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Entomopathogenic Nematodes as Biological Control Agents of Ticks

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**השפריה המרכזית
למדעי החקלאות
בית דגן**

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Abstract

This research project was aimed to create a basis for the use of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of ticks. The specific objectives were- to determine: 1. Nematode virulence to various . 2. Host-parasite interactions of nematodes and ticks. 3. Effect of environmental factors of tick habitats on nematode activity. 4. To test nematodes (anti tick activity) in defined field trials.

Throughout the project 12 nematode strains from five species were tested in laboratory assays against all developmental stages of eight tick species. All tick species were found susceptible to nematode infection. The nematode strains IS-5 and IS-12 of *Heterorhabditis bacteriophora* were found to be the most virulent. Engorged adults, particularly females, were the most susceptible stages.

Despite the high susceptibility, ticks are not suitable hosts for nematode development and propagation. Entomopathogenic nematodes enter ticks and kill them by releasing the symbiotic bacteria from their foregut.

Under favorable conditions, i.e. moist soil, moderate temperature (22-27°C) and sandy soil, nematode efficacy against *B. annulatus* engorged females was very high (>90% control). However, low soil moisture (> 5% w/w) and high animal manure concentration in soil adversely effect nematode efficacy. In field trials, nematodes were effective when soil moisture was maintained at high levels.

The results indicate that under favorable conditions the nematodes show promise as a biological control method for ticks. However, we still face several potential obstacles to the use of nematodes under less favorable conditions.

Original objectives:

Objective 1: To determine the virulence of nematodes for various species of ticks of economic importance to animal health in participating countries.

Objective 2: To study the development and host-parasite interactions of entomopathogenic nematodes in ticks.

Objective 3: To characterize environmental factors of tick habitats that may affect nematode persistence and activity.

Objective 4: To test use of virulent nematodes for control of susceptible ticks in defined field trials.

Relevant data:

Objective 1: Virulence of nematodes to various species of ticks.

Field survey of new efficient anti tick nematode isolates (Israel)

Soil samples from various areas in Israel were collected in order to search for more efficient anti tick entomopathogenic nematodes. The samples were from areas with domestic animals or from other agricultural locations. Altogether 144 soil samples were collected from 19 settlements. The samples from various potentially infected places were brought to the laboratory. Larvae of *Galleria mellonella* and engorged *B. annulatus* females were placed in small net cages. The cages were covered with soil and arthropod mortality was recorded on days 2, 4, 6, 8, 10. The dead bait arthropods were placed into collecting petri dishes in order to observe the release of nematodes.

Three new isolates; *Heterorhabditis bacteriophora* (H.b.) -IS-5 (found in the Negev near Kibbutz Nir-Izhak), *H. bacteriophora* -IS-3(found in the Arava near Kibbutz Yahel) and *Steinernema feltia* (S.f.) -TG (found in the Israeli Judean plains) near Tel Goded in a cattle yard as well as *Steinernema carpocapsa* (S.c.) – All (a commercial strain) and other strains listed in the first annual report which appeared to be more promising were studied in more detail. See bellow.

Infectivity of nematodes to different tick species and developmental stages (Israel & USA)

See details in appendix 1,3-5.

The ticks tested in Israel belonging to the species *Boophilus annulatus*, *Rhipicephalus sanguineus*, *R. bursa* and *Hayalomma exavatum*. In the US the tick species *A. americanum*, *A. cajennense*, *A. maculatum*, *Dermacenter variabilis* as well as *R. sanguineus*.

The susceptibility of different developmental stages of these ticks (larvae, nymphs, and adults) were tested with a variety of nematode species and strains which were applied at different concentrations. The initial procedures for evaluation of nematode infectivity against the ticks was developed by the Israeli team. This knowledge was then transferred to the US team by reciprocal visits of Dr. Kocan at Dr. Glazer laboratory at Rutgers (where he spend a sabbatical) and Dr. Glazer and dr. Samish at Oklahoma State University during the first year of the project. The Israeli team also provided nematode strains to Dr. Kocan.

The details of the procedures and the results are given in the enclosed appendixes.

In a laboratory assay, the susceptibility was tested of different larval, nymph and adult stages of four tick species to 12 nematode strains from the families Steinernematidae and Heterorhabditidae, including 5 Israeli isolates. The nematodes were reared at 25°C in the last instar of *Galleria mellonella* according to the method of Kaya and Stock (1997). The techniques in insect nematology in "Manual of techniques in insect pathology. Academic Press.

The effect of nematode strains upon the mortality of the various tick species and stages was determined in petri dish bioassays. The nematodes were applied on a surface of moist filter paper at different concentrations (200, 1000, 5000 IJs/dish. Then ticks were placed in these dishes. Tick mortality was recorded daily. Control treatment consisted of nematode-free dishes.

Susceptibility of all three tick species, to nematode infection ranged from poor (< 35%), and moderate (35-65%) to high (>65%) depending on the combination between the developmental stage of the ticks and nematode strain.

