Delayed Effect of Anisaldehyde on Feeding Behavior and Reproduction of Adult *Aedes albopictus* (Diptera: Culicidae)[1]

Huiling Hao,[2,4] Jianqing Dai,[3] and Jingcheng Sun[2]

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ABSTRACT Anisaldehyde may be useful as a spatial repellent against *Aedes albopictus*, and it could play a major role in new repellent technology for mosquito management. This study characterized the delayed effects of anisaldehyde on *Ae. albopictus* adult host-seeking behavior, feeding behavior, and reproduction. Anisaldehyde was applied as a fumigant (0.25 μg/cm^3) during different developmental stages of the mosquito (larvae from 1st instar to pupation, larvae from 1st instar to adult emergence, pupae, and two-day-old adults). Except when treated as pupae, the resulting adult females (at 5 days of age) exhibited significantly lower host-seeking capability and lower repeated-feeding rates than untreated females. Anisaldehyde treatments also significantly reduced the number of eggs and hatchability, and this chemical prolonged the gonotrophic cycle and egg development. These results showed that anisaldehyde acted as a spatial repellent, where it not only reduced host-seeking capability, but also greatly reduced vectoring capacity and reproduction potential, which is significant for mosquito control and disease prevention.

KEY WORDS Mosquitoes, plant volatiles, spatial repellent

Spatial repellents are compounds that when dispensed into the atmosphere can inhibit the ability of insects, such as mosquitoes (Diptera: Culicidae), to locate and track a host (Nolen et al. 2002). These airborne compounds have attracted increasing interest (Dogan & Rossignol 1999, Kline et al. 2003) as an effective protective barrier between humans/animals and mosquito transmitted diseases. A variety of plant volatiles, such as citral (Oyedele et al. 2002), eugenol (Ngoh et al. 1998), citronella, geraniol (Barnard & Xue 2004), and anisaldehyde (Hao et al. 2008), have been reported as potential spatial repellents. Hao et al. (2008) showed that anisaldehyde could be a more effective spatial repellent against the Asian tiger mosquito, *Aedes albopictus* (Skuse), than other tested compounds. Anisaldehyde caused a significant inhibition of *Ae. albopictus* host-seeking ability at every stage of blood-feeding behavior. Their research also showed that some affected individuals did not recover and never had normal blood-feeding behavior (Hao et al. 2008). These results suggest that anisaldehyde has great potential for further development as a spatial repellent against mosquitoes.

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[2] Naval Medical Institute, 880 Xiangyin Road, Shanghai, 200433, China.
[3] Guang Dong Entomological Institute, Guangzhou, 510260, China.
[4] Corresponding author, haohuiling@hotmail.com

As early as 1928, anisaldehyde was known as a common plant volatile that attracted insects (Morgan 1928). Kirk et al. (1985) found that anisaldehyde attracted many types of thrips. Toshihiro et al. (2000) demonstrated that anisaldehyde was attractive to *Anthrenus verbasci* (L.) (Coleoptera: Dermestidae) in the same manner as a sex pheromone, especially for females. As a secondary plant compound, anisaldehyde is present in essential oils or plant extracts that are insecticidal, and this compound has both contact and fumigant toxicity against many insects, including mosquito larvae (Marcus & Lichteustein 1979). Ndomo et al. (2010) tested anisaldehyde-treated cowpeas and beans against *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchinae) and *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae: Bruchinae) and found that this compound not only was toxic to adults, but it also reduced the number of F_1 progeny. The effects of anisaldehyde on mosquitoes is not fully known, especially when the compound is applied as a spatial repellent in areas where mosquitoes propagate. Considering the potential use of anisaldehyde as a spatial repellent for mosquito control, the objective of the current study was to investigate the delayed effects on host-seeking and repeated-feeding behaviors and reproduction of adult *Ae. albopictus* treated during different development stages. The study characterized the effect of anisaldehyde as a spatial repellent and provides fundamental information for its future use in mosquito management.

