EFFICACY OF ENTOMOPATHOGENIC NEMATODES (NEMATODA: RHABDITIDA) AGAINST *CULEX GELIDUS* (DIPTERA: CULICIDAE) LARVAE

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EFFICACY OF ENTOMOPATHOGENIC NEMATODES (NEMATODA: RHABDITIDA) AGAINST CULEX GELIDUS (DIPTERA: CULICIDAE) LARVAE.

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ABSTRACT

The breeding sites of *Culex gelidus,* a secondary vector of Japanese Encephalitis, are close to agricultural areas and homes, such as temporary and semi-permanent fresh ground water from pig farms and rice fields. Entomopathogenic nematodes (EPN) are an alternative bio-control for insects. Therefore, the application of EPN to control *Cx. gelidus* larvae was studied with the objectives of 1) determining the efficacy of EPN between 2 genera against $3^{rd} – 4^{th}$ instars larvae of *Cx. gelidus* under laboratory conditions and 2) determining the dosages of EPN effective against *Cx. gelidus* larvae. The experiment was carried out in the laboratory under room temperature of 29 ± 2 ° C and relative humidity (RH) 70 – 80 %.

Results indicated that mortality rates of $3^{rd} – 4^{th}$ instars *Cx. gelidus* larvae caused by *S. carpocapsae* (Weiser) EPN were greater than *H. indica* (Local Thai strain) 63% and 13%, respectively. The mortality of both control groups was 5%. Infection rates between the 2 genera were 14.5% and 2%, respectively. The thorax of dead *Cx. gelidus* larvae were the site where EPN were mostly found, more than other parts of their bodies. Comparing mean difference for mortality rates of *Cx. gelidus* larvae between 2 genera at 48 and 72 hours post exposure found significant difference by T-Test (p-value < 0.05). *S. carpocapsae* (Weiser) kills more than 50% at dosage 2000 and 4000 IJs per larvae, but there was no significant difference in number of $3^{rd} – 4^{th}$ instars larvae *Cx. Gelidus* killed at either dosage. There was significant interaction between the 2 genera at the various dosages (p-value < 0.01, analysis by 2 way ANOVA).

The results showed that under laboratory conditions, *S. carpocapsae* (Weiser) EPN have potential as a bio-control against $3^{rd} – 4^{th}$ instars *Cx. gelidus* larvae. Further study should involve water depth, temperature, pH of water and feeding behavior of target host prior to use in field trials.

KEY WORDS : ENTOMOPATHOGENIC NEMATODE / STEINERNEMA CARPOCAPSAE (WEISER) / HETERORHABDITIS INDICA (LOCAL THAI STRAIN) / CULEX GELIDUS LARVAE

60 pp.

ประสิทธิภาพของไส้เดือนฝอย (Nematoda: Rhaditida) ต่อการเข้าทำลายลูกน้ำยุงรำคาญ *Culex gelidus* (Diptera: Culicidae)
(EFFICACY OF ENTOMOPATHOGENIC NEMATODES [NEMATODA: RHABDITIDA] AGAINST CULEX GELIDUS [DIPTERA: CULICIDAE] LARVAE)

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บทคัดย่อ

ยุงรำคาญ (*Culex gelidus*) เป็นพาหะที่สองของโรคไข้สมองอักเสบ แหล่งเพาะพันธุ์ส่วนใหญ่อยู่ใกล้กับบ้านเรือนที่เป็นลักษณะเชิงเกษตรกรรม โดยเฉพาะน้ำขังบริเวณรอบคอกสัตว์ และน้ำขังในนาข้าว ปัจจุบันมีการใช้ไส้เดือนฝอยในการควบคุมแมลงทางชีวภาพ ดังนั้นจึงได้มีการประยุกต์ใช้ไส้เดือนฝอยในการควบคุมลูกน้ำยุงรำคาญ โดยมีวัตถุประสงค์คือ เปรียบเทียบประสิทธิภาพระหว่างไส้เดือนฝอย 2 สกุล คือ *Steinernema carpocapsae* (Weiser) และ *Heterorhabditis indica* (Local Thai strain) ที่มีต่อการควบคุมลูกน้ำยุงรำคาญในระยะที่ 3 - 4 และ ปริมาณของไส้เดือนฝอยที่เหมาะสมต่อการควบคุมลูกน้ำยุงรำคาญ ทั้งนี้เป็นการทดลองภายในห้องปฏิบัติการ ที่อุณหภูมิห้อง 29 ± 2 องศาเซลเซียส และ ความชื้นสัมพัทธ์ 70 – 80 %

ผลการทดลองพบว่า อัตราการตายของลูกน้ำยุงรำคาญ ระยะที่ 3 - 4 จากไส้เดือนฝอยสกุล *S. carpocapsae* (Weiser) สูงกว่า *H. indica* (Local Thai strain) กล่าวคือ 63% และ 13% ตามลำดับ ในขณะที่กลุ่มควบคุม อัตราการตายอยู่ที่ 5% ทั้ง 2 สกุล และพบว่าอัตราการติดเชื้ออยู่ที่ 14.5% และ 2% ตามลำดับ ส่วนใหญ่แล้วลูกน้ำที่ตายจะพบไส้เดือนฝอยอยู่บริเวณช่องอกมากกว่าบริเวณอื่น และเมื่อเปรียบเทียบค่าเฉลี่ยการตายของลูกน้ำยุงรำคาญ ระยะที่ 3 - 4 ใน เวลา 48 และ 72 ชั่วโมง พบว่าลูกน้ำที่ตายจาก *S. carpocapsae* (Weiser) สูงกว่าลูกน้ำที่ตายจาก *H. indica* (Local Thai strain) อย่างมีนัยสำคัญทางสถิติ (p-value < 0.05) ปริมาณของไส้เดือนฝอยต่อลูกน้ำยุงรำคาญ สกุล *S. carpocapsae* (Weiser) ที่ทำให้ อัตราตายของลูกน้ำมากกว่า 50% คือ ที่ 2000:1 ซึ่งไม่แตกต่างกับที่ 4000:1 จากการวิเคราะห์โดยใช้ 2 way ANOVA พบว่า มีปฏิกิริยาร่วม ระหว่าง ไส้เดือนฝอย 2 สกุล (p-value < 0.001)

ผลการทดลองแสดงให้เห็นว่า ไส้เดือนฝอยสกุล *S. carpocapsae* (Weiser) มีความเป็นไปได้ในการนำมาใช้ควบคุมลูกน้ำยุงรำคาญ *Cx. gelidus* ระยะที่ 3 – 4 แต่ถึงอย่างไรควรมีการศึกษาถึงระดับความสูงของน้ำ อุณหภูมิของน้ำ ความเป็นกรดด่าง และพฤติกรรมการกินของลูกน้ำยุงรำคาญก่อนที่จะนำไปใช้ทดลองในภาคสนาม ต่อไป

60 หน้า .

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LIST OF ABBREVIATIONS

e.g.	Exempli gratia(Latin), for example
EPN	Entomopathogenic Nematode
et al.	Et alli (Latin), and others
gm	Gram
IJs	Infective Juvenile stage
Min	Minute
ml	Millilitre
No.	Number
pH	Negative logarithm of hydrogen ion activity
RT	Room temperature
spp.	Species

CHAPTER I
INTRODUCTION

Background

Japanese encephalitis (JE), a central nervous system (CNS) infection caused by a flavivirus, is the most important public health problem among other encephalitis in Thailand. The vector of the disease is Culicine mosquitoes which in Thailand they are *Culex tritaeniorhynchus Cx. gelidus* and *Cx. fuscocephala.* Among these three species, *Cx. tritaeniorhynchus* is the main vector of the disease while *Culex gelidus* Theobald is secondary vector (1).

In Thailand, JE virus was reported to be isolated from *Cx. gelidus* in 1972 (2) and this virus was also found in *Cx. gelidus* in suburban area of Bangkok (3). This mosquito is close to man and domestic animals such as pigs, cows and buffaloes in rural and urban communities of Thailand. Their larvae have been collected from various habitats such as temporary and semi-permanent fresh ground water from pig farms and rice fields.

Biological control, a natural control method, is one of the alternative means that has been used to control pests and one example of this method used pathogen from natural like bacteria, protozoa, virus, fungi and nematode(4). Entomopathogenic nematodes (EPN), which are insect pathogens, have become increasingly popular in insect control since the infective juveniles kill insects in 24 - 48 hr. and they are proven to be safe for plants, animals and environment by The United States Environmental Protection Agency(5).

In Thailand, most of the studies regarding the efficacy of EPN against mosquito larvae are for control of *Aedes aegypti* (L.) and the results show they are significantly effective(6). The aim of this study is to determine the efficacy of EPN, i.e. *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai

strain), under laboratory conditions, as to whether they can be used as an alternative mean in controlling *Cx.gelidus* larvae or not.

Research problem

Can EPN be used for control of *Cx.gelidus* larvae?

Objectives of study

1.3.1 General objective

To determine the efficacy of entomopathogenic nematodes against *Cx.gelidus* larvae under laboratory conditions.

1.3.2 Specific objectives

To compare the efficacy of entomopathogenic nematodes of the genera *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain) against *Cx.gelidus* larvae.

To determine the dosages of entomopathogenic nematodes which is effective against *Cx.gelidus* larvae.

Hypotheses of the study

1. The efficacy of EPN against *Cx.gelidus* laravae between *Steinernema carpocapsae*(Weiser) and *Heterorhabditis indica* (Local Thai sp.) are different.

2. The efficacy of EPN against *Cx.gelidus* larvae is related to their dosages (Numbers of EPN / *Cx.gelidus* larvae).

Variable of the study

1.5.1 Independent variables

Genera of entomopathogenic nematodes

- *Steinernema carpocapsae* (Weiser)
- *Heterorhabditis indica* (Local Thai strain)

Dosages of EPN (Numbers of EPN / *Cx.gelidus* larvae)

- 500 nematodes (500 : 1)
- 1000 nematodes (1000 : 1)
- 2000 nematodes (2000 : 1)

- 4000 nematodes (4000 : 1)

1.5.2 Dependent variables

Mortality rates and infection rates of *Cx.gelidus* larvae.

1.5.3 Control variables

The experiment was carried out at room temperature (29 ± 2 º C) and relative humidity (RH) 70 – 80 %.

Scope of study

1. The EPN used in this study was supplied by Entomology and Zoology Group, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operative, Bangkok.

2. *Cx. gelidus* larvae used were collected from breeding place rice fields in Nonthaburi Province and pig farm in Rachaburi Province. All larvae were reared under the same condition in the laboratory until used.

Limitation of study

1. This study was carried out in laboratory of Parasitology Department, Faculty of Public Health, Mahidol University. Sometime the conditions were not stabled, in particular room temperature and relative humidity are not controlled.

2. Only 3^{rd} – 4^{th} instar larvae were tested under laboratory condition. Since experiment on the 1^{st} and 2^{nd} instars larvae was very difficult to observe.

3. This study was not tested on pupa of *Cx. gelidus* because they were not fed and the structure of *Cx. gelidus* pupa made them untestable.

Definition of Terms

1. Efficacy of entomopathogenic nematodes referred to the number of *Cx. gelidus* larvae died in test container by different level of *Steinernema carpocapsae*(Weiser) and *Heterorhabditis indica* (Local Thai strain) at 24 - 72 hours post exposure.

2. Genera of entomopathogenic nematodes referred to genera of nematodes parasite of insect *Steinernema carpocapsae*(Weiser) and *Heterorhabditis indica* (Local Thai strain).

3. The dosages of EPN referred to four levels of EPN againts *Cx. gelidus* larvae (i.e. 500 nematodes per larva, 1000, 2000 and 4000). Using dilution counting technique to approximate the nematodes tested.

4. Larvae of *Cx. gelidus* were collected from rice fields and pig farms. All larvae were reared under the same condition in laboratory condition until used. Selected 3^{rd} – 4^{th} instars larvae to test because they were maximum mortality and easier identify. (7)

5. Test water referred to dechlorinated tap water and rice straw infusion. Analytical pH and temperature of the water before tests.

Conceptual framework

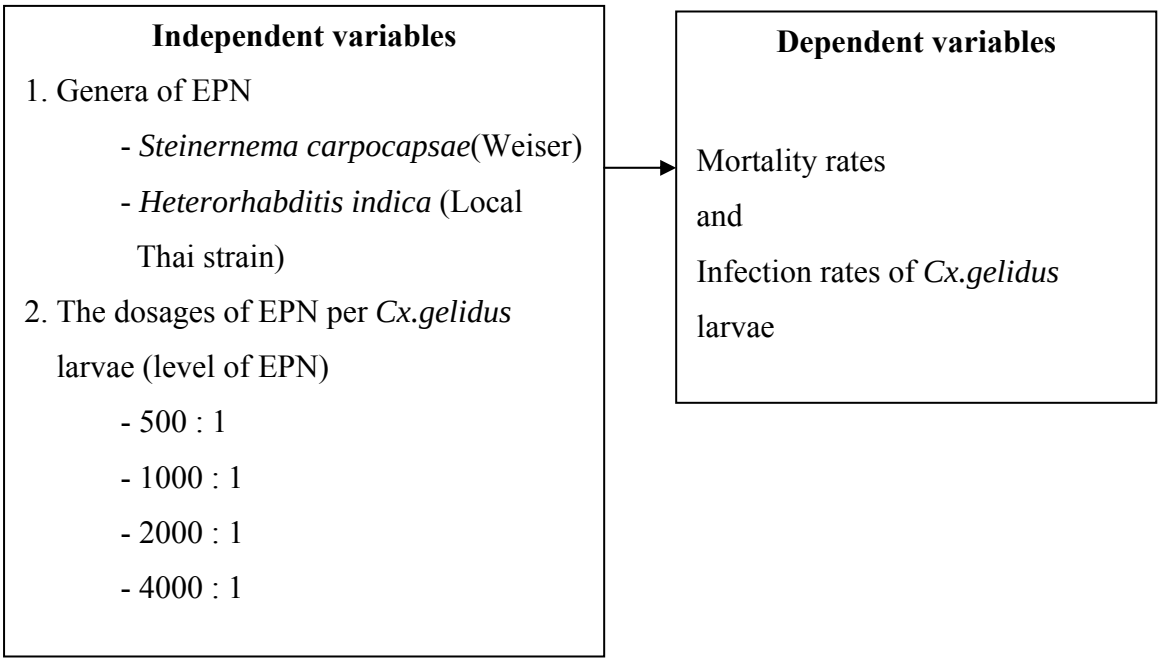


Figure 1 Conceptual framework

CHAPTER II
LITERATURE REVIEW

Japanese encephalitis

Japanese encephalitis (JE) is serious health problem in Thailand. JE has been known to be epidemic disease for many years. The first outbreak occurred sporadically in 1969 in Chiang Mai province during rainy season that caused 655 cases with 152 deaths (8). JE is a disease caused by the mosquito-borne Japanese encephalitis virus. The Japanese encephalitis virus is a virus from the family Flaviviridae like dengue virus. JE is principally a disease of rural agricultural areas, where vector mosquitoes proliferate in close association with pigs, chickens, and ducks, the principal vertebrate amplifying hosts especially pigs. The mosquitoes principally culicine species are vectors born disease and they can be transovarial transmission. Humans may become ill after infection but they are incidental to the transmission cycle. Culicine group are main vector of JE virus in Thailand. The mosquitoes are transmitted mainly by *Culex tritaeniorhynchus Cx. gelidus* and *Cx. fuscocephala*. The recorded breeding sites of these mosquitos include puddles, pools, ponds, ditches, drains streams, rice fields and marshy depression. The principal hosts for viral amplification are domestic animals such as pigs, cows and buffaloes, especially piggy are important amplifying hosts. Periods of viremia after infection are several days and the vast majority of infections are unapparent and only one in 300 infections results in symptomatic illness. Thus, in Thailand the patients are infections but non symptomatic illnesses have many case. Although the patients survived after infection just have immunities as long term. (8)

Japanese encephalitis vectors

The main vectors in Thailand are *Cx. tritaeniorhynchus Cx. gelidus* and *Cx. fuscocephala* play an important role as vector transmission for JE viral to host. Thailand is traditionally an agricultural country and the majority of more than 60 percent of Thai people are involved in production of some forms of agricultural products. The breeding sites of *Culex* spp. are rice fields and farms of pigs. The reported breeding place of *Cx. gelidus* and *Cx. fuscocephala* in Bangkok in 1920 (9) and *Cx. tritaeniorhynchus* was discovered in Bangkok and some provinces, e.g. Uttaradit, Chumporn, Chiang Mai, Lopburi and Ayuthaya in 1930 by Sinton. In 1963 first diagnosed patient of JE virus was in Phisanulok province while researchers of Mahidol University and SEATO separated JE virus from *Cx. tritaeniorhynchus* and *Cx. gelidus* collected from Tambol Bangpha, Chonburi province. The studies involve mosquitoes are actively pursued after an epidemic in Chiang Mai province in 1969 (10) and separated JE virus in *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. fuscocephala* was done in 1964 from endemic areas. Recently, the three vectors can be found throughout regions in Thailand, especially in agricultural areas, rice fields and husbandries.

