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SCIENTIFIC NOTE

SENSITIVITY OF THE LARVIVOROUS COPEPOD SPECIES, *MESOCYCLOPS PEHPEIENSIS* AND *MEGACYCLOPS VIRIDIS*, TO THE INSECT GROWTH REGULATOR, PYRIPROXYFEN

SHANQING WANG,[1,2] TRAN VU PHONG,[1] NOBUKO TUNO,[1,3] HITOSHI KAWADA[1] AND MASAHIRO TAKAGI[1]

ABSTRACT. The effects of the insect growth regulator pyriproxyfen were evaluated on the mortality, fecundity, longevity, and predation capability of 2 species of copepods, *Mesocyclops pehpeiensis* Hu and *Megacyclops viridis* (Jurine), under laboratory conditions. Pyriproxyfen showed no significant effects on either the development or reproduction of *M. pehpeiensis* at 0.1 ppm, which is a 10-fold greater concentration than the reported effective dosage for controlling mosquito larvae (0.01 ppm). In contrast, the development of *M. viridis* was impaired by pyriproxyfen at 0.1 ppm. An 80% reduction in nauplius survivorship was observed in the experimental (treated) group compared with the control group. Although the application of pyriproxyfen caused high mortality in the nauplius stage of this species, the pyriproxyfen-treated group developed faster, killed more mosquito larvae, yielded more eggs per oviposition event, and survived longer than the control group. These results indicate that pyriproxyfen caused mortality in the earlier stages of this sensitive species but that the surviving individuals were those that were selected for significantly faster development, better predation ability, and greater longevity during their reproductive stage. Therefore, under natural conditions, pyriproxyfen would cause modifications in the characteristics of a copepod population rather than its complete loss. Our results suggest that the combined application of copepods and pyriproxyfen to control *Aedes* populations is feasible. However, repeated application of pyriproxyfen may cause changes in copepod populations and communities in the affected ecosystem.

KEY WORDS Biocontrol, copepod, insect growth regulator, *Aedes*, dengue virus vector

For the past 2 decades, cyclopoid copepods have been successfully used to control the aquatic larval stages of *Aedes* mosquitoes, which are vectors of dengue virus (Riviere et al. 1987, Marten et al. 1994, Tietze et al. 1994, Nam et al. 1998, Schaper 1999, Kay et al. 2002). Copepods have an advantage as biologic control agents in that they continue to reproduce in situ, and therefore continue to be effective for as long as the environmental conditions allow. They selectively predate younger mosquito larvae, thus gradually reducing the population of mosquito larvae after their introduction (Marten et al. 1993). It is therefore desirable for copepods to be able to survive and function in conjunction with other mosquito control measures such as insecticides, which work to instantly reduce mosquito populations.

Pyriproxyfen, 4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy) propyl ether, is a photostable insect growth regulator that is effective against the immature stages of mosquitoes, flies, cockroaches, and fleas (Hirano et al. 1998, Wilson 2004). Pyriproxyfen has detrimental effects on the growth and reproduction of the crustacean *Daphnia* spp. (Trayler and Davis 1996), but these negative effects have not been noted in the copepod *Cyclops* spp. (Hirano et al. 1998). Bircher and Ruber (1988) reported that the nauplii of the copepod *Apocyclops spartinus* were more sensitive to the insect growth regulator methoprene than were adults. Marten et al. (1993) also studied the sensitivity to methoprene of 4 copepod species from 3 genera (*Acanthocyclops, Macrocyclops,* and *Mesocyclops*) and concluded that methoprene is probably compatible with cyclopoids as long as it is not applied at concentrations above the label rate because cyclopoids are less sensitive than mosquitoes. Schaefer and colleagues (Schaefer et al. 1988, Schaefer and Miura 1990) reported no significant effects on mixed populations of cladocerans and copepods treated with 0.01 ppm pyriproxyfen during a 2-week test period. However, a minor suppression of the reproductive capacity of daphnid cladocerans and ostracods was observed when pyriproxyfen was applied twice at high rates (8 and 20 times greater than the effective dosage, respectively) to experimental rice plots.

