Effect of temperature on survival of Australian entomopathogenic nematodes and their virulence against the Queensland fruit fly, *Bactrocera tryoni*

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Abstract Entomopathogenic nematodes (EPNs) are commonly used biocontrol agents of insect pests, with a wide range of commercially available isolates targeting specific pests. New isolates are, however, required to improve pest control across a wider range of environmental conditions for target pests, including emerging threats. We assessed the effect of temperature on survival and virulence of 17 Australian isolates of five EPN species (*Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelatus*, *Heterorhabditis zealandica* and *Steinernema feltiae*) against larvae and pupae of the Queensland fruit fly, *Bactrocera tryoni*. All isolates still infected and killed larvae after infective juveniles (IJ) had been kept without insect hosts at 15 °C, 25 °C or 30 °C for two weeks, indicating their potential to remain viable under field conditions. However, the mean LD$_{50}$ value ranged from 35 to 150 and was generally lower at 15 °C than at 25 °C and 30 °C. Similarly, after IJs had been kept at 25 °C for 1–3 weeks without insect hosts, all isolates infected *B. tryoni* larvae, with mean LD$_{50}$ values ranging from 25 to 144. Interestingly, 15 isolates infected and killed *B. tryoni* pupae after one week, with a mean LD$_{50}$ value between 130 and 209, but only two isolates after two weeks, with a mean LD$_{50}$ value between 229 to 209. No pupal mortality was seen after three weeks. In absence of hosts, EPNs survived longer at 15 °C and 25 °C than at 30 °C. Complete EPN mortality occurred after nine weeks at 30 °C, and after 18 weeks at 15 °C and 25 °C, except for some survival in one *S. feltiae* isolate (Sf.ECCS). Overall, six isolates of *H. indica* (Hi.HRN2, Hi.LMI2, Hi.QF6), *H. bacteriophora* (Hb.HIE), *H. zealandica* (Hz.NAR1) and *S. feltiae* (Sf.ECCS) performed best and need further testing as potential biocontrol agents against *B. tryoni* under semi-field and field conditions.

Keywords Entomopathogenic nematode · Persistence · Temperature · Queensland fruit fly
Introduction

The family of tephritid fruit flies (Diptera: Tephritidae) contains many important and widespread pest species (White and Elson-Harris 1992). They can cause substantial losses in fruit and fruiting vegetable crops, and limit market access of fresh produce because of biosecurity restrictions imposed by many countries (Clarke 2019). The Queensland fruit fly, *Bactrocera tryoni* (Frogatt), is the most destructive horticultural pest in Australia, and has a high invasion potential, being one of the most polyphagous tephritids (Clarke et al. 2011) with a high climatic adaptability (Popa-Báez et al. 2020). Adult females lay their eggs into the fruit where the larvae develop causing the fruit to rot. The eggs and developing larvae are protected within the fruit and therefore more difficult to target in pest control. Thus the focus of fruit fly control is mostly on adult fruit fly and reproduction. However, late instar larvae leave the fruit prior to the pre-pupal stage and then pupate within a few hours in the top layer (1 to 5 cm) of the soil (Hulthen and Clarke 2006), and adult flies emerge from the pupae and then crawl to the soil surface (Bateman 1972). These developmental stages outside the fruit and in the soil can be targeted by soil-borne natural enemies and biological control agents, including entomopathogenic nematodes (EPNs).

EPNs of the families Heterorhabditidae and Steinernematidae with their symbiotic bacteria *Photorhabdus* (in *Heterorhabditis* spp.) and *Xenorhabdus* (in *Steinernema* spp.) are well studied and effective biological control agents of diverse pest insects (Kaya and Gaugler 1993; Tailliez et al. 2010). The dauer juveniles, also known as infective juveniles (IJ), leave infected insect cadavers after the depletion of resources, and can survive a short time in the soil while seeking new insect hosts. The symbiotic bacteria of EPNs provide IJs with the capacity to kill an insect host in which the EPNs can then develop (Waterfield et al. 2009). While EPNs are successful biological control agents of a diversity of pest insects with developmental stages in the soil, they have also been proven effective against several fruit fly pests (Sirjani et al. 2009; Godjo et al. 2018), including *B. tryoni* (Langford et al. 2014; Aryal et al. 2022b). However, for effective biological control there is a need for EPN strains that can survive and remain virulent under local conditions.

