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Use of cyclopoid copepods for control of *Anopheles* (Diptera: Culicidae) mosquito larvae to prevent re-emergence of malaria in Sri Lanka

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ABSTRACT

Background & objectives: Although malaria is eliminated from Sri Lanka, there is a possible risk of spread from infected persons coming from malaria endemic countries. The presence of major and potential vectors in several parts of the country along with drug resistance, necessitates the identification of effective and novel control methods. The present study focused on identifying effective biological control agents for anopheline larvae using carnivorous copepods under laboratory and field conditions to prevent re-introduction of malaria in the country.

Methods: Three copepod species, namely *Mesocyclops scrassus*, *Cyclops varicans* and *C. languides* collected from different areas in the country were cultured by adding supplementary food, and their predatory efficacy was evaluated under laboratory and field conditions.

Results: Significant variation (p <0.05) was observed in predation rates of studied copepod species. The species *M. scrassus* showed the highest predacious efficiency, and consumed the highest number of anopheline larvae under laboratory and field conditions. Further, *M. scrassus* had higher survival rate than *C. varicans* and *C. languides*.

Interpretation & conclusion: The results of the study suggest that the predatory copepod *M. scrassus* can be used as a bio-control agent for the control of *Anopheles* mosquitoes to prevent re-emergence of malaria in the country. Additional research is suggested to identify naturally available copepod species and their predatory efficacy.

Key words *Anopheles*; biological control; copepods; malaria vectors; predatory efficacy; Sri Lanka

INTRODUCTION

Malaria is the most important health issue in the world manifesting very high morbidity and mortality. There were an estimated 228 million cases and 405,000 deaths[1] in the year 2018. Further there are almost 80 countries with ongoing malaria transmission, and the children <5 yrs is most vulnerable group[1]. Although, Sri Lanka has been declared as a malaria-free country on 5th September 2016, by the WHO (since no indigenous cases were reported for over three years from November 2012); considerable number of imported malaria cases were recorded from some areas in the country[2]. Imported malaria cases may increase the risk for local malaria transmission due to the persistence of malaria vectors in the country[2–4].

Anopheles culicifacies s.l. (Diptera: Culicidae) is well-established as the major vector of both falciparum and vivax malaria in Sri Lanka[5]. Recently, several studies have reported natural *Plasmodium* infections in 11 other anopheline species in addition to *An. culicifacies* using the enzyme-linked immunosorbent assay (ELISA) and sporozoite dissection methods[6]. These include *An. aconitus*, *An. annularis*, *An. barbirostris*, *An. nigerrimus*, *An. pallidus*, *An. subpictus*, *An. tessellatus*, *An. vagus* and *An. varuna*[7–8]. Species that have consistently been incriminated among these are *An. annualaris*, *An. subpictus*, *An. varuna* and *An. tessellatus*[9]. When considering secondary vectors, *An. subpictus* is considered the most efficient secondary vector in Sri Lanka. In addition *An. annularis*, *An. varuna* and *An. tessellatus* are also reported and incriminated as important secondary vectors.

Treatment with antimalaria drugs and vector control are integrated approaches for malaria prevention. However, the use of antimalarial drugs over a long period has contributed to the development of drug-resistant malarial parasites in endemic areas[10]. Thus, vector control has played an important role in the substantial reduction of malaria[11–12]. Different methods of vector control have been used throughout history, which include chemical and biological methods[10]. Continuous use of chemicals for mosquito control can affect non-target populations, increase environmental pollution, develop resistance in mosquitoes and ill effects to humans and other biota[13]; however, they are safe if used at the recommended dosages. Therefore, to overcome these problems emphasis has been placed recently on eco-friendly and economically viable methodologies for vector control[14].

Biological methods involve the use of natural ene-

mies and bio-toxins to eradicate mosquito vectors. Many organisms have or are being evaluated as potential biological control agents against anopheline mosquito larvae. A few of these agents have been used to control mosquitoes for many years. The WHO has used larvivorous fish, parasitic nematodes, beetle larvae, certain fly larvae, aquatic bugs, dragonfly, damselfly and cyclopoid copepods, in many parts of the world since the 1940's[10,15–16]. The bacteria *Bacillus thuringiensis israelensis* (*Bti*) has been in the market for several years and is one of the most successful biological control agents currently used[17]. *Bacillus sphaericus* is also used either alone or in combination with *Bti*.