1. Breeding place

1.1 *Cx. tritaeniorhynchus*, breeding place are rice fields after flood and puddles from foot animal. However, number of larvae in rice field are reduced after emergent vegatation. Some breeding place of *Cx. tritaeniorhynchus* have brown color like iron oxide on surface.

1.2 *Cx. gelidus*, breeding places include puddles, pools, ponds, ditches, drains, streams, rice fields and marshy depressions which usually contain abundant aquatic vegetation, such as water lily, hyacinths, duckweeds and grasses. The property of the water ranges from fresh and clear to strongly colored with contamination from decayed organic matter and mud particles. On occasion, it has also been found in breeding places in tanks, barrels, earthenware pots and coconut shells.

1.3 *Cx. fuscocephala*, which has similar breeding habitats to *Cx. tritaeniorhynchus*.

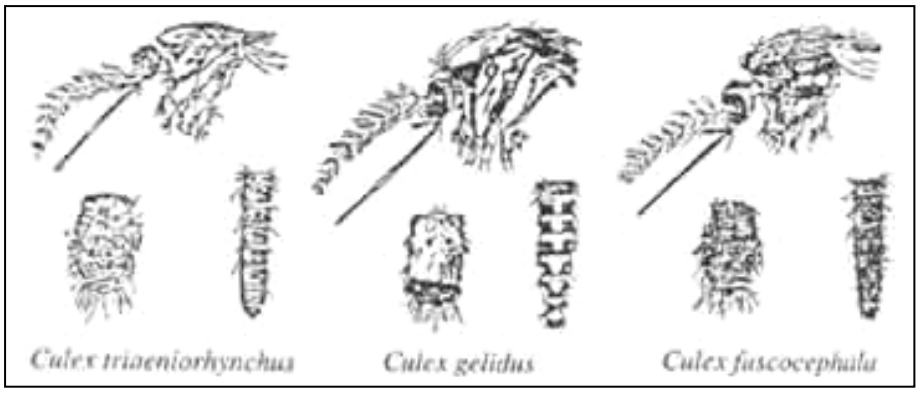


Figure 2 Encephalitis vector mosquitoes
(Cite: http://www.aars-acrs.org/acrs/proceeding/ACRS1997/Papers/PS197-19_files/ps2022b.jpg)

2. Life cycle of *Culex* mosquitoes

Life cycle of *Culex* spp. is holometabolous 4 developmental stages: Egg, Larva, Pupa and Adult.

Egg: *Culex* mosquitoes: lay their eggs on surface of fresh and clear to strongly color with contamination from decay organic matter and mud particles. The *Culex* mosquitoes usually lay eggs looks like a raft. Numbers of eggs per raft were 60 to 250 eggs. Period average 1-3 days.

Larva: Fertilize of eggs have been disrupted and tiny larvae (1^{st} instars) emerge from the eggs and development to 2^{nd} instars, 3^{rd} instars and 4^{th} instars. The larvae of Culex have a long siphon. They hang upside down at the water surface with breathing by siphon tube. They feed on algae, plankton, fungi, bacteria and microorganisms. Period average 6 – 10 days depend on species and environment (e.g. temperature, humidity and feed)

Pupa: No feeding and sensitive to vibration. They breathe by siphon-likes "Trumpet". Period average 1 – 2 days.

Adult: The body with 3 sections: head, thorax and abdomen. *Culex* mosquitoes usually are preferred bite animal especially pigs, cows, buffaloes and horses. They are preferred to attack at dusky. Female *Culex* life span about 4 week while male life span about 1 week. Their chances of survival depend on temperature and humidity.

3. Prevalence and infection rate

The immature and adult occur throughout the years. Adult of *Culex* mosquitoes have been frequently collected in numbers from light trap and animal baited trap. Population of *Culex* mosquitoes was highest in rain season (June - October). The experimental studies and field observations suggest that antigen JE virus in vectors was increase in rainy same as numbers of vectors.

4. Biting time and biting habitat

Biting time of JE vectors was highest at dusky between 19.00 – 22.00 p.m. They usually prefer to bite domestic animals and prefer biting outdoors than indoors (Exophilic).

5. Feeding Habit

Vectors of JE are preferred to bite animals (zoophilic) such as cows, buffaloes, pigs, chickens and some bird. The studies of host preference by light trap suggest that most prefer bite cows, buffaloes and pigs. Female mosquitoes begin feeding bloods 4 days after emergence in adult stage, before or after fertilized. They lay eggs about 3rd - 4th days after feeding blood.

6. Resting habitat

The survey adult mosquitoes by aerial net and aspirator suggest that catch many mosquitoes in around domestic animal and less indoors.

7. Flight range

Culex mosquitoes can fly for a distance of up to 1.5 kilometers, depending on species of mosquitoes. The studies by probe fluorescent for measurement distance was 1.8 kilometers. In Japan, *Culex* can fly about 8.4 kilometers (1). Potential of flying depend on species and nature (wind, temperature and humidity).

Nematodes

1. Introduction of Nematodes

Nematodes are simple roundworms, colorless, invertebrate, bilateral, pseudocoelomate, non-segmented and with elastic cuticle. Many of the parasitic species cause important diseases of plants, animals, and humans. Nematodes have systems consisting of excretory system, nervous system, muscular system, but not in circulatory and respiratory systems. They have simple roundworm or cylindrical

forms in some species, or even filiform. Other names of nematodes are roundworm, eelworm and threadworm. The nematodes are separated into 4 groups as determined by habitat and feeding: marine nematode, free-living nematode, plant parasitic nematode and animal parasitic nematode. Groups of separate animal parasitic nematodes are parasitic to insects found in more than 40 families, but 2 families are found like insecticidal nematodes. Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae have been used control many insects (11).

2. Biology and Taxonomy

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been used to control important insect pests. The mechanism by which these nematodes are able to infect and reproduce in the insect host involves a mutual relationship between the nematode and the symbiotic bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. These 2 families were classified as Phylum Nematode and Order Rhabditida because they feed bacteria but are different nematodes from those families Steinernematidae and Heterorhabditidae which are merely symbiotic bacteria (12).

Table 1 Classification of Entomopathogenic Nematodes (Families Steinernematidae and Heterorhabditidae)

Phylum Nematoda
Class Adenophorea
Class Secernentea
Order Rhabditida
Sub-order Rhabditina
Superfamily Rhabditoidea
Family Steinernematidae
Family Heterorhabditidae
+ 15 other families

Entomopathogenic nematodes from two families Steinernematidae and Heterorhabditidae within *Steinernema*, species have been recognized by morphological differences and some problems of misidentification and nomenclature. Within Heterorhabditis it is arduous to obtain cross breeding data and there has been

difficulty in defining taxonomically useful morphological characters. Recently, the molecular techniques use to identification of sibling species, subspecies, and other intra specific groupings. Besides contributing to understanding the biology and evaluation of entomopathogenic nematodes.

Table 2 Species and Strains of Steinernematidae

Genus	**Species**	**Strain**
Steinernema	*carpocapsae*	All
	carpocapsae	DD-136
	carpocapsae	Mexican
	carpocapsae	Agriotos
	glaseri	N.J.
	scapterisci	Uruguay
	kushidai	Japan
	feltiae	Newzealand
	rara	Argentina
	riobravis	Texas
	siamkayai	Thailand

In families of Heterorhabditidae, the taxonomy identifications are little difficultly than Steinernematidae. Recently, they have been divided eight species *Heterorhabditis bacteriophora, H. argentinensis, H. bravicaudis, H. hawaiiensis, H. indica, H. marelata, H. megidis* and *H. zealandica*.

3. Infective Juvenile

Steiner described the first steinernematid isolated from Germany in 1923. Significant developments have continued the last 80 years. The relationship between nematodes and their symbiotic bacteria has been revealed and explored. *Xenorhabdus* and *Photorhabdus* are motile gram-negative bacteria belonging to the family Enterobacteriaceae was present in the anterior region of the infective juvenile (IJ/IJs) of *Steinernema carpocapsae*. Since then it has been known that *Steinernema* spp. carry bacteria of the genus *Xenorhabdus* while *Heterorhabditis* nematodes harbour a species of the genus *Photorhabdus* (13-14). The symbioses are related between

bacteria and nematode. They are carried as symbionts in intestinal of infective juvenile stage of nematodes. The nematodes penetrate into the insect body cavity, usually via natural body openings (mouth, anus, spiracles) or areas of thin cuticle and subsequently penetrate into the hemocele of host. The nematodes release the bacteria into the hemolymph, septicemia becomes established and insect death occurs within 48 hour. As soon as the infective juveniles enter the host hemocoel, they initiate development. The alimentary tract becomes functional and cells of the symbiotic *Xenorhabdus* bacteria are released through the anus and start to multiply in the insect's hemocoel. These are consumed and digested by the developing nematodes. Amphimitic is reproduction between male and female infective juveniles. The nematodes develop and multiply in hemocoel of cadavers about 2 or 3 generations, but they depending on size of insects. Ultrastructural investigations reveal the presence of numerous mitochondria and large amount of food reserve stored (lipid) in enlarged hypodermal glands and intestinal cells. The new infective juveniles emerge in cadaver and movement search of new hosts (15).

The entomopathogenic nematodes are considered and received by The United States Environmental Protection Agency (EPA). Safety nontargets, plant animal and human involve nature. Steinernematids and heterorhabditids nematodes are becoming accepted as biological control agent *Bacilus thuringiensis* (Bti) and nuclear polyhedrosis virus (NPV). They have survived the tests production, application, field efficacy, and safety standard. A number of commercial enterprises worldwide are now producing them and alternative selected to use biological control reduce pesticides (5).

4. Life Cycle

Steinernematids and heterorhabditids have similar life cycle histories. They are consisting of egg stage and 4 larval stages and development by molting to adult stage. The third juveniles stage are infective to host (Infective Juvenile, IJ) and resist in environment. *Xenorhabdus* and *Photorhabdus* spp. are carried as symbionts in the intestine of the infective juvenile stage of nematodes belonging to the families Steinernematidae and Heterorhabditidae, respectively. The infective juveniles enter digestive tract of larva stage and subsequence into the hemocele of host insects. Depend on temperature and moisture they can persist in environment enter host

insect. The juveniles stage establish in group of free living nematodes. Average length of the infective stage about 400 – 1500 μm which depend on species of nematode. They can penetrate into the insect body cavity, usually via natural body openings (mouth, anus, spiracles) or areas of thin cuticle (16).

The reproductions of nematodes are differences between *Steinernema* and *Heterorhabditis.* In *Steinernema*, the infective juveniles develop into amplimictic females or males but never hermaphrodites. In *Heterorhabditis*, each infective juveniles develops into a hermaphroditic female and never an amphimitic female or male. However, the second generation consists of amphimictic females or males in both genera. The studies involve development and reproduction suggest one male of *S. carpocapsae* can mate with more than one female. The female can deposit eggs about 300 – 3050 per one female nematode in vivo condition (rearing procedures and sources for *Galleria mellonella*) (17).

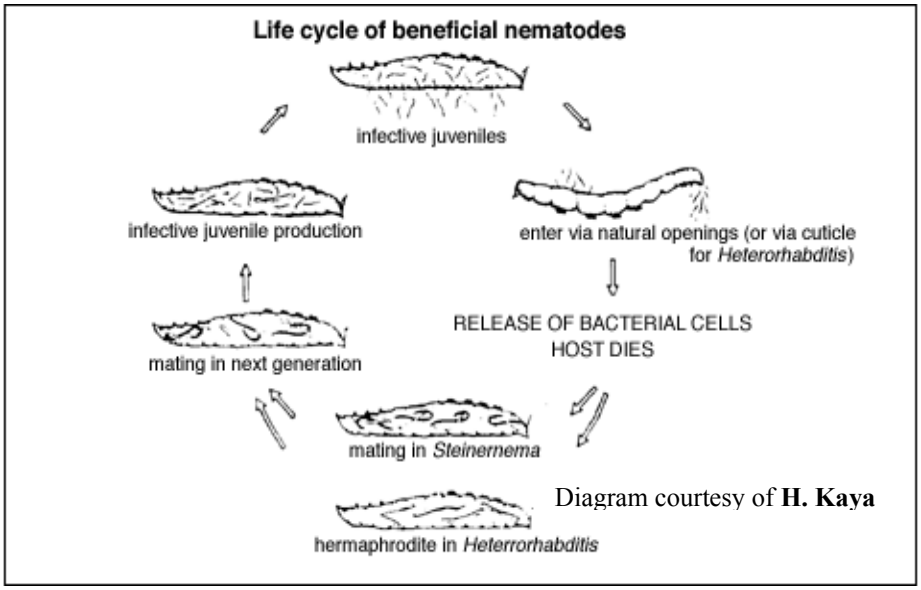


Figure 3 Life cycle of *Steinernema* and *Heterorhabditis* nematodes (Cite: http://www.ento.psu.edu/extension/factsheets/nematodePics/nematodeLifeCycle2.jpg)

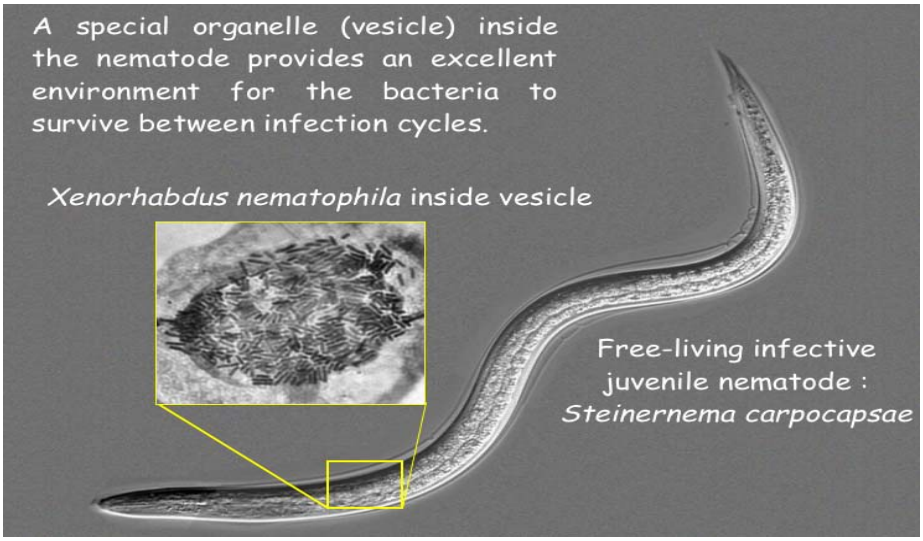


Figure 4 The bacteria to survive in intestinal of nematodes (Cite: http://xenorhabdus.danforthcenter.org/pix/fig3.jpg)

5. Penetration

The infective stage (L3) of both genera (Steinernematidae and Heterorhabditidae) can move more than one centimeters or many kilometer that depend on wind, rain, soil, human and insect (18).

