Gaseous Requirements for Postparasitic Development of Romanomermis culicivorax[1]

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Abstract: The development of postparasitic stages of *Romanomermis culicivorax* was studied under various concentrations of oxygen and carbon dioxide. The nematode developed poorly if only nitrogen was supplied; only one-third molted and all died eventually. In the presence of 5% CO_2–95% N_2, development was normal; most nematodes molted and oviposited with respective mean developmental times of 32 and 50 d. Addition of 0.2% O_2 stimulated development; molting and oviposition commenced at days 18 and 41, respectively. There was an additional stimulation of development by increasing amounts of O_2 up to 1%, but concentrations greater than 1% produced no additional stimulation. Carbon dioxide was required for development after exsheathment under anaerobic conditions or O_2 concentrations less than 1%. Oxygen or CO_2 were not required for embryological development or egg hatch. It is suggested that postparasitic stages function as facultative anaerobes. *Key words:* Mermithidae, nematoda, oxygen, carbon dioxide, biological control.

Romanomermis culicivorax, a mermithid parasite of mosquitoes, has shown great potential as an applied biological control agent. Since its discovery by Petersen in Louisiana (14), and the subsequent development of culture methods (15), there has been considerable research on the biology and usefulness of the nematode for mosquito control. Preparasites have been shown to be effective inoculum in the field, but their usefulness in polluted water is limited by their sensitivity to salts (2) and dissolved oxygen (3). Recent work indicates that postparasites also may be effective field inoculum (23), but little is known about the physiology of this stage of the nematode.

The postparasitic development of *R. culicivorax* begins with emergence of the third-stage larvae from the host. The larvae settle to the bottom of a mosquito pond, molt twice, copulate, and produce eggs. The nematodes penetrate into the sediment where the eggs are deposited, embryonate, and hatch after formation of the second-stage larvae. The purpose of the experiments described here was to determine the effects of various concentrations of oxygen and carbon dioxide on the postparasitic development of *R. culicivorax* and to elucidate the environmental requirements of these life stages.

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MATERIALS AND METHODS

Romanomermis culicivorax cultures were maintained in an autogenous strain of *Culex pipiens* using the procedures of Petersen and Willis (15) as modified by Platzer and Stirling (17). Postparasites were collected over a 24-h period as they emerged from the host and experiments were initiated within the following 24–72 h. Three females and four males were placed in 30-ml plastic tissue culture flasks modified to permit gassing with the experimental mixtures (Fig. 1). Each flask contained 10 ml of 1.0% water agar (WA). Five or six replicative flasks per experimental group were connected in a series with latex tubing and gassed with mixtures of oxygen, carbon dioxide, and nitrogen (Certified Standard Matheson). Compositions of the mixtures are listed in Tables 1, 2, and 3.

Gases were humidified by passage through distilled water before entry into the experimental flasks. A constant flow rate of 80 cc per minute, determined with a flowmeter, was maintained throughout the experiments. Experiments were limited to 55 d at 27 C, since the agar dried after 60 d. The flasks, temporarily isolated from the gas source by clamping the exhaust and inlet tubes, were examined daily for development of female nematodes and eggs. Data were expressed as percentage of females that molted, initiated, and completed oviposition and mean time required for these events to occur in each treatment. Nematodes which died accidently (e.g., trapped on top or side of flasks and dehy-