In general, EPNs have limited ability to survive in the soil without insect hosts, and, therefore, ability of EPNs to survive and remain infective in the soil before finding new insect hosts remains a major constraint of EPN field applications (Strong 2002). IJs survival in the absence of hosts depends largely on temperature, moisture, soil type and pH (Kung et al. 1990a, b; Griffin 1993; Koppenhöfer and Fuzy 2007), the susceptibility of the targeted insect pest species and its developmental stages (Bedding et al. 1993). Moreover, survival and infectivity differ between nematode species and strains (Georgis and Gaugler 1991), and are also influenced by bacterial strain identity (Grewal et al. 1997), bioassay methods (Grewal et al. 1994) and lipid reserves, with higher lipid content allowing IJs to survive longer (Patel et al. 1997; Hass et al. 2002; Fitters and Griffin 2004). Thus increased long term survival and infectivity improve the efficiency of EPN-based biological control, allow lower application doses and rates (Bedding et al. 1993) and, therefore, reduce the associated cost.

A challenge in the development of EPNs as a biocontrol agent of fruit flies is that fruit flies can vary considerably in abundance and distribution across seasons. For example, *B. tryoni* has several generations per year and a diverse host range, and can respond to variable resource availability (Tasnin et al. 2021). Furthermore, earlier studies have shown that larval and adult stages of fruit flies are more susceptible to EPN infection than pupae (Yee and Lacey 2003; Kamali et al. 2013; Langford et al. 2014), yet larvae and adults are less likely to be exposed to EPNs because they spend most time above ground. In contrast, the pupal stage is the longest developmental stage of fruit flies in the soil (Bateman 1972), but may be less susceptible to EPNs (Langford et al. 2014; Aryal et al. 2022b). Therefore, in order to have an effect on fruit fly populations, EPNs must be able to survive when no fruit fly individuals are in the soil and maintain high levels of infectivity, or infect alternative hosts in the meantime (Kurtz et al. 2007; Susuruluk and Ehlers 2008). IJs may also need to be able to infect individuals quickly as the available time window of infection of larvae (prior to pupation) and adults (after emergence) in the soil is with a few hours in *B. tryoni* (Hulthen and Clarke 2006) relatively short. Alternatively, IJs should have the capacity to also infect pupae (Aryal et al. 2022b). Furthermore, EPN isolates with longer survival might need to be
applied less frequently and may be more economic. Likewise, more virulent isolates might allow application of lower doses.

We investigated the survival and virulence of EPN isolates at different temperatures to identify potential candidates for the biological control of *B. tryoni*. We assessed these traits in 15 EPN isolates that have recently been isolated from Australian soils and molecularly characterized (Aryal et al. 2022a, 2022b). We also compared these traits between the new EPN isolates and two commercially available EPN strains previously isolated from Australian soils but since then kept in long-term laboratory culture. We further aimed to identify EPN isolates that have superior survival and virulence and are less affected by variation in temperature.

**Materials and methods**

**EPN production and storage**

This study used 15 EPN isolates recently isolated from eastern Australia (Aryal et al. 2022a) and two commercial isolates obtained from Ecogrow Environment Pty Ltd (Table 1). The EPNs were reared on *T. molitor* larvae kept at 25 °C and were harvested from white traps as described previously (Aryal et al. 2022a) and stored in Ringer’s solution (9.0 g NaCl, 0.42 g KCl, 0.37 g CaCl₂·2H₂O and 0.2 g NaHCO₃ dissolved in 1 l of distilled water) at a concentration of 1000 IJs ml⁻¹ in a culture flask at 15 °C until use.

**Table 1** The EPN isolates used in this study included 15 recently isolated from soils in New South Wales (NSW), Australia, and Queensland (QLD), Australia (Aryal et al., 2022a) and two commercially available Australian isolates (*) obtained from Ecogrow Pty Ltd

