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ORIGINAL ARTICLE

Effects of the nutrition of different diets and lipid content of the insect host larvae, *Galleria mellonella* on the efficacy of indigenous entomopathogenic nematodes

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Abstract

Entomopathogenic nematodes (EPNs) are promising as biocontrol agents for the most economically important insect pest attacking a wide range of host plants. Therefore, the aim of this work was to study the impact of four artificial diets and one natural food on numbers, weights, and total lipid content of the greater wax moth larvae, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) as well as the impact of these diets on the ability of nematode species *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* to infect insects and multiply inside an insect host which had been reared on one of five different diets (D1, D2, D3, D4 and D5). The correlation between larvae weight and total lipid content, pathogenicity or multiplication of nematodes was also studied. The obtained results indicated that D2, D5 and D3 gave the highest growth or weights of larvae. The larvae produced weighed 3.71, 3.67 and 3.25 g from 50 g media, respectively. Statistically, larvae weights had a positive and significant correlation with the lipid content in larvae where $r = 0.732$. On the other hand, infective juveniles (IJs) of nematodes produced from insect hosts reared on D2 and D5 revealed more pathogenicity on larvae, since they caused the highest percent of mortality, 53.33 and 50.0% for *H. bacteriophora,* and 56.67 and 53.33% for *S. carpocapsae*, respectively. The total lipid content had a positive and highly significant correlation with the pathogenicity of the two nematode species where $r = 0.97$ and 0.971, respectively. Ultimately, the supplied foods of the artificial diets D2, D3 and natural beeswax (D5) gave the most suitable chance for developing insect growth and increasing the EPN quality and enhancing the potential of EPNs as biological control agents against different insect pests.

Keywords: diets, *Galleria mellonella, Heterorhabditis bacteriophora,* lipids, multiplication, pathogenicity, *Steinernema carpocapsae*

Introduction

Entomopathogenic nematodes (EPNs) are promising as biocontrol agents of the most economically important insect pest attacking a wide range of different host plants such as: turf grass, vegetable crops, nurseries, fruit orchards and greenhouses. Nematodes provide eco-friendly and sustainable crop protection. EPNs belonging to genera *Heterorhabditis* and *Steinernema* are obligatory parasites and lethal to insects and can be applied easily (El-Wakeil and Hussein 2009; Saleh *et al.* 2009; San-Blas 2013; Nouh and Hussein 2014; Hussein *et al.* 2015; Hussein and El-Mahdi 2019). These two EPNs genera can be mass-produced by *in vivo* or *in vitro* culture methods (Rahoo *et al.* 2019; Hussein and El-Mahdi 2020). *In vivo* methods for producing EPNs by using insect host *Galleria mellonella* (Lepidoptera: Pyralidae) (Testa and Shields 2017) were restricted to laboratory production on a small scale (Shapiro-Ilan *et al.* 2012).

The greater wax moth, *G. mellonella* is a serious pest attacking beeswax in both hives and stores during storage (Jorjao *et al.* 2018). This insect was suitable for nematode production purposes due to their high susceptibility to EPNs (Fuchs *et al.* 2010; Ramarao *et al.* 2012), ease of rearing on artificial diets, suitable size,

relatively rapid life cycle and high production yield of nematodes (van Zyl and Malan 2015; Testa and Shields 2017; Pereira *et al.* 2018; Rahoo *et al.* 2018). Furthermore, they do not require feeding during both pupae and adult stages and their activity for reproduction is in the dark (Ellis *et al.* 2013; Jorjao *et al.* 2018).

In vivo production of EPN is faced with the expensive problem of rearing insects (Divya and Sankar 2009). Therefore, it is necessary to find alternative solutions and economic diets to rear insects by replacing a natural diet with a less expensive one (Cohen 2003). It is important to assess the efficiency of new diets on EPNs' emergence from insects and infected hosts which reared on these diets, kill and reproduce in any insect species. Ramakuwela *et al.* (2016) and Zhen *et al.* (2018) demonstrated that the quality of insect host and insect nutrition can affect reproduction, pathogenicity and persistence of EPNs produced *in vivo*. Selection of ingredients (types and quants) play a significant role in growth and development of insect larvae; and in the nematode, yield, fitness and quality (Shapiro-Ilan *et al.* 2004). Flanders *et al.* (1996), Kaya and Stock (1997) determined that nematode yield is correlated with insect host size and weight. Unfortunately, many natural ingredients of diets, such as glycerol, powdered milk and yeast are expensive and decrease the efficiency of *in vivo* production of EPNs on a commercial scale.

