Divergent thermal specialisation of two South African entomopathogenic nematodes

Matthew P Hill, Antoinette P Malan, John S Terblanche

Thermal physiology of entomopathogenic nematodes (EPN) is a critical aspect of field performance and fitness. Thermal limits for survival and activity, and the ability of these limits to adjust (i.e. show phenotypic flexibility) depending on recent thermal history, are generally poorly established, especially for non-model nematode species. Here we report the acute thermal limits for survival, and the thermal acclimation-related plasticity thereof for two key endemic South African EPN species. Results indicate *Steinernema yirgalemense* has greater high temperature tolerance, but poorer low temperature tolerance in comparison to *Heterorhabditis zealandica*, suggesting these two EPN species occupy divergent thermal niches to one another. Acclimation had both negative and positive effects on survival of both species, although the overall variation meant that many of these effects were non-significant. There was no indication of a consistent loss of plasticity with improved basal thermal tolerance for either species at upper lethal temperatures. At lower temperatures measured for *H. zealandica*, the 5°C acclimation lowered survival until below -12.5°C, where after it increased survival. Such results indicate that the thermal niche breadth of EPN species can differ significantly depending on recent thermal conditions, and should be characterized across a broad range of species to understand the evolution of thermal limits to performance and survival in this group.
Divergent thermal specialisation of two South African entomopathogenic nematodes

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Abstract

Thermal physiology of entomopathogenic nematodes (EPN) is a critical aspect of field performance and fitness. Thermal limits for survival and activity, and the ability of these limits to adjust (i.e. show phenotypic flexibility) depending on recent thermal history, are generally poorly established, especially for non-model nematode species. Here we report the acute thermal limits for survival, and the thermal acclimation-related plasticity thereof for two key endemic South African EPN species. Results indicate *Steinernema yirgalemense* has greater high temperature tolerance, but poorer low temperature tolerance in comparison to *Heterorhabditis zealandica*, suggesting these two EPN species occupy divergent thermal niches to one another.

Acclimation had both negative and positive effects on survival of both species, although the overall variation meant that many of these effects were non-significant. There was no indication of a consistent loss of plasticity with improved basal thermal tolerance for either species at upper lethal temperatures. At lower temperatures measured for *H. zealandica*, the 5°C acclimation lowered survival until below -12.5°C, where after it increased survival. Such results indicate that the thermal niche breadth of EPN species can differ significantly depending on recent thermal conditions, and should be characterized across a broad range of species to understand the evolution of thermal limits to performance and survival in this group.
Introduction

Temperature plays a key role in both the survival and activity of terrestrial invertebrates. Thermal tolerance may be characterized through traits such as thermal maxima and minima (absolute limits), processes or rates (e.g. development), as well as the optima thereof (e.g. temperatures for which growth rate and reproduction are maximised). Such basal thermal tolerance of terrestrial invertebrate species may also be adjusted through plastic responses induced through acclimation, hardening or acclimatization, allowing for phenotypic flexibility in the species relationship to temperature (Chown & Terblanche, 2006; Angilletta, 2009). Plasticity may thus allow for environmental extremes to be buffered against, although sometimes this comes with a trade-off with basal thermal tolerance (Calosi et al., 2008; Nyamukondiwa et al., 2011). Information on both basal thermal tolerance and the plasticity thereof can compared to give insight into hierarchical levels of, and the magnitude and direction of, variation that may exist between species and across different groups (e.g. Hoffmann et al., 2013; Faulkner et al., 2014), and thus constraints or trade-offs that may be significant for understanding adaptive evolution.

Entomopathogenic nematodes (EPNs) are soil inhabiting insect parasites that fall within two monogenic families: Heterorhabditidae and Steinernematidae (Rhabditida). The families do not share a common ancestry, but have nonetheless developed a similar lifestyle (Blaxter et al., 1998). Heterorhabditids and steinernematids each have corresponding mutualistic bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively. These bacteria are held within the nematode intestine and released once the infective juvenile (IJ) has penetrated the insect host through the natural openings (spiracles, mouth or anus) (Griffin et al., 2005). The bacteria suppress the host’s immune system, typically killing the host within 24-48 h, subsequently providing nutrition for the nematodes and their offspring (Gaugler et al., 1997; Griffin et al., 2005). The EPNs are then able to complete multiple (e.g. up to three) generations within the insect cadaver (depending on the size of the host) before releasing a new cohort of IJs to start a new cycle (Gaugler et al., 1997). This life history strategy has allowed for the development of different biocontrol programmes using soil and aerial application of the IJs on agricultural pest insects (Lacey & Georgis, 2012).

