Acute toxicity of sodium chloride to first and fourth instar *Aedes albopictus* larvae

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Abstract: The increase in the number of imported cases of dengue fever in Japan is of particular concern as *Aedes albopictus* is a vector of dengue fever. Due to the potential for insecticide resistance and the impact of insecticides on non-target species, increased attention is being paid to alternative methods of pest control. Placing salt in used tires has been recommended by the Ministry of Health, Labor and Welfare in Japan as a means of controlling mosquitoes. However, the effectiveness of salt as a larvicide against *Ae. albopictus* are currently unclear. This study examined the acute toxicity of sodium chloride against first and fourth larval instars of *Ae. albopictus*. Acute toxicity tests were conducted according to World Health Organization guidelines. The susceptibility of *Ae. albopictus* larvae was tested against 0.25%, 0.5%, 0.75%, 1.00%, 1.25% and 1.5% NaCl solutions. Larval mortality was correlated with an increase in NaCl concentration and exposure duration. First instar *Ae. albopictus* larvae were more sensitive to NaCl than fourth instar, and 72-h LC₉₀ values for first and fourth larval instars were 0.49% and 1.01% NaCl, respectively. Our results suggest that the application of 0.5% NaCl to a habitat for 3 days is effective for *Ae. albopictus* control.

Key words: *Aedes albopictus*, larvicide, LC₉₀, sodium chloride

INTRODUCTION

Originally restricted to Asia, the distribution range of *Aedes albopictus* (Skuse) has expanded to include Europe, North and South America and Africa (Hawley, 1988; Loumibos, 2002; Wilke et al., 2020). Considered to be a pernicious pest throughout its range, *Ae. albopictus* may also be a vector for several pathogens. In particular, *Ae. albopictus* is a competent vector of arboviruses, such as dengue, chikungunya, Zika and yellow fever (Pagès et al., 2009; Lambrechts et al., 2010; Amraoui et al., 2016; Chouin-Carneiro et al., 2016; Smartt et al., 2017; Wilke et al., 2018). In Asia, the mosquitoes *Aedes aegypti* (L.) and *Ae. albopictus* are the main vectors of dengue hemorrhagic fever (DHF), a prostrating, sometimes epidemic disease of humans, that is often fatal (Simmons et al., 2012). Dengue outbreaks occurred in Japan during World War II, and *Ae. albopictus* was identified as the vector (Hotta, 1998; Kurihara, 2003). Although the disease was no longer thought to be endemic to Japan, a total of 162 people contracted dengue fever via *Ae. albopictus* in 2014 (Seki et al., 2015; Shimada et al., 2016). Recently, there has been a marked increase in the number of travelers infected with the dengue virus in Japan, and a total of 868 cases were reported between 1999 and 2010 (Takasaki, 2011). In 2019, two domestic infected persons were reported in Japan (Ministry of Health, Labor and Welfare, 2019). This increase in the prevalence of dengue fever implies that climate change and globalization have accelerated the risk of contracting dengue fever in temperate regions (Gubler, 2011; Ebi and Nealson, 2016).

To date, prevention measures for dengue have focused on decreasing the chance of being bitten by a carrier mosquito (World Health Organization, 2009). Given the problems associated with insecticide resistance (Vontas et al., 2012) and the effects of chemicals on non-target species (Karunarante and Hemingway, 2000; Milam et al., 2000), increased attention is being paid to alternative or combined methods (e.g., source reduction and insecticide
application) of pest control. Source reduction has been reported to be the single most effective control technique against container-inhabiting Aedes species (Hawley, 1988; Faraji and Unlu, 2016). Furthermore, the application of salt to used tires and other possible habitats is recommended by the Ministry of Health, Labour and Welfare of Japan (2015). However, the effectiveness of salt as a larvicide and the amount of salt required is unknown.