Cyclopoid copepods are predatory crustaceans[14]. The potential of copepods for mosquito control was first recognized by Riviere and Thirel[18] in a study carried out in Tahiti. They observed that the number of *Aedes aegypti* and *Ae. polynesiensis* larvae greatly reduced in ovitraps that contained *Mesocyclops aspericornis,* accidentally introduced with creek water. Similar observations were reported independently by Marten[19], with *M. aspericornis* for *Ae. albopictus* larvae in artificial containers in Hawaii. Since then, copepods have proved to be particularly effective at eliminating *Aedes* sp. production from water storage tanks and other container breeding habitats that have water for extended periods. In fact, the use of copepods in *Aedes* container habitats has been responsible for virtually all published instances of mosquito eradication in recent years[16, 20]. However, the use of copepods to control anopheline larvae has not been sufficiently tested in Sri Lanka. Therefore, the present study focused on identifying effective biological control agents for anopheline larvae using carnivorous copepods under laboratory and field conditions to prevent re-introduction of malaria in the country.

MATERIAL & METHODS

Establishment of Anopheles tessellatus colony

Eggs of *An. tessellatus* were obtained from the existing insectary at the Anti Malaria Campaign, Narahenpita, Sri Lanka and kept for one generation. Adults were housed in adult mosquito rearing cages ($24 \times 24 \times 24$) cm^3 with mesh screening on top and were fed 10% sugar solution using cotton wool saturated with water. Mosquitoes were starved of sucrose solution for 24 h and allowed to feed on cattle-blood using an artificial membrane feeding system[21]. Females were held for two days at 27 ± 2 °C, 75 ± 5% relative humidity (RH) and a 12:12 (L:D) photoperiod.

After two days, the females were placed in screen topped oviposition plastic cups (9.7 cm in diameter and 11.5 cm in depth) with the inner-side lined with cotton on which the eggs were laid, and filled with approximately 150 ml of filtered dechlorinated tap water. The eggs hatched within 2–3 days after oviposition and the I instar larvae were transferred daily from the oviposition cups to plastic trays ($40 \times 25 \times 6$ cm), containing 2000 ml of water. One batch of immature stage of mosquitoes was reared to the IV instar (larvae in 500 ml plastic cups with a larval diet containing 50% tuna meal, 36% bovine liver powder, and 14% yeast[22] to maintain mosquito colony. Another batch of I instar larvae was taken for the predatory studies.

Field collection of copepods

Copepods were collected from ponds, ditches and other standing water sources to establish a culture. They were collected by quickly dipping a standard mosquito dipper (1000 ml capacity) in water, particularly near submerged vegetation. After dipping, 2/3 of water in the container was slowly filtered through a zooplankton net (100 μm). The net was inverted over a container with clean dechlorinated water and the filtrate with copepods was washed using a squirt bottle into the container.

Identification of field collected copepods in the laboratory

A few individual live copepods were preserved from different sites with 70% alcohol. Dead copepods were placed individually on the microscopic glass slide with a minimum amount of water and identified under a light microscope (Olympus Optical Co. Ltd., Tokyo) with an objective (10×) using interactive keys[23–25].

Maintaining copepod cultures in the laboratory

Copepod cultures were initiated using a single gravid female to assure consistency once an appropriate species was identified. Gravid females were recognized by the presence of their eggs externally on both the sides of the body. To establish pure cultures, an individual gravid female was picked up with a pipette and transferred to a shallow dish with de-chlorinated water. This was subsequently transferred again to a culture container filled ¾ with 500 ml of dechlorinated water. This minimized the chances of unintentionally introducing more than one species into the culture containers. The containers were monitored continuously for 2–3 weeks to determine the growth of the copepod population. The copepods were fed with a *Paramecium* culture and wheat grain when necessary. The cultures were kept open during day-time to receive light for their development, but were not exposed to sunlight directly.