There appears to be a wide range of responses to environmental stress exhibited across EPN species and strains, including variation in desiccation tolerance and hypoxia tolerance, and freeze tolerance (Morton & Garcia-del-Pino, 2009; Salame et al., 2012; Shapiro-Ilan et al., 2014). Some of the variation exhibited for freeze tolerance is most likely attributed to different adaptive strategies including cryoprotective dehydration, anhydrobiosis and freeze avoidance or tolerance strategies, for example, cold tolerance (Wharton, 2011), although the mechanisms for cold tolerance are typically the primary focus of investigation (e.g. Ali & Wharton, 2014). While EPNs are able to withstand adverse environmental conditions inside their host cadavers, the IJ stage is required to seek out new hosts and thus may be
particularly vulnerable to environmental variability in this life-stage (Brown & Gaughler, 1997). Much of the work looking at thermal performance of EPNs has focused on cold tolerance and how this relates to long-term cold storage solutions, or overwinter survival of free-living nematodes in polar environments (Perry and Wharton, 2011), although there have been assessments of heat tolerance in EPNs (e.g. Shapiro et al., 1996; Jagdale & Gordon, 1998).

In addition to variation in basal thermal tolerance, acclimation-related adjustments in tolerance may be substantial in nematodes. For example, higher temperatures experienced during propagation increase upper thermal limits, while diminishing lower thermal ones (Grewal et al. 1996; Shapiro et al., 1996; Jagdale & Gordon, 1998). Further, short term exposure to lower temperatures prior to freezing has been shown to enhance freezing survival (Ali & Wharton, 2012). Previous EPN cold tolerance work has included findings that *Heterorhabditis bacteriophora* Poinar, 1976 and *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 (Rhabditida: Heterorhabditidae) have demonstrated an increase in freezing tolerance after acclimation to temperatures below their propagation temperature (Brown & Gaugler, 1996; Ali & Wharton, 2012). Thus, testing thermal tolerances and associated plasticity or physiological adjustments in response to propagation or short-term temperature treatments to estimate thermal niche breadth across EPN species should help identify potential trade-offs between basal and acute responses. From an evolutionary perspective, understanding whether specialization to a particular environment has evolved at the expense of poorer performance in another environment is a significant avenue for forecasting and managing climate change responses over longer timescales (see discussion in Angilletta, 2009; Gilchrist, 1995).

*Heterorhabditis zealandica* and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler and Adams, 2005 have demonstrated high virulence against a range of pest insect species in South Africa, including the mealybugs, *Planococcus ficus* (Signoret) and *P. citri* (Risso) (Hemiptera: Pseudococcidae) (Van Niekerk & Malan, 2012; Le Vieux & Malan, 2013) and tortricid moths (De Waal et al., 2011; Malan et al., 2011). *Heterorhabditis zealandica* was originally described in New Zealand, and though it is not a common species, it has been reported from the USA, Lithuania, Russia, Australia and South Africa (see Malan et al., 2006). *Steinernema yirgalemense* was first described from Ethiopia (Nguyen et al., 2004), and is highly prevalent there (Mekete et al., 2005), and also found in Kenya (Mekete et al., 2005) with only one isolate from South Africa (Malan et al., 2011). Importantly, these two species have demonstrated higher efficacy in host mortality than other EPN species and thus provide two potential candidates for ongoing pest management programmes (Van Niekerk & Malan, 2012). There are however, important differences between the species that require further investigation. *Steinernema yirgalemense* is two times more tolerant to low levels of free water than *H. zealandica* and has been demonstrated to detect and infect *P. citri* hosts quicker (Van Niekerk & Malan, 2012). *Heterorhabditis zealandica* and *S. yirgalemense* may therefore have contrasting thermal tolerance profiles.
In this paper we aim to characterise and compare the lethal upper and lower temperatures for *H. zealandica* and *S. yirgalemense* by using an accurately controlled thermal stage. We extend on estimating the thermal niche breadth and examine whether short term induced thermal acclimation is able to alter basal thermal resistance in these EPN species and discuss potential evolutionary trade-offs.