We therefore examined the effect of sodium chloride (NaCl) on Ae. albopictus larvae. Although the larvae of many mosquito species can develop in salt water, and several species actually prefer oviposition sites that contain relatively high salt levels (Wallis, 1955; Nicolson, 1972), a strong negative correlation was reported between the number of eggs deposited by Ae. albopictus and the NaCl concentration of ovitraps (Panigarah et al., 2014; Gunathilaka et al., 2017). Saline water with a concentration of 0.5% NaCl has a strong repellent effect on the oviposition of overwintering eggs of Ae. albopictus (Jinguij et al., 2020). We hypothesized that Ae. albopictus larvae cannot tolerate salty water. To test this hypothesis, we assessed the acute toxicity of NaCl against Ae. albopictus larvae.

**Materials and Methods**

**Mosquitoes**

All of the mosquitoes used in our experiments were Ae. albopictus from six colonies that were originally collected in Sendai city, Miyagi prefecture, Japan during the summer of 2019. The sampling locality was approximately 13 km from the coast. Adults were collected from July 10 to 20. Adults from each colony were kept separately in cages (320×220×220 mm) covered with surgical cotton stocking. The adult mosquitoes were provided a 10% sucrose solution ad libitum under conditions of high-humidity (>60% RH) at 25–28°C and natural photoperiod conditions. We provided them with the opportunity to feed on human blood for at least 15 min, daily. This research was approved by the Research Ethics Committee at Miyagi University, Japan (Approval No.: 2019-969).

Gravid solution, which has been widely used to investigate the distribution of Ae. albopictus in the field, was used as an oviposition medium (Fay and Eliason, 1966; Moriya, 1974). Ovitraps are plastic cylinders measuring 85 mm high and 75 mm wide lined with black paper on the inside. Gravid solution was composed of rice straw submerged in filtered water for 10 days at 25°C. Aedes albopictus females oviposited their eggs on the black paper in the ovitraps containing the gravid solution in the cage. We then collected eggs and reared the larvae separately by colony in an enamel tray (200×250×50 mm) containing filtered water at room temperature. The number of larvae kept in each enamel tray was maintained at approximately 200 individuals. Larvae were supplied continuously with yeast-based rat feed. Fourth instar larvae were reared from the eggs that hatched in the enamel tray in the laboratory under natural light conditions.

**Acute toxicity testing**

Acute toxicity testing was conducted on Ae. albopictus larvae in 2019 using standard acute toxicity tests that were performed according to WHO guidelines (WHO, 2005). Briefly, the susceptibility status of immature stages of Ae. albopictus in the laboratory were tested against 0.25%, 0.5%, 0.75%, 1.00%, 1.25% and 1.5% (w/v) solutions of pure NaCl. Pure NaCl was purchased from Sendai Wako Pure Chemical Industries, Ltd. (Sendai, Japan). Stock solutions were prepared by dissolving 2.5 g, 5 g, 7.5 g, 10.0 g, 12.5 g and 15.0 g of NaCl in 1000 mL of deionized water. We considered that small larvae were more susceptible to the influence of the osmotic pressure of NaCl than larger larvae. Consequently, in addition to fourth instar larvae, we also used first instar larvae for the acute toxicity tests, which were performed in 300 mL beakers containing 200 mL of each stock solution. Specifically, 20 first-instar larvae and 25 fourth-instar larvae were placed in separate beakers containing 200 mL of 0.25%, 0.5%, 0.75%, 1.0%, 1.25% and 1.5% NaCl solution. As a control, 20 first-instar larvae and 25 fourth-instar larvae were placed in separate beakers containing 200 mL of deionized water. Six replicates for the first and fourth instar larvae were then performed at each concentration (i.e., 20 first-instar larvae ×6 concentrations ×6 replicates; total of 720 larvae, and 120 larvae were used as a control). Each beaker was covered with plastic film to prevent evaporation and no food was provided during the acute toxicity test. Larval mortality was observed at 24, 48 and 72 h after starting the test. The beakers were incubated at 25±0.5°C and kept under a photoperiod of 12:12 h (L:D).

