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SUCCESSFUL ESTABLISHMENT AND SUBSEQUENT RECYCLING OF *ROMANOMERMIS CULICIVORAX* (MERMITHIDAE: NEMATODA) IN A CALIFORNIA RICE FIELD FOLLOWING POSTPARASITE APPLICATION[1]

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Abstract. An early season application of postparasites of the mosquito-parasitic nematode *Romanomermis culicivorax* demonstrated that the nematodes could mature to adults, mate, and lay eggs in the rice field environment. Preparasites hatching from these eggs provided continuous partial control of larval *Anopheles freeborni* and *Culex tarsalis* throughout the rice growing season. Control of *An. freeborni* was comparable to that obtained in the past by direct applications of preparasites and exceeded that achieved previously for *Cx. tarsalis*. The mean weekly infection level for both species exceeded 60%. Infection was observed up to 12 m from the original point of application. *R. culicivorax* successfully overwintered within the rice check and again parasitized mosquitoes the following summer.

Each year in California over 220,000 ha of agricultural land are flooded during a 4-month period (May–August) for the production of rice. The warm, shallow waters provide an ideal breeding habitat for the mosquitoes *Culex tarsalis* Coquillett and *Anopheles freeborni* Aitken. In addition to their roles as major pests of man and livestock, the former is a vector of western equine and St. Louis encephalitis and the latter is probably the most important potential vector of malaria in western North America (Bohart & Washino 1978).

Approximately 10 years ago, Petersen & Willis (1970) began work with a nematode parasite of mosquitoes, *Romanomermis culicivorax* Ross & Smith, which appeared to have promise for use in integrated control programs. Most subsequent field studies (which have been recently reviewed by Poinar 1979) have involved the application of the preparasitic larval stage, even though application of either the preparasitic or postparasitic stage of the nematode has shown promise in mosquito control. Following application of preparasites, the nematode penetrates the cuticle of a susceptible mosquito larva and enters the hemocoel; there it remains for several days, absorbing nutrients from the host and increasing in length from 0.1 to 2 cm. The host dies following egression of the nematode larva. Because of their small size, preparasites can be applied with conventional spray equipment. This provides an economical control program in breeding areas that produce only 1 or a few generations of mosquitoes each year.

In situations such as California rice fields, which produce mosquitoes on a continuous basis during the 4 months that fields are flooded, the application of the larger postparasitic stage offers the potential of achieving continuous partial control of mosquito populations with only 1 application. In favorable habitats this stage burrows into the substrate, molts to the adult stage, mates, and lays eggs that hatch to produce preparasites on a continuous basis beginning ca. 3 weeks after application. The present study was conducted to determine the ability of postparasitic nematodes to become established in California rice fields and the ability of preparasites reared within the rice field to parasitize larvae of *Cx. tarsalis* and *An. freeborni*.

MATERIALS AND METHODS

The nematodes used in this study were obtained from the *R. culicivorax* mass-rearing facility at the University of California at Riverside. Starting cultures for this facility were originally obtained from J. J. Petersen, Gulf Coast Mosquito Research, SEA, USDA, Lake Charles, Louisiana, in 1973 and were reared in a hybrid autogenous strain of *Culex pipiens* L. obtained from A. R. Barr, University of California at Los Angeles. The postparasitic nematodes were shipped by air from Riverside to Davis in a small amount of tap water in polyethylene bags placed inside styrofoam containers. Nematodes were applied to rice fields within 24 h of their arrival in Davis.