In this study, we assessed the effects of pyriproxyfen on 2 copepod species to evaluate the feasibility of their combined application for integrated pest management focusing on the most effective, least-risk option including natural, biologic, cultural, and chemical methods. We evaluated the effects of pyriproxyfen on the mortality, fecundity, longevity, and predation capability of 2 species, *Mesocyclops pehpeiensis* Hu and *Megacyclops viridis* (Jurine), under laboratory conditions.

[1] Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan.
[2] Hainan Provincial Institute of Tropical Diseases, Haikou 570203, China.
[3] To whom correspondence should be addressed.

MATERIALS AND METHODS

Copepods

Colonies of *M. pehpeiensis* and *M. viridis* were established from natural populations occurring in rice fields in Nagasaki Prefecture, southwestern Japan. The laboratory colony was fed with laboratory-cultured *Paramecium* infused with tiny pieces (ca. 3 mm^2) of commercial sea algae (nori, a common Japanese food) at temperatures of 25–28°C and relative humidities of 60–80%. Details are described in the study by Dieng et al. (2003).

Prey mosquitoes

Aedes albopictus (Skuse), our laboratory strain of mosquitoes, were used to evaluate the predation efficiency of the copepods. The mosquitoes were originally collected in Nagasaki in 1998 and were subsequently bred in the laboratory at temperatures of 25–28°C and relative humidities of 60–80%. First instars were used as prey in this study.

Effects of pyriproxyfen on immature copepods

The effects of pyriproxyfen on the immature stages, mortality, and development period of the 2 species of copepods were studied. Copepod females bearing pairs of egg sacs were isolated from the laboratory colonies in 100-ml plastic cups containing 50 ml of water and a sufficient food supply. Ethanol was used as the solvent to achieve dilution in water. Pyriproxyfen was added to each container for a final concentration of 0.1 ppm, a concentration 10-fold higher than the reported effective dosage for controlling mosquito larvae. Preliminary experimentation has proved that our colony of *Paramecium* are nonsensitive to pyriproxyfen at 0.1 ppm, which predicted the same quality of the food supply among treatment and control groups. In the control group, an equivalent volume of ethanol was added to the water (0.007%). Isolated females were removed from each container after they had dropped their egg sacs. As a result, 1 pair of egg sacs (a brood) was left in each container, and their development was monitored. The emergence of larvae was examined daily under a stereomicroscope. The presence of at least 1 live individual was recorded as positive, and was noted per brood as nauplius-0/1 or copepodite-0/1. Eclosed adults were removed from the container and were sexed and counted. Monitoring continued for 14 days after the 1st adult eclosion was observed in each brood. The development period of each stage (nauplius, copepodite, and adult) was represented by the minimum period (in days) per brood. It has been speculated that pyriproxyfen adsorbs onto organic matter (Schaefer et al. 1987), which may cause unexpected heterogeneity of its concentration among replications. To maintain the test concentration of pyriproxyfen, the liquid in each container was renewed every 5 days.

Effects of pyriproxyfen on the reproductive stage

The effects of pyriproxyfen on copepod fecundity (frequency of egg-bearing event through life span, and number of eggs per egg-bearing event from the 1st 4 events), longevity (days from 1st egg-bearing event to death), and predation ability (mean number of mosquito larvae killed within 10 days after the 1st egg-bearing) were evaluated. Offspring born by 18 females of each copepod species were mass bred in a tray containing ca. 2.4 liters of rearing solution, with and without ethanol-dissolved pyriproxyfen (0.1 ppm). This density of copepods was about 5% of the laboratory-maintained supply, and was far from the saturated population that may yield a density-dependent effect. The control group was held in the same concentrations of ethanol as the experimental group.