Materials and Methods

Insects. *Aedes albopictus* were obtained from a colony that has been maintained for more than 15 years at 28 ± 1°C, 70 ± 5% RH, and a 14:10 (L:D) photoperiod at the Institute of Plant Physiology & Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. An artificial diet developed for the house mouse, *Mus musculus* L., was fed to larvae, and a 6% glucose solution was fed to adults.

Chemicals. Anisaldehyde (99%, CAS-No. 123-11-5) and methanol (99.5%, reagent grade) were purchased from Shanghai Chemical Reagent Co., Inc. (Shanghai, China).

Indirect effects of anisaldehyde on adult host-seeking ability. *Aedes albopictus* of different developmental stages were treated with anisaldehyde vapor at a concentration of 0.25 μg/cm^3 in a closed 17-liter glass container. The developmental stages treated were: (1) larvae for the duration of the larval period (from 1st instar to pupation), (2) larvae for the duration of the larval and pupal periods (larvae from 1st instar to adult emergence), (3) pupae for the duration of the pupal period, and (4) two-day-old adults. This concentration of anisaldehyde has been shown to be effective for reducing the host-seeking ability of adult *Ae. albopictus* (Hao et al. 2008). The desired airborne concentration was established by hanging a piece of filter paper (15 × 30 mm) impregnated with anisaldehyde in the container for 24 hours. This concentration was achieved according to the specific gravity and corrected for the total volume by the weight loss of the filter paper-borne chemicals in the closed container during a 24-h period. The desired concentration of anisaldehyde (0.25 μg/cm^3) was monitored by SPME (Solid Phase Micro Extraction) analysis to ensure a relatively consistent concentration throughout the treatments (Hao et al. 2008).

Larvae or pupae (10 individuals) in small dishes containing 20 ml water, or 10 adults in a nylon net cage (100 mm $\times$ 20 mm), were introduced into 17-liter glass containers to expose them to anisaldehyde for different lengths of time as described above. The filter paper was removed immediately before placing test mosquitoes into the containers. Throughout the experiment, the containers with mosquitoes were maintained under the same conditions that were used for colony rearing. After treatment, the mosquitoes were transferred to clean arenas and reared until adults reached the age of five days. At that time, host-seeking ability was measured by individually introducing the adults into a gauze cage (300 $\times$ 300 $\times$ 300 mm) containing a mouse. Host-seeking behaviors were observed for the first 8-minute period. Inhibition of host seeking behavior was defined as a mosquito's inability to land on the host's skin surface or its unwillingness to insert its stylets into the host within the first eight minutes after landing on it.

Indirect effects of anisaldehyde on repeated blood-feeding behavior. Mosquitoes are vectors of several important virus diseases. Mosquitoes become infected after sucking blood from humans or animals carrying a virus, but the acquired virus can be transmitted to another host only after an incubation period. Because only infected mosquitoes that feed again after the incubation period can spread the disease, repeated blood-feeding behavior is important in determining their vectoring capabilities. In this experiment, we treated *Ae. albopictus* with anisaldehyde as described above at 0.25 μg/cm^3 over (1) the duration of the larval period, (2) the duration of the larval and pupal periods, (3) the duration of the pupal period, and (4) the duration of the adult period (from one day of age). After treatment, mosquitoes were transferred to clean arenas and reared until adults had reached five days of age. At this time, female mosquitoes were offered a blood meal on a shaved mouse. Mosquitoes that had a successful first engorgement were individually caged and offered a mouse every 24 hours for seven days to observe the occurrence of repeated blood-feeding. The effects of anisaldehyde on adults was evaluated by exposing five-day-old females to anisaldehyde (0.25 μg/cm^3) for 24, 48, or 72 hours, giving them a blood meal, and then observing repeated blood-feeding behavior. Additionally, the size of the blood meal was calculated by weighing mosquitoes before and after feeding using an analytical balance.