A total of 4 separate nematode applications (Fig. 1, Sites A, B, C and D) were made in 1978 in a

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15 × 140-m field in Colusa Co., California, USA, which had a history of producing large populations of mosquito larvae and did not contain a natural population of *R. culicivorax*. Approximately 15,000 nematodes (male : female ratio of ca. 1:1) were used for each application. The postparasites were divided into 10 groups of ca. 1500 nematodes (1 ml) each. The 1st application (Site A) was made 2 weeks (2 June) after flooding of the rice check. Ten wooden stakes were placed at 1-m intervals in a line perpendicular to the long axis of the rice check. A Plexiglas® tube (4 cm diam) that extended through the water into the substrate was placed next to each stake. One group of postparasites was poured into each tube. After allowing 3 min for the nematodes to settle onto the substrate, the tubes were removed. An uninoculated control site was established in an adjacent field. Three additional applications were made in a similar manner during the 5th, 6th and 7th weeks (Sites B–D, respectively) after flooding of the check. Water temperatures within the rice check were monitored weekly with maximum-minimum thermometers. Two methods were used to determine if the postparasites had become established in the field and were producing infective preparasites. These began 3 weeks after the 1st application and continued on a weekly or biweekly basis until the field was drained at the end of August.

In the 1st method, 4 sentinel cages (Case & Washino 1976) (2 containing 20 2nd-instar *Cx. tarsalis* and 2 containing 20 2nd-instar *An. freeborni*) were attached to the stakes at each of the 4 application sites and in the control site. After 72 h all mosquitoes were removed from the sentinel cages, returned to the laboratory, and dissected to determine the level of parasitization. Beginning the 5th week after application, 2 additional sentinel cages (1 with *Cx. tarsalis* and 1 with *An. freeborni* larvae) were placed at distances of 1.5, 3, 6, 12, and 18 m from the point of application at Site A to determine the extent to which parasitization could spread from the initial application point. Levels of parasitization indicate the number of mosquito larvae that would be killed by the postparasite application because infected larvae do not progress to the pupal stage.

The 2nd method utilized to evaluate parasitization was dipping for resident larvae around the 4 application sites using a polyethylene tub (Nagamine et al. 1979). Each site was sampled by 1 operator for 15 min or until 35 resident larvae of each species had been collected. The mosquitoes collected were returned to the laboratory where they were classified as to species and instar and dissected to determine the level of parasitization.

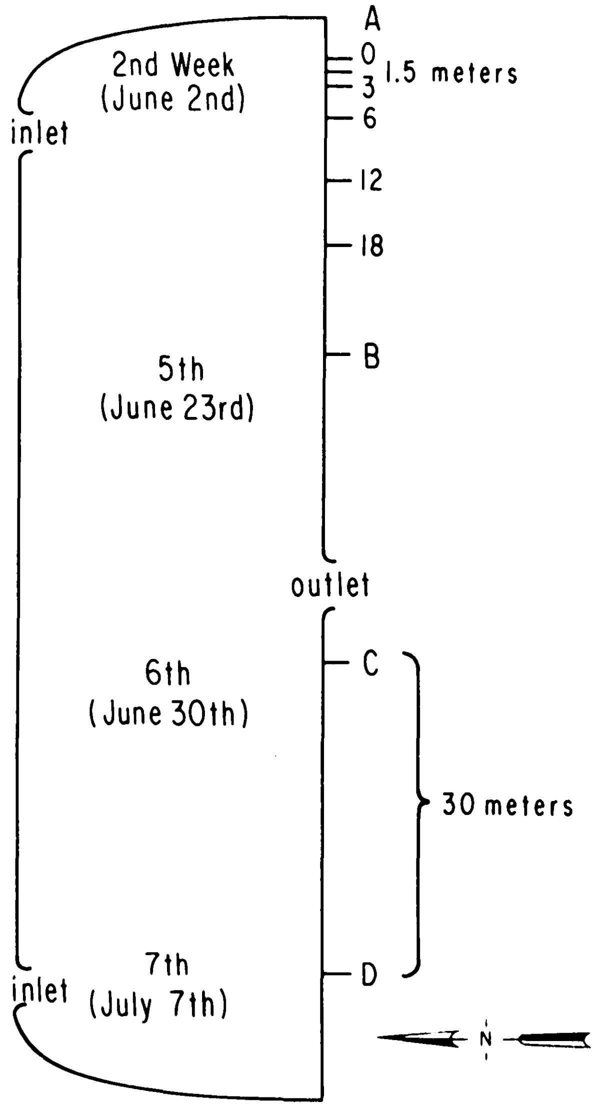

FIG. 1. Diagram of rice field in Colusa Co., California, in which the study was conducted, indicating locations and times of postparasite applications.