Newly matured females (identified as egg-bearing individuals) were isolated to record their productivity until death. All copepods were individually reared in 100-ml plastic cups filled with 50 ml of rearing water containing a *Paramecium*-algae infusion; they were fed daily with 2 ml of the infusion. Pyriproxyfen (0.1 ppm) or ethanol solvent was added to the rearing water to replicate the treatments received by their mothers. The rearing solution was renewed every 5 days to maintain the test concentrations. Copepods were observed twice a day to record egg-bearing events. Half of the copepods were supplied daily with 20 1st-stage mosquito larvae, and predation ability was defined as the number of *Ae. albopictus* larvae killed. The presence of larval head capsules or mangled dead bodies was considered evidence of predation by copepods. The control group was treated in the same manner. Each treatment was repeated in more than 9 females.

Females (n = 122) were initially isolated to count the number of eggs per egg batch for the 1st 4 egg-bearing events. The pair of egg sacs was carefully dissected under a stereomicroscope, and the number of eggs was counted under a microscope.

Data analysis

Binary (presence/absence) data on developing stages (egg, nauplius, copepodite, adult) were totaled and were compared among treated and control groups for the 2 copepod species using Fisher's exact 2-tailed test. The number of emerged adults and the sex ratio of each brood of the treated and control groups was compared using Mann-Whitney U-tests for the 2 species (the probabilities were sequentially Bonferroni corrected). Longevity and the number of egg-bearing events were compared among groups using multivariate analysis of vari-