Maintaining of paramecium culture

About 25 ml of a start paramecium culture was introduced to a one gallon jar containing spring water. Approximately 12 g of wheat flour per gallon of culture and a small pinch of yeast was added. The culture was kept for two weeks at room temperature before using to feed the copepods.

Identification of predatory efficacy of copepods in the laboratory

Predatory efficacy of copepod on *An. tessellatus* larvae was assessed under laboratory conditions. About 100 newly hatched mosquito larvae were placed in a 100–200 ml tissue culture plate wells (35 mm diameter, 18 mm depth). The containers were filled with dechlorinated water and 10 adult copepods were introduced. Different species of copepods were introduced to separate tissue culture plates . The larvae remaining in each container and dead larvae were observed at 3 h intervals until 24 h at 27 °C, under constant artificial lighting conditions. Finally the numbers of dead *An. tessellatus* larvae were counted using a magnifying glass (10×) every 24 h for 3 days after which the number of larvae consumed was replenished in each of the container to maintain the prey density for another interval. Predatory efficiency of a copepod was calculated by the following formula[14].

$$\text{Predatory efficiency} = \frac{\text{No. of prey/No. of predator introduced}}{\text{Total no. of prey introduced}} \times 100$$

This experiment was repeated 10 times to identify the best candidate for effective larval control.

Identification of the predatory efficacy of copepods in the field

Selection of study area: The identification of predatory efficacy of carnivorous copepods in the field was carried out in the District of Kandy, Sri Lanka. District Kandy was selected due to the presence of a large number of river bed rock pools along the Mahaweli river with high prevalence of anopheline mosquitoes including *An. culicifacies*[26–27]. Therefore, rock pools located along the Mahaweli river bed in Theldeniya area (below the Kotmale dam) (7°4' 30.46"N, 80°34' 32.28" E) were selected for intervention with copepods.

Pre-intervention: Each breeding pool in the Theldeniya area was monitored from April to June 2015 as a preliminary survey for mosquito larvae. Anopheline larvae were collected from each rock pool by a standard dipper. The same procedure was repeated for three times in each pool. From each breeding pool, approximately 5 dips were collected depending on the size of the breeding pools. The volume of single dip was around 200–250 ml. The number of dips and anopheline positive dips were recorded. Among the positive dips, number of different larval stages (I, II, III, IV) were recorded.

Collected anopheline mosquito larvae were reared until the adults emerged. Emerged adults were identified to the species level by using achromatic magnifying lens (10×) following the taxonomic keys prepared by Carter [28] and Amerasinghe[29].

Intervention: A total of 20 adults of three copepod species, namely M. scrassus, C. varicans and C. languides, which proved to be effective anopheline mosquito controlling agents in the laboratory, were introduced separately into nine rock pools with 10 gravid females of each species for one pool. Three rock pools were maintained as control without intervention with copepods.

Post-intervention: Survival of the introduced copepods in the respective rock pools was evaluated (in terms of number) before and after 24, 48 and 72 h. The abundance of the anopheline mosquito larvae in each rock pool was monitored after the introduction of copepods once a fortnight. For collection of mosquito larvae, water samples were systematically taken by dipping every rock pool (3 scoops/rock pool). From the collected water samples anopheline mosquito larval counts were made. Water samples were (5 dips/rock pool) poured away slowly through a zooplankton net and the copepods retained on the mesh were counted. Counted copepods and mosquito larvae were re-introduced into the respective breeding pools. The percentage of larval reduction was calculated by the following formula[14].

$$\text{Percentage reduction} = 100 - (C_1/T_1) \times (T_2/C_2) \times 100$$

where, C_1 and C_2 are the pre- and post-treatment densities of mosquito larvae in the control group and T_1 and T_2 are density of mosquito larvae in pre- and post-treatment in treated habitats.

Data analysis

The numbers of mosquito larvae predated by the copepods were analyzed with one-way ANOVA to evaluate the predatory efficacy of copepod species, and the most effective copepod species were identified. The significant differences between pre- and post-intervention by the copepod species in the field conditions were analyzed using the general linear modelling of SPSS software (ver. 15).

Ethical statement

Ethical clearance (No. P92/09/2014) to conduct the

study was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka.

