Effect of Temperature on Different Stages of *Romanomermis iyengari*, a Mermithid Nematode Parasite of Mosquitoes

KP Paily, K Balaraman

Vector Control Research Centre (Indian Council of Medical Research),
Indira Nagar, Pondicherry-605 006, India

The effect of temperature (20°-35°C) on different stages of Romanomermis iyengari *was studied. In embryonic development, the single-cell stage eggs developed into mature eggs in 4.5-6.5 days at 25-35°C but, required 9.5 days at 20°C. Complete hatching occurred in 7 and 9 days after egg-laying at 35 and 30°C, respectively. At 25 and 20°C, 85-96% of the eggs did not hatch even by 30th day. Loss of infectivity and death of the preparasites occurred faster at higher temperatures. The 50% survival durations of preparasites at 20 and 35°C were 105.8 and 10.6 hr respectively. They retained 50% infectivity up to 69.7 and 30.3 hr. The duration of the parasitic phase increased as temperature decreased. Low temperature favoured production of a higher proportion of females which were also larger in size.*

The maximum time taken for the juveniles to become adults was 14 days at 20°C and the minimum was 9 dyas at 35°C. Oviposition began earlier at higher temperature than at lower temperature. However, its fecundic period was shorter at 20°C than at 35°C indicating enhanced rate of oviposition at 20°C. Fecundity was adversely affected at 20°C and 35°C. It is shown that the temperature range of 25°-30°C favours optimum development of R. iyengari.

Key words: *Romanomermis iyengari* - embryo - preparasite - parasitism- postparasite - temperature effect

Romanomermis iyengari (Nematoda: Mermithidae) is a tropical species originally found in the haemocoels of *Anopheles subpictus* larvae from India (Welch 1964, WHO 1980). This species has been mass produced in the laboratory for many years (Gajanana et al. 1978, Paily 1990) and preliminary observations on the life cycle have been reported (Gajanana et al. 1978). The effects of temperature and parasite density on the parasitism and sex determination of *R. iyengari* have been studied and are similar to observations reported for *R. culicivorax* (Paily & Balaraman 1990).

The duration of different phases in the life cycle of mermithids varies between species, with the species of mosquito host and also with the temperature of the aquatic environment (Petersen 1985). These factors are important for the successful production and use of mermithid nematodes for mosquito control. Thornton and Brust (1979) studied the embryonic development of *R. communensis* at temperatures ranging from 5-20°C and found faster development at higher temperatures. In *R. culicivorax*, the survival and infectivity of preparasites was age and temperature dependent (Petersen 1975, Kurihara 1976). The optimum temperature range for development of its parasitic stage was 20-32°C (Hughes & Platzer 1977). Similarly, development and fecundity of postparasites of *R. culicivorax* and *R. nielseni* have been studied at different temperatures and were found to differ with the species and temperature (Petersen 1975, 1976). Comparable data are not available on the biology of *R. iyengari*. In this study, the effects of temperature on embryonic development, survival and infectivity of preparasites; parasitic and postparasitic development and female fecundity and oviposition were investigated.

MATERIALS AND METHODS

All stages of *R. iyengari* were obtained from a cyclic colony maintained at the Vector Control Research Centre, Pondicherry, following the method of Petersen and Willis (1972). The host larvae used were laboratory reared *Culex quinquefasciatus* II instar, the most susceptible larvae to *R. iyengari* infection under laboratory conditions (Paily 1990). The source colonies of the mosquito and the nematode were raised at a temperature of 30±2°C.

Embryonic development - About 500 gravid females of *R. iyengari* were placed in 100 ml chlorine-free tap water in a beaker for egg-laying. After 4 hr, the nematodes were removed and the egg

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suspension was diluted to 25 eggs/ml. Ten ml of this suspension (250 eggs) was transferred to a cavity block (5 cm diameter x 1 cm height). Sixteen such cavity blocks containing eggs were covered with glass plates and four each were incubated at 20, 25, 30 and 35°C in BOD incubators. Embryonic development was monitored at 12 hr intervals for 30 days. For this, 1 ml of the suspension was withdrawn from each cavity block, placed into cavity slides and examined under a microscope. The number of eggs matured i.e., eggs that reached the late coil stage, and the number of eggs hatched, were determined following the method of Thornton and Brust (1979). After examination, the samples were returned to the cavity blocks. The percentage of eggs matured and hatched was calculated from the total number of eggs present on the slides. Because of poor rate of hatching of mature eggs at 20 and 25°C, these cavity blocks were transferred to 30±2°C after 30 days and observation was continued.