| EPN isolates | EPN species | Locality               |
|--------------|-------------|------------------------|
| Hb.HIE1      | *H. bacteriophora* | Richmond, NSW          |
| Hi.ECC1H     | *H. indica*   | Richmond, NSW          |
| Hi.HRN2      | *H. indica*   | Heron Island, QLD      |
| Hi.LMBT      | *H. indica*   | Lady Musgrave Island, QLD |
| Hi.LMI2      | *H. indica*   | Lady Musgrave Island, QLD |
| Hi.QF6       | *H. indica*   | Palmwoods, QLD         |
| Hi.QGL       | *H. indica*   | Duingal, QLD           |
| Hm.ENCBF2    | *H. marelatus*| Somersby, NSW          |
| Hm.GOS1      | *H. marelatus*| Somersby, NSW          |
| Hz.ECOGROW*  | *H. zealandica*| Australia              |
| Hz.BB1       | *H. zealandica*| Batemans Bay, NSW      |
| Hz.BB3       | *H. zealandica*| Batemans Bay, NSW      |
| Hz.NAR1      | *H. zealandica*| Narara, NSW            |
| SF.ECOGROW*  | *Steinernema feltiae* | Australia             |
| SF.ECCS      | *S. feltiae*  | Richmond, NSW          |
| SG.G1        | *S. feltiae*  | Gundagai, NSW          |
| SF.Y13       | *S. feltiae*  | Temora, NSW            |

All new isolates had been baited with larvae of *Tenebrio molitor* except Hi.LMBT which had been baited with *Bactrocera tryoni* larvae. The two commercially available isolates had originally been baited with *Galleria mellonella* (Aryal et al., 2022b).

**Insect rearing**

We used a laboratory population of *B. tryoni* (HAC), established from flies collected on the Hawkesbury campus of Western Sydney University in Richmond, New South Wales (NSW), in summer 2009 (Morrow et al. 2015), and maintained in a glasshouse chamber at 25 °C and 70% RH as described previously (Langford et al. 2014). Eggs were collected from adult flies kept in cages (30 cm each side) by offering them a 120 ml oviposition cup filled with larval diet covered with perforated parafilm as described previously (Meats et al. 2004). The larval diet cups with eggs were then placed on a thin layer of sterile (autoclaved) washed sand contained within a container with fly mesh on top. Late third instar larvae left the larval diet cup for pupation on the sand layer. Larvae which had recently jumped from the larval diet cup...
Effect of temperature on EPN survival and virulence against fruit fly larvae

The effect of three temperatures (15 °C, 25 °C and 30 °C) on EPN survival and virulence against *B. tryoni* larvae was assessed using a sand plate assay. These temperatures include the temperature range of top soil in Australia throughout the year (Bureau of Meteorology), and a majority of sites previously assessed across southeastern Australia have been categorized with a thermic soil temperature regime (Watson 1980). Washed sand was autoclaved, oven dried and maintained at 10% moisture (w/w). Petri dishes (150 mm) were filled with 250 g of sand. Each EPN strain was tested as five replicates over time, with IJs for each replicate harvested from a different white trap. IJs were harvested from white traps, and, prior to inoculation of the sand, were stored in Ringer’s solution at 15 °C, 25 °C and 30 °C for two weeks. Then, solutions containing 50, 100, 200, 500 and 1000 IJs were prepared in 1 ml of Ringer’s solution and inoculated at the center of each plate. The control received 1 ml of Ringer’s solution without IJs. Immediately after EPN inoculation, 20 *B. tryoni* larvae were added to the center of each plate on top of the sand, and the larvae then dispersed across the plate. The plates were then incubated at 25 °C. Insect mortality due to EPN infection was recorded after one week, and EPN infection of individuals confirmed by dissection. The LD50 was calculated for each replicate to characterize the variation for each EPN isolate.

Effect of storage time at 25 °C on EPN survival and virulence against fruit fly larvae and pupae

The ability of EPNs to infect and kill *B. tryoni* larvae and pupae after one, two and three weeks at 25 °C and without the provision of an insect host during this time was assessed using another sand plate assay. Petri dishes (150 mm) were filled with 250 g sterile washed sand and maintained at 10% moisture as described earlier. Each EPN isolate was tested as five replicates over time, with IJs for each replicate harvested from a different white trap. EPN solutions containing 50, 100, 200, 500 and 1000 IJs in 1 ml of Ringer’s solution were inoculated at the center of the plates and incubated at 25 °C. After one, two and three weeks of storage, 20 *B. tryoni* larvae were added to the center of each plate (and larvae then dispersed across the plate), or 20 pupae were evenly distributed across each plate. The plates were then incubated at 25 °C. Insect mortality due to EPN infection was recorded after one week, and EPN infection of individuals confirmed by dissection. The LD50 was calculated for each replicate to characterize the variation for each EPN isolate.