Nematode strains. Nematode virulence is highly influenced by the target host. Since larval and nymph stages showed poor susceptibility, only nematode virulence to adults was compared. Furthermore, since we found in several cases differences in mortality levels between engorged and unfed adults the data concerning each feeding state was analyzed separately. High mortality levels (75-95%) were recorded with the IS-3 and IS-5 strains of *H. bacteriophora*. Whereas, low or moderate mortality was recorded with HP88 of *H. bacteriophora* and the DT and Mexican strains of *S. carpocapsae*.

In most treatments tick mortality was proportional to the nematode concentrations. However with engorged adults of *R. sanguineus* and *R. bursa*, moderate to high levels of mortality were recorded even with the lowest nematode concentrations.

Tick species. Highest mortality for all four investigated tick species was recorded among engorged adults (average >73.2%). Engorged *R. bursa* adults are comparatively less susceptible and most susceptible one is the *B. annulatus* tick. Moderate to poor mortality rates were recorded for all other non-adult developmental stages of unfed and engorged ticks of all three species

Tick developmental stages. The engorged adults, particularly females of *R. sanguineus* were most susceptible. No marked difference was found between the mortality of engorged ticks and unfed adult stages of *R. sanguineus* or *H. excavatum* whereas the mortality of engorged males and unfed females of *R. bursa* was significantly higher ($P < 0.01$) than that of unfed males or of engorged females.

Techniques were developed in the laboratory at Oklahoma State University to propagate several strains of entomopathogenic nematodes, including *Steinernema glaseri* (SG), *S. riobravensis* (SR), *S. carpocapsae* (DT), *S. feltiae* (SF) and *Heterorhabditis bacteriophora* (HP88). Stocks of these nematodes were obtained from Dr. I. Glazer, ARO, The Volcani Center, Bet-Dagan, Israel and from Dr. R. Gaugler at the Department of Entomology, Rutgers University, NJ, USA, where Dr. Glazer spend his sabbatical. *Galleria* moth larvae were obtained from Northern Bait Co., Chetek, WI and

used as hosts for production of the various species of entomopathogenic nematodes according to the procedures of Kocan *et al.* (Appendix 4,5). Each of the five species of nematodes were then used to expose replete *Ambliomma americanum* ticks and all five killed a percentage of these ticks. Egg mass weight of exposed ticks of each species were significantly lower than those of the control. Two species of nematodes, SRs and SFs, were selected for further studies because they appeared to be the most effective in killing ticks. The SR nematodes appeared to be most readily attracted to the replete ticks and clustered around the mouthparts, spiracles, anus and genital opening shortly after being placed in the exposure dishes (Appendix 4).

Before testing these two species of nematodes on several species of ticks, we first conducted experiments to determine an effective dose. Preliminary experiments were done using a nematode exposure dosage of 10,000 per dish. Three exposure doses were tested (10,000, 5,000 and 1,000/dish) and the 5,000 dose was found to be comparable to the 10,000 dose while the killing rate was greatly reduced in the 1,000 exposure dose group (Appendix 4).

Replete females of several tick species of economic importance in the United States were then tested with the SR and SF entomopathogenic nematodes (Appendix 5), including *Amblyomma cajennense*, *A. maculatum*, *Dermacentor variabilis* and *Rhipicephalus sanguineus*. Although the percentage of ticks killed varied among tick species, the nematodes killed all species of ticks and thus did not appear to be host specific. The variable killing rate of the tick nematode combinations may have resulted from undefined factors, such as variations in the viability of the various nematode stocks.

Objective 2: Host-parasite interactions of nematodes and ticks.

In this part of the project we determined aspects related to the different parts of the infectivity process, i.e. attraction of nematodes to ticks, penetration and penetration rate and final establishment and development of bacteria or nematodes in the tick body. We used the particular experience of each laboratory to achieve the best result. In Israel we concentrated on studying the

biological aspects, based on the vast experience gained already with nematode-insect interactions. In the US we utilized our great experience in electron microscopy and tick ultrastructure study for this purpose. The specific results are:

Attraction of Nematodes to Ticks (Israel)

