



Low labor “in vivo” mass rearing method for entomopathogenic nematodes



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HIGHLIGHTS

- Low labor “in vivo” rearing system to mass rear EPNs.
- Field and laboratory tested for 8 years.
- 100 billion EPNs reared for field release.
- Easily adapted for many species of EPNs.

ARTICLE INFO

Article history:

Received 28 March 2016
Revised 6 January 2017
Accepted 7 January 2017
Available online 9 January 2017

Keywords:

EPN
Entomopathogenic nematode
Low labor in vivo rearing

ABSTRACT

The wide spread use of biological control agents in commercial agriculture is frequently limited by the high cost of the biological control agents. We describe in this paper an entomopathogenic nematode (EPN) “in vivo” mass rearing method, not based on the White trap concept, which is both “low technology” and “low labor”, reducing the cost of producing large quantities of EPN IJ to the level conducive for use in an area-wide biocontrol program. Rearing temperature has a significant impact on the number of EPN IJ produced per host larvae and the IJ viability at any point in time. Emerged IJs remain within a non-aqueous, high humidity environment with improved oxygen access without extra steps or equipment. Depending on storage conditions, IJs remain viable for 7–14 d without extra labor. Our laboratory has used this rearing method to rear more than 100 billion IJ in the past 8 years to support an area wide biological control program.

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1. Introduction

The wide spread use of biological control agents in commercial agriculture is frequently limited by the high cost of the biological control agents compared to conventional pesticides and this is especially true for entomopathogenic nematodes (EPN) (Shapiro-Ilan et al., 2014).

Laboratory and small scale EPN production usually employ some “in vivo” variation utilizing the concept of the White trap (White, 1927) as a device to harvest emerging IJs, taking advantage of the IJs natural migration away from the host cadaver upon emergence. The White trap consists of a dish or tray (usually of plaster of Paris) on which the cadavers rest. The cadaver surface is surrounded by water and contained in a larger container. Emerging IJs leave the cadaver and enter the water. Harvesting IJs

requires rinsing the cadavers with water and draining the water. Variations of the white-trapped based in vivo rearing method are reported by various authors (Dutky et al., 1964; Poinar 1979; Woodring and Kaya, 1988; Lindegren et al., 1993; Flanders et al., 1996; Kaya and Stock 1997; Shapiro-Ilan et al., 2002). “In vivo” mass culturing of EPNs is expensive due to high labor costs and the use of live insects as production hosts. However, “in vivo” mass rearing operations require minor capital outlay, nominal expertise to achieve good product quality and the system is easily adapted to multiple EPN species. Without significant reduction of costs, primarily the cost of the host insect and labor, there is little cost benefit of scale (Shapiro-Ilan et al., 2014).

In contrast, “in vitro” rearing systems require highly technical expertise to produce the required monoxenic culture of the symbiont bacteria and the establishment of bacteria-free nematodes (Shapiro-Ilan et al., 2014). Once achieved, the “in vitro” mass rearing has economy of scale but requires a significant increase in required capital outlay and expertise while reducing the labor

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costs. “In vitro” systems are specialized and are difficult to adapt to additional EPN species (Shapiro-Ilan et al., 2014).

In both types of mass rearing systems, care must be taken to assure the final project is a viable biological control agent in the field. Biological control programs utilizing EPN strains which persist in the environment for multiple years from a single application face additional supply roadblocks, because few if any commercial EPN suppliers are interested in culturing an EPN strain which persist for multiple years in the field and therefore discouraging the establishment of a stable retail market (Shields unpublished).

We describe in this paper an EPN “in vivo” mass rearing method, not based on the White trap concept, which is both “low technology” and “low labor”, reducing the cost of producing large quantities of EPN IJ to the level conducive for use in an area-wide biocontrol program. Emerged IJs remain within a non-aqueous, high humidity environment with improved oxygen access without extra steps or equipment. Depending on storage conditions, IJs remain viable for 7–14 d without extra labor. Our laboratory has used this rearing method to rear more than 100 billion IJ in the past 8 years to support an area wide biological control program utilizing persistent EPNs against alfalfa snout beetle, *Otiorynchus ligustici* (L.) (Shields and Testa, 2015). In 2015 alone, this rearing method was used to rear 60 billion IJs for field release and were used to inoculate 1700 hectares of agricultural fields in Northern NY against alfalfa snout beetle, *Otiorynchus ligustici* (Shields unpublished, Shields and Testam 2015).