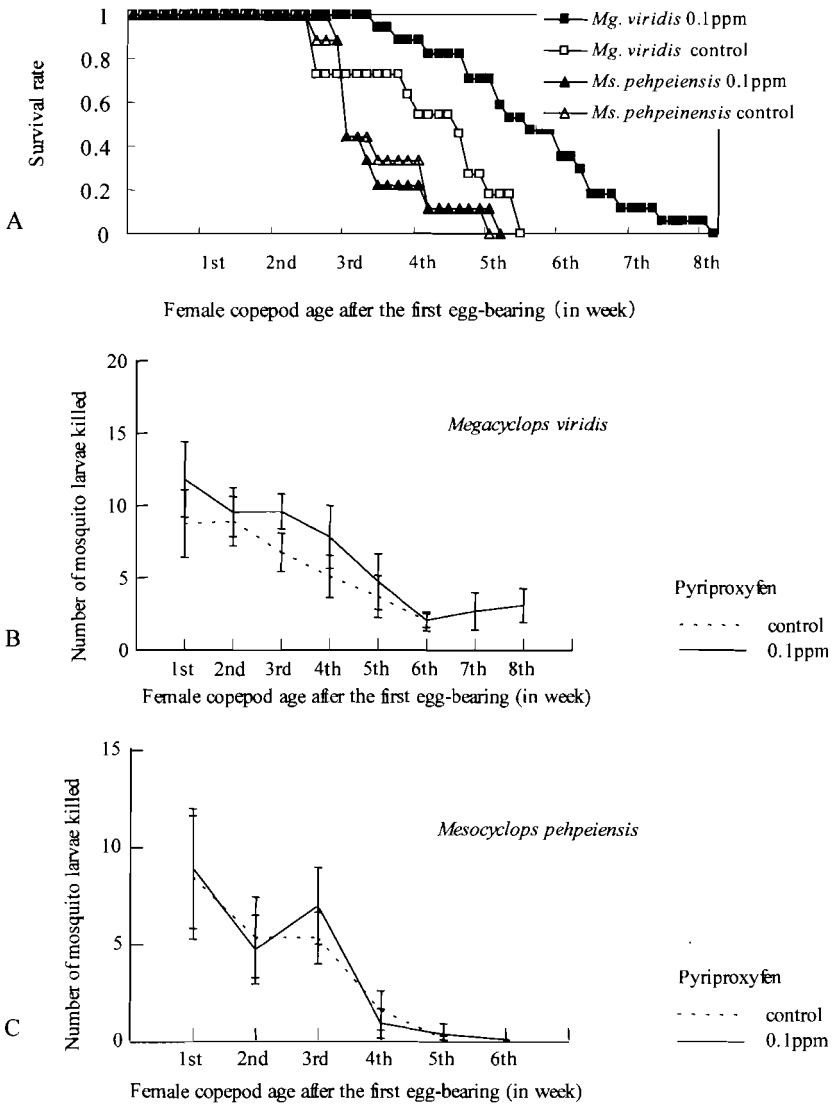

Fig. 1. (A) Proportion of surviving copepods (in weeks) that was isolated to measure predation efficiency. (B, C) Individual predation efficiency in relation to predator age and pyriproxyfen application. The *y* axis shows the mean number of *Aedes albopictus* larvae killed by female *M. viridis* (B) and *M. pehpeiensis* (C) of 20 larvae that were supplied daily. The *x* axis shows the age of copepods (in weeks) after the 1st egg-bearing event.

ance (ANOVA). Predation ability was compared using ANOVA. The mosquito larvae-supplied groups were provided with 20 larvae daily throughout their life span. Both species showed some degree of reduction in their predation activities with increasing age (Fig. 1). In order to exclude these aging effects, only data from the 1st 10 days of life of each copepod were analyzed. The data on the number of eggs per brood of the 1st to 4th egg-bearing events were compared using a 2-way ANOVA (pyriproxyfen presence and absence and order of egg-bearing event). The purpose of our analysis was to examine the effect of pyriproxyfen within the respective copepod species, not to compare among species. When significant differences were detected by the MANOVA or ANOVA, a posteriori Tukey-Kramer HSD tests (Hayter 1984) were applied to determine which means were significantly different ($P < 0.05$). All statistical analyses were performed using JMP® software version 5.0 (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

There were no differences in mortality during the developing stages, number of emerged adults, or sex ratio of each brood among treated and control groups of *M. pehpeiensis* (Table 1). In contrast, we

Table 1. Effects of pyriproxyfen on developing-stage survival of 2 copepod species.[1]

	M. pehpeiensis					*M. viridis*				
	Control		Pyriproxyfen (0.1 ppm)			Control		Pyriproxyfen (0.1 ppm)		
	Survival ratio	No. of brood	Survival ratio	No. of brood	P*	Survival ratio	No. of brood	Survival ratio	No. of brood	P*
Survival ratio	1	20	0.8	20	NS	0.95	18	0.17	30	<0.001
Developing stage	Mean ± SD	No. of brood	Mean ± SD	No. of brood	P**	Mean ± SD	No. of brood	Mean ± SD	No. of brood	P**
Nauplius	1	20	1	20		1	20	0.2	30	
Copepodite	1	20	0.8	16		0.95	19	0.83	6	
Adult	1	20	1	16		0.95	18	1	5	
No. of eclosed adults	32.2 ± 10.6	20	36.8 ± 18.9	16	NS	7.7 ± 4.4	18	3.0 ± 1.9	5	<0.05
Female ratio	0.64 ± 0.15	20	0.65 ± 0.14	16	NS	0.61 ± 0.12	18	0.34 ± 0.34	5	NS

* Fisher's exact test probability (2-tailed).
** Mann–Whitney *U*-test probability.
[1] NS, not significant; $P > 0.05$.

observed high mortality in *M. viridis* during the egg-hatching-nauplius stage in the treated group (Table 1). Survivorship was reduced from 100% ($n = 20$) in the control group to 20% ($n = 30$) in the treated group ($P < 0.001$; Table 1). There were no significant differences among the groups in either the later developing stages or in sex ratios, although the number of emerged adults in the treatment group was reduced to 39% of the control group ($P < 0.05$; Table 1). The minimum developmental duration of *M. pehpeinsis* from egg to 1st egg-bearing event was 14.7 ± 3.5 days for the control group and 13.7 ± 2.0 days for the treated group, showing no statistical difference ($F = 0.84$, $P = 0.475$; Table 2). The duration of *M. viridis* was 16.2 ± 3.0 days for the control group and 13.0 ± 0.8 days for the treated group (Table 2). We observed a specific effect again, i.e., a shorter development time, in treated *M. viridis* ($F = 15.23$, $P < 0.001$; Table 2).