The testing technique is a modification of that described previously (Lewis *et al.*, 1992; Parasitology, 105:315-319). A 2% Gel Write Agar was poured into a 9 cm Ø petri dish and kept refrigerated until use. One hour before use the petri dish was opened in a sterile hood to dry out. Nematodes were placed on a 5 mm Ø filter paper (Whatman No. 1) and sucked by low vacuum through the paper. The filter paper with the semi dry nematodes was placed on the agar in the center of the petri dish (Fig. 1-III) and attractant/repellent arthropods or water used for washing tick cuticles were placed on 2 opposite sides near the rim of the plate (Fig. 1, I&II) The arthropods were inserted into a 5 ml pipette tip and its top closed with modeling clay. The pipette tip was placed through an opening in the petri dish 1 mm above the agar. In some experiments the arthropods were placed directly on agar or on 1.5 cm Ø aluminum foil (so as to observe the possible influence of volatile compounds) and covered with a 1 ml plastic cap 1 cm Ø with 3 tiny holes near the agar to prevent the arthropods from wandering on the agar. The influence of arthropod extracts on the movement of nematodes was observed by placing 5 arthropods in 2 ml water for 2 h, discarding the arthropods and transferring a 3µl aliquot of the water used for washing the cuticle on 0.5 cm Ø filter paper (Whatman #3) placed on the agar (Fig. 1-I). Filter paper with water served as control (Fig. 1-II). After 2 h in darkness at 26°C the filter paper with the adherent nematodes was removed and the number of nematodes recorded in areas α and β (Fig. 1). The average amount of nematodes counted in areas α and β after 2 h of incubation was calculated ($\pm$ SD). In some instances almost all of the nematodes remained for unknown reasons outside of sections α and/or β . Dishes where we found in sections α and β less than a total of 5 nematodes were not taken into account.

Fig.1 - Design of a nine cm. diameter petri dish with agar.

Nematodes placed on 0.5 cm filter paper circle put in center (III), and attractants/repellents in sector I and/or II. After 2h. incubation the nematodes in the shaded sectors were counted

Table 1 The influence of volatile compound(s) excreted by arthropods placed in the tip of a pipetter or on aluminium foil (I) on the movement of *H.Bacteriophora* -HP88 nematodes on agar.

Arthropodes and nematodes placed simultaneously and incubated for 2h at 26° C

Arthropods					# petri dishes	Avr. # nematodes in sector		Avr. x	± SE	t
	Species	Stage	Weight(mg)	#/ dish		x α	x β			
In tip of pipetter	<i>B.annulatus</i>	female ¹	280.0	3	12	67.3	11.2	70.6	8.7	2.35*
	<i>G.mellonela</i>	larvae	139.0	3	11	131.3	6.8	91.3	4.23	9.78**
	<i>H.excavatum</i>	nymph	16.7	8	24	35.7	7.2	64.7	18.6	0.78
	<i>H.excavatum</i>	unfed adults	8.5	32	4	93.0	8.0	84.8	8.68	4.01**
	<i>R.sangvineus</i>	nymph	8.2	32	8	33.0	8.0	75.1	8.62	2.91**
On aluminium foil	<i>B.annulatus</i>	female ¹	280.0	3	10	20.5	0.5	96.4	5.58	12.96**
	<i>G.mellonela</i>	larvae	139.0	3	8	62.0	1.1	97.2	1.99	23.7**

1-engorged *P<0.05

Table 2 The influence of arthropodes (3/ dish) or their cuticle extracte in contact with the agar on the movment of *H.Bacteriophora* -HP88 nematodes.

Arthropodes placed on agar 0 or 2 h before the nematodes and incubated for 2 h at 26° C

	Area		Arthropod	# petri	Avr. # nematodes		Avr.	± SE	t
	I	II	h. before	dishes	in sector		×		
	(test)	(control)	nematodes		× α	× β			
Live arthropod	<i>B.annulatus</i>	Air	0	16	10.4	16.6	43.7	9.66	-0.65
	(engorged female)	Air	2	18	6.4	21.3	28.6	5.92	-3.6**
	<i>G.mellonela</i>	Air	0	10	11.6	4.2	58.9	12.04	-0.74
	(larvae)	Air	2	4	3.0	6.8	34.4	17.21	-0.91
cuticle wash water	<i>B.annulatus</i>	Water	0	36	8.4	26.0	36.3	5.67	-2.42*
	(engorged female)								
	<i>H.excavatum</i>	Water	0	5	20.4	28.0	47.6	14.85	-0.16
	(engorged female)								
	<i>H.excavatum</i>	Water	0	5	7.2	34.0	25.8	11.27	-2.15*
	unfed adults								

*P<0.05

**P<0.01

The X value for each dish was calculated as $X=100.X\alpha/(X\alpha+X\beta)$. The average of X of all dishes $\pm$ SE of the mean are presented in tables 1 & 2. To test for significance of the results we calculated $t=(X-50)/SE$. The significance of repellence (negative t) or attraction (positive t) was taken from the t distribution.

All the ticks which we tested excreted a volatile compound(s) which attracted nematodes from the species H.b.-HP88 (Table 1). Three larvae of *G. mellonella* averaged more nematodes than the tested ticks. However when *B. annulatus* females or *G. mellonella* larvae were placed simultaneously with nematodes in contact with the agar their ability to attract the nematodes H.b.-HP88 could not be demonstrated (Table 3). If the female ticks were placed on the agar 2 h before the nematodes they repelled them. Water from washed cuticles of engorged *B. annulatus* females or of unfed *H. excavatum* adults also repelled the nematodes (Table 2).

