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.

2   **Divergent thermal specialisation of two South African entomopathogenic nematodes**

3   Matthew P. Hill[1], Antoinette P. Malan[2] & John S. Terblanche[1]

4   [1]DST-NRF Centre of Excellence for Invasion Biology, Department of Conservation Ecology and

5   Entomology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, South

6   Africa

7   [2]Department of Conservation Ecology and Entomology, Faculty of AgriSciences, Stellenbosch

8   University, Private Bag X1, Matieland 7602, South Africa

9   email: hillmp@sun.ac.za

10   **Abstract**

11   Thermal physiology of entomopathogenic nematodes (EPN) is a critical aspect of field performance and

12   fitness. Thermal limits for survival and activity, and the ability of these limits to adjust (i.e. show

13   phenotypic flexibility) depending on recent thermal history, are generally poorly established, especially

14   for non-model nematode species. Here we report the acute thermal limits for survival, and the thermal

15   acclimation-related plasticity thereof for two key endemic South African EPN species, *Steinernema*

16   *yirgalemense* and *Heterorhabditis zealandica*. Results including LT_{50} indicate *S. yirgalemense* (LT_{50} =

17   40.8±0.3°C) has greater high temperature tolerance than *H. zealandica* (LT_{50} = 36.7±0.2°C), but *S.*

18   *yirgalemense* (LT_{50} = -2.4±0°C) has poorer low temperature tolerance in comparison to *H. zealandica*

19   (LT_{50} = -9.7±0.3°C), suggesting these two EPN species occupy divergent thermal niches to one another.

20   Acclimation had both negative and positive effects on temperature stress survival of both species,

21   although the overall variation meant that many of these effects were non-significant. There was no

22   indication of a consistent loss of plasticity with improved basal thermal tolerance for either species at

23   upper lethal temperatures. At lower temperatures measured for *H. zealandica*, the 5°C acclimation

24   lowered survival until below -12.5°C, where after it increased survival. Such results indicate that the

25   thermal niche breadth of EPN species can differ significantly depending on recent thermal conditions, and

26   should be characterized across a broad range of species to understand the evolution of thermal limits to

27   performance and survival in this group.

28

29

Introduction

30

31 Temperature plays a key role in both the survival and activity of terrestrial invertebrates. Thermal

32 tolerance may be characterized through traits such as thermal maxima and minima (absolute limits),

33 processes or rates (e.g. development), as well as the optima thereof (e.g. temperatures for which growth

34 rate and reproduction are maximised). Such basal thermal tolerance of terrestrial invertebrate species may

35 also be adjusted through plastic responses induced through acclimation, hardening or acclimatization,

36 allowing for phenotypic flexibility in the species relationship to temperature (Chown & Terblanche, 2006;

37 Angilletta, 2009). Plasticity may thus allow for environmental extremes to be buffered against, although

38 sometimes this comes with a trade-off with basal thermal tolerance (Calosi *et al*., 2008; Nyamukondiwa

39 *et al*., 2011). Information on both basal thermal tolerance and the plasticity thereof can be compared to

40 give insight into hierarchical levels of, and the magnitude and direction of, variation that may exist

41 between species and across different groups (e.g. Hoffmann *et al*., 2013; Faulkner *et al*., 2014), and thus

42 constraints or trade-offs that may be significant for understanding adaptive evolution.

43 Entomopathogenic nematodes (EPNs) are soil inhabiting insect parasites that fall within two monogeneric

44 families: Heterorhabditidae and Steinernematidae (Rhabditida) (Hunt, 2007). The families do not share a

45 common ancestry, but have nonetheless developed a similar lifestyle (Blaxter *et al*., 1998).

46 Heterorhabditids and steinernematids each have corresponding mutualistic bacteria in the genera

47 *Photorhabdus* and *Xenorhabdus*, respectively. These bacteria are held within the nematode intestine and

48 released once the infective juvenile (IJ) has penetrated the insect host through the natural openings

49 (spiracles, mouth or anus) (Griffin *et al*., 2005). The bacteria suppress the host's immune system,

50 typically killing the host within 24-48 h, subsequently providing nutrition for the nematodes and their

51 offspring (Gaugler *et al*., 1997; Griffin *et al*., 2005). The EPNs are then able to complete multiple (e.g. up

52 to three) generations within the insect cadaver (depending on the size of the host) before releasing a new

53 cohort of IJs to start a new cycle (Gaugler *et al*., 1997). This life history strategy has allowed for the

54 development of different biocontrol programmes using soil and aerial application of the IJs on agricultural

55 pest insects (Lacey & Georgis, 2012).