6. Survival

The infective stage (L3) can persist several weeks although not feed. They can survive many months in dehydration conditions. Persistencies of infective juveniles depend on several factors such as temperature, moisture, predators and soil profiles. In low moisture, they can survive in sand and fine sand and temperature from 15-25 °C than clay soil and temperature upper 25 °C or lower 15 °C (19)(20).

7. Commercial production and development

Steinernematids and Heterorhabditid have been produced by insect infection or on artificial media, by axenic or monoaxenic culture, in solid and liquid phase fermentation (21-24). In artificial media, solid and liquid phase use sterilize plastic foam impregnated with homogenize animal tissue and inoculate symbiotic bacteria store 24 hour. When cultures are already available, inoculate the nematodes with plastic foam impregnated and rear 15 days. Harvesting and separate infective juveniles stage from plastic foam before to use biological control insect host. The mass products in large scale have been produced by solid media in tank fermentation. Because, save power to produce and low cost (23). Recently, department of Agriculture and faculty of science and technology, Thammasart University has been produced mass product of *S. carpocapsae* with nutrient in fermentation tank 100 liters and development procedure storage in form of powder and tablet substitution synthesis sponge (25).

Biological control

Biological control is defined as the reduction of a target population by using natural predators such as copepods, some fish and parasites or by using pathogens such as bacteria, protozoa and virus. It is one of the alternative control methods. Using biological control to control insects was increased, on an average 10-15 percent per year while chemical pesticides was increase average 1-2 percent per year,

especially in USA (1). The efficacy of the biological control should be considered in several components as follow:

1. The efficacy of biological control agents should not be less than the chemical pesticides.

2. Low cost similar to conventional methods, especially in field studies and natural conditions.

3. Formulations are specific to the target organism. They are formulated considering behavioral feeding and difference of habitat.

4. Application and equipment are easy to use.

5. Safety is not considered and was passed by applicator reception of safety and consideration of impact to environment and human.

6. Public education and awareness.

7. Resistance possibility, such as from inorganic chemicals.

The studies related entomopathogenic nematodes

Kondo and Ishibashi, 1987 (26) studied size and age of host insect related to and capable of hosting entomopahogenic nematode. Small size enhances ability to enter host at a lower percent than a large size.

Kung, Gaugler and Kaya, 1990 (27) studied efficacy of entomopathogenic nematodes reduced when the potential of hydrogen (pH) lower than 4 and *S. carpocapsae* none potential pathogen with *G. mellonella* and has an attractive base better than acidic. Avoid pH lower 2.5 (28)

Giffin and Downes, 1991 (29) suggested a factor of temperature tends to inhibit ability to enter host and a low temperature inhibits activity between the symbiosis bacteria and the nematode.

Georgis and Hague, 1988 (30) studied using biology controls by showing entomopathogenic nematodes was unsuccessful in a low temperature between 12-14 °C. Temperature survival of *Heterorhabditis* spp. was greater at 16-28 °C.

Kaya 1977 (31) indicated that entomopathogenic nematodes were unable to develop at temperature lower 10 °C and higher 30 °C

Survival temperature of entomopathogenic nematodes was set at -10 to 35 °C (32). The infective juveniles had ability to enter host at temperature between 9-33 °C and the temperature reproduction was between 25-28 °C. (31,33)

Poiner and Thomas, 1965 (34) established that a single species of bacterium in the family Enterobacteriaceae was present in the anterior region of the infective juvenile (IJ/IJs) of *Steinernema carpocapsae*. Since then it has been discovered that *Steinernema* spp. carry bacteria of the genus *Xenorhabdus* while *Heterorhabditis* nematodes harbour a species of the genus *Photorhabdus*. *Xenorhabdus* spp. is a gram-negative facultative anaerobic, non-spore forming bacterium with numerous peritrichous flagella of the non-resistance stage.

Grenier, Catzeflis and Abad, 1997 (35) studied genome size and complexity in entomopathogenic nematodes. *S. carpocapsae* and *H. bacteriophora* were estimated at 2-3 × 10^8 and 3-9 × 10^7 for base pair, respectively.

Forst and Clarke, 2002 (36) studied the entomopathogenic nematode *Steinernema carpocapsae* and its mutualistic symbiont, the γ-proteobacterium *Xenorhabdus nematophila* which parasitize various insect species that they kill and use for reproduction.

Manit, Chaiyaporn and Anu, 2004 (6) studied biology control of five entomopathogenic nematode species that was carried out to control five mosquito larvae species in the laboratory. The results showed that *Steinernema carpocapsae* (Weiser), *Steinernema siamkayai*, *Steinernema feltiae*, *Heterorhabditis indica* and *Heterorhabditis bacteriophora* had the effect of controlling the larvae of *Aedes aegypt* (L.), *Culex quinquefasciatus*, *Culex gelidus*, *Anopheles dirus* and *Anopheles minimus*. All of the treatments were effective in significantly controlling *Ae. aegypti* (L.), *Cx. quinquefasciatus*, *Cx. gelidus* larvae when compared with the untreated control.

CHAPTER III
MATERIALS AND METHODS

Research design

Experimental 2 × 4 factorial research design were used in this study. Each experiment had 4 replications. Mortality of 3^{rd} – 4^{th} instar larvae of *Culex gelidus* by entomopathogenic nematodes, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain), were then determined. Factors A are two genera of entomopathogenic nematodes and factors B are different dosage of nematode (numbers of EPN per *Cx.gelidus* larvae) which is 500, 1000, 2000 and 4000.

Experimental place

The experiments were carried out at :

1. Laboratory of Parasitology Department, Faculty of Public Health, Mahidol University, Bangkok and

2. Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operative, Bangkok.

Materials

The materials and equipment used in the experiment were provided by Bureau of Vector-Borne Disease Control, Department of Disease Control, Ministry of Public Health; Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operative and Parasitology Department, Faculty of Public Health, Mahidol University. All materials and equipment used were as follows:

1. Equipment for preparing entomopathogenic nematode.

1.1 *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai sp.) stored in form of plastic foam and kept at temperature 7 – 10 °C.

1.2 Dechlorinated tap water

1.3 Glass beaker, size 250 and 1000 ml

1.4 Micropipette, size 1 and 10 ml

1.5 Tip

1.6 Glass slide

1.7 Microscope

1.8 Counting plate

1.9 Counter

1.10 pH meter

2. Equipment for rearing *Culex gelidus* larvae

2.1 Dipper net

2.2 Glass beaker, size 250 and 1000 ml

2.3 Pipettes

2.4 Stereoscope

2.5 Dechlorinated tap water

2.6 Dog biscuit

2.7 Plastic trays, size 30 × 25 × 5 cm

2.8 Rice straw

2.9 Dropper

2.10 Cages, size 30 × 30 × 30 cm

3. Equipment for laboratory bioassay

3.1 Plastic cups with 8 – cm diameter and 3.5 – cm height

3.2 Digital camera for stereoscope

3.3 Dissecting needle

3.4 Pipettes

3.5 Glass slide

3.6 Digital timer

Methods

The study was divided into three parts as follows :

1. **Preparation of the entomopathogenic nematodes**, *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain)

The procedure for preparing nematodes of the two genera were followed the manual recommended by the Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture. Using dilution counting technique (see appendix B on page 56) they were identified under microscope. 4 million nematodes are stored in synthetic sponge sealed in plastic bag. Rinse these nematodes off synthetic sponge using dechlorinated tap water.

2. **Preparation of 3rd – 4th instar larvae of *Cx. gelidus***.

Engorged females of *Cx. gelidus* were collected at rice fields in Bangbuathong district Nonthaburi province using aspirator from man baits and light trap. The mosquitoes were then transferred into paper cups (approximately 100 individuals per cup). Cotton wool soaked with 5% sugar solution was provided as the source of food during transportation. At the laboratory, female mosquitoes were released into 30 × 30 × 30 cm cages. Cotton wool soaked with a mixture of 5% sugar solution and multivitamin syrup (1:1) was supplied as food.

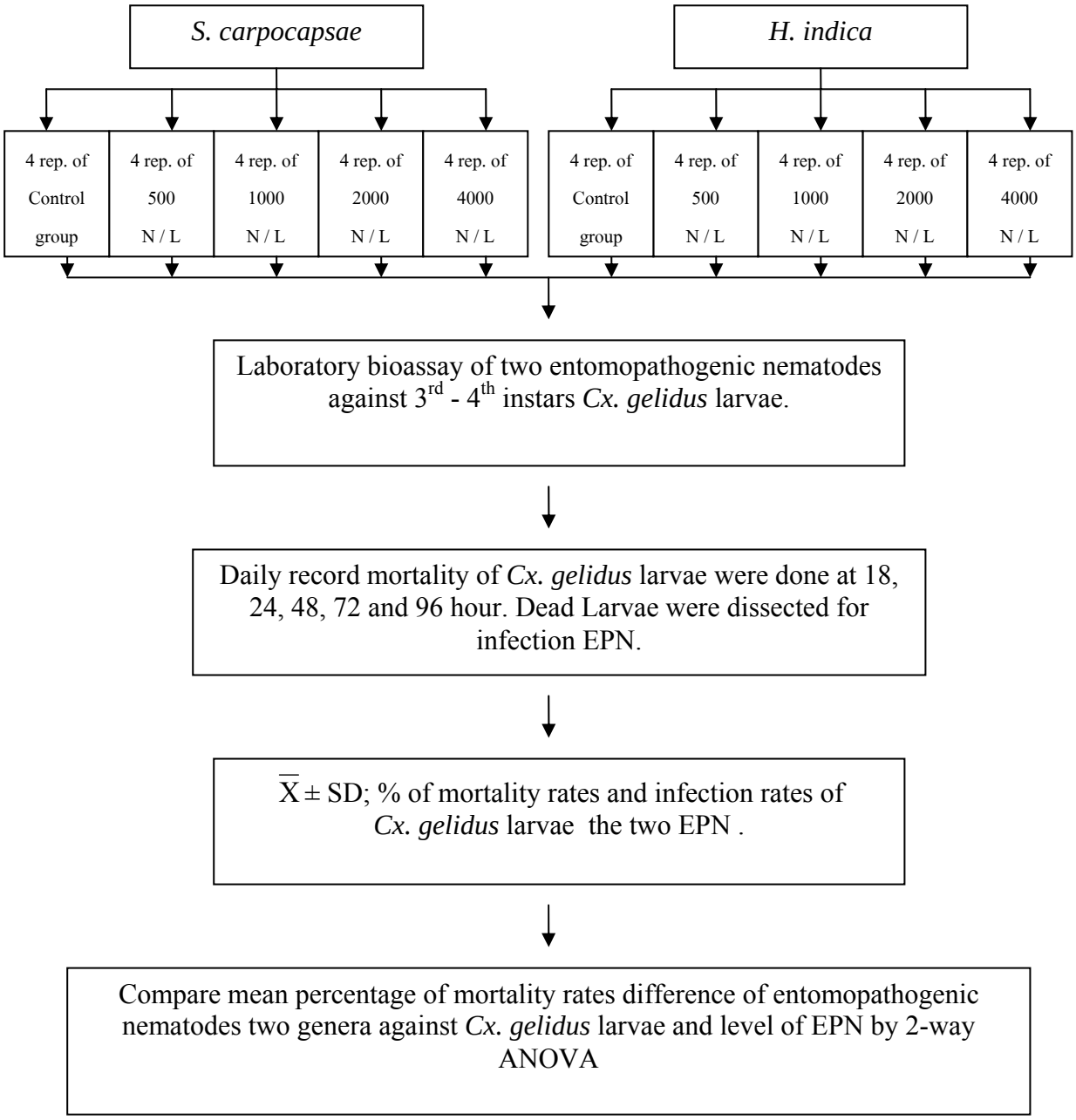
2-3 days later, plastic cups containing rice straw infusion water were placed in cage for oviposition. The female mosquitoes lay egg rafts in plastic cups. About 200-300 hatched larvae were introduced into an enamel tray containing about 1.5 liters of 3 days fermented rice straw infusion water. Add homogenized dog biscuit which were provided larvae as food. (37)

The larvae which are suitable for the test are 3 - 4 instars larvae. This is done by observation of their body length which are 0.4-0.5 cm and 0.8-1.0 cm respectively.