Rate of nematode penetration into ticks (Israel)

Engorged *Boophilus* females were exposed to various nematodes strains for 6, 12 or 22 h at 26°C, washed thoroughly and kept for further observations at 26°C. Tick mortality on day 6 is shown in Fig 2. With 5 out of 7 nematode strains a contact with nematodes for 22 h was sufficient for the mortality of over 90% of the ticks. Exposure for 6 h to either H.b.-IS-5, H.b.-HP88 or S.c.-All killed ca-80% of the nematodes, S.c.-DT and S.f.-TG killed only ca 20% after contact with nematodes for 6 h.

Amount of nematodes penetrating into *B. annulatus* engorged females (Israel)

See appendix 2

After exposure of the ticks to 9 nematode strains (5000/dish) for 36 h washing and dissecting them either immediately or after 6 days the average amount of recovered nematodes was recorded (Table 3). An average of over hundred nematodes penetrated into each tick infested for 36 h with 4 strains and only less than 20 nematodes were recovered from ticks infested with 3 other strains. When the ticks were kept until day 6 the number of nematodes recovered was lower. Thus

Fig.2 - Mortality of *B.annulatus* females (n=20) on day 6 after 6-12-22 h. contact with various nematode strains (5000 nematodes/dish).

IS-5, IS-12, HP-*H.bacteriophora*; TG-*S.feltia*; SR-*S.riobravus*; All, DT-*S.carpocapsae*

Table 3 - Amount and stages of nematodes recovered within *B.annulatus* engorged females (5000IJ/dish). Ticks exposed to nematodes for 36 h. and dissected 36h. or 6 d. after exposure.

Dissected time after exposure started			36 h.	6 d.			
Nematodes		# ticks dissected	IJ	IJ&L4		Adult	
species	strain		#/tick	#/tick	% live	#/tick	% live
<i>H.bacteriophora</i>	HP88	10	104	~	~	~	~
<i>H.bacteriophora</i>	IS-5	15	116.8	72	21	0	0
<i>H.bacteriophora</i>	IS-12	15	4.3	3.2	2	0	0
<i>S.carpocapsae</i>	All	15	60.8	~	~	~	~
<i>S.carpocapsae</i>	DT	15	139.1	32.9	4.9	0.06	0
<i>S.carpocapsae</i>	MEX	15	105.7	7.2	4.6	0	0
<i>S.carpocapsae</i>	S20	15	18.5	2.5	0	0	0
<i>S.feltia</i>	TG	15	6.7	0.5	0	1	6.6
<i>S.riobravus</i>	SR	15	96.7	~	~	~	~

nematodes apparently disintegrate with time in the tick. However when the nematode-tick contact lasted for 6 d and the tick cadavers were dissected after 6 an additional days more nematodes had a chance to penetrate (Table 4).

Table 4 - The amount of nematodes recovered from *B. annulatus* engorged females(5000 nematodes/dish). Ticks exposed to nematodes for 6d. and dissected on day 6.

Species	Strain	Nematodes		Avr. #nematodes recovered/tick
		Diameter(μ m)	# ticks	
<i>H.bacteriophora</i>	IS-5	23	30	141
<i>H.bacteriophora</i>	IS-12	23	30	36
<i>S.carpocapsae</i>	ALL	25	15	70
<i>S.carpocapsae</i>	DT	25	20	36
<i>S.feltia</i>	TG	26	25	115
<i>S. glaseri</i>	SG	43	25	130

The effect of axenic nematodes on survival of *B. annulatus* engorged females (Israel)

Producing axenic nematodes - Nematodes were reared axenically according to the procedure described by Lower & Buecher 1970 (Nematologica 16: 563-566). The Axenic S.c.-DT nematodes were suspended in saline and injected into ticks as well as into larvae of *Galleria mellonella*.

Control arthropods were injected with normal nematodes from the same strain or with saline only.

Ten axenic nematodes were sufficient to kill within one week post injection all the insects but injecting 75 axenic nematodes into ticks killed only 7% which is similar to the mortality in the control group. (Table 5).

Survival and development of the nematodes in ticks (Israel & USA)

Development of inter tick nematodes - Ticks infested with nematodes were thoroughly washed and torn into very small pieces in petri dishes with 5 ml saline. When the solution was turbid it was diluted and divided into 2-3 more petri dishes. Each dish was carefully checked for nematodes under a stereoscope. The number, stage and survival of the nematodes was recorded. Nematodes without movement were touched with a fine pin to ascertain whether they are alive.

Ticks were exposed to nematodes for 36 h and washed thoroughly. At least 15 females were

Fig.3 - Mortality of IJ nematodes in engorged *B.annulatus* females(n=15-45) expressed as % of live IJ in ticks dissected after 1.5, 3 or 6 days
(Ticks exposed to nematodes for 36 h.)