2. Materials and methods

To evaluate the viability of this low labor ‘in vivo’ mass rearing technique, all three EPN species were cultured under three different temperatures (20 °C, 25 °C, 30 °C) and 50% relative humidity (RH). The total number of infective juveniles produced and the duration of their survival in the sawdust media was recorded for each temperature/species over a 30–35 d period.

2.1. Source of EPNs

Three isolates of entomopathogenic nematodes infective juveniles (IJs) were used in our laboratory study. *Heterorhabditis bacteriophora* Poinar (‘Oswego’), and, *Steinernema carpocapsae* (Weiser) (‘NY 001’), were isolated in the fall of 1990 from soil in Oswego County, New York. The third nematode, *Steinernema feltiae* (Filipjev) (‘NY 04’), was isolated in 2004 in soil collected in Jefferson County, New York. Nematodes have been re-isolated from the field every second year beginning in 2009, and used to restart the laboratory culture. The IJs used in this trial were re-isolated from field plots in 2012. Nematode cultures are maintained in greater wax moth, *Galleria mellonella* (L.), larvae (Woodring and Kaya, 1988) between field isolations. To protect against the loss of the EPN to persist in the environment, culturing methods have been adapted to preserve the persistent genetics in the population during the two years of laboratory culturing (Shields and Testam 2015).

2.2. Inoculation of hosts

Two hundred and eighty-eight (288) plastic containers (450 ml) were filled ¾ full of sawdust and 250 6th instar wax moth, *Galleria mellonella*, larvae were added (Morning Dew Bait Company, Trumansburg, NY). Containers were capped with a vented lid. Larvae were allowed 24 h to redistribute throughout the sawdust in the container. Containers containing larvae were then randomly divided into 3–96 container lots. Each 96 container lot was inoculated with a different species of EPN. Each container was

inoculated with 15,000 IJ suspended in 20 ml of distilled water and pipetted onto the surface of the sawdust in a double figure 8 pattern. Nematode suspensions were prepared by (1) counting nematodes in five subsamples from the nematode stock suspension, and (2) adjusting the concentration to 15,000 IJs/20 ml water. Inoculated containers were held at room temperature for 24 h and then randomly selected for placement in each of the three temperatures (32 containers/species/temperature). Death of the wax moth larvae was verified in each container after 36 h.

Beginning seven days after inoculation, cups were checked daily for IJ emergence from cadavers by observing the sides and lids of cups for infective juvenile presence. Once, emergence was observed, four randomly selected cups from each 32-cup series representing each EPN species/temperature were rinsed through screens to separate the IJs from the organic material. Sawdust and cadavers were removed from each container (one container at a time) by dumping contents into a No. 20 mesh sieve stacked on top of a No. 60 mesh sieve (U.S.A. Standard Testing Sieve), and rinsed for 2–3 min using distilled water flushing IJs into a second container. The inside of the cup and lid were also rinsed into the second container.

The number of IJs in each sample was estimated using the standard serial dilution protocol. The percent of the IJs alive was estimated by counting the number of alive IJs and the number of dead IJ in 5 0.1 ml droplets. The remaining cups in the series were left at the experimental temperature. This procedure was repeated every 4 days for 8 extraction cycles.

2.3. Statistical analysis

The production of IJs and viability between experimental temperatures within a species was evaluated for significant differences using ANOVA (Systat Software Inc, 2009).

3. Results

Production yields of EPN IJs in wax moth larvae are influenced by temperature regime. Temperature also influenced IJ survival in the sawdust rearing media.