For this purpose, the main objectives of this study were (i) to determine which diet, out of five different diets, was the best for rearing *G. mellonella*; (ii) to evaluate the total lipid content of *G. mellonella* from each diet; (iii) to assess under laboratory conditions, the ability of infective juveniles (IJs) of *H. bacteriophora* and *S. carpocapsae* to infect and kill an insect host *G. mellonella* larvae and (vi) to assess the multiplication ability of EPNs inside an insect host for producing a good yield of nematodes.

Materials and Methods

Insect host, *Gallería mellonella* source

Larvae of the greater wax moth, *G. mellonella* used for nematode production were obtained from the permanent culture of susceptible strains in the laboratory of Nematology, Pests and Plant Protection, Agricultural and Biological Research Institute, NRC, Cairo, Egypt. Larvae were transferred to transparent 1 liter glass rearing jars containing 250 g diet secured with filter paper discs and metal screen; and maintained under rearing conditions (28 ± 2°C; a relative humidity of 65 ± 5% and full darkness) till adult moth emergence. Females laid their eggs on the filter paper discs. They were then collected, and introduced into new, clean jars containing fresh food.

Entomopathogenic nematodes source

The two endemic nematode isolates used in this study, *H. bacteriophora* BA1 and *S. carpocapsae* BA2 were isolated from sandy soil of South Sinai Governorate, Egypt and identified by Hussein and Abo El-Sooud (2006).

Entomopathogenic nematode culturing

For *in vivo* cultures of the two nematodes on *G. mellonella*, 10^4 IJs in 5 ml distilled water were inoculated on 200 last instar larvae, which were put on double discs of filter paper and deposited in Petri dishes (20 cm diameter). The larvae were dead within 48 h after inoculation. Nematodes were harvested using the method described by Kaya and Stock (1997). The harvested IJs obtained from white traps were washed twice with distilled water and used in the experiments.

Formulated diets for rearing *Gallería mellonella*

To optimize the efficiency of diets for rearing insect hosts and mass-producing nematodes, four formulated diets (D1, D2, D3 and D4) were compared with a natural food (beeswax D5) (Table 1). The diet suitability was based on numbers, weights, and lipid content in the last instar larvae for mass production of EPNs.

Table 1. Ingredients of each diet suggested for nutrition of *Galleria mellonella* larvae

Diets	Ingredients	Amount [%]
D1	wheat flour	35
	corn flour	20
	milk powder	13
	baking yeast powder	7
	honey	10
	glycerin	15
D2	wheat flour	35
	corn flour	20
	milk powder	13
	baking yeast powder	7
	honey	10
	sorbitol	15
D3	wheat flour	40
	corn flour	28
	baking yeast powder	7
	honey	10
	sorbitol	15
D4	wheat flour	35
	corn flour	20
	milk powder	13
	fodder yeast	7
	honey	10
	sorbitol	15
D5	beeswax (natural food)	100

These four artificial diets were adapted from diets of Poinar (1975), Woodring and Kaya (1988), Bhatnagar and Bareth (2004), Meyling (2007), Birah *et al.* (2008) and Huang *et al.* (2010). The adaptation involved substituting one natural ingredient in each diet with another less expensive one, such as substituting glycerol with sorbitol or baking yeast with fodder yeast.

Impact of different diets on *Gallería mellonella* production

To test the response of rearing *G. mellonella* on these tested diets, 30 newly hatched larvae were introduced into 120 ml cups filled partially with 50 g diet by using an artist brush and repeated 10 times for each diet. Cups were kept in an incubator at 30°C ± 2°C and 60–65% relative humidity (RH). When fully developed 6th instar larvae (0.22 g) began to spin cocoons, they were removed from each tested diet, counted, and weighed individually.

Impact of different diets on total lipid content in *Gallería mellonella* larvae

To evaluate the relationship between the nutrition of different diets and total lipid content, full-grown larvae (6th instar) were prepared as follows:

Galleria tissues homogenate preparation: one gram about (5–6 larvae) was homogenized by grinding in 10 ml normal physiological saline solution (0.9%) to form a homogenate (10%).

Total lipid extraction: the total lipid was extracted from homogenate *Galleria* tissue using the method of Littlefield *et al.* (1955).

