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Parasitism of Kenyan Mosquito Larvae (Diptera: Culicidae) by *Romanomermis culicivorax* (Nematoda: Mermithidae)[1]

NAYNA VYAS-PATEL[2]

Abstract: The ability of *Romanomermis culicivorax* to infect, develop, and emerge from Kenyan mosquito hosts was evaluated in the laboratory. Host species tested were *Aedes aegypti, Ae. dentatus, Ae. hirsutus, Anopheles arabiensis, An. coustani, An. funestus, An. gambiae, An. pharoensis, Culex duttoni, Cu. ethiopicus, Cu. poicilipes, Cu. quinquefasciatus, Cu. tigripes, Cu. univittatus, Coquillettidia metallica, Mansonia africana, Ma. uniformis, Mimomyia splendens, Mi. uniformis, Toxorhyncites brevipalpis,* and *Uranotaenia balfouri. R. culicivorax* penetrated all the host species tested and developed and emerged from most of the hosts. Both penetration and some development, but not nematode emergence, were observed from all instars of *Ma. uniformis. T. brevipalpis* exhibited signs of resistance in the form of melanization of *R. culicivorax* within 48 hours of infection in all four instar stages. Nematode melanization, especially in older hosts, was observed in *Ae. dentatus, Ae. hirsutus, Cu. duttoni, Cu. tigripes,* and *Mi. splendens.* When melanization occurred, the melanized carcass of the nematode was passed on from instar to instar. The implications for field release of *R. culicivorax* in Kenya are still good, especially in habitats where different mosquito species occupy the same niche at different times, which would allow for nematode recycling.

Key words: biological control, host resistance, host susceptibility, melanization, Mermithidae.

Natural mermithid parasitism of mosquitoes in Africa has been reported. *Anopheles funestus* adults are parasitized by *Empidomermis cozii* in West Africa (3,16), the tree-hole breeding mosquito larvae by *Octomyomermis muspratti* (11,12) and the rice-field inhabiting mosquitoe larvae by mermithids in East Africa (18). This indicated the potential for natural nematode recycling in suitable habitats in Africa. Culturing these mermithids has not been pursued, or it has proved difficult (17). *Romanomermis culicivorax* Ross and Smith, a nematode indigenous to America, is readily cultured (13) and has already shown some recycling potential (10,19).

A few mosquito species from different parts of the world have been shown to be refractory to *R. culicivorax* (8,14,15). As a prerequisite to field trials using *R. culicivorax* for tropical mosquito biocontrol, the susceptibility of 21 Kenyan mosquito species to *R. culicivorax* was tested under laboratory conditions at Mbita Point field station, Kenya.

Many mosquito species, both medically important and unimportant, share the same habitat, and their populations can peak at different times (1). Therefore, information on the susceptibility of all the mosquito species present in a system was needed to determine the host species which could act as reservoirs for recycling of *R. culicivorax* at different periods in a biological program.

Little is known about the susceptibility of East African mosquito species to *R. culicivorax.* The few host species included in the 1979 review by Petersen (15) which listed the susceptibility of 87 different mosquito species to *R. culicivorax,* needed to be tested in order to eliminate possible mosquito strain differences in susceptibility to *R. culicivorax.*

MATERIALS AND METHODS

Mosquito collection: Larval sampling of mosquitoes was undertaken at localities in the South Nyanza (Mbita Point and Rusinga Island) and Nyanza Provinces (Kisumu and the Ahero Rice Irrigation scheme). Two species collected from Taveta and reared at the Kenya Medical Research Laboratory (KEMRI) in Kisumu were exposed to *R. culicivorax.* Larvae were collected in

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[2] University of Reading, Department of Agriculture, Earley Gate, P.O. Box 236, Reading RG6 2AT, UK.

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a dipper (10 × 5 cm) and transported to the laboratory in pond water. For mosquito species associated with plants (*Mansonia* and *Mimomyia* spp.), whole *Pistia* plants were collected and their roots washed over a fine sieve which retained the mosquito larvae. *Anopheles funestus* larvae were obtained by collecting gravid adult females from indoor resting sites in Ahero and releasing them into cages containing oviposition bowls. Adult mosquitoes were identified using Gillett's description (5), and larvae were identified using the keys of Hopkins (7) and Gillies and De Meillon (6). *Anopheles arabiensis* was distinguished from its sibling species by the method of Coluzzi and Sabatini (2).