Survival and infectivity of preparasites - Preparasitic nematodes were collected from two month old sand cultures of *R. iyengari* as per the method of Petersen and Willis (1972). Briefly, the culture was flooded with chlorine-free water for 12 hr and the water containing the preparasitic nematodes was collected in beakers. This was allowed to stand for 30 min to facilitate settling of eggs, if any. Then, 75% of the water was aspirated off and diluted with tap water to get 75 preparasitic nematodes/ml. This suspension was divided into four portions (1 litre each), transferred to beakers and incubated at 20, 25, 30 and 35°C. The preparasitic nematodes were examined for survival and infectivity. To determine the survival rate, 1 ml samples (n=10) were withdrawn from each beaker at 24 hr interval and examined for actively swimming ones. The percentage survival was calculated from the initial count and the number of survivors at different time intervals. The infectivity of preparasitic nematodes was determined by withdrawing 150 from each beaker at 24 hr interval. These were incubated at room temperature (28°C) for 2 hr and then *C. quinquefasciatus* second instar larvae (50) were exposed to them for 24 hr in enamel bowls containing 50 ml of tap water. The proportion of host larvae infected by the preparasitic nematodes was observed under a microscope. All the tests were replicated four times. The median time of the preparasitic nematode survival and infectivity at different temperatures were determined by probit regression analysis (Finney 1971).

Parasitic phase and postparasite emergence - Host larvae (4000) were exposed to freshly hatched preparasitic nematodes at a host-parasite ratio of 1:5, to ensure about 90% parasitism (Paily & Balaraman 1990), at 28°C for 2 hr in enamel trays (25x20 cm) containing 1 litre of chlorine-free tap water. The larvae were washed with tap water, divided into 16 equal lots (250 larvae/lot), transferred to enamel trays containing 1 litre of water and reared at 20, 25, 30 and 35°C, in quadruplicate.

The mortality and pupation of the host larvae and emergence of the postparasites were monitored at 12 hr intervals. Dead larvae and pupae were dissected and examined for parasitic stages of *R. iyengari*. Dead larvae with a ruptured cuticle and characteristically shrunken body-wall were classified as dead due to nematode emergence. The sex ratio of postparasites collected from each tray was determined by examining them for the presence or absence of genital opening (vulval anlagen) of the female and spicules of the male (Welch 1964). Emergent postparasitic juveniles of *Romanomermis* spp. are identifiable as male or female but do not become sexually active until they moult to adult (Tingley & Anderson 1986). At the end of the experiment, 100 male and 100 female postparasites from each temperature group were selected randomly, killed and fixed in hot (60°C) 70% alcohol and their length was measured with a calibrated ocular micrometer.

The percentage parasitism was calculated after taking into account the dead and pupated larvae that were negative for parasites and infections that did not produce postparasites. The parasite burden (mean number of parasites per host larva) was estimated from the number of postparasites emerged and the number of parasites dissected from dead and surviving mosquitoes. The proportion of female postparasites was calculated from the total number of males and females emerged.

Postparasitic development, oviposition and fecundity - Postparasitic juveniles of *R. iyengari* were obtained from the source colony and cultured in distilled water using standard techniques (Paily 1990). The male and female postparasitic juveniles were separated under a dissection microscope within 12 hr of their emergence from the host (*C. quinquefasciatus*) larvae. One hundred individuals of each sex were placed in 250 ml conical flasks containing 200 ml distilled water, in quadruplicate. These were incubated at 20, 25, 30 or 35°C. Males are polygamous (Tingley & Anderson 1986) and a male to female ratio of 1:1 facilitates mating if the nematodes are cultured in water (Paily 1990). The water in the flasks was changed every second day so as to remove the cast skin. Clumps of the nematodes were loosened by stirring the water with a glass rod. From the second day of seeding, 25 males and 25 females were withdrawn randomly from each flask at 24 hr intervals, observed for

moulting (exsheathment of the last larval cuticle as indicated by the absence of tail filament) and returned into the same flask. This was done until the last postparasitic juvenile had moulted.

These cultures were monitored for oviposition at 4 days interval from the time of initiation of egg-laying until the death of all the females. For this, the flasks were shaken to suspend the eggs and preparasites and ten samples of 1 ml each were withdrawn from each flask, examined for the presence of eggs and preparasitic nematodes and recorded after counting. After such observations, the nematodes were transferred to a new set of flasks containing 200 ml of water. The total number of eggs produced during the observed period was calculated by multiplying the number present in 1 ml with the volume of water in the flask (200 ml).

Mortality of postparasites in the flasks, if any, was noted on different days. The dead females seen after the commencement of oviposition were examined under microscope to determine whether or not they had completed egg-laying. Females that had completed egg-laying were identified by the presence of empty follicles and absence of trophosome. The experiment was repeated four times and the results presented are the average of the replicates. The number of eggs laid/female in flasks incubated at different temperatures was calculated from the total number of eggs produced in each flask and the number of females that completed egg-laying.