Total lipid determination: the level of total lipids in larval tissue extraction was determined using the method of Zöllner and Kirsch (1962). The test kit was supplied by the Bio-diagnostic Company, Egypt.

$$\text{Total lipid concentration in } G.\ mellonella \text{ larval tissue } [\text{mg} \cdot \text{g}^{-1}] = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard used [g]}} \times \frac{1}{\text{tissue weight}}.$$

Impact of different diets on total lipid content of nematode reproduction (multiplication)

The last instar larvae of *G. mellonella* from each diet were used as a host to determine the counts of *H. bacteriophora* BA1 and *S. carpocapsae* BA2 IJs that represented the multiplication of nematodes. One gram of larvae from each diet was placed on Petri dishes (5 cm in diameter) containing 50 g diet and lined with filter paper. Each treatment was replicated three times and inoculated with 500 IJs and nematode species in 1 ml of water at 25°C ± 2°C. After 2 days, larvae were transferred to white traps (Kaya and Stock 1997). Harvested nematodes from respective host/diet/replicate/nematode species were counted using the dilution count method (Glazer and Lewis 2000).

Pathogenicity of EPNs emerged from infected *Gallería mellonella* larvae fed different diets

The ability of nematodes to infect an insect host, propagate inside it and finally, cause mortality was assessed under laboratory conditions by exposing the full-grown larvae from each diet to the IJs of nematode species. Both larvae and IJs used for a pathogenicity test must be produced from the same diet. So, 10 larvae from each diet were infected with 1,000 IJs for *H. bacteriophora* or *S. carpocapsae*; each treatment was replicated three times. In each replicate, larvae were put on 5 cm diameter Petri dishes and padded with moist filter paper, then incubated at 25°C ± 2°C. After 24 h, the larvae were checked to determine the mortality %. Infected cadavers were transferred to a white trap and kept in the dark at 25°C for 10 days to permit the nematodes to complete their development and propagation. The harvested IJs were collected and counted using the dilution count method.

Data analysis

The obtained data were analyzed using one-way ANOVA according to Gomez and Gomez (1984). Nematode yield from the different tested diets were subjected to logarithm transformation. The correlation coefficient (r) and the slope (b) were calculated between these parameters.

Results

Impact of different diets on weights and total lipid content of *Gallería mellonella* larvae

The artificial diet D2 produced the highest weight of larvae (3.71 g) from 50 g diet and resembled that produced from the natural food beeswax (D5) (3.67 g); followed by larvae from D1 (3.09 g), D3 (3.25 g) and D4 (2.57 g) (Table 2). Diets D2 and D5 produced larvae with the highest content of total lipid in their tissues (118.92 and 106.39 mg), respectively. The lowest lipid content was obtained from larvae fed the artificial food D3 (71.54 mg · g^{-1} of larvae). The influence of each tested diet on the larval weights and their lipid

Table 2. Mean counts of larvae, weights and their contents of lipid produced in four artificial diets and natural food; as well as the ability of infective juveniles (IJs) to pathogen and multiplicate on the larvae of *Galleria mellonella*

Diets	Mean counts of larvae	Weights of larvae [g]	Total lipids [g · g^{-1} of larvae]	Pathogenicity of IJs [%]		Multiplication of nematodes in insect host[a]	
				Heterorhabditis bacteriophora	*Steinernema carpocapsae*	*Heterorhabditis bacteriophora*	*Steinernema carpocapsae*
D1	17 ± 0.19	3.09 ± 0.19	76.12 ± 2.29	16.67	3.33	5,205.69 ± 185.83 (6.72)	2,580.31 ± 383.19 (6.41)
D2	19.1 ± 0.39	3.71 ± 0.37	118.92 ± 10.35	53.33	56.67	4,758.63 ± 682.28 (6.68)	1,856.36 ± 343.16 (6.27)
D3	19.6 ± 0.31	3.25 ± 0.31	71.54 ± 10.14	3.33	3.33	5,249.94 ± 430.20 (6.63)	1,230.04 ± 147.83 (6.09)
D3	16.7 ± 0.21	2.57 ± 0.21	80.55 ± 4.74	10.00	3.33	4,214.43 ± 61.91 (6.63)	1,378.89 ± 145.15 (6.14)
D4	19.70 ± 1.47	3.67 ± 0.34	106.39 ± 9.21	50.00	53.33	6,500.41 ± 709.00 (6.813)	2,943.51 ± 436.951 (6.47)
f-value	1.22 ns	2.98	6.68			2.944 ns	5.57

Figures in parenthesis are logharitmic transformed values; [a]the nematodes counts multiplied by 1000 (x1000)

content was significantly different (f = 2.98 and 6.68), respectively (Table 2).