Effect of temperature on IJ survival

Survival of IJs of the 17 EPN isolates was assessed in 24-well plates. For each isolate, 10,000 IJs in 2 ml Ringer’s solution were placed into each well and stored at 15 °C, 25 °C and 30 °C. Each isolate was tested as five replicates over time, with IJs for each replicate harvested from a different white trap. Dead nematodes were counted every week for 18 weeks or till 100% mortality was observed. Confirmation of IJ mortality was done by probing IJs with a fine needle to check for nematode movement (Kaya and Stock 1997). Before counting, the IJ solutions were homogenously mixed. Then, IJs contained in 100 µl were counted for each well. This was repeated three times to obtain the average mortality. The counted EPNs were placed back into their respective well and the volume was maintained at 2 ml after each count by adding additional Ringer’s solution.

Statistical analysis

The dose required to kill 50% of the insects (LD50) for each replicate was calculated and the averages were compared between different isolates (Strauch et al. 2004). The LD50 values were calculated by fitting a saturation model (mortality = a [1-ln(-bx)] + c) to the mortality data by minimization of the $\chi^2$ value for the comparison of the theoretical and observed distribution using Microsoft Excel Solver. In the model $a$ is equal to the total number of insects corrected by the control mortality, $b$ is the slope, $c$ is the control mortality, and $x$ is the dose used per insect. The data for the temperature effect on survival and virulence (LD50) were analysed for normality with the Shapiro–Wilk test. For normally distributed data,
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Fig. 1   \(LD_{50}\) of EPN isolates (a) Heterorhabditis bacteriophora, (b) Heterorhabditis indica, (c) Heterorhabditis marelatus, (d) Heterorhabditis zealandica and (e) Steinernema feltiae tested against Bactrocera tryoni larvae after IJs had been kept for two weeks without insect hosts at 15 °C, 25 °C and 30 °C. Error bars indicate SD across five replicates. Different letters on top of the error bars indicate that means are significantly different from each other among all isolates and temperatures as per Tukey’s HSD test (\(p<0.05\)). Asterisks on top of the braces denote statistically significant differences among EPN isolates at different temperatures (\(* * * p<0.0001\)), and N.S stands for non-significant (\(p>0.05\)). Lower \(LD_{50}\) values correspond to higher virulence.

Results

Effect of temperature on EPN survival and virulence against B. tryoni larvae

After two weeks in Ringer’s solution at 15 °C, 25 °C and 30 °C, all 17 EPN isolates were able to infect B. tryoni larvae at these temperatures (Fig. 1). The larval mortality caused by EPNs were significantly different among the temperatures (\(F_{2, 255} = 536.9; p<0.0001\)), isolates (\(F_{16, 255} = 7.9; p<0.0001\)) and their interaction (\(F_{32, 255} = 3.1; p<0.0001\)). Across all isolates, the mean \(LD_{50}\) value at 15 °C was lower than at 25 and 30 °C and ranged from 34.56 (±4.6) to 62.44 (±9.74) IJs per insect, 48.26 (±3.1) to 85.26 (±4.86) IJs per insect, and 82.16 (±12.54) to 149.92 IJs (±19.79) IJs per insect, respectively. The lowest and highest \(LD_{50}\) were found for Hi.LMI2 and Hz.NAR1 at 15 °C, for Hz.NAR1 and Hm.ENCBF2 at 25 °C, and for Hz.NAR1 and Hm.ENCBF2 at 30 °C. The commercial strain Hz.EG was more virulent than Hm.GOS1 at 15 °C and 30 °C, and more virulent than Hm.ENCBF2 at 25 °C. In contrast, the commercial strain Sf.EG was less virulent than Hz.QF6, Hz.HRN2 and Hi.LMI2 at 15 °C, and less virulent than Hz.BB1, Hz.BB3, Hi.QF6 and Hz.NAR1 at 25 °C.
IJ survival and virulence in sand against B. tryoni larvae and pupae

All EPN isolates were still able to infect and kill B. tryoni larvae after IJs had been kept at 25 °C without hosts for one, two and three weeks (Fig. 2), while 15 and two isolates still infected and killed B. tryoni pupae after one and two weeks, respectively (Fig. 3). However, no pupae were infected by EPN isolates after three weeks. Overall, the larval mortality caused by the EPN isolates increased with the length of incubation, with the highest virulence observed in isolates kept without insect hosts for three weeks.