Mosquito rearing: Anopheles gambiae, An. arabiensis, Culex quinquefasciatus, Aedes aegypti, and *Toxorhyncites brevipalpis* were maintained as colonies with standard methods of larval and adult mosquito rearing adapted for local conditions (22). Anopheline larvae were fed ground yeast and dog biscuit, and culicines were fed ground rabbit chow. *T. brevipalpis* larvae were reared separately in microwell plates, and each one was fed about 20 *An. arabiensis* larvae daily. The adults were given a 20% solution of honey on cotton gauze, and the eggs were collected from a 37-cm-d bowl half full of tap water.

Nematode rearing: A laboratory culture of *R. culicivorax* was established from a stock sent from the Gulf Coast Mosquito Laboratory, Lake Charles, Louisiana. The procedures for mass rearing of *R. culicivorax* (13) were modified to suit the tropical conditions at Mbita. *Culex quinquefasciatus* larvae were used as the principal hosts for nematode rearing. Infected hosts were kept in tap water in clean bottles (50 per bottle). This reduced developmental time, because of the higher temperature in the bottles, and prevented contamination. Emerged nematodes were sieved and placed on sterile, moist sand in plastic boxes (16 × 11 cm and 21 × 14 cm) with lids. The surface of the sand was covered with a plastic sheet and then with sponge cut to size to prevent condensation. Cultures were stored at room temperature (28–30 C). The first nematode preparasites hatched 23 days after postparasite emergence.

Host infection: Lots of 50 or 100 first-instar and second-instar larvae of *An. funestus, An. gambiae, An. arabiensis, Ae. aegypti, Cu. univittatus,* and *Cu. quinquefasciatus* were exposed to *R. culicivorax* for 24 hours in 10 ml distilled water per host used. After infection, the larvae were reared normally in boiled tap or lake water (colonized species) or in pond water (larvae of species from the wild). Host-to-nematode ratios of 1:5 or 1:10 were used.

Some species of mosquito larvae were limited in availability, so the instars and numbers of hosts used varied. Postcollection mortality of mosquito larvae was sometimes a problem caused by epibiotic *Vorticella* spp. Laboratory mortality due to *Vorticella* has been observed in other studies, but these epibionts are believed to cause little mosquito mortality under natural conditions (21).

Each host species was exposed separately by instar and species type. With each set of experimental exposures, *Cu. quinquefasciatus* larvae were exposed to ascertain the viability of the nematode culture.

Exposed hosts were transferred to fresh water each day to prevent fungal growth. When hosts were present in sufficient numbers (i.e., 50–100 per set), the penetration and infection rate were ascertained by dissection (24 hours postinfection) of 50% of the mosquito larvae exposed. If fewer than 50 individuals of a mosquito species were available, nematode penetration was checked microscopically at 4, 16, and 24 hours postexposure. Whereas penetration was readily observed in culicines and younger hosts of some genera, the darker coloring of many anophelines and older hosts obscured the host haemocoel.

Before nematode emergence, each host was placed in a microwell cell, and nematode emergence per host was noted.

Results

The mosquito species collected from Mbita and Rusinga were *Anopheles gambiae*

TABLE 1. *Romanomermis culicivorax* percentage of penetration in and emergence from larvae of Kenyan mosquito species.

	Host : parasite ratio	Penetration		Emergence	
		First instars	Second instars	First instars	Second instars
Anopheles gambiae	1:5	100	90	90	94
	1:10	96	99	94	96
An. arabiensis	1:5	94	90	96	84
	1:10	92	98	88	92
An. funestus	1:5	68	80	68	84
	1:10	84	88	84	80
Aedes aegypti	1:5	88	94	92	96
	1:10	96	100	96	100
Culex quinque fasciatus	1:5	96	98	98	96
	1:10	100	100	100	98
Cu. univittatus	1:5	92	80	92	88
	1:10	96	96	96	100

Giles (species A of the *An. gambiae* complex), *Aedes hirsutus* Theobald, *Ae. dentatus* Theobald, *Culex quinquefasciatus* Say, *Cu. univittatus* Theobald, *Cu. duttoni* Theobald, and *Cu. tigripes* Grandpre. Those collected from Kisumu and the Ahero Rice Irrigation Scheme were *Anopheles arabiensis* Patton (species B of the *An. gambiae* complex), *An. funestus* Giles, *An. coustani* Laveran, *An. pharoensis* Theobald, *Culex ethiopicus* Edwards, *Cu. poicilipes* Theobald, *Coquillettidia metallica* Theobald, *Mansonia africana* Theobald, *Mansonia uniformis* Theobald, *Mimomyia splendens* Theobald, *Mi. uniformis* Theobald, and *Uranotaenia balfouri* Theobald. *Aedes aegypti* Linnaeus and *Toxorhyncitis brevipalpis* Theobald from Taveta were obtained from colonies established at the Kenya Medical Research Institute, Kisumu.