S.20,Mex,DT,All-S.carpocapsae;S.f-S.feltiae

Table 5 -Effect of injecting axenic or normal nematodes (5 ml ;S.c.-DT) into arthropods

Arthropod		Nematodes		% mortality		
species	(n)	Type	Avr. #/Arthropod	d. post infestation		
				3	5	7
<i>Boophilus annulatus</i>	25	Normal	2.5	96	96	100
	29	Axenic	75	7	7	7
	40	Control	0	0	5	7.5
<i>Galleria mellonela</i>	5	Normal	1.5	100	100	100
	10	Axenic	10	40	70	100
	20	Control	0	5	10	10

Fig. 4 - Effect of temperature on mortality of *B.annulatus* in Petri dishes (n=20)

A. kept 12 d. i with various nematode strains (500 nema./dish).

dd, S20-*S.carpocapsae*, TG-*S.feltia*, IS5-*H.bacteriophora*, SR-*S.riobravus*
B-kept 12 d. with various nematode strains (500 nema./dish).

S.c.-*S.carpocapsae*, Hb-*H.bacteriophora*, S.f.-*S.feltia*

dissected immediately after washing (36 h), and others 3 d or 6 d after their exposure started. The live and the dead IJ form of the nematodes was recorded (Fig. 3).

Some 60% of the S.c.-DT nematodes penetrating into ticks were still alive 3 d after exposure of the ticks to nematodes while less than 20% of the other 4 strains studied (Fig. 3) survived on day 3. On day 6 only few of the IJ of the nematodes from the S.c.-Mex strain survived while practically all other strains died or were transformed into other developing stages. Ticks were washed after being exposed for 36 h. to nematodes and their cadaver dissected on day 6. Some nematodes developed into their L₄ stage and some into adults (Table 3). The strain S.f.-TG, found originally in the soil of a cattle yard and strain S.c.-DT seems to be relatively better adapted towards development within the tick.

Experiments in the USA were conducted to determine (1) if nematodes actually entered the ticks, (2) whether the nematodes multiplied within the ticks as they do in the insect larvae, and (3) whether the symbiotic bacteria released from the nematodes were involved in tick death. Partially fed female *A. americanum* and *D. variabilis* that were exposed to 5,000 SR nematodes in petri dishes were collected 8,24,48 and 96 hrs post-exposure (PE) (Trial 1) and 1,2, 3,4, 7 and 9 days PE (Trial 2) and fixed, processed and embedded in resin for microscopy studies. Nematodes were found within these ticks within 24 hrs PE but they did not appear to multiply within ticks; the number of nematodes observed in half of tick sections did not increase over time. Only a few nematodes were observed in tick hemocoel and tick tissues, [and these nematodes were] surrounded by a clear space. Bacteria, presumed to have been released from the nematodes, were present in the exposed ticks and appeared to increase daily resulting in a generalized bacterial infection. Degeneration of tick tissues and death, therefore, appeared to result from bacterial proliferation. Nematode guts were filled with the bacteria, suggesting that the bacteria were a food source. Nematodes did not appear to multiply within ticks as they do in insect larvae. These studies will be published in the Journal of Medical Entomology in Kocan *et al.* (Appendix 5)

Fig. 7 -The effect of various concentrations of extracts from 4 types of manure on the survival of *S.riobravis* SR and *S.carpocapsae* - MEX at 26°C

We attempted to study and capture the invasion of nematodes into ticks using (1) time-lapse video photography and (2) an environmental scanning electron microscopy (USEM) in which live specimens can be observed and photographed. Both systems proved to have inadequacies that would not allow for the observation of nematode entry. In these studies, the SR nematodes were used that clustered heavily around tick orifices. Although we obtained good photographs of the clustering nematodes, we were unable to confirm nematode penetration.

Factors influencing the development of nematodes within ticks (Israel)

a. Influence of oxygen on nematode inter tick development

The amount of oxygen in the tick body was increased by either of two methods.

1. Injecting 5 μ l of pure O₂ or air into ticks on days 0, 2, 4 post nematode infestation.
2. Destroying the cuticle by washing ticks in acetone or by scratching the cuticle with an abrasive before infesting with nematodes.

All the tick cadavers were dissected on day 6 post infestation and the stage of nematode development was recorded. In these preliminary tests no significant differences in nematode inter tick development between treated and untreated ticks could be observed.

b. Influence of tick protection factors on nematode inter tick development

In an attempt to explain why the nematodes do not develop well within ticks we also surveyed the possible involvement of a tick self protection mechanism in hindering the inter tick development of the nematodes. This was done either by infesting ticks which had been killed shortly before contact with the nematodes or by injecting live nematodes or live bacteria into ticks killed previously by nematodes so as to bypass the possibility that a tick self protection system has damaged the normal development of the nematodes and/or the symbionts.

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Factors influencing the development of nematodes within ticks (Israel)

a. Influence of oxygen on nematode inter tick development

The amount of oxygen in the tick body was increased by either of two methods.

1. Injecting 5 µl of pure O₂ or air into ticks on days 0, 2, 4 post nematode infestation.
2. Destroying the cuticle by washing ticks in acetone or by scratching the cuticle with an abrasive before infesting with nematodes.