The data were analyzed by one-way ANOVA to find out whether significant differences existed in the parasitism, production of females, parasite burden, length of the postparasites and the number of eggs laid by female postparasites due to incubation at different temperatures. For this, the results in percentages were transformed into angular values and the least significant difference or critical difference was used for comparison at 5% level (P=0.05). The median day of completion of parasitic life (day by which 50% of the nematodes completed the parasitic phase) and the median day of oviposition (day by which 50% of the eggs are laid) were calculated using the method of Snedecor and Cochran (1968). The values were plotted on a graph and free-hand smooth curves were drawn to see the relative frequency (%) of embryonic development, survival and infectivity of preparasites, and moulting, oviposition and mortality of the postparasites (Hills 1974).

RESULTS

The maturation of embryos from single-cell stage into late coil stages (mature eggs) (Fig. 1) and initiation and completion of egg hatching

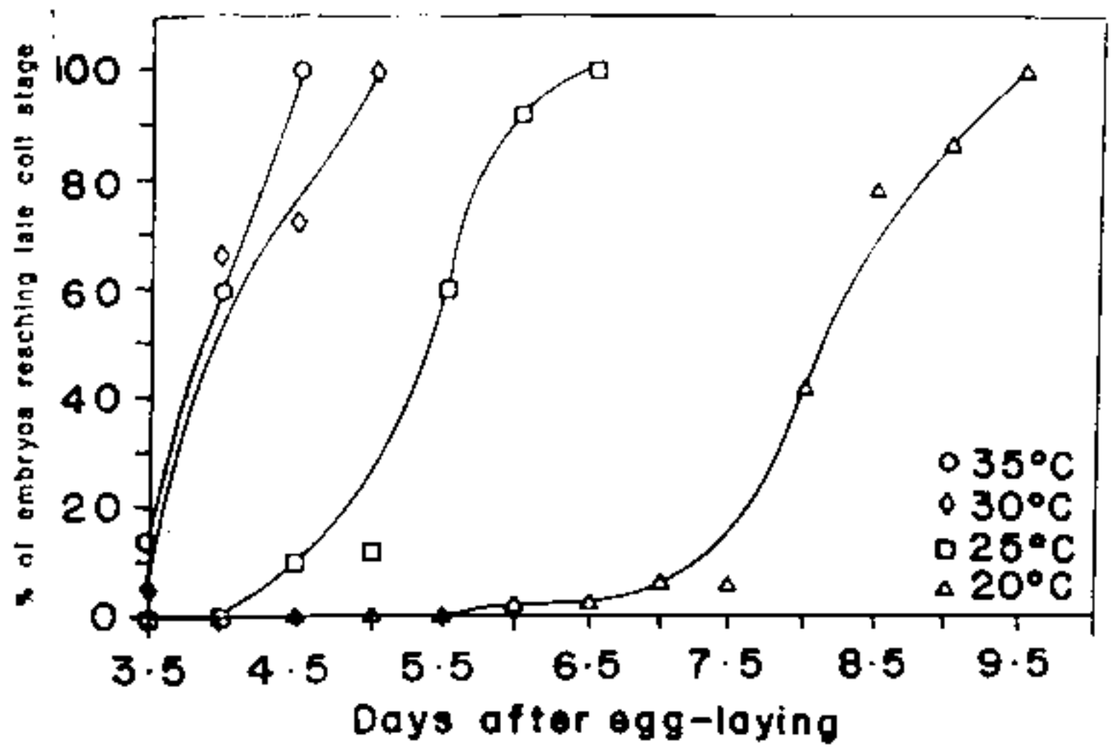

Fig. 1: embryonic development of *Romanomermis iyengari* at different temperatures.

TABLE I

Percentage of *Romanomermis iyengari* eggs hatching on different days after laying

Days after egg-laying	% of hatching			
	20°C	25°C	30°C	35°C
4.5	0.0	0.0	0.0	2.0
5.0	0.0	0.0	0.0	8.0
5.5	0.0	0.0	0.0	14.0
6.0	0.0	0.0	0.0	26.0
6.5	0.0	0.0	2.0	56.0
7.0	0.0	0.0	20.0	100.0
7.5	0.0	0.0	30.0	
8.0	0.0	0.0	38.0	
8.5	0.0	0.0	72.0	
9.0	0.0	0.0	100.0	
11.5	0.0	0.0		
12.0	0.0	2.0		
13.0	0.0	4.0		
15.0	0.0	12.0		
17.0	0.0	15.0		
18.0	2.0	15.0		
23.0	4.0	15.0		
30.0	4.0	15.0		
31.0	10.0[a]	28.0[a]		
32.0	25.0[a]	70.0[a]		
33.0	40.0[a]	100.0[a]		
34.0	40.0[a]			
35.0	40.0[a]			

