



Engineered expression of the invertebrate-specific scorpion toxin AaHIT reduces adult longevity and female fecundity in the diamondback moth *Plutella xylostella*

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Abstract

BACKGROUND: Previous genetic pest management (GPM) systems in diamondback moth (DBM) have relied on expressing lethal proteins ('effectors') that are 'cell-autonomous', that is, they do not leave the cell in which they are expressed. To increase the flexibility of future GPM systems in DBM, we aimed to assess the use of a non-cell-autonomous, invertebrate-specific, neurotoxic effector – the scorpion toxin AaHIT. This AaHIT effector was designed to be secreted by expressing cells, potentially leading to effects on distant cells, specifically neuromuscular junctions.

RESULTS: Expression of AaHIT caused a 'shaking/quivering' phenotype that could be repressed by provision of an antidote (tetracycline): a phenotype consistent with the AaHIT mode-of-action. This effect was more pronounced when AaHIT expression was driven by the *Hr5/ie1* promoter (82.44% of males, 65.14% of females) rather than *Op/ie2* (57.35% of males, 48.39% of females). Contrary to expectations, the shaking phenotype and observed fitness costs were limited to adults in which they caused severe reductions in mean longevity (–81%) and median female fecundity (–93%). Quantitative polymerase chain reactions of AaHIT expression patterns and analysis of *piggyBac*-mediated transgene insertion sites suggest that restriction of the observed effects to the adult stages may be due to the influence of the local genomic environment on the tetO-AaHIT transgene.

CONCLUSION: We demonstrated the feasibility of using non-cell-autonomous effectors within a GPM context for the first time in Lepidoptera, one of the most economically damaging orders of insects. These findings provide a framework for extending this system to other pest Lepidoptera and to other secreted effectors.

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1 INTRODUCTION

The diamondback moth (DBM; *Plutella xylostella*) is a highly invasive and economically important pest of brassicas, costing upwards of US \$5 billion in damage and control measures each year.^{1,2} Contributing to the notoriety of this pest is its extreme insecticide resistance, with populations having developed resistance to most classes of insecticides, including *Bacillus thuringiensis* (Bt) Cry toxins and Dichlorodiphenyltrichloroethane.³ As such, research into novel, more environmentally friendly control methods is required.

Advances in molecular biology have allowed DBM to function as a model for the development of genetic pest management (GPM) tools in Lepidoptera. Previously, we developed a transgenic, tetracycline-repressible, female-specific lethality system in DBM based on the sex-specific alternative splicing circuitry of the

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Pectinophora gossypiella (pink bollworm) *doublesex* gene.⁴ This female-lethal system is repressed in the laboratory or rearing facility when larvae are fed a diet containing sufficient quantities of an antidote (tetracycline or suitable analogues) (Figure 1). In the generation prior to release, tetracycline is withdrawn from the diet resulting in the death of female larvae, allowing the release of adult males homozygous for the female lethal transgene which they then pass on to their progeny in the field after mating with wild females.^{5,6} Because tetracycline is not present in sufficient quantities under field conditions, female progeny inheriting the transgene will die prior to adulthood, whereas male heterozygotes will survive to pass the transgene on to subsequent generations.⁷ This technology has been transferred to other lepidopterans including pink bollworm,⁴ silkworm (*Bombyx mori*)⁸ and fall armyworm (*Spodoptera frugiperda*; <https://www.oxitec.com/fall-armyworm>). A related technology not utilizing the *doublesex* cassette and thus resulting in lethality of both males and females off tetracycline has also been developed in pink bollworm.⁹ An orthogonal system in *B. mori* to the female-lethal system described above utilizes a W-chromosome linked (female-linked) germline expressing Cas9 insertion, and an autosomal sgRNA (single guide RNA) targeting the *transformer 2* gene essential for embryonic viability. Crosses between these two lines resulted in embryonic death of all female offspring.¹⁰ In terms of the *doublesex*-based female-lethal system developed in DBM, glasshouse trials have shown that repeated releases of these

homozygous males can result in eradication of caged wild-type populations and, additionally, delay evolution of *Bt* resistance,^{11,12} a finding in agreement with previous modelling.^{13,14} Wind tunnel experiments have demonstrated that these males retain the ability to locate and respond to female pheromone plumes¹⁵ and open-field trials have shown that these males are able to disperse within a realistic crop setting.¹⁶

Despite the rapid advance of this technology, a limitation – shared with a similar field-tested dominant lethal genetic system in the dengue fever mosquito *Aedes aegypti*¹⁷ – is that it is based on the expression of ‘cell-autonomous’ effectors, for example the transcriptional activator tTAV from the ‘tet-off’ expression system.¹⁸ In an unrepressed state (for example, in transgenic DBM female larvae in the field), expression of tTAV results in an uncontrolled positive feedback loop causing wide-scale gene misexpression/deregulation. This misexpression/deregulation is hypothesized to be severely deleterious to the cell, eventually resulting in organismal lethality.¹⁹ However, because the tTAV protein does not leave the cell in which it is expressed, the deleterious effects of its expression are limited to the cells in which its expression is directed (that is, it is cell-autonomous), usually necessitating a design with a broad spatial expression range to ensure that this results in a sufficiently deleterious phenotype at the whole-organism level. This reliance on cell-autonomous effectors including tTAV has restricted the utility of more complex and

