between workers and brood after treatment (Kaya *et al*, 1882; Taha & Abdelmegeed, 2016).

As the vitality of the entire colony and the expected safety of the honey bee workers and brood should be considered during decision making. The main objective of this study is to answer the question, is it safe option to use entomopathogenic nematodes as bio-control agents inside honey bee colonies without any side effects on the vitality of the honey bee workers and brood?

MATERIALS AND METHODS

Bees and Nematodes:

Four entomopathogenic nematode isolates, *Steinernema sp*. (S1) & *S. riobrave* (S2) and *Heterorhabditis bacteriophora* (H1) & *Heterorhabditis sp.* (H2) were obtained from Applied Center of Entomonematodes (ACE), Faculty of Agriculture, Cairo University. The nematodes were cultured in the last instar larvae of *G. mellonella* L. and were maintained according to the method of Woodering & Kaya (1988). The stock suspension of the infective juveniles (IJs) was stored in sterile distilled water at 15ºC, and should be used for experiments within two weeks.

In order to examine the effect of entomopathogenic nematode isolates in the honey bee colonies, the apiary experiments were carried out in Bee Research Department, Plant Protection Research Institute (PPRI), Agric. Res. Center (ARC), Giza, Egypt, while the laboratory experiments were carried out in Bio-Insecticide Production Unit, PPRI, ARC, Giza, Egypt.

Susptibility of Workers Brood under the Bee Colonies Conditions:

In order to examine the effect of the EPN species on the workers brood under the bee colonies conditions, thirteen honey bee combs covered with unknown numbers of hybrid workers bees (*A. mellifera carnica*) from each side were containing unsealed brood, honey, and bee bread, each comb of them was placed inside wooden cage covered with metal net from both sides (fig.1), the combs were sprayed directly with three concentrations of nematodes suspensions (200, 400, 600 IJs/ml). Distilled water without nematodes was used as control. Four unsealed workers brood areas were selected (fig. 1) from each comb as a replicate, each replicate was four inches (100 cells). All cages were transferred into strong and healthy colonies after spraying directly. The empty cells, dead workers and infected larvae were counted daily for five days after treatment.

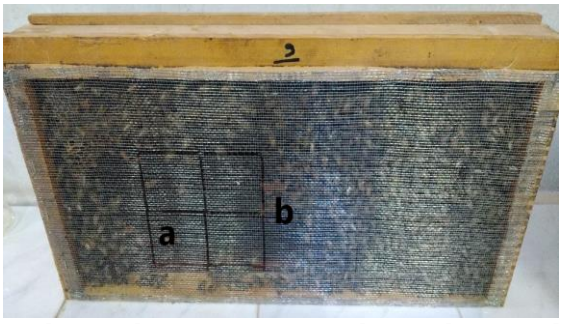

Fig1. The picture represent the used cage containing honey bee comb (containing unsealed brood, honey, and bee bread) covered with hybrid workers bees from each side.

a. Treated areas divided into four replicates each about four inches (100 cells).
b. Untreated area.

Susptibility of Bee Workers and Larvae under the Laboratory Conditions:

In order to examine the direct effect of EPN species on adult workers and larvae in laboratory, thirty wooden cages (11×15×18 cm) with two sides of metal net were used in the

laboratory. Fifty individuals of bee workers were inserted into each cage, the cages have been divided into two groups according to ways of treatments, direct exposure with spraying (direct exposure assay) and indirect exposure during feeding (indirect exposure assay). The first group was sprayed directly with 10 ml of both nematode sp. suspensions (400 IJs/ml), cotton plugs soaked in a sugary solution were provided as feeding for the bees. The second group was treated indirectly by applying the cotton plug that soaked first in a sugary solution then contaminated with the same concentration of the examined nematode isolates. Sugary solution was used for control. After three days the dead bees were counted and dissected and the rest were placed on White nematode traps (White, 1927) at 23±2 ºC for one week.

In the case of bee larvae treatments, thirty bee workers larvae were transferred gently on glass petri-dish lined with tissue paper that moistened with 5 ml of 400 IJs/ml nematode isolates. Five replicates were used for each isolate; all the treatments were kept at laboratory conditions at 27±2ºC. After 48 hours the dead larvae were removed in all treatments, placed on the White nematode traps at 23±2ºC for one week to assess the successful recycling of all tested nematode isolates.