[a]: hatching after the eggs transferred to room temperature (30±2°C)

(Table I) was faster at higher than at lower temperatures. The time taken for 50% of the embryos to become mature eggs ranged from 3.9 days at 35°C to 8.1 days at 20°C. The initiation of egg hatching at 20°C was 13.5 days later than at 35°C. All eggs hatched within 7 and 9 days of egg-lay-

TABLE II

Probit regression of time against mortality (A) and infectivity (B) of preparasites of *Romanomermis iyengari* at different temperatures

	Temperature (°C)	a	b	X^2	LST-50[a]/ LIT-50[b]	LCL[c]	UCL[d]
A	35	3.06	0.82	1.63	10.59	5.38	20.86
	30	-0.73	1.48	10.84[e]	48.12	39.22	59.03
	25	-0.63	1.40	8.39	56.13	50.59	62.26
	20	-3.85	1.90	21.13[e]	105.77	92.04	121.53
B	35	9.45	-1.30	10.44[e]	30.29	19.11	48.02
	30	9.74	-1.33	6.77	34.79	29.07	41.63
	25	10.05	-1.35	7.17	42.13	36.11	49.15
	20	9.52	-1.06	18.50[e]	69.73	53.33	91.17

[a]: 50% survival duration of preparasites
[b]: 50% infectivity duration of preparasites
[c]: lower confidential limit
[d]: upper confidential limit
[e]: heterogenity is significant at 5% level. For this the LCL and UCL were adjusted

TABLE III

Effect of temperature on the parasitic stage of *Romanomermis iyengari*

Tempe-rature (°C)	Mean % infection ±SD	Mean parasite burden/ larva±SD	Mean % of female produced ±SD	Mean length of postparasites (mm±SD)		Duration between		Median day of completion parasitic stage[a]
				male	female	infection and 1st emergence (days)	1st and last of emergence (h)	
35	89.3±2.52	2.0±0.01	48.7±1.46	9.5±1.59	13.3±3.99	3	24	3.4
30	88.3±2.11	2.3±0.64	51.6±1.49	9.3±1.46	16.1±3.64	4	48	4.8
25	90.2±2.62	2.5±0.25	54.4±6.51	10.4±1.47	15.5±3.30	7	60	7.4
20	87.8±0.44	2.2±0.23	62.9±1.36	11.6±1.50	18.8±3.52	10	84	10.7

[a]: day by which 50% of the nematode completed the parasitic phase

ing at 35 and 30°C, respectively. At 25 and 20°C, 85-96% of the eggs had not hatched by day 30. Transfer of unhatched eggs from 25° to 30±2°C resulted in 100% hatching. However, the hatch was only 40% when unhatched eggs were transferred from 20° to 30±2°C.

Preparasites died earlier at higher temperatures (Fig. 2). While the maximum decrease in their survival was 78% during 0-24 hr period at 35°C, it was 27% during 120-144 hr period at 20°C. The maximum decrease was 28% during 24-48 hr at 30°C and 24% during 48-72 hr at 25°C. Infectivity of the preparasitic nematodes was less and lost faster at higher than at lower temperatures (Fig. 2). The maximum decrease was 47% during 0-24 hr period at 35°C while it was 23% during 72-96 hr period at 20°C. It was 39% during 0-24 hr at 30°C and 33% during 48-72 hr at 25°C. Probit regression analysis of the data showed (Table II) a 50% survival duration (LST50) of 10.6 and 105.8 hr and infectivity duration (LIT50) of 30.3 and 69.7 hr at 35 and 20°C, respectively.

The mean percentage infection and parasite burdens of the host larvae reared at different tem-