After the rice field was reflooded in the spring of 1979, placement of sentinel cages and dipping for resident larvae was resumed at Site A. Two sentinel cages, 1 containing *An. freeborni* and 1 containing *Cx. tarsalis*, were placed at the shoreline and across the field parallel to the initial line of application at distances of 3, 6 and 9 m from shore.

RESULTS

Evidence from both sentinel and dipping samples indicates that the postparasites applied at Site A were more successful at establishing themselves and producing infective preparasites than those at

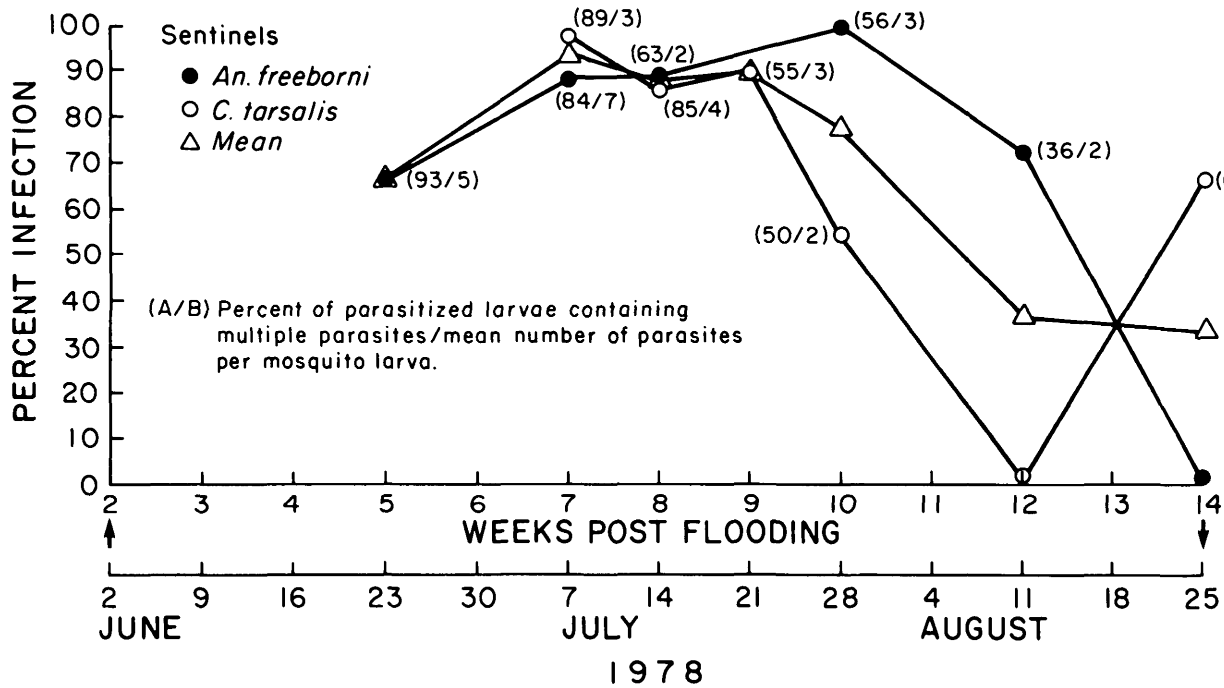

FIG. 2. Levels of parasitization obtained in sentinel cages containing 2nd-instar larvae of *An. freeborni* and *Cx. tarsalis* (2 cages/species with 20 mosquitoes each), and mean for both species at Site A.

Sites B, C and D. Parasitization at Sites B and C never exceeded 5% and no infected mosquitoes were recovered at Site D.