RESULTS

Field collection of copepods

The copepod species, namely *M. scrassus*, *C. varicans* and *C. languides* were collected from streams, lakes, small ponds and rivers. The species collected and their locations are given in Table 1.

Predatory efficacy of copepods in the laboratory

Predatory efficacy of *M. scrassus*, *C. varicans* and *C. languides* was tested in the laboratory. All the three copepod species consumed the anopheline larvae, but at different rates. *Mesocyclops scrassus* showed the highest predacious efficiency on I instar larvae of *An. tessellatus* within 24 h while *C. varicans*, and *C. languides* showed the lowest (Table 2).

Predatory efficacy of copepods under field conditions

Pre-intervention: During the preliminary survey 808 anopheline mosquito larvae were collected from 1560 dips comprising 10 species from the rock pools of the study area (Table 3). The majority consisted of *An. maculatus* (38.61%), and the rest were *An. karwari* (29.70%) and *An. vagus* (12.50%) larvae.

Table 1. Collected copepod species, locations and GPS points

Copepod species	Location	GPS points
M. scrassus	Attanagalu Oya, Meerigama	7° 9'12.25"N, 80°10'27.89"E
C. varicans	Kurunegala lake, Kurunegala	7°29'28.52"N, 80°21'51.14"E
	Wendaru lake, Kurunegala	7°27'37.67"N, 80°22'27.07"E
	Below the Kotmale dam, Mahawali river, Kandy	7° 4'30.46"N, 80°34'32.28"E
C. languides	Between Victoria dam and power station, Mahawali river, Kandy	7°12'7.56"N, 80°48'19.37"E

Table 2. Predatory efficacy of three copepod species on the larvae of malarial vector *An. tessellatus*

Species	No. of copepod introduced	Duration			Predation (%)	Predatory efficiency of a single copepod/day
		Day 1	Day 2	Day 3		
M. scrassus	10	53±2.3	49±1.7	42±2.1	49±5.4[a]	4.9
C. varicans	10	48±2.1	37±1.6	35±1.3	41±5.1[b]	4.1
C. languides	10	46±2.2	23±1.4	39±1.4	38±4.8[c]	3.8

Different superscript letters in column show significant differences (p <0.05), calculated using Tukey's pairwise tests after one-way ANOVA.

Table 3. The abundance of anopheline species breeding in the river bed pools in Theldeniya area below the Kotmale dam (study site) in District Kandy

Anopheline species	Number of larvae
An. maculatus	312 (38.61)
An. karwari	240 (29.70)
An. vagus	101 (12.50)
An. jamesii	52 (6.43)
An. barbirostris	18 (2.22)
An. peditaeniatus	44 (5.44)
An. nigerrimus	16 (1.98)
An. elegans	3 (0.37)
An. culicifacies	0 (0)
An. varuna	2 (0.24)
An. pseudojamesii	20 (2.47)
Total	808 (100)

Table 4. The predatory efficacy of three copepod species on larvae of *Anopheles* mosquitoes under field conditions

Species	Mean No. of anopheline larvae				Percentage reduction
	Pre-intervention	Post-intervention			
		Day 1	Day 2	Day 3	
Control	54±2.8	52±2.6	48±2.4	46±2.3	–
M. scrassus	56±2.6	34±2.8	22±1.2	9±06	81.22[b]
C. varicans	64±2.9	42±2.1	29±1.6	18±0.9	67.13[c]
C. languides	59±3.1	41±1.8	34±1.8	25±0.8	50.32[d]

Different superscript letters in a column show significant differences (p <0.05), calculated using tukey's pairwise tests after one-way ANOVA.

Post-intervention through copepods: The highest *Anopheles* larvae count was reported in control rock pools. The abundance of anopheline larvae differed significantly (p <0.05 at 95% level of confidence) among the control and intervention sites as indicated by the results of general linear model (Table 4). Prior to the intervention larval population of *Anopheles* mosquito were higher in all the study sites. The breeding capacity and survival rate were high for all the three copepods species at all study sites.

Rock pools with *M. scrassus* indicated the lowest abundance of mosquito larvae. Therefore, it can be suggested that, *M. scrassus* has the highest degree of predatory efficacy. Meanwhile, *C. languides* indicated the lowest predatory efficacy among the studied copepod species.