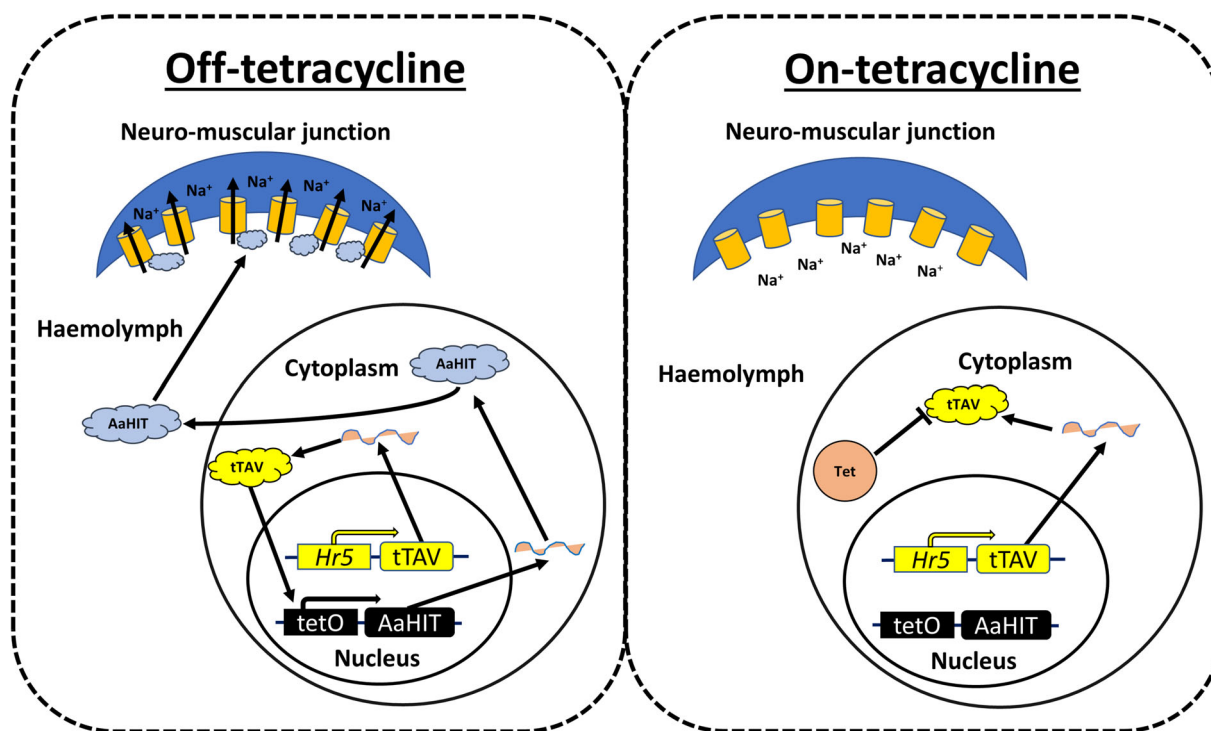


FIGURE 1. Schematic representation of the bipartite ‘tet-off’ system and its use in secreted neurotoxic effector (AaHIT) expression. This system is composed of two components: (1) a transgene expressing tTAV (here represented by *Hr5*-tTAV); and (2) a transgene which in the presence of active tTAV will express the neurotoxic effector AaHIT (here represented by *tetO*-AaHIT). (Left) ‘Off tetracycline’: the system in its unrepressed state. In the absence of the antidote (tetracycline, Tet), tTAV protein expressed from the *Hr5*-tTAV transgene will bind to *tetO* and drive expression of AaHIT. The secretory peptide encoded within AaHIT allows it to leave the cell in which it is expressed and travel through the haemolymph where it will encounter, bind and overstimulate neuromuscular junctions resulting in a loss of muscular control. (Right) ‘On-tetracycline’: the system in its repressed state. Here, Tet is provided to the organism as it is developing. Tet binds the tTAV protein and prevents it binding to *tetO*, thus inhibiting expression of AaHIT and allowing individuals bearing this system to retain muscular control. In the context of using these systems as population control tools, the ‘On-tetracycline’ situation describes how strains bearing these transgenes would be mass-reared in the laboratory/rearing facility. The ‘Off-tetracycline’ situation describes how the progeny of released individuals developing in the field would be subject to fitness costs brought about through loss of muscle control.

intricate GPM systems utilizing transcriptional regulatory elements (that is, promoters and enhancers) that display useful temporal or spatially explicit expression patterns, for example if expression of tTAV in these areas would not necessarily result in organism death/non-viability.^{20–22}

To circumvent this limitation, we recently demonstrated a novel mechanism for the expression of non-cell-autonomous effectors in the mosquito *Ae. aegypti*.²³ There, fusion of the invertebrate-specific neurotoxic protein AaHIT – a component of the venom from the scorpion *Androctonus australis hector* – and the gp67 secretory signal peptide from the *Autographa californica* baculovirus resulted in a synthetic gp67–AaHIT effector protein (henceforth AaHIT) that could be secreted from the cells in which it was expressed and subsequently bind to voltage-gated sodium channels at neuromuscular junctions, resulting in rapid onset of paralysis after expression (see Figure 1 for example of mechanism). The advantage of this system over those based on traditional cell-autonomous components is that because the effector is secreted, its ultimate effect (overstimulation of voltage-gated sodium channels) was independent of its original expression location (in the mosquito example, the cells of the adult female fat-body immediately post blood-feeding). This allowed use of the female fat-body-specific promoter from the *VitellogeninA1* gene even though expression of cell-autonomous effectors (Michelob, Reaper^{KR}) using this promoter had no observable effect.²³ The AaHIT gene has also been used to increase the potency of a variety of biocontrol technologies targeting lepidopteran pests including engineering its expression in recombinant *Bt* and *Autographa californica* baculovirus, both tested against DBM,²⁴ and thus we hypothesized that such an AaHIT-based non-cell-autonomous system could also function in this species. Here, we extend our previous work in mosquitos to DBM using the same bipartite ‘tet-off’ gene expression system utilized in the DBM female-lethal GPM system. Utilizing two transcriptional regulatory elements of known function in DBM (*Hr5/ie1* and *Op/ie2*)²⁵ to drive strong and ubiquitous tTAV production (henceforth *Hr5/ie1*-tTAV and *Op/ie2*-tTAV), we directed expression of a tetO-AaHIT

transgene in DBM resulting in a tetracycline-repressible ‘shaking’ phenotype in adults that negatively affected their longevity and fecundity. These results lay the foundation for further research into a new generation of non-cell-autonomous effector-based GPM strategies in this global pest and other lepidopterans.

2 MATERIALS AND METHODS

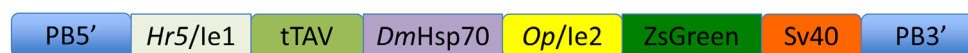
2.1 Insect rearing

DBM larvae were reared on beet armyworm artificial diet (Frontier Biosciences) under a 16:8 h light/dark photoperiod, 25°C and 50% relative humidity. Permissive conditions (henceforth on-tet) in which the tet-off system is repressed were created by the addition of tetracycline hydrochloride (Sigma) to this diet to a final concentration of 1 µg ml⁻¹. Tetracycline was mixed into the diet during assembly and unused diet was kept at 4°C prior to use. Restrictive conditions (henceforth off-tet) in which the tet-off system is unrepressed were created by making diet without tetracycline hydrochloride. Adult stages were supplied with 10% sugar water *ad libitum* via soaked cotton wool. For on-tet conditions, tetracycline hydrochloride was added to this sugar water to a final concentration of 1 µg ml⁻¹.

2.2 Details of lines

Plasmids were generated using standard molecular biology techniques. Sequences for the three plasmids used [*Hr5/ie1*-tTAV (OX5378), *Op/ie2*-tTAV (OX4585) and tetO-AaHIT (AGG1074) were deposited into GenBank under Accession numbers MT533615, MT533614, and MT533613, respectively] and simplified schematics are given in Figure 2. Transgenic DBM lines were generated for each construct, and insertion copy number was assessed using previously published methods.²⁵ Lines showing strong marker fluorescence were maintained as heterozygotes and a randomly selected line for each construct utilized in subsequent experiments. The genomic sequence flanking the *piggyBac* insertion site for each of the lines used here was identified using previously published methods^{23,25} (Table 1) and the online NCBI BLAST programme (NCBI <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Hr5/ie1-tTAV



Op/ie2-tTAV



tetO-AaHIT



FIGURE 2. Simplified schematic representations of the three constructs used. Components are as follows: PB5'/PB3' = *piggyBac* transposable element ITRs. *Hr5/ie1* = Homologous region 5 enhancer/immediate early gene 1 promoter + 5'-UTR from *Autographa californica* baculovirus. tTAV, tetracycline-controlled transactivator; *DmHsp70*, 3'-UTR from *D. melanogaster Hsp70* gene; *Op/ie2*, immediate early gene 2 promoter + 5'-UTR from *Orgyia pseudotsugata* baculovirus; ZsGreen, green fluorescent protein central transformation marker; Sv40, 3'-UTR from Simian virus 40; *BmVasa*, 3'-UTR from *Bombyx mori vasa* gene; DsRed, red fluorescent protein central transformation marker; tetO, seven repeats of the Tet operator sequence; AaHIT, gp67 secretory signal peptide from the *Autographa californica* baculovirus fused to *Androctonus australis hector* neurotoxic venom protein.

TABLE 1. Genomic sequence flanking 60 bp either side of TTAA insertion site (shown in red) for each of the lines used here

Line	Genomic sequences flanking transgene insertion	Scaffold no.
<i>Op/ie2-tTAV</i>	TGTAAGGAGCTGGCCAGATGGTATCCCGCCAATATTTATAAAAAGATGTGCTAAG TATTTAACACAGCCCTTAAACTTATATTTAACAAAGTCTCTCTAGCTCG ACTTTCCATCTACTT	NW_011952220
<i>Hr5/ie1-tTAV</i>	TCCCGCCAATATTTATAAAAAGATGTGCTAAGTATTTAACACAGCCCTTAAACTTATATTTAACAAAGTC TCTCTAGCTCGACTTTTCCATCTACTTGAAGCTAGCAAATGTAATACCAAT	NW_011952220
<i>tetO-AaHIT</i>	CGTTCATACTGATCGTTGATACTGACATATAGTTTGCAACTGTGTAAATAGATTGAATT TTAACAGATTGTGGTGAACGAACATTATATCGGTCAAAA ATTTATCCATCAAACATCTTTG	AHIO01014277

2.3 Survival to eclosion experiment

Heterozygous *tetO-AaHIT* adult females and heterozygous *Hr5/ie1-tTAV* adult males were mated en masse, with the reciprocal cross also conducted in a separate cage. Adults were supplied with cabbage juice-painted Parafilm (Bemis Company Inc.) to act as an oviposition substrate. After 24 h, egg sheets were removed from each cage and divided into two pieces. One piece from each cross was then placed onto either an on-tet or off-tet diet. On- and off-tet cohorts were kept separate for the remainder of the experiment and were analysed separately. At pupation, all individuals were screened for fluorescence and subsequently separated into the four possible genotypes: *Hr5/ie1-tTAV* single heterozygotes; *Hr5/ie1-tTAV* + *tetO-AaHIT* double heterozygotes (henceforth *Hr5/ie1 > AaHIT*); *tetO-AaHIT* single heterozygotes; and wild-type (WT). Cohorts were allowed to eclose and numbers of pupae and subsequent adults were compared between genotypes based on an expected 1:1:1:1 ratio (chi-square goodness of fit). The same experiment was performed using the *Op/ie2-tTAV* line in place of *Hr5/ie1-tTAV*.

