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Original Research Article

The consociated use of the copepod *Mesocyclops ogunnus* (Copepoda: Cyclopoidea) and Bti bacteria in controlling *Aedes albopictus* (Diptera: Culidae) mosquito larvae

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Copepods of the genus *Mesocyclops* and *entomotoxic* bacteria of the *Bacillus thuringiensis varisraelensis* (Bti) strain have been utilized with success in the control of mosquito larvae. We tested the efficiency of the consociated use of the copepod *M. ogunnus* with Bti to control *Aedes albopictus* larvae. The experiments were performed within an Atlantic forest fragment in the municipality of João Pessoa, Paraíba State, Brazil, using consociated as well as individual treatments with these two biological agents. The association of copepods and the Bti was found to be significantly more effective in controlling *Aedes albopictus* larvae than either of these agents alone during the 21 experimental days. Copepod densities were significantly greater in the treatments containing Bti. Our results indicate that this consociation is a viable low-impact alternative for controlling *Aedes* larvae.

Key words: Biological control, dengue, copepods, Bti, Aedes.

INTRODUCTION

The mosquito *Aedes aegypti* (Linnaeus,1792) is considered the principal vector of the dengue virus in Brazil, but the species *Aedes albopictus* (Skuse, 1894) is also a potential vector of the arbovirose and is responsible for the transmission of this disease throughout the Asian continent (Forattini 2002). The first records of *A. albopictus* in Brazil date to the 1986s, and these mosquitoes are now encountered throughout the country, especially in forested areas (Alencar *et al*. 2008). These two mosquito vectors have similar morphologies and ecological requirements (Forattini 2002), allowing the use of *A. albopictus* as a biological model to test methodologies for controlling *Ae. aegypti*. *Aedes* mosquitoes are generally combated using chemical insecticides and larvicides, but these insects are gradually becoming resistant to these products (Maciel-de-Freitas *et al*. 2012) Additionally, these substances can cause serious problems for humans and the environment (Pereira *et al*. 2009). In light of these problems, low-impact alternatives are being sought for controlling these insects, such as the use of mosquito traps (Resende *et al*. 2012) or biological control with Essential oils or extracts the plants (Amer and Mehlhorn 2006; Rahuman *et al*. 2008).

A number of different classes of organisms have been tested and used in the biological control of mosquitoes, including bacteria, fish, platyhelminthes, crustaceans, even reptiles and plants (Forattini 2002; Dias and Moraes 2013; Zhang *et al*. 2014). The bacteria *Bacillus thuringiensis var israelensis* (Bti) (Polanczyk *et al*. 2003), and cyclopoid copepods have shown considerable promise in this area (Nam *et al*. 1998). Bti produces crystals containing toxins that provoke the disintegration of the median intestine of mosquito larvae when ingested, rapidly killing them (Polanczyk *et al*. 2003). One of the principal characteristics of this bacterium is its specific toxicity to insect groups considered pests. Additionally, the environmental impacts associated with Bti use are quite low, as studies have shown that its toxins do not have negative effects on vertebrates (Schnepf et al. 1998). Copepods are micro-crustaceans found in diverse aquatic habitats. Some species of the order Ciclopoida are known predators of the larvae of *Aedes, Anopheles*, and *Culex* mosquitoes. These animals feed on the first instar larvae of mosquitoes and have the capacity to consume up to 40 larvae per day, thus reducing infestations of *Aedes* larvae by up to 99-100% (Marten and Reid 2007).

Copepods demonstrate a number of advantages in terms of their use as biological control agents, for they are naturally abundant and easily cultivated in captivity (Marten et al. 1994).

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Promising results were seen in the use of copepods in the biological control of *A. aegypti* in Vietnam (together with community health education efforts) when local species of copepods were inoculated into water tanks – resulting in reductions of 76.7% in the number of cases of dengue in the focal communities (Nam *et al*. 1998). In spite of their efficiency, biological control agents demonstrate certain limitations in terms of their use. The efficiency of Bti lasts only for an average of 30 days under laboratory conditions (Vilarinhos and Monnerat 2004), which implies the necessity of monthly visits by health agents to reapply the product.