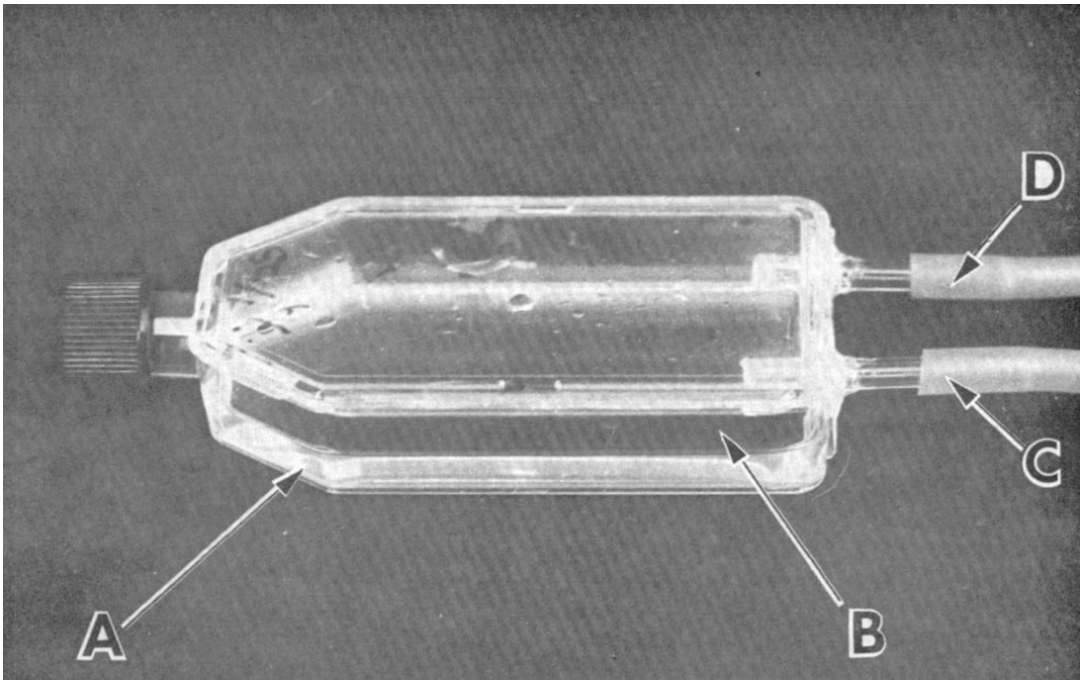

Fig. 1. Flask for exposing *Romanomermis culicivorax* postparasites to various O_2:CO_2:N_2 atmospheres. Nematodes were placed on 1% water agar (WA) and gas mixtures passed through space above agar (B) at 80 cc per min. Rubber tubing at gas inlet (C) and exit (D) was clamped off to permit microscopic observation of developing nematodes.

drated) were not included as part of the experimental group. The times required for embryological development (20) to multicell, cresent, first-stage larvae and hatch were also determined.

Statistical significance was determined by analysis of variance and Duncan's multiple-range tests.

Table 1. The percentage of postparasitic *Romanomermis culicivorax* females that molt, initiate and complete oviposition under various O_2:CO_2:N_2 atmospheres.

Atmosphere, %*			Oviposition	
O_2	CO_2	Molt	Initiation	Completion
0	0	33.3	0†	0†
0	5.0	93.3	46.6	0
0.2	5.0	93.3	86.7	7.7
1.0	5.0	100	100	100
2.0	5.0	93.3	93.3	64.3
5.0	5.0	80.0	73.3	73.3
21.0	0.03 (air)	93.3	93.3	93.3

*Plus N_2 to equal 100%.
†All animals died.

RESULTS

Males and females molt equally well and at the same time at the various O_2 and

Table 2. Time required for postparasitic development of *Romanomermis culicivorax* under various O_2:CO_2:N_2 atmospheres.

Atmosphere, %†		Mean development time in days*		
			Oviposition	
O_2	CO_2	Molt	Initiation	Completion
0	0	21.4 a	0‡	0‡
0	5	32.4 b	50.4 b	0§
0.2	5	17.8 c	41.4 c	47.0‖
1.0	5	13.5 d	21.8 d	37.7 a
2.0	5	11.7 d	23.8 d	41.0 a
21.0	0.03 (air)	10.6 d	19.9 d	36.0 a

*Numbers followed by different letters are significantly different according to Duncan's multiple-range test ($P = 0.01$).
†Plus N_2 to equal 100%.
‡All animals died.
§Oviposition not completed during 53-day experiment.
‖One nematode, statistical analysis not possible.

Table 3. Effect of carbon dioxide on development of *Romanomermis culicivorax*.

Atmosphere, %*		Molting		Oviposition			
				Initiation		Completion	
O_2	CO_2	%	Time†	%	Time†	%	Time†
0.5	5.0	100	12.2 a	46.7	32.6 a	20.0	39.0 a
0.5	0.0	100	13.6 b	16.7	27.0‡	0§	0§
2.0	5.0	100	11.2 c	100	17.8 b	83.3	31.5 a
2.0	0.0	100	11.2 c	85.7	17.1 b	85.7	32.9 a
21.0	0.03 (air)	100	11.0 c	100	18.2 b	85.7	29.3 a
21.0	0.0	100	11.1 c	83.3	15.7 b		

*Plus N_2 to equal 100%.