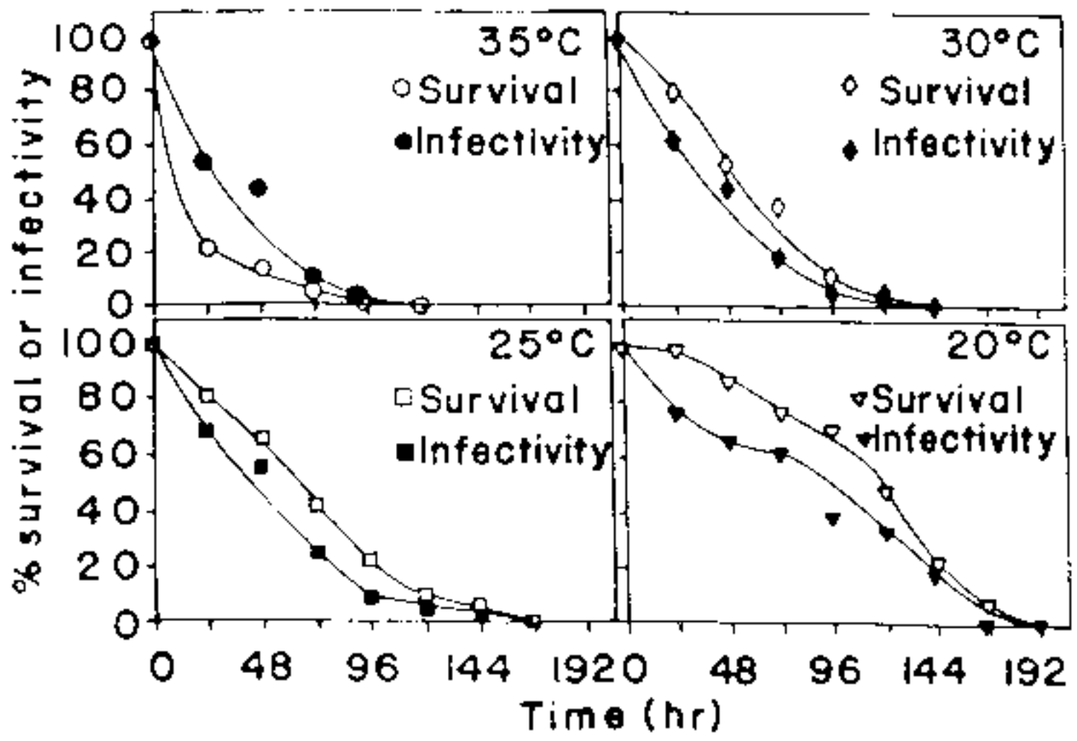

Fig. 2: survival and infectivity of preparasites of *Romanomermis iyengari* at different temperatures.

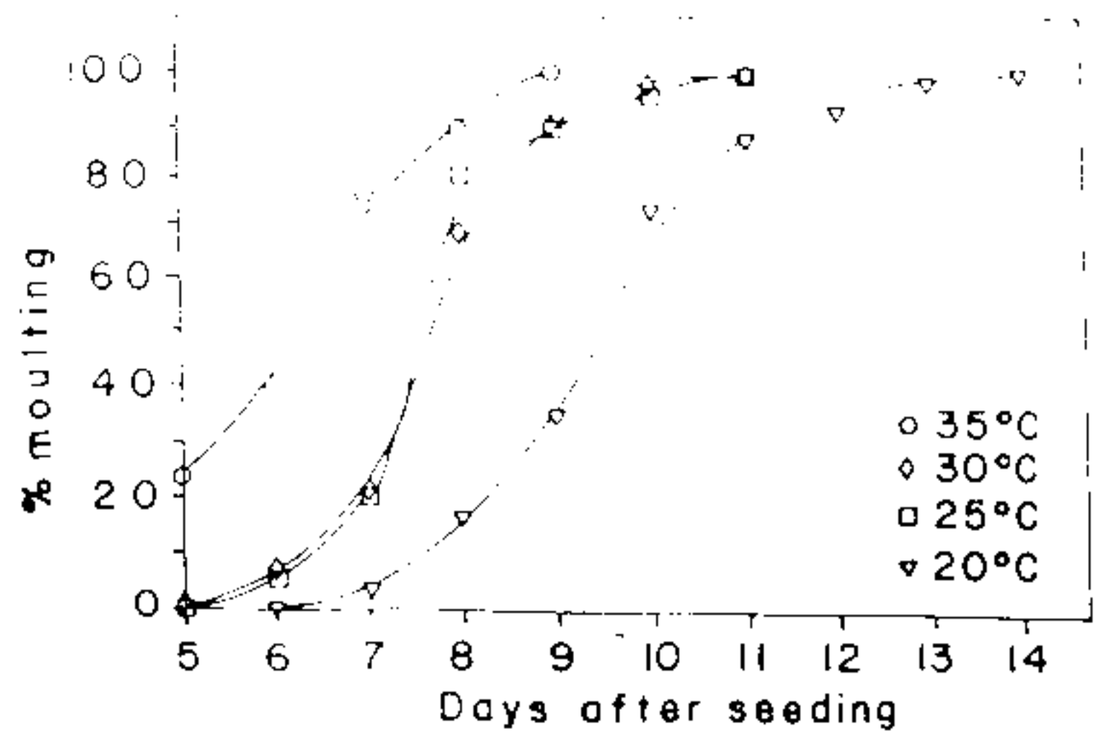

Fig. 3: moulting of postparasites of *Romanomermis iyengari* in cultures maintained at different temperatures.

