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Characterization of an adulticidal and larvicidal interfering RNA pesticide that targets a conserved sequence in mosquito G protein-coupled *dopamine 1* receptor genes

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Abstract

G protein-coupled receptors (GPCRs), key regulators of a variety of critical biological processes, are attractive targets for insecticide development. Given the importance of these receptors in many organisms, including humans, it is critical that novel pesticides directed against GPCRs are designed to be species-specific. Here, we present characterization of an interfering RNA pesticide (IRP) targeting the mosquito GPCR-encoding *dopamine 1 receptor (dop1)* genes. A small interfering RNA corresponding to *dop1* was identified in a screen for IRPs that kill *Aedes aegypti* during both the adult and larval stages. The 25 bp sequence targeted by this IRP is conserved in the *dop1* genes of multiple mosquito species, but not in non-target organisms, indicating that it could function as a biorational mosquito insecticide. *Aedes aegypti* adults treated through microinjection or attractive toxic sugar bait delivery of small interfering RNA corresponding to the target site exhibited severe neural and behavioral defects and high levels of adult mortality. Likewise, *A. aegypti* larval consumption of dried inactivated yeast tablets prepared from a *Saccharomyces cerevisiae* strain engineered to express short hairpin RNA corresponding to the *dop1* target site resulted in severe neural defects and larval mortality. *Aedes albopictus* and *Anopheles gambiae* adult and larval mortality was also observed following treatment with *dop1* IRPs, which were not toxic to non-target arthropods. The results of this investigation indicate that

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dop1 IRPs can be used for species-specific targeting of *dop1* GPCRs and may represent a new biorational strategy for control of both adult and larval mosquitoes.

Keywords

Aedes ; *Anopheles* ; vector; insecticide; RNAi; G protein-coupled receptor

1. Introduction

Mosquito control is the primary means of preventing the spread of existing and the emergence of new mosquito-borne illnesses. However, resistance to every class of chemical insecticides has been documented in many mosquito species globally (Airs and Bartholomay, 2017), and the potential impacts of pesticides on non-target organisms are of ongoing concern (EPA, 2019). Discovery of new insecticides and mosquito control strategies that pose no risk to human health and which do not harm the environment is of critical importance (WHO, 2009). GPCRs, which comprise a large family of membrane bound receptors that regulate numerous intracellular signaling cascades and mediate a variety of critical biological processes, are attractive yet underexploited pesticide targets (Hill et al., 2016; Meyer et al., 2012; Ngai and McDowell, 2017; Nowling et al., 2013). Recent advances in mosquito genome sequencing and reference genome assemblies (Arensburger et al., 2010; Holt et al., 2002; Matthews et al., 2018; Neafsey et al., 2015; Nene et al., 2007) have revealed a mosquito GPCR superfamily consisting of hundreds of new putative insecticide targets in *Aedes* (dengue, Zika, chikungunya, and yellow fever vectors), *Anopheles* (malaria vectors), and *Culex* (lymphatic filariasis and West Nile virus vectors) mosquitoes (Hill et al., 2016; Hill et al., 2018; Ngai and McDowell, 2017; Nowling et al., 2013). Roughly one third of human pharmaceutical drugs impact GPCRs (Sriram and Insel, 2018; Wise et al., 2002), indicating that these receptors can be readily targeted, but highlighting the importance of identifying novel pesticides that are specific to mosquito GPCRs yet do not impact these receptors in humans or other non-target species (Hill et al., 2016).

RNAi, an endogenous regulatory pathway in eukaryotic cells that silences gene expression through the production of small interfering RNAs (siRNAs), has traditionally been applied for functional characterization of mosquito genes in the laboratory (Airs and Bartholomay, 2017), but has attracted the interest of the insect agricultural pest control community (Zhang et al., 2017). We recently initiated a large-scale effort to assess the potential use of RNAi technology for mosquito control. These studies began with high-throughput screens that led to the identification of siRNAs which function as mosquito larvicides (Hapairai et al., 2017; Mysore et al., 2017), several of which recognize conserved target sites in multiple mosquito species and function as broad-based mosquito pesticides (Mysore et al., 2019a,b). A proportion of the siRNA larvicides identified in these screens target genes with orthologs that are known to be required for both larval and adult viability in *Drosophila melanogaster,* a well-characterized genetic model insect. Here, through the development and evaluation of IRPs corresponding to a conserved target site in mosquito *dop1* genes, we examine the hypothesis that IRPs can function as insecticides that target both developing and adult mosquitoes.

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Previous characterization of *A. aegypti dop1* demonstrated that this gene (the subject of this investigation) and *dop2*, a second GPCR family member in *A. aegypti,* encode D1-like dopaminergic GPCRs that respond to dopamine in a dose-dependent manner (Meyer et al., 2012). A screen for lead chemistries active at Dop2 identified amitriptyline and doxepin as Dop2-selective compounds that induce high levels of *A. aegypti* larval mortality (Meyer et al., 2012). Dop2 antagonists also induce high rates of mortality in *Culex quinquefasciatus* (Nuss et al., 2015) and *Anopheles gambiae* larvae (Hill et al., 2016). Likewise, manipulation of dopamine levels in *D. melanogaster* larvae through inhibition of tyrosine hydroxylase activity results in larval death (Neckameyer, 1996). RNAi-mediated targeting of *dop1R2*, the *D. melanogaster* ortholog of *dop2*, as well as treatments with the D1-like receptor antagonist flupenthixol resulted in late larval/prepupal defects and developmental arrest in developing fruit flies (Regna et al., 2016), and silencing a dopamine-2 like receptor (D2R) in *Tribolium castaneum* caused larval death (Bai et al., 2011). These findings suggest that silencing *A. aegypti dop1* could also result in larval mortality. Furthermore, neural expression of dsRNA targeting *dop1R1* in *D. melanogaster* adults results in a flightless phenotype and adult lethality (Dietzl et al., 2007). Given that the *A. aegypti dop1* gene is expressed in developing and adult mosquitoes (Akbari et al., 2013), and that dopamine signaling is required for viability at multiple stages of the fruit fly life cycle (Dietzl et al., 2007; Mummery-Widmer et al., 2009; Neckameyer, 1996; Regna et al., 2016), it was hypothesized that silencing *dop1* in *A. aegypti* would induce mortality at multiple life cycle stages.

In this study, we describe a scalable attractive toxic sugar bait- (ATSB-) based delivery system that was used for effective delivery of siRNAs targeting *dop1* to adult *A. aegypti* mosquitoes under simulated deployment conditions. We also discuss the development and characterization of a yeast-based system for delivery of *dop1* IRPs to *A. aegypti* larvae. We demonstrate that silencing *dop1* in *A. aegypti* results in high levels of mortality in adults and larvae and show that IRPs which silence *dop1* can be used for selective, biorational targeting of multiple species of disease vector mosquitoes.

2. Materials and Methods

2.1 Mosquito rearing

A. aegypti Liverpool-IB12 (LVP-IB12) strain mosquitoes, *A. albopictus* (obtained from BEI Resources, NIAID, NIH: *A. albopictus*, Strain Gainesville, MRA-804, contributed by Sandra A. Allan), and *A. gambiae* G3 strain mosquitoes (obtained through BEI Resources, NIAID, NIH: *A. gambiae*, Strain G3, Eggs, MRA-112, contributed by Mark Q. Benedict) were reared as described (Clemons et al., 2010a). Adult females of each species were provided with blood meals through use of a Hemotek artificial membrane feeding system (Hemotek Limited, Blackburn, UK) that was used to deliver sheep blood that had been purchased from HemoStat Laboratories (Dixon, CA). The mosquitoes were cultured in an insectary that is maintained at 26° C, ~80% relative humidity, and which has a 12 hr light/12 hr dark cycle with 1hr crepuscular periods at the beginning and end of each cycle.

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2.2 Discovery of siRNA #462:

siRNA #462, which was not described previously, was identified in larval soaking (Hapairai et al., 2017; Mysore et al., 2017) and adult microinjection (see below) screens for genes essential for mosquito viability. A subset of the siRNAs tested in these screens, including siRNA #462, uncover target sites conserved in orthologous genes of multiple mosquito species, but not humans. These genes were screened in *A. aegypti* larvae and adults if the *D. melanogaster* orthologs were known to be required for larval viability (Thurmond et al., 2019), when the *A. aegypti* orthologs were known to be expressed throughout larval development (Akbari et al., 2013), if the genes are required for adult *D. melanogaster* survival (Thurmond et al., 2019), and if the target sites were not known to reside in the genomes of organisms other than mosquitoes [per blastn searches (Giraldo-Calderon et al., 2015; Johnson et al., 2008)]. Larval soaking screen experiments were performed as described (Singh et al., 2013) using first instar (L1) larvae and the following siRNAs purchased from Integrated DNA Technologies (Coralville, Iowa): #462: 5'- AUAUCAUCGCCGCGUUCUGCAAGAC –3' in *dop1 (AAEL019437)* and a control sequence that has not been identified in mosquito or other genomes (Tomchaney et al., 2014): 5'-GAAGAGCACUGAUAGAUGUUAGCGU-3'. The larval soaking screen was performed in duplicate experiments in which 20 L1 larvae were placed in 20 ul of 0.5 μg/μl siRNA for four hours, then placed in sterile distilled water, reared, and assessed as described in the larvicide testing guidelines of the World Health Organization (WHO, 2005), with the experiment concluding when mosquitoes had either died or emerged as adults. Larval screen data were evaluated using the Fisher's exact test.