Copepods, on the other hand, can establish more or less permanent populations, although they do not prey upon later instar larval stages that may be present when they are first introduced (Marten and Reid 2007). In light of the individual limitations of these two agents, some preliminary experiments have been undertaken to examine their consociated use. Chansang *et al*. (2004) and Kosiyachinda *et al*. (2003) tested the consociated use of the copepods *M. thermocyclopoides* and *M. aspericornis*, respectively, with Bti in controlling *A. Aegypti* larvae in Thailand. Their laboratory experiments demonstrated that the use of a short-duration controlling agent (Bti) together with an organism capable of long-term effects (a copepod) could be more efficient than the use of either agent separately. However, experiments to test the efficiency of the consociation of copepods and Bti under natural conditions have not yet been undertaken in the Americas. The present work reports the results of field-condition tests of the efficiency of the consociation between the copepod *Mesocyclops ogunnus* and Bti bacteria in controlling larvae of the mosquito *A. albopictus*. We also evaluated variations in the population densities of this copepod species in small artificial recipients.

Methodology

Study area

The experiments were undertaken in an Atlantic Forest fragment located on Campus I of the Universidade Federal da Paraíba, João Pessoa, Paraíba State, Brazil (7° 08'21" Sx34° 50'38'' W). Preliminary studies found that *A. albopictus* was the dominant species of Culicidae in the area, indicating this site as an adequate environment for our experiments.

Field experiments

Forty transparent plastic recipients (1 L capacity) were filled with tap water and received a flake of fish food weighing approximately 0.03g and a 12 x 2 cm strip of plywood to aid in ovipositioning. The 40 recipients were divided equally into four treatment categories: Control (water), with copepods, with Bti, and with the two organisms in consociation (copepods + Bti). The recipients were first left in the forest fragment for 30 days (receiving a flake of fish food every two weeks) without any other experimental manipulation to be naturally colonized by *A. albopictus* larvae. Copepods of the species *M. ogunnus* were collected in the Três Lagoas reservoir in the city of João Pessoa (7°9'56"Sx 34°53' 44"W) and maintained in monospecific colonies in the laboratory. The Bti utilized were obtained in a commercial formulation (VectobacAS) packaged in concentrated solutions (10 L). After 30 days, the recipients left in the field were removed to the laboratory to count the mosquito larvae, and were subsequently inoculated with the biological agents or left as controls. Bti treatments consisted of the addition of 9 ppm of the Bti formulation; the copepod treatments consisted of the introduction of 20 individuals of *M. ogunnus* into each recipient; the copepod/Bti consociation consisted of the two organisms being added together in the same proportions as above; the control recipients did not receive any biological agent. Forty-eight hours after the addition of the copepods and – or the Bti, the mosquito larvae and the copepods were counted; counts were made on a weekly basis thereafter. The recipients were examined on a daily basis, however, and any pupae encountered were removed to prevent adult mosquitoes from being liberated into the environment.

Statistical analyses

The effects of the copepods and the Bti on the densities of A. albopictus larvae were tested on a weekly basis using ANOVA to determine when (and if) each agent significantly affected larval development. Additionally, a global test was performed using One-way ANOVA analysis of variance followed by a Fisher's PostHoc LSD test. The densities of copepods present in the treatments with and without Bti were compared using the Mann-Whitney test. The normality of the data was examined using the Shapiro-Wilk test. All of the statistical calculations were performed using R Development Core Team software (2012), version 2.15.1.

Results

Consociation of copepods with Bti - During the 21 days of the experiment, a total of 673 *A. albopictus* larvae were sampled. The greatest densities of larvae were found in the control treatment recipients, with an average of 16.7 (± 3.0 SE) larvae/200 ml. The consociated treatment with copepods and Bti demonstrated the lowest larval density (1.1 ± 0.3 larvae/200 ml). Treatments with only copepods or only Bti demonstrated intermediate larval densities (9.0± 1.7and 6.4± 1.2 larvae/200 ml respectively). Figure 1 presents the average total densities of larva encountered in the different treatments.

The One-way Anova demonstrated that there were significant differences between the treatments ($F= 12.695$, $p = 0.000008$). Fisher's LSD Post Hoc test (table 1) indicated that there were significant differences between treatments when compared with the control, and the consociation of Bti with copepods had an effect that was significantly greater in terms of reductions in the numbers of *A. albopictus* larva in comparisons with treatments containing only a single biological agent.