3. **Determination of the efficacy of EPN against *Cx. gelidus* larvae**. Four dosage of EPN per larva, i.e. 500, 1000, 2000 and 4000 were tested against *Cx. gelidus* larvae, each dosage were four replications and control group (see procedure on page 20). The total volume of water test in each cup was 38.5 cm^2 and water depth was 2.5 cm. The observations time for mortality and infection were done at 18, 24, 48, 72 and 96 hours after the larvae are exposed to EPN. Dead larvae (no movement and

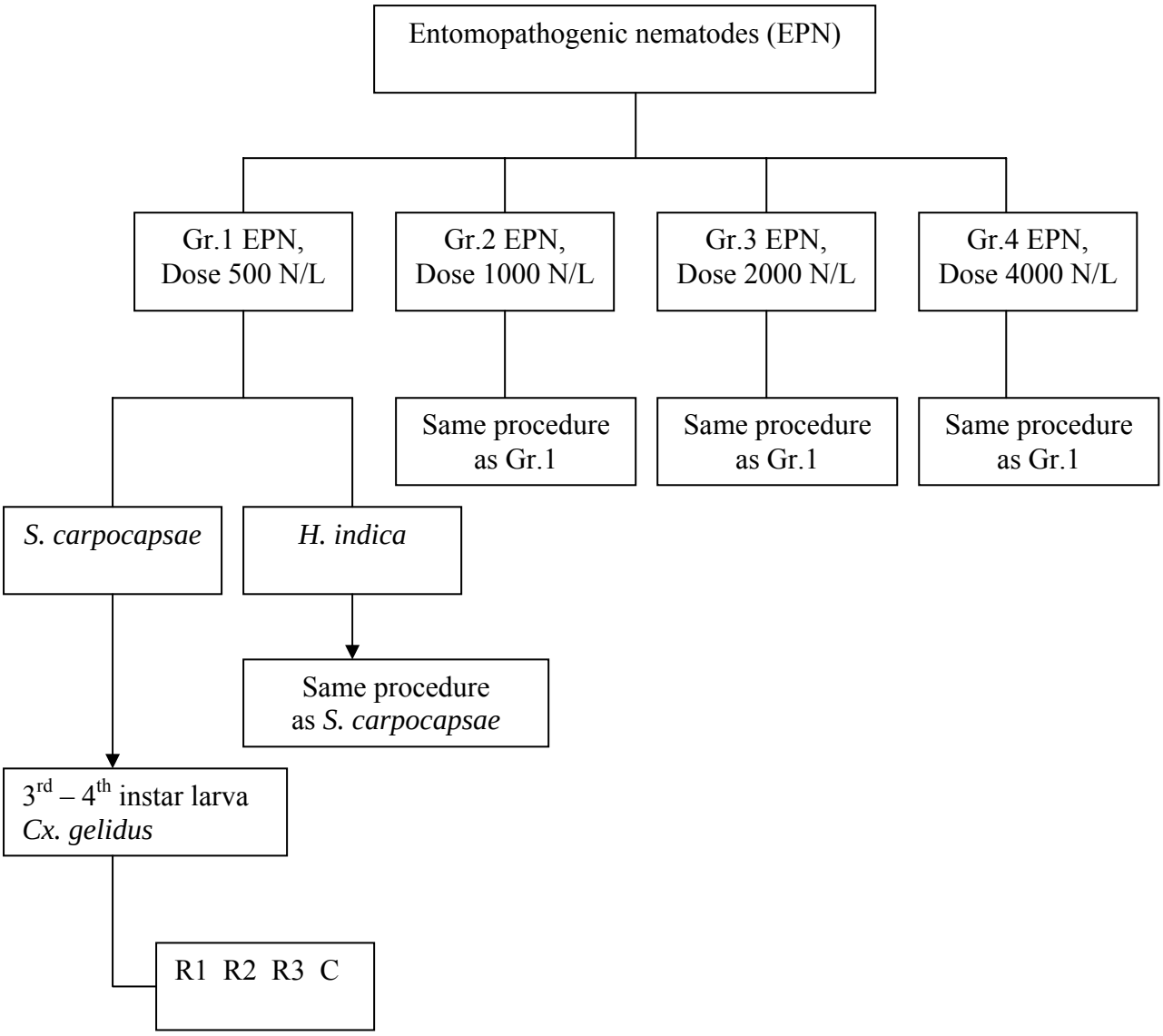
no feed) were dissected in saline under microscope. Record and take photographs of dissected larvae.

Procedure of experiment



* L = Larvae, N = Nematodes and rep. = Replications

Layout of experiment



Data Analysis

1. The results of four replicates in exposure time observed was calculated by mean ($\overline{X}$) and standard deviation (S.D.), percentage (%). The control test will not be calculated. Data presentation was performed by using the graphical method and tables.

2. Comparatives mean percentage difference of *Culex gelidus* larvae mortalities by using entomopathogenic nematodes between two genera, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) and the mean percentage difference level of larvae per nematode was analyzed by 2 – way ANOVA. The determination of significant difference p-value level was at 0.05

CHAPTER IV
RESULTS

This study was carried out to determine the mortality and infection of the 3rd - 4th instars larvae of *Culex gelidus* by two genera of entomopathogenic nematodes, *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain). The experiment was done at the Parasitology Department laboratory, Faculty of Public Health, Mahidol University.

Experimental results were observed daily for five consecutive days and recorded the mortality of 3rd - 4th instars *Culex gelidus* larvae were recorded at 18, 24, 48, 72 and 96 hours exposure time.

The mortality and infection

4.1 The mortality and infection of the 3rd – 4th instars *Culex gelidus* larvae by two genera of entomopathogenic nematodes.

4.1.1 The mortality of *Culex gelidus* larvae by two genera of entomopathogenic nematodes.

The mortality of third and fourth instars *Cx. gelidus* larvae caused by *S. carpocapsae* (Weiser). The average numbers of mortality observed at 18, 24, 48, 72, 96 hours exposure time to dosage 4000 IJs per larva were 0.0±0.0, 1.0±0.8, 7.3±1.9, 13.0±1.4 and 15.8±1.7 (mortality rate more than 60%), respectively. The average mortality at dosage 2000 IJs per larvae were 0.0±0.0, 0.5±0.6, 5.8±3.1, 11.3±2.8 and 13.3±2.2 (mortality rate more than 50%), respectively. The average mortality at dosage 1000 IJs per larvae were 0.0±0.0, 0.0±0.0, 4.3±1.0, 6.0±1.4 and 7.8±1.7, respectively. The mortality averages at dosage 500 IJs per larvae were 0.0±0.0, 0.3±0.5, 3.3±2.2, 5.3±2.5 and 7.3±1.9, respectively. The control treatment was died 1.3±0.5 (average 5%) (Table 3).

The mortality rates either dosage were different between 4000, 2000, 1000, 500 and 0 IJs per larvae and were 63.0%, 53.0%, 31.0%, 29.0% and 5.0%, respectively (Figure 5,7).

The results indicated that the mortality in *Cx. gelidus* third and fourth instars larvae in part of *H. indica* (Local Thai strain) had a least difference in each level of nematodes per larvae and exposure times. The average number of mortality that observed and recorded at 18, 24, 48, 72, 96 hour and total mortality had a highest in 4000 IJs per larvae namely 0.0±0.0, 0.0±0.0, 0.5±0.6, 1.8±1.0 and 3.3±2.1, respectively. The mortality averages at dosage 2000 IJs per larvae were 0.0±0.0, 0.0±0.0, 0.0±0.0, 0.8±0.5 and 2.0±0.8, respectively. The mortality averages at dosage 1000 IJs per larvae were 0.0±0.0, 0.0±0.0, 0.5±0.6, 0.8±1.0 and 1.3±1.0, respectively. The mortality averages at dosage 500 IJs per larvae were 0.0±0.0, 0.0±0.0, 0.3±0.5, 0.5±0.6 and 1.0±0.8, respectively. The control treatment was died 1.3±0.5 (average 5%) (Table 3).

The mortality rates either dosage were different at 4000, 2000, 1000, 500 and 0 IJs per larvae and were 13.0%, 8.0%, 5.0%, 4.0% and 5.0%, respectively (Figure 6,8).

Table 3 Mean and standard deviation mortality of 3rd – 4th *Culex gelidus* instars larvae by two genera of entomopathogenic nematodes.

Species of nematodes	Dosage of nemato-des per larva	n	Cumulative No. of mortality larvae in duration of exposure time (Hours)					Mortality rates (%)
			18	24	48	72	96	
S. carpocapsae (Weiser)	4000	4	0.0±0.0	1.0±0.8	7.3±1.9	13.0±1.4	15.8±1.7	63.0
	2000	4	0.0±0.0	0.5±0.6	5.8±3.1	11.3±2.8	13.3±2.2	53.0
	1000	4	0.0±0.0	0.0±0.0	4.3±1.0	6.0±1.4	7.8±1.7	31.0
	500	4	0.0±0.0	0.3±0.5	3.3±2.2	5.3±2.5	7.3±1.9	29.0
	0	4	0.0±0.0	0.0±0.0	0.0±0.0	0.5±0.6	1.3±0.5	5.0
H. indica (Local Thai strain)	4000	4	0.0±0.0	0.0±0.0	0.5±0.6	1.8±1.0	3.3±2.1	13.0
	2000	4	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.5	2.0±0.8	8.0
	1000	4	0.0±0.0	0.0±0.0	0.5±0.6	0.8±1.0	1.3±1.0	5.0
	500	4	0.0±0.0	0.0±0.0	0.3±0.5	0.5±0.6	1.0±0.8	4.0
	0	4	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.5	1.3±1.0	5.0

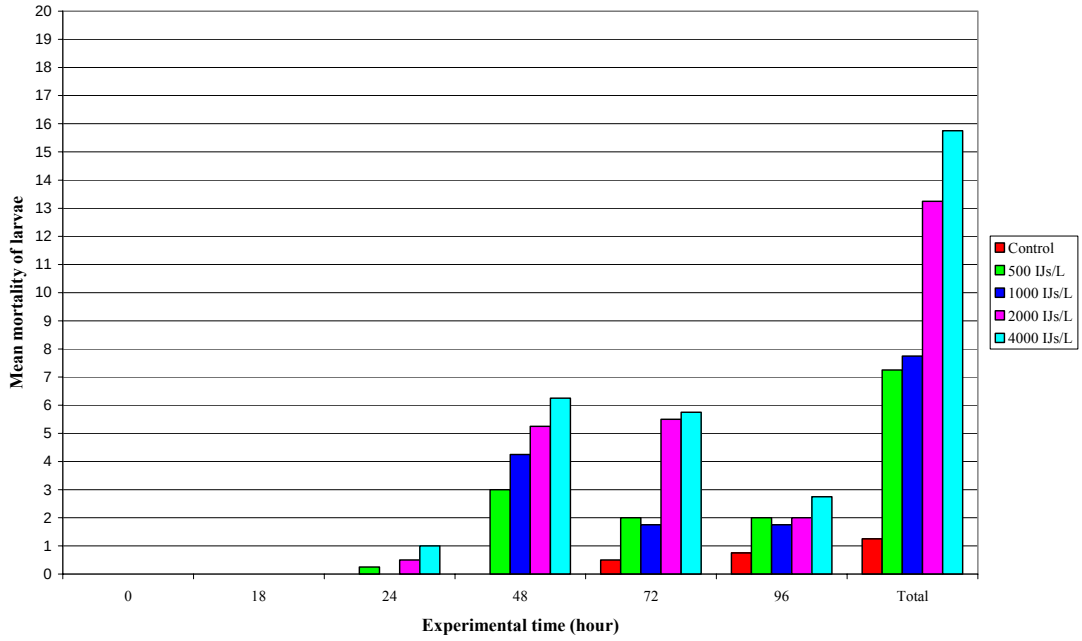


Figure 5 Mean mortality of larvae in each exposure time (Hours), *S. carpocapsae* (Weiser)

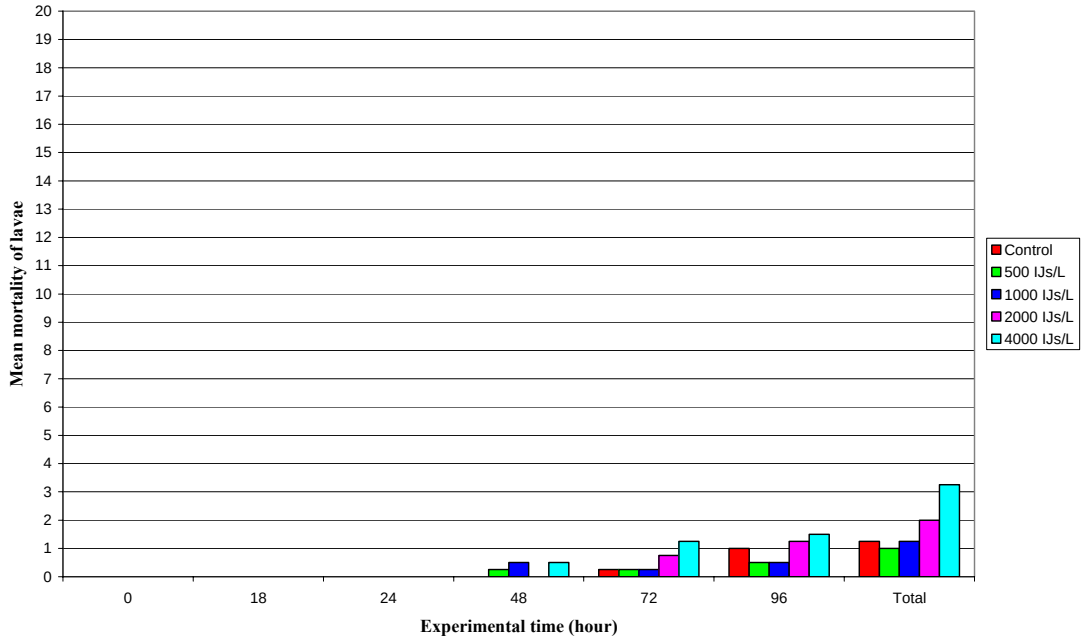


Figure 6 Mean mortality of larvae in each exposure time (Hours), *H. indica* (Local Thai strain)

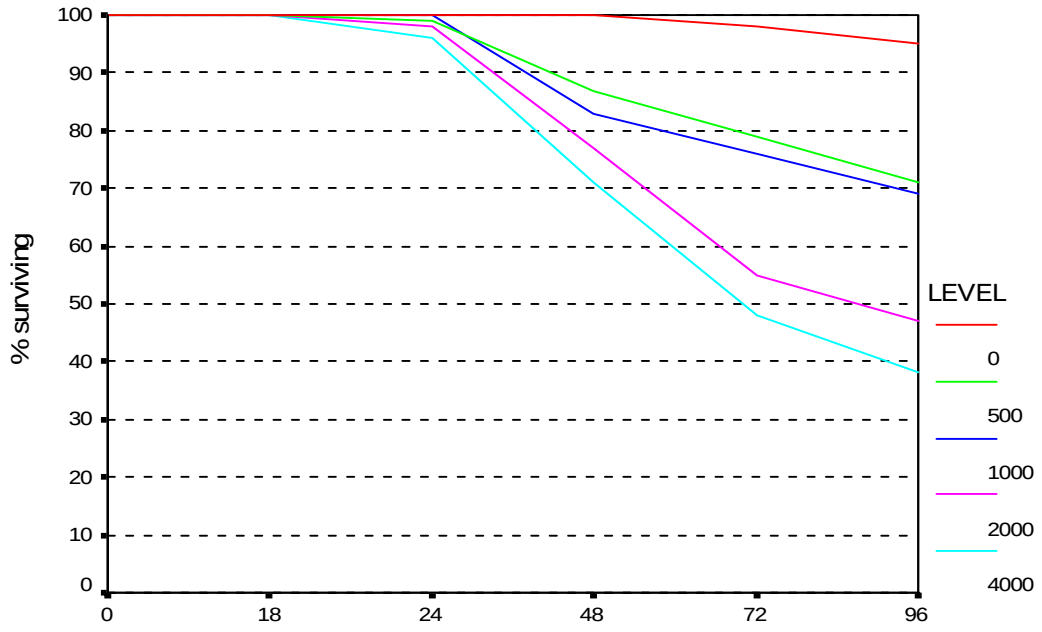


Figure 7 Percentage surviving curves of larvae from EPN, *S. carpocapsae* (Weiser) in each dosage.