We evaluated the effects of pyriproxyfen on copepod reproduction in those individuals that survived through their developing stages. The longevity and the frequency of egg-bearing events were 30.4 ± 6.0 days (treated) and 31.2 ± 6.3 days (control), and 4.3 ± 0.9 times (treated) and 4.2 ± 1.3 times, respectively, with a supply of mosquito larvae; the longevity and the frequency of egg-bearing events were 35.1 ± 8.4 days (treated) and 34.1 ± 6.4 days (control), and 3.6 ± 0.9 times (treated) and 3.2 ± 0.7 times, respectively, without a supply of mosquito larvae for *M. pehpeiensis* (Table 3). The values for the longevity and the frequency of egg-bearing events for *M. viridis* were 48.0 ± 13.9 days (treated) and 33.3 ± 9.4 days (control), and 9.4 ± 3.0 times (treated) and 8.5 ± 2.7 times, respectively, with a supply of mosquito larvae, and 42.4 ± 14.0 days (treated) and 38.8 ± 11.2 days (control), and 8.4 ± 2.8 times (treated) and 9.6 ± 2.4 times, respectively, without a supply of mosquito larvae (Table 3). The longevity and the frequency of egg-bearing events did not differ between the 2 groups of *M. pehpeiensis* with or without larval supply (P of whole model = 0.215; Table 4). There were differences between groups in the longevity and the frequency of egg-bearing events of *M. viridis* (P of whole model = 0.042, P of pyriproxyfen effect = 0.019; Table 4). The treated group of the copepod species showed a longer life span than the control group (q ratio = 2.00, $P < 0.05$) with no difference of the frequency of egg-bearing events. The predatory ability of *M. pehpeiensis* did not differ between treated and control groups ($\chi^2 = 1.18$, $P > 0.05$; Table 4), whereas the treated group of *M. viridis* killed more mosquito larvae than did the control group ($\chi^2 = 6.84$, $P = 0.009$; Table 4).

The number of eggs in the 1st 4 egg-bearing events was examined and compared by 2-way ANOVA for 14 or 16 individuals of the 2 species in the treated and control groups, and in the 1st to 4th egg-bearing events. There were no significant ef-

Table 2. Effects of pyriproxyfen on development duration (days) of 2 copepod species.

	M. pehpeiens						*M. viridis*					
	Control			Pyriproxyfen (0.1 ppm)			Control			Pyriproxyfen (0.1 ppm)		
	Mean	SD	No.	Mean	SD	No.	Mean	SD	No.	Mean	SD	No.
Minimum duration from egg per brood												
Copepodite	4.0	1.0	20	4.0	1.3	16	4.6	0.6	19	4.0	0	4
Adult	10.0	1.7	20	9.8	1.7	16	10.6	2.7	18	8.0	0.8	4
1st egg bearing	14.7	3.5	20	13.7	2.0	15	16.2	3.0	18	13.0	0.8	4
ANOVA of pyriproxyfen effect (development stage nested)												
df	3, 103						3, 64					
F	0.84						15.23					
P	0.475						<0.001					

fects of pyriproxyfen on *M. pehpeiensis* fecundity ($F = 2.595$, $P = 0.110$). The treated group of *M. viridis* yielded more eggs than did the control group ($F = 8.037$, $P = 0.005$). Both species produced significantly different numbers of eggs between the 1st and 4th egg-bearing events ($F = 5.475$, $P = 0.001$ for *M. pehpeiensis;* $F = 4.699$, $P = 0.004$ for *M. viridis*). Although the reasons for these differences are unknown, they may have been the result of differences in food quality. We fed the copepods with the same *Paramecium* culture throughout the experiment; however, the density of the protozoa fluctuated to some degree. There was no significant cross-effect of pyriproxyfen and the order of the egg-bearing event for either species ($F = 0.143$, $P = 0.934$ for *M. pehpeiensis;* $F = 0.831$, $P = 0.480$ for *M. viridis*).