3.1. *S. carpocapsae*

Temperature influenced emergence of *S. carpocapsae* IJs from *Galleria* larvae. At 20.0 °C, IJ emergence was initiated 9 d after inoculation, 7 d after inoculation at 25.0 °C and 8 d after inoculation at 30.0 °C. On the first rinse date, which was initiated on the date of first IJ emergence, 4.7 ± 0.6 million IJ (mean ± SE) were present at 20.0 °C with a viability of 78 ± 4%, 21.0 ± 2.7 million with a viability of 76 ± 3% at 25.0 °C, and 0.37 ± 0.05 million at 30.0 °C with a viability of 94 ± 6% at 30.0 °C. On the first rinse date, significantly more IJ were present at 25.0 °C than the other two temperatures ($F = 13.9$; $df = 3$; $P < 0.01$) (Fig. 1).

Peak number of IJs were available at 21 d at 20.0 °C (16.9 ± 3.1 million IJ) with a viability of 78 ± 0.02%, IJ production diminished gradually over the final 14 d of the trial. At 35 d, cadavers within the wax worm cups were badly disintegrated, <1 million IJs were emerging and the trial was discontinued. At 25.0 °C, peak IJ were recorded on day 9 at 37.0 ± 6.1 million IJs with a viability of 72 ± 2%. Two more peaks of 37 million IJs were recorded on day 16 and 23, but the viability decreased to 64 ± 2% and 51 ± 4% respectively. The three peaks of production were not significantly different but the decline in viability was significant ($F = 5.3$; $df = 2$; $P < 0.01$). IJ production declined to 2.5 ± 0.9 million IJ at 30 d post inoculation with the viability declining to 45 ± 5%. At