**Fig. 2** LD₅₀ of EPN isolates tested against *Bactrocera tryoni* larvae after (a) one week, (b) two weeks and (c) three weeks of incubation of IJs without insect hosts at 25 °C. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey’s HSD test (p < 0.05). Lower LD₅₀ values correspond to higher virulence.

**Fig. 3** LD₅₀ of EPN isolates tested against *Bactrocera tryoni* pupae after (a) one week and (b) two weeks of incubation of IJs without insect hosts at 25 °C. IJs incubated for three weeks at 25 °C were not able to infect pupae (and are therefore not shown). Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey’s HSD test (p < 0.05). Lower LD₅₀ values resemble higher virulence.
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by EPNs kept at 25 °C without hosts for one, two and three weeks was significantly different among the isolates \( (F_{16, 255} = 27.6; \ p < 0.0001) \), the time after EPN inoculation \( (F_{2, 255} = 778.7; \ p < 0.0001) \) and their interaction \( (F_{32, 255} = 4.6; \ p < 0.0001) \). The mean LD\(_{50}\) value against \( B. \ tryoni \) larvae ranged from 24.72 (± 3.29) to 70.82 (± 10.6) IJs per insect, 51.02 (± 4.57) to 117.24 (± 20.71) IJs per insect and 84.62 (± 12.45) to 144.02 (± 15.93) IJs per insect after IJs had been kept at 25 °C without hosts for one, two and three weeks, respectively.

Furthermore, the pupal mortality caused by IJs of EPN isolates kept at 25 °C without hosts for different time periods was also significantly different among isolates \( (F_{16, 255} = 38.2; \ p < 0.0001) \), the time after EPN inoculation \( (F_{2, 255} = 1220.1; \ p < 0.0001) \) and their interaction \( (F_{32, 255} = 30.78; \ p < 0.0001) \). The pupal mortality caused by EPN isolates after one week without hosts at 25 °C ranged from 129.64 (± 16.5) to 209.28 (± 46.49) IJs per insect. Two EPN isolates, Hz.NAR1 \( (LD_{50} \ 228.82 ± 27.8 \ IJs \ per \ insect) \) and Hi.ECCH \( (LD_{50} \ 209.42 ± 20.48 \ IJs \ per \ insect) \), killed pupae after two weeks without hosts at 25 °C. The Hb.HIE isolate was more virulent than both.

### Table 2

The survival % of EPN isolates were analysed using Kruskal–Wallis test and the corresponding results \( (\chi^2, \ df \ and \ p\text{-values}) \) are presented for each isolate

| S.N | EPN isolates | \( \chi^2 \) | df | p-value |
|-----|--------------|--------------|----|---------|
| 1   | Hi.ECCH     | 266.3        | 53 | < 0.0001|
| 2   | Hb.HIE      | 266.5        | 53 | < 0.0001|
| 3   | Hi.HRN2     | 267.4        | 53 | < 0.0001|
| 4   | Hm.ENCBF2   | 266.7        | 53 | < 0.0001|
| 5   | Hi.LMBt     | 266.8        | 53 | < 0.0001|
| 6   | Hi.LMII2    | 267.3        | 53 | < 0.0001|
| 7   | Hi.QF6      | 266.6        | 53 | < 0.0001|
| 8   | Hi.QGL      | 267.2        | 53 | < 0.0001|
| 9   | Hm.GOS1     | 264.9        | 53 | < 0.0001|
| 10  | Hz.BB1      | 267.2        | 53 | < 0.0001|
| 11  | Hz.BB3      | 267.3        | 53 | < 0.0001|
| 12  | Hz.EG       | 266.5        | 53 | < 0.0001|
| 13  | Hz.NAR1     | 267          | 53 | < 0.0001|
| 14  | Sf.ECCS     | 262.9        | 53 | < 0.0001|
| 15  | Sf.EG       | 266.3        | 53 | < 0.0001|
| 16  | Sf.GG1      | 265.2        | 53 | < 0.0001|
| 17  | Sf.Y13      | 265.4        | 53 | < 0.0001|

\( \chi^2 \) tests compared the survival (%) of individual isolates at three temperatures across storage time (18 weeks for each temperature)

![Fig. 4](image-url) Survival (%) of the EPN isolates of (a) *Heterorhabditis bacteriophora*, (b-g) *Heterorhabditis indica*, (h-i) *Heterorhabditis marelatus*, (j-m) *Heterorhabditis zealandica* and (n-q) *Steinernema feltiae* at 15 °C (black filled squares), 25 °C (grey filled squares) and 30 °C (open squares) across time. The name and origin of the isolates are listed in Table 1. Error bars indicate SD across five replicates.
commercial strains after all three time periods without hosts at 25 °C.