56 There appears to be a wide range of responses to environmental stress exhibited across EPN species and

57 strains, including variation in desiccation tolerance and hypoxia tolerance, and freeze tolerance (Morton

58 & García-del-Pino, 2009; Salame *et al*., 2012; Shapiro-Ilan *et al*., 2014). Some of the variation exhibited

59 for cold tolerance is most likely attributed to different adaptive strategies including cryoprotective

60 dehydration, anhydrobiosis and freeze avoidance or tolerance strategies (Wharton, 2011), although the

61 mechanisms underlying cold tolerance responses are typically the primary focus of investigation (e.g. Ali

62   & Wharton, 2014). While EPNs are able to withstand adverse environmental conditions inside their host

63   cadavers, the IJ stage is required to seek out new hosts and thus may be particularly vulnerable to

64   environmental variability in this life-stage (Brown & Gaughler, 1997). Much of the work looking at

65   thermal performance of EPNs has focused on cold tolerance and how this relates to long-term cold

66   storage solutions, or overwinter survival of free-living nematodes in polar environments (Perry and

67   Wharton, 2011), although there have been assessments of heat tolerance in EPNs (e.g Shaprio *et al*.,

68   1996; Jagdale & Gordon, 1998).

69   In addition to variation in basal thermal tolerance, acclimation-related adjustments in tolerance may be

70   substantial in nematodes. For example, higher temperatures experienced during propagation increase

71   upper thermal limits, while diminishing lower thermal ones (Grewal *et al*. 1996; Shaprio *et al*., 1996;

72   Jagdale & Gordon, 1998). Further, short term exposure to lower temperatures prior to freezing has been

73   shown to enhance freezing survival (Ali & Wharton, 2012). Previous EPN cold tolerance work has

74   included findings that *Heterorhabditis bacteriophora* Poinar, 1976 and *Steinernema feltiae* (Filipjev,

75   1934) Wouts, Mráček, Gerdin & Bedding, 1982 (Rhabditida: Heterorhabditidae) have demonstrated an

76   increase in freezing tolerance after acclimation to temperatures below their propogation temperature

77   (Brown & Gaugler, 1996; Ali & Wharton, 2012). Thus, testing thermal tolerances and associated

78   plasticity or physiological adjustments in response to propagation or short-term temperature treatments to

79   estimate thermal niche breadth across EPN species should help identify potential trade-offs between basal

80   and acute responses. From an evolutionary perspective, understanding whether specialization to a

81   particular environment has evolved at the expense of poorer performance in another environment is a

82   significant avenue for forecasting and managing climate change responses over longer timescales (see

83   discussion in Angilletta, 2009; Gilchrist, 1995).

84   *Heterorhabditis zealandica* and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler and Adams,

85   2005 have demonstrated high virulence against a range of pest insect species in South Africa, including

86   the mealybugs, *Planococcus ficus* (Signoret) and *P. citri* (Risso) (Hemiptera: Pseudococcidae) (Van

87   Niekerk & Malan, 2012; Le Vieux & Malan, 2013) and tortricid moths (De Waal *et al*., 2011; Malan *et*

88   *al*., 2011). *Heterorhabditis zealandica* was originally described in New Zealand, and though it is not a

89   common species, it has been reported from the USA, Lithuania, Russia, Australia and South Africa (see

90   Malan *et al*., 2006). *Steinernema yirgalemense* was first described from Ethiopia (Nguyen *et al*., 2004),

91   and is highly prevalent there (Mekete *et al*., 2005), and also found in Kenya (Mekete *et al*., 2005) with

92   only one isolate from South Africa (Malan *et al*., 2011). Importantly, these two species have

93   demonstrated higher efficacy in host mortality than other EPN species and thus provide two potential

94   candidates for ongoing pest management programmes (Van Niekerk & Malan, 2012). There are however,

95   important differences between the species that require further investigation. *Steinernema yirgalemense* is

96   two times more tolerant to low levels of free water than *H. zealandica* and has been demonstrated to

97   detect and infect *P. citri* hosts quicker (Van Niekerk & Malan, 2012). *Heterorhabditis zealandica* and *S.*

98   *yirgalemense* may therefore have contrasting thermal tolerance profiles.