Dead Ae. albopictus larvae were defined as larvae that could not be induced to move when they were probed with a needle near the siphon or in the cervical region. The number of dead larvae was confirmed under a stereomicroscope at 24, 48 and 72 h after initiating acute toxicity tests. In accordance with OECD test guidelines, the exposure period used was 48 h. In this study, the acute toxicity value after 72 h of exposure was also recorded in consideration of outdoor use. All of the acute toxicity tests were conducted from August 20 to 27.

Basic water chemistry parameters, pH, dissolved oxygen (DO), electric conductivity (EC) and NaCl concentration, were measured immediately before (0 h) and after acute toxicity testing (72 h) using a water quality meter (WQC-24, TOA, Japan).
Statistical analysis

For the number of dead *Ae. albopictus* larvae, mean values obtained for the treatment groups were compared to the mean of the respective control by one-way ANOVA and multiple comparison tests (Dunnett’s test). The lethal concentrations that kill 90% of the population (LC₉₀) of the 24-, 48- and 72-h acute toxicity tests were estimated by probit analysis using the actual NaCl concentration. The software package R, version 3.1.1 (R Development Core Team, 2014) was used for all statistical analyses. Multiple comparisons were performed using the “multcomp” library, version 1.1-1 (Hothorn et al., 2015).

Results

Acute toxicity of sodium chloride to *Ae. albopictus* larvae

In the acute toxicity tests, physicochemical parameters did not change significantly over the course of the experiment. Larval mortality increased with increasing NaCl concentration and exposure duration. The mortality observed in first instar larvae in the 24-h acute toxicity test at 0.75, 1.0, 1.25 and 1.5% NaCl was significantly higher than the larval mortality observed in the control (Dunnett’s test; *p*<0.001, Fig. 1). With increasing exposure duration, lower NaCl concentrations, such as 0.5%, had a highly toxic effect in the 72-h acute toxicity test. Fourth instar larvae were less sensitive to NaCl than first instar larvae. The mortality observed in fourth instar larvae in the 24-h acute toxicity test at 1.25 and 1.5% NaCl was significantly higher than the mortality observed in the control (Dunnett’s test; *p*<0.001, Fig. 2).

Basic water chemistry parameters, pH, DO (mg/L) and EC (s/m) measured at the beginning and end of the test period for first instar larvae showed changes in pH of 6.57±0.26 to 6.32±0.33, DO of 10.25±1.01 to 11.21±0.41 and EC of 0.54±0.47 to 0.48±0.33. pH, DO and EC measured at the beginning and end of the test period for fourth instar showed changes in pH of 6.08±0.15 to 6.56±0.08, DO of 8.60±0.93 to 7.53±0.18 and EC of 1.05±0.85 to 1.07±0.90. In the acute toxicity test, physicochemical variables did not show significant increases or decreases over the
Table 1. LC₉₀ for NaCl in first and fourth instar Ae. albopictus larvae.

| LC₉₀ (95% CI) (%) | 24h | 48h | 72h |
|-------------------|-----|-----|-----|
| First instar      | 0.98 (0.91–1.06) | 0.77 (0.69–0.86) | 0.49 (0.43–0.55) |
| Fourth instar     | 1.19 (1.16–1.23) | 1.08 (1.04–1.12) | 1.01 (0.96–1.06) |

experiment period.

**LC₉₀ for NaCl solution in Ae. albopictus larvae**

The LC₉₀ values for NaCl in *Ae. albopictus* larvae are shown in Table 1. For first instar larvae, the lowest NaCl concentration was 0.49% (0.43–0.55%) in the 72-h LC₉₀ treatment. For fourth instar larvae, the lowest NaCl concentration was 1.01% (0.96–1.06%) in the 72-h LC₉₀ treatment.

