ENHANCEMENT OF THE EFFICACY OF A COMBINATION OF MESOCYCLOPS ASPERICORNIS AND BACILLUS THURINGIENSIS VAR. ISRAELENSIS BY COMMUNITY-BASED PRODUCTS IN CONTROLLING Aedes aegypti Larvae in Thailand

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Abstract. Prolonged efficacy of a combination of bacteria (Bacillus thuringiensis var. israelensis [Bti]) and copepods (Mesocyclops aspericornis) in controlling immature forms of Aedes aegypti in peridomestic water containers was achieved by adding various products from local villages as supplementary food for copepods. In all experiments, 100 first-instar larvae were added into the breeding containers every day for eight weeks. Combinations of biological control agents and each local supplementary food were applied once at the beginning of the experiment. At the end of the experiment, the average number of mosquito larvae in containers with a combination of copepods and Bti with one gram of rice grain had decreased to only 0.5% of that with no control agent. In comparison, the average numbers of mosquito larvae in containers with Bti only, or copepods only, were approximately 10% and 33% of those in containers with no control agents, respectively. In addition, the number of copepods in containers with mosquito larvae and supplementary food was at least three times higher than those with mosquito larvae alone.

INTRODUCTION

Dengue fever is a serious infectious disease in several tropical and subtropical countries in Asia, Africa, and the Americas. It causes more illness and death than any other arboviral infection, and no commercial vaccine for dengue is currently available. Dengue fever is caused by an RNA flavivirus. The spread of dengue throughout the world is mainly attributed to proliferation and adaptation of its mosquito vectors. Once infected by the virus, the mosquito vectors remain viruliferous for the rest of their life. The main vector of dengue flavivirus is Aedes aegypti L., which is anthropophilic and common in urban and suburban environments. In nature, Aedes aegypti relies mainly on rainfall for breeding. However, it has adapted to urban and suburban environments as well, where it uses human-made peridomestic containers such as clay jars and vases for breeding regardless of the rains. Therefore, attempts to control dengue have been aimed mostly at controlling the Aedes aegypti mosquito vectors.

A variety of methods have been used to control Aedes mosquitoes. Application of oil and sulfur fumigation, larvicide fogging, and other insecticides work well in the short-term, but after a few weeks mosquito populations inevitably increase and eventually develop resistance against these chemicals. Biocontrol efforts have included the use of predatory fish, plankton management, and the application of copepods7,8 and Bacillus spp.9,10 Cultural methods, such as removal of breeding sites, are sometimes effective, depending on the scale of urbanization. Integration of these methods could provide a more cost-effective and environmentally friendly approach in controlling mosquito vectors.12,13

Copepods are small aquatic crustaceans. Observations of copepod predation on first-instar mosquito larvae led to the first investigation in 1981 of their potential as biological control agents. Since then, various species of predacious copepods have been tested for their potential to control mosquitoes. Most of them are omnivorous and prey on immature mosquitoes, especially first-instar larvae, but rarely on later stages. Several species of copepods, including Mesocyclops aspericornis, M. thermocyclopoides, M. guangxiensis, and M. longisetus, have been reported as potential biological control agents of Ae. aegypti. Entomopathogenic bacteria have been alternatives to chemical insecticides for decades. Several soil bacteria have been isolated and characterized, including Bacillus thuringiensis var. israelensis (Bti). Proteinaceous endotoxins in the crystalline spores of Bti are lethal to mosquito larvae upon ingestion. A number of formulations have been developed and tested against Ae. aegypti and many other mosquito larvae. The endotoxins produced by Bti are very specific to certain types of dipterans, including mosquitoes (Culicidae) and black flies (Simuliidae). Application of Bti in a natural setting is relatively safe and ecologically acceptable to non-target organisms and has no detrimental effects on humans. However, its limitation is the high sensitivity of Bacillus spp. to environmental factors.

An effective control of mosquito larvae was attained when M. aspericornis and Bti were combined. Nevertheless, re-application of Bti may be necessary to achieve satisfactory control levels because the controlling efficacy of Bti decreases a few days after application under natural settings. In addition, effectiveness of using copepods to control mosquito larvae greatly depends on the availability of their food. To prolong the efficiency of a combination of M. aspericornis and Bti in controlling Ae. aegypti, we investigated the
potential of using products readily available from the local community as copepod supplementary food in peridomestic containers. The copepods prey mostly on the newborn mosquito larvae due to size limitation, whereas Bti could kill most mosquito larvae upon application. Addition of food for the copepods could then supplement their natural food source, sustain the copepod population, and enhance controlling efficacy of the combination to achieve a practical long-term control of *Ae. aegypti* vector.