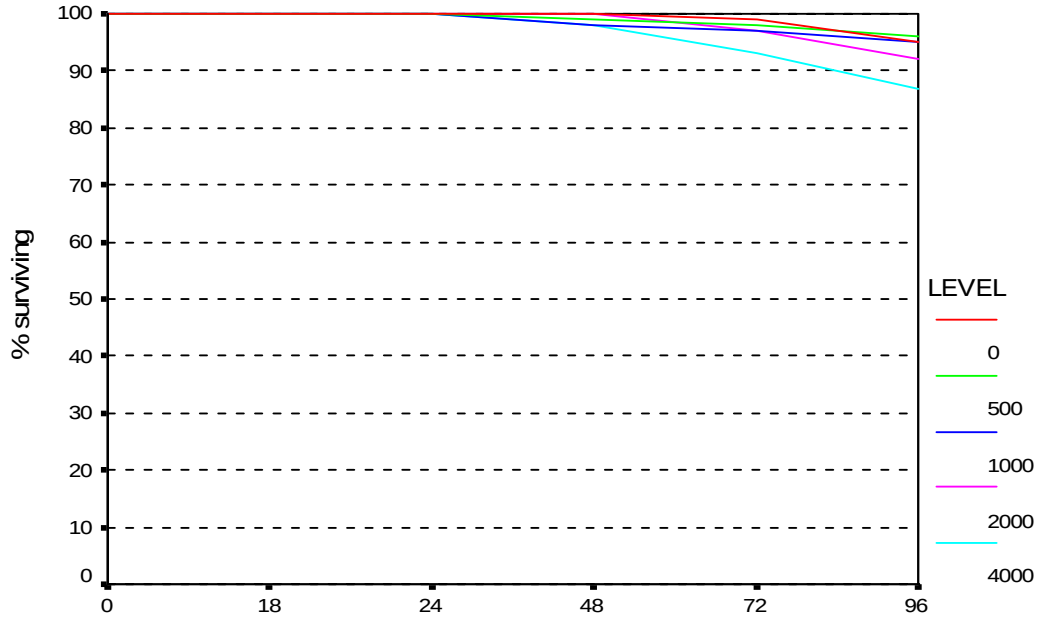


Figure 8 Percentage surviving curves of larvae from EPN, *H. indica* (Local Thai strain) in each dosage.

4.1.2 The infection of *Cx. gelidus* larvae by two genera of entomopathogenic nematodes.

S. carpocapsae (Weiser), after dissected larvae the total infection rate was 14.5%. The infection rates for these genera were highest in dosage 4000 IJs per mosquito larvae that individual observed at 24, 48, 72 and 96 hour namely 3.0%, 11.5%, 11.3% and 18.8%, respectively. The infection rates in dosage 2000 IJs per mosquito larvae that individual observed at 24, 48, 72 and 96 hour were 2.0%, 11.2%, 6.5% and 3.6%. The infection rates were lowest in dosage 1000 IJs per mosquito larvae that only found at 72 and 48 hour namely 3.6% and 4.0%. The dosage 500 IJs per mosquito larva were no infection (Table 4).

H. indica (Local Thai strain), after dissected larvae the total infection rate was 2.0%. The infection rates for these genera were low. The maximum infection rates were 2.0% in dosage 4000 IJs per mosquito larvae at 48 and 72 hour (Table 5).

Table 4 Infection rates, *S. carpocapsae* (Weiser)

Exposure time	Dosage of nematodes per larva	No. of larvae test	No. of larvae died	No. of larvae with nematodes	Infection rates %
18	4000	100	0	0	0.0
	2000	100	0	0	0.0
	1000	100	0	0	0.0
	500	100	0	0	0.0
24	4000	100	4	3	3.0
	2000	100	2	2	2.0
	1000	100	0	0	0.0
	500	100	1	0	0.0
48	4000	96	25	11	11.5
	2000	98	21	11	11.2
	1000	100	16	4	4.0
	500	99	13	0	0.0
72	4000	71	23	8	11.3
	2000	77	22	5	6.5
	1000	84	7	3	3.6
	500	86	8	0	0.0
96	4000	48	11	9	18.8
	2000	55	8	2	3.6
	1000	77	7	0	0.0
	500	78	8	0	0.0
Total		400	176	58	14.5

Infection rates; (No. of larvae with nematodes / No. of larvae test) x 100

Table 5 Infection rates, *H. indica* (Local Thai strain)

Exposure time	Dosage of nematodes per larva	No. of larvae test	No. of larvae died	No. of larvae with nematodes	Infection rates %
18	4000	100	0	0	0.0
	2000	100	0	0	0.0
	1000	100	0	0	0.0
	500	100	0	0	0.0
24	4000	100	0	0	0.0
	2000	100	0	0	0.0
	1000	100	0	0	0.0
	500	100	0	0	0.0
48	4000	100	2	2	2.0
	2000	100	0	0	0.0
	1000	100	2	2	2.0
	500	100	1	1	1.0
72	4000	98	5	2	2.0
	2000	100	3	1	1.0
	1000	98	1	0	0.0
	500	99	1	0	0.0
96	4000	93	6	0	0.0
	2000	97	5	0	0.0
	1000	97	2	0	0.0
	500	98	2	0	0.0
Total		400	30	8	2.0

The nematodes genera *S. carpocapsae* (Weiser) were found mostly in thorax than head and abdomen of infected mosquitoes larvae 83.0%, 10.0% and 7.0%, respectively. At dosage 4000 IJs per mosquito larvae individual observed times were found mostly in thorax in each time after infection at 24, 48, 72 and 96 hour namely 75%, 92%, 94% and 67%, respectively. Dosage 2000 IJs per mosquito larvae were found mostly in thorax in each time at 24, 48, 72 and 96 hour namely 83%, 83%, 78% and 25%, respectively. Dosage 1000 IJs per mosquito larvae were found mostly in thorax in each time at 48 and 72 hour namely 82% and 94% (Table 6) (Figure 8 - 11).

The nematode genera *H. indica* (Local Thai strain) were found only in thorax (Table 7).

Table 6 Numbers and percentage of *S. carpocapsae* (Weiser) in different part of *Cx. gelidus* larvae.

Exposure time	Dosage of nematodes per larva	No. and % of nematodes in different parts of larvae					
		Head	%	Thorax	%	Abdomen	%
18	4000	-	-	-	-	-	-
	2000	-	-	-	-	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
24	4000	3(2)	19.0	12(3)	75.0	1(1)	6.0
	2000	-	-	5(2)	83.0	1(1)	17.0
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
48	4000	1(1)	4.0	23(11)	92.0	1(1)	4.0
	2000	3(3)	10.0	24(10)	83.0	2(1)	7.0
	1000	2(2)	18.0	9(4)	82.0	-	-
	500	-	-	-	-	-	-
72	4000	2(2)	6.0	34(8)	94.0	-	-
	2000	-	-	7(3)	78.0	2(2)	22.0
	1000	-	-	15(3)	94.0	1(1)	6.0
	500	-	-	-	-	-	-
96	4000	3(2)	13.0	16(7)	67.0	5(3)	21.0
	2000	3(2)	75.0	1(2)	25.0	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
Total		17(14)	10.0	146(53)	83.0	13(10)	7.0

The numbers of dead mosquito larvae are in parenthesis.

Table 7 Numbers and percentage of *H. indica* (Local Thai strain) in difference part of *Cx. gelidus* larvae.

Exposure time	Dosage of nematodes per larva	No. and % of nematodes in different parts of larvae					
		Head	%	Thorax	%	Abdomen	%
18	4000	-	-	-	-	-	-
	2000	-	-	-	-	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
24	4000	-	-	-	-	-	-
	2000	-	-	-	-	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
48	4000	-	-	5(2)	100.0	-	-
	2000	-	-	-	-	-	-
	1000	-	-	3(2)	100.0	-	-
	500	-	-	2(1)	100.0	-	-
72	4000	-	-	5(2)	100.0	-	-
	2000	-	-	1(1)	100.0	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
96	4000	-	-	-	-	-	-
	2000	-	-	-	-	-	-
	1000	-	-	-	-	-	-
	500	-	-	-	-	-	-
Total		-	-	16(8)	100.0	-	-

The numbers of dead mosquito larvae are in parenthesis.

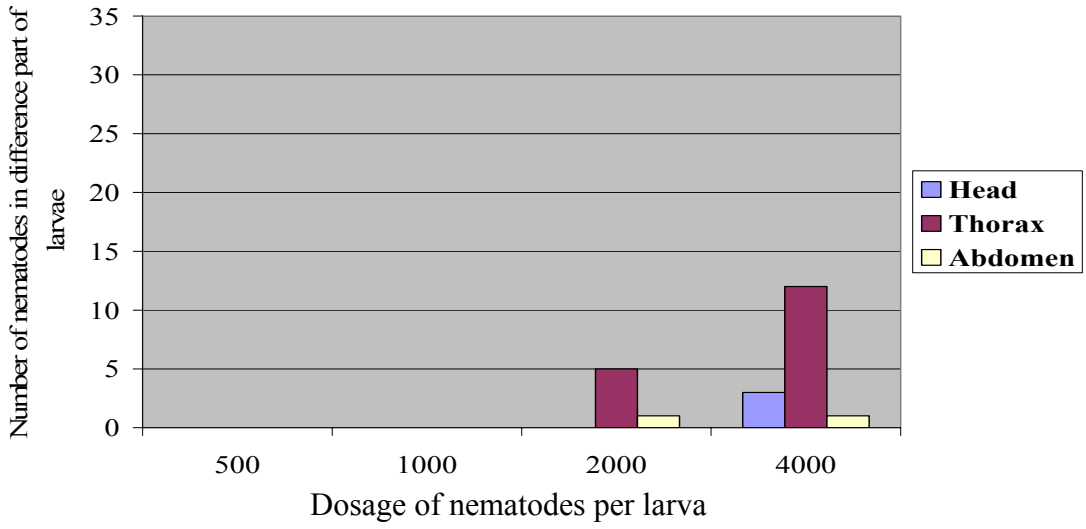


Figure 9 Numbers of *S. carpocapsae* (Weiser) nematodes recovered from different parts of mosquitoes larvae, 24 hour post exposure.

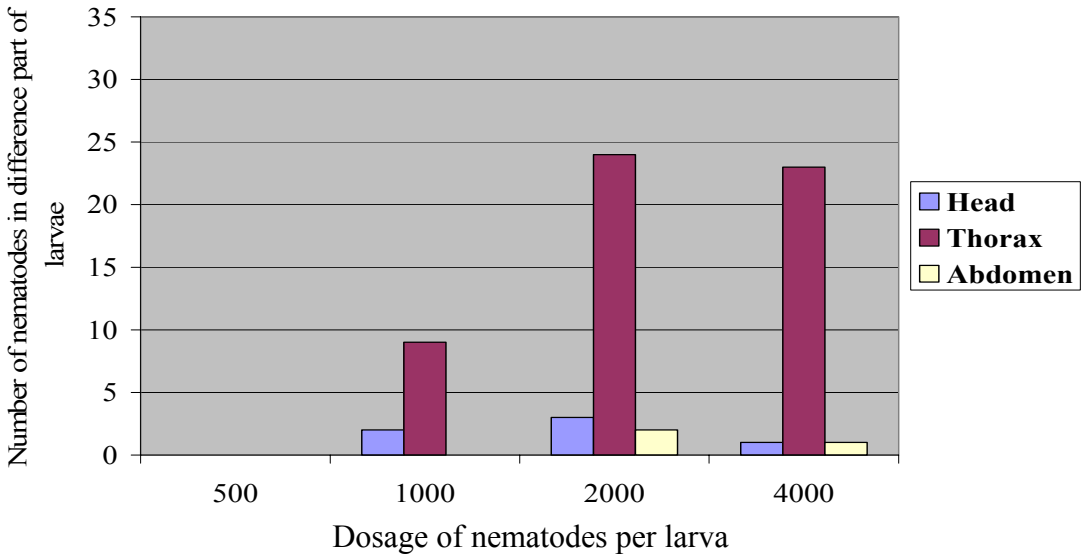


Figure 10 Numbers of *S. carpocapsae* (Weiser) nematodes recovered from different parts of mosquitoes larvae, 48 hour post exposure.

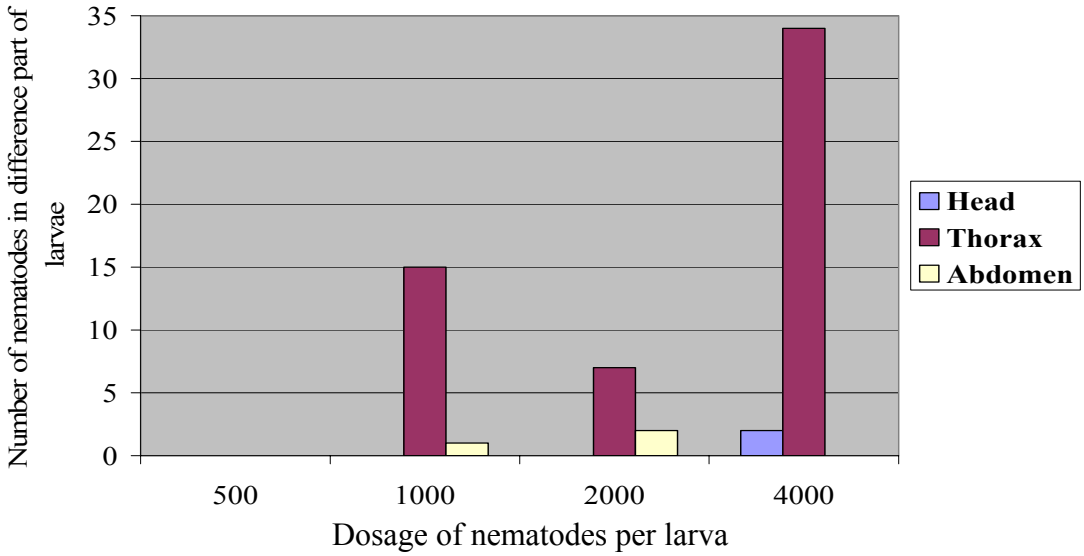


Figure 11 Numbers of *S. carpocapsae* (Weiser) nematodes recovered from different parts of mosquitoes larvae, 72 hour post exposure.