**Materials & Methods**

**Codling moth cultures**

Recently the virulence of *H. zealandica* and *S. yirgalemense* on codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) was demonstrated to result in above 90% mortality and rapid EPN development time, thus providing a suitable *in vivo* host for these EPN species (De Waal et al. 2011). We obtained *C. pomonella* eggs and diet from Entomon Technologies (Pty) Ltd, Stellenbosch, Western Cape province, South Africa. After hatching, *C. pomonella* larvae were reared on this diet under diapausing conditions in temperature controlled cabinets (10:14h L:D photoperiod, 25°C, 60% RH).

**Nematode cultures**

We used IJ of *H. zealandica* and *S. yirgalemense* (Malan *et al*., 2006; 2011) from Stellenbosch University stocks (isolates SF41 and 157-C respectively). Both of these species were originally collected within South Africa (SF41 from Patensie, Eastern Cape and 157-C from Friedenheim, Mpumalanga [Malan *et al*. 2006; 2011]). Fifth instar *C. pomonella* larvae were used as host for culturing IJ of the two EPNs species. Development period was held at 25°C and lasted for around 10 days. Emerging IJ were collected over a 3 day period using modified White traps (White, 1927) and stored at 14°C in distilled water, in horizontal placed, vented culture flasks, until use. We quantified the IJs to 100 individuals per 50 µl prior to assays (Navon & Ascher, 2000). The experiments were repeated with fresh batches of recycled nematodes, in an attempt to control for any cohort effects (see Supplementary Material 1).

**Experimental setup**

All temperature survival assays were performed using the stage design of Hill *et al*. (2013). This was originally used to measure critical thermal limits of mites, but was suited to temperature assays of EPNs due to being an open-well design. Briefly, this stage is an aluminium double-jacketed block (100 mm * 100 mm * 10 mm) with 19 wells (5 mm diameter). The wells are arranged in a circle and the middle well was used as a control, with a thermocouple (type K) secured in place. A perspex lid covers this stage so that each well is kept sealed during experimentation. Samples can be easily pipetted to and from each well and thoroughly rinsed between replicates to prevent contamination.
This stage was connected to a thermoregulator controller and waterbath (Huber cc410wl) filled with either ethanol for low temperature assays or 50:50 propylene glycol:water for high temperature assays. The temperature controlled fluid is pumped through channels in this block and allows for accurate temperature control, and verified independently.

Upper Lethal Temperatures

As the number of nematodes taken in each pipette draw was quite variable we took 100 µl of each quantified sample and nematode samples where then transferred into individual wells in the thermal stage. We used two replicates of each species for each different temperature assay.

The initial temperature was set and held for 5 min at 25°C before being quickly ramped up at 0.5°C min⁻¹ to the set high temperature. After being held at this temperature for 60 min, the temperature was ramped back down to 25°C at 0.5°C min⁻¹. While this rate is different from temperature change experienced in the field in this region (e.g. Nyamukondiwa & Terblanche, 2010), it still allows for physiological differences between species to be contrasted, and has been employed for characterising nematode tolerances elsewhere (e.g. Ali & Wharton, 2013; 2014), thus maximising possibility of comparison between studies. While this was still relatively quick, ramping assays are likely to be more ecologically relevant than static assays, especially in capturing elements of the daily thermal cycles and whether these may pose survival limits (Terblanche et al., 2011).

Temperature was recorded every second during the run using a PicoLog TC-08 USB datalogger and PicoLog software. We took the observed temperature to be the average temperature for the period between 3000 and 4000 seconds as this allowed for stabilization of the waterbath away from ramping temperature times.

Following assays each sample was drawn from the well and placed in a 500 µl Eppendorf tube, together with 0.025g/100mL Meldola’s blue dye to stain dead cells (Ogiga & Estey, 1974). Nematode survival was scored 24 hours post assay with the aid of a dissection microscope (Leica MZ7s). By using a combination of dye penetration, mechanical stimulus (probing with dissection needle) and nematode shape it was possible to record mortality with a high level of accuracy.