2.4 Adult time series experiment (analysis of shaking phenotype)

Heterozygous *Hr5/ie1-tTAV* and heterozygous *tetO-AaHIT* individuals were crossed in the same way as above to give pupae of the four possible genotypes, reared on and off tetracycline and separated by sex (16 cohorts total). However, as pupae, each cohort was placed into a cage (BugDorm) with added cotton wool soaked in sugar water of the appropriate tetracycline condition. These pupae were observed daily for eclosion with the first day of adult eclosion counted as day 0 of the experimental period for that cage; analysis was conducted from the first day post eclosion (1 dpe). Because cohorts were separated by sex, eclosion timing was relatively simultaneous in each cage. From this period, all cages were observed daily and any adults that displayed a shaking phenotype were removed from the cage, placed in RNA_{later} (Invitrogen) and frozen in liquid nitrogen for subsequent analysis. Observations continued until all individuals were dead or collected in the *Hr5/ie1 > AaHIT* off-tet cage. The same experiment was also performed using the *Op/ie2-tTAV* line in place of *Hr5/ie1-tTAV*. Individuals that failed to eclose were removed from each cohort data set prior to analysis. Analysis of cohorts that had shown a phenotype was conducted using a Cox's proportional hazards test comparing between tTAV lines and sexes.

2.5 Adult longevity experiment

This experiment was conducted using the *Hr5/ie1-tTAV* and *tetO-AaHIT* lines. The 16 cohorts were created as described above. Thirty

pupae from each of the *Hr5/ie1 > AaHIT* on- and off-tet, male and female cohorts were placed in individual observation pots, supplied with sugar water of the appropriate tetracycline condition and observed daily. Recorded for each individual was the day of eclosion and the day of death. From this data a longevity analysis was performed using a Cox's Proportional Hazards test.

2.6 Female fecundity experiment

This experiment was conducted using the *Hr5/ie1-tTAV* and *tetO-AaHIT* lines. The 16 cohorts were created as described above. Thirty pupae of each of the *Hr5/ie1 > AaHIT* on- and off-tet female cohorts were placed in individual observation pots, supplied with sugar water of the appropriate tetracycline condition and observed daily. On the day each female eclosed, three freshly eclosed, virgin WT males were placed in each individual pot. Females were allowed to mate and lay eggs on Parafilm painted with cabbage juice for 72 h after which time egg sheets were collected. Eggs were counted on each sheet and hatch rates were calculated. Statistical differences in egg laying and hatch rate were assessed using a quasipoisson and quasibinomial glm, respectively.

2.7 qPCR experiment

2.7.1 Total RNA extraction and cDNA synthesis

Total RNA from individual whole DBM from various life stages (L2, L3, L4 = 2nd, 3rd and 4th larval instars) (L2 off-tet *n* = 3, L2 on-tet *n* = 3, L4 off-tet *n* = 3, L4 on-tet *n* = 3, pupae off-tet *n* = 3, pupae on-tet *n* = 3, 2 dpe off-tet *n* = 4, 2 dpe on-tet *n* = 4, 3 dpe off-tet *n* = 4, 3 dpe on-tet *n* = 3, 4 dpe off-tet *n* = 2, 4 dpe on-tet *n* = 3) was extracted using a Qiagen RNeasy Mini Plus Kit that includes a column-based genomic DNA (gDNA) removal step. Extracted RNA was quantified using a NanoDrop 2000/2000c spectrophotometer (ThermoFisher Scientific). Complementary DNA (cDNA) was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. After reverse transcription cDNA was stored at -20°C.

2.7.2 RT-qPCR assay design

Primers for 40S ribosomal protein S17 gene (17S; NM_001305512.1) were designed manually after interrogation of the genomic sequence (downloaded from <http://iae.fafu.edu.cn/DBM/>). Primer sets for elongation factor 1 gene (*EF1*; XM_011562844.1), ribosomal protein L32 gene (*Rpl32*; NM_001309136.1) and ribosomal protein S13 gene (*RpS13*; NM_001305523.1) were designed previously.²⁶ Primers for *AaHIT* were designed manually in-house (Table 2).

TABLE 2. Description of RT-qPCR primer sequences, genomic location and amplicon characteristics

Gene	Accession number	Nucleotide sequence	Amplicon size (bp)	Ref.
<i>17S</i>	NM_001305512.1	F: 5'-CAAGCCTCTTCGTAACAAGATCG-3' R: 5'-CAGCTTGATGGAGATACACGC-3'	91	This study This study
<i>EF1</i>	XM_011562844.1	F: 5'-GCCTCCCTACAGCGAATC-3' R: 5'-CCTTGAACAGGGCATCT-3'	161	Fu <i>et al.</i> ²⁶ Fu <i>et al.</i> ²⁶
<i>RpL32</i>	NM_001309136.1	F: 5'-CCAATTTACCGCCCTACC-3' R: 5'-TACCCTGTTGTCAATACCTCT-3'	120	Fu <i>et al.</i> ²⁶ Fu <i>et al.</i> ²⁶
<i>RpS13</i>	NM_001305523.1	F: 5'-TCAGGCTTATTCTCGTCG-3' R: 5'-GCTGTGCTGGATTCGTAC-3'	100	Fu <i>et al.</i> ²⁶ Fu <i>et al.</i> ²⁶
<i>AaHIT</i>		F: 5'-GCCCGGAATGCCTGCTGTC-3' R: 5'-AGGCAGCAGTATCCCTTATCGGC-3'	80	This study This study

Each quantitative reverse transcription polymerase chain reaction (RT-qPCR) run was setup manually by the same operator in MicroAmp fast optical 96-well reaction plates with barcode 0.1 ml (Applied Biosystems) and sealed with MicroAmp optical adhesive film (Applied Biosystems) at The Pirbright Institute and run on the QuantStudio 3 PCR system (Life Technologies) on 'fast' cycling conditions using QuantStudio™ Design and Analysis Software v. 1.3.1. Each reaction was run in 10-μl volumes and included 1× Luna Universal qPCR Master Mix (New England Biolabs) and 1 μg of template; optimal forward and reverse concentrations were determined experimentally (200 nmol L⁻¹ for both forward and reverse primers for *17S*, *EF1*, *RpS13* and *AaHIT*; 150 nmol L⁻¹ for both forward and reverse primers for *RpL32*). Reactions were run in triplicate, including no template and no-RT (no-Reverse Transcriptase) controls. The cycling conditions were as follows: initial 95°C for 1 min, followed by 40 cycles of 95°C for 5 s, and 60°C for 20 s.

2.7.3 RT-qPCR analysis

Standard curves, including the slope, y-intercept, correlation coefficient and the PCR efficiency for each primer set were constructed using QuantStudio™ Design and Analysis Software v. 1.3.1.