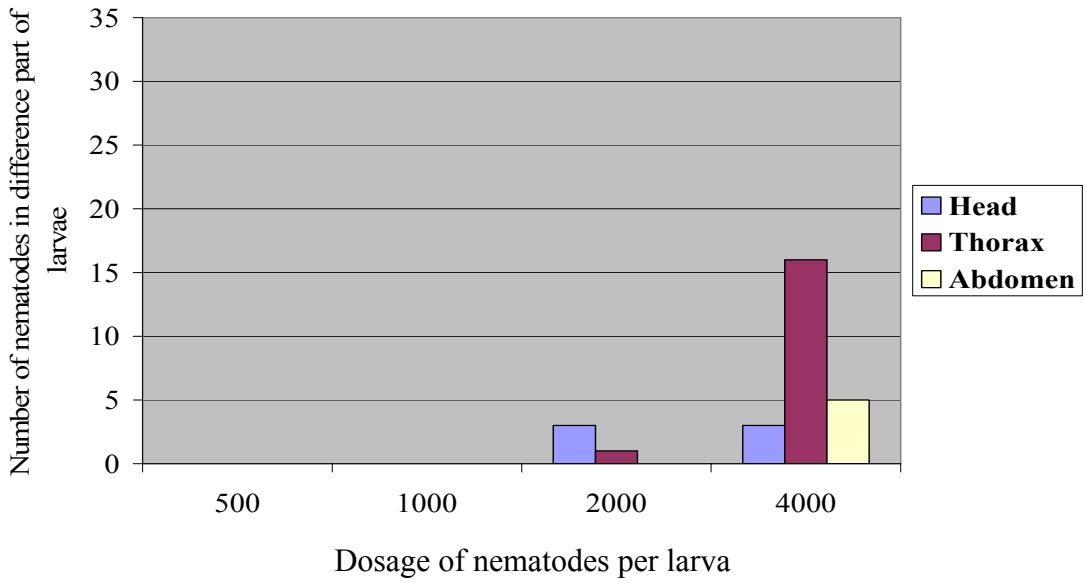


Figure 12 Numbers of *S. carpocapsae* (Weiser) nematodes recovered from different parts of mosquitoes larvae, 96 hour post exposure.

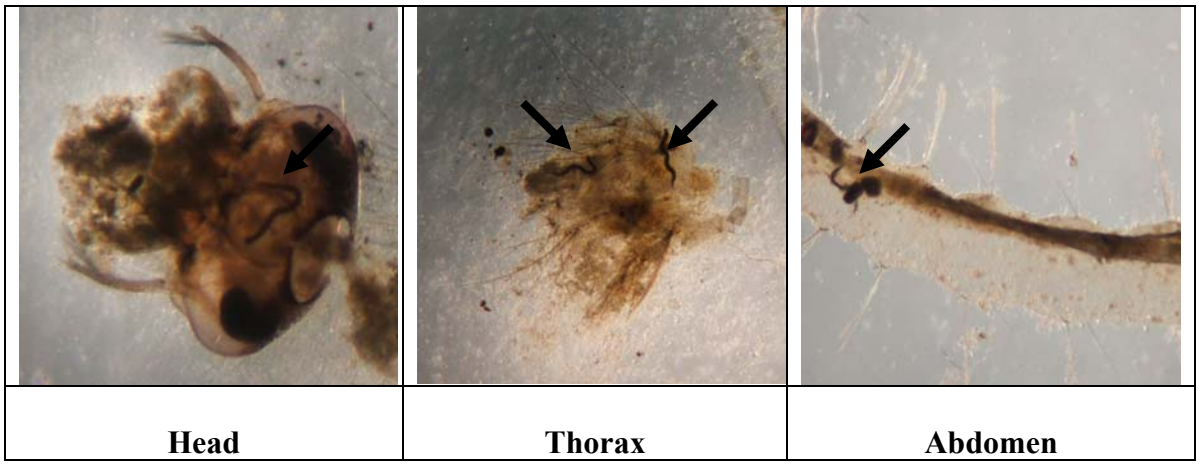


Figure 13 Infective stages of nematodes in different parts of *Cx. gelidus* larvae. × 100

4.2 Analytical mortality of odds ratio of EPN applied against third and fourth instars *Cx. gelidus* larvae

The odds ratio of *S. carpocapsae* (Weiser) applied against larvae of *Cx. gelidus* in each dosage compared to control group (0 IJs per larvae) at 4000, 2000, 1000 and 500 were 32.35 (95% CI 11.32-99.62), 21.43 (95% CI 7.55-65.50), 8.54 (95% CI 2.96-26.44) and 7.76 (95% CI 2.68-24.13), respectively (Table 8).

Table 8 Odds ratio of *S. carpocapsae* (Weiser) applied against third to fourth instars *Cx. gelidus* larvae

Dosage of nematodes per larvae	*S. carpocapsae* Died	Survived	Total	OR	95% CI
0	5	95	100	1	
500	29	71	100	7.76	2.68-24.13
1000	31	69	100	8.54	2.96-26.44
2000	53	47	100	21.43	7.55-65.50
4000	63	37	100	32.35	11.32-99.62
Total	181	319	500		

The odds ratio of *H. indica* (Local Thai strain) applied against larvae of *Cx. gelidus* in each dosage compared to control group (0 IJs per larvae) at 4000, 2000, 1000 and 500 were 2.84 (95% CI 0.89-9.56), 1.65 (95% CI 0.47-6.07), 1 (95% CI 0.24-4.14) and 0.79 (95% CI 0.17-3.53), respectively (Table 9).

Table 9 Odds ratio of *H. indica* (Local Thai strain) applied against third to fourth instars *Cx. gelidus* larvae

Dosage of nematodes per larvae	*H. indica* Died	Survived	total	OR	95% CI of OR
0	5	95	100	1	
500	4	96	100	0.79	0.17-3.53
1000	5	95	100	1	0.24-4.14
2000	8	92	100	1.65	0.47-6.07
4000	13	87	100	2.84	0.89-9.56
Total	35	465	500		

4.3 The comparative efficacy of EPN between two genera applied against third to fourth instars *Cx. gelidus* larvae

4.3.1 Mean comparison of mortality of *S. carpocapsae* (Weiser) and *H. indica* (Local Thai sp.) applied against third to fourth instars *Cx. gelidus* larvae

The results indicated that there was significant different in each dosage in genus of *S. carpocapsae* (Weiser) ($F = 43.99$; $df = 4$; $p < 0.001$) (Table 10).

Each dosage was compared to control group and that was significant difference. However, between dosage at 500 and 1000 IJs per larvae there was no significant difference ($p > 0.05$) and between dosage at 2000 and 4000 IJs per larvae there was no significant difference ($p > 0.05$) (Table 10).

The results of *H. indica* (Local Thai strain) indicated that there was no significant difference in each dosage ($F = 1.449$; $df = 4$; $p = 0.267$) (Table 11).

Table 10 Mean comparison of mortality larvae by *S. carpocapsae* (Weiser)

Dosage of IJs per larvae	n	$\overline{X}$	SD	F	*df*	p-value
0	4	1.3^a	0.5	43.99	4	<0.001
500	4	7.3^b	1.9			
1000	4	7.8^b	1.7			
2000	4	13.3^c	2.2			
4000	4	15.8^c	1.7			

The same alphabet in column $\overline{X}$ represent no significant difference at $\alpha = 0.05$ (LSD)

Table 11 Mean comparisons (translated to 10 base logarithms) of mortality larvae by *H. indica* (Local Thai strain).

Dosage of IJs per larvae	n	$\overline{X}$	SD	F	*df*	p-value
0	4	1.3^a	1.0	1.45	4	0.27
500	4	1.0^a	0.8			
1000	4	1.3^a	1.0			
2000	4	2.0^a	0.8			
4000	4	3.3^a	2.1			

The same alphabet in column $\overline{X}$ is represent no significant difference at $\alpha = 0.05$(LSD)

4.4 The comparative mean different of mortality between *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) applied against third to fourth instars *Cx. gelidus* larvae at either dosage.

The result at 48 and 72 hour showed between two genera in either post exposed time and dosage of nematodes per larvae were effect mortality applied against third to fourth instars *Cx. gelidus* larvae. The variances of two genera of EPN were heterogeneity.

The results that found between two genera in each exposed time had affected to mortality of *Cx. gelidus* larvae ($p < 0.001$). EPN of genera *S. carpocapsae* (Weiser) had highest affected mortality than genera *H. indica* (Local Thai strain) at 48 and 72 hour (Table 12).

Table 12 Mean comparison of mortality of *Cx. gelidus* larvae in either exposed time by EPN two genera.

Exposed Time	n	$\overline{X}$	SD	t	*df*	p-value
48 hour				5.570	20	< 0.001
S. carpocapsae	20	4.1	3.1			
H. indica	20	0.3	0.4			
72 hour				5.765	20	< 0.001
S. carpocapsae	20	7.2	4.9			
H. indica	20	0.8	0.8			

The result at finite time to exposed that found between two genera were significant difference ($p < 0.001$; $df = 1$; $F = 82.867$) and level of nematodes per larvae were significant difference ($p < 0.001$; $df = 4$; $F = 11.925$) (Table 13).

The two way interaction test found that there were interactions between 2 factors ($p = 0.008$; $df = 4$; $F = 4.178$) (Table 13) (Figure 14).

Table 13 Result 2 way ANOVA of the mortality between *S. carpocapsae* (Weiser) and *H. indica* (Local Thai sp.) applied against third to fourth instars *Cx. gelidus* larvae at either dosage.

Source	Sum of Squares	*df*	Mean Square	F	p-value
Corrected Model	6.501	9	.722	16.364	<0.001
Intercept	13.018	1	13.018	294.932	<0.001
LEVEL	2.105	4	.526	11.925	<0.001
GENERA	3.658	1	3.658	82.867	<0.001
LEVEL * GENERA	.738	4	.184	4.178	0.008
Error	1.324	30	.044		
Total	20.843	40			
Corrected Total	7.825	39			

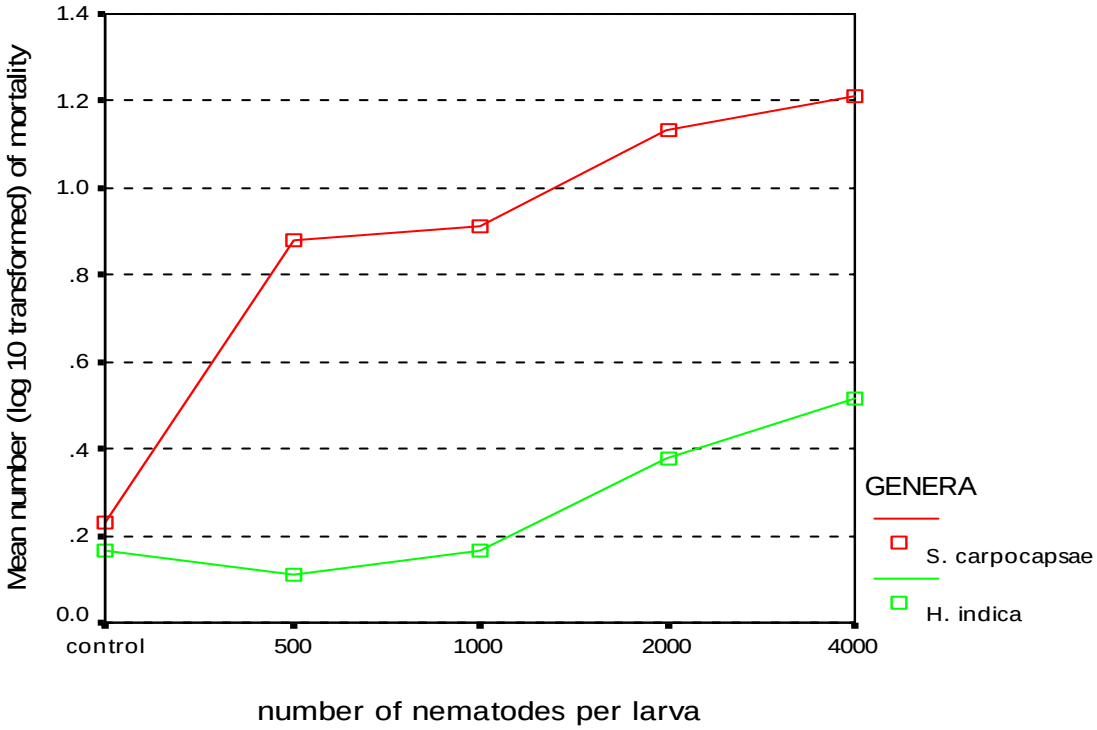


Figure 14 Mean number (10 log transformed) of mortality between *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) applied against third to fourth instars *Cx. gelidus* larvae at each dosages.

Multiple comparison of interaction between two genera in each dosages of EPN per larvae that found significant difference interaction in varies dosages χ^2 = 48.435, *df* = 15, p < 0.001 (Kruskal Willis Test) and multiple comparison to determine mean rank difference between groups namely (A,I) (A,J) (A,K) (A,L) (A,M) (A,N) (A,O) (A,P) (B,I) (B,J) (B,K) (B,L) (B,M) (B,N) (B,O) (B,P) (C,I)(C,J) (C,K) (C,L) (C,M) (C,N) (C,O) (C,P) (D,I) (D,J) (D,K) (D,L) (D,M) (D,N) (D,O) (D,P) (E,J) (E,K) (E,L) (E,M) (E,N) (E,O) (E,P) (F,L) (F,M) (F,N) (F,O) (F,P) (G,K) (G,L) (G,M) (G,N) (G,O) (G,P) (H,L) (H,M) (H,N) (H,O) (H,P) (I,O) (I,P) (J,P) and (K,P) (Figure 15).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A (12.0)									*	*	*	*	*	*	*	*
B (13.5)									*	*	*	*	*	*	*	*
C (14.5)									*	*	*	*	*	*	*	*
D (15.5)									*	*	*	*	*	*	*	*
E (16.75)										*	*	*	*	*	*	*
F (19.5)												*	*	*	*	*
G (21.5)											*	*	*	*	*	*
H (25.63)												*	*	*	*	*
I (38.13)	*	*	*	*											*	*
J (39.38)	*	*	*	*	*											*
K (42.88)	*	*	*	*	*		*									
L (48.38)	*	*	*	*	*	*	*	*								
M (49.25)	*	*	*	*	*	*	*	*								
N (50.5)	*	*	*	*	*	*	*	*								
O (53.88)	*	*	*	*	*	*	*	*	*							
P (58.75)	*	*	*	*	*	*	*	*	*	*						

* Significant difference (p-value < 0.05),
The numbers in parenthesis are mean rank of dead mosquito larvae interaction dosage

Figure 15 Difference and similar of interaction between two genera of EPN in each dosage of EPN per *Cx. gelidus* larvae. *(A = S500+H500, B = S500+H1000, C = S1000+H500, D = S1000+H1000, E = S500+H2000, F = S1000+H2000, G = S500+H4000, H = S1000+H4000, I = S2000+H500, J = S2000+H1000, K = S2000+H2000, L = S2000+H4000, M = S4000+H500, N = S4000+H1000, O = S4000+H2000 and P = S4000+H4000)

CHAPTER V
DISCUSSION

This research was designed factorial analysis for answering question concerning available to use EPN control 3^{rd} – 4^{th} larva *Cx. gelidus* and emphasize various dosage of EPN per larvae.

1. Efficacy of entomopathogenic nematodes of the genera *Steinernema carpocapsae* (Weiser) and *Heterorhabditis indica* (Local Thai strain) against *Cx.gelidus* larvae. This study has showed that the infective juveniles (IJs) of *S. carpocapsae* (Weiser) had significant higher mortality than control ($P<0.001$). The mortality rate at highest dosage of nematodes per larvae was more than 60%. In contrast, the mortality of larvae by *H. indica* (Local Thai strain) compared to control was not significant difference. The mortality rate at highest dosage was lower than 15%. Comparative two genera had significant ($P<0.05$). Infection rate from *S. carpocapsae*(Weiser) was higher than that from *H. indica* (Local Thai strain), being 14.5 and 2%, respectively. Mortality rates had strongly started at 48 hour in genera *S. carpocapsae* (Weiser) but not seem in genera *H. indica* (Local Thai sp). The reasons for these differences remain unclear and may be related to nematode behavior, efficiency of nematodes, and adaptation to a given host (attraction to the host of ability to overcome defense mechanism) (38). In study of Fallon et al., 2006 (39) found similarity my studied that efficacy of EPN against the cottonwood borer, *Plectrodera scalator* (Fabricius), *Steinernema feltiae* SN and *S. carpocapsae* All killed 58% and 50% of larvae, while *H. indica* MG-13 killed less than 10% of larva in both assays. The host diet was compared to that of *Anoplophora glabripennis*, a host against which *S. carpocapsae* Sal and *S. feltiae* produced 71 – 100% mortality in similar bioassay (40 - 41).