Long-term survival of IJs in Ringer’s solution

Temperature had a significant effect on the survival of tested EPN isolates (Fig. 4, Table 2). All isolates survived without hosts for up to 16 weeks at 15 and 25 °C, and nine weeks at 30 °C. The *S. feltiae* isolates had the highest survival and were the only species to survive ≥ 18 weeks at 15 °C and 25 °C and nine weeks at 30 °C. Among the *Steinernema* isolates, *Sf.ECCS* had the highest survival with over 17 weeks at 15 °C (54.28 ± 5.26) and 25 °C (56.6 ± 7.5), and with over eight weeks at 30 °C (11.78 ± 2.91). Among the *Heterorhabditis* isolates, *Hi.ECCH* had the highest survival, with up to 18 weeks at 15 °C (8.28 ± 5.07) and 25 °C (14.2 ± 1.92), and *Hz.BB1*, with up to nine weeks at 30 °C (15.2 ± 4.64).

Discussion

We found that IJs of Australian EPN isolates can survive without hosts and remain virulent at different temperatures for a considerable time. This situation can commonly arise in EPN applications, but also naturally in the soil. Specifically, IJs of all isolates survived and remained virulent after two weeks without a host at all temperatures (15 °C, 25 °C and 30 °C). Furthermore, at 25 °C IJs survived for three weeks without a host to still infect larvae, and two weeks to still infect pupae. In the absence of hosts, we recorded higher IJ survival rates at lower temperatures, indicating that IJs may be better able to preserve energy stores at the lower temperatures. Similarly, IJs survived without hosts for a maximum of nine weeks at 30 °C and up to 18 weeks at 15 °C and 25 °C. After IJs had been kept at 15 °C for two weeks without hosts and then provided with *B. tryoni* larvae, the *H. indica* isolates were the most virulent followed by the *H. zealandica* isolates. At 25 °C, the *H. zealandica* isolates were the most virulent followed by the *H. indica* isolates, and at 30 °C, the *H. zealandica* isolates were the most virulent followed by the *H. bacteriophora* isolates. The *H. marelatus* isolates were the least virulent at all temperatures. After they had been kept without hosts for two weeks at the three temperatures, IJs of all isolates were able to infect *B. tryoni* larvae, but virulence was highest after they had been kept at 15 °C, whereas there was a reduction in virulence with increase in storage temperature. All isolates were still able to infect *B. tryoni* larvae after IJs had been kept at 25 °C for one, two and three weeks prior to the provision of hosts. However, we found higher infection rates after one week than two and three weeks.

Our results demonstrated that EPNs stored at lower temperatures and for shorter time periods showed higher survival and virulence against *B. tryoni*. Therefore, storage temperature and time prior to experiments can affect the virulence of EPN strains. Previous studies found that ageing of EPNs reduced the ability to locate and penetrate insect hosts, thereby reducing EPN infectivity and virulence (Yoder et al. 2004; Lee et al. 2016; Alonso et al. 2018). Furthermore, the EPN isolates tested in our study had been collected from warm-temperate and subtropical regions of eastern Australia, and, therefore, we expected them to be well adapted to these climatic conditions. Irrespective of this, and based on our study, we can imply that EPN isolates originating from warmer regions survive well at cool temperatures and can still infect hosts after a considerable time. A similar result was obtained by El Khoury et al. (2018) who recorded higher mortality of *G. mellonella* caused by *S. feltiae* and *H. bacteriophora* at 15 °C and 20 °C than at temperatures of up to 35 °C. An important contributing factor may be lipid reserves which are the EPNs’ sole energy source until they find a new host (Patel et al. 1997; Andaló et al. 2011). Higher temperatures might result in increased physiological activity leading to consumption of stored energy resulting in restricted movement and mortality of EPNs in absence of host resources (Smits, 1996; Hass et al. 2002). Conversely, in the presence of host, many studies reported increased EPN infectivity at higher temperatures (Yul et al. 2002; Hussaini et al. 2005; Rohde et al. 2010). For example, Rohde et al. (2010) observed an increase in EPN-caused mortality of *C. capitata* with increases in temperature, with highest mortality seen at 31 °C and lowest mortality at 19 °C. Similarly, Kepenekci et al. (2015) found that temperature had a significant effect on EPN virulence, with higher virulence at higher temperatures. Previous reports suggested that higher temperature not only affects virulence, but also progeny development. Hazir et al. (2001) recorded
100% mortality of *G. mellonella* at temperatures between 8 and 28 °C but no progeny was found at 28 °C, even with tropical *S. feltiae* isolates. Similarly, Larkin et al. (2020) found absolute mortality of the potato cutworm (*Agrotis deprivata*) due to *S. feltiae* at 28 °C, but no EPN progeny was obtained.