Parasitization in sentinel mosquito larvae placed at Site A beginning 5 weeks after the field was flooded averaged greater than 60% throughout the mosquito breeding season and reached a maximum of 90% (Fig. 2). In general, parasitization in *An. freeborni* tended to be ca. 10% greater than in *Cx. tarsalis* throughout the season. During the season, parasitization in both mosquito species ranged from 0 to 100% and a large proportion of the parasitized mosquitoes contained several *R. culicivorax* larvae.

A similar pattern of infection was seen in the weekly dipping samples taken at Site A (Fig. 3). Levels of parasitization for *An. freeborni* ranged from 2 to 100% and for *Cx. tarsalis* from 7 to 65%, with partial control for both species being the rule throughout the season. Again, parasitization was greater in *An. freeborni* than in *Cx. tarsalis* and a large proportion of the mosquito larvae contained several *R. culicivorax* larvae.

The sentinel cages placed at distances of 1.5, 3, 6, 12 and 18 m from Site A showed that parasitization was obtained up to 12 m from the point of application and decreased with increasing distance from this point (Fig. 4).

Infected sentinel mosquitoes were first found at Site A 7 weeks after the check was reflooded in 1979 (Fig. 5). Highest levels of parasitization were obtained close to the shoreline and decreased towards the center of the check. No infected mosquitoes were recovered at the sampling site 9 m from shore. As in the previous season, highest levels of infection were obtained in *An. freeborni*. Only 4 *An. freeborni* (3 on 10 August and 1 on 24 August) and 5 *Cx. tarsalis* (all on 22 June) were collected during the entire season, although dipping samples were taken each week. Two of the 3 *An. freeborni* collected on 10 August were parasitized by *R. culicivorax*.

DISCUSSION

Petersen & Willis (1972a) established the first mass-rearing facility for *R. culicivorax* and conducted the first field trials with this nematode (Petersen et al. 1972b, Petersen & Willis 1975). The rapid development of this biological control agent has been aided by Petersen's willingness to distribute the nematode in large quantities to scientists in all parts of the world. This has led to advances in mass-rearing techniques, to increased understanding of the biology of this nematode, and to field trials in a variety of habitats. Petersen recommended that this nematode be utilized by application of