2. The dosage of entomopathogenic nematodes which is effective against *Cx.gelidus* larvae.

This study has showed that the infective juveniles (IJs) of *S. carpocapsae* (Weiser) in the dosage had significant different mortalities of mosquito larvae than control ($P<0.001$). Although between dosage 2000 and 4000 IJs per larvae had highest mortality rates (more than 50%), but there was not significant different among the two dosage in the mortality. Therefore, the dosage suitable for control larvae is 2000 IJs per larvae. In contrast, the mortality of larvae by *H. indica* (Local Thai strain) was so low that it may be unsuitable for control larvae *Cx.gelidus*.

George, Pionar and Kaul, 1981(42) showed the results of similarity study when the nematode concentration increases, a higher number are ingested and more reach the body cavity of host. The larval mortality rates of *Aedes aegypti* was a positive linear function of nematode dosage and exposure time. (50) Size (stage) of host, parasitism in general was highest in fourth-instar larvae. (42,43) Host reaction, *Cx. pipiens* larvae are able to melanize nematodes that have entered their hemocoels. The melanization process is mush more rapid and strong in third and fourth-instar larva than second. In the latter, a newly entered nematode is often able to initiate development and liberate the bacterium. Only after the maximum melanization number is reached can additional nematodes develop freely in the hemocoel and release their bacteria. (43) Then, involve behavior of larvae feeding (bottom feeding). Level of depth water that was found related feeding behavior of larvae mosquito. Manit, Chaiyaporn and Anu, 2004 (6) showed that five nematodes had most effect in controlling the larvae of *Aedes aegypt (L.)* than other mosquito larvae because feeding behavior are “Collecting-gathering” that mean highly expose than other larvae. The aquatic habitat offers an excellent environment for nematode survival. However, steinernema and heterorhabditids are soil organisms and are not adapted for directed motility in the aquatic environment (52).

3. Responses interaction to *Cx. gelidus* larvae infected between two genera in combined each dosage of EPN

This study has showed multiple comparison had mean rank difference significant ($p<0.001$) namely theses interaction between two genera of EPN in each dosage of EPN per larvae

S. carpocapsae and *S. glaseri* co-invade *Galleria* larvae in the laboratory (53, 54). Koppenhofer et al. (1995)(53) found no effect on number of nematodes establishing in mixed versus single infections, whereas (54) found that more *S. carpocapsae* invaded when mixed with *S. glaseri* than when alone. But Normally *Heterorhabditis* and *Steinernema* cannot co-exist within a host, though clearly they can co-infect (51) In addition, responses by *Heterorhabditis* to insects harboring heterospecifics have received less attention than those of steinernematids.

Mosquitoes and black flies would appear to be prime candidates for control with nematodes because they readily ingest nematodes. However, a number of factors reduce efficacy, including damage to the nematode during ingestion, (44-45) host immune response (46-48), and spatial separation of host and nematode. (46, 49)

Gaugler and Molloy, 1981(45) demonstrated that the nematodes were physically excluded during feeding of the first through third instars, rendering the host resistant to infection. Older instars were susceptible to infection. The principle regulating susceptibility was nematode injury caused by the larva mouthparts during ingestion, Dadd, 1971 (44) observed that larvae size excluded nematode ingestion by early instars and that some nematode injury occur during ingestion.

Mosquito feeding behavior and spatial of the nematodes also affect efficacy. The substrate type influences uptake of nematodes by the mosquito larvae. Nematodes settle quickly to the bottom. Mosquitoes will easily remove them from a smooth surface, but when debris is added, the nematodes are less available to the mosquitoes larvae (46,49).

Insects are capable of recognizing a diversity of foreign objects in their hemolymph and initiating a myriad of non-self responses to contain the aliens, including phagocytosis, nodule formation, cellular and humeral encapsulation, and induce the induction of antibacterial proteins. The types of made to the *Steinernema/Xenorhabdus* and *Heterorhabditis/Xenorhabdus* complexes and

effectiveness vary with the insect species, physiological status, and the strain of nematodes/bacteriam complex. The bacteria eventually destroy the host hemocytes by releasing the hemocytotoxin, lipopolysaccharide, from bacteria outer membrane into the hemolymph. The toxin binds to the hemocytes, in part, by lipid A moiety; lipid A contain the toxic fatty acids that damage the hemocytes. In immune insects the antibacterial proteins, cecorpins, are capable of lysing *Xenorhabdus*. This is prevented by protein secretions from the nematodes destroying the cecropins (55).

CHAPTER VI
CONCLUSION AND RECOMMENDATION

The study was a factorial designed as an experimental research. The experiment was carried out in laboratory condition. The aims of this study are to compare the efficacy of entomopathogenic nematodes of the genera *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) against *Cx. gelidus* larvae and to determine the dosage of entomopathogenic nematodes which is effective against *Cx.gelidus* larvae at 18, 24, 48, 72 and 96 hour exposure time. These results were computed were by statistical analysis and concluded as follows:

6.1 Conclusion

The experiments showed that the larva of Japanese encephalitis vector (*Cx. gelidus*) is susceptible to entomopathogenic nematode in genera *S. carpocapsae* (Weiser). In laboratory condition of this study that found *Steinernema carpocapsae* (Weiser) had most potential as biological control agent *Cx. gelidus* larvae than *Heterorhabditis indica* (Local Thai strain) under laboratory conditions. However, factors of timing to expose between nematodes and larvae that has effect mortality of larvae, including feeding behavior of larvae, host immune mechanism and exposure time had strongly affective at 48 hour exposure time in genera *S. carpacapsae* (Weiser) .

Dosage of nematodes per larva had increase direct related in genera of *S. carpocapsae* (Weiser). Our preliminary experiment showed that 2000 – 4000 IJs/larva of genera *S. carpocapsae* (Weiser) was the most effective against third to fourth *Cx. gelidus* larvae (mortality > 50%) and at 500 – 1000 IJs/larva mortality was lower than 50%, while genus *H. indica* (Local Thai strain) that found all dosage had no significant difference from control.

6.2 Recommendation

6.2.1 Recommendation for the applications

The information from this study has to apply for the control of mosquito larvae in field trial. Genus *S. carpocapsae* (Weiser) should be the EPN of choice for control larvae of mosquitoes or larva of another pest in rice filed, paddy in domestic animal farm. However, Dosage of nematodes per larvae, temperature of water is important, pH of water and timing for test yet, before use nematode for control larva in the field.

6.2.2 Recommendations for the further research.

1. This study was tested under laboratory conditions in plastic cup containers. However, it should be tested in field trial such as stagnant water surrounding domestic farm, rice field.

2. The further research should be on the level of depth water and timing for control effecting mortality of mosquito larvae for the better understanding in mosquito control program.

3. The further research should be to evaluate effectiveness and benefit before use of this method for the better understanding in mosquito control program.

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APPENDIX

APPENDIX A

IDENTICAL CHARACTERISTICS OF THE SPECIES WITHIN *GELIDUS* SUBGROUP.(Bram, 1967)

CULEX GELIDUS THEOBALD

The adult female may be recognized by the presence of a dense covering of silver-white scales on the scutellum. The male is distinguished by the scales of the scutum and by the simple inner division of the phallosome of the terminalia, which is covered with fine sculpturings. The fourth stage larva exhibits an expanded siphon and the subventral tufts are all inserted in line.

FEMALE. *Head.* Proboscis dark brown, with a moderately broad median band of paler scales; palpus dark brown, in some specimens with several white scales randomly scattered among the dark decumbent scales of the vertex silver-white at the occiput, then with a narrow area of dark brown scales, with dull brownish white scales posterolaterally; erect scales silver-white at the occiput and dark brown posterolaterally. *Thorax.* Scutum covered with a dense pattern of silver-white scales which terminate at approximately the level of the wing base; within the silver-white scale pattern are 2 or 4 variables, round patches of dark brown scales. The posterior third of the scutum and the scutellum more sparsely covered with dark brown scales, integument of the pleuron light brown, with some faintly darker brown areas on the sternopleuron; distinct but sparse patches of white scales on the sternopleuron; distinct but sparse patches of white scales on the upper and posterior sternopleuron. *Wing.* All dorsal wing scales light brown. *Legs.* Anterior surface of the hind femur lightly scales proximally, with a dark apical band which extends along the dorsal margin; hind tibiae clothed light brown scales and with a narrow apical light band, hind tarsus light brown, with narrow white basal white bands on tarsomeres I-V; fore and mid femora darker; fore and mid tibiae and tarsi marked as the high legs. *Abdomen*. Terga dark scaled with moderately broad basal light bands which reach to the lateral margins and with a median, posteriorly directed “V” which in some cases

almost reaches the posterior margin of the tergum and with lateral triangular patches which are barely visible from above (specimens with a straight posterior margin are occasionally found); sterna primarily pale, but with dark scales at the anterior and posterior margins.

MALE. *Head.* Proboscis with a well defined pale band near the middle, without a basomedian patch of conspicuous setae; palpus dark, with narrow basal and broad median pale bands on segment III, and narrow basal pale bands on IV and V. *Terminalia.* Subapical lobe of the basimere well developed with 3 basal rods followed by 3 accessory setae (one of which is very broad), a broad symmetrical leaf, and a gently curved seta; distimere expanded medially with minute annulations on the convex surface at the apex; inner division of the phallosome short, blunt, slightly curve laterally and heavily sculptured; outer division evenly rounded, speculate; proctiger crowned with a tuft of strong spine and with 2 or 3 cercal setae; basal sternal process long, darkly pigmented, and strongly curved.

LARVA. *Head.* Antennae pale, with a narrow, dark basal ring ; head hair 1-C darkly pigmented, tapering to a sharp point, its length approximately half the distance between the bases of the pair; 4-C single, simple, 5,6-C trifid, pectinate. *Thorax.* Integument glabrous; hairs 1,2,3-P single, pectinate; 7-P trifid, pectinate; 8-P bifid, pectinate; 14-P single, simple. *Abdomen.* Integument glabrous; comb consisting of from 30 – 40 fan-shaped scaled arranged in a broad, triangular patch; siphon index variable, ranging from 3:1 to 3.5:1, the siphon expanded medially; 4 pairs of sub ventral tufts inserted in a straight line on the siphon; individual tufts with from 3 to 6 branches, their length less than width of siphon at the point of the insertion; pecten consisting from 7 to 12 teeth restricted to the basal third of siphon; individual pecten tooth elongated, with a sharp apical spine and from 4 to 7 sharp lateral barbs.

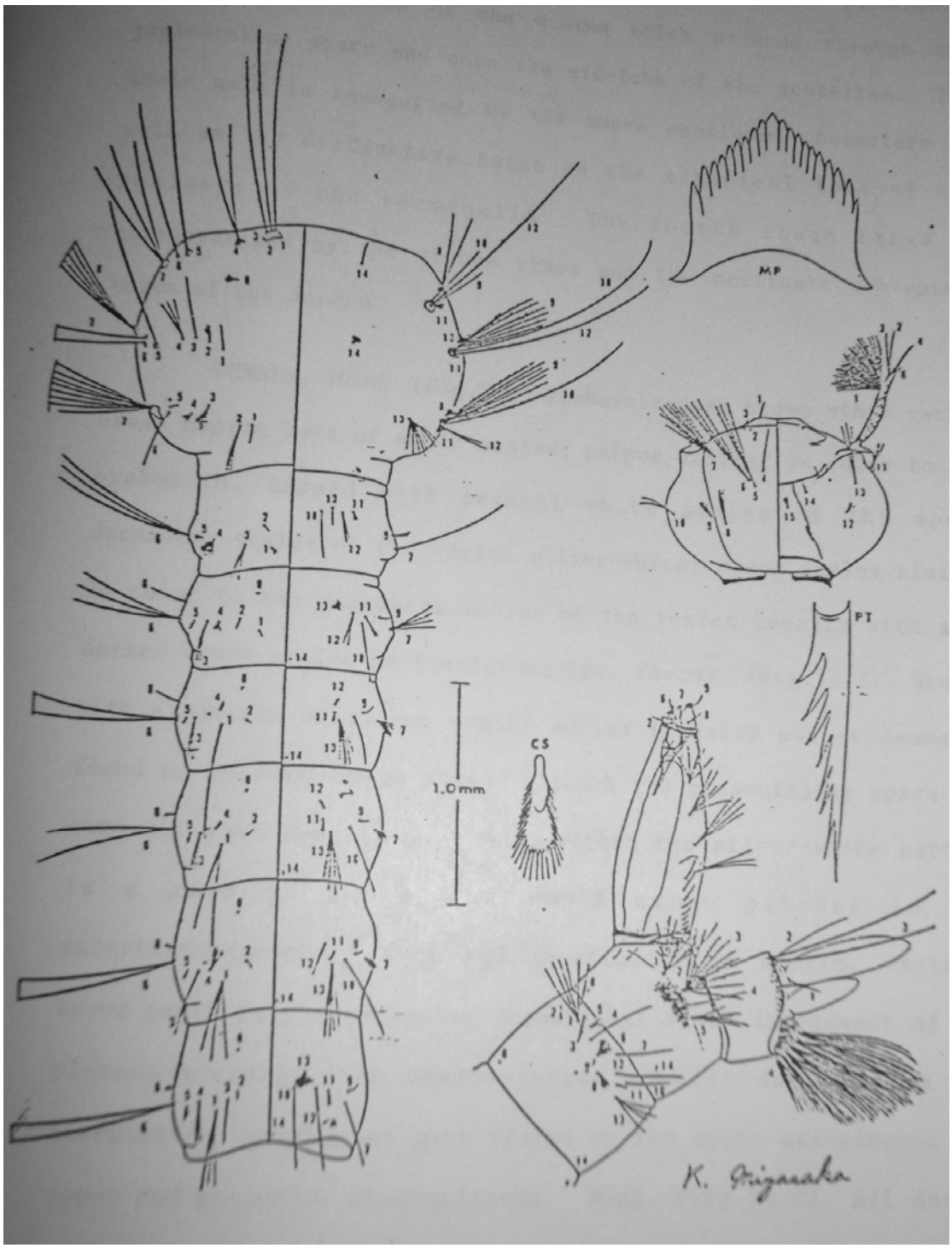

Figure 1 *Culex gelidus*. Fourth stage larva: dosoventral view of the head, thorax and abdomen, and lateral aspect of the terminal abdominal segments.