†Mean postemergent developmental time in days. Numbers followed by different letters are statistically significant according to Duncan's multiple-range test ($P = 0.01$).

‡One nematode produced eggs, others died.

§Oviposition not completed during 55-day experiment.

CO_2 concentrations tested (data not shown). The males lived longer, however, and were still active after 55-d exposure to 1% and higher O_2 concentrations, whereas the females apparently had exhausted food reserves and moved infrequently. In all treatments except 100% N_2, copulation occurred immediately following exsheathment and continued intermittently. Males continued to copulate with females throughout the experiments, even after females completed oviposition.

The effect of various gas mixtures on the development of *R. culicivorax* females is given in Table 1. When the nematodes were maintained in the presence of 100% nitrogen, only one-third lived long enough to molt and these died without producing eggs.

Nearly all nematodes molted when 5% CO_2 was included in the anaerobic environment. About half had started to produce eggs when the experiment was terminated, and all of the nematodes survived. Molting and oviposition were stimulated by the addition of O_2. At 0.2% and all higher O_2 concentrations, nearly all of the nematodes which molted initiated egg production. About 9% of the exsheathed nematodes completed oviposition in the presence of 0.2% oxygen and 5% CO_2. There was an additional stimulation at 1% and higher O_2 concentrations, with most of the nematodes completing egg laying.

The mean time required for female development is given in Table 2. Significant differences ($P = 0.01$) in molting times were found between treatments containing 1% or less O_2; there were no differences between treatments containing more O_2. Oxygen greatly stimulated molting; at 0.2% and 1–21%, molting was 1.8 and 2.7 times faster, respectively, than under anaerobic conditions. Similar decreases occurred in times required for initiation and completion of oviposition with the addition of oxygen.

The requirement of CO_2 for development of postparasitic females under various oxygen concentrations is given in Table 3. At 2% and 21% O_2 there was no requirement for CO_2. The nematodes developed normally, and no differences in time required for molting and initiation and completion of oviposition were found. However, at 0.5% O_2 significant differences were found. At this low O_2 concentration, CO_2 had no effect on the number of nematodes that molted, but in the absence of CO_2 the time required for exsheathment was about 10% greater. Development beyond the molt was severely retarded by lack of CO_2, with only one female ovipositing and the remainder dying.

Nematodes exposed to oxygen until exsheathment started oviposition at the same time regardless of the gaseous atmosphere following exsheathment, but this stimulation was lost prior to the completion of egg production, since none of the nematodes in 0.2% O_2 and only one in 95% N_2–5% CO_2 atmosphere completed egg production (Table 4). Also, the requirement for CO_2 under anaerobic conditions is unaffected

Table 4. Effect of exposure to aerobic conditions until exsheathment on subsequent development of *Romanomermis culicivorax*.

Atmosphere, %†		Mean postemergent developmental time in days*	
		Oviposition	
O_2	CO_2	Start	Completed
0.0	0.0	22.5 a	0‡
0.0	5.0	19.2 a	53.0§
0.2	5.0	19.2 a	>55‖
1.0	5.0	23.4 a	34.0 a
2.0	5.0	26.6 a	37.3 a
21.0	0.03 (air)	19.9 a	36.0 a

*Numbers followed by different letters are statistically significant at 1%.
†Plus N_2 to equal 100%.
‡All animals died.
§One nematode, statistical analysis not possible.
‖Oviposition not completed during 55-day experiment.

because the nematodes died in 100% nitrogen.

The effect of various concentrations of O_2 and CO_2 on the embryological development of the eggs is shown in Table 5. No statistical significance was found between any of the gassing mixtures of various O_2 concentrations with and without CO_2

DISCUSSION

Previous investigators suggested that a sand substrate was required for normal postemergent development of *R. culicivorax* (16). However, Finney (6) reported maturation and oviposition by the nematodes in pans containing only water, and J. Eby in our laboratory found that third-stage larvae placed on water agar plates appeared to develop normally. These observations were confirmed by our experiments. One percent WA provides a suitable substrate for casting of molted cuticles, copulation, and egg production, and the use of tissue culture flasks permits easy observation. This system would permit additional studies on the effects of various environmental factors on the postparasitic development of *R. culicivorax*.