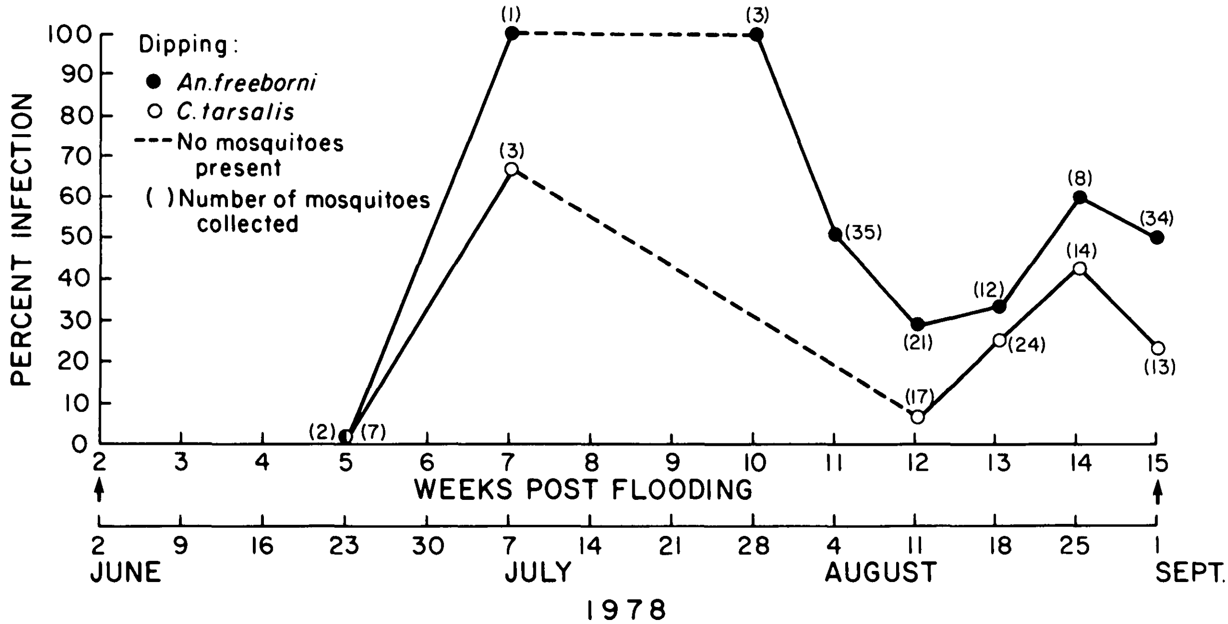

FIG. 3. Levels of parasitization obtained in native larvae of *An. freeborni* and *Cx. tarsalis* at Site A.

either the free-swimming infective stage as a microbial insecticide or in the postparasitic stage that could become established in a breeding habitat and provide continuous partial control throughout the mosquito season. However, only 1 previous study (Petersen & Willis 1972b) has dealt with the latter approach.

The first application of preparasitic larvae of *R. culicivorax* in a California rice field was made by Petersen et al. in 1972. The results indicated that good levels of control were possible in *An. freeborni* but were inconclusive for *Cx. tarsalis* because of the absence of this species from the field at the time of application. Previous studies by Petersen & Willis (1975) had shown that *R. culicivorax* could become established (recycle) in a number of aquatic habitats. The finding of an infected *An. freeborni* larva 5 weeks after the initial application suggested that *R. culicivorax* was able to recycle within California rice fields as well (Hoy & Petersen 1973).

Additional field trials with preparasites were conducted in California rice fields by Brown et al. (1979). These studies provided additional evidence of the value of *R. culicivorax* in the control of *An. freeborni* and showed that although infection could be obtained in *Cx. tarsalis* (30–50%), the levels of parasitization were lower than with *An. freeborni* (50–90%).

In the present study, the results of the application at Site A have shown that postparasites can become established in California rice fields and provide effective partial control of both *An. freeborni* and *Cx. tarsalis* throughout the mosquito breeding season. In addition, the single application of postparasites provided continuous control of *An. freeborni* at a level comparable to a single application of preparasites and higher than has been achieved in the past against *Cx. tarsalis*. Also, the actual level of mosquito control resulting from the nematode application is likely to be greater than the level of parasitization reported because of mosquito death resulting from predators, other parasites present within the field, other environmental factors and from the early death of multiply-infected host mosquitoes.

The differences in the levels of control achieved at Site A (postparasites applied the 2nd week postflooding) and at Sites B, C and D (postparasites applied the 5th, 6th and 7th weeks postflooding, respectively) indicate the need for additional research on the timing of applications. (In this study nematodes were not available for application during the 3rd and 4th weeks postflooding.) Future studies will probably show that, for maximum effectiveness, postparasites should be applied soon after the field has been flooded. The lack of establishment at Sites B, C and D was probably caused by an increase in the numbers of predators in the field between the 2nd and 5th weeks after flooding. The number of predators was not quantitatively determined in this study. However, a marked increase in predators was noted by investi-