Lower Lethal Temperature

We conducted these assays as we did for upper lethal temperatures, but with a few changes. Experiments were started at 5°C, held at this temperature for 5 min and then ramped down at 0.5°C min⁻¹ to the different set temperatures. While other studies have required seeded freezing of samples (e.g. Ali & Warton 2014; Shapiro-Ilan et al., 2014), our stage design allows for freezing to occur passively, instead of supercooling taking place. Freezing was observed to occur in the wells through detection of an exothermic
release on the temperature logs. The temperature was then raised back up at the same rate to 5°C before
the nematodes taken to be held at 25°C for 24 hours prior to scoring survival. Observed temperature was
calculated as the average between 1000 and 2000 seconds from recordings of the thermocouple in middle
well. Survival was scored as for the upper lethal temperature assays.

Acclimation

To investigate the effects of induced acclimation on EPNs we placed samples of both species into different
constant temperature incubators (and cold rooms) and held for 24 hours prior to experimentation (Ali &
Wharton, 2013). We held samples at 5, 20 and 30°C and included two replicates of each acclimation
treatment with two control temperature samples (25°C) for both species. For the acclimation treatments,
we selected a number of temperatures appropriate to each species based on results from upper lethal
temperature (ULT) and lower lethal temperature (LLT) experiments. We also added the control replicate
data from these acclimation experiments back to our ULT and LLT datasets to increase their sample size.

Statistical analysis

All statistical analyses were performed in R (version 3.1.2; R core team, 2014). For the upper and lower
lethal temperature experiments, without acclimation treatments, we modelled percentage survival as a
function of observed temperature for both species separately using generalised linear models (glm())
function with a binomial distribution and a logit link function. We tested for over-dispersion using the
dispmod (version 1.1) package and then rescaled deviance to 1 when necessary. We then calculated LT_{10},
LT_{50} and LT_{90} temperature profiles for each species using the dose.p function in the MASS package.

For the acclimation experiments we again considered each species separately and performed generalised
linear models using binomial distributions and logit link function. In this case we used set temperature
rather than observed temperature so that we could examine acclimation between cohorts, and then added
acclimation treatment as an additional effect and an interaction term between acclimation and set
temperature. Temperature and acclimation treatments were used as categorical variables and then Wald’s
$\chi^2$ test in the “arm” package was used to test the significance of these predictors.
Results

*Heterorhabditis zealandica* and *S. yirgalemense* displayed contrasting lethal temperature responses (Fig. 1). Overall, *S. yirgalemense* displayed greater survival at higher temperatures than *H. zealandica* (*p* < 0.001). For lower lethal temperatures, this pattern was reversed: *H. zealandica* had higher survival at low temperatures than *S. yirgalemense* (*p* < 0.001). These differences are also reflected in lethal temperature values derived from the generalized linear models (Table 1). The predicted curves for survival of the two species show distinct and contrasting responses to upper and lower lethal temperatures (Fig. 1).

For both species, acclimation of thermal limits in both species resulted in both negative and positive responses to experimental temperatures as seen in both lower and higher survival limits at different temperatures (Fig. 2). There was considerable variation between acclimation treatments and the overall effect of acclimation and the interaction with temperature was non-significant (Table 2). For *H. zealandica* ULT performance was increased by the 30°C acclimation treatment, especially at the 39°C test temperature, although this was not significant. There was no increase in survival at 41°C test temperatures from any of the acclimation treatments. For *S. yirgalemense*, the 30°C acclimation increased survival at 41°C and even slightly at 43°C, although this again was not significant.

For lower lethal temperatures, *H. zealandica* individuals acclimated at 5°C have decreased performance until the experimental temperatures dropped below -12.5°C, when this acclimation treatment gave increased survival down to -15°C (significantly so from 20 and 30°C acclimations; *p* = 0.014 and 0.011, respectively). Acclimations treatments of 5 and 30°C were significantly different from one another for *H. zealandica* (*p* = 0.013) and this was driven by the differences at -7 and -13°C (*p* = 0.004 and *p* = 0.011, respectively). At the -7°C test temperature the 5°C acclimation treatment yielded significantly decreased survival from the 20 and 30°C acclimation treatments (*p* = 0.025 and *p* = 0.004, respectively) and marginally significant (*p* = 0.052) from the 25°C acclimation treatment. At the -13°C test temperature, this trend was reversed and the 5°C acclimation treatment gave increased survival over the other treatments, though only significantly so from the 20°C and 30°C acclimation treatments (*p* = 0.014 and *p* = 0.011, respectively). While there was insufficient data to test for acclimation and LLT for *S. yirgalemense*, there appears to be a well-defined threshold of survival around -5°C, with temperatures below this resulting in complete mortality and acclimation treatments unable to elicit a shift this threshold.