The oxygen and carbon dioxide concentrations encountered by postparasitic stages of *R. culicivorax* under natural conditions can be estimated from the limnological literature. The temperature, salinity, depth of water, stratification, and biological characteristics will effect the concentration of dissolved oxygen. Oxygen diffuses slowly in still water, and unless there is mixing in a pond, thermal stratification leads to a lack of oxygen in the lower layers (4,12,18,24). If the pond is shallow or if there is good mixing, dissolved oxygen concentrations approaching saturation may occur throughout the water profile (4,12), and with abundant light O_2 production by photosynthetic organisms results in supersaturation of the water (4,18,24). Relatively high concentrations of O_2 may be present down to the water-sediment interface, but with increasing depths within the sediment oxygen disappears rapidly as a result of biological and chemical utilization. Unless the sediment is very coarse with extensive water flow through it, anaerobic conditions are usually found within 1 to 3 mm of the

Table 5. Embryological development of *Romanomermis culicivorax* under various O_2:CO_2:N_2 atmospheres.

Atmosphere, %†		Embryological development time in days*			
O_2	CO_2	Multicell	Cresent	Larvae	Hatch
0.0	5.0	1.0	2.0	5.5	11.7
0.2	5.0	1.0	2.0	4.5	9.5
1.0	5.0	1.0	2.7	6.0	10.8
2.0	5.0	1.0	2.4	7.3	10.2
2.0	0.0	1.0	2.4	7.0	10.0
5.0	5.0		3.0	4.3	10.0
21.0	0.03 (air)	1.0	2.4	5.8	11.2

*Mean of six replicates. None of the differences were statistically significant.
†Plus N_2 to equal 100%.

sediment surface (5,8,9,12,19,24). Carbon dioxide occurs in water as the dissolved gas, bicarbonate, and carbonates, depending on the pH of the water (4,18,24). Bicarbonate prevails under neutral conditions, and in slightly acid waters, free carbon dioxide concentrations may be as high as 200 ppm (18). It is rare to find a lack of CO_2 in water except when the water is extremely hard and alkaline resulting in the precipitation of CO_2 as carbonates. The distribution of CO_2 in sediments is the inverse of O_2. As the amount of oxygen declines quickly below the uppermost layers of the sediment, the amount of CO_2 increases (5).

Assuming diffusion in 1% WA is equal to that in water, the amounts of oxygen and carbon dioxide dissolved in 1% WA at 27 C and the various partial pressures of the gas mixtures used in the experiments are given in Table 6. Less than 1/2 mg/liter dissolved oxygen would be found in water in equilibrium with 1% O_2 in the gas phase. This concentration or higher has been frequently reported in shallow ponds throughout the water profile (4,12,18,24) and in the upper, oxidized portion of pond sediments (5,8,9,12). Oxygen concentrations of less than 1% reflect the amounts of O_2 found in the major portion of the oxidized sediment layer. The deeper portions of the sediment are probably anaerobic. Amounts of CO_2 dissolved in water at the experimental concentrations (Table 6) are reasonable approximations of the quantities of CO_2 encountered in ponds and sediments (4,5,18).

Table 6. Theoretical concentrations of oxygen and carbon dioxide in 1% water agar at 27 C.

Gas	% in gas phase	Amount in solution, mg/L*
Oxygen	20.9 (air)	8.2
	5.0	2.0
	2.0	0.8
	1.0	0.4
	0.5	0.2
	0.2	0.08
Carbon dioxide	0.03 (air)	0.4
	5.0	70.5

*Calculated on basis of solubility coefficient of gas in distilled water at 27 C and 1.0 atmosphere (Handbook of Chemistry and Physics, 41st ed., p. 1706-1707).