Statistical Analysis:

Data was analyzed using Proc. ANOVA in SAS (Anonymous. 2003). Means separation was conducted using LSD in the same statistical program

RESULTS

The Effect of EPNs on the Brood Mortality in Honey Bee Combs under the Colony Conditions:

The mortality percentage of the brood treated with the tested isolates of *Heterorhabditis species* (H1&H2) and *Steinernema species* (S1&S2) under colony conditions were determined according to the percentages of the empty cells (uncapped) in the treated areas of combs.

Data resulted in table (1) showed that nearly all of the mortality percentages were recorded during the 1st and 2nd days among all isolates. During the 2nd day, the highest mortality percentages were recorded. It was noticed that all the infected larvae were removed regularly by the workers inside the colony. However, five days post-treatment, the total uncapped cells in honey bee brood areas that treated with *Steinernema* isolates were significantly higher than that treated with *Heterorhabditis* isolates. Regarding to *Steinernema* isolates the total percentages of uncapped cells in honey bee brood areas that treated with 200, 400 and 600 IJs of the isolate S1 were 57%, 71.9%, and 77.6%, respectively, that were higher than treated with S2 isolate (49.2%, 63.5%, and 75.3%, respectively). While, in case of treatment with 200, 400 and 600 IJs of *Heterorhabditis* isolates, the total percentages of uncapped cells of honey bee brood areas that treated with the isolate H2 were 39.8%, 51.3%, and 55.2%, respectively, that were higher than that of H1 isolate (44.8%, 48.7 and 50%, respectively). It was noticed that throughout the whole experiment, some of dead workers were noticed inside the treated cages, but there weren't any symptoms of infection with nematodes in the entire colonies except the treated areas inside the cages and the colony still healthy with normal appearance.

The Effect of EPNs on the Mortality of Honey Bee Workers and Larvae under the Laboratory Conditions:

Data tabulated in table (2) showed that the mortality percentages of bee workers which treated directly by spraying in the miniature cages were significantly lower than that of the indirect exposure during feeding. The cages that treated with *Heterorhabditis* isolates showed significantly lower mortality percentages than those treated with *Steinernema*

isolates. The percentages of dead bee workers for the indirect exposure assay were 47.91, 40.83, 60 and 54.35% for each of H2, H1, S2, and S1, respectively, while for direct exposure assay were 18.29, 18.89, 32.81 and 29.33%, respectively. After dissection of dead bee workers that treated with all isolates under investigation, it was observed that there weren't any individuals of nematodes. After one week, there weren't any infective juveniles observed in the White trap and no signs of recycling process were observed.

In the case of direct larval treatment with 400 IJs/ml of both *Heterorhabditis* and *Steinernema* isolates, 100% mortality percentages were recorded. After one week, the infected larvae that transferred on the White traps showed high number of IJs of nematodes in case of *Heterorhabditis* species, while in *Steinernema* species the number of IJs of nematodes were very low.

Table 1. Mortality percentages of bee brood after treatment with EPN isolates under colony conditions.

Days	***Heterorhabditis* sp.**						**Control**	**Mean**
	H1			**H2**				
	200 IJ/ml	**400 IJ/ml**	**600 IJ/ml**	**200 IJ/ml**	**400 IJ/ml**	**600 IJ/ml**		
Day 1	13.30	16.20	18.50	16.20	22.90	24.20	6.50	18.55 b
Day 2	29.70	29.60	27.60	23.60	26.80	28.70	3.40	27.7 a
Day 3	1.50	2.10	2.10	0.00	1.10	1.50	0.50	1.4 c
Day 4	0.30	0.80	1.00	0.00	0.50	0.80	0.00	0.66 c
Day 5	0.00	0.00	0.80	0.00	0.00	0.00	0.00	0.13 c
Total uncapped cells	44.80%	48.70%	50.00%	39.80%	51.30%	55.20%	10.40%	-
Mean	8.96 bc	9.74 abc	10.00 abc	7.96 cd	10.26 abc	11.04 abc	2.08 d	-
Days	***Steinernema* sp.**						**Control**	**Mean**
	S1			**S2**				
	200 IJ/ml	**400 IJ/ml**	**600 IJ/ml**	**200 IJ/ml**	**400 IJ/ml**	**600 IJ/ml**		
Day 1	18.50	28.90	33.30	14.60	23.70	32.00	6.50	25.17 b
Day 2	37.50	39.90	40.40	31.00	34.10	35.50	3.40	36.4 a
Day 3	0.50	2.00	1.80	2.10	3.10	3.10	0.50	2.1 c
Day 4	0.50	1.10	1.30	1.50	2.10	3.10	0.00	1.6 c
Day 5	0.00	0.00	0.80	0.00	0.50	1.60	0.00	0.48 c
Total uncapped cells	57.00%	71.90%	77.60%	49.20%	63.50%	75.30%	10.40%	-
Mean	11.40 abc	14.38 ab	15.52 a	9.84 abc	12.7 abc	15.06 a	2.08 d	-

*Means followed by the same letter do not differ significantly at the 5% level of probability.