There were two sets of experiments in this study. The first experiment was conducted to determine a suitable local product as copepod supplementary food. The second experiment was conducted to study the effects of supplementary food on the efficacy of copepods and Bti for controlling *Ae. aegypti* mosquito larvae.

**MATERIALS AND METHODS**

**Test facilities.** We used human-made 200-liter peridomestic containers for the experiment. These containers are the same kind that villagers in rural Thailand use for storing rainwater for their everyday use. The containers were maintained under ambient temperature within a greenhouse of the Faculty of Science, Mahidol University (Bangkok, Thailand). Each container was filled with 150 liters of dechlorinated water and covered with mosquito net to prevent any contamination from local mosquitoes. The first experiment was performed once in summer (July) with two replicates for each treatment. The second experiment was performed once in summer (July–August) and once in winter (December–January) with two replicates for each treatment. The mean ± SD water temperature during the experiment conducted in summer was 28.8°C ± 0.38°C. The mean ± SD water temperature during the experiment conducted in winter was 26.53°C ± 2.49°C.

**Sampling methods.** Copepods and living mosquito larvae were sampled from each container with a very fine round sweep net with a diameter of 25 cm. The net was swept gently 10 times in a spiral manner from the surface to the bottom of the water. Copepods and mosquito larvae were separated and counted. After counting, third-instar and fourth-instar mosquito larvae and pupae were discarded since they were too large for successful predation by the copepods, whereas the copepods and first-instar and second-instar mosquito larvae were re-inoculated into the same container from which they were taken. The net was then put in boiling water for five minutes and washed before each use to avoid cross contamination of the samples.

**Liquid Bti preparation and application.** Liquid Bti was produced at the Department of Biotechnology, Faculty of Science at Mahidol University. Bti IPS82 was used as a standard to titrate the Bti used in this experiment because it has been produced since 1982 and its potency is considered stable.\(^3\)\(^2\) The International Toxic Unit (ITU) of Bti used in this study was 531 ITU/mg when compared with Bti IPS82 at 25 ± 2°C (mean ± SD) (Chansang U, Bhumiratana A, Kittayapong P, unpublished data). Its 50% and 90% lethal concentrations (LC\(_{50}\) and LC\(_{90}\)) were 2.3 × 10\(^{-4}\) and 5.3 × 10\(^{-4}\) ml/L, respectively (Chansang U, Bhumiratana A, Kittayapong P, unpublished data). A concentration 20 times that of the LC\(_{90}\) was used in this study.

**Mass rearing of predatory copepods.** Copepods were collected from their natural breeding sites at Ban Laem Hin, Hua Sam Rong Subdistrict, Plaeng Yao District, Chachoengsao Province in eastern Thailand. Isofemale lines were established from gravid females and maintained at the Department of Biology, Faculty of Science, Mahidol University (Bangkok, Thailand). More than 90% of the collected samples were identified and confirmed by Dr. Janet W. Reids (National Museum of Natural History, Smithsonian Institution, Washington, DC) to be *Mesocyclops aspericornis* (Daday) (Vihokto S, 1994, Preliminary survey and experimental studies of *Mesocyclops* spp. as biological control agents of dengue vectors in a rural Thai community. M.S. Thesis, Bangkok, Thailand: Mahidol University). Once identified, gravid female *M. aspericornis* from different isofemale lines were pooled and mass-reared in dechlorinated water (pH 7) in a 15-liter fish tank at 27 ± 2°C (mean ± SD). *Paramecium* spp. prepared from boiled rice straw water extract and commercial powdered fish food were used to support the copepod cultures. We sampled 100 copepods to apply as the initial number to each treatment that contained copepods as a control agent.

**Mosquito larvae.** First-instar larvae of *Ae. aegypti* were kindly provided by the Center for Vectors and Vector-Borne Diseases, Faculty of Science at Mahidol University. The colony was established in 1994 from field-collected larvae. The original location was from Hua Sam Rong Subdistrict, Plaeng Yao District, Chachoengsao Province, This colony has never been exposed to any insecticides or Bti products.

**Test of suitable local supplementary food for copepods.** This experiment was conducted in peridomestic containers similar to those that villagers in rural Thailand use to store rainwater for their everyday use. The water in these containers was generally kept clean, but contained a limited amount of organic materials. Limited organic sediments and mosquito larvae may not be sufficient to sustain a population of *M. aspericornis* used to control mosquito larvae in a long-term control strategy. Thus, products from the local community used as supplementary food for the copepods might help sustain the numbers of copepods when their natural food is scarce.