The rates of *R. culicivorax* penetration, development, and emergence from *An. gambiae, An. arabienses, An. funestus,* and *Ae. aegypti* were high (Table 1), as were those of the *Cu. quinquefasciatus* check. Premature host deaths of *Mansonia uniformis, Ma. africana, Mimomyia uniformis, Coquillettidia metallica,* and *Culex poicilipes* did not allow for the recording of nematode emergence or possible melanization. Dissection of the cadavers indicated that some nematode development prior to host death had occurred in *Ma. uniformis* and *Ma. africana,* but observations from the other species were inconclusive (Table 2). Nematode penetration was observed in all mosquito species tested.

Host resistance in the form of nematode melanization was observed in *Ae. dentatus, Ae. hirsutus, Cu. duttoni, Cu. tigripes,* and *Mi. splendens,* usually about day 3 or day 4 postinfection, and in *T. brevipalpis* within 48 hours postinfection. *R. culicivorax,* however, developed and emerged from *Cu. tigripes, Cu. duttoni,* and *Ae. hirsutus* if they were infected as first instars and (or) were heavily infected with the nematode. In one case, when a second-instar *Cu. tigripes* was infected by five nematodes, four healthy male nematodes emerged and the remaining one was found melanized in the host. Female nematodes did not emerge from host species showing physiological resistance. The melanized body of the nematode was passed on from instar to instar, a few even being found in the haemocoel of the pupal stage. *T. brevipalpis* (colonized species), which was infected at all four instar stages (15 larvae per instar), first and second instars, but not third or fourth instars, reached out for the attacking preparasites with their mouth parts and ingested or damaged them. Consequently, third-instar and fourth-instar *T. brevipalpis* harbored more nematodes per host than first or second instars. Not a single nema-

TABLE 2. *Romanomeris culicivorax* development and emergence from Kenyan mosquito hosts.

		Number of hosts					
	Instar	Exposed	Infected	Nematode developed	Nematode emerged	Premature death	Melanization
Anopheles pharoensis	2, 3	8	3	4	8	0	No
An. coustani	2, 3	6	2	4	4	0	No
Aedes dentatus	2	4	4	2	0	1	Yes
Ae. hirsutus	All	18	10	3	4	3	Yes
Culex ethiopicus	2, 3, 4	– 5	3	4	5	0	No
Cu. duttoni	All	32	17	6	5	10	Yes
Cu. poicilipes	3, 4	14	10	0	0	14	
Cu. tigripes	All	29	15	4	9	2	Yes
Coquillettidia mettalica	4	3	3	1	0	3	
Mimomyia uniformis	2, 3	4	2	1	0	4	
Mi. splendens	2, 3	8	5	1	0	4	Yes
Mansonia africana	2, 3, 4	10	7	4	0	10	
Ma. uniformis	All	15	9	6	0	9	
Uranotaenia balfouri	1	3	3	3	3	0	
Toxorhyncites brevipalpis	All	60	25	0	0	0	Yes

tode emerged from any *T. brevipalpis*; melanization was swift and complete in this host.

Natural nematode infections were found in mosquito larvae sampled from the Ahero Rice fields. Six of the *An. pharoensis* from the rice fields had natural mermithid nematode infections. Nematodes emerged from all six hosts, and all were males; therefore, detailed taxonomic studies were not possible.

DISCUSSION

Most studies, especially field trials, of *R. culicivorax* as a biocontrol of mosquitoes have been conducted in the United States. Recycling of the nematode has been reported from field releases of *R. culicivorax* in permanent water habitats or irrigated areas like rice fields (10,19). This study provides baseline data for future release of *R. culicivorax* in a tropical country in both permanent and temporary water sites.