Table 2. Mortality percentages of honey bee workers in the laboratory using direct and indirect exposure to EPN isolates.

Treatments	**Direct exposure assay**	**Indirect exposure assay**
H2	18.29% f	47.91% bc
H1	18.89% f	40.83% cd
S2	32.81% de	60% a
S1	29.33% e	54.35% ab
Control	5.26% g	

*Means followed by the same letter do not differ significantly at the 5% level of probability.

DISCUSSION

The main objective of the present work was to examine the possibilities of using EPN isolates, *Heterorhabditis sp.* and *Steinernema sp.* inside the bee colonies and discuss the consequences on colony vitality in case of using them as biological agents to suppress the infestation of *G. mellonella* in the bee colonies.

Under the bee colony conditions, all of the tested isolates caused high larval mortalities levels in the treated areas in honey bee combs through the first 48 hours with the first deaths evident after 24 hours of treatment compared to the control frames. The total uncapped cells in honey bee brood areas that treated with tested isolates of nematodes were significantly high. Also, some of dead workers were observed in the treated cages without any symptoms of nematode recycling on the White trap. There was a difference in nematode proliferation within larvae than adult workers and also between *Heterorhabditis sp.* than in *Steinernema sp*. The unsuccessful recycling of the EPNs inside adult workers might refer to the failure of the EPNs to proliferate well inside worker's bodies and to overcome their immune defense system. Goodrich-Blair & Clarke, 2007 explained the successful proliferations of *Heterorhabditis sp.* inside the larval body, and the period that needed for the symbiotic bacteria, *Photorhabdus* in *Heterorhabditis* and *Xenorhabdus* in *Steinernema*, to perform successful proliferation to overcome insect immune defenses, causing septicemia and death. Nielsen-Le Roux *et al.*, 2012 and Dutka *et al*., 2015 also discussed the differences between the symbiotic bacteria *Xenorhabdus and Photorhabdus* to overcome the insect host immune system, clarifying that the *Xenorhabdus* symbiotic bacteria of *Steinernema* was able to overcome the bee immune system and caused septicemia, but couldn't sufficiently break down the tissues to allow nematode feeding and proliferation, while the *Photorhabdus*, symbiotic bacteria of *Heterorhabditis sp.*, might be able to digest the bee tissues more effectively and provide a better supply of nutrients to the nematodes.

It was noticed throughout the whole experiment that the bee workers detecting and removing the diseased individual from a colony that reflected social immunity against nematode infection which was keeping the colony healthy with normal appearance. Spivak, 1996 clarified the hygienic behaviour as a genetically immune mechanism of resistance to different pathogens infecting honey bees.

The high temperatures inside the colonies might be also another reason for the incomplete cycle of the nematode proliferation inside the carcass of workers and the inability of the symbiotic bacteria to overcome the bee immune system even after invading the adult body and causing death, that was in coincidence with Kaya *et al*., 1982 who confirmed the inability of the nematodes to infect the brood in the high temperatures of the center of the hive. This remarkable unsuccessful recycling of EPNs and the hygienic behavior of bees in the colony might be the reasons that the nematode infection inside the entire colony was restricted to the treated frames and didn't widespread through the other frames, that keeps the vitality of the colony. On the contrary, Neumann *et al*., 2013 proved that the infected individuals with *Heterorhabditis sp.* may release infective juveniles that succeed to proliferate inside the carcass and would widespread through the entire colony with great risk on the colony vitality.

Due to it was too hard under colony circumstances to count exactly the number of healthy and infected workers inside the bee colonies; that was a good reason to use the miniature cages with known number of workers in the laboratory. The obtained results reflected the sever effects of EPNs in case of direct exposure and in direct exposure during feeding on the honey bee workers. While, the mortality percentages were significantly higher in case of treatments during feeding than that of direct spraying group. The time required to the EPNs to invade the body of the workers during the feeding period through the natural

opening might be the main reason for that difference in mortality percentages. Poinar, (1990) also proved that adult honey bees that treated during oral feeding with honey or fruit concentrate showed high infection rates by invasive forms of *S. carpocapsae*. Higher mortality percentages between workers were observed in the cages that treated with *Steinernema* isolates than that treated with *Heterorhabditis.* However, while the direct brood treatment with both *Heterorhabditis and Steinernema* isolates initiated 100% mortalities but the yields of IJs in case of *Heterorhabditis* were higher than that in *Steinernema* isolates and might reflect the ability of *Steinernema* isolates to invade the larval tissues with higher penetration rates. The same results were obtained by Rosa *et al*., 2002 who confirmed the ability of the infective stage of *S. carpocapsae* to invade hosts and their higher penetration rate than *H. bacteriophora*.