We tested low-cost local natural products that seemed of practical use for villagers. In this experiment, we used clean 200-liter peridomestic containers filled with 150 liters of dechlorinated water. The experiment was conducted by inoculating 100 adult *M. aspericornis* to every container. One hundred first-instar *Ae. aegypti* larvae were then supplied to each container daily as the regular copepod food. There were five treatments in this experiment and two replications for each treatment. In the control containers, there was no supplementary food. We used one and five grams of rice grain (*Oryza sativa* L. [Poaceae]); five grams of commercial fish food granules; or five grams of sesban leaves (*Sesbania grandiflora* L. [Fabaceae]) as the supplementary food for the copepods. All materials were sterilized before applying to the treatment containers. The supplementary food was supplied only once at the beginning of the experiment. Copepods were sampled and counted every seven days. This experiment was terminated at the end of the fourth week.

**Copepods and Bti with supplementary food for controlling mosquito larvae.** We tested the efficacies of Bti, copepods, and their combination with local products as copepod supplementary food for controlling *Ae. aegypti* larvae in peridomestic-
tic containers. We divided containers into five treatments with two replications.

The experiment was conducted by inoculating 100 first-instar *Ae. aegypti* larvae into each container daily to simulate high numbers of *Ae. aegypti* in nature. The first treatment (L) was the control containers in which 0.1 grams of commercial fish food granules was added with mosquito larvae, but without Bti nor copepods. In the second treatment (L + C), we inoculated 100 adult *M. aspericornis* as the only control agent. In the third treatment (L + B), liquid Bti was used as the only control agent. In the fourth treatment (L + C + B + R), a combination of Bti and 100 adult copepods were used as control agents and one gram of rice was added as supplementary food for the copepods at the beginning and during the fourth week of the experiment. In the fifth treatment (L + C + R), we inoculated 100 adult *M. aspericornis* as the only control agent and one gram of rice was supplied at the beginning and during the fourth week of the experiment. The fifth treatment was performed only in the winter experiment. Living mosquito larvae and copepods were sampled and counted every seven days. This experiment was terminated at the end of the eighth week.

**RESULTS**

**Experiment on supplementary food for copepods.** The average number of copepods that survived in each treatment is shown in Figure 1. At the end of the fourth week when this experiment was terminated, the average number of copepods in the treatment with sesban leaf (367) was more than twice that of the control (168). The number of copepods in the treatment with one gram of rice (609) was more than three times that of the control (168). The numbers of copepods in the treatment with five grams of rice (1,154) or five grams of commercial fish food (1,214) were each more than six times that of the control (168). Nevertheless, the water appeared cloudy and polluted in the latter two treatments.

**Supplementary food and controlling efficacy of copepods and Bti.** The numbers of living mosquito larvae (M) and copepods (C) from each treatment of the summer and winter experiments are shown in Figure 2A and B, respectively. By the end of the experiment, the number of living mosquito larvae in the control, L (M), was at least twice as many as those of other treatments. The number of living mosquito larvae from the treatment that combined Bti and copepods with supplementary food, L + C + B + R (M), was the least throughout both summer and winter experiments. The treatment with Bti only (L + B) kept the number of living mosquito larvae very low, although the number of living mosquito larvae, L + B (M), increased in the fifth week of the winter experiment and in the seventh week of the summer experiment. These seemed to be signs of loss of larvicidal activity of Bti in the treatment containers. The numbers of living mosquito larvae in the treatments, in which Bti or copepods was the only control agent, L + B (M) and L + C (M), respectively, were not significantly different. However, there was a persistent contamination problem with ostracods in the treatment of mosquito larvae with copepods in the winter experiment that clearly affected the numbers of copepods, L + C (C), and living mosquito larvae, L + C (M). For example, there was an increase in the number of copepods in the fifth week of the summer experiment, but the number of copepods in the same treatment, L + C (C), in the winter experiment was at least five times less. The major decrease in the number of copepods could be attributed to the increases of living mosquito larvae and the lack of controlling agents. The number of living mosquito larvae, L + C (M), in the fifth week of the winter experiment was at least eight times higher than that of the summer experiment during the same period.

Effects of seasonal changes on the number of copepods
FIGURE 2. Average number of *Mesocyclops aspericornis* copepods (solid lines [C]) and living *Aedes aegypti* mosquito larvae (dotted lines [M]) from A, summer (July–August) and B, winter (December–January) experiments. L = *Ae. aegypti* larvae; L+B = *Ae. aegypti* larvae and *Bacillus thuringiensis* var. *israelensis* (Bti); L+C = *Ae. aegypti* larvae and *M. aspericornis*; L+C+R = *Ae. aegypti* larvae, *M. aspericornis*, and one gram of rice; L+C+B+R = *Ae. aegypti*, *M. aspericornis*, Bti, and one gram of rice.
were observed, especially in the treatment of mosquito larvae, copepods, and Bti with supplementary food (L + C + B + R). Fluctuations in the numbers of copepods and living mosquito larvae were also observed (Figure 2). Supplementary food had noticeably positive effects on the numbers of copepods. The average number of copepods of the treatment provided with supplementary food, L + C + B + R (C), was generally greater than the average number of copepod with supplementary food, L + C + R (C), and at least twice that of the treatment provided with mosquito larvae alone, L + C (C). Conversely, the average number of living mosquito larvae in the treatment provided with supplementary food, L + C + B + R (M), was the least throughout the experiment when compared with that of other treatments: L + C + R (M), L + C (M), and L + B (M).