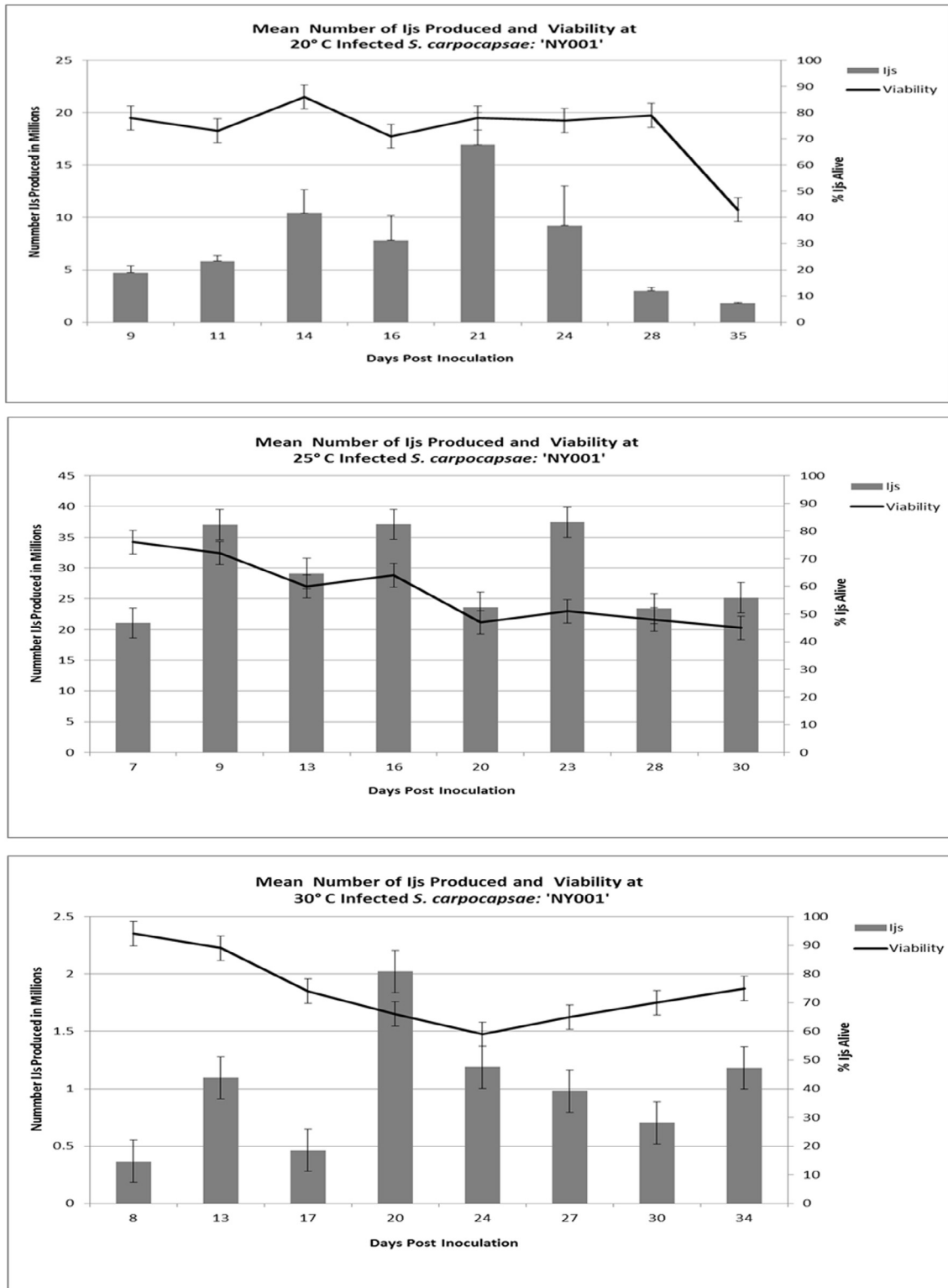


Fig. 1. Production and viability of *Steinernema carpocapsae* 'NY 001' infective juveniles at 20 °C, 25 °C and 30 °C.

30.0 °C, peak IJs were recorded at day 20 at 2.0 ± 0.8 million with a viability of $66.0 \pm 7.0\%$. The final four rinse date production plateaued to less than 1 million IJs. Peak production was significantly greater at 25.0 °C at 37 million IJs than 20.0 °C at 16.9 million IJs and 2 million IJ at 30.0 °C ($F = 25.3$; $df = 3$; $P < 0.01$). The two cooler temperatures had the same viability ($F = 01.1$; $df = 3$; $P > 0.1$) (Fig. 1).

3.2. *S. feltiae*

Temperature influenced emergence of *S. feltiae* IJs from *Galleria* larvae. At 20.0 °C, IJ emergence was initiated 9 d after inoculation, 7 d after inoculation at 25.0 °C and 8 d after inoculation at 30.0 °C. On the first rinse date which was initiated on the date of first IJ emergence, 1.9 ± 0.5 million IJ were present at 20.0 °C with a

viability of $80 \pm 4\%$, 0.02 ± 0.01 million with a viability of $43 \pm 25\%$ at 25.0°C , and 0.03 ± 0.03 million at 30.0°C with a viability of $25 \pm 25\%$ at 30.0°C . Significantly more IJs were present at the first rinse date at 20.0°C than the other two temperatures. ($F = 33.3$; $df = 3$; $P < 0.01$) (Fig. 2).

Peak number of IJs were available at 21 d at 20.0°C (15.9 ± 1.6 million IJ) with a viability of $76 \pm 1\%$. IJ numbers declined to

12.5 ± 0.7 million on day 24, but the viability remained at $75 \pm 4\%$. By day 35 at the end of the trial, the number of IJs had declined to 1.6 ± 0.2 million with a viability of $34 \pm 2\%$. At 25.0°C , peak number of IJs were available at day 16 (5.2 ± 0.6 million IJ) with a viability of $95 \pm 5\%$. Available IJs declined to 2.1 ± 0.3 million on day 20 with a viability of $83 \pm 5\%$ and declining to 1.2 ± 0.1 million by day 30 with a viability of $64 \pm 6\%$. At 30.0°C , IJ production