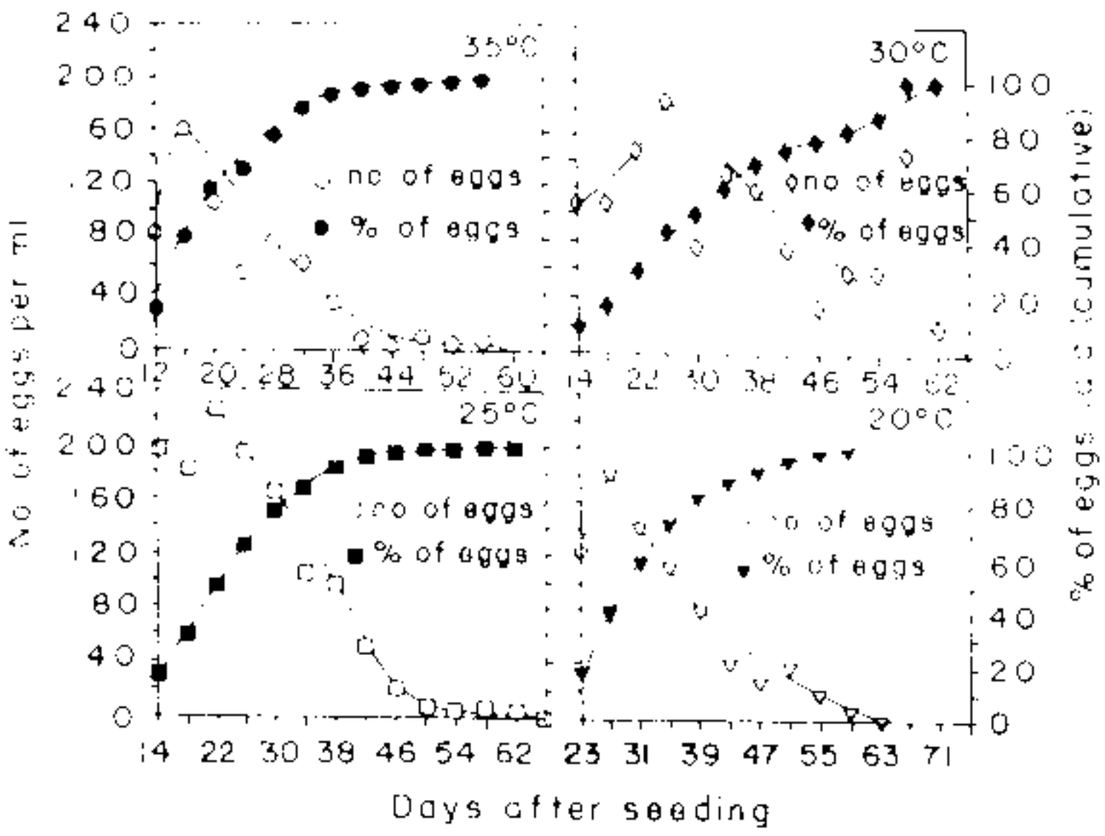

Fig. 4: oviposition pattern of *Romanomermis iyengari* in cultures maintained at different temperatures.

peratures were 87.8-90.2% and 2.0-2.5 respectively, at 20-35°C (Table III). There was no significant difference in the percentage parasitism (F=2.57; P=0.19) and parasite burden (F=0.53; P=0.68) at different temperatures. As the infection was done in a single tray at room temperature, there should not be any difference in parasitism and parasite burden unless host/parasite mortality occurs at certain temperature. The production of females was significantly higher at 20°C than at other temperatures (F=8.96; Critical difference=0.10; P=0.03). Significant difference was noticed in the length of female postparasites between 20° and 25°C, and 30° and 35°C (F=39.22; Critical difference=1.00; P<0.01). The length of male postparasites was significantly greater at 20°C than at all other temperatures (F=48.82; Critical difference=0.42; P<0.01).

TABLE IV
Fecundity of *Romanomermis iyengari* in cultures maintained at different temperatures

Temperature (°C)	No.of nematodes completing oviposition	Total no. of eggs laid	Mean no. of eggs /female ± SD	Duration of oviposition	
				No. of days	Median day[a]
35	95	121400	1278± 79.4	44	17.7
30	96	252000	2625±281.3	48	22.6
25	95	255000	2684±444.3	48	22.6
20	91	155200	1705±160.9	36	28.7

[a]: day by which 50% of the eggs are laid

Postparasite emergence after infection began earlier and was completed faster at higher temperatures (Table III). It commenced on the 3rd day at 35°C and 10th day at 20°C and was completed within 24 and 84 hr, respectively. The median day of completion of parasitic phase was 3.4 at 35°C and 10.7 at 20°C.