A geNORM analysis was conducted to determine which reference genes of the four measured (*17S*, *EF1*, *RPL32*, *RPS13*) were most stably expressed across all life stages and treatment groups. Raw RT-qPCR data were read into the statistical computing software R v. 1.2.5033 using the package 'ReadqPCR',²⁷ and the geNORM analysis was conducted using the package 'NormqPCR'.²⁷ The four reference genes were ranked across all life stage by

treatment group combinations based on their gene stability measure (M), and the gene with the highest M value was excluded. Average expression stability was then calculated for each life stage and treatment group combination, as was the pairwise variation. The three most stable (*17S*, *RPL32*, *EF1*), and therefore most suitable, reference genes were used to normalize the C_q (quantification cycle threshold) values using the qBase relative quantification framework.²⁸ Normalized relative quantification values were calculated by making each normalized biological sample (mean of technical triplicates) relative to an independent calibrator consisting of equimolar pooled cDNA from all 38 samples, itself also a mean of technical triplicates. For comparisons between life stages on- and off-tet, only sampling points where at least three biological replicates were present in both on- and off-tet treatments were included (33 samples).

2.8 Double homozygous line generation

A line homozygous for both *Hr5/ie1*-tTAV and tetO-*AaHIT* was generated as follows. All larval stages during this process were fed with on-tet diet. F₀ individuals from each of the two lines were crossed, reared on-tet and F₁ *Hr5/ie1* > *AaHIT* individuals isolated at the pupal stage. These F₁ double heterozygotes were then pool crossed to give F₂ individuals possibly homozygous for both transgenes (double homozygotes). F₂ individuals that showed the strongest fluorescence intensity for both transgene markers under fluorescence microscopy were sexed and isolated as pupae in 1.5-ml tubes, although observed individual variation was not great. Shortly after eclosion, individuals were immobilized using CO₂ and a single rear leg removed for genomic extraction using

TABLE 3. Description of primer sequences, amplicon characteristics and cycling conditions for use in the transhomozygous screening experiment

PCR purpose	Primers used	Amplicon size (bp)	Cycling conditions
gDNA positive control	F: 5'-CTTTAGAAATGGTTACCAAGA TAGGACAGAC-3' R: 5'-CAGTGACACTTACCGCATTGACAAG-3'	337	95°C, 3 min 35× (95°C, 10 s; 60°C, 30 s; 72°C, 1 min) 72°C, 5 min
<i>Hr5/ie1</i> -tTAV genomic insertion site	F: 5'-GTCTGATTCATTGCCACCTTTATGTATTGGTATTAC-3' R: 5'-CACTACAGGAGGTGACTGGT GTAATC-3'	770	95°C, 3 min 35× (95°C, 10 s; 67°C, 30 s; 72°C, 1 min) 72°C, 5 min
tetO- <i>AaHIT</i> genomic insertion site	F: 5'-CGGTAACAATAGCACCAAATT ACCTAAACG-3' R: 5'-ACACAGACACACTTCGGACCAC-3'	578	95°C, 3 min 35× (95°C, 10 s; 67°C, 30 s; 72°C, 1 min) 72°C, 5 min

TABLE 4. Description of primer sequences, amplicon characteristics and cycling conditions for use in the tTAV expression RT-PCR experiment

PCR purpose	Primers used	Amplicon size (bp)	Cycling conditions
tTAV expression	F: 5'-CAACAACCGATTGCGTCC-3' R: 5'-CCCGACTCGCATTTTCAGTTG-3'	136	95°C, 3 min 35× (95°C, 10 s; 50°C, 30 s; 72°C, 1 min) 72°C, 5 min
17S expression	F: 5'-CAAGCCTCTTCGTAACAAGATCG-3' R: 5'-CAGCTTGATGGAGATACACGC-3'	90	95°C, 3 min 35× (95°C, 10 s; 53°C, 30 s; 72°C, 1 min) 72°C, 5 min

the Phire Animal Tissue Direct PCR Kit (ThermoFisher Scientific). Three PCRs were performed on each gDNA extraction. A positive control PCR for gDNA quality (designed to amplify between the genomic flanking sequence of the *Hr5/ie1*-tTAV and the 5' *piggy-Bac* flank), a PCR across the *Hr5/ie1*-tTAV genomic insertion site and a similar PCR across the tetO-*AaHIT* genomic insertion site, designed so that wild-type sequence would generate a product, but the large transgene insertion would prevent amplification from an insertion allele. Primers within genomic flanking sequences were designed using information gained during the previous flanking assessment (Section 2.2 and Table 1). Any individuals that did not show amplification in the positive control were not assessed further. Of the remaining individuals, those that showed no amplification in either of the other PCRs were taken to be homozygous for both transgenes, with amplification of one or other insertion site PCR taken to indicate a heterozygous state at that locus. Individuals that fulfilled the above criteria for homozygosity were crossed together to form a homozygous colony. Primer sequences used and PCR cycling conditions are provided in Table 3. PCR was conducted using Dreamtaq polymerase (ThermoFisher Scientific) according to the manufacturer's instructions.

2.9 RT-PCR of larval stages for tTAV expression

Males from each of the *Hr5/ie1*-tTAV and *Op/ie2*-tTAV lines were crossed to WT females. For each cross, offspring carrying the respective transgene marker were selected (transgene

heterozygotes). Fifteen transgenic individuals were collected at stages L2 and L3 and a single male and single female pupa was also collected from each heterozygous pool. These were stored in RNAlater at -80°C and subsequently all samples were processed simultaneously. RNA from each of the four samples was extracted using the Qiagen RNeasy Mini Plus Kit as previously described for qPCR samples. Extracted RNA was quantified using a NanoDrop 2000/2000c spectrophotometer. cDNA was synthesized from total RNA using the LunaScript RT SuperMix kit (New England Biolabs) following the manufacturer's protocol. A single pooled RNA sample was used as template for a no-RT control. After reverse transcription, cDNA was stored at -20°C. Two PCRs were run on each cDNA sample: one for tTAV and one for the 17S ribosomal RNA gene as a positive control. Both PCRs were also run on a WT cDNA sample (extracted in the same way as previous samples but using a single male pupa). A no template control was also conducted by replacing the cDNA template with H₂O. Primers and cycling conditions for each reaction are given in Table 4. PCR was conducted using Dreamtaq polymerase according to manufacturer's instructions.

2.10 Statistical analysis

Data analysis was conducted with R v. 3.6.2 (R Core Team 2019). Survival analyses were conducted with the *survival*²⁹ and *survminer* (Kassambara A, Kosinski M, Bieckel P, <https://CRAN.R-project>.

TABLE 5. Results of chi-square test of given probabilities comparing numbers of pupae recorded across the four genotypes and an expected 1:1:1:1 ratio

tTAV line used	On-tet	Off-tet
<i>Hr5/ie1</i> -tTAV	$\chi^2 = 6.75$, d.f. = 3, $P = 0.080$, <i>Hr5/ie1</i> -tTAV = 152, tetO- <i>AaHIT</i> = 118, <i>Hr5/ie1</i> > <i>AaHIT</i> = 135, WT = 115	$\chi^2 = 3.64$, d.f. = 3, $P = 0.303$, <i>Hr5/ie1</i> -tTAV = 244, tetO- <i>AaHIT</i> = 288, <i>Hr5/ie1</i> > <i>AaHIT</i> = 266, WT = 268
<i>Op/ie2</i> -tTAV	$\chi^2 = 0.065$, d.f. = 3, $P = 0.996$, <i>Op/ie2</i> -tTAV = 152, tetO- <i>AaHIT</i> = 157, <i>Op/ie2</i> > <i>AaHIT</i> = 138, WT = 122	$\chi^2 = 5.21$, d.f. = 3, $P = 0.157$, <i>Op/ie2</i> -tTAV = 131, tetO- <i>AaHIT</i> = 133, <i>Op/ie2</i> > <i>AaHIT</i> = 132, WT = 135

Each combination of tTAV line and tetracycline treatment was analysed separately. No significant differences from the expected ratio were observed. Actual number of individuals counted is shown after statistical estimates.