All the tick cadavers were dissected on day 6 post infestation and the stage of nematode development was recorded. In these preliminary tests no significant differences in nematode inter tick development between treated and untreated ticks could be observed.

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1. Killing ticks by freezing (-20°C) or by keeping them in an atmosphere saturated with ether before their contact with nematodes (S.c.-DT strain) did not change their inter tick development.

2. Injecting nematodes (S.c.-DT-strain) or symbiotic bacteria (*Xenorhabdus*) into ticks on days 0, 2, 4 post infesting with nematodes showed no significant differences in nematode inter tick development between treated and untreated ticks.

Objective 3: Environmental factors affecting nematode persistence and activity against ticks.

Most of the project was done in Israel. We determined temporal and special aspects of nematode survival and efficacy in sand containers simulating natural conditions. The methodology and the results are detailed bellow.

The effect of temperature on anti-tick virulence of nematodes (Israel)

Five different nematode strains were scattered in petri dishes (500/dish) with 5 engorged *B. annulatus* (4 dishes/repetition) and placed into 6 dark incubators at various constant temperatures.

Tick mortality on day 12 is demonstrated in Fig. 4-A. All strains tested were most efficient at 26°C. A large variation in the virulence of nematode strains is seen at higher and lower t°. The virulence of the S.c.-IS-5 strain is least affected by temperature (Fig. 4-A&B). At 14°C the female ticks didn't start laying eggs until the end of the test on day 12 (Fig. 4-A). At 18°C as well as at 26°C H.b.-IS-5 was efficient enough to kill the ticks before they had a chance to lay eggs.

The effect of time of exposure in soil on anti-tick virulence of nematodes (Israel)

Different nematode strains were scattered on soil in 10 l buckets (50 or 200 nema./cm²) and ticks were added on top of the soil either on the same day or 3 d after the nematodes.

Table 6 - Mortality (%) of *B.annulatus* engorged females (n=80) exposed to nematodes in 10 l buckets with sandy soil (26°C) (10 d. post infestation).

Ticks added days after spraying nematodes	#IJ/cm ²	Nematodes strain*				
		H.b.-HP88	S.c.-DT	S.c.-MEX	S.f.-TG	S.r.-SR
0	50	50	75	88.8	22.5	78.3
0	200	87.5	98.4	100	63.2	97.5
3	200	66.6	76.1	68.7	60	86.2

*in control (no nematodes) 0% mortality

The loss in nematode virulence during 3 d was only 3.2% for S.f.-TG and 11.3% for S.r.-SR strains respectively (Table 6). However three other investigated strains lost between 20.9-31.3% of their virulence during 3 d in sandy soil at 26°C.

The effect of soil humidity on the anti-tick virulence of nematodes (Israel)

Oven dried sandy soil was mixed with a known amount of water and placed into 10 l buckets. Nematodes were scattered on the soil surface (200/cm²), and *B. annulatus* engorged females were added on the same day or 3 d later. The buckets (20 ticks/buckets, 4 buckets for each concentration) were covered with a perforated plastic bag and kept stored in a glasshouse at 26°C.

A reduction in concentration of the water content of soil from 100% to 40% of its field capacity had no influence on nematode virulence even when they had been previously kept in soil for 3 d (Fig. 5). But a reduction of soil moisture to zero seems to reduce all nematode activity after 3 d.

The effect of animal manure on virulence of nematodes (Israel)

(A) manure mixed with sand

Sandy soil was mixed with animal manure (3/1-V/v) and put in 10 l buckets. Nematodes (200/cm²) were scattered on the mixture and engorged *B. annulatus* females placed in the buckets either on the same day or 3 days later. Mortality of ticks was recorded daily. The manure had relatively little influence on nematode virulence when the nematodes and the ticks were placed into the buckets on the same day (Fig. 6). If the ticks were however added 3 d after the nematodes, only 40% of the ticks were killed in the buckets with added cattle manure in comparison to 100% mortality in buckets without manure (control).

(B) Water extract of manure

Fresh horse, , cattle, chicken or sheep manure were mixed with water (1 part dry manure/5 parts water) for 12 h at 4°C. The supernatant was centrifuged and frozen until use (called 100% concentration). Nematodes were kept with the manure extract (3 dishes/concentration) at 26°C and their mortality recorded on days 1, 2, 3 of the experiment.

S.r.-SR nematodes seem to suffer to a similar degree from all 4 types of manure extracts studied i.e. ca 50% mortality after exposure for 3 d to the various extracts (Fig. 7). The strain S.c.-Mex was more susceptible to chicken manure extract than S.r.-SR.

The effect of antibiotics upon the survival of nematodes *in vitro* (Israel)

Fifteen antibiotics from different groups which are used commonly during the care of farm animals were tested at their estimated maximum concentration when extracted via the urine of treated animals. Ten ml of the solution to be tested (water served as control) were poured into 5 cm Ø petri dishes with about 1500 live nematodes and kept at 26°C. At known

intervals 0.5 ml aliquots were removed and the percentage of dead nematodes recorded. Each combination was carried out in 3 replicates.