The adulticidal capacity of siRNA #462 (hereafter referred to as dop1.462 siRNA) was assessed in a microinjection screen conducted in *A. aegypti*. For the microinjection experiments, adult female mosquitoes were injected using an embryo microinjection protocol (Clemons et al., 2010b) that was adapted for adults. In summary, non-blood fed three day old females were anesthetized with carbon dioxide and injected with a dose of 2.25 μg siRNA (250 nl of 9 μg/μl dop1.462 or control siRNA) vertical to the body axis in the thoracic region. After injection, the mosquitoes were placed in a cage where they recovered and were observed for behavioral defects and death over the course of the next week. 20 adult females/treatment were injected in each of three replicate experiments, and data were evaluated with the Fisher's exact test. The susceptibility of *A. albopictus* and *A. gambiae* adult females to dop1.462 siRNA was evaluated in the same manner, except that the siRNA dose was reduced to 150 nl of 6 μg/μl siRNA per mosquito for *A. gambiae* adult females, which are smaller than *A. aegypti* and *A. albopictus* adult females.

2.3 ATSB simulated field trials:

ATSB feedings were performed using a protocol modified from Coy et al. (2012). To create the bait station, the pointed end of a 0.2 ml plastic tube (Eppendorf, Hauppauge, NY) was cut off using a razor blade, creating a 1 mm opening through which a small piece of cotton (~4 mg) that served as a wick was placed. 64 μl of 10% sucrose in sterile DEPC-treated water with 0.5% of blue tracer dye (McCormick) alone, or containing 2.5 μg/μl of dop1.462 or control siRNA, was added to the tube. The tube was then capped and hung with the wick facing down at the top of a 3.75 L cage (Berry Global, Evansville, IN). 25 non-blood fed 4–5

day old adult females that had been sugar-starved for 48 hrs were permitted to feed from the wick for four hrs beginning at dawn. Females that were semi-engorged or that had failed to feed were discarded, while fully engorged females were collected as individuals and placed in fruit fly rearing vials. After 24 hrs, the mosquitoes were fed with 10% sucrose solution, which was subsequently provided every two days over the next six days, at which time behavioral phenotypes and mortality were assessed. The G-test of independence was used to compare feeding rates among treatments in each of three biological replicate experiments, while the log-rank test was used for comparison of survival rates among the control or experimental sugar bait treatments.

2.4 Yeast engineering and culturing

Custom DNA oligonucleotides (Invitrogen Life Technologies, Carlsbad, CA) encoding a short hairpin RNA (shRNA) expression cassette corresponding to *dop1* target sequence 5'-AUAUCAUCGCCGCGUUCUGCA-3' were used to produce stably transformed *S. cerevisiae* as previously described (Hapairai et al., 2017). In short, the dop1.462 shRNA-encoding nucleotide was ligated downstream of the galactose-inducible *Gal1* promoter (Bassel and Mortimer, 1971) and upstream of the *cyc1* terminator. The resulting construct was inserted into the multiple cloning sites of the *pRS404* and *pRS406* integrating shuttle vectors (Sikorski and Hieter, 1989), which are marked by *TRP1* and *URA3*, respectively. The resulting plasmids facilitated chromosomal integration and selection of recombinant *S. cerevisiae CEN.PK* strain yeast [genotype *MATα ura3–52 trp1–289 leu2–3_112 his3 1 MAL2–8C SUC2* (van Dijken et al., 2000)] which were able to grow on synthetic complete media lacking tryptophan and uracil. PCR and sequencing were used to verify integration of the dop1.462 shRNA expression cassettes at both loci. This strain, which is hereafter referred to as dop1.462 yeast IRP, as well as a previously described control shRNA expression strain (Hapairai et al., 2017), were cultured as described (Hapairai et al., 2017). The yeast cultures were used in the preparation of 40 mg dried inactivated yeast interfering RNA larvicide tablets as detailed in a methods protocol (Mysore et al., 2019c).

2.5 Larvicide trials

Lab assays: Laboratory larvicide trials which conformed to the WHO larvicide testing guidelines (WHO, 2005) were performed as described previously (Mysore et al., 2017). In short, in each of three biological replicate experiments, 20 first instar (L1) larvae were placed in 50 ml of distilled water in each of three 500 ml replicate containers per condition (control or dop1.462 treatment). In each replicate container, the 20 larvae were fed a single 40 mg control or dop1.462 yeast tablet at the beginning of the trial, which permitted *ad libitum* larval feeding throughout the experimental trial period. Fourth instar (L4) larvae were given a dietary supplement, which consisted of 150 μl of 6% w/v liver powder (MP Biomedicals) mixed in distilled water as described (Mysore et al., 2019a). Larval mortality was assessed, and the percentages of larval mortality were arcsine transformed prior to analyzing data from replicate experiments using Student's t-test.

For generation of dose-response curves, which were produced as described (Hapairai et al., 2017), three biological replicate experiments, each with three larvicide-treated replicate containers per dose, were performed. To generate the different doses of dop1.462 IRP yeast,

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various proportions of the larvicidal yeast culture were mixed with the control shRNA yeast. Replicate data were pooled for analysis, and LD_{50} values with 95% confidence intervals were determined using SPSS 25 software (IBM, Armonk, NY) and log dosage-probit mortality regression as previously described (Hapairai et al., 2017; Mysore et al., 2017). The data were also assessed separately using linear regression analysis that was performed using Microsoft Excel (Microsoft Corp, Seattle, WA).

2.6 Semi-field larvicide trials:

Larvicide trials were also conducted outdoors in a rooftop laboratory in Notre Dame, IN during May and June 2019. These semi-field trials, which were completed in accordance with the WHO larvicide testing guidelines (WHO, 2005), were conducted as previously described (Mysore et al., 2019a,b) on LVP-IB12 strain *A. aegypti* mosquitoes. To prepare each replicate container, 20 L1 larvae, 3.7 L of distilled water (water height of 10 cm), and one dop1.462 or control yeast larvicide tablet were placed in a 10 L plastic container (height = 25 cm, diameter = 23 cm). To prevent mosquito escape and the entrance of macrobiota into the test site, the containers were covered with mesh and placed in a screened (472 openings per centimeter) SansBug 1-Person Free-Standing Pop-Up Mosquito-Net tent (Hakuna Matata Tents, Ontario, Canada) located underneath an overhang. A total of 14 replicate containers/condition were evaluated over the course of three biological replicate trials. At the conclusion of the trials, the percentages of larval mortality were arcsine transformed, and Student's t-test was used to evaluate data from multiple replicate experiments. During the trial period, humidity levels averaged 75±15%, while temperatures ranged from 9° C to 35° C, with mean daytime temperatures of 23.5±5° C and mean nighttime temperatures of 19±4° C.

2.7 Evaluation of *dop1* transcript levels

A riboprobe corresponding to bases 272–708 of the *A. aegypti dop1* gene *(AAEL019437)* was prepared as described (Patel, 1996) and used in *in situ* hybridization experiments that were conducted as described (Haugen et al., 2010) and used to assess transcript levels in adult and L4 larval brains. For analysis of adult transcripts, brains from 20 adult females that had been microinjected with dop1.462 or control siRNAs were evaluated in each of three biological replicate experiments. For analysis of larval transcripts, the brains of 20 larvae that had been fed with either dop1.462 or control yeast tablets were assessed in each of three biological replicate experiments. Following imaging of the processed brains using a Zeiss Axioimager (Carl Zeiss Microscopy, LLC, Thornwood, NY) that is equipped with a Spot Flex camera (Diagnostic Instruments, Inc. Sterling Heights, MI), FIJI ImageJ software (Schindelin, 2019) was used to calculate mean gray values (average signal intensity over the selected area). This permitted quantification of digoxigenin-labeled *dop1* transcript signals in the brains of control or dop1.462-treated L4 or adult mosquitoes as described (Mysore et al., 2015). Data from the three larval or adult biological replicate experiments were combined and statistically evaluated with Student's t-test.

2.8 Immunohistochemical analysis of mosquito brains:

Three biological replicate immunohistochemical staining experiments were performed on the brains of control or dop1.462 IRP-treated adult or L4 mosquitoes as described

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(Clemons et al., 2010c; Mysore et al., 2011). The following reagents were used in these studies: anti-Bruchpilot mAb nc82 antibody (Wagh et al., 2006) (Developmental Studies Hybridoma Bank, Iowa City, Iowa, Product nc82, which was deposited by E. Buchner) and TO-PRO-3 iodide (Molecular Probes, Eugene, OR). Three biological replicate experiments were performed on the brains of 20 L4 larvae or adults per control or experimental treatment in each biological replicate experiment. Processed brain tissues were mounted and imaged using a Zeiss 710 confocal microscope and Zen software, and the images were analyzed with Adobe Photoshop CC 2018 and FIJI ImageJ (Schindelin, 2019) software. This permitted quantification of mean gray values, which were calculated and statistically analyzed using Student's t-test as described (Mysore et al., 2015).

2.9 Evaluation of dop1.462 pesticide in non-target species:

Tribolium castaneum: Adult *Tribolium castaneum* were obtained from Carolina Biologicals (Burlington, NC), and the beetles were reared according to the provider's instructions. For the toxicity assays, in each of two biological replicate experiments, 20 newly hatched *T. castaneum* larvae were reared in a plastic tube (provided by Carolina Biologicals) on 10 g of an 8:8:1:1 mixture of white flour, brown flour, nutritional yeast (provided by Carolina Biologicals), and control or dop1.462 yeast. The tubes were stored in an incubator maintained at 32° C, and beetle survival was monitored throughout development. The number of adults that eclosed per replicate tube was observed and recorded, and combined data from two biological replicate experiments were analyzed with the Fisher's exact text.

Daphnia: *Daphnia pulex* and *Daphnia magna* were acquired from Carolina Biologicals (Burlington, NC) and evaluated as previously described (Mysore et al., 2019c) at 22° C, under ambient laboratory illumination (12 hr light/12 hr dark), and in COMBO medium containing 0.0001% sodium selenium (Kilham SS, 1998) and control or dop1.462 yeast. In each of three biological replicate trials, a 40 mg dop1.462 or control yeast tablet was dissolved in 50 mL of distilled water, and this solution was fed to 20 *Daphnia* over a five day period, with 10 ml of solution provided each day. A Fischer's exact test was used to analyze combined survival data from each of three 10 day biological replicate trials.

D. melanogaster: Survival of Oregon R (Thurmond et al., 2019) *D. melanogaster* larvae that fed on control or dop1.462 yeast was assessed in assays that were performed at 22° C under ambient laboratory illumination (12 hr light/12 hr dark). In summary, for each assay performed as previously described (Mysore et al., 2019b), a tablet of control or dop1.462 yeast was resuspended in 200 μl of distilled water and 10 μl of red food dye (McCormick's) and mixed with 10 ml of standard fly media. In each replicate trial, 20 L1 larvae were placed in a vial of food containing control or larvicidal yeast, and yeast consumption was verified through observation of red food dye in the larval guts. The number of adults that emerged from each of seven biologial replicate trials was recorded as a measurement of survival, and data were evaluated using the Fischer's exact test.

For analysis of adults, 20 females were placed in a cage with a 100×150mm petri dish with 32 μl of 10% sucrose solution containing 0.5% of blue tracer dye (McCormick) alone

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or with 2.5 μg/μl of control or dop1.462 siRNA that was divided into four 8 μl droplets. The adult flies were permitted to feed overnight before being transferred into plastic vials containing standard rearing media. These assays were performed under ambient laboratory illumination (12 hr light/12 hr dark) at 22° C. Mortality was recorded daily for six days, and data from two biological replicate experiments were combined and analyzed using a G-test of independence.

3. Results

3.1 Discovery of dop1.462 siRNA, a dual-action adulticidal/larvicidal mosquito IRP:

siRNA #462, hereafter referred to as dop1.462 siRNA, was prioritized for testing in screens for genes required at multiple stages of the mosquito life cycle (see methods for detail). dop1.462 siRNA corresponds to a target sequence located in exon six of *A. aegypti dop1* (Meyer et al., 2012) that is conserved in multiple *Aedes* and *Anopheles* mosquito species (Giraldo-Calderon et al., 2015), but has not yet been identified outside of mosquitoes (Supplementary files 1, 2). Injection of *A. aegypti* adult females with 9 μg/μl of dop1.462 siRNA induced 48±1% mortality in *A. aegypti* adult females (Fig. 1A; P=0.00015253 vs. control siRNA treatment). Soaking L1 *A. aegypti* larvae in 0.5 μg/μl dop1.462 siRNA resulted in 70±7% larval mortality (Fig. 1B; $P=9.2733\times10^{-5}$ vs. control siRNA treatment). The results of these screening studies support the hypothesis that dop1.462 siRNA has both adulticidal and larvicidal activity in *A. aegypti.*

3.2 Silencing the *Aae dop1* gene results in neural defects in the *A. aegypti* adult brain

The functions of *dop1* were characterized in the *A. aegypti* adult brain, in which it was hypothesized that silencing of *dop1* expression by dop1.462 siRNA would impact neural activity. In wild type and control-injected animals, *Aae dop1* is expressed broadly throughout the adult brain (the control-injected animal is shown for reference in Fig. 2A1). A significant reduction in *dop1* transcripts was observed in the brains of *A. aegypti* adult females that were injected with dop1.462 siRNA (Fig. 2A2; 86±1.5% reduction with respect to control siRNA-treated brains, $P=1.42X10^{-103}$). Although neural density, which was evaluated through quantification of TO-PRO nuclear staining (Fig. 2C3), was not significantly different (P>0.05) in dop1.462 (Fig. 2C2) vs. control-treated brains (Fig. 2C1), levels of Bruchpilot expression, a marker of active neural synapses (Wagh et al., 2006), were reduced by 82±2% (Fig 2B3, $P=3.741X10^{-54}$) in the brains of adults injected with dop1.462 siRNA (Fig. 2B2,C2; compare to control-treated brains in Fig. 2B1,C1). These experiments confirmed that dop1.462 siRNA effectively silences *dop1* and demonstrated that this silencing results in disruption of neural function in the *A. aegypti* adult brain.

3.3 ATSB-mediated delivery of dop1.462 IRPs

The adulticidal activity of dop1.462 siRNA observed in microinjection assays (Fig. 1A) suggested that it may be useful to identify a mechanism for delivery of dop1.462 that could potentially translate to the field. To this end, a sugar-baited delivery system was evaluated in simulated deployment trials conducted in the laboratory in which adult females were fed 10% sucrose sugar bait marked with blue tracer dye alone, with control siRNA, or with dop1.462 siRNA. From a total of 75 female mosquitoes subjected to each treatment

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(25/treatment in each of three biological replicate trials), 55± 4% fed on sugar bait, 55± 4% on sugar bait containing control siRNA, and 45±4% on sugar bait with dop1.462 ATSB (resulting in an average dose of ~12 μg siRNA/mosquito); no significant differences were observed in feeding rates among the three treatments (G=1.75, d.f=2, P=0.413). Although negligible mortality was observed in mosquitoes fed with sugar bait or sugar bait containing control siRNA, 88±4% of mosquitoes that fed on dop1.462 ATSB died [Fig. 3A; P<0.001 compared to sugar (χ^2=53.21) or sugar with control siRNA (χ^2=49.73)] over the course of a six day trial period (see survival curve in Fig. 3B). All dop1.462 ATSB-treated mosquitoes (n=34 individuals combined from three biological replicate experiments), including the 12% of dop1.462-treated mosquitoes that recovered and survived treatment (Fig. 3A), failed to fly and exhibited very limited, uncoordinated walking behavior (Video 1). 100% morbidity, defined here as a failure to respond to stimuli for 10 sec, was observed in mosquitoes that consumed dop1.462 ATSB (n=34). In summary, ATSB-mediated delivery of dop1.462 resulted in severe behavioral deficits and high levels of adult mortality (Fig. 3A,B) that exceeded those observed in adult microinjection experiments (Fig. 1B).

3.4 Targeting *dop1* induces neural defects and death in *A. aegypti* larvae:

The impact of silencing *dop1* in *A. aegypti* larvae was examined using a yeast delivery system (Duman-Scheel, 2019; Hapairai et al., 2017) for dop1.462 IRP. *S. cerevisiae* engineered to express shRNA corresponding to dop1.462 siRNA (hereafter referred to as dop1.462 yeast) were generated. Larval consumption of inactivated dop1.462 yeast tablets prepared from this strain resulted in 92±1% *A. aegypti* larval mortality in indoor laboratory trials (Fig. 4A; P=8.754X10^{-18} vs. control yeast interfering RNA treatment; LD_{50}=34 mg, Fig. 4D). Likewise, dop1.462 yeast induced 93±1% larval death in semi-field experiments conducted in an outdoor rooftop laboratory in Notre Dame, IN (Fig. 4C; P=9.6608X10^{-18} vs. control). Larvae treated with dop1.462 yeast (beginning in L1) died as fourth instar (L4) larvae or as early pupae (Fig. 4B). Comparable to adults (Fig 2A1,A2), broad expression of *dop1* throughout the L4 larval brain (Supplementary File 3.A1) was reduced by 93±1% in dop1.462 yeast-treated larvae (Supplementary File 3.A2; P=9.0902X10^{-130}) that were harvested in early L4 just prior to the time that treated animals typically die (Fig. 4B). As observed in adults (Fig. 2), silencing of *dop1* transcripts correlated with significant loss (78±1.5%) of Bruchpilot expression (P=1.803X10^{-46}) in L4 brains (Supplementary File 3.B2,C2 vs. control in 2.B1,C1), but no significant differences in neural density were detected (Supplementary File 3.C3, P>0.05; compare dop1.462 treated brain in Supplementary File 3.B2,C2 vs. control-treated brain in 2.B1,C1). This loss of neural activity in the L4 nervous system correlated with the timing of larval death (Fig. 4B). These experiments, combined with analyses of adults (Figs. 2, 3), indicated that Dop1 neural activity is required at multiple stages of the *A. aegypti* life cycle.

3.5 Dop1.462 IRPs function as broad-range mosquito insecticides and are not toxic to a selection of non-target arthropods:

The target site of dop1.462 IRPs is conserved in *A. albopictus* and multiple species of *Anopheles* malaria vector mosquitoes, but not in the sequenced genomes of other arthropods, humans, or other non-target organisms (Supplementary file 1). Given the conservation of this target site in multiple mosquito species, it was hypothesized that

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dop1.462 yeast would function as a biorational broad-range mosquito IRP. In support of this hypothesis, as observed in *A. aegypti* adult microinjection experiments (Fig. 1A), in adult female mosquitoes, microinjection of dop1.462 siRNA resulted in 55±1% adult female *A. albopictus* mortality (Fig. 5A; P=1.8246X10^{-12} vs. control siRNA treatment) and 42±1% adult female *A. gambiae* mortality (Fig. 5B; P=3.6901X10^{-7} vs. control siRNA treatment). Likewise, dop1.462 yeast larvicide treatments induced 91±1% mortality in *A. albopictus* (Fig. 5C; P=1.607X10^{-23} vs. control yeast treatment) and 91±1% mortality in *A. gambiae* (Fig. 5D; P=3.277X10^{-18} vs. control yeast treatment). This adulticidal and larvicidal activity of dop1.462 appears to be restricted to mosquitoes, as no significant mortality (P>0.05) was observed in *Daphnia pulex* (Fig. 6A) and *Daphnia magna* adults (Fig. 6B), *Tribolium castaneum* larvae (Fig. 6C), or *D. melanogaster* larvae (Fig. 6D) or adults (Fig. 6E), all of which have known *dop1* orthologs (Kriventseva, 2019) that lack the dop1.462 target site (Supplementary file 1) and survived treatment with dop1.462 IRPs. Combined, these results support the hypothesis that dop1.462 IRPs function as a biorational broad-range mosquito IRP.

4. Discussion:

4.1 RNAi-based approaches for mosquito-specific GPCR targeting:

GPCRs, which have been successfully targeted for human drug development (Wise et al., 2002), have been described as underexploited pesticide targets (Hill et al., 2016; Ngai and McDowell, 2017). As discussed by (Hill et al., 2016), new insecticides that target GPCRs will ideally be mosquito-selective, yet effective against multiple species of disease vector mosquitoes. Genome sequencing efforts (Arensburger et al., 2010; Holt et al., 2002; Matthews et al., 2018; Neafsey et al., 2015; Nene et al., 2007) have facilitated the design of RNAi-based pesticides that recognize conserved targets in multiple mosquito species, but which are not conserved in non-target species (Supplementary file 1), such as honey bees and other pollinators, suggesting that RNAi pesticides could represent a “green” approach for mosquito-specific GPCR targeting. The results of this investigation demonstrate that RNAi-mediated silencing of *dop1* causes mosquito mortality at both the adult (Figs. 1, 3) and larval (Figs. 1, 4) stages of the mosquito life cycle. Severe defects in synaptic activity in the mosquito adult (Fig. 2) and larval (Supplementary file 3) brain correlate, and are likely primary contributors to the high levels of mortality induced by dop1.462 IRPs. The adult behavioral defects observed are comparable to those resulting from neural silencing of *dop1R1* in *D. melanogaster*, in which a flightless phenotype and adult lethality were also reported (Dietzl et al., 2007). The results of this investigation also demonstrate that the requirement for *dop1* function is conserved between *A. aegypti, A. albopictus,* and *A. gambiae* mosquitoes (Fig. 5). Conservation of the *dop1* target site in multiple other *Anopheles spp.* (Supplementary file 1) suggests that dop1.462 IRPs will have activity in multiple species of malaria vector mosquitoes, indicating that IRPs targeting mosquito *dop1* genes could one day be used in integrated control programs targeting multiple malaria vectors. Although the dop1.462 target site is not identically conserved in the *C. quinquefasciatus dop1* ortholog (Kriventseva, 2019), which was consequently not included in this investigation, the design of siRNAs or shRNAs to match the *C. quinquefasciatus*

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dop1 gene, as well as the genes of other *Anopheles* species with known *dop1* orthologs (Kriventseva, 2019), would likely permit successful targeting of these species as well.

In silico analyses failed to detect the dop1.462 IRP target sites outside of mosquitoes (Supplementary files 1, 2). It is difficult, however, to rule out the potential for non-target impacts solely on the basis of a lack of sequence similarity (Jackson and Linsley, 2010). To begin to address this, dop1.462 activity was evaluated in several non-target arthropods that lack dop1.462 target sites (Supplementary file 1, Fig. 6). *D. melanogaster* (Fig. 6D,E), a dipteran insect relative of vector mosquitoes, as well as *Tribolium castaneum* (Fig. 6C), a more distantly related insect, were not impacted by consumption of dop1.462 IRPs. *T. castaneum*, in which a variety of tissue types are known to respond to extracellular dsRNA (Miller et al., 2008), is sensitive to oral RNAi pesticides (Alshukri et al., 2019). *T. casteneum* is known to have a stronger systemic RNAi response than *D. melanoagaster,* in which most tissues do not take up extracellular dsRNA (Miller et al., 2008), but neither insect died following dop1.462 treatment (Fig. 6C). Likewise, dop1.462 yeast consumption was not toxic to two species of *Daphnia* (Fig. 6A). *Daphnia* were selected for these non-target assays because these distantly related aquatic arthropods are often used in U.S. Environmental Protection Agency (EPA) toxicity assays (EPA, 2002) and are known to be sensitive to RNAi (Hiruta et al., 2013; Guo et al., 2016; Schumpert et al., 2015). The results of these initial toxicity assays suggest that dop1.462 may have little, if any activity in non-target organisms. These preliminary results suggest that RNAi-based insecticides could help overcome challenges associated with discovery of vector selective chemistries targeting GPCRs, perhaps helping to eliminate a need for timed pesticide applications that would avoid pollinator activities (Hill et al., 2018). If dop1.462 insecticides were ever to be commercialized, it will of course still be important to pursue additional requisite toxicity assays with commercial-ready dop1.462 formulations in additional species, including vertebrate organisms. However, the present results indicate that dop1.462 IRPs, which appear to have a desirable safety profile with respect to current chemical pesticides, may represent a new tool for the biorational control of multiple species of disease vector mosquitoes during both the adult and larval stages of the mosquito life cycle.

4.2 ATSB-mediated delivery of dop1.462:

In recent years, we have worked to develop a yeast-based IRP production and delivery system (Duman-Scheel, 2019; Hapairai et al., 2017) that was used to successfully characterize the larvicidal capacity of dop1.462 activity in this investigation (Fig. 4). This yeast-based system, one of the most effective methods for larval gene silencing evaluated in our laboratory to date (Hapairai et al., 2017), has many advantages. For example, interfering RNA is produced through relatively inexpensive yeast culturing, significantly reducing RNA production costs. Yeast, a non-toxic natural product that is used for food and beverage preparation and sold as a dietary supplement, has been cultivated worldwide for thousands of years, and yeast interfering RNA technology, which could be implemented in the field, could be readily scaled for use in global larvicide programs (Duman-Scheel, 2019). In this investigation, microinjection experiments demonstrated that dop1.462 siRNA can also function as an adulticide (Fig. 1A), suggesting that identification of a field-appropriate method for delivery of IRPs to adult mosquitoes would be beneficial. Unfortunately,

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effective methods for topical application of IRPs to insects have not yet been developed. We therefore turned to sugar-baited delivery, a system that enabled successful delivery of dop1.462 to *A. aegypti* in the laboratory (Fig. 3) and which could one day translate to the field.

ATSBs, an emerging new paradigm for vector control (Fiorenzano et al., 2017), exploit the sugar feeding behavior of female and male mosquitoes, which are attracted to feed on sugar sources containing toxins. ATSBs, which are delivered through sprays or at bait stations, can be used both outdoors and indoors for mosquito control (Fiorenzano et al., 2017; Müller, 2016). Significant reductions in disease vector mosquitoes were observed in ATSB field trials (Fiorenzano et al., 2017; Müller, 2016; Sissoko et al., 2019), suggesting that this technology will significantly advance integrated mosquito control programs. Although sugar baits facilitate more targeted pesticide delivery, pesticide resistance is still of concern (Faraji and Unlu, 2016), and efforts to develop IRPs as a new class of pesticides which are compatible with ATSB technology may therefore be beneficial. Furthermore, many of the IRPs that are currently in use, for example garlic oil and boric acid insecticides, are not specific to mosquitoes and could impact non-target organisms (Fiorenzano et al., 2017). Advancements such as the addition of protective barriers to ATSB bait stations and limiting ATSB application to non-flowering vegetation (Fiorenzano et al., 2017; Müller, 2016) have improved the specificity of this application. The added specificity of IRPs, which appear to have a highly desirable safety profile (MonSanto, 2014) (Supplementary file 1, Fig. 6), could further enhance ATSB technology. At this time, dop1.462 ATSBs have only been tested on *A. aegypti,* but it would be interesting to further develop this technology for use in *A. albopictus* and *A. gambiae,* both of which are also sensitive to dop1.462 microinjection treatment (Fig. 6A,B). Given the increased insecticidal capacity of dop1.462 observed when it was delivered as an orally consumed ATSB (Fig. 3) rather than through injection (Fig. 1A), it is anticipated that ATSB-mediated delivery of dop1.462 to *A. albopictus* and *A. gambiae* would result in high levels of mortality in these species, as well. The higher levels of mortality observed following ATSB delivery likely result from the increased dose of siRNA delivered in an average sugar meal (~12 μg siRNA per mosquito in an average ATSB treatment, opposed to 4.5 μg siRNA per mosquito through microinjection). Moreover, given that the small percentage of mosquitoes which recover and survive dop1.462 treatment in the laboratory have severe locomotor and flight defects (Video 1) that would likely prohibit their survival in the wild, it is anticipated that field mortality rates could approach 100%.

4.3 Prospects and considerations for future field implementation of mosquito IRPs:

In addition to demonstrating the potential for using IRP-ATSBs to control adult mosquitoes, the results of this investigation support continued efforts to develop yeast IRPs for control of mosquito larvae. Activity of dop1.462 yeast, like that of several other IRP yeast larvicides recently developed in our laboratory (Mysore et al., 2019a,b) was retained when the yeast was used outdoors, where it was exposed to temperatures that reached 35° C (Fig. 4C). These results, combined with previous studies in which it was demonstrated that yeast IRP larvicide activity levels were retained in different types of water, in different sizes of containers that held 50 ml to 26 L of water, in both laboratory and field strains of mosquitoes, and when *A. aegypti* larval numbers and densities were varied (Hapairai

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et al., 2017; Mysore et al., 2019a,b), suggest that these larvicides could become useful components of integrated mosquito control programs. However, yeast IRP technology would be further enhanced by the development of robust formulations that are resistant to extreme conditions (i.e. high heat and humidity) that may be encountered during shipment to and long-term storage in the tropics. Moreover, the development of long-lasting formulations with residual activities that extend beyond two weeks in water, the residual activity of the present formulation (Hapairai et al., 2017), would also be beneficial. We are presently working to address these concerns and are also pursuing efforts to scale yeast production to commercially-appropriate levels (Duman-Scheel, 2019). Likewise, the use of ATSB systems for delivery of insecticidal RNAs would also require scaled IRP production, as well as the development of shelf-stable robust formulations with residual activities that are sufficient for deployments in bait stations or as foliar sprays in the tropics. San Miguel and Scott (2016) demonstrated that foliar application of dsRNA targeting actin in the Colorado potato beetle was active for at least a month and was not easily removed after it had dried on plant leaves. Furthermore, in field trials, Hunter et al. (2010) successfully delivered dsRNA in sugar solutions to honey bees, which were subsequently protected from Israeli Acute Paralysis Virus infections. These investigations suggest that ATSB-mediated IRP delivery systems could be effectively deployed for mosquito control.

Following development of commercial-ready dop1.462 yeast and ATSB formulations, field testing in the United States must be conducted to support future EPA registry applications. Moreover, in addition to the United States, the use of RNAi-based pesticides will need to be approved in each country of intended use. Approvals for use of yeast-based IRPs, which are genetically modified organisms, may be challenging, particularly given that some nations lack a regulatory body equivalent to the United States EPA and have no mechanism to review requests to use IRP technology. The demonstrated ability to use heat-killed yeast for RNAi-based applications (Hapairai et al., 2017), as was the case in the present study, may help to overcome these challenges. Moreover, the pursuit of further toxicology testing, field testing in the United States, and EPA registry approval for both larvicidal and adulticidal RNAi-based pesticides would likely help to facilitate approval for these technologies elsewhere.

In advance of further development of RNAi-based pesticides for mosquito control, concerns for the potential to develop resistance to RNAi-based pesticides have also been raised. With the addition of dop1.462 to the growing arsenal of IRPs, we continue to develop new pesticides that could effectively replace any specific IRP to which mosquitoes become resistant. Furthermore, given that RNAi machinery regulates endogenous cellular mechanisms, one could argue that resistance to RNAi as a whole might not be likely to develop. However, Khajuria et al. (2018) recently reported on the development and characterization of a dsRNA-resistant *Diabrotica virgifera virgifera* (western corn rootworm) population. This raised the question of whether the ~5–10% of larvae in some containers that survive treatment with dop1.462 (Fig. 4) or other larvicidal yeast strains (Hapairai et al., 2017; Mysore et al., 2019a,b) are resistant to dsRNA. This does not appear to be the case, as all larvae die when reared as individual larvae following yeast IRP treatments (Mysore et al., 2019a,b), suggesting that survivors are likely eating dead larvae (which are rarely observed in the containers) rather than yeast. Likewise, detection of severe behavioral phenotypes or

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death in all mosquitoes that consumed dop1.462 ATSB (Fig. 3, Video 1) suggests that adults are not resistant to this insecticide. Finally, in ongoing studies with two different yeast IRPs, no evidence of resistance has been observed following 10 generations of selection (MDS, unpublished). Given that the evolution of insecticide resistance must be considered with every new class of pesticides under consideration (Khajuria et al., 2018), further research on this topic is nevertheless warranted.

It should also be noted that although siRNAs and shRNAs 21–25 bp in length appear to work well in *A. aegypti, A. albopictus, A. gambiae, and C. quinquefasciatus* (this study and Mysore et al., 2019a,b), short interfering RNAs do not perform as well in some other insects. For example, siRNAs are not effectively taken up by *D. melanogaster* S2 cells (Saleh et al., 2006). Likewise, oral RNAi studies in *Diabrotica virgifera virgifera* demonstrated that a 21 bp siRNA targeting the *DvSnf7* gene did not appear to be taken up in midgut cells. By contrast, midgut uptake of a longer 240 bp dsRNA was noted in *Diabrotica virgifera virgifera* and supported a size-activity relationship in bioassays (Bolognesi et al., 2012). Thus, mosquitoes appear to have more efficient uptake of siRNAs than the western corn rootworm. The endocytic pathway mediates dsRNA entry in *D. melanogaster* (Saleh et al., 2006), and *T. castaneum* processes ingested dsRNA via clathrin-dependent endocytosis (Xiao et al., 2015). It is presumed that mosquito siRNA and microbe/shRNA uptake is through endocytosis, but the mechanism will need to be further investigated in mosquitoes. Likewise, the overall mechanisms leading to spreading of the RNAi response in tissues beyond the mosquito midgut, for example the larval or adult brain in which *dop1* transcript levels were significantly reduced (Fig 2A2, Supplemental file 3A2), will need to be further investigated in mosquitoes and other insects (Cooper et al., 2018). Evaluation of the mechanisms of siRNA and yeast/shRNA uptake and systemic spreading of the RNA response in mosquitoes could help to explain the differences in RNAi efficiency noted between mosquitoes and other insects. Such research could facilitate the enhancement of RNAi methodology in insects that are less amenable to RNAi (Cooper et al., 2018). The mosquito RNAi research agenda should therefore include elucidation of the cellular machinery that permits siRNA and microbe-shRNA uptake, how these species are processed and degraded, whether the mechanisms differ between various delivery methods, and evaluation of the potential for developing RNAi resistance when utilizing various interfering RNA delivery strategies.

4.4 Conclusions and Future Directions:

In conclusion, these studies demonstrated that dop1.462, a dual-action adulticidal and larvicidal IRP with target sites conserved in the orthologous genes of multiple species of mosquitoes (Supplementary file 1), may represent a new method of controlling *Aedes* and *Anopheles* mosquitoes (Figs. 1, 3, 4, 5) at multiple stages of the mosquito life cycle. Characterization of dop1.462 indicated that the mode of action for this IRP is through silencing of the *dop1* gene, resulting in disruption of neural function in adults (Fig. 2) and larvae (Supplementary file 3). In silico data which indicate that the dop1.462 target site is only present in mosquitoes (Supplementary file 1), combined with the lack of dop1.462 toxicity observed following treatments of non-target organisms (Fig. 6), indicate that this IRP may offer a method for specifically targeting mosquito *dop1* GPCRs that will not

impact GPCR activity in non-target species. Moreover, the results of simulated field trials demonstrated that adulticidal dop1.462 siRNA can be delivered to adult mosquitoes in the form of an ATSB (Fig. 3), suggesting that use of IRPs could promote the development of ATSBs with increased species-specificity. Efforts to generate and characterize the dop1.462 yeast IRP (Figs. 4, 5) in this investigation have resulted in the addition of an additional larvicide to the growing arsenal of yeast larvicidal IRPs (Hapairai et al., 2017; Mysore et al., 2017, 2019a,b). Future studies will aim to further build upon this arsenal of larvicides and to develop a comparable arsenal of adulticidal IRPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ATSB	attractive toxic sugar bait
dop1	dopamine 1 receptor
EPA	Environmental Protection Agency
L1	first instar
GPCR	G protein-coupled receptor
IRP	interfering RNA pesticide
LVP-IB12	Liverpool-IB12
shRNA	short hairpin RNA
siRNA	small interfering RNA
WHO	World Health Organization

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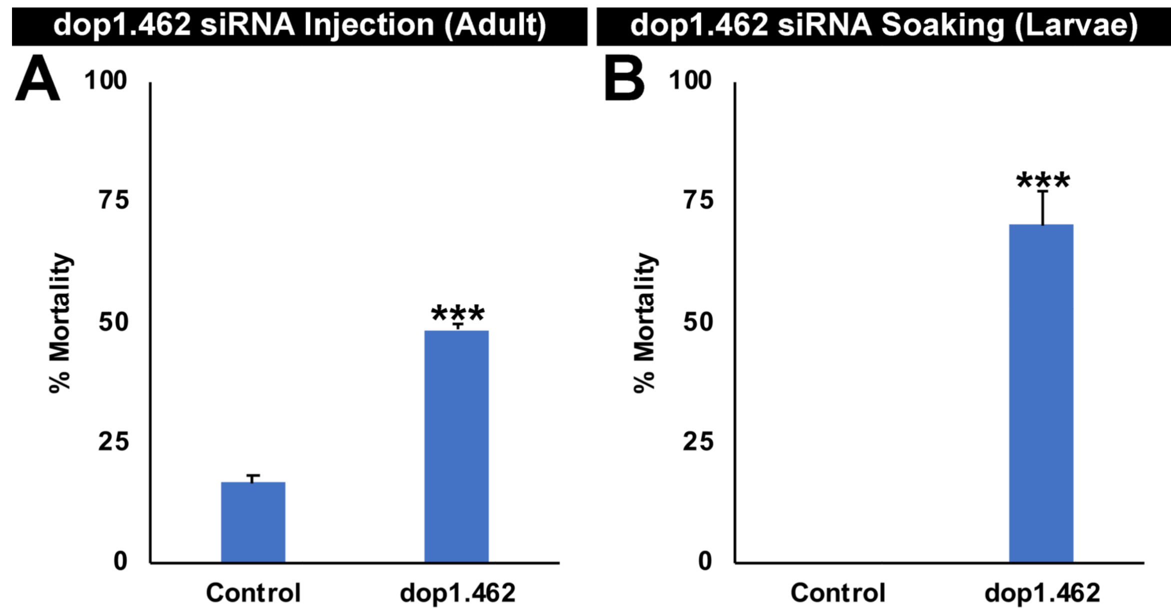

Fig. 1. Adulticidal and larvicidal activity of dop1.462 in *A. aegypti*.

siRNA dop1.462 was discovered in screens for mosquito adult lethal (A) and larval lethal (B) genes. Significant adult mortality was observed following microinjection of adult females with 250 nl of 9 μg/μl dop1.462 siRNA (A; data compiled from three biological replicate experiments, each with 20 adults/treatment are shown; mortality levels were recorded after six days of observation). Significant larval mortality was observed after four hour L1 soaking treatments with 0.5 μg/μl dop1.462 siRNA (B; the soaking screen was performed in duplicate with 20 larvae/treatment, with the experiment concluding when all mosquitoes had either died or emerged as adults). Data from dop1.462 vs. control siRNA-treated *A. aegypti,* represented here as mean percentage mortality, were statistically analyzed using the Fischer's exact test; error bars denote standard errors of the mean (SEM), and *** denotes $P<0.001$ vs. control.

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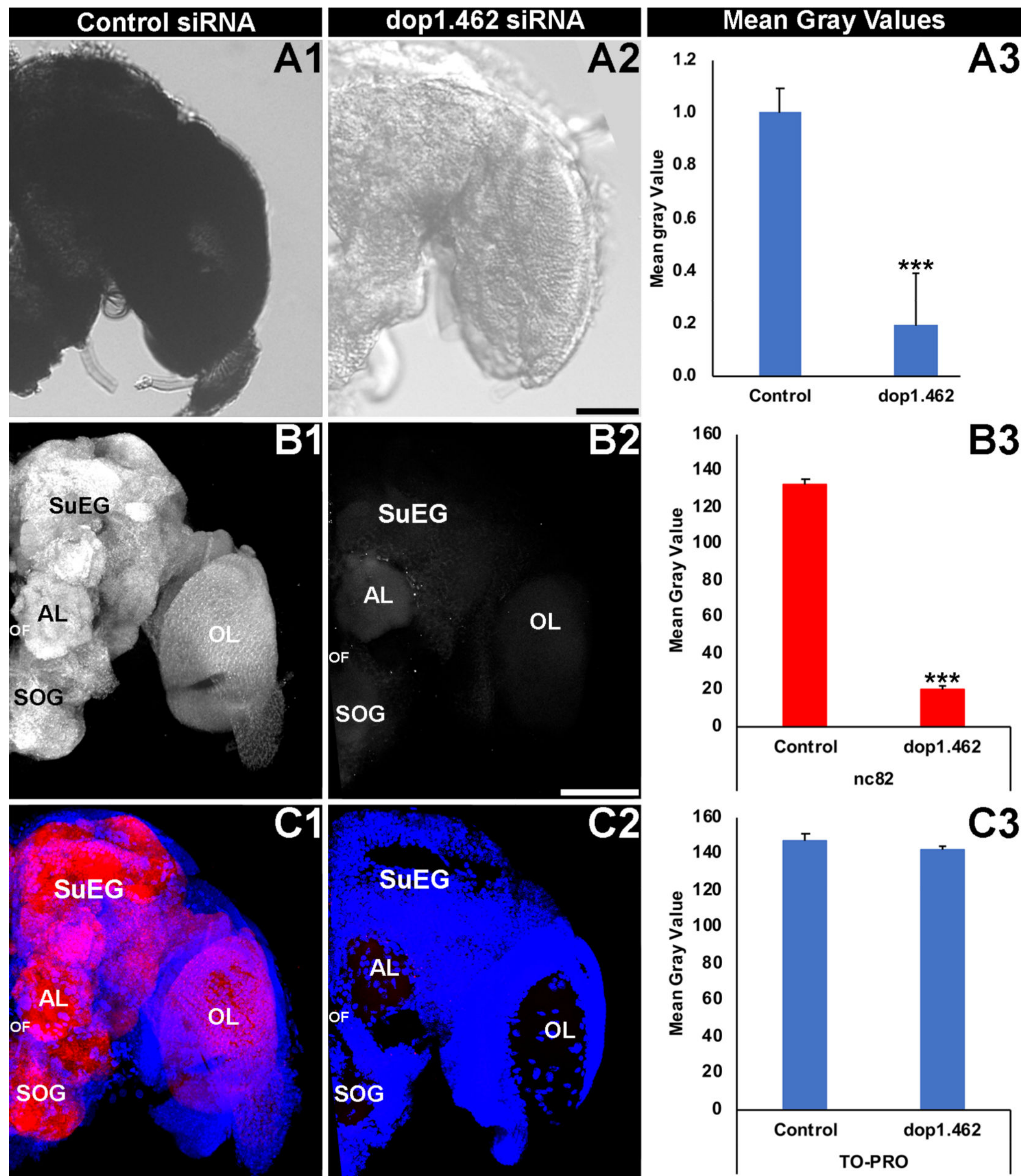

Fig. 2. Neural defects are observed in dop1.462-treated *A. aegypti* adults.

Broad expression of dop1.462 transcripts detected at high levels throughout the control-injected and wild-type *A. aegypti* adult female brain (a brain from a control siRNA-microinjected animals is shown in A1) was significantly reduced in the brains of adults injected with dop1.462 siRNA (A2; mean gray value results from three biological replicate experiments are shown in A3; n = 50 control-treated brains, and n = 60 dop1.462-treated brains). Although levels of TO-PRO nuclear staining (blue in C1, C2) were not significantly different (C3) in the brains of adults injected with control (Cl) or dop1.462 (C2) siRNA, levels of Bruchpilot (white in B1, B2; red in C1, C2), a marker of synaptic active zones (labeled by mAb nc82) were significantly reduced (B3) in the synaptic neuropil following microinjection of dop1.462 siRNA (B2, C2; compare to control siRNA treatment in B1,

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C1). Data were compiled from three biological replicate experiments performed on a total of 43 control-treated brains and 37 dop1.462-treated brains (B3, C3) and are represented as average mean gray values in A3, B3, and C3, in which error bars represent SEM. In all panels, brains were dissected and fixed 24 hours following injection of the mosquitoes with a 2.25 μg dose of siRNA. Student's t-tests were used for statistical analyses of control vs. dop1.462 siRNA injected animals; ***=P<0.001 vs. control. Representative adult brains are oriented dorsal upward; scale Bar=100 μm. Labels are as follows: **AL:** antennal lobe; **OF:** oesophageous foramen; **OL:** optic lobe; **SOG:** sub-esophageal ganglion; **SuEG:** supra-esophageal ganglion.

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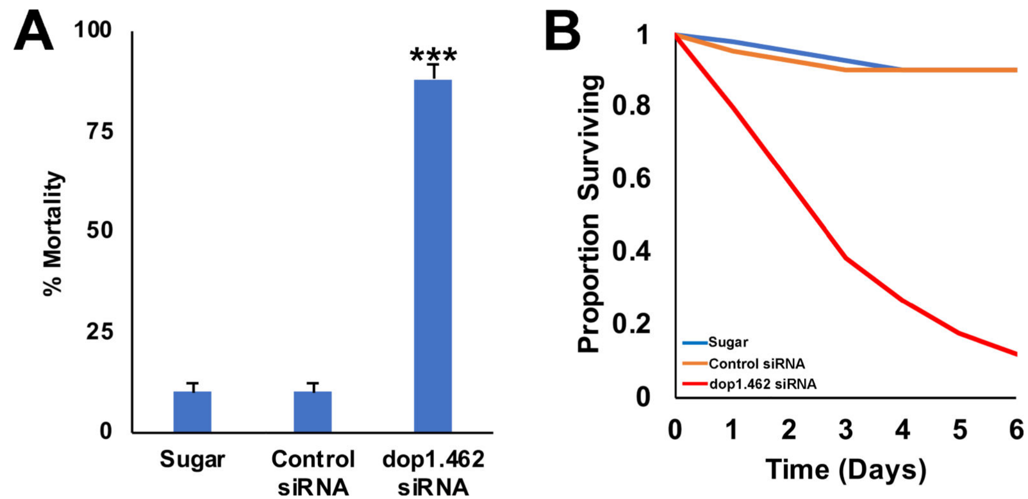

Fig. 3. Delivery of dop1.462 siRNA as an ATSB results in high levels of *A. aegypti* adult mortality.
A. In simulated field trials, high levels of adult mortality were observed in adult females that fed on ATSB with 2.5 μg/μl dop1.462 siRNA (n=34) vs. sugar bait alone (Sugar; n=41) or sugar bait with 2.5 μg/μl control siRNA (Control siRNA, n=41). The average dose was ~12 μg siRNA per mosquito. The data shown, which were compiled from three biological replicates trials, are represented as mean percentage mortality and were analyzed using the log-rank test; error bars represent SEM; ***=P<0.001 in comparison to sugar alone or control siRNA treatments. B. The survival curves over a six day trial period are shown for adult females that fed on sugar bait, control siRNA sugar bait, or dop1.462 ATSB.

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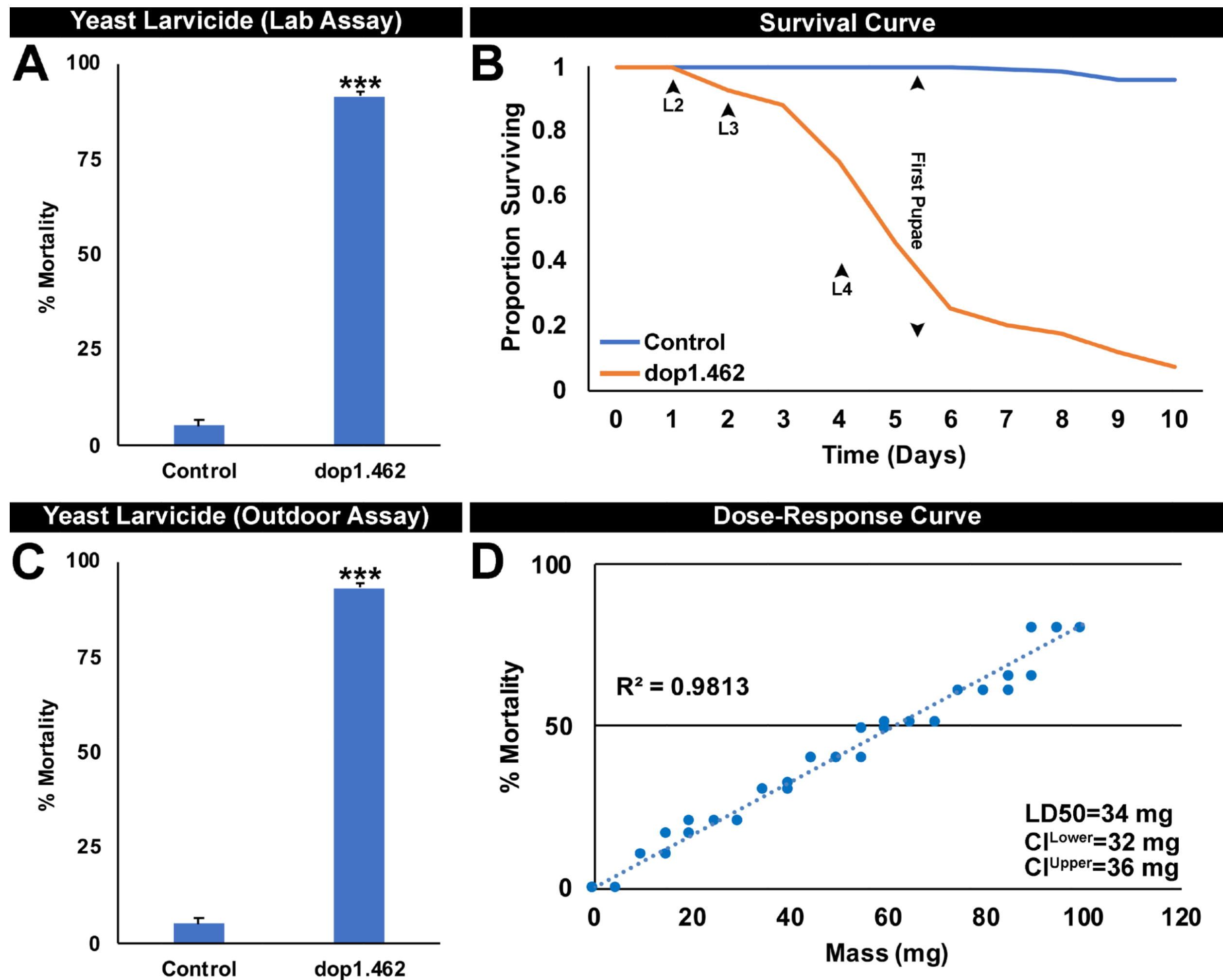

Fig. 4. Oral consumption of dop1.462 yeast by *A. aegypti* larvae results in high levels of mortality.
Larval consumption of inactivated dried dop1.462 yeast tablets resulted in significant larval mortality in laboratory (A) and outdoor semi-field larvicide trials conducted in Indiana (C). Data shown in panels A (n=260 total larvae/treatment) and C (n=280 total larvae/treatment), were compiled from three biological replicate experiments and are represented here as mean percentage mortality; error bars represent SEM. Mortality data from dop1.462 vs. control yeast IRP-treated larvae were arcsine transformed and analyzed using Student's t-test (***=$P<0.001$ vs. control). Consumption of inactivated dried dop1.462 yeast larvicide tablets beginning in L1 resulted in death beginning in L3 or during the L4 or early pupal stages (B; compare to larvae fed with control yeast IRP that survived). In A-C, a single 40 mg yeast tablet was provided to 20 larvae in each replicate container at the onset of each experiment. D. A dose-response curve shows the mass of dop1.462 yeast vs. the resulting percentage of *A. aegypti* mortality following treatment; LD_{50} = 34 mg. Regression analysis (D) indicated that these dose-response data are linearly correlated (R^2=0.9813).

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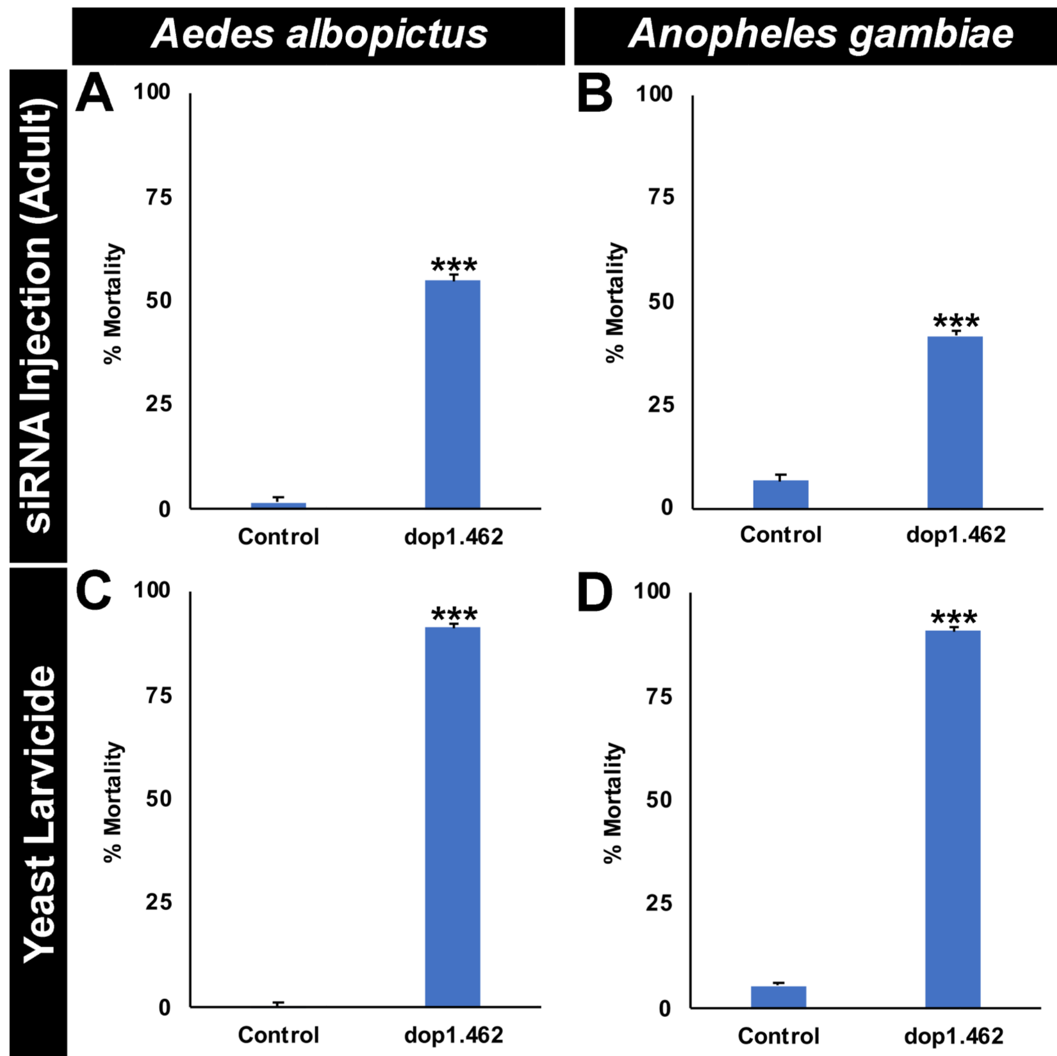

Fig. 5. Broad-range activity of dop1.462 IRP in mosquitoes.

Microinjection of dop1.462 siRNA induces mortality in *A. albopictus* (A; n=60/treatment; dose = 250 nl of 9 μg/μl siRNA per mosquito) and *A. gambiae* (B; n=60/treatment; dose = 150 nl of 6 μg/μl siRNA per mosquito) adult females (compare to control siRNA injections of the same doses). For the experiments in A and B, adult mortality was evaluated for six days following injection, after which time the final results were recorded. Likewise, larval consumption of inactivated dop1.462 yeast tablets (C, D) induces high levels of mortality in *A. albopictus* (C; n=240/treatment) and *A. gambiae* (D; n=240/treatment). In the experiments shown in A and B, one 40 mg dop1.462 or control yeast tablet was provided to 20 larvae in each replicate container trial; the data shown were compiled from a total of three biological replicate experiments, with the experiment concluding when all animals had either died (typically during L4) or emerged as adults. Data were arcsine transformed and analyzed with a Fisher's exact test (A, B) or Student's t-test (C, D); ***=P<0.001 vs. control.

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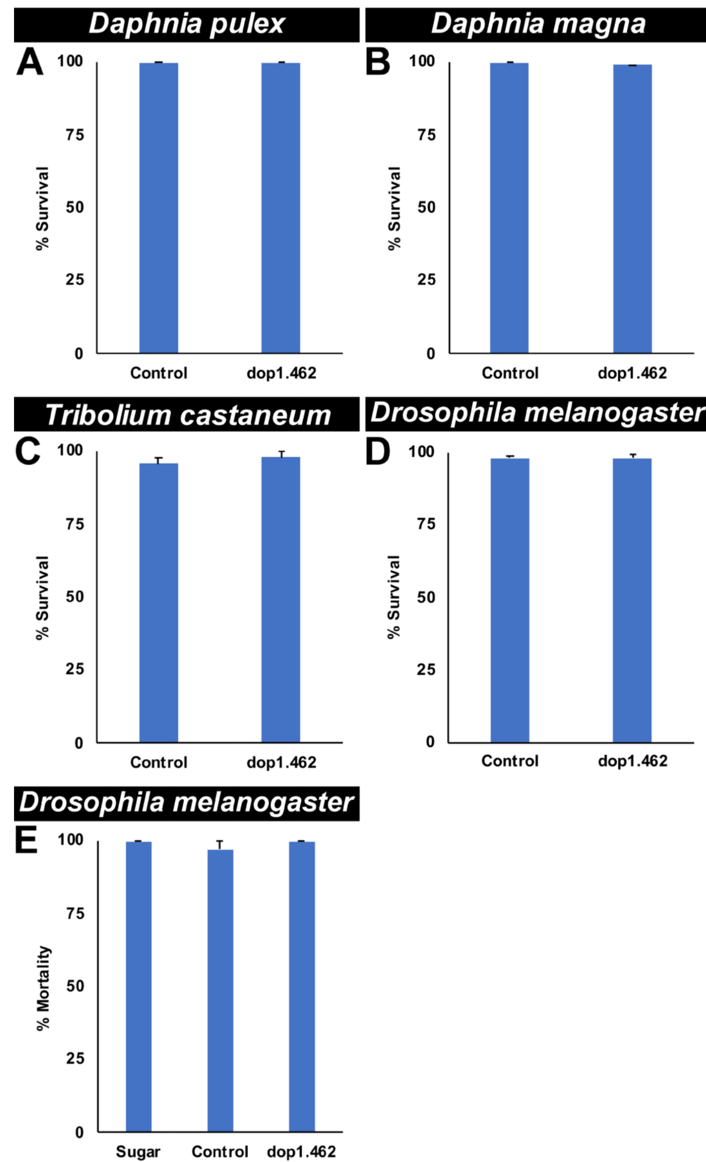

Fig. 6. Non-target arthropods survive treatments with dop1.462 IRPs.

Consumption of dop1.462 yeast by *D. pulex* (A; n=60/treatment), *D. magna* adults (B; n=60/treatment), *Tribolium castaneum* larvae (C; n=120/treatment), or *D. melanogaster* larvae (D; n=140/treatment) had no significant impact on survival (P>0.05 with respect to arthropods treated with control IRPs). In all replicate trials conducted on these larvae (A-D), 20 individuals were fed 40 mg of yeast at the onset of the trial, which concluded when all insects had emerged as adults. Likewise, no significant differences in mortality were observed in adult *D. melanogaster* fed with dop1.462 ATSB, sugar bait alone, or sugar bait with control siRNA, (E; n=40/treatment; 20 adults were fed 32 μl of 2.5 μg/μl siRNA at the onset of the trial, with mortality assessed six days following treatment). Data in A-E are displayed as mean percentages of survival with error bars representing SEM. The survival data shown in A-D were compiled from multiple biological replicate trials (three in A and B,

six in C, seven in D) and analyzed using the Fisher's exact test, while survival data shown in E were compiled from two replicate experiments and analyzed using the G-test.

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Video 1. Defective motor behavior observed in mosquitoes that consumed dop1.462 ATSB.
Defective locomotor behavior was observed in adult female mosquitoes that consumed dop1.462 ATSB. In the video, individual females that consumed sugar bait (right) or sugar bait with control siRNA (center) display typical locomotor behaviors, including flight and exploration of their local environments. The dop1.462-treated female (left) attempts to perform these activities, but fails (well beyond the recording session). The video can be viewed at this link.