DISCUSSION

The present study investigated the effectiveness of three locally available species of copepods, namely *M. scrassus*, *C. varicans* and *C. languides*, collected from

different areas in the country as biological control agents of *Anopheles* vectors. *Mesocyclops scrassus* was found in Meerigama area in the District of Gampaha, *C. varicans* are common species in the districts of Kurunegala and Kandy and *C. languides* was recorded only from the district of Kandy. The three predatory copepod species showed significant effects against the larvae of the malaria vector, in both the laboratory and field conditions.

Earlier studies have reported *Mesocyclops* sp. such as, *M. thermocyclopoides*[30], *M. aspericornis*[31], *M. albidus*[32], *M. longisetus* and *M. albidus*[33], as efficient control agents of mosquito larvae. *Mesocyclops* is both a predator and competitor for mosquito larvae. Ramanibai and Kanniga[34], reported that *Mesocyclops* are good biological control agents against *Aedes* mosquito larvae under laboratory conditions. In their study the survival rate of *M. ruttneri* and *M. edax* was inferior, but their predatory efficacy was high in the field. Some species such as *Diacyclops navus* was a weak predator while showing a high survival rate. Further, *M. annulatus* effectively preyed on larvae of *Aedes* and *Culex* larvae.

In the present study also, it was observed that *M. scrassus* is a more effective predator than the other two species under laboratory and field conditions. Copepods are successful predators of I and II instar mosquito larvae than the III and IV instars. Due to the swimming behaviour and small size of the copepods, the predatory capacity on III and IV instar mosquito larvae was almost zero.

However, the presence of alternative food sources in breeding habitats, reduce the predation effectiveness of copepods[12]. Therefore, under field conditions, many factors need to be evaluated, such as survival rates, predatory efficacy, physico-chemical parameters of water, reproductive capacity and food preferences[12].

Anopheles culicifacies, which is considered as the primary vector of malaria in Sri Lanka, breeds in natural streams and rivers, especially in rock pools that are created during dry periods, or during high rainfall periods along previously dried stream and river-beds3[4]. Although earlier studies have recommended introducing larvivorous fish into rock pools as an effective biological controlling agent for *Anopheles* mosquito control[27–35], limitations in the size and depth of such breeding places often restrict the survival of the introduced fish. Therefore, *Mesocyclops* that can easily survive even in the small rock pools, could be introduced to control anopheline mosquito larvae.

Although, use of copepods to control anopheline larvae has not been sufficiently tested in Sri Lanka, studies from other countries have shown its effectiveness in control of malaria vector. Among them, Roe *et al*[36] have investigated the abundances of cyclopoid species and the malaria vector *An. aquasalis* to certain abiotic parameters and vegetation features in Venezuela. They observed that the abundances of *M. meridianus* and *An. aquasalis* larvae were negatively correlated each other. Confirming the copepods predate on anopheline larvae Pernia *et al*[37] reported that *M. meridianus* preys on I and II instar larvae of *An. aquasalis*.

Use of a mixture of copepods species gave poor results and best results can be obtained by introducing a single species[31]. Some species are specific in some areas and particular breeding habitats. Therefore, the key factors which should be considered before use of copepods for mosquito control are copepod species, area, habitat type, how long the population lasts in that kind of container and the number of copepods in the container. Use of synthetic chemicals for mosquito control, increases concomitant damage to the environment. Biological control of mosquito larvae is more efficient and eco-friendly than chemicals such as insecticides[38].

CONCLUSION

The study findings indicate that copepods can be employed for biological control of anopheline larvae. Among the three copepod species which were used to evaluate predatory efficacy on anopheline mosquito larvae, *M. scrassus* showed the highest predacious ability under laboratory and field conditions. Thus, fish and copepods could be used as a complementary agent in an integrated mosquito control programme.

It is crucial to identify native/indigenous copepod species that thrive under the conditions prevalent in mosquito breeding sites in different parts of country for use in the control of mosquitoes. Anopheline mosquito control using copepods should be used in addition to other strategies already in places.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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