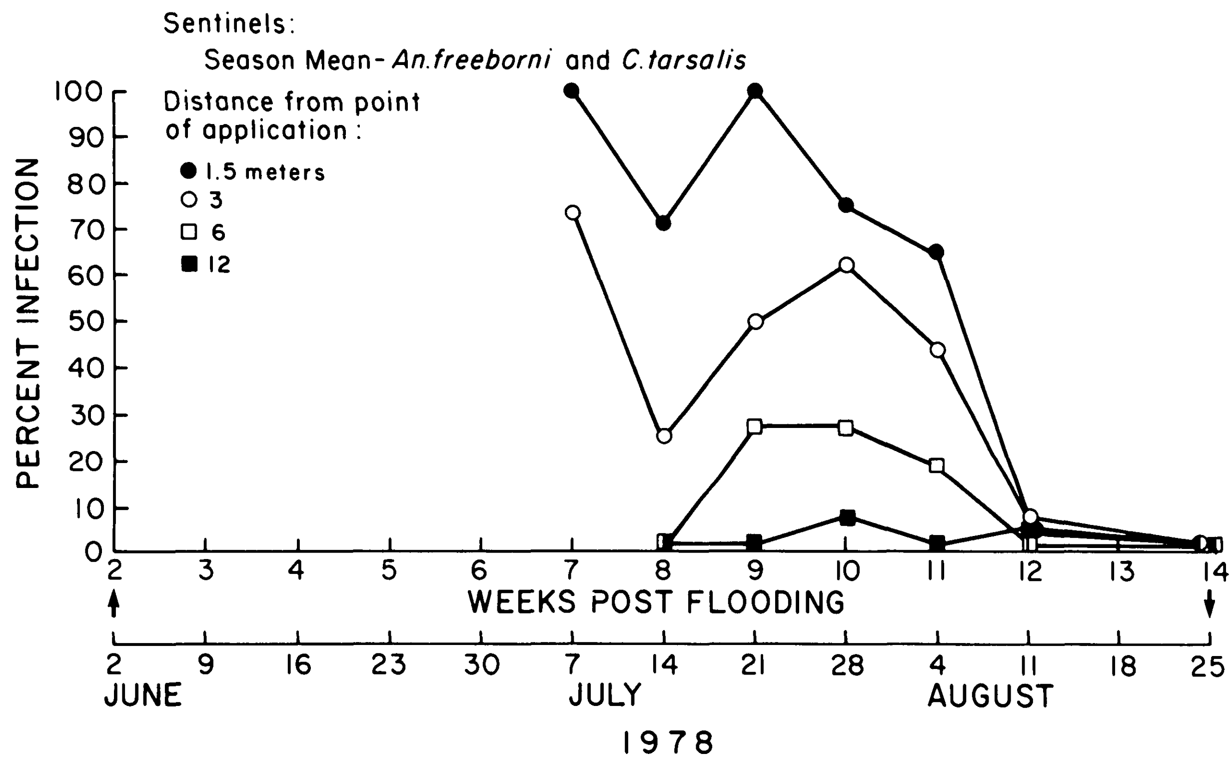

FIG. 4. Dispersal of infection from initial line of application at Site A. Each point is the mean of 2 sentinel cages, 1 of which contained 20 2nd-instar *An. freeborni* larvae and the other 20 2nd-instar *Cx. tarsalis* larvae.

gators between the 1st and subsequent applications. Previous studies (Washino, Ahmed et al. 1972) have shown that rice fields are relatively free of predators immediately after flooding, but that numbers of predators increase rapidly as the season progresses. In laboratory studies, Platzer & MacKenzie-Graham (1978) have shown that a number of invertebrates will prey on postparasites of *R. culicivorax*.

The temperatures present within the rice check during this study were within the optimum range for infection by *R. culicivorax* (Brown & Platzer 1977) and for development within mosquito larvae (Hughes & Platzer 1977).

Although the number of resident larvae collected in this study appears low, a number of studies have shown similar levels to be the rule rather than the exception in California rice fields, where numbers less than 1 larva per dip are common (Washino, Whitesell et al. 1972). Although larval densities within individual checks are often low, the vast acreage under cultivation results in a significant mosquito problem.

The ability of *R. culicivorax* to overwinter in various habitats has been reported by Petersen & Willis (1975) in Louisiana and by Nickle (1976) in Maryland. The present study is the first in which overwintering has been reported in a cultivated habitat. The rice check in which this study was conducted was subject to normal cultivation procedures by the grower, which included draining of the field, mechanical harvesting of rice, burning of stubble, discing and replanting of the field.