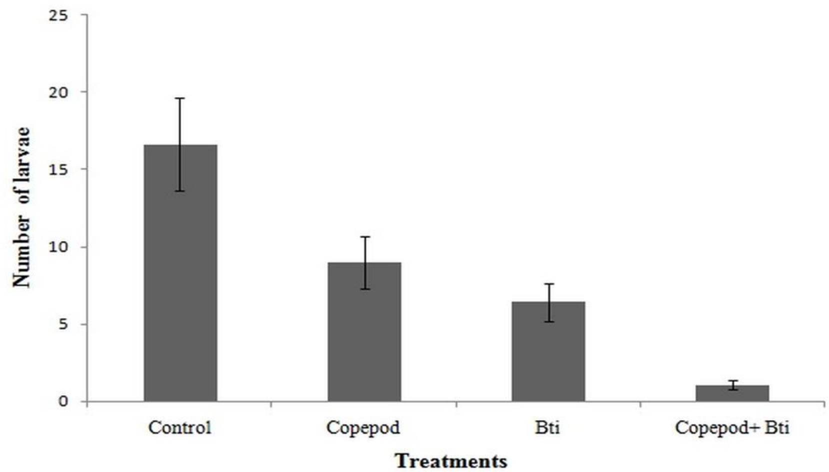

Figure 1: Average densities of *A. albopictus* larvae (±SE) in treatments with copepods, Bti, the two agents consociated, and controls during the 21 day experimental period

Table 1

Results of the Fisher's LSD Post Hoc test comparing the efficacy of different biological agents in *reducing A. albopictus* larval densities during the course of the experiment (underlined results are significant, α < 0.05).

Treatments	Control	Copepod	Bti	Copepod+Bti
Control				
Copepod	0.00523			
Bti	0.00032	0.31960		
Copepod + Bti	<0.00001	0.00389	0.04503	

Factorial Anova per week comparing the effects of Bti and copepods demonstrated that there were no significant differences among treatments on day 0 (before the application of biological control agents) (p>0.05). Significant differences were found between Bti treatment and controls on larval populations on days 2, 7 and 14, and between copepods treatments and controls on days 14 and 21. The interactions between the copepods and Bti were not significant, indicating that the effects of the consociation of these two agents were additive and not multiplicative (table 2).

Table 2

Factorial analysis of variance per week of the effects of Bti and copepods on A. albopictus mosquito larvae (underlined results are significant, α < 0.05).

Day	Bti (F-value)	Bti (p-value)	Copepod (F-value)	Copepod (p-value)	Bti*copepod (F-value)	Bti*copepod (p-value)
2	45.15928	<0.00001	3.35225	0.07564	1.22456	0.27602
7	11.70331	0.00160	0.16685	0.68541	0.84470	0.36435
14	5.32792	0.02701	27.36830	0.00032	0.88645	0.35290
21	1.76812	0.19222	12.84113	0.00102	21.46030	0.15187

The Bti treatment demonstrated decreasing larval numbers immediately after the addition of the bacteria, and larval numbers remain low for 7 days (Fig. 2A). The copepod treatment demonstrated decreasing larval numbers after 14 days (Fig. 2B). The consociation treatment of copepods and Bti demonstrated low larval densities during the entire experiment period (Fig 2C).

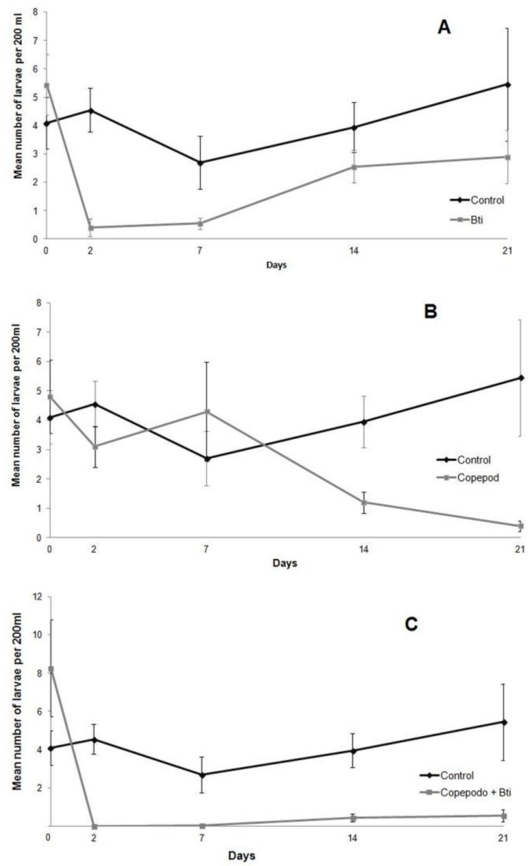

Figure 2: Temporal variations in the average densities (±SE) of A. albopictus larvae in the treatments containing Bti (A), Copepods (B), and the consociation of these two agents (C),compared with the control treatment