**DISCUSSION**

Our findings suggest that the mortality of first and fourth instar *Ae. albopictus* larvae increased with increasing NaCl concentration and exposure duration. Our results are consistent with previous studies (Wigglesworth, 1933; Mukhopadhyay et al., 2010), which showed that exposure to NaCl can kill *Ae. aegypti* larvae. In our experiments, fourth instar *Ae. albopictus* larvae exposed to a 1.25% NaCl concentration showed approximately 100% mortality after 48h (Fig. 2). Total mortality was also observed when laboratory-reared, fourth instar *Ae. aegypti* larvae were exposed to a 1.25% NaCl concentration for 84h (Mukhopadhyay et al., 2010). Similarly, 100% mortality was observed in fourth instar *Ae. aegypti* larvae at 1.4% NaCl within 48 hours (Wigglesworth, 1933). In laboratory experiments, 100% mortality was observed sooner in *Ae. albopictus* larvae at low NaCl concentrations compared to *Ae. aegypti* larvae. Thus, our findings suggest that *Ae. albopictus* larvae are more sensitive to NaCl exposure than *Ae. aegypti* larvae. We also found that first instar *Ae. albopictus* larvae were more sensitive to NaCl than fourth instar larvae. Brito-Arduino et al. (2015) reported that 100% mortality of *Ae. aegypti* larvae occurred at a 1.75% NaCl concentration from egg hatching until 48h. Their results showed that larvae are less tolerant of high salt concentrations immediately after hatching. Further, a strong negative correlation was observed between the number of eggs deposited by *Ae. albopictus* females and the NaCl concentration of ovitraps (Panigrahi et al., 2014; Gunathilaka et al., 2017; Jinguji et al., 2020).

Since the amount of salt to be sprayed at potential oviposition sites has not yet been clarified, our findings need further research on *Ae. albopictus* control and reduce the associated costs. In our experiments, the 72-h LC₉₀ of first and fourth instar larvae was estimated to be 0.49% and 1.01% NaCl, respectively (Table 1). This implies that if an NaCl concentration of 0.49% is applied to an area such as used tires for 3 days, approximately 90% of the *Ae. albopictus* larvae at the site would be killed. Exposure to NaCl solutions also affects subsequent larval growth. For example, the growth rate of first instar *Ae. aegypti* larvae that were exposed to 1.0% NaCl and survived was either extremely slow or not evident (Wigglesworth, 1933). Less than 50% of *Ae. aegypti* larvae reached the pupal stage when a 1.4% NaCl concentration was used to rear larvae (Brito-Arduino et al., 2015). Therefore, survivor *Ae. albopictus* larvae are expected to finally decrease more in the future.

*Aedes albopictus* is a highly anthropophilic species whose dispersal around the world is known to have been mediated by humans (Kotsakiozi et al., 2017). This species has adapted well to urban environments where larvae have been found in artificial containers, such as discarded tires, cemetery urns, plastic buckets, flower pots, pans, gutters and water storage containers (Simard et al., 2005; Bagny et al., 2009; Rochlin et al., 2013; Li et al., 2014). Some studies have shown that source reduction is labor-intensive, time-consuming and costly (Zhou et al., 2009; Rao, 2010; Fonseca et al., 2013), and might not be effective in isolation (Wheeler et al., 2009). Thus, adding NaCl or table salt to disused tires could potentially be employed as a measure for controlling *Ae. albopictus*. For example, adding 5g of salt per 1000g water to disused tires could potentially be employed as a measure for controlling *Ae. albopictus*. However, since our findings are the result of laboratory studies, we need to clarify the efficacy of NaCl in the field. To date, several studies have shown that *Ae. albopictus* larvae were not found in outdoor areas where the average salt concentration ranged from 0.11 to 0.73% (Watanabe et al., 2012; Kobayashi et al., 2012). However, the application of salt to these environments needs to be carefully considered. Firstly, the application of salt to many discarded tires costs labor-intensive and future studies need to consider simplification of the application method. Secondly, salt damage to crops needs to be considered. Simply spraying a saline solution into vegetation may be effective for mosquito control, but it would harm the vegetation.

It has been shown that *Ae. albopictus* can adapt to brackish water conditions and oviposit and undergo preimaginal development in unused wells and discarded artificial containers (Ramasamy et al., 2011). We consider that *Ae. albopictus* has the ability to lay eggs that develop into adults in saline water. We therefore need to continuously monitor *Ae. albopictus* populations after adding salt to their optimal habitat.

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