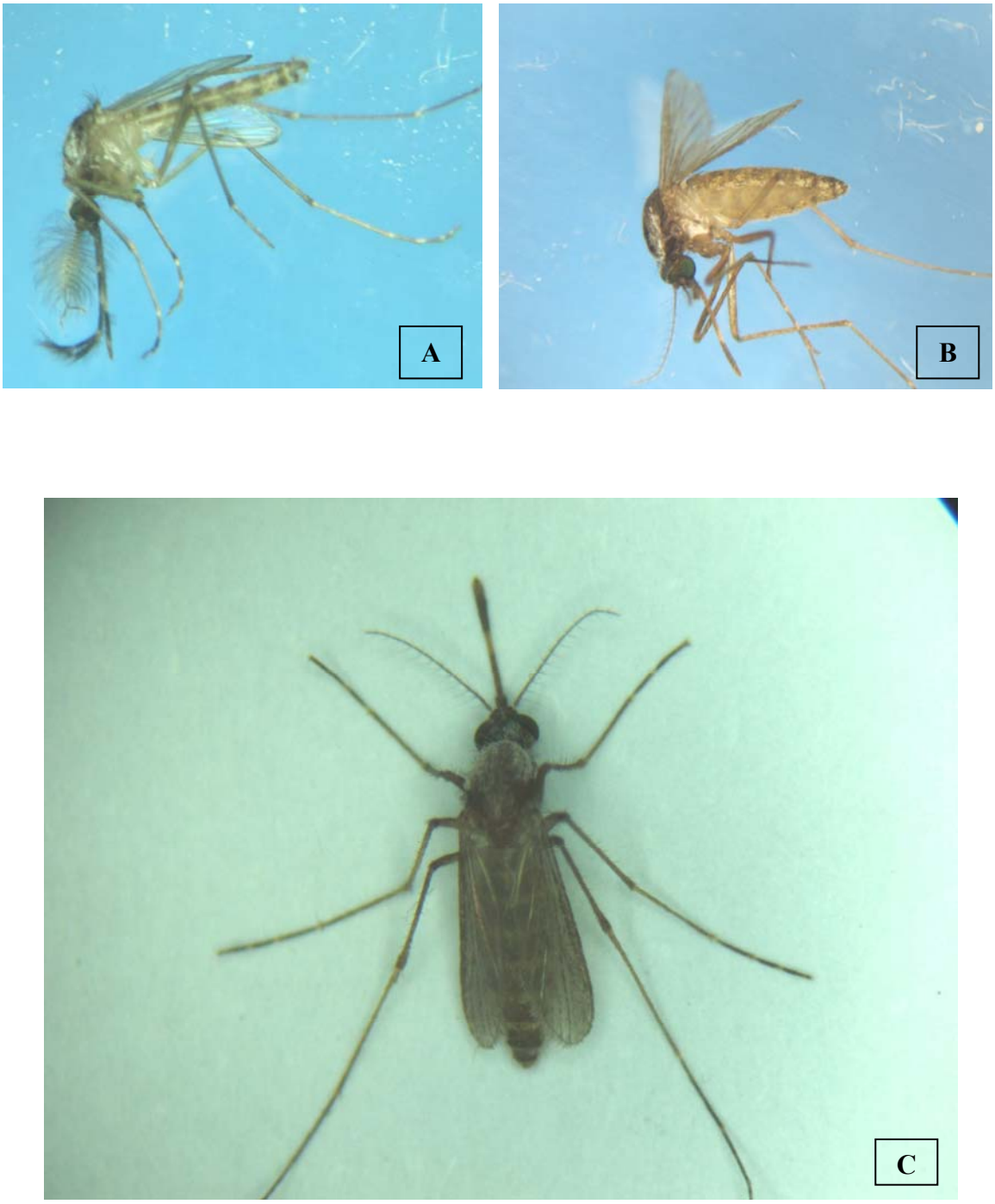

Figure 2 Morphology of *Culex gelidus*. (A) Male (B,C) Female

APPENDIX B

Dilution Counting Technique (12)

The procedures were carried out as recommended by Entomology and Zoology Division, Plant Protection Research and Development Office, Department of Agriculture Ministry of Agriculture and Cooperative. Dilution technique were use in theses study.

Equipment and Material

1. 15 ml flask
2. Micropipette 1000 µl.
3. Counting plate
4. Dechlorinated tap water
5. 10 ml Slender tube
6. 250 ml glass beaker
7. Counter
8. Compound Microscope

Methods

1. The *S. carpocapsae* (Weiser) and *H. indica* (Local Thai strain) were stored at 7 -10 °C before to test. The nematodes were container in plastic foam (Total approximate 4,000,000 per bag). The nematodes suspension was prepared on site by mixing with 200 ml dechlorinated tap water.

2. Shake and transferred suspension nematodes between 2 beakers, 5 time before counting nematodes

3. Add dechlorinated tap water into 3 flasks (to give 9 ml per flask)

4. Pipette 1 ml was removed from 200 ml suspension nematodes (shaking 5 times before pipette) transferred into 1° flask. Shaking 5 times and pipette 1 ml transferred into 2° flask. Shaking 2° flask about 5 times and pipette 1 ml transferred into 3° flask. Finally, pipette 1 ml into counting plate (do not over than 100 nematodes in 1 ml) and then using a 1 ml counting chamber and a stereoscope. To check the accuracy of the serial dilution, this procedure was repeated for a total of

three from each sample. The concentration of nematodes in the original suspension was calculated from the mean.

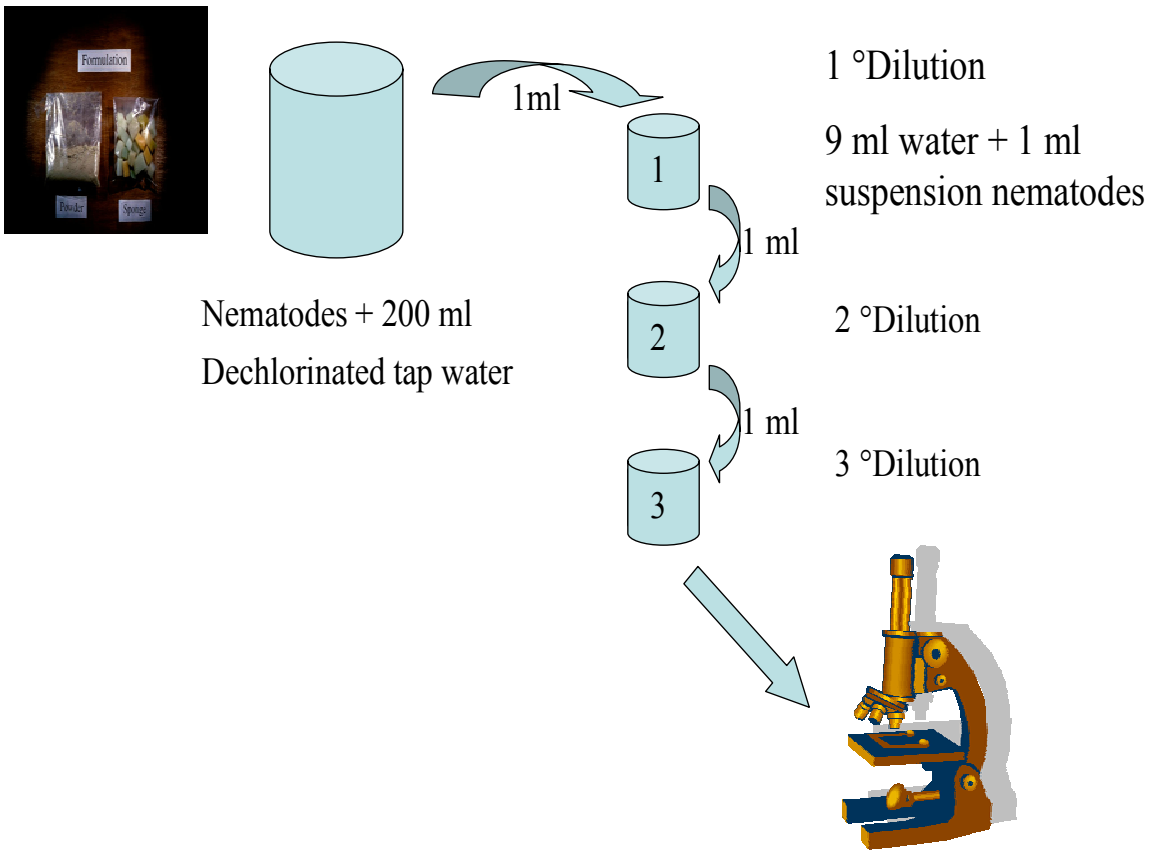

Figure 3 Dilution Counting Technique

APPENDIX C

Species of Nematode	EPN	0		24		48		72		96	
		pH	C	pH	C	pH	C	pH	C	pH	C
S. carpocapsae	Control	6.66	26.8	7.54	23	7.76	26.2	8.03	23	8	26.2
	Control	6.66	26.8	7.6	23	7.5	26.3	7.98	23.7	7.96	26.4
	Control	6.66	26.8	7.6	23.2	7.47	26.4	7.97	23.5	7.98	26.8
	Control	6.66	26.8	7.58	23.1	7.68	26.6	7.96	23.8	7.99	26.8
	Mean	6.66	26.8	7.58	23.075	7.60	26.38	7.99	23.5	7.98	26.55
	SD	0.00	0.00	0.03	0.10	0.14	0.17	0.03	0.36	0.02	0.30
	500	6.66	26.8	7.54	22.7	7.62	25.2	8	23	7.91	25.8
	500	6.66	26.8	7.56	22.8	7.72	25.1	8.03	23.2	7.92	25.9
	500	6.66	26.8	7.5	22.7	7.77	25.5	7.95	23.5	7.92	26
	500	6.66	26.8	7.53	22.8	7.82	25.5	7.98	23.3	7.96	26
	Mean	6.66	26.8	7.53	22.75	7.73	25.325	7.99	23.25	7.93	25.93
	SD	0.00	0.00	0.02	0.06	0.09	0.21	0.03	0.21	0.02	0.10
	1000	6.66	26.8	7.48	22.7	7.69	24.8	7.98	22.4	7.82	25.6
	1000	6.66	26.8	7.43	22.6	7.84	25.1	7.95	22.9	7.85	25.7
	1000	6.66	26.8	7.45	22.7	7.86	25.1	7.92	23.1	7.84	25.6
	1000	6.66	26.8	7.45	22.8	7.64	25.3	7.95	23	7.85	25.9
	Mean	6.66	26.8	7.45	22.7	7.76	25.075	7.95	22.85	7.84	25.7
	SD	0.00	0.00	0.02	0.08	0.11	0.21	0.02	0.31	0.01	0.14
	2000	6.66	26.8	7.41	22.3	7.74	24.8	7.9	23	7.72	25.4
	2000	6.66	26.8	7.39	22.4	7.75	24.8	7.89	23	7.73	25.1
	2000	6.66	26.8	7.39	22.5	7.75	24.7	7.9	22.9	7.72	25.2
	2000	6.66	26.8	7.44	22.5	7.43	24.8	7.74	23.3	7.65	25.4
	Mean	6.66	26.8	7.41	22.42	7.67	24.78	7.86	23.05	7.71	25.28
	SD	0.00	0.00	0.02	0.10	0.16	0.05	0.08	0.17	0.04	0.15
	4000	6.66	26.8	7.35	22.3	7.56	24.2	7.86	22.8	7.6	25
	4000	6.66	26.8	7.32	22.4	7.59	24.4	7.83	22.9	7.64	25
	4000	6.66	26.8	7.33	22.5	7.62	24.4	7.8	23	7.62	25
	4000	6.66	26.8	7.33	22.4	7.43	24.6	7.72	22.9	7.55	25.1
	Mean	6.66	26.8	7.33	22.4	7.55	24.4	7.80	22.9	7.60	25.03
	SD	0.00	0.00	0.01	0.08	0.08	0.16	0.06	0.08	0.04	0.05

Species of Nematode	EPN	0		24		48		72		96	
		pH	C	pH	C	pH	C	pH	C	pH	C
H. indica	Control	6.66	26.80	7.61	22.60	7.64	25.70	8.06	23.00	7.96	26.10
	Control	6.66	26.80	7.60	22.90	7.69	25.70	8.03	23.30	7.93	26.10
	Control	6.66	26.80	7.60	22.80	7.46	25.90	7.95	23.00	7.90	26.20
	Control	6.66	26.80	7.51	22.80	7.33	25.90	7.92	23.30	7.86	26.50
	Mean	6.66	26.80	7.58	22.78	7.53	25.80	7.99	23.15	7.91	26.23
	SD	0.00	0.00	0.05	0.13	0.17	0.12	0.07	0.17	0.04	0.19
	500	6.66	26.80	7.47	22.20	7.26	24.30	7.56	22.50	7.36	25.00
	500	6.66	26.80	7.47	22.30	7.38	24.50	7.83	22.50	7.53	25.00
	500	6.66	26.80	7.43	22.10	7.48	24.30	7.95	22.80	7.54	24.90
	500	6.66	26.80	7.38	22.30	7.46	24.50	7.93	22.90	7.52	25.30
	Mean	6.66	26.80	7.44	22.23	7.40	24.40	7.82	22.68	7.49	25.05
	SD	0.00	0.00	0.04	0.10	0.10	0.12	0.18	0.21	0.09	0.17
	1000	6.66	26.80	7.43	22.30	7.33	24.10	7.79	22.90	7.42	25.00
	1000	6.66	26.80	7.44	22.40	7.42	24.20	7.81	23.00	7.56	24.60
	1000	6.66	26.80	7.44	22.40	7.43	24.10	7.87	22.50	7.50	24.70
	1000	6.66	26.80	7.43	22.20	7.33	24.20	7.77	22.70	7.49	25.00
	Mean	6.66	26.80	7.44	22.33	7.38	24.15	7.81	22.78	7.49	24.83
	SD	0.00	0.00	0.01	0.10	0.06	0.06	0.04	0.22	0.06	0.21
	2000	6.66	26.80	7.41	22.10	7.34	24.20	7.88	22.40	7.40	24.70
	2000	6.66	26.80	7.47	22.10	7.42	24.20	7.84	22.70	7.41	24.70
	2000	6.66	26.80	7.43	22.00	7.42	24.00	7.82	22.40	7.42	24.80
	2000	6.66	26.80	7.50	22.10	7.44	24.00	7.88	22.70	7.46	24.60
	Mean	6.66	26.80	7.45	22.08	7.41	24.10	7.86	22.55	7.42	24.70
	SD	0.00	0.00	0.04	0.05	0.04	0.12	0.03	0.17	0.03	0.08
	4000	6.66	26.80	7.34	22.10	7.43	24.10	7.69	22.50	7.36	24.70
	4000	6.66	26.80	7.41	22.20	7.29	24.10	7.58	22.70	7.25	24.10
	4000	6.66	26.80	7.39	22.10	7.66	23.80	7.86	22.70	7.37	24.30
	4000	6.66	26.80	7.49	22.00	7.36	23.90	7.85	22.80	7.27	24.60
	Mean	6.66	26.80	7.41	22.10	7.44	23.98	7.75	22.68	7.31	24.43
	SD	0.00	0.00	0.06	0.08	0.16	0.15	0.13	0.13	0.06	0.28

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