The increase in infection competency of preparasites reared within the rice field (following postparasite application) compared to those reared within the laboratory (and applied as preparasites in previous studies) seen in this study with regard to *Cx. tarsalis* represents a strong argument for the application of postparasites rather than preparasites in California rice fields. The reason for this increase is not known but it is probably related to a loss of energy by preparasites. These are commonly stored in the egg stage in laboratory cultures of moist sand for several months until the preparasites are needed, at which time the sand is flooded with water and the nematodes hatch. Although it has become common to speak of preparasites hatching from eggs following flooding in laboratory cultures, in actuality a large proportion of preparasites are already hatched and are in a quiescent state by the time cultures are several

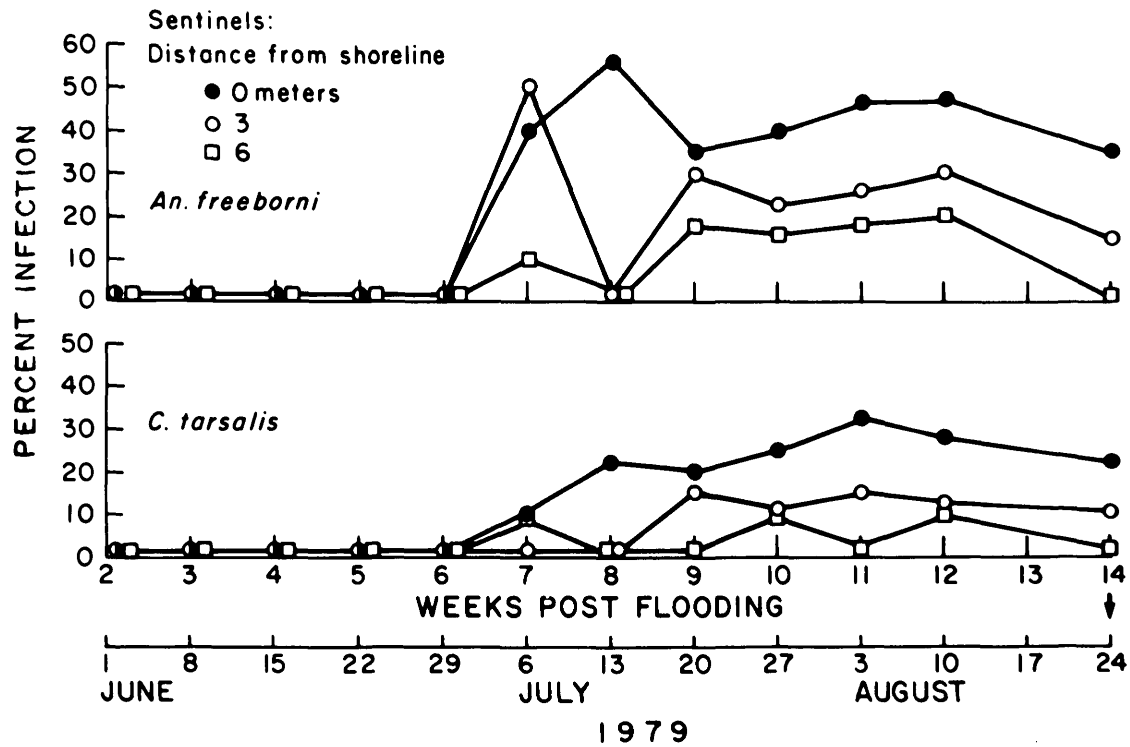

FIG. 5. Levels of parasitization in sentinel cages containing *An. freeborni* and *Cx. tarsalis* following overwintering of nematodes at Site A. Cages were placed in a line parallel to original line of application.

months old (Westerdahl unpubl. observ.). This has been observed in cultures produced by U. C. Riverside, Nutrilite Inc., and the USDA. One possible explanation is that the quiescent, nonfeeding preparasites within cultures are slowly utilizing stores of energy and so have less available with which to seek out and penetrate *Cx. tarsalis* larvae; these larvae are more active and have tougher cuticles than those of *An. freeborni,* which spend more time in a quiescent state parallel to the water surface.