Our laboratory experiments showed that pyriproxyfen had no significant effects on the development and reproduction of *M. pehpeiensis* at concentrations of 0.1 ppm. In contrast, *M. viridis* was significantly affected by pyriproxyfen during the developmental stage, as evidenced by the 80% reduction in nauplii survivorship over the control group. The age of 1st egg-bearing event was 3.2 days earlier in the treated group than in the control group. During the reproductive stage, the treated group with a mosquito larval supply killed more mosquito larvae and survived longer than did the control group. Also, the treated group yielded more eggs per egg-bearing event. The reason for these changes is unknown. However, these results indicate that the pyriproxyfen-induced mortality in the earlier stages of development of this species allowed for a greater proportion of selected individuals exhibiting significantly faster development, superior predation ability, and greater longevity among the reproductive stages.

There is an alternate possibility that the survived *M. viridis* enjoyed a better nutritious condition in lower density that was thinned by pyriproxyfen. This is less likely because the rearing condition in our experimental condition was far from saturation. However, the density-dependent effect may be important and may present difficulties in influencing a natural community. Under natural conditions, the application of pyriproxyfen would retard short-term copepod population increases; however, populations would recover from this detrimental effect over time, unless they were driven to extinction. When the populations are saturated under natural conditions, the thinned-out population may also represent density-dependent changes.

In summary, our experiment demonstrated a specific response of copepods to pyriproxyfen. The repeated application of this compound under natural

Table 3. Effects of pyriproxyfen on copepod longevity, frequency of egg-bearing event, and predation ability.

Species	Mosquito larvae[1]	Pyriproxyfen	No.	Longevity[2]		Egg bearing[3]		Predation[4]	
				Mean	SD	Mean	SD	Mean	SD
M. pehpeiens	+	0.1 ppm	9	30.4	6.0	4.3	0.9	6.93	1.20
	+	Control	9	31.2	6.3	4.2	1.3	6.43	0.81
	−	0.1 ppm	9	35.1	8.4	3.6	0.9		
	−	Control	9	34.1	6.4	3.2	0.7		
M. viridis	+	0.1 ppm	17	48.0	13.9	9.4	3.0	10.47	2.27
	+	Control	11	33.3	9.4	8.5	2.7	8.51	1.12
	−	0.1 ppm	14	42.4	14.0	8.4	2.8		
	−	Control	14	38.8	11.2	9.6	2.4		

[1] Reared with or without daily supply of *Ae. albopictus* larvae (20 1st instars).
[2] Days from 1st egg-bearing event to death.
[3] Frequency of egg-bearing event through life span.
[4] Daily averaged number of *Ae. albopictus* larvae killed within 10 days after the 1st egg-bearing event.

Table 4. Summary of multivariate ANOVA and ANOVA for effects of pyriproxyfen in 2 copepod species.

	M. pehpeiensis			*M. virdis*		
	df	F value	*P*	df	F value	*P*
Results of MANOVA on longevity[1] and egg-bearing event[2]						
Whole model	3, 32	1.57	0.215	3, 52	2.9	0.042
Source variables						
Pyriproxyfen				1, 52	5.9	0.019
Mosquito larvae[3]				1, 52	0.0	0.988
Pyriproxyfen × larvae				1, 52	2.9	0.097
Results of ANOVA for effects of pyriproxyfen on predation ability[4]						
	df	χ^2	*P*	df	χ^2	*P*
Pyriproxyfen	1	1.18	NS[5]	1	6.8	0.009

[1] Days from 1st egg-bearing event to death.
[2] Frequency of egg-bearing event through life span.
[3] Reared with or without daily supply of *Ae. albopictus* larvae (20 1st instars).
[4] Daily averaged number of *Ae. albopictus* larvae killed 10 days after the 1st egg-bearing event.
[5] NS, not significant.

conditions would not likely cause the extinction of sensitive copepod species, but it may modify certain characteristics of their population. In our results, these modifications were desirable for the integrated pest management of immature mosquitoes. However, we should caution that any new actions for vector control, not just insecticides, may trigger changes in abundance and composition in the vector community.

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