TABLE 6. Results of chi-square test of given probabilities comparing numbers of adults recorded across the four genotypes and an expected 1:1:1:1 ratio

tTAV line used	On-tet	Off-tet
<i>Hr5/ie1</i> -tTAV	$\chi^2 = 5.49$, d.f. = 3, $P = 0.139$, <i>Hr5/ie1</i> -tTAV = 143, tetO- <i>AaHIT</i> = 115, <i>Hr5/ie1</i> > <i>AaHIT</i> = 119, WT = 109	$\chi^2 = 5.43$, d.f. = 3, $P = 0.143$, <i>Hr5/ie1</i> -tTAV = 228, tetO- <i>AaHIT</i> = 274, <i>Hr5/ie1</i> > <i>AaHIT</i> = 243, WT = 231
<i>Op/ie2</i> -tTAV	$\chi^2 = 0.010$, d.f. = 3, $P = 0.992$, <i>Op/ie2</i> -tTAV = 143, tetO- <i>AaHIT</i> = 155, <i>Op/ie2</i> > <i>AaHIT</i> = 134, WT = 122	$\chi^2 = 4.22$, d.f. = 3, $P = 0.238$, <i>Op/ie2</i> -tTAV = 131, tetO- <i>AaHIT</i> = 128, <i>Op/ie2</i> > <i>AaHIT</i> = 130, WT = 122

Each combination of tTAV line and tetracycline treatment was analysed separately. No significant differences from the expected ratio were observed. Actual number of individuals counted given after statistical estimates.

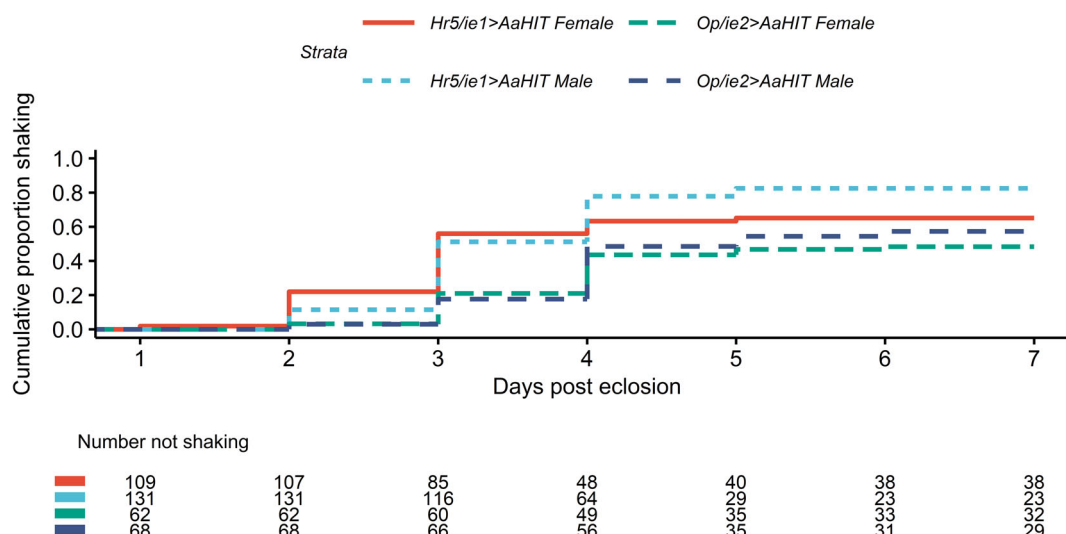


FIGURE 3. Expression of AaHIT causes an adult 'shaking' phenotype. (Upper) Cumulative proportion of adults in each cohort recorded as shaking each day over the 7-day observation period. This figure only displays results for the four double heterozygous cohorts (i.e. *Hr5/ie1* > *AaHIT* and *Op/ie2* > *AaHIT* males and females) as no shaking events were observed in any of the other 28 cohorts assessed. (Lower) Data from which cumulative proportions were calculated. Results analysed using a Cox's proportional hazards test suggesting *Hr5/ie1* > *AaHIT* individuals were significantly more likely to display a shaking phenotype than *Op/ie2* > *AaHIT* individuals ($n = 370$, $Z = -5.11$, $P < 0.005$) but no significant effect of sex.

org/package=survminer) packages. Figures were created with *ggplot2* from the tidyverse range of packages.³⁰

3 RESULTS AND DISCUSSION

Previous studies involving the engineering of *AaHIT* into baculoviruses and *Bt* have demonstrated a significantly increased lethality to larval stages of DBM.²⁴ As such, we first set out to assess

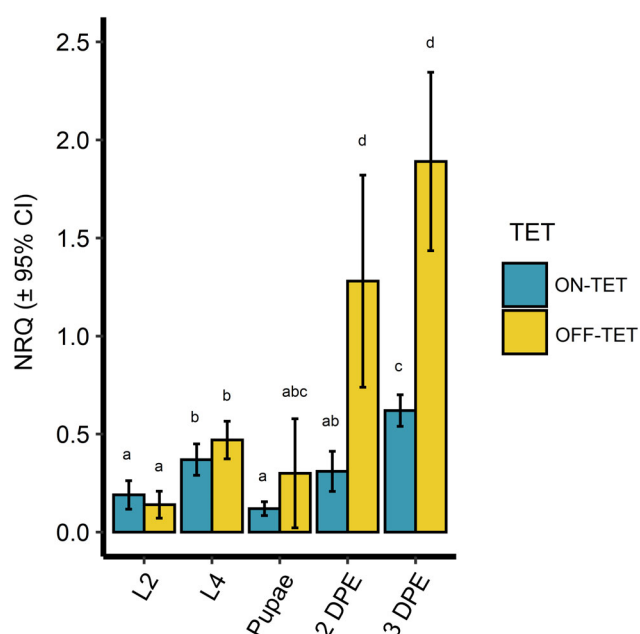


FIGURE 4. qPCR analysis of AaHIT expression. Normalized relative quantification (NRQ) values of AaHIT expression from *Hr5/ie1* > *AaHIT* DBM reared on- and off-tet across several life stages: L2 larvae, L4 larvae, pupae, 2 and 3 days post eclosion adults. Bars represent the geometric means of biological replicates for relative gene expression. Error bars are 95% confidence intervals and lowercase letter groupings denote significantly different groups as denoted by non-overlapping confidence intervals. Sample sizes provided in methods.

whether the transgenic expression of the synthetic AaHIT neurotoxin affected survival to pupation and/or adult eclosion.

Hr5/ie1-tTAV and *Op/ie2*-tTAV heterozygotes were crossed to tetO-*AaHIT* line heterozygotes en masse (for example, *Hr5/ie1*-tTAV males × tetO-*AaHIT* females and vice versa). Egg sheets from each cross were divided in half and reared either on-tet or off-tet. Pupae from each cross were collected, separated by genotype by screening for fluorescence markers, counted and then allowed to eclose. From each cross, given equal survival, pupae of four genotypes should have been present (*Hr5/ie1*-tTAV single heterozygotes, tetO-*AaHIT* single heterozygotes, *Hr5/ie1* > *AaHIT* double heterozygotes, and wild-type) in a 1:1:1:1 ratio. Deviation from this ratio indicates potential fitness costs in the under-represented genotypes. Overall, we found no significant deviation from this ratio for any of the treatment conditions (Table 5). Similarly, the number of adults eclosing from these pupae did not significantly differ from the expected 1:1:1:1 ratio (Table 6).