Moulting of postparasitic juveniles into adults started earlier at higher temperatures (Fig. 3) i.e., on 5th day at 35°C and was completed within 5 days. Similarly, oviposition commenced on the 12th day at 35°C and was delayed by 11 days at 20°C (Fig. 4). However, the whole oviposition process was completed within 44 and 36 days at 35 and 20°C respectively, in contrast to 48 days at 30° and 25°C (Table IV). Most eggs were laid 4-8 days after the commencement of oviposition at 35 and 20°C (160 and 180 eggs/ml, respectively), 8-12 days at 25°C (226/ml) and 12-16 days at 30° (186/ml). The median day of oviposition was 17.7 and 28.7 respectively, at 35 and 20°C. However, at 25° and 30°C, it was 22.6 days.

The mean number of eggs produced per female at 20 and 35°C were significantly lower than at 25 and 30°C (F=24.97; Critical difference=418.58; P<0.01). While the eggs laid per female at 20°C

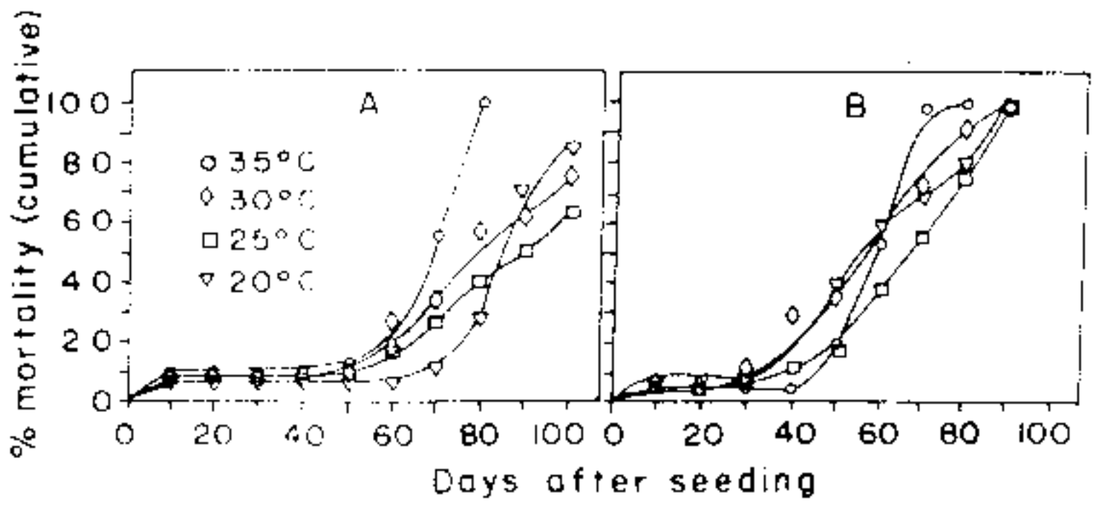

Fig. 5: mortality of male (A) and female (B) postparasites of *Romanomermis iyengari* in cultures maintained at different temperatures.

was significantly higher than at 35°C, there was no significant difference in the per female egg production at 25 and 30°C.

The mortality of postparasites before egg-laying was higher at 20°C than at other temperatures (Fig. 5). The mortality rate of spent females was higher at 35°C and all the males and females died by the 80th day. At 20-30°C, all the females died by the 90th day, while 15-37% of the males survived to 100 days.

DISCUSSION

The duration of embryonic development in *R. iyengari* was 9.5 days at 20°C and decreased as the temperature increased to 25, 30 and 35°C. Influence of temperature on embryonic development has been reported in the case of *R. communensis* and it was found to be 16.5 days at 20°C, 22 days at 15°C and 32 days at 10°C (Thornton & Brust 1979). However, as the data indicate, there is a difference of 7 days in the duration of embryonic development between these two species at comparable incubation temperatures (20°C) and it may be due to the inherent differences in their developmental periods.

The percentage egg hatching of *R. iyengari* was 4 at 20°C and it increased as the temperature increased to 25, 30 and 35°C. The optimum temperature for 100% egg hatching was 30°C. The low level of egg hatching observed at 20°C is similar to that of 5% hatching reported for *R. communensis* (Thornton & Brust 1979). When eggs incubated at 25°C were transferred to 30±2°C, the hatch increased from 15% to 100% indicating that 85% of the eggs that were dormant at the former temperature were activated. Similar activation did not occur in the case of eggs incubated at 20°C and the hatch was only 40%. This may be possibly due to irrepairable damage the eggs suffered at the time of incubation at 20°C.