Delayed effects of anisaldhyde on reproduction. *Ae. albopictus* were exposed to anisaldehyde (0.25 μg/cm^3) for 24–96 h as (1) larvae for the duration of the larval period, (2) larvae for the duration of the larval and pupal periods, (3) pupae for the duration of the pupal period, and (4) five-day-old adults, as described above. After treatment, insects were transferred to clean arenas and reared normally with blood supplement. The number of eggs laid, egg development, hatchability, and the gonotrophic cycle were observed.

All experiments for this two-year study were conducted at 25 $\pm$ 1°C, 70 $\pm$ 5% RH, 4500 Lux, and 14:10 (L:D) photoperiod. For all experiments, there were three replications for each treatment, and an untreated control was included for each procedure.

Statistical Analysis. SPSS 11.5 (SPSS Inc., Chicago, IL) was used. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P <$ 0.05) were performed to evaluate the indirect effects of anisaldehyde on *Ae. albopictus* host-seeking behavior during different developmental stages. Independent sample *t*-tests were performed to evaluate the indirect effects of

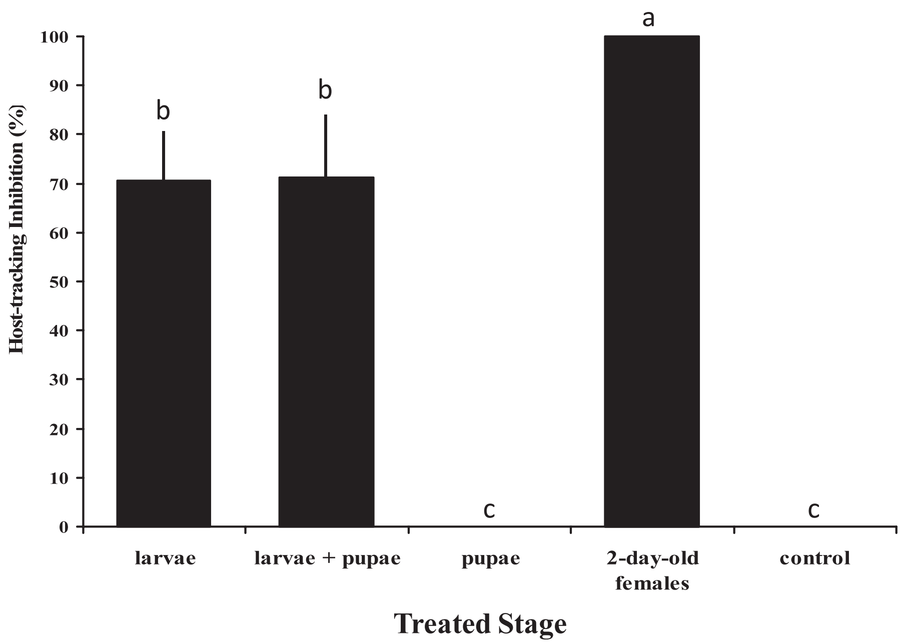

Fig. 1. Host-seeking ability of *Aedes albopictus* treated with anisaldehyde during different developmental stages. Bars with the same letter are not significantly different from each other according to Duncan's multiple range test ($P < 0.05$, n = 30).

anisaldehyde on *Ae. albopictus* repeated blood-feeding behavior, size of blood meal, and the measured reproduction parameters.

Results

Delayed inhibition effect on adult host-seeking ability. Figure 1 shows the delayed inhibition effects of anisaldehyde fumigant treatments on host-seeking ability of five-day-old adult *Ae. albopictus* that had been treated at different developmental stages. When mosquitoes were treated with anisaldehyde over the duration of the larval period, over the duration of the larval and pupal periods, or as adults two days old or younger, female mosquito host-seeking ability was reduced by 70.6%, 71.2%, and 100%, respectively. However, when *Ae. Albopictus* were treated during the pupal stage, no delayed inhibitory effects on adult host-seeking ability were observed (Fig. 1).