With the absence of an observed effect on survival at larval/pupal stages, we next tested whether a phenotype consistent with the mode of action of AaHIT could be detected in adult stages. Reciprocal crosses were again performed as described previously giving eight pupal genotype cohorts for each of the two tTAV line (for example, for the *Hr5/ie1*-tTAV line: four genotypes on- and off-tet = eight cohorts). All pupae were subsequently sexed giving a total of 16 cohorts per tTAV line (32 cohorts in total). Pupae from each cohort were placed in a separate cage and observed daily. No abnormal phenotype was observed in any of the single heterozygote or wild-type cages of either sex or tetracycline treatment. However, beginning at 1 dpe for the *Hr5/ie1* > *AaHIT* cohort and 2 dpe for the *Op/ie2* > *AaHIT* cohort, adults were observed displaying a 'shaking' phenotype typified by uncontrolled and rapid vibration of the wings and body and uncoordinated locomotion (Figure 3 and Videos S1–S12). Additionally, it was anecdotally observed that these cages had fewer flying individuals (more individuals were stationary on the floor/walls of the cage), although the numbers of such individuals were not recorded or analysed further. After being recorded as shaking and sexed by eye, adults displaying this phenotype were placed

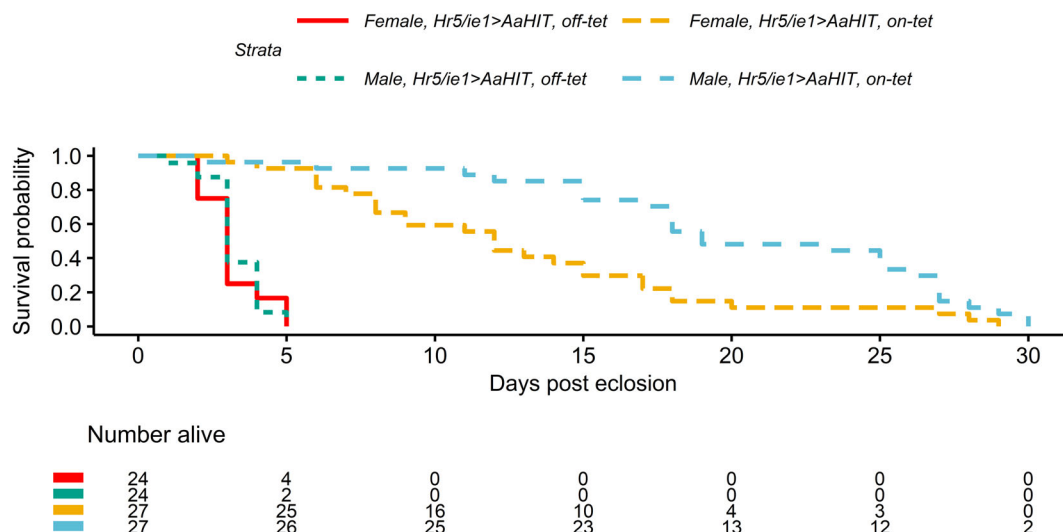


FIGURE 5. Effect on adult longevity of AaHIT expression. (Upper) Daily survival probability for adults from four cohorts (*Hr5/ie1* > *AaHIT* male and females, on- and off-tet). (Lower) Data from which survival probabilities were calculated. Results analysed using a Cox's proportional hazards test suggesting a significant effect of tetracycline treatment on adult longevity ($n = 102$, $Z = -6.00$, $P < 0.005$) but no significant interaction between sex and tetracycline treatment at this level of replication.

individually in RNA_{later} (Invitrogen) and stored at -80°C for subsequent analysis. In each cohort, the number of individuals displaying this phenotype (per day) increased for 2 days at which point it reached its peak: *Hr5/ie1* > *AaHIT* at 3 dpe, 39.9% of males and 33.9% of females started shaking; *Op/ie2* > *AaHIT* at 4 dpe, 30.9% of males and 22.6% of females started shaking. In both cases, the percentages are of the starting number of individuals of that sex in that cage, not the number of remaining individuals.

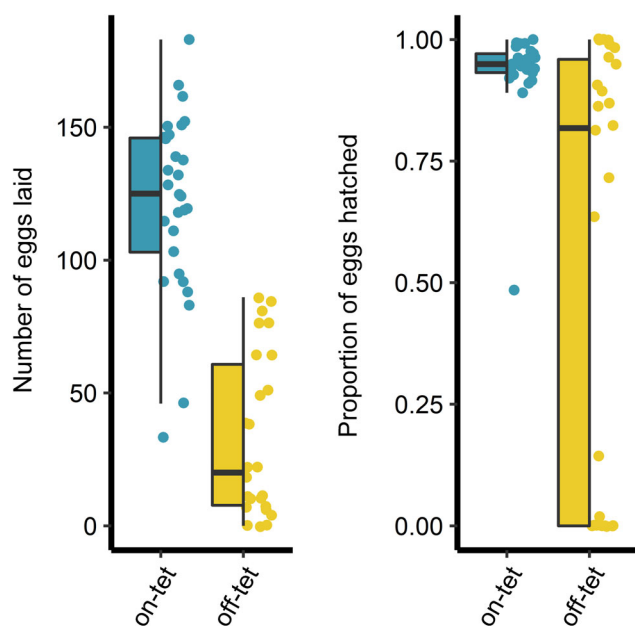


FIGURE 6. Effects on female fecundity of AaHIT expression. (Left) Number of eggs laid by *Hr5/ie1* > *AaHIT* females reared either on- or off-tetracycline. Off-tet females laid significantly fewer eggs (median 20) than on-tet females (median 124.5), $n = 55$, $Z = 7.54$, $P < 0.005$ – quasipoisson glm. (Right) Of these eggs, a significantly lower proportion hatched when laid by off-tet females (median 0.82) than on-tet females (median 0.95), $n = 55$, $Z = 4.39$, $P < 0.005$ – quasibinomial glm. Box limits represent the 25th and 75th percentiles, whiskers represent $1.5 \times$ the interquartile range, middle tendency estimates represent the medians.

Over the 7 days of observation, a total of 82.4% of males and 65.1% of females from the *Hr5/ie1* > *AaHIT* cohort and 57.4% of males and 48.4% of females from the *Op/ie2* > *AaHIT* cohort were observed shaking. Observations ceased at 6 dpe and 7 dpe for *Hr5/ie1* > *AaHIT* and *Op/ie2* > *AaHIT*, respectively, because at this point all adults in these cages had either been recorded as shaking (and thus removed) or were dead. An analysis of the shaking behaviour observed (number of shaking events over time) using Cox's proportional hazards test suggested a strongly significant effect of tTAV line on shaking hazard [*Hr5/ie1* > *AaHIT* cohorts were 1.57–2.7 times (57–170%) more likely to display shaking behaviour than *Op/ie2* > *AaHIT* cohorts] ($n = 370$, $Z = -5.11$, $P < 0.005$) but no significant effect of sex on shaking hazard at this level of replication ($n = 370$, $Z = 1.8$, $P = 0.073$). From an applied standpoint, these results would suggest further work into non-cell-autonomous expression systems in DBM may benefit from prioritization of the *Hr5/ie1* promoter over the *Op/ie2* promoter. However, to assess the generality of this conclusion, further analysis of other insertion sites bearing these constructs to account for any potential positional effects would be required.

Given that the *Hr5/ie1* and *Op/ie2* promoters are known from previous work to express from early stages in DBM (for example, from eggs), including as directors of fluorescent protein marker expression in the two lines used here (Figure S1), we were intrigued as to why the shaking phenotype observed was confined to adult stages. To explore this we performed qPCR analysis of AaHIT expression using a selection of those individuals that had been collected from the on- and off-tet *Hr5/ie1* > *AaHIT* cohorts during the previous experiment (Figure 4).

These results aligned with the observed trends in shaking behaviour. In off-tet samples, levels of AaHIT transcript were relatively low at pre-adult stages before rising in the post-eclosion (adult) samples. The highest levels of AaHIT expression coincided with the peak of the shaking phenotype observed in the previous experiment (3 dpe). In general, levels of AaHIT expression in on-tet samples were relatively low and did not overlap with levels observed in any off-tet shaking individuals. A caveat of these experiments is that although pre-adult samples were selected at

random from individuals of the relevant genotype in the developing population, adult samples represent a random selection from individuals that were observed shaking on that day (in the off-tet groups). As such, the estimated normalized relative quantification values of these adult samples may represent the upper level of AaHIT expression among the *Hr5/ie1* > AaHIT adults as a whole.