The effect on preparasites was the least at 20°C as it did not cause rapid decrease in survival until 120 hr and infectivity until 72 hr. Petersen (1975) has reported that the survival and infectivity of the preparasites of *R. culicivorax* decreased as time passed after hatching, at 24-27°C. Low temperatures were found to prolong the life span of the preparasites of *R. culicivorax*, the LST50 values were being 105.8, 43.0 and 25.2 hr, respectively at 20, 25 and 30°C (Kurihara 1976). Tingley (1980) observed that the life span of the preparasites of this species decline linearly with increase in temperature from 10-35°C. He has also noted that the ability to infect hosts was retained by the preparasites until the end of their life span.

In the case of *R. iyengari*, although the LST50 duration of survival of the preparasites at 20°C is more or less the same as that reported for *R. culicivorax*, at 25 and 30°C, it was higher by 13.7 and 22.9 hr respectively. This indicates that the preparasites of *R. iyengari* can survive longer at 25 and 30°C compared to *R. culicivorax*. When the LST50 and LIT50 durations of the preparasites of *R. iyengari* are compared, the infectivity was more adversely affected than survival at 20, 25 and 30°C. Whereas the reverse was the case at 35°C. Infectivity of preparasites being determined by various behavioural/ physiological factors such as the ability to locate and penetrate the host may be affected by environmental temperatures more drastically than survival. Although the preparasites survive longer, their ability to infect the host is not retained till the end of their life span and no relationship exists between survival and infectivity.

Fewer females were produced at higher temperatures and these were also smaller. The reduction in the body length is reported to reduce the reproductive potential of the females of *R. culicivorax* (Tingley & Anderson 1986). It has also been observed that smaller postparasites of *R. culicivorax* from nutritionally deprived hosts had very little trophosomal storage material which is required for energy metabolism, egg development etc. (Gordon et al. 1981).

The duration of the parasitic phase of *R. iyengari* and also the total time taken for emergence of all the postparasites was longer at lower temperatures. This observation is in conformity with that noted by Hughes and Platzer (1977) for *R. culicivorax* in *Culex pipiens*. However, they reported median developmental durations of 11.5, 7.1 and 5.8 days for this mermithid species respectively at 20, 27 and 32°C which are relatively longer than that of *R. iyengari*. As reported in an earlier study on *R. iyengari* (Paily & Balaraman 1990), lower temperatures favour the emergence of a higher proportion of females. In the case of *R. culicivorax*, the availability of food resources to the parasite in the host has been attributed as the reason for the occurrence of such a phenomenon (Tingley & Anderson 1986). That is, the availabil-

ity of nutrients to the parasite might be greater at lower temperature favouring production of more number of females.

The moulting of *R. iyengari* juveniles to adults was faster at higher temperature and was slower at lower temperature. There was no difference in the rate of moulting between male and female postparasites. On the other hand, Petersen (1975) has reported that at 24-27°C, the males of *R. culicivorax* matured 10-14 days earlier than females.

The rate of oviposition of *R. iyengari* at 25° and 30°C was, by and large, unaffected. However, oviposition began and ended earlier at the extreme higher (35°C) temperature. This may be due to earlier moulting/ maturation of juveniles into adults and/ or earlier mating/ egg development at higher temperature. At the extreme low temperature (20°C), though the commencement of oviposition was delayed, the time taken for completion was shorter due to an increase in the rate of oviposition. That is, eggs were laid as soon as they developed which compensated for the postponed commencement of oviposition. With respect to *R. culicivorax*, the first group of females exhibiting egg development and oviposition were observed 25-30 days (at 24-27°C) after emergence and the oviposition period was 18 days (Petersen 1975). This indicates that *R. iyengari* starts oviposition earlier than *R. culicivorax* but complete it in a much longer period.

The mean egg production per female at 25 and 30°C corresponds to the earlier reported number of 2480 eggs per female of *R. culicivorax* (Petersen 1975). However, the fecundity of *R. iyengari* was adversely affected at the highest and lowest temperature tested and why the egg production is reduced at these temperatures is not known. Perhaps, at extreme temperatures more energy is used for survival rather than for egg production. Mermithids do not feed after emergence from the host and their energy requirements are obtained from stored food reserves in the trophosome. The females are semelparous (Wharton 1986), ie., they breed once and then die. The females of *R. iyengari* have died by 90 days after emergence, a few days after completing egg-laying. Males remained active longer, but did not live up to 180 days as reported for *R. culicivorax* (Petersen 1975).

Results of the present study indicate that the duration of different stages in the life cycle of *R. iyengari* varies from other species such as *R. culicivorax* and *R. communensis* and it can be differentially influenced by the environmental temperature. Though the embryonic development of this species was not studied at temperatures lower than 20°C, a more pronounced adverse effect might be observed at temperatures of 15, 10 or 5°C. The information obtained from this study is important in mass production of *R. iyengari* because maintenance of the culture under suitable temperature (25-30°C) can increase the yield through optimum levels of female production and fecundity.

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