Temporal variations of copepod densities

The population densities of the copepod *M. ogunnus* increased until the 14th day of the treatments, with and without Bti (figure 3). After the 14 days, copepod densities decreased in the treatment without Bti; copepod densities continued to increase, however, in the consociation treatment with Bti. The average number of copepods encountered in the treatments with the added Bti at the end of the experimental period was 31.45 ± 4.27/200 ml; in the treatment without Bti, copepods averaged 17.5 ± 2.76/200 ml. The Mann-Whitney test demonstrated that the observed difference in copepod numbers on the last day of the experiment was significant (U= 20; p= 0.02319).

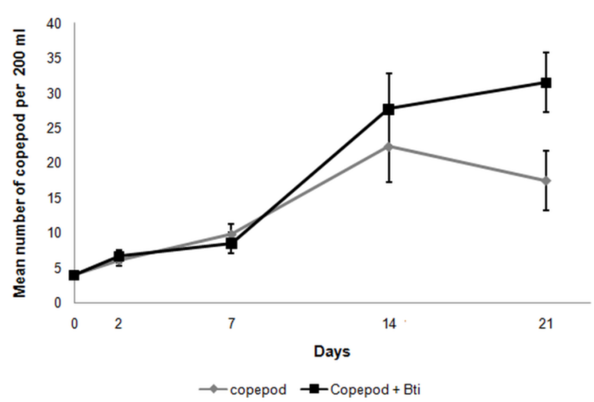

Figure 3: Temporal variations in the average densities (±SE) of copepods (*M. ogunnus*) in treatments with and without the addition of Bti

Discussion

Consociation of copepods with Bti

Our results demonstrated that the consociation between Bti and the copepod *M. ogunnus* reduced *A. albopictus* mosquito larval densities more efficien than either of these biological agents alone. This increased efficiency is the result of the temporal complementarity of the actions between these two agents, as Bti demonstrates highly efficient larvicidal activity immediately after its application (table 2), with its effects becoming significantly reduced after the 14th day, while copepods demonstrated significant control effects over *A. albopictus* larvae only after the 14th day. The consociation of these two agents resulted in low larval densities during the entire experiment. These results were similar to those of Chansang *et al*. (2004) and Kosiyachinda *et al*. (2003), who reported positive effects of the consociation between Bti and the copepod species *M. thermocyclopoides* and *M. aspericornis*, respectively, in the control of *A. aegypti* larvae.

The capacity of copepods to complement the early larvicidal effects of Bti should provide important cost reductions to programs of biological control of *Aedes* larvae by increasing the time between the re-applications (Braga and Valle 2007a). Additionally, as these two biological agents result in larval deaths through quite distinct processes (intoxication in the case of Bti, and predation in the case of the copepods), so there would be a reduced chance of selecting for two different successful resistance strategies simultaneously, as has occurred in other treatments with single larvicides (Lima *et al*. 2006, Cadavid-Restrepo *et al*. 2012)

Temporal variations in copepod densities

The populational increases noted for *M. ogunnus* (figure 2) demonstrated that these copepods are able to adapt and reproduce in relatively small artificial recipients exposed to outdoors conditions. Additionally, the copepods demonstrated greater densities in the recipients with added Bti, which again demonstrates the specificity of Bti toxicity to mosquito larvae but not to micro-crustaceans (Schnepf *et al*. 1998). Increases in the densities of copepods in the presence of Bti was also reported by Chansang *et al*. (2004) in experiments undertaken in Thailand. Populational increases (which also can increase the effectiveness of the consociation) may occur because nauplius larvae (the initial phase of copepod development) can feed on those bacteria or take advantage of the extra nutrients supplied by the Bti culture media (Kumar and Rao 1998).

Our results demonstrated the viability of using consociations of copepods and Bti to control *Aedes* larvae under natural conditions. In light of the apparent low environmental impacts of this technique and its economic advantages, we suggest that this consociation be considered a viable alternative to controlling *A. albopictus* and *A. aegypti*.

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