In the replication of acclimation experiments we observed some significant differences between experiments (Supplementary Material 1). Particularly for *H. zealandica* ULTs, the second cohort displayed significantly higher survival at 37°C (*p* = <0.001; Supplementary Material 1). Likewise, at 41°C for *S. yirgalemense*, the first cohort was significantly different from the second (*p* = <0.021) and the acclimation response was also more evident in the first experiments (Supplementary Material 1). For *H. zealandica* LLTs the cohorts were significantly different at -5, -7 and -13°C (*p* = 0.002, 0.39 and <0.001 respectively).
While these differences in response between the experimental cohorts were significant, the pattern of interaction between acclimation and temperature was largely preserved within both cohorts and on the combined data of these experiments (Supplementary Material 1).

Discussion

Our results here demonstrate contrasting thermal tolerance profiles for two EPN species and support findings that thermal tolerance varies widely across different EPN species (e.g. Morton & García-del-Pino, 2009; Shapiro-Ilan et al., 2014). The two contrasting thermal tolerance curves were obtained on isolates that have been kept under the same laboratory conditions for many generations (Malan et al., 2006; 2011) and thus the differences observed here are likely to be due to fixed genetic differences rather than recent environmental influences (i.e. acclimatization). However, inadvertent directional selection during laboratory propagation may have influenced the estimates of thermal tolerance for these species (Grewal et al., 1996). The two species are also from separate monogeneric families within the Rhabditida and have thus have had disparate evolutionary histories. It has been proposed that Heterorhabditis species are endemic to warmer climates, whilst Steinernema species more common in temperate regions (see Grewal et al., 1994), however our estimates of tolerances from the laboratory-propagated lines did not reflect this. Further studies on field collected EPNs are therefore required to examine if patterns of thermal resistance are consistently different between the two families in line with biogeographical hypotheses.

While the overall effects of acclimation were complex, both species exhibited some improved survival after being acclimated at 30°C for high temperature assays, and consequently, do not support evidence of a direct trade-off between plastic and basal ULT. Previous studies in Drosophila have identified tradeoffs between basal low-temperature tolerance and acute low-temperature plasticity, but at high temperatures increased basal tolerance was accompanied by increased plasticity (Nyamukondiwa et al., 2011). However, thermal acclimation may provide other costs and benefits to performance, such as increased resistance to desiccation or reduced fecundity or longevity, which were not the focus of investigation here (Kleynhans et al., 2014; Terblanche, 2014).

Unlike most EPN species, which are freeze tolerant, H. zealandica has been shown to exhibit a freeze avoidance strategy, enabled through a protective sheath (Wharton & Surrey, 1994), and cold acclimation has been found to decrease freezing survival in H. zealandica (Surrey, 1996). We also found evidence for acclimation to decrease survival at some low temperatures on the isolate used: however, at the lowest temperatures the 5°C acclimation treatment actually increased survival. In addition to the presence or absence of a protective sheath which greatly influences H. zealandica survival at low temperatures (Wharton & Surrey, 1994), variation in results may also arise from differences in how acclimation and freezing are implemented into experiments, as substantial variation in results of acclimation experiments appear to be common for EPNs (Ali & Wharton, 2013). Differences from previous studies may also be
related to our thermal stage design and reliance on freezing to occur spontaneously, as opposed to samples
being seeded (inoculated) with ice crystals in other studies (e.g. Ali & Wharton, 2013). This is likely to
introduce further variation and have implications for species comparisons, especially between freeze
tolerant and freeze avoidance strategies that require cryoprotective dehydration to occur (Brown &
Gaugler, 1998; Ali & Wharton, 2013). For instance, Ali & Wharton (2013) suggest that how long IJs are
exposed to the temperature of ice nucleation has implications on survival of freeze-tolerant species such as
*S. feltiae*. This is something that should be considered in broader comparisons of cold tolerance across
EPN species.