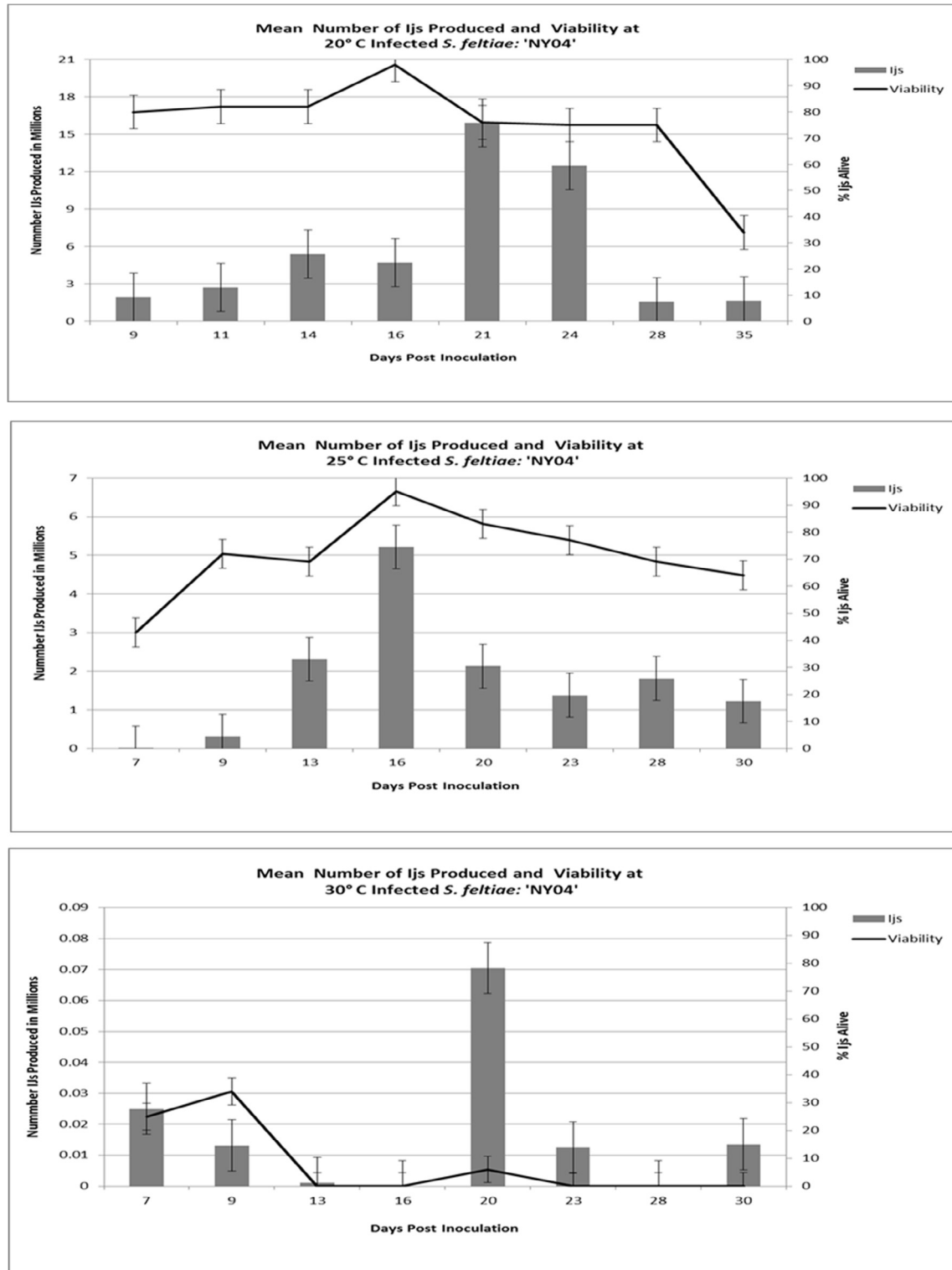


Fig. 2. Production and viability of *Steinerema feltiae* 'NY 04' infective juveniles at 20°C , 25°C and 30°C .