Impact of different diets on the pathogenicity and multiplication of EPNs

On *Heterorhabditis bacteriophora* BA1

The IJs of this nematode species emerged from *G. mellonella* larvae reared on diets D2 and D5 recorded the highest ability to infect the larvae, and cause their mortality but with different percentages at 24 h after inoculation. These percentages, which referred to the ability of EPNs to attack an insect host, were 53.33 and 50% from D2 and D5, respectively (Table 2). However, this ability decreased for the same nematode species which emerged from D1, D3 and D4 and were 16.67, 3.33 and 10.0%, respectively. The highest production of *H. bacteriophra* IJs was obtained from Galleria larvae reared on diets D3, D1 and D5. They yielded 5,249.940; 5,205.690 and 6,500.410 IJs from larvae weights 3.25, 3.09 and 3.67 g, respectively. However, those reared on D2 and D4 yielded less (4,758.630 and 4,241.430 IJs) from larvae weights (3.71 and 2.57 g), respectively. There was very little variation between these nematode counts and ranged from 6.63 to 6.813 between different diets.

On *Steinernema carpocapsae* BA2

The IJs of *S. carpocapsae* from *G. mellonella* larvae which were mainly reared on diets D2 and D5 revealed the highest pathogenic ability to infect the last instar larvae of *G. mellonella*; causing their mortality of about 56.67 and 53.33%, respectively (Table 2). For multiplication and propagation of this nematode species, larvae reared on D5, D1 and D2 exhibited the highest multiplication for IJs of *S. carpocapsae* and yielded 2,943.510; 2,580.310 and 1,856.360 IJs, respectively. On the other hand, those emerged from D4 and D3 yielded less (1,378.890 and 1,230.040 IJs) when larvae weighed 2.57 and 3.25 g, respectively. Transformed values of nematode counts revealed very little variation in the nematode counts between different diets and ranged from 6.09 to 6.47.

Correlation between larval weights and the studied parameters

The correlation coefficient (r) and the slope (b) values between the larval weights (as independent factor) and the tested parameters (as dependent factor) were explained in Table 3. Total lipid content of *G. mellonella* larvae was affected significantly and positively by the larval weights where r = 0.732. The pathogenicity of two nematode species, *H. baceriophora* and *S. carpocapsae* on *G. mellonella* larvae were affected significantly and positively by their weights (r = 0.8 and 0.854), respectively. In contrast, the multiplication of both species revealed insignificant and positive correlation with the larval weights where r = 0.195 and 0.28, respectively (Table 3).

Correlation between total lipid content of larvae and certain parameters

The pathogenicity of both nematode species, *H. bacteriophora* and *S. carpocapsae* on the last instar larvae of *G. mellonella* were affected positively and highly significantly with increasing total lipid content in the larval

Table 3. Calculated correlation coefficient (*r*) and the slope (*b*) values between larvae weights (*X*) and other studied parameters (pathogenicity and multiplication) (*Y*)

Larvae weights *X*	Total lipid content *Y*1	Pathogenicity of nematodes		Multiplication of nematodes	
		Heterorhabditis bacteriophora *Y*2	*Steinernema carpocapsae* *Y*3	*Heterorhabditis bacteriophora* *Y*4	*Steinernema carpocapsae* *Y*5
r	0.732	0.8	0.854	0.195 ns	0.28 ns
b	32.644	40.072	51.94	0.097	0.173

Table 4. Calculated correlation coefficient (*r*) and the slope (*b*) values between the lipids content of *Galleria mellonella* larvae and both the pathogenicity and multiplication of nematodes

Total lipid content of larvae *X*	Pathogenicity of nematodes		Multiplication of nematodes	
	Heterorhabditis bacteriophora *Y*1	*Steinernema carpocapsae* *Y*2	*Heterorhabditis bacteriophora* *Y*3	*Steinernema carpocapsae* *Y*4
r	0.97	0.971	0.063	–0.409
b	10.923	13.295	0.007	–0.057

bodies where $r = 0.97$ and 0.971, respectively (Table 4). This indicated that each diet contained ingredients which improved the growth, increased their weight and the lipid content in body tissues of *G. mellonella* larvae, and thus increased the pathogenicity and the quality of IJs of both nematode species for infecting the insect host and finally causing the mortality in their population after 24 h of inoculation. However, the multiplication of nematodes revealed insignificant correlation with the total lipid content of its insect host (Table 4).