99   In this paper we aim to characterise and compare the lethal upper and lower temperatures for *H.*

100   *zealandica* and *S. yirgalemense* by using an accurately controlled thermal stage. We extend on estimating

101   the thermal niche breadth and examine whether short term induced thermal acclimation is able to alter

102   basal thermal resistance in these EPN species and discuss potential evolutionary trade-offs.

103   **Materials & Methods**

104   *Codling moth cultures*

105   Recently the virulence of *H. zealandica* and *S. yirgalemense* on codling moth Cydia pomonella L.

106   (Lepidoptera: Tortricidae) was demonstrated to result in above 90% mortality and rapid EPN

107   development time, thus providing a suitable in vivo host for these EPN species (De Waal et al. 2011). We

108   obtained *C. pomonella* eggs and diet from Entomon Technologies (Pty) Ltd, Stellenbosch, Western Cape

109   province, South Africa. The codling moth diet consists of agar, carrageenan, yeast, wheat germ, brown

110   bread flower, ascorbic acid, benzoic acid, nipagin and formalin (Stenekamp, 2011). After hatching, *C.*

111   *pomonella* larvae were reared on this diet under diapausing conditions in temperature controlled cabinets

112   (10:14h L:D photoperiod, 25°C, 60% RH).

113   *Nematode cultures*

114   We used IJ of *H. zealandica* and *S. yirgalemense* (Malan *et al.*, 2006; 2011) from Stellenbosch University

115   stocks (isolates SF41 and 157-C respectively). Both of these species were originally collected within

116   South Africa (SF41 from Patensie, Eastern Cape and 157-C from Friedenheim, Mpumalanga [Malan et al.

117   2006; 2011]). Fifth instar *C. pomonella* larvae were used as host for culturing IJ of the two EPNs species.

118   Development period was held at 25°C and lasted for around 10 days. Emerging IJ were collected over a 3

119   day period using modified White traps (White, 1927) and stored at 14°C in distilled water, in horizontally

120   placed, vented culture flasks, until use. We quantified the IJs to 100 individuals per 50 µl prior to assays

121   (Navon & Ascher, 2000). The experiments were repeated with fresh batches of recycled nematodes, in an

122   attempt to control for any cohort effects (see Supplementary Material 1).

123   *Experimental setup*

124 All temperature survival assays were performed using the stage design of Hill *et al*. (2013). This was
125 originally used to measure critical thermal limits of mites, but was suited to temperature assays of EPNs
126 due to being an open-well design. Briefly, this stage is an aluminium double-jacketed block (100 mm *
127 100 mm * 10 mm) with 19 wells (5 mm diameter). The wells are arranged in a circle and the middle well
128 was used as a control, with a thermocouple (type K) secured in place. A perspex lid covers this stage so
129 that each well is kept sealed during experimentation. Samples can be easily pipetted to and from each well
130 and thoroughly rinsed between replicates to prevent contamination.

131 This stage was connected to a thermoregulator controller and waterbath (Huber cc410wl) filled with
132 either ethanol for low temperature assays or 50:50 propylene glycol:water for high temperature assays.
133 The temperature controlled fluid was pumped through channels in this block and allows for accurate
134 temperature control, and verified independently.

135 *Upper Lethal Temperatures*

136 As the number of nematodes taken in each pipette draw was quite variable, we took 100 µl of each
137 quantified sample to ensure a minimum number of individuals ($n > 50$) and then nematode samples were
138 transferred into individual wells in the thermal stage. We used two replicates of each species for each
139 different temperature assay.