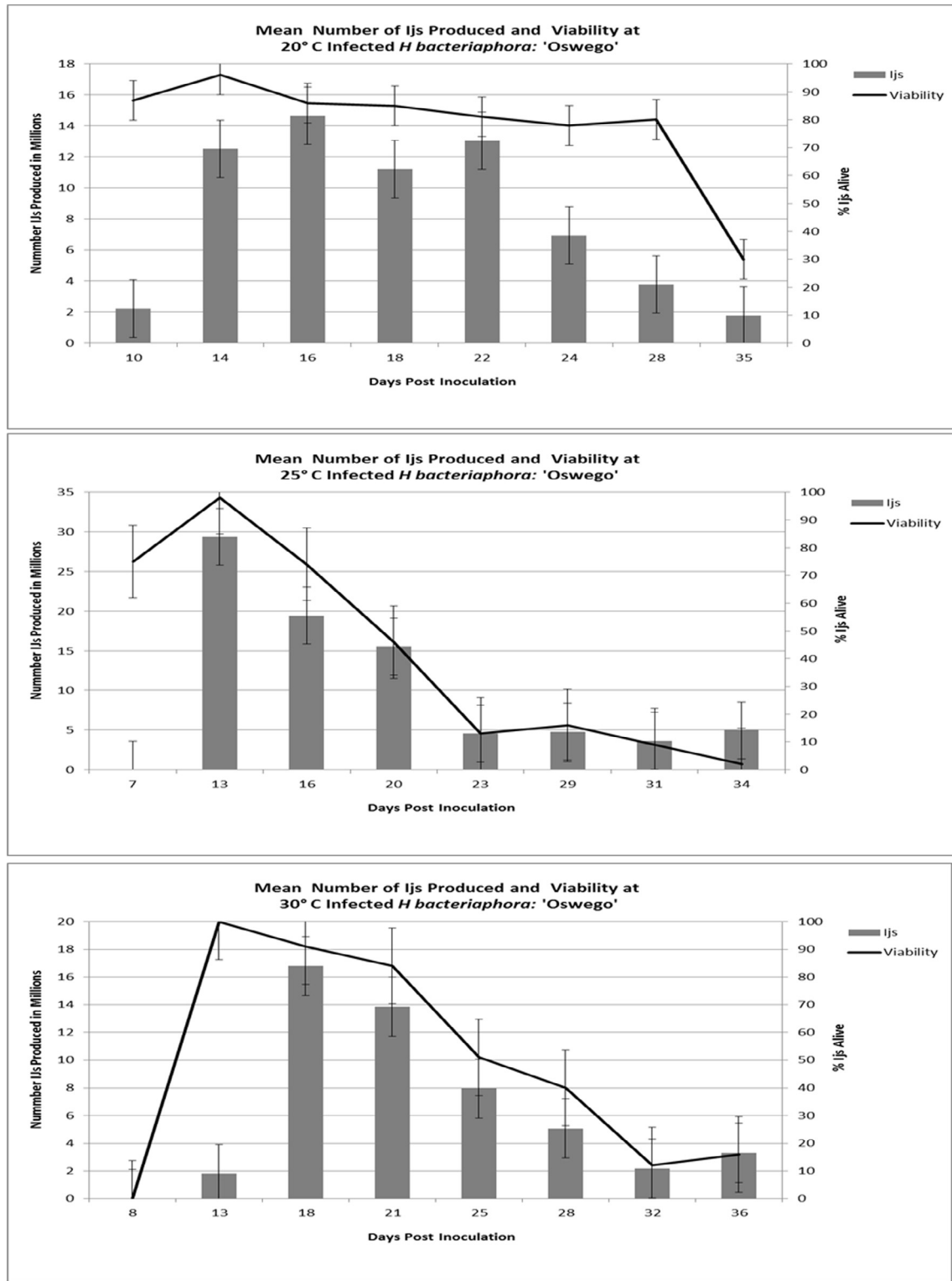


Fig. 3. Production and viability of *Heterorhabditis bacteriophora* 'Oswego' infective juveniles at 20 °C, 25 °C and 30 °C.

never exceeded 70,000 at any point during the experimental period. Peak number of IJs were significantly higher at 20.0 °C than the other two temperatures with the peak number of IJ at 25.0 °C ranking second ($F = 22.3$; $df = 3$; $P < 0.01$). While the total number of IJ present at peak production was higher at 20.0 °C than 25.0 °C, the viability was significantly lower (76% vs 94%) ($F = 21.9$; $df = 3$; $P < 0.01$) (Fig. 2).