These results suggest that the lack of observed shaking or other deleterious effects observed in pre-adults was possibly due to a lack of significant upregulation in AaHIT expression during these stages. An alternative explanation is insensitivity of the pre-adulthood stages to the AaHIT toxin; however, this is deemed unlikely given the previously successful transgenic use of this toxin against early larval stages of DBM and other lepidopterans.²⁴

Normally, it would be expected that expression of a tetO transgene would mirror the expression profile of the tTAV transgene(s) present, possibly with some additional basal expression of the tetO transgene. The baculovirus promoters chosen here to regulate tTAV are known to display strong, constitutive and ubiquitous expression in DBM and indeed conformed to these expectations when driving the fluorescent marker (*ZsGreen*) in each tTAV line, which could be observed from late egg stages onwards (Figure S1) and also when tTAV mRNA was assessed at early larval stages using RT-PCR (Figure S2). It is therefore possible that positional effects caused by the tetO-AaHIT insertion site may have played a role in restricting substantial AaHIT expression to adults (for example through chromatin structure reducing access of tTAV or other transcriptional machinery to relevant areas prior to these stages). Characterization of the tetO-AaHIT insertion site revealed it was located within the putative 5' regulatory region of the *Aldehyde oxidase 1-like* gene (LOC105383938). The closest *B. mori* match to this gene, *xanthine dehydrogenase 1* (LOC101738209), shows substantial upregulation immediately prior to pupation. If this upregulation was related to a heterochromatin-euchromatin transition, and this situation was mimicked in DBM, this may explain the adult-specific response of the tetO-AaHIT line observed here. However, if this was the case, it did not seem to completely restrict expression from the *Hr5/ie1* promoter at this locus, as DsRed expression from the fluorescent marker was visible, even at early pre-adult stages (Figure S3). Additional characterization of other tetO and tTAV transgene insertion sites would be required to address this question further.

In addition to being of fundamental interest, the above results are consistent with previous findings in *Ae. aegypti* in demonstrating the speed with which convulsion/paralysis phenotypes can be brought about using this synthetic non-cell-autonomous system: in both cases, almost coincident with significant AaHIT upregulation. These findings bode well for use of this system in situations where rapid-onset of toxicity in the pest species is required.

Although we did not observe a deleterious phenotype in larvae or pupae, we hypothesized that the rapid onset of the shaking phenotype in adult stages may negatively impact one or more fitness components. To explore this, we next assessed relative adult male and female longevity and female fecundity (number of eggs laid and hatch rate) in *Hr5/ie1* > AaHIT individuals.

To assess effects of AaHIT expression on adult longevity, on- and off-tet cohorts of *Hr5/ie1* > AaHIT pupae were first generated and sexed as described previously. A minimum of 24 pupae from each of the four cohorts (on- and off-tet, male and female) were placed individually in observation pots and their dates of eclosion and death recorded. Using Cox's proportional hazards test we found a highly significant effect of tetracycline provision on adult longevity ($n = 102$, $Z = -6.00$, $P < 0.005$) with off-tet adults living

an average of 3.23 ± 0.137 SE days and on-tet adults an average of 16.7 days ± 1.10 SE (reduction in average longevity of approximately 81%) (Figure 5). We observed no significant interaction between tetracycline provision and sex at this level of replication – marginal effect ($n = 102$, $Z = -1.89$, $P = 0.059$).

To assess the effects of AaHIT expression on female fecundity, sexed *Hr5/ie1* > AaHIT cohorts were first produced as above. A minimum of 26 individual female pupae from each tetracycline provision were placed in observation pots and on the day of eclosion, provided with three virgin, WT adult males. Females were allowed to mate and lay eggs for 72 h (approximately the average lifespan of off-tet individuals from the previous experiment) after which time laid eggs were removed and counted with hatch rates also recorded. Using a quasipoisson glm we found a significant effect of tetracycline provision on the number of eggs laid by females over this period ($n = 55$, $t = 8.06$, $P < 0.005$) with on-tet females laying a median of 124.5 eggs and off-tet females a median of 20 eggs (reduction in median eggs laying of approximately 83.9%; Figure 6). We further observed a significant effect of tetracycline provision on proportion of eggs hatched ($n = 55$, $t = 4.05$, $P < 0.005$ – quasibinomial glm) with a median of 0.95 of eggs laid by on-tet females hatching but only 0.82 of eggs laid by off-tet females hatching (reduction in median proportion hatched of 13.7%). Overall, the median fecundity (number of larvae produced) of off-tet females (8.5) was reduced by approximately 93% compared with on-tet females (119.5).

These results contrast with those previously observed in *Ae. aegypti* in which expression of AaHIT did not result in significant effects on adult female survival or egg-laying (hatch rates were not recorded in that experiment). A possible reason for this lies in the difference in how AaHIT was expressed in these two experiments. In *Ae. aegypti* we employed the *VitellogeninA1* promoter element to drive tTAV (and therefore AaHIT) production.^{31–33} This promoter shows a short window of upregulation (approximately 24 h) in adult female fat-body cells immediately following a blood meal. However, here we used the less-restricted baculovirus promoters *Hr5/ie1* and *Op/ie2*. Although the expected expression profiles of these two promoters did not drive a concomitant upregulation of AaHIT in stages prior to adults (possible reasons for this addressed above) it is possible that, once active, the tetO insertion will have expressed in a larger number of cells and over a longer period than in our *Ae. aegypti* experiments. Therefore, where previously we achieved a short period of adult female paralysis followed by almost total recovery, here the induction of the tetO-AaHIT transgene resulted in reduced egg production and early death. This complex interplay between component choice and the confounding effects of transgene genomic context highlights both the flexibility possible in developing such GPM strategies and the importance of individual component testing.

Finally, given the bipartite nature of the system presented here, it would be desirable if such a strain destined for field use were made homozygous at both loci, primarily to facilitate and economise mass-rearing prior to release. Additionally, the release of double homozygotes would likely lead to more efficient population suppression, although mathematical modelling suggests a similar situation, in which a female-lethal bipartite system is allowed to segregate in male progeny in the field, can still be an efficient population suppression mechanism.³⁴ Although our initial goal was simply to investigate the possibility of developing an effective, repressible, non-cell-autonomous lethal system, for which single-copy (heterozygous) transgenes are the most

relevant genotype, we nonetheless set out to determine whether such a 'double homozygous' strain could be generated. We were able to generate a strain simultaneously homozygous for both the tetO-*AaHIT* and *Hr5/ie1*-tTAV transgenes by conventional rearing on-tet, with potential homozygotes selected by fluorescence intensity and confirmed by PCR. Fluorescence screening of all individuals in the line the generation after creation did not reveal any WT individuals at either transgene locus. This strain was successfully maintained on-tet, indicating that neither the additional copies of tTAV and tetO-*AaHIT* nor recessive insertion effects caused major changes to viability or fertility, although we did not undertake the more sophisticated analysis that would be required to assess the possibility of more subtle effects.

4 CONCLUSION

We have demonstrated the first use of a non-cell-autonomous transgene-based effector in a lepidopteran, causing adult-specific, tetracycline-repressible, neurotoxic effects. DMB adults experiencing these neurotoxic effects were observed to be less mobile, often sitting on the bottom or wall of their enclosure with a shaking/shivering phenotype. On its own, it is possible that such a phenotype would impose significant fitness costs under real-world conditions where it could impede their ability to avoid an array of biotic and abiotic dangers. However, even in the relatively safe and stable environment of the laboratory, these adults experienced substantial decreases in longevity and female fecundity – both relevant parameters in terms of population control. These effects would, for the same reasons outlined above, likely be exacerbated in the field where even the average longevity of wild adults is estimated to be less than 5 days.³⁵ Although such phenotypes would likely prove deleterious to those inheriting them in the field, the system as demonstrated here remains a 'proof-of-principle'. For true 'field-ready' strains both a higher degree of penetrance and an earlier-acting phenotype would be required, especially for protection of cash crops such as brassicas where aesthetic damage by younger instars can prove economically damaging.² Achieving this may prove as simple as the generation of a larger number of tetO-*AaHIT* insertions to test for loci that allow earlier expression. For ease and economy of mass-rearing, it may also be desirable to combine the bipartite tet-off components (for example, *Hr5/ie1*-tTAV and tetO-*AaHIT*) into a single construct and genomic insertion site, although recent modelling has indicated that use of two-part systems can still function effectively at population regulation, at least when deleterious effects are limited to a single sex.³⁴ Further work to develop true field-ready strains is clearly required. Although recognizing these caveats, our findings, alongside others, confirm the utility of the invertebrate-specific neurotoxic protein *AaHIT* as a means of inducing effective insect control phenotypes. More specifically, our results extend the potential of non cell-autonomous effectors as potential tools for GPM to the Lepidoptera which include some of the world's most damaging pest species.