Seven of these i.e. tetracycline, sulfadimidine, lyncomycine, chloramphenicol, erythromycine, trimethoprim and flumequine appear to be toxic to S.c.-DT nematodes (Fig. 8) while the nematode strain H.b.-IS-5 seems to be resistant to nearly all the antibiotics used during this experiment.

Depth of nematodes in soil (Israel & USA)

Nematodes were counted in 0-6 cm depth of soil 3 d post topical application of 200 nema.cm² according to the following procedure: Three days after the nematodes had been dispersed on top of wet soil in 10 l buckets a plastic cylinder (28 mm diam.) was forced vertically into the soil. The column of soil in the cylinder was pushed out and cut into 1 cm slices. Slices from depth of 0-1, 1-2, 2-3, 4-5 cm were placed into petri dishes and 2 *G. mellonella* larvae were added to each dish. Each repetition included 4 larvae. After incubation of the soil samples with the larvae for 3 d at 26°C the larvae were dissected and the # of intra insect nematodes recorded.

(1) Nematode strains - while by far most of the nematode strains studied live at the very top of the soil (Sr-SR, S.c.-Mex. S.c.-DT) some will be found in deeper layers in the ground (S.f.-TG, H.b.-HP88). Therefore will probably be less efficient against ticks which live on its upper layer (Table 7).

Table 7 -Parameters influencing the relative amount of nematodes found in various depth (cm.) of soil 3 d after sprayng 200 IJ/cm² (10 l buckets). Expressed as % of total amount of nematodes in *Galleria mellonela* used as biological trap.

a Effect of nematode strain

Depth	Nematode strain						
	Hb-HP88	Hb-IS-5	S.c.-DT	S.c.-MEX	S.c.-S-20	S.f.-TG	S.r.-SR
0-1	8	71	73	99	26.6	19.5	86.2
1-2	58	29	11.4	1	50.9	42.9	4.6
3-4	17	0	10.9	0	22.5	4.5	4.6
5-6	17	0	4.7	0	0	33.1	4.6

b Effect of soil humidity

Depth	Humidity of soil (% relative to field capacity)					
	100	80	60	40	20	0
0-1	92.7	92.4	98.5	87.8	92.4	0
1-2	7.3	6.8	1.5	10.3	5.8	0
3-4	0	0.8	0	1	1.8	0
5-6	0	0	0	0.9	0	0

c Effect of soil type

Depth	Soil type			(% fraction 2 μ >)
	Sandy soil(0.05)	Sand stone(0.1)	Clay soil(40-50)	
0-1	82.7	76.4	14.3	
1-2	11.5	20.4	24.5	
3-4	5.8	3.2	40.8	
5-6	0	0	20.4	

d Effect of manure type

Depth	Type of manure					
	Cattle	Chicken	Horse	Rabbit	Sheep	None (control)
0-1	11.5	0	9.7	22.4	0	73
1-2	16.2	51.7	19.5	53.8	71	11.4
3-4	36.9	30.5	55.2	22.4	29	10.9
5-6	35.4	17.8	15.6	1.4	0	4.7

* Buckets in trials a,b & c covered with plastic, trial d not covered

** Nematodes used in trial b,c & d S.c.- DT

Hb-HP88-*H.bacteriophora*, Hb-IS5, IS-12- *H.bacteriophora*, S.c-*Steinernema*

carpocapsae, S.f.-*S.feltia*, S.r.-*S.riobris*

(2) Humidity - oven dry soil was mixed with known amounts of water to obtain a known % of field capacity. The buckets were covered with a perforated plastic bag to keep humidity rather constant throughout the experiment and homogenous in various depths of soil. The results appear to be that a uniform soil humidity between 20-100% makes barely any difference in nematode virulence to ticks (Table 7b).

(3) Soil type - less nematodes were found when the upper layer of soil which contained [a higher amount of] more silt (Table 7c).

(4) Manure: In soil with a higher concentration of organic compounds more active nematodes are found at 1-5 cm depth than at 0-1 cm (Table.7d).

The studies in the USA on the interactions of ticks and nematodes in petri dishes or while ticks were feeding on cattle showed that moisture was an important factor that facilitated the longevity and movement of the nematodes and apparently affected the ability of nematodes to kill ticks. When petri dishes filled with sand were too moist with small pools of water or when little moisture was present, nematode survival and tick killing was reduced or absent. We received a moisture resistant strain of nematode from Dr. Glazer (IS5) but were not successful in propagating this strain in the laboratory. The one tick exposure trial that was done with the IS5s on replete *A. americanum* did result in tick mortality. We were unable to follow up on these studies because soon thereafter all companies that raise and supplied *Galleria* moth larvae were plagued by a virus that caused a temporary halt in our ability to purchase moth larvae for our studies. We were, therefore, unable to propagate nematodes for approximately 6 month until they resolved the problem and replenished the moth larvae stocks.