Delayed effect on repeated blood-feeding behavior. Female mosquitoes that were not treated with anisaldehyde (controls) exhibited repeated blood-feeding behavior after first engorgement that reached 100% after six days (Table 1). When treated with anisaldehyde during different development stages, the resulting adults exhibited lower ($P < 0.01$) repeated blood-feeding rates compared to the control, except for adults treated during the pupal stage. The greatest anisaldehyde effect was observed when mosquitoes were treated as

Table 1. Blood-feeding behavior of *Aedes albopictus* that were treated with anisaldehyde at different developmental stages.

Days after first engorgement	Second blood-feeding rate ± SE (%) (n = 30)						
	Control	Treated as larvae	Treated as larvae & pupae	Treated as pupae	Treated as adults 24 h	48 h	72 h
1	19.4 ± 1.5	0.0**	0.0**	15.2 ± 1.2	0.0**	0.0**	0.0**
2	25.0 ± 5.0	20.0 ± 1.5	0.0**	20.2 ± 2.5	0.0**	0.0**	0.0**
3	65.4 ± 1.2	20.0 ± 1.5**	12.0 ± 3.0**	45.3 ± 1.5	0.0**	0.0**	0.0**
4	75.0 ± 3.5	20.0 ± 1.5**	12.0 ± 3.0**	60.5 ± 5.2	15.3 ± 2.0**	0.0**	0.0**
5	88.0 ± 4.0	20.0 ± 1.5**	12.0 ± 3.0**	80.0 ± 6.0	18.4 ± 2.0**	0.0**	0.0**
6	100.0	20.0 ± 1.5**	12.0 ± 3.0**	100.0	18.4 ± 2.0**	0.0**	0.0**
7	100.0	20.0 ± 1.5**	12.0 ± 3.0**	100.0	18.4 ± 2.0**	0.0**	0.0**

**Significantly different from control, $P < 0.01$ (independent sample *t*-test).

adults, followed by mosquitoes treated during both the larval and pupal stages, followed by mosquitoes treated in the larval stage alone. Adults that had been treated for 48 or 72 h with anisaldehyde completely stopped secondary blood-feeding behavior (Table 1). Anisaldhyde treatment had no significant effects on the size of blood meals, which ranged from 1.37 to 1.48 mg/female (Table 2).

Delayed effect of anisaldhyde on reproduction. Anisaldhyde treatments during different developmental stages significantly affected the number of eggs laid ($P < 0.01$) (Table 3). Compared to the untreated control, females in all treatment groups, except for adults treated for 24 h, produced fewer eggs. Egg hatchability also was significantly reduced ($P < 0.05$) for all of the treatment groups, except for individuals treated for the duration of the larval period. The delayed anisaldhyde treatments also significantly affected the gonotrophic cycle and egg development. All treatment groups had prolonged gonotrophic cycles and egg development compared with untreated control ($P < 0.01$) (Table 3). The adverse effect of anisaldhyde on reproduction intensified as the treatment time was lengthened, so that when adult females were treated for 96 h, none of their eggs hatched (Table 3).

Discussion

Previously, we reported that anisaldehyde exposure had immediate inhibition effects on adult host-seeking ability and blood-feeding behavior of *Ae. albopictus* (Hao et al. 2008). The current study showed that similar exposures to anisaldehyde during development had delayed effects on *Ae. albopictus* adult host-seeking capability. When treated during the larval stage or during the larval and pupal stages, the resulting adults had significantly lower host-finding rates, indicating lower capabilities for sensing host odors. Additionally, the anisaldehyde treatments adversely affected the rate of repeated-feeding, but not the size of blood meals. These results suggest that anisaldehyde likely affects the olfactory system of mosquitoes and that such effects can be initiated not only

Table 2. Size of blood meals of *Aedes albopictus* that were treated with anisaldehyde at different developmental stages.