The differences between the cohorts in the replication of our acclimation experiments could be due to a
number of factors. Given that nematodes are propagated under controlled conditions, the most likely
sources of variation are perhaps differences in age of the nematodes on a given experimental day and/or
variation in host health and nutritional content, e.g. lipid composition (Grunder & Jurg, 2005) which may
have a marked effect on plasticity and basal tolerance, especially if linked to IJ ability to synthesise heat
shock proteins and cryoprotectants such as trehalose and glycerol (Grewal & Jagdale, 2002; Jagdale *et al*.,
2005). Observer bias is also unlikely as a consistent directional influence since observers were well
trained and produced inconsistent differences/similarities in direction of effects on the same treatment (see
e.g. discussions in Terblanche *et al*., 2011; Castaneda *et al*., 2012; Blackburn *et al*., 2014). The cultures are
maintained in a highly stable environment so environmental differences in terms of temperature and
humidity are unlikely. And while we only conducted experiments on IJs that were less than 2 weeks old,
the age of the IJ EPNs prior to experiment should be further investigated. We attempted to use all EPNs at
the same time, but our experiment setup is limited to one stage and thus we are required to run our
experiments in series, spanning over two weeks to complete them all. Time post emergence may be an
important factor in thermal stress resistance.

Thermal tolerance of EPN species plays a key role in components of its life history, including mobility of
the IJ, infection, development and reproduction (Grewal *et al*., 1994; Morton & García-del-Pino, 2009;
Salame *et al*., 2012; Shapiro-Ilan *et al*., 2014). These results encourage for more EPN species to be
characterized using these methods to better understand thermal tolerance in these nematodes and how this
relates to biogeographical patterns as well as the evolution of thermal resistance in this group.

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Table 1. GLM/ logit model predictions for 10, 50 and 90% survival of the population (i.e. LT<sub>90</sub>, LT<sub>50</sub>, LT<sub>10</sub>) of the two EPN species at upper and lower temperatures. LT = lethal temperature, SE = standard error. All values reported are for nematodes kept at 25°C.

<table>
<thead>
<tr>
<th>% Survival</th>
<th>Upper Lethal Temperatures (Deg C)</th>
<th>Lower Lethal Temperatures (units)</th>
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<tbody>
<tr>
<td></td>
<td>H. zealandica</td>
<td>S. yirgalemense</td>
</tr>
<tr>
<td></td>
<td>LT</td>
<td>SE</td>
</tr>
<tr>
<td>10</td>
<td>38.2</td>
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<tr>
<td>50</td>
<td>36.7</td>
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<td>90</td>
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<td>50</td>
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<tr>
<td>90</td>
<td>-5.4</td>
<td>0.5</td>
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Table 2. Upper lethal limit – Wald’s χ<sup>2</sup> test with categorical acclimation and set temperature variables. The generalized linear model used a binomial distribution for survival data with a logit link function and deviance was rescaled to 1.

### H. zealandica

<table>
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<tr>
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### S. yirgalemense

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<td>Acclimation x Temp</td>
<td>4.8</td>
<td>11</td>
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Table 3. Lower lethal limit – Wald’s $\chi^2$ test with categorical acclimation and set temperature variables. The generalized linear model used a binomial distribution for survival data with a logit link function and deviance was rescaled to 1. Test not possible to be performed on S. yirgalemense LLT data.

**H. zealandica**

<table>
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<th></th>
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Figure 1. Thermal performance curves for two entomopathogenic nematodes. (a) Lower lethal temperatures (LLTs) as a function of percentage survival(0-1). Curves glm logit model fits. Green is *Heterorhabditis zealandica*, orange is *Steinernema yirgalemense*. (b) Upper lethal temperatures (ULTs) as a function of percentage survival(/10). Curves represent glm logit model fits. Green is *Heterorhabditis zealandica*, orange is *Steinernema yirgalemense*. 
Figure 2. Acclimation and lethal thermal tolerances for two species of entomopathogenic nematodes, *Heterorhabditis zealandica* and *Steinernema yirgalemense*. Data represents both batches of experiments pooled together. Error bars reflect ±1 standard error. (a) Upper lethal temperatures and acclimation for *H. zealandica*. (b) Upper lethal temperatures and acclimation for *S. yirgalemense*. (c) Lower lethal temperatures and acclimation for *H. zealandica*. (d) Lower lethal temperatures and acclimation for *S. yirgalemense*. 