140 The initial temperature was set and held for 5 min at 25°C before being quickly ramped up at 0.5°C min^{-1}
141 to the set high temperature (set at: 35, 37, 39, 41, 43°C). After being held at this temperature for 60 min,
142 the temperature was ramped back down to 25°C at 0.5°C min^{-1}.While this rate is different from
143 temperature change experienced in the field (for the temperate regions of collection for these EPN
144 species, e.g. Nyamukondiwa & Terblanche, 2010), it still allows for physiological differences between
145 species to be contrasted, and has been employed for characterising nematode tolerances elsewhere (e.g.
146 Ali & Wharton, 2013; 2014), thus maximising possibility of comparison between studies. While this was
147 still relatively quick, ramping assays are likely to be more ecologically relevant than static assays,
148 especially in capturing elements of the daily thermal cycles and whether these may pose survival limits
149 (Terblanche *et al*., 2011).

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

154 Following assays each sample was drawn from the well and placed in a 500 µl Eppendorf tube, together

155 with 0.025g/100mL Meldola's blue dye to stain dead cells (Ogiga & Estey, 1974). Nematode survival

156 was scored 24 hours post assay with the aid of a dissection microscope (Leica MZ7s). By using a

157 combination of dye penetration, mechanical stimulus (probing with dissection needle) and nematode

158 shape it was possible to record mortality with a high level of accuracy.

159 *Lower Lethal Temperature*

160 We conducted these assays as we did for upper lethal temperatures, but with a few changes. Experiments

161 were started at 5°C, held at this temperature for 5 min and then ramped down at 0.5°C min⁻¹ to the

162 different set temperatures (set at: -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15°C). While other studies have

163 required seeded freezing of samples (e.g. Ali & Wharton 2014; Shapiro-Ilan *et al.*, 2014), our stage

164 design allows for freezing to occur passively, instead of supercooling taking place. Freezing was observed

165 to occur in the wells through detection of an exothermic release on the temperature recordings. The

166 temperature was then raised back up at the same rate to 5°C before the nematodes taken to be held at

167 25°C for 24 hours prior to scoring survival. Observed temperature was calculated as the average between

168 1000 and 2000 seconds from recordings of the thermocouple in the middle well. Survival was scored as

169 for the upper lethal temperature assays.

170 *Acclimation*

171 To investigate the effects of induced acclimation on EPNs we placed samples of both species into

172 different constant temperature incubators (and cold rooms) and held for 24 hours prior to

173 experimentation. In previous studies examining thermal tolerance in EPN species, it was found that

174 periods of time ranging from a few hours through to two days was sufficient to induce an acute effect of

175 acclimation, hence we chose a period of 24 hours (see discussion). We held samples at 5, 20 and 30°C

176 and included two replicates of each acclimation treatment with two control temperature samples (25°C)

177 for both species. For the acclimation treatments, we selected a number of temperatures appropriate to

178 each species based on results from upper lethal temperature (ULT) and lower lethal temperature (LLT)

179 experiments (*H. zealandica*: LLT: -5, -6, -7, -13, -14, -15°C, ULT: 35, 37, 39°C; *S. yirgalemense*: LLT: -

180 5, -6°C, ULT: 35, 37, 39, 41, 43°C). We also added the control replicate data from these acclimation

181 experiments back to our ULT and LLT datasets to increase their sample size.

182 *Statistical analysis*

183 All statistical analyses were performed in R (version 3.1.2; R core team, 2014). For the upper and lower

184 lethal temperature experiments, without acclimation treatments, we modelled percentage survival as a

185 function of observed temperature for both species separately using generalised linear models

186 (glmfunction) with a binomial distribution and a logit link function. We tested for over-dispersion using

187 the dispmod (version 1.1) package and then rescaled deviance to 1 when necessary. We then calculated

188 LT_{10}, LT_{50} and LT_{90} temperature profiles for each species using the dose.p function in the MASS package.

189 For the acclimation experiments we again considered each species separately and performed generalised

190 linear models using binomial distributions and logit link function. In this case we used set temperature

191 rather than observed temperature so that we could examine acclimation between cohorts, and then added

192 acclimation treatment as an additional effect and an interaction term between acclimation and set

193 temperature. Temperature and acclimation treatments were used as categorical variables and then Wald's

194 χ^2 test in the "arm" package was used to test the significance of these predictors.

195 **Results**

196 *Heterorhabditis zealandica* and *S. yirgalemense* displayed contrