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ORIGINAL CONTRIBUTION

Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA

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Abstract

The induction of the naturally occurring phenomenon of RNA interference (RNAi) to study gene function in insects is now common practice. With appropriately chosen targets, the RNAi pathway has also been exploited for insect control, typically through oral delivery of dsRNA. Adapting current methods to deliver foreign compounds, such as amino acids and pesticides, to mosquitoes through sucrose solutions, we tested whether such an approach could be used in the yellow fever mosquito, *Aedes aegypti*. Using a non-specific dsRNA construct, we found that adult *Ae. aegypti* ingested dsRNA through this method and that the ingested dsRNA can be recovered from the mosquitoes post-feeding. Through the feeding of a species-specific dsRNA construct against vacuolar ATPase, subunit A, we found that significant gene knockdown could be achieved at 12, 24 and 48 h post-feeding.

Introduction

Mosquitoes (Diptera: Culicidae) are the most medically important arthropods worldwide, vectoring numerous agents that negatively affect humans, including dengue, arboviral encephalitides and malaria parasites. These mosquito-borne pathogens adversely affect millions of people annually, and the total burden of diseases associated with mosquitoes is well documented (e.g. see Sachs and Malaney 2002; Hemingway et al. 2006; Suaya et al. 2009). Mosquito control relies heavily on the use of chemical insecticides, from aerial spraying to impregnated bed nets. However, because of increasing insecticide resistance induced by selection pressure, chemically based control of mosquito populations is becoming increasingly difficult. For example, a clear relationship has been demonstrated between the reduced efficacy of pyrethroid-impregnated bed nets and residual sprays to the increased frequencies of the *kdr* allele in Benin *Anopheles gambiae* populations (N'Guessan et al. 2007; Yadouleton et al. 2010). Resistance is not limited to Anophelines as surveillance programmes demonstrate a general trend in the rise of insecticide resistance in numerous mosquito species of medical importance (e.g. da-Cunha et al. 2005; Cui et al. 2006; Montella et al. 2007). Other negative impacts of insecticide usage include accumulation of the insecticide within the environment and the widespread killing of non-target organisms. Thus, insecticides can create a long-term burden on species diversity and ecosystem sustainability.

Double-stranded RNA (dsRNA) is an attractive alternative as a potential bio-insecticide because it avoids the negative effects of chemical insecticides. Specifically, it poses no risk of accumulation within the environment because it is readily degraded by ubiquitous bacterial enzymes. In addition, it is highly sequence-specific and can be designed to avoid non-target species toxicity. Within cells, dsRNA induces a cellular response called RNA interference (RNAi), a naturally occurring process by which transcriptional messages (mRNA) are silenced prior to translation by a large protein complex guided by complementary small interfering RNAs (siRNAs) that are generated

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14. ABSTRACT

The induction of the naturally occurring phenomenon of RNA interference (RNAi) to study gene function in insects is now common practice. With appropriately chosen targets, the RNAi pathway has also been exploited for insect control, typically through oral delivery of dsRNA. Adapting current methods to deliver foreign compounds, such as amino acids and pesticides, to mosquitoes through sucrose solutions, we tested whether such an approach could be used in the yellow fever mosquito Aedes aegypti. Using a non-specific dsRNA construct, we found that adult Ae. aegypti ingested dsRNA through this method and that the ingested dsRNA can be recovered from the mosquitoes post-feeding. Through the feeding of a species-specific dsRNA construct against vacuolar ATPase subunit A, we found that significant gene knockdown could be achieved at 12, 24 and 48 h post-feeding.

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from the original dsRNA molecules that induced the response. The RNAi response can be induced experimentally through the delivery of dsRNA through a variety of means, including injection, topical application or oral delivery. Through these techniques, RNAi has been used for a wide range of experimental and practical applications, from functional gene expression studies (Agrawal et al. 2003) to gene therapy (Davidson and McCray 2011). In most studies investigating functional genomics in insects, dsRNA was delivered by injection. However, for insect control, a remote method of introduction must be utilized, such as oral delivery (Huvenne and Smagghe 2010). Indeed, there are numerous studies in which dsRNA was delivered orally with the intent of developing it as a bio-insecticide against a variety of insect orders including Coleoptera (Baum et al. 2007; Whyard et al. 2009), Diptera (Walshe et al. 2009; Whyard et al. 2009; Zhang et al. 2010; Li et al. 2011a), Hemiptera (Araujo et al. 2006; Shakesby et al. 2009; Whyard et al. 2009), Homoptera (Li et al. 2011b; Upadhyay et al. 2011), Hymenoptera (Aronstein et al. 2006; Nunes and Simoes 2009), Isoptera (Zhou et al. 2008), Lepidoptera (Rajagopal et al. 2002; Turner et al. 2006; Mao et al. 2007; Griebler et al. 2008; Bautista et al. 2009; Kumar et al. 2009; Tian et al. 2009; Whyard et al. 2009) and Orthoptera (Meyering-Vos and Muller 2007).

Likewise, experimental induction of the RNAi pathway in mosquitoes has relied largely on the injection of dsRNA for the purpose of elucidating gene function (Blandin et al. 2002; Attardo et al. 2003; Zhu et al. 2003; Boisson et al. 2006; Magalhaes et al. 2008). However, the utilization of dsRNA as a bio-insecticide against mosquitoes has only recently begun to be evaluated. Double-stranded RNA targeting chitin synthase mRNA was fed to *An. gambiae* larvae, resulting in gene silencing and significant mortality (Zhang et al. 2010). In addition, it was demonstrated that mortality of adult *Aedes aegypti* mosquitoes could be induced by the topical application of dsRNA against *IAP* (inhibitor of apoptosis; Pridgeon et al. 2008). Although topical application remains an attractive option for mosquito control, both production cost and function dictate that more a direct method of dsRNA delivery be developed. Building on the development of mosquito adulticiding devices with attractive toxic sugar baits (Xue et al. 2008; Muller et al. 2010a,b,c; Allan 2011), and by the recent success of Walshe et al. (2009) to induce mortality in the tsetse fly *Glossina morsitans morsitans* through the delivery of dsRNA through a blood meal, we developed a method to orally deliver dsRNA in liquid sucrose in which we show that dsRNA is ingested by adult female *Ae. aegypti* mosquitoes and that oral delivery of gene-specific dsRNA results in the reduction in the cognate mRNA.

Experimental Procedures

Mosquitoes

Aedes aegypti (Linnaeus) mosquitoes were obtained and reared as described in Pridgeon et al. (2008) and were between 3 and 5 days post-emergence for all experiments. Twenty-four hours prior to being placed in feeding assays, mosquitoes were starved by placing them into large paper cups and provided with sterile water only. Mosquitoes were held in a Percival Scientific Incubator (Perry, IA) equipped with an Intellus Environmental Controller set on a 12/12 light-dark cycle and 28°C before and during experimentation. All experiments were conducted on male and female mosquitoes unless otherwise noted.

Oral delivery of dsRNA and RNA isolation

One assay unit was comprised of ten *Ae. aegypti* mosquitoes in an 8 oz Solo paper cup (Highland, IL). RNase/DNase-free 1.5-ml microcentrifuge tubes with the caps removed were used as reservoirs for the sucrose/dsRNA solutions, with dental cotton used as a wick for the solution. The tubes were pushed through a hole cut in the side of the cup, with the wick facing inwards to enable mosquitoes to feed from the wick. All sucrose concentrations were 10% (w/v) and were prepared with nuclease-free water. Mosquitoes were allowed to feed *ad libitum* on sucrose solutions for 24 h, after which the tubes were removed and cotton balls soaked in 10% sucrose were provided. For collection, mosquitoes were cold-anesthetized, transferred to 1.5-ml RNAse-free microcentrifuge tubes and placed at −80°C for storage until RNA extraction. RNA was isolated from the pool of ten mosquitoes using TRIzol Reagent (Invitrogen, Carlsbad, CA), unless otherwise noted. Insects dead at the time of collection were removed from the assay cup prior to cold anaesthesia and were not processed. Total RNA concentration and purity was determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA). All experiments were repeated three times unless otherwise noted.

Double-stranded RNA

Remebee®, provided by Beeologics, Inc., is a blend of two dsRNA molecules of approximately 480 base pairs

each, which are homologous to the sequence of Israeli Acute Paralysis Virus (IAPV) genome, and was used to investigate the ingestion of dsRNA in *Ae. aegypti*, as well as to serve as a negative control in experiments where species-specific dsRNA was utilized. The sequences of the Remebee® were BLASTed against the *Ae. aegypti* genome to insure that no more than a 20-bp homology existed between the sequences and any gene within the mosquito. For the specific silencing experiments, a 667 base-pair dsRNA fragment homologous to the N-terminus region of the v-ATPase catalytic A-subunit of *Ae. aegypti* (GenBank accession no. XP_001659520.1) was designed and manufactured by Beeologics Inc. using a proprietary platform. To establish the degree of similarity and conservation between different orthologs of v-ATPase, sequences were analysed using BLAST (Altschul et al. 1990) and ClustalW multiple alignment tool (Thompson et al. 1994).

Ingestion and subsequent recovery of dsRNA from the insects

Mosquitoes were allowed to feed on 0, 100, 500, 1000 or 2000 ng/μl of Remebee® in 10% sucrose for 24 h, and after which were collected, and RNA was isolated and quantified as described earlier. Nuclease digestion was performed using 5 μg of total RNA with RNase I_f (NEB, Ipswich, MA) or ShortCut RNase III (NEB) per manufacturer's instructions. Mock digests were set-up using supplied buffers minus enzyme. Digestion products (2.5 μg) were electrophoresed through a non-denaturing, 2% agarose gel containing 0.5 μg/ml ethidium bromide.

v-ATPase gene expression knockdown

The endogenous gene expression of v-ATPase levels over a 9-day period was determined by isolating RNA from mosquitoes that were collected 1–2, 2–3, etc., through 8–9 days post-emergence, and performing quantitative PCR (qPCR). Expression levels of v-ATPase were determined as compared to the endogenous control, *Ae. aegypti* 60s ribosomal protein L24 (GenBank accession no. XM_001659098), in arbitrary units using the standard curve method. Data were analysed using a one-way ANOVA with the Student–Newman–Keuls as the *post hoc* test. For gene knockdown experiments, mosquitoes were fed 680 μl of 10% sucrose solutions containing either 0, 1000 ng/μl Remebee® or 1000 ng/μl v-ATPase for up to 24 h and were subsequently collected at 12, 24 and 48 h post-exposure. Mortality was recorded at 24 and 48 h. Total RNA was isolated using Ambion's RNaqueous 4-PCR Kit (Grand Island, NY). Complementary DNA was synthesized from 150 ng of total RNA using oligo-dT primers in Invitrogen's Cloned AMV First-Strand cDNA Synthesis Kit (Carlsbad, CA). Quantitative PCR was conducted using an Applied Biosystems 7300 Real Time PCR System with Platinum SYBR Green qPCR SuperMix-UDG w/ ROX from Invitrogen (Carlsbad, CA). Changes in gene expression were determined according to Pfaffl (2001) expressed as proportional change with comparison to values obtained from insects treated with 10% sucrose alone. L24 was used as the endogenous control. The primer sequences for v-ATPpase gene quantification are as follows: AA_VATP_F2 TGA AGT TCA AGG ACC CAG TG and AA_VATP-R2 TTT CGC ATT GAA GAG ACC AC, which produce an amplicon of 140 base pairs in length. Primers were designed to fall outside of the dsRNA construct, and the forward primer spans an exon–exon junction to reduce error introduced by genomic DNA contamination. Primers for L24 amplification are L24-F GAG GCA GTA AAA TTT CGC CA and L24-R AGG TGA AAG TCT TGC CAT CG and result in a 113 base-pair amplicon that spans an intron. L24 suitability as a reference gene was evaluated using BestKeeper-1 (Pfaffl et al. 2004). Specificity of the primer sets and detection of primer-dimer products were evaluated via melt-curve analysis and agarose gel electrophoresis. Data from gene knockdown experiments were analysed using an unpaired *t*-test conducted with SigmaStat 11.0 (Systat Software Inc., San Jose, CA, USA), with significance assumed at $P < 0.05$.

Results

dsRNA is ingested by mosquitoes and is recovered in RNA isolation

Adult *Ae. aegypti* mosquitoes were fed a dsRNA construct (Remebee®) with no greater than a 20-bp homology to any mosquito gene in a 10% sucrose solution. After 24 h, RNA was isolated from the mosquitoes and subjected to nuclease digestion to determine the nature of the nucleic acid species recovered (fig. 1). A band the size of Remebee® (480 bp) was recovered in a concentration-dependent manner according to the amount of Remebee® included in the sugar meal (fig. 1a). Digestion with RNase I_f (fig. 1b) left the 480-bp band intact, while digestion with RNase III (fig. 1c) eliminated this band, demonstrating that the nucleic acid species isolated is dsRNA. No digestion products were observed in mock reactions, and similar results were

obtained for male mosquitoes (data not shown). These data show that dsRNA can be delivered orally to mosquitoes in a sucrose meal, that it is taken up by the mosquitoes through feeding and that it can be isolated from the mosquitoes. However, because the entire insect was used to perform these experiments, the suborganismal location from which the ingested dsRNA was recovered (e.g. diverticula, midgut, etc.) remains unknown.

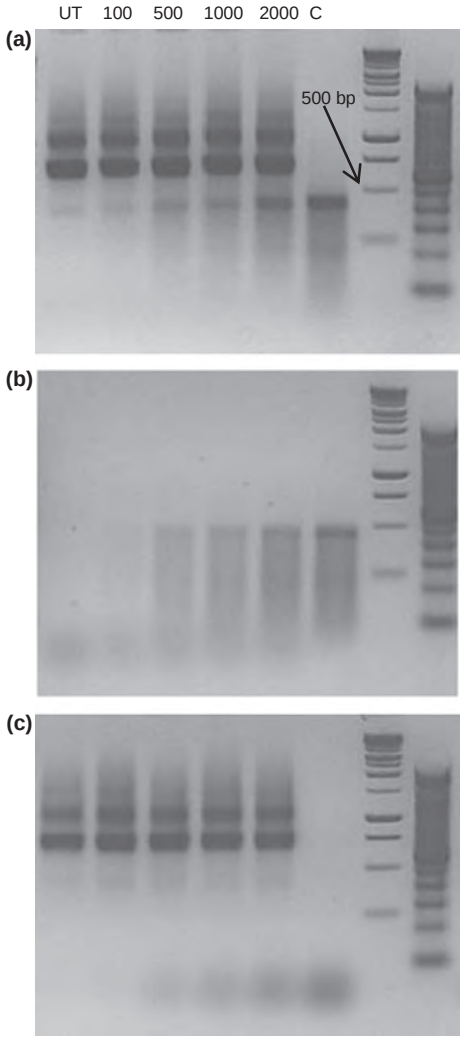

Fig. 1 RNA recovered from mosquitoes after 24 h of free access to Remebee®. Reverse-contrast photograph of an ethidium bromide-stained 2.0% agarose-TAE gel showing nuclease digests of RNA isolated from adult *Ae. aegypti* female mosquitoes given free access to dsRNA in a 10% sucrose solution for 24 h. Total mass of RNA loaded on the gel is 2.5 μg. (a) Control (no digest); (b) ssRNA Digestion (RNase I_f); and (c) dsRNA digestion (RNase III). UT, 10% sucrose; 100–2000, 100–2000 ng/μl of Remebee® (non-targeting dsRNA) in 10% sucrose solution; C, control: dsRNA Remebee® run for comparison. Markers from left to right are 1 kb and 100 bp, respectively. Remebee® is approximately 480 base pairs in length and is a blend of two similarly sized molecules. Trials run with adult male mosquitoes produced the same results. See text for details and discussion.

Specific gene knockdown in adult mosquitoes

Double-stranded RNA targeting v-ATPase, subunit A, was provided to adult female *Ae. aegypti* mosquitoes in sucrose meals to evaluate gene knockdown of its cognate mRNA. A 2.4–2.5 fold reduction in gene expression was observed for all three time points surveyed (12, 24 and 48 h; fig. 2). This reduction was statistically significant over expression observed in the negative control, Remebee®, with 12 and 24 h being highly significant ($P < 0.01$). A survey of endogenous gene expression of v-ATPase over time post-emergence showed that its level is highest immediately after emergence and gradually drops off as the female mosquito ages (fig. 3), but remains relatively stable during the time period of experimentation with the mosquitoes 3–5 days post-emergence. Regardless, any normal fluctuation in v-ATPase mRNA abundance would be cancelled out because of the fact that the fold difference in abundance was determined by normalizing the data against sucrose-fed mosquitoes of the same age.

Discussion

Knockdown of a gene target through the oral delivery of dsRNA has been demonstrated in numerous

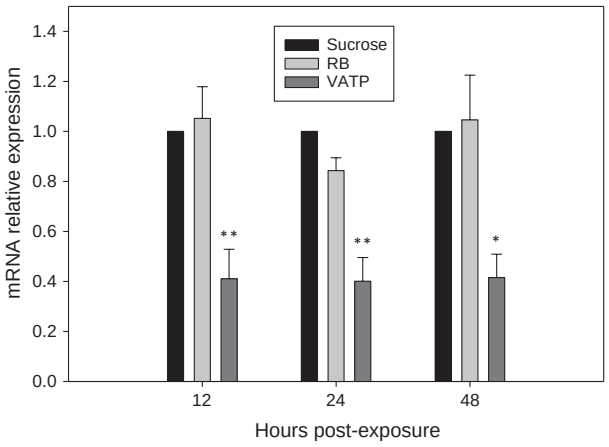

Fig. 2 Reduction in cognate v-ATPase mRNA abundance in adult female *Ae. aegypti* mosquitoes in response to ingestion of dsRNA against v-ATPase, subunit A. Significant knockdown was observed for all three time points collected, 12, 24 and 48 h post-exposure ($P < 0.05$; marked with a single asterisk), with high statistical significance observed for 12 and 24 h ($P < 0.01$; marked with double asterisks). Time points 12 and 24 are represented with five replicates and time point 48 H with four. Error bars represent standard error of the mean.

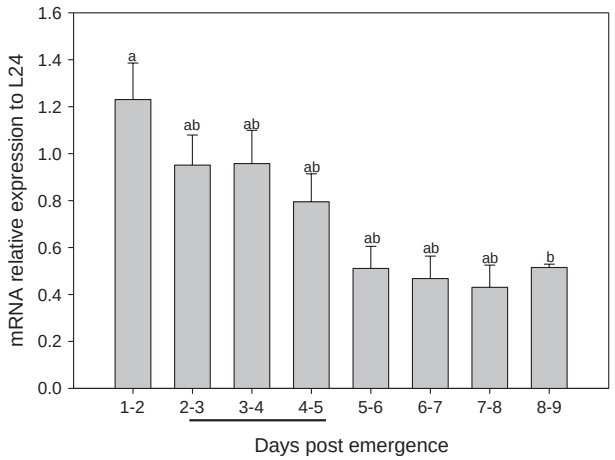

Fig. 3 Endogenous expression levels of v-ATPase gradually drop over time post-emergence in adult female *Ae. aegypti* mosquitoes. Levels are expressed relative to L24 levels in arbitrary units using the standard curve method. Differences in expression levels were tested for statistical significance using a one-way ANOVA with the Student–Newman–Keuls Method as the *post hoc* test. Bars with the same letter are not significantly different from one another ($P < 0.05$). Underlined is the age range of the mosquitoes used in this study. Error bars represent standard error of the mean (SEM).

insect species, including larval mosquitoes, but this is the first report of knockdown in adult mosquitoes through oral delivery of dsRNA. Specifically, we demonstrated that dsRNA can be delivered to *Ae. aegypti* adult mosquitoes through a liquid sucrose meal and that oral delivery of dsRNA targeting a specific gene results in the reduction in its cognate mRNA levels. This is the first step towards the development of dsRNA as a bio-insecticide to be used against adult mosquitoes via oral delivery, which can have far reaching implications for the general field of vector-borne disease prevention, especially if combined with other control measures.

Vacuolar ATPase was chosen as a target because it is expressed by the epithelial cells of the gut and Malpighian tubules in *Ae aegypti* (Patrick et al. 2006) and because of demonstrated vulnerability to RNAi-induced gene knockdown through oral delivery in several other insect species including *Diabrotica virgifera virgifera*, *D. undecimpunctata howardi*, *Leptinotarsa decemlineata* and *Anthonomus grandis* (Baum et al. 2007); *Tribolium castaneum*, *Acyrthosiphon pisum* and *Manduca sexta* (Whyard et al. 2009); *Bactrocera dorsalis* (Li et al. 2011b) and *Nilaparvata lugens* (Li et al. 2011a). Increased mortality was seen in some species (*D. virgifera virgifera, D. undecimpunctata*, *L. decemlineata*, *T. castaneum*, *A. pisum* and *M. sexta*) and not in others (*A. grandis*, *N. lugens* and *B. dorsalis*). Like these latter species, we did not observe increased mortality associated with v-ATPase mRNA knockdown. The reason may have a biological basis such as life stage tested or a difference in vulnerability to dsRNA knockdown, or a logistical basis such as concentration of dsRNA that was tested, the region of the dsRNA construct within the gene or length of construct, or a combination of multiple factors. Future work will be focused on teasing out differences between biological and logistical aspects of the RNAi response in *Ae. aegypti*, which will be greatly facilitated by the verification of knockdown at the protein level using immunochemical and/or enzymatic analyses.

In comparison with other insect species, the development of dsRNA into a bio-insecticide against mosquitoes is at its beginning, and much ground work needs to be covered in order to optimally exploit the RNAi process for insect control. One of the most important questions remaining to be answered is whether or not the silencing signal is amplified and disseminated throughout the mosquito as it is in other organisms such as *Caenorhabditis elegans* (Fire et al. 1998). Future research efforts will include testing a construct against a gene that is not expressed inside the gut to determine whether dsRNA introduced orally can reach other tissue targets. If knockdown is limited to genes expressed within the gut, oral delivery still holds promise with carefully chosen targets. For example, by combining knockdown of a gut-expressed detoxification enzyme and application of a pesticide that is metabolized by that enzyme, Bautista et al. (2009) were able to reduce resistance to permethrin in *Plutella xylostella*.

To develop dsRNA as a means of population control of mosquitoes, either alone or in conjunction with other control measures, a better understanding of the RNAi process in these insects must be undertaken. In addition, gene targets that can induce physiological changes that affect survivorship, fecundity and/or behaviour must be identified. Both of these research areas are currently being investigated, and with this information, the use of dsRNA for a means of mosquito population control may become a reality. Functional aspects of this methodology are also being refined, such as optimal length of the dsRNAs, region of the gene for the dsRNA construct and effective concentration of dsRNA within the sucrose solution. Another area of research being undertaken is the identification of constituents to provide protection to the dsRNA from degradation while it is in the bait station so that it provides the maximal impact on the target species. Once outside

of the bait station, dsRNA is readily degraded by ubiquitous bacterial RNAse III enzymes which is the desired consequence so that there is no accumulation in the environment and, therefore, reduced risk to non-target species. Other future directions will focus on the development and application of this methodology to other medically important mosquitoes, particularly Anophelines. Finally, inspired by the success of Remebee® in targeting the virus implicated in colony collapse disorder in honeybees (Maori et al. 2009; Hunter et al. 2010), we are identifying and will be testing potential gene targets against disease-causing agents transmitted by mosquitoes such as dengue virus.

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