The obtained results were in coincidence with Zóltowska *et al*., 2003 who proved that the adult workers and larvae *A. mellifera mellifera* of different ages that treated with two tolerant broad-range nematodes, *S. affinis* and *S. feltiae* in brood combs and in the laboratory in petri dishes were highly infected. Also, Cantwell *et al*., 1974 found that the honey bee larvae are susceptible to invasion by *S. dutkyi,* when placed on moist paper inoculated with (IJs) under laboratory conditions.

In contrast, some researches reflected another hypothesis that the EPNs showed negligible effects on colony individuals. Kaya *et al*., 1982 found that the direct spraying of the infective stage of the nematode *S. carpocapsae* Weiser on the frames containing *A. mellifera* L. causing limited mortality between workers with percentage mortalities that happened only during the first three days after direct spraying with normal muscle tissue appearance in both thorax and abdomen of honey bee. Also Taha & Abdelmegeed, 2016 proved that high inoculum levels of both *H. bacteriophora* and *Steinernema spp.* caused zero mortality percentages in adult worker and larvae and can be used as safe alternative to control *G. mellonella* in a natural environment of the bee hives. Baur *et al*., 1995 also confirmed that there wasn't any evidence of infection in dead bee workers after treatment with high-temperature sensitive nematodes *S. carpocapsae* and *H. bacteriophora* within the miniature hives within the incubators due to the hygienic behavior of bee colonies and confirmed that strong colonies can protect combs from wax moth infestation damage.

In summary, our findings strongly supported that the biological control using EPNs isolates as bio-control agents inside the honey bee colonies is unpredictable and may also have off-target effects and might potentially harm honey bee individuals surrounding the treated area. Our results proved that both *Heterorhabditis sp.* and *Steinernema sp.* isolates in this study might cause significant mortalities between honey bee workers and brood under both colony and laboratory conditions and it might not be considered as a safe bio-control agent to *G. mellonella* in bee colonies even at low concentrations and spraying precautions. Further research is needed to cover briefly the possibilities of using EPNs isolates for infected wax combs during storage before reusing it in the bee colonies.

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ARABIC SUMMARY

هل من الممكن إستخدام النيماتودا الممرضة للحشرات داخل طوائف نحل العسل؟

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1-قسم بحوث النحل-معهد بحوث وقاية النباتات-مركز البحوث الزراعية-الدقي-جيزة

2-وحدة إنتاج المبيدات الحيوية-قسم دودة ورق القطن-معهد بحوث وقاية النباتات-مركز البحوث الزراعية-الدقي-جيزة

أيدت بعض الفرضيات مفهوم أن النيماتودا الممرضة للحشرات آمنة كإسلوب مكافحة بيولوجية مسببة إصابة طفيفة محتملة لشغالات وحضنة نحل العسل تحت ظروف الطائفة وإنها لا تؤثر على قوة الطائفة ويمكن إستخدامها كوسيلة مكافحة حيوية ضد دودة الشمع الكبيرة. علي الرغم من أن النتائج المتحصل عليها أوضحت أن نسب الموت في يرقات النحل بعد المعاملة بالنيماتودا الممرضة للحشرات بلغت من 49,2% إلى 77,6% في حالة المعاملة بعزلات *Steinernema sp.* وبلغت من 39,8% إلى 55,2% في حالة المعاملة بعزلات *Heterorhabditis sp.* وذلك تحت ظروف الطائفة، فإن الإصابة بالنيماتودا داخل الطوائف كانت محصورة في المساحات المعاملة ولم تنتقل إلي باقي الإطارات. المعاملة المباشرة في المعمل أكدت أيضا حساسية شغالات ويرقات النحل للإصابة بكل عزلات النيماتودا المختبرة مع إختلاف نجاح النيماتودا في إتمام دورة حياتها.

وقد أثبتت الدراسة الحالية بقوة أن كلا من عائلات Steinernematidae و Heterorhabditidae لا يفضل إستخدامها داخل طوائف نحل العسل كوسيلة للمكافحة الحيوية وينبغي إجراء المزيد من الدراسة لمعرفة مدى إمكانية إستخدامها على الأقراص الشمعية المصابة أثناء التخزين قبل إعادة إستخدامها داخل طوائف النحل.