Additional research will be required to determine the most effective and economical rate for application of postparasites. If the area of acceptable control at Site A is assumed to be 6 × 15 m (Fig. 4) to which a total of 15,000 (10 ml) postparasites were applied, the level of application in this study is equivalent to 165 (0.1 ml) postparasites per square metre of surface area. The high percentage of multiply-infected mosquitoes and the large number of preparasites per mosquito found early in the season indicate that lower levels of application would be effective and might contribute to the success of recycling within the field. Studies (Petersen & Willis 1972b, 1974) have shown that superparasitization results in the production of large numbers of male nematodes and often causes early death of mosquitoes with subsequent death of the immature nematodes. A recent laboratory study by Petersen (1980) has shown that the optimum production of preparasites in laboratory cultures is related to the density of postparasites within the culture. This finding could also be applicable to field situations. The present study did not show whether infectivity throughout the season was from preparasites arising from successive generations of postparasites or from those applied initially.

Laboratory studies conducted in a 2-m diam indoor pool have shown that preparasites are able to travel only 0.5 m from the point of application (Hughes & Platzer unpubl. observ.). Therefore, the spread of nematodes to distances of up to 12 m from the point of application probably occurs because of the existence of currents within the field (although none was noticeable or measurable in this study) or to the migration of infected mosquito larvae.

With current rearing procedures, a commercial enterprise would place postparasitic nematodes on a substrate covered with water for 3 weeks, after which the water would be drained and the culture stored for 3 months prior to sale. Those cultures resulting from postparasites applied to sand in May and June (the months when they could be utilized for applications in California rice fields) would not be ready for sale until September or October when mosquito larvae are on the decline in most areas of the country. Because these cultures have a shelf life of 6 months, they would not be usable the following spring. Therefore, the use of postparasites in California rice fields would not preclude or compete with the sale of preparasites but rather offer an advantage to commercial enterprises by expanding their market and extending their commercial rearing season by ca. 2 months.

As promising as this and previous studies with *R. culicivorax* have been, it is unlikely that any single biological agent will routinely be able to achieve population reductions greater than about 80%. This is because parasite-host relationships have evolved to permit the survival of the host population to help ensure the survival of the parasites. Evidence for the validity of this theory with respect to *R. culicivorax* is seen in studies by Petersen & Willis (1970) that showed that a 2.4:1 ratio of preparasites to hosts produced 80% infection in *Cx. pipiens quinquefasciatus* but a 5:1 ratio was required to achieve 95% infection, and the finding by Brown et al. (1979) that increasing treatment rates from 1000 to 25,000 preparasites per square metre of surface area did not significantly increase the number of *Cx. tarsalis* larvae infected. Excessively high rates of application of *R. culicivorax* are relatively ineffective, are not cost effective, and are likely to result in high levels of superparasitization. This will lessen the chance of the nematode becoming established in a habitat because early death of multiply-infected hosts (Petersen & Willis 1974) and/or high production of male nematodes results from superparasitization (Petersen 1972). Although 80% control is usually considered a significant reduction in the number of pests on agricultural crops, it is often not an acceptable level of mosquito control to health officials or to the general public who demand a kill close to 100% (Legner et al. 1974).

Laboratory studies that have been conducted with pesticides and *R. culicivorax* suggest that this nematode could be used successfully in combination with pesticides commonly used against insects and with algicides (Mitchell et al. 1974, Gordon et al. 1976, Platzer & Brown 1976, Finney et al. 1977). Future studies should emphasize the development of integrated programs of mosquito control utilizing the nematode with other biological agents as well as pesticides.

The results of the present study have shown that postparasites of *R. culicivorax* can become established in California rice fields, provide partial continuous control of *An. freeborni* and *Cx. tarsalis* throughout the breeding season and overwinter to provide partial control the following season.

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