	Control*	Treated as larvae	Treated as larvae & pupae	Treated as pupae	Treated as adults		
					24 h	48 h	72 h
Size of blood meals (mg/ female)	1.42 ± 0.09	1.38 ± 0.05	1.40 ± 0.06	1.48 ± 0.06	1.37 ± 0.08	1.42 ± 0.11	1.39 ± 0.02
t value	-	0.95	0.25	1.80	0.95	0.07	0.89
P value	-	0.37	0.81	0.11	0.37	0.95	0.40

*Means ± SE (n = 20), no significant differences from the control were detected for any developmental stage using the independent sample *t*-tests ($P > 0.11$).

during the adult stage, but also during larval stages. No effects were observed when pupae were treated, suggesting that pupae are the most insensitive stage for anisaldehyde to have an effect on host-seeking ability.

The current study also showed that anisaldehyde had negative effects on *Ae. albopictus* reproduction, indicated by longer gonotrophic cycles, reduced numbers of eggs, lower hatchability, and slower egg development. Anisaldehyde effects on reproduction were observed previously where the F_1 progeny of two Coleoptera species were significantly reduced (Ndomo et al. 2010). Our study confirmed that anisaldehyde affects insect reproduction systems and the effect can be initiated with treatment during immature stages. Interestingly, this delayed adverse effect on reproduction was observed for all treatment regimens (development stages), including the pupal stage. In this case, pupae were as sensitive to anisaldehyde exposure as larvae and adults, suggesting that the pupal stage is not completely immune to adverse effects of anisaldehyde.

The most significant findings of the current study are the negative effects of anisaldehyde on reproduction potential and the delayed effects of this chemical on the host-finding and repeated-feeding behaviors of *Ae. albopictus*. One can imagine a scenario where a slow release formulation of anisaldehyde is applied to an area to serve as a spatial repellent. Any adults that emerged from larvae grown in this anisaldehyde-enriched environment would have a lower host-seeking capability, a reduced rate of repeated-feeding behavior (lower disease transmitting capability), and a lower reproductive potential, even for individuals that flew away from the area. The overall result could be significantly lower mosquito population and less human/animal contact. More and more evidence indicates that anisaldehyde could serve as an excellent spatial repellent, and it could be a new and revolutionary tool for comprehensive mosquito management programs. Of course, to realize the above scenario, more research is needed on multiple topics, such as optimal release techniques, proper application concentration, and effective coverage area by unit dosage. We plan to investigate all of these research objectives in our laboratory, in addition to conducting proof-of-concept field tests.

Table 3. Reproductive ability of *Aedes albopictus* that were treated with anisaldehyde at different developmental stages.

Parameter measured	Control	Treated as larvae	Treated as larvae & pupae	Treated as pupae	Treated as adults			
					24 h	48 h	72 h	96 h
# of eggs/♀ (SE)	120.5 (25.0)	22.2** (20.2)	23.4** (12.5)	27.6** (8.2)	114.5 (30.0)	38.5** (8.3)	42.0** (15.0)	12.0** (5.0)
Hatchability (%) (SE)	92.8 (10.2)	91.6 (15.0)	50.3* (5.0)	53.6* (10.2)	77.2* (10.5)	42.6* (12.5)	19.1** (2.5)	0**
Gonotrophic cycle (days) (SE)	3.2 (0.5)	6.2** (1.0)	6.0** (0.5)	5.0** (0.0)	5.2** (0.5)	5.5** (1.2)	6.5** (1.5)	7.6** (3.3)
Eggs development (days) (SE)	4.0 (0.5)	6.8** (1.0)	7.0** (1.0)	5.2** (1.5)	15.0** (3.0)	14.3** (2.8)	14.5** (3.5)	-

*$P < 0.05$, **$P < 0.01$ (independent sample t-test).

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