Discussion

This study revealed the impact of four artificial diets and one natural food on producing *G. mellonella* larvae suitable for pathogenicity by IJs of *H. bacteriophora* and *S. carpocapsae* as well as suitability for multiplying nematode stages. It is clear that the food supplied in diets 2 and 3 efficiently produced a greater number of larvae with higher weights than natural beeswax. Previous studies were conducted to find the most suitable diets for rearing *G. mellonella* (Coskun *et al.* 2006; Birah *et al.* 2008; Huang *et al.* 2010; Ellis *et al.* 2013; Mohamed and Amro 2022). The suggested artificial diets in our study contained the same ingredients as the diets of Birah *et al.* (2008) and Huang *et al.* (2010) except for the absence of wheat bran and beeswax in our diets. Our results were in agreement with previous findings of Dadd (1963) who found that diets rich in carbohydrates resulted in developed larvae similar to those produced from diets containing beeswax. Moreover, van Zyl and Malan (2015) observed that the effect of milk powder on larval development is unclear, but it might influence larval weight. Also, yeast has been reported as a necessary compound in the diets of *G. mellonella* rearing (Gross *et al.* 1996; Singh *et al.* 2014; van Zyl and Malan 2015; Kotchofa and Baimey 2019). In addition, a beeswax diet revealed a good production for *G. mellonella* larvae and can be used as an alternative suitable food instead of diets 2, 3 for rearing insects, in spite of the nutritional deficiency of a beeswax diet which forces larvae to pupate earlier (Coskun *et al.* 2006; Jorjão *et al.* 2018). Niemierko and Wlodawer (1950) found that beeswax can supply the larvae with large amounts of energy, but it cannot provide larvae with other nutrients such as protein, which was necessary for good development. Therefore, it is necessary to know insect biology in detail in order to evaluate the required modifications of artificial diets (Birah *et al.* 2008; Kulkarni *et al.* 2012).

For the first time, this study clearly showed that larval weight had a positive significant correlation with the total lipid content in body tissues. This illustrated that each diet which increased the weights was followed by an increase in lipid content. The larvae fed diet D2 and beeswax D5 had the highest weight and the highest lipid content in their tissues. Also, the same diets improved the quality of IJs of both nematode species in pathogenicity and propagation, thus causing mortality of the last instar larvae of *G. mellonella*. These results were verified by calculating the correlation between the total lipid content and the pathogenicity of nematode IJs, wherever it was clear that lipid content in larvae had a positive and highly significant correlation with pathogenicity of nematodes to *G. mellonella* larvae.

It was concluded that the diet used for nutrition and rearing *G. mellonella* can significantly affect the sensitivity of an insect host to EPNs and affect and improve the efficiency of IJs to kill an insect host. These findings were in accordance with the results recorded by Finke (2002) who showed that when *G. mellonella* larvae had high percentages of fats and lipid components, the developmental rate and multiplication (yield) of *Heterorhabditis* were increased. In this regard, Andaló *et al.* (2011) and Salem *et al.* (2021) determined that lipid is the main source of energy for EPNs; the level of lipid may affect the infectivity (pathogenicity) of IJs. Also, Shapiro-Ilan *et al.* (2008, 2012) noticed that the presence of certain lipids in insect diets increased the host's susceptibility and its infection rates by EPNs.

Concerning the nematode multiplication inside an insect host when fed the tested artificial diets or natural food, it was seen that the natural beeswax D5 produced the highest numbers of two nematode species, *H. bacteriophora* and *S. carpocapsae,* followed by or equal to that of the D1 diet. In addition, D3 and D2 diets came after D5 and D1 in producing *H. bacteriophora*.

In general, each rearing diet increased *G. mellonella* larval weights and may be able to increase nematode yield. This concept was similar to the results observed previously by van Zyl and Malan (2015), Kotchofa and Baimey (2019) and Rahoo *et al.* (2019) who found that the number of nematode production from *G. mellonella* is proportional to the insect host body weight and size. Dadd (1963), Lee *et al.* (2007), Shapiro-Ilan *et al.* (2008) and Mohamed and Amro (2022) stated that the proportion and/or selection of ingredients in diets play a vital role in larval development, fitness and in turn the quality of nematodes. In this study, the multiplication of *H. bacteriophora* inside *G. mellonella* larvae was always greater than *S. carpocapsae* which agrees with that reported previously by Raj Kumar *et al.* (2003).

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