3.3. *H. bacteriophora*

Temperature influenced emergence of *H. bacteriophora* IJs from *Galleria* larvae. At 20.0 °C, IJ emergence was initiated 10 d after inoculation, 7 d after inoculation at 25.0 °C and 13 d after inoculation at 30.0 °C. IJ emergence was significantly shorter at 25.0 °C than the other two temperatures ($F = 11.93$; $df = 3$; $P < 0.01$). On

the first rinse date which was initiated on the date of first IJ emergence, 2.2 ± 0.2 million IJ were present at 20.0 °C with a viability of $87 \pm 5\%$, 0.009 ± 0.004 million with a viability of $75 \pm 25\%$ at 25.0 °C, and 1.8 ± 0.4 million at 30.0 °C with a viability of $100 \pm 0\%$ at 30.0 °C. Number of IJs present on the first rinse date was identical for 20.0 °C and 30.0 °C but significantly larger than the number of IJ at 25.0 °C ($F = 7.3$; $df = 3$; $P < 0.01$) (Fig. 3).

Peak number of IJs were available on day 16 at 20.0 °C (14.6 ± 2.1 million IJ) with a viability of $86 \pm 3\%$. IJ numbers remained above 10 million for rinse days 18 and 22 with viability above 80% before dropping to 7.0 ± 0.4 million (viability $78 \pm 2\%$) on day 24 and 1.7 ± 0.2 million with viability falling to $30 \pm 1.0\%$ on day 35. Peak numbers of IJ at 25.0 °C were present at day 13 (29.3 ± 2.2 million IJ, viability = $98 \pm 1\%$) and declined after that peak. At 30.0 °C, IJ numbers peaked at 16.8 ± 4.7 million on day 18 with a viability of $91 \pm 2\%$ and then declined rapidly after the peak. Peak IJ production at 25.0 °C was significantly higher than the other two temperatures which had similar production ($F = 17.3$; $df = 3$; $P < 0.01$). Under all three temperatures, IJ viability at peak emergence was very similar and above 95%. Peak production numbers are very similar to numbers of IJ produced in an earlier study (29 million IJ at 25.0 °C vs 23 million IJ at 23.0 °C) (Flanders et al., 1996) (Fig. 3).

4. Discussion

The single most critical variable in this mass rearing method is temperature control during the nematode rearing/reproduction process. Temperature needs to be maintained within a few degrees of the desired rearing temperature for the best results. Based on this data, *S. feltiae* 'NY 04' is best reared near 20 °C. In comparison, *S. carpocapsae* 'NY 001' and *H. bacteriophora* 'Oswego' is are best reared at 25 °C.

To remove the IJs from the sawdust and associated organic debris in preparation to application through a conventional pesticide sprayer with all screens and filters removed was a simple process. Rearing containers were dumped and rinsed into a No. 20 mesh screen stacked on top of a No. 40 mesh screen positioned atop a collection container. Rearing debris was rinsed using unchlorinated water for approximately 5 min. The resulting solution was free of debris large enough to clog spray nozzles and applied easily through a conventional pesticide sprayer having all screens and filters removed.

Using this low labor "in vivo" rearing method, we estimate the total cost of rearing 1 billion alive IJ ready for field application is approximately \$350 USD (\$0.35 per million IJs). The major driving expense is the cost of the insect larvae used to rear the nematodes.

The cost structure is estimated in the following manner using easily obtainable *Galleria* larvae as the rearing host. To rear 1 billion alive IJ requires 12,500 *Galleria* larvae, because each larva produces on average 80 million IJs under the preferred rearing temperatures reported in this study. *Galleria* larvae are easily obtainable from bait suppliers in 450 ml plastic containers with 250 larvae, filled $\frac{3}{4}$ with sawdust and capped with vented lids. Rearing 1 billion IJ requires 50 containers of larvae with a price ranging between \$5–7 per container delivered ($50 \times \$6 = \300). Total labor which includes time to prepare the IJ to inoculate the 50 containers, container inoculation, checking containers during the rearing process and harvesting the IJ for field application ranges between three and four hours ($4 \text{ h} \times \$12/\text{h} = \48).

Acknowledgments

Funding for this research was provided by Northern NY Agricultural Development Program, NY Farm Viability Institute and Cornell University.

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