Objective 4: Field trials.(Israel)

On host experiments (USA)

Laboratory studies were conducted to determine whether entomopathogenic nematodes would kill female ticks while they were feeding on cattle in order to (1) provide preliminary evidence that tick populations on infested cattle in the field could be reduced by nematode application and (2) to determine whether nematodes could penetrate ticks via orifices other than the mouthparts. Female ticks remain attached during the lengthy feeding process and normally do not detach until they are replete. In our studies, we removed all ticks that were not attached before nematode application and observed that the females remained attached during this time. The feeding ticks were exposed to SR nematodes (10,000 per tick; 250,000 per tick feeding cell) on 3, 6, or 9 days post-attachment. After the females were replete, they were collected and placed in individual petri dishes. The effect of the nematodes on these ticks was judged by whether they caused tick death or reduced egg production.

Tick mortality and reduced egg production resulted when the ticks had fed 6 and 9 days prior to nematode exposure, but not when ticks were exposed at 3 days of feeding. Exposure of feeding female ticks demonstrated that the nematodes were able to penetrate tick orifices other than via the hypostome which was embedded in the bovine epidermis for the duration of the feeding process.

Moisture was found to be an important factor in the cattle exposures. In Trial 1, where moisture was not effectively maintained via moistened cotton balls placed in the tick feeding cells, ticks were not killed by the nematodes nor was egg production significantly affected. However, when water-soaked pieces of sponge were placed in the tick cells in Trials 2 & 3, nematodes penetrated and killed a percentage of ticks in the 3- and 9 day exposure groups and the

subsequent egg production in these exposed ticks was significantly reduced. We believe that dependence on moisture may be an important factor in the ability of entomopathogenic nematodes to be effective biological control agents of ticks.

We are continuing to conduct further experiments in which ticks are exposed to nematodes while feeding on cattle in which the infected moth larvae were placed in the tick feeding cells while the ticks are in the latter stages of engorgement. We hypothesized that the vigor of the nematodes for seeking and killing ticks may be improved when they are newly emerged from moth larvae rather than after being stored in the refrigerator for a short period of time. Preliminary data suggests that our hypothesis was correct because we observed much higher rates of tick killing and significantly reduced egg production. In all of the feeding tick/cattle exposures, the ticks died after they had become replete from feeding. Nematode penetration did not appear to interrupt the feeding process.

Off host experiments (Israel)

A preliminary outdoor experiment was performed. Open cylinders of 19 cm diameter (arenas) were buried outdoors 4 cm deep in native sandy soil or in a premixed mixture of 1/3 cattle manure +2/3 sandy soil. Fully engorged *B. annulatus* females (15/arena) were placed on the ground in the arenas 1 h after spraying of the nematodes. Once a day the arenas were sprayed with water to retain a high humidity. All ticks died within 5 d when the spray contained 140 nematodes/cm² or more (Fig. 9) while 30 nematodes/cm² caused a 50% mortality. These preliminary results are highly encouraging. Generally the amount of nematodes used against soil inhabiting plant pest insects is about 100-150 nematodes/cm².

Fig. 9 Mortality of engorged *B.annulatus* females (15,2 replica) that were placed on sandy soil open cylinders(280 cm²) outdoor.
Nematodes (*S.ccrpocapsae* -DT) were sprayed on the soil 1 hour before introducing the ticks.

*Mixture 2/3 sandy soil +1/3 cattle manure

Concluding remarks

The original aim of this project was "to create a basis for the use of entomopathogenic nematodes for the biological control of ticks". We achieved that by accumulating a large amount of information on the tick-nematode interaction and on the effect of environmental factors on nematodes as anti-tick agents.

All species of ticks that we tested were susceptible to nematodes. The results obtained from the present project indicate that under favorable conditions the nematodes show promise as a biological control method for ticks. However, we still face several potential obstacles to the use of nematodes under less favorable conditions. For instance:

- Low soil humidity reduces nematode anti-tick activity- However, selection of desiccation tolerant nematodes and addition of anti desiccants to the nematode suspension may reduce low humidity effect.
- The susceptibility of nematodes to external compounds and conditions such as manure, antibiotics, and soil composition varies considerably for different nematode strains. This finding indicate that far more resistant nematode strain could be found after screening.

Based on these results it is recommended to start field experiments at optimal conditions (susceptible ticks, humid zones, etc.) simultaneously with further studies designed to adapt the system to less favorable areas.

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5. Samish, M., E. Alekseev & I. Glazer. Interaction between ticks and pathogenic nematodes: The susceptibility of various tick species at different development stages (in preparation (enclosed)).
6. Samish, M., E. Alekseev & I. Glazer. Effect of different entomopathogenic nematode strains on mortality rate of various tick species (in preparation).
7. Additional publication as result of this project prepared during 1988-1999.

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