The *An. gambiae* complex is the major cause of endemic malaria in Kenya (20). Typically, the species breeds in temporary pools, but it has successfully adapted to areas of intense irrigation like the rice fields at Ahero (1,18,20). The use of postparasites has been advocated for permanent water breeding mosquito species (19). Such a measure could be wasteful, however, if applied to temporary water sites such as those found at Mbita and Rusinga, most of which dry up during the dry season. Under these conditions, application of preparasites soon after long rains, when *An. gambiae* populations peak, may prove more beneficial. Nematodes would be applied at least twice a year to coincide with the long and short rains.

In the Ahero rice fields, anophelines and other *Culex* species peak during the early and latter part of the rice cycle, respectively, whereas *Mansonia* species and *Cu. poicilipes* breed throughout most of the rice cycle (1). Thus, the formal succession of mosquitoes as the season progresses should be favorable for recycling of *R. culicivorax* following release of postparasites, especially if it were known that *Mansonia* species and *Cu. poicilipes* were susceptible to the nematode. *Cu. poicilipes* and *Mansonia* spp., the most abundant species at Ahero, are present throughout the year (1). Confirmation of their susceptibility to *R. culicivorax* is therefore extremely important for nematode recycling in this area. There is no conclusive evidence from this investigation to support Petersen's rating (15), based on Kerdpibule's study (8), of the resistance of *Ma. uniformis* to *R. culicivorax*, since resistance can operate at a later stage of nematode development in the host. Sim-

ilar resistance is evident in the present study with *Cu. tigripes.* Unlike in Kerdpibules' study (9), however, all four larval stages of *Ma. uniformis* were penetrated by the nematode.

The six hosts with varying degrees of resistance to *R. culicivorax* are not medically important, but resistance does exclude them as alternative or reservoir hosts for nematode recycling when they occur with susceptible hosts. Their resistance may be due to the evolution of defense mechanisms to a previous long, and ultimately unsuccessful, parasitism by indigenous mermithids. Nevertheless, such effective defense reactions to a foreign parasite in these hosts was surprising. Alternatively, these hosts may never have experienced such nematode infections before, and thus they express acute host defense actions on first infections. The latter is felt to be unlikely, as both the resistant and susceptible hosts found in this study share the same habitats and are often found together. Knowing whether these resistant hosts would show any humoral defenses to an indigenous mermithid, such as the one found at Ahero, or to other species of *Romanomermis* (e.g., *R. iyengeri*) could be useful. A few mosquito species in America are resistant to *R. culicivorax* (15), and it is possible that such resistance could have evolved for protection through long association with the nematode.

The observations from this study also suggest that the susceptibility of older hosts (i.e., third and fourth instars) should not be assumed from the susceptibility data of younger host infections (i.e., first and second instars). Nematodes may develop in hosts parasitized as first or second instars (especially if hyperparasitized) but show melanization in third-instar or fourth-instar infections, as in *Cu. tigripes.* This should be noted for the host *U. balfouri,* where only first instars were infected.

High rates of nematode penetration were observed in *Ae. aegypti* (Table 1), comparable to the penetration rate of *Cu. quinquefasciatus.* This is in contrast to Petersen's repeated observations with *Ae. aegypti* (14,15), where very low nematode penetration rates were found in *Ae. aegypti* compared with *Cu. quinquefasciatus.* In the absence of explanatory data, such differences could have resulted from the mosquito strain of *Ae. aegypti* used in the tests.

Development and emergence of *R. culicivorax* has been reported previously from two other Toxorhynchitine mosquitoes, *T. amboinensis* from the Pacific (4) and *T. rutilus septentrionalis* from North America (15). Infection has not been observed in *T. rutilus rutilus,* even at high parasite densities (unpubl. data, 1980, from Insects Affecting Man and Animals Research Laboratory, Gainesville, FL). In this study, penetration but no development occurred in *T. brevipalpis.* Although this observation does not really affect the future use of *R. culicivorax* in habitats such as tested in this study, *T. brevipalpis* may serve as a host model for studies to determine resistance mechanisms to *R. culicivorax.*

Significantly, the naturally occurring mermithid nematode found in Ahero rice fields was from *An. pharoensis* larvae. Mermithid infections of mosquito larvae had been reported 9 years previously in Ahero rice fields (18). The nematode species from both findings remains unidentified, but it indicates the suitability of the Ahero rice field environment for recycling of mermithid nematodes. This potential for nematode recycling in suitable habitats in tropical Africa could be harnessed in a biocontrol program using mermithids. Thus, the importance of carrying out field trials using mermithids for larval mosquito biocontrol in Africa should be emphasized.

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