier estimate, differences in the survival of groups was determined by log-rank analysis, and 95% confidence intervals were calculated using Greenwood’s method. To test for differences in single-pair male courtship, a one-way chi-square test for contingency tables were used to calculate *P* values. Confidence intervals for the single-pair male courtship, as well as the mating competitiveness of *antr*-GAL4/UAS:NbVHH05 males, were calculated using the Wilson method. No statistical method was used to pre-determine sample sizes, and no data were excluded from the analyses. The Investigators were not blinded to allocation during experiments and outcome assessment.

Figures

Kaplan–Meier survival curves and cumulative biting rates of model *Ae. aegypti* populations were plotted using the *Matplotlib* (v3.9.0)⁸⁵ Python package. The model sensitivity heatmap and partial dependence plots were plotted using the *Matplotlib* and *seaborn* (v0.13.2)⁸⁶ packages.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data are provided with this paper or have been made available through Zenodo under accession code 13927099 (<https://doi.org/10.5281/zenodo.11465042>)⁸⁷. Source data are provided with this paper.

Code availability

GAMA 1.9 model files have been made available through Zenodo under accession code 13927094 (<https://doi.org/10.5281/zenodo.11439089>)⁸⁸. All codes used to perform statistical analysis and generate plots used in this study have been made available through Zenodo under accession code 13927099 (<https://doi.org/10.5281/zenodo.11465042>)⁸⁷.

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Author contributions

S.J.B. and M.M. conceived this study, designed experiments and analysed data. S.J.B. performed the experiments and developed the model. All authors contributed to writing the manuscript.

Competing interests

M.M. and S.J.B. have submitted a patent application (AU2023903662A0) to the Australian patent office pertaining to the enablement of the Toxic Male Technique.

Additional information

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