Control of pests in the soil can be challenging, thus EPN isolates with ability to survive for longer periods in the soil are better biocontrol agent candidates. In our study EPNs were still able to kill *B. tryoni* larvae after three weeks at 25 °C without host availability, and some isolates were able to still kill pupae after two weeks at 25 °C without host availability. In contrast, many previous studies reported no pupal infection with EPNs (Yee and Lacey, 2003; Langford et al. 2014), while other studies found lower infection rates in pupae than larvae (Aryal et al. 2022b). Several studies have been carried out to find EPN isolates with better persistence in soil after field application. Blatt et al. (2021) showed that *S. feltiae* and *H. bacteriophora* survived and remained infective in soil without irrigation for nine weeks. We found that the survival rate of *Steinernema* was higher than that of *Heterorhabditis*, and this corroborates previous studies (Abate et al. 2019). The higher survival rate of *Steinernema* might be the result of higher lipid reserves in this species when compared to *Heterorhabditis* (Selvan et al. 1993). Moreover, EPNs can persist for up to 150 days (21.4 weeks) in the field, with higher persistence of *H. bacteriophora* than *S. feltiae* (Şahin and Gözel, 2021). Furthermore, two years after EPN application, Harvey and Griffin (2016) recovered *S. carpocapsae* from soil and underneath the bark of EPN treated tree stumps, and EPN presence in treated tree stumps was correlated with the number of insect hosts emerging from untreated tree stumps. This indicates the importance of host availability for EPN persistence in ecosystems.

EPN isolates may be adapted to the environmental conditions at their original location of isolation, and, in those environments, may be more virulent compared to introduced or commercial isolates (Abate et al. 2019). As the efficacy of EPNs can depend on several abiotic and biotic factors (including EPN species and target pest species), proper bioassays are important, in particular ones that consider the specific life cycle aspects of the target pest in the soil. EPN persistence can also be improved by managing the soil environment to be more suitable for EPN survival and dispersal. For example maintaining a neutral to acidic soil pH, adequate soil moisture and increasing soil organic matter can improve EPN persistence (Campos-Herrera et al. 2019). Besides environmental factors, species interactions with soil bacteria, fungi, mites and collembolans can also be important as several of these can act antagonistically and cause mortality in EPNs (Karthik Raja et al. 2020). Moreover, to improve the survival and persistence of EPNs after application, application of EPN-infected cadavers in place of aqueous suspension as suggested by Gulzar et al. (2020) may be useful. Furthermore, adult fruit flies could also play an important role for EPN dispersal in the field as indicated by Garriga et al. (2020) who found that around 21% of EPN infected adults of *Drosophila suzukii* could fly, therefore aiding EPN dispersal. Hence, more detailed research on the role of adult flies for the dispersal and persistence of EPNs in the field will be important.

In conclusion, high temperature in the absence of hosts significantly reduced the survival and virulence of EPNs against *B. tryoni*, and EPN virulence was higher in larvae than in pupae. Overall, based on our research, *H. indica* (Hi.HRN2, Hi.LMI2, Hi.QF6), *H. zealandica* (Hz.NAR1), *H. bacteriophora* (Hb.HIE) and *S. feltiae* (Sf.ECCS) are potential candidates for the control of *B. tryoni*. Next, the best performing isolates should be evaluated in their efficacy against *B. tryoni* under semi-field and field conditions, as well as their potential for large scale production, before the development of cost-effective application strategies can begin.

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Data availability All data is contained within the manuscript.

Conflicts of interest The authors do not have a conflict of interest, except that CW works at Ecogrow, a company that supplied two EPN isolates for this study.

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