**DISCUSSION**

*Mesocyclops aspericornis* belongs to the lower food chains of complex aquatic ecosystems. In a complex system of a large body of water, this copepod may have little direct impact on mosquito larval populations. In restricted conditions, however, where the copepods become dominant, *M. aspericornis* could be both a predator and a competitor for food of mosquito larvae. In some instances, introduction of *M. aspericornis* in conjunction with other controlling methods resulted in the eradication of *Ae. aegypti* in both laboratory and natural settings. In this study, we investigated a combination of *M. aspericornis* and Bti in the long-term control of the anthropophilic *Ae. aegypti* mosquito in peridomestic containers by adding natural supplementary food available from the local community.

One of the important factors influencing the efficacy of using *M. aspericornis* to control mosquito larvae has been its ability to subsist in containers regularly used by people. In rural Thailand, villagers use large peridomestic containers to store rainwater for their everyday use and keep the containers relatively clean, resulting in minimal contamination with organic materials. Application of *M. aspericornis* to these containers to effectively control the mosquito larvae may require the addition of supplementary food to sustain *M. aspericornis* populations for the long-term control of *Ae. aegypti*.

Based on the results of this study, a good candidate as copepod supplementary food without compromising the usability of the water was one gram of rice per 150 liters of water, although re-application was necessary as the supplementary food was depleted. Although the containers with commercial fish food granules and those with five grams of rice produced the very high numbers of copepods, the water became so polluted that it was not suitable for domestic use by villagers. There was little response of copepods to sesban Supplementary food was provided.

A combination of *M. aspericornis* and Bti for the long-term control of *Ae. aegypti* in rural Thailand has great potential. The copepods are very successful as predators of the first-instar mosquito larvae, but are not effective predators of larger mosquito larvae. Thus, a combination of approaches may lead to more satisfactory control of mosquitoes. *Bacillus thuringiensis* var. *israelensis* has been used in conjunction with *M. aspericornis* because of its high toxicity and high specificity to mosquito larvae. In addition, Bti shows no detrimental effects on either copepods or humans and has relatively minor effects on non-target organisms.

We showed that the number of surviving mosquito larvae was lowest and the number of copepods was highest in the treatment containing copepod supplementary food. This implies that efficacy of the combination of copepods and Bti to control *Ae. aegypti* mosquito larvae in peridomestic containers could be prolonged and enhanced by addition of copepod supplementary food.

It is possible that seasonal differences between summer (July–August) and winter (December–January) experiments could influence copepod populations within the containers. Decreases in copepod numbers in the second week of the summer experiment and in the third week of the winter experiment may be due to depletion of food sources. In the summer experiment, copepods in the treatment with Bti and copepods with supplementary food may have consumed the supplementary food faster than they did in the winter experiment. In addition, the copepod populations from the winter experiment seemed to take a longer time to replenish, after addition of supplementary food during the fourth week of the experiment, than those in the summer experiment. It has been reported that the duration of *Ae. aegypti* larval development varies depending on the availability of food resources, but inversely with the temperature. This could subsequently affect the population dynamics of the copepods, since the copepods responded to the availability of the supplementary food more rapidly in the summer experiments than in the winter ones.

The ostracods interfered with the ability of the copepods to control the mosquito larvae. Contamination with ostracods was observed in the containers with copepods as the sole control agent in the winter experiments. The ostracod contamination apparently decreased the number of copepods, since the ostracods may have competed for food with early larval stage of copepod or nauplii. As a result, it increased the number of surviving mosquito larvae. It was suggested that using leaves to cover the water surface could help alleviate contamination problems with the ostracod *Cypretta globulus* because the ostracod adheres to leaves that can be easily removed and replaced.

A density-dependent relationship among juvenile insects is often directly associated with the availability of food resources and consequently with increased juvenile mortality, delayed maturity, and reduced adult size. Addition of suitable supplementary food helped prolong the controlling efficacy of a combination of *M. aspericornis* and Bti in controlling *Ae. aegypti* mosquito larvae in peridomestic containers. Implementation of a combination of *M. aspericornis* and Bti with supplementary food provided by the local community to control *Ae. aegypti* mosquito larvae under field conditions should be investigated.

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