It was found in our experiments that the rate of development of *R. culicivorax* at 1% O_2 is the same as at higher O_2 concentrations. This rate declined at lowered O_2 and under anaerobic conditions. It would be reasonable to hypothesize that upon emergence the nematode would have access to 1% or greater O_2 concentrations. In addition, the stimulation of development by these higher O_2 concentrations continued for some time even when the nematodes are placed under anaerobic conditions. Therefore, it appears that upon emergence from the host, the nematode functions aerobically if very small amounts of O_2 are available. Under these conditions development is stimulated and the nematode undergoes physiological changes which continue under anaerobic conditions.

Experiments on penetration of postparasites into the bottom of ponds indicated that the nematodes are found as deep as 15 cm (B. B. Westerdahl, unpublished). At this depth anaerobic conditions are certain to exist.

If the nematode is exposed to anaerobic conditions immediately upon emergence (e.g., a still pond with large amounts of decaying organic matter), it is capable of completing development and egg production, although at a lower rate. The requirement for CO_2 at extremely low O_2 tensions and anaerobic conditions is not a physiological problem, for significant amounts are found typically in pond waters and sediments.

The lack of a requirement for O_2 by the eggs for embryological development and hatch is consistent with the anaerobic environment of the sediment in which they are deposited. This is unusual, for it is generally believed that some O_2 is required for the development of all nematode eggs even though eggs of many species can withstand anoxia (11,22). Oxygen is required for the hatching of eggs of soil-inhabiting nematodes, but the eggs of some animal parasites hatch under anaerobic conditions (11,22). It is not known what physiological adaptations allow *R. culicivorax* eggs to embryonate under anaerobic conditions.

Our observations that the eggs hatched

without delay conflicts with the results of some investigators. Thornton and Brust (20) reported that only 5% of *R. communensis* eggs hatched at 20 C and suggested a state of dormancy prevented additional hatch. Finney (6) found that *R. culicivorax* eggs hatched intermittently and at a slow rate when stored in water at 27 C, but the addition of a mosquito larval homogenate stimulated hatch. Our results agree, however, with those of Petersen (13) who found a rapid hatch of *R. culicivorax* within 7 h of flooding cultures.

There are significant physiological implications from the experiments on postemergent development under various gaseous environments. After emergence *R. culicivorax* completes its development without feeding and therefore is dependent upon stored nutrient reserves in the trophosome for energy production. The trophosome contains large amounts of triacylglycerols, and it has been suggested that these function as substrate for catabolic energy production (7,10). Lipid catabolism, however, does not occur under anaerobic conditions whereas carbohydrate catabolism does (11). When O_2 is available, even at low levels, our results suggest that development is stimulated. Under anaerobic conditions development is somewhat slower, probably because of the reduced energy available during anaerobic carbohydrate catabolism. Lower O_2 tensions resulted in a decreased rate of movement and probably represented conservation of available energy. The requirement for CO_2 when very little or no O_2 is available is consistent with the idea of carbohydrate catabolism via a fumarate reductase pathway which requires CO_2 for the phosphoenolpyruvate carboxykinase reaction.

Von Brand suggested in an early review (21) that fresh water and marine muds were anaerobic and mentioned the importance of knowing the availability of O_2 in an animal's surroundings in deciding whether it leads an anaerobic existence. The experiments described here, along with the large body of experimental evidence demonstrating the lack of oxygen in sediments, indicate that the postparasitic stages of *R. culicivorax* function as facultative anaerobes. The more rapid development and requirement of CO_2 at 0.5% O_2 probably resulted from the use of anaerobic pathways to supplement aerobic pathways under low O_2 tensions as suggested by Atkinson (1) for other nematodes. The continued stimulation of development after exposure to aerobic conditions is probably the result of increased carbohydrate stores during aerobic lipid catabolism.

The results of the experiments suggest that when used as inoculum in field experiments, the postemergent stages of *R. culicivorax* would be less susceptible to anoxic conditions than the preparasites would be.

Current culture methodology is compatible with the gaseous requirements of the nematode. It appears that development is stimulated under the culture conditions which are aerobic. The times required for molting and initiation and termination of oviposition determined by Petersen (13) for cultures are close to times observed for nematode development in agar at 1% and higher O_2 concentrations. These times probably represent the minimum developmental period for the nematode at 27 C. Anaerobic atmospheres could serve to slow postemergent development of the nematode in mass-rearing systems for winter or offseason storage.

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