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CONFLICTS OF INTEREST

V.C.N., A.W., T.D., L.Ak. and J.T. were employees of OXITEC Ltd during the period of this work.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- 1 Furlong MJ, Wright DJ and Dosdall LM, Diamondback moth ecology and management: problems, progress, and prospects. *Annu Rev Entomol* **58**:517–541 (2013).
- 2 Zalucki MP, Shabbir A, Silva R, Adamson D, Liu S-S and Furlong MJ, Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): just how Long is a piece of string? *J Econ Entomol* **105**:1115–1129 (2012).
- 3 Talekar NS and Shelton AM, Biology, ecology, and management of the diamondback moth. *Annu Rev Entomol* **38**:275–301 (1993).
- 4 Jin L, Walker AS, Fu G, Harvey-Samuel T, Dafa'lla T, Miles A *et al.*, Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synth Biol* **2**:160–166 (2013).
- 5 Alphey L, Re-engineering the sterile insect technique. *Insect Biochem Mol Biol* **32**:1243–1247 (2002).
- 6 Black WC, Alphey L and James AA, Why RIDL is not SIT. *Trends Parasitol* **27**:362–370 (2011).
- 7 Harvey-Samuel T, Ant T, Gong H, Morrison NI and Alphey L, Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects. *Evol Appl* **7**:597–606 (2014).
- 8 Tan A, Fu G, Jin L, Guo Q, Li Z, Niu B *et al.*, Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*. *Proc Natl Acad Sci U S A* **110**:6766–6770 (2013).
- 9 Morrison NI, Simmons GS, Fu G, O'Connell S, Walker AS, Dafa'lla T *et al.*, Engineered repressible lethality for controlling the pink bollworm, a lepidopteran pest of cotton. *PLoS One* **7**:e50922 (2012).
- 10 Zhang ZJ, Niu BL, Ji DF, Li MW, Li K, James AA *et al.*, Silkworm genetic sexing through W chromosome-linked, targeted gene integration. *Proc Natl Acad Sci U S A* **115**:8752–8756 (2018).
- 11 Harvey-Samuel T, Morrison NI, Walker AS, Marubbi T, Yao J, Collins HL *et al.*, Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biol* **13**:49 (2015).
- 12 Zhou LQ, Alphey N, Walker AS, Travers LM, Morrison NI, Bonsall MB *et al.*, The application of self-limiting transgenic insects in managing resistance in experimental metapopulations. *J Appl Ecol* **56**:688–698 (2019).
- 13 Alphey N, Bonsall MB and Alphey L, Combining pest control and resistance management: synergy of engineered insects with Bt crops. *J Econ Entomol* **102**:717–732 (2009).
- 14 Alphey N, Coleman PG, Donnelly CA and Alphey L, Managing insecticide resistance by mass release of engineered insects. *J Econ Entomol* **100**:1642–1649 (2007).
- 15 Bolton M, Collins HL, Chapman T, Morrison NI, Long SJ, Linn CE *et al.*, Response to a synthetic pheromone source by OX4319L, a self-limiting diamondback moth (Lepidoptera: Plutellidae) strain, and field dispersal characteristics of its progenitor strain. *J Econ Entomol* **112**:1546–1551 (2019).
- 16 Shelton AM, Long SJ, Walker AS, Bolton M, Collins HL, Revuelta L *et al.*, First field release of a genetically engineered, self-limiting agricultural pest insect: evaluating its potential for future crop protection.

- Front Bioeng Biotechnol* **7** (2020). <https://doi.org/10.3389/fbioe.2019.00482>.
- 17 Phuc HK, Andreassen MH, Burton RS, Vass C, Epton MJ, Pape G *et al.*, Late-acting dominant lethal genetic systems and mosquito control. *BMC Biol* **5**:11 (2007).
 - 18 Gossen M and Bujard H, Tight control of gene-expression in mammalian-cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89**:5547–5551 (1992).
 - 19 Bryk J, Reeves RG, Reed FA and Denton JA, Transcriptional effects of a positive feedback circuit in *Drosophila melanogaster*. *BMC Genomics* **18**:990 (2017).
 - 20 Fu GL, Lees RS, Nimmo D, Aw D, Jin L, Gray P *et al.*, Female-specific flightless phenotype for mosquito control. *Proc Natl Acad Sci U S A* **107**:4550–4554 (2010).
 - 21 Labbe GMC, Scaife S, Morgan SA, Curtis ZH and Alphey L, Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*. *PLoS Negl Trop Dis* **6**:e1724 (2012).
 - 22 Marinotti O, Jasinskiene N, Fazekas A, Scaife S, Fu G, Mattingly ST *et al.*, Development of a population suppression strain of the human malaria vector mosquito, *Anopheles stephensi*. *Malar J* **12**:142 (2013).
 - 23 Haghighat-Khah RE, Harvey-Samuel T, Basu S, StJohn O, Scaife S, Verkuil S *et al.*, Engineered action at a distance: blood-meal-inducible paralysis in *Aedes aegypti*. *PLoS Negl Trop Dis* **13**:e0007579 (2019).
 - 24 Deng SQ, Chen JT, Li WW, Chen M and Peng HJ, Application of the scorpion neurotoxin AaIT against insect pests. *Int J Mol Sci* **20** (2019). <https://doi.org/10.3390/ijms20143467>.
 - 25 Martins S, Naish N, Walker AS, Morrison NI, Scaife S, Fu G *et al.*, Germline transformation of the diamondback moth, *Plutella xylostella* L., using the piggyBac transposable element. *Insect Mol Biol* **21**:414–421 (2012).
 - 26 Fu W, Xie W, Zhang Z, Wang SL, Wu QJ, Liu Y *et al.*, Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). *Int J Biol Sci* **9**:792–802 (2013).
 - 27 Perkins JR, Dawes JM, McMahon SB, Bennett DLH, Orenge C and Kohl M, ReadqPCR and NormqPCR: R packages for the reading, quality checking and normalisation of RT-qPCR quantification cycle (Cq) data. *BMC Genomics* **13**:296 (2012).
 - 28 Hellemans J, Mortier G, de Paepe A, Speleman F and Vandesompele J, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**:R19 (2007).
 - 29 Therneau MT and Grambsch PM, *Modeling Survival Data: Extending the Cox Model*. Springer, New York, NY (2000).
 - 30 Wickham H, Averick M, Bryan J, Chang W, D'agostino McGowan L, Francois R *et al.*, Welcome to the tidyverse. *J Open Source Software* **4**:43 (2019).
 - 31 Kokoza V, Ahmed A, Cho WL, Jasinskiene N, James AA and Raikhel A, Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci U S A* **97**:9144–9149 (2000).
 - 32 Romans P, Tu ZJ, Ke ZX and Hagedorn HH, Analysis of a vitellogenin gene of the mosquito, *Aedes aegypti* and comparisons to vitellogenins from other organisms. *Insect Biochem Mol Biol* **25**:939–958 (1995).
 - 33 Kokoza VA and Raikhel AS, Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4–UAS system. *Insect Biochem Mol Biol* **41**:637–644 (2011).
 - 34 Vella MR, Gould F and Lloyd AL, Mathematical modeling of genetic pest management through female lethality with independently segregating alleles. *bioRxiv* (2020). <https://doi.org/10.1101/2020.04.06.028738>.
 - 35 Furlong MJ, Pell JK, Pek Choo O and Abdul Rahman S, Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bull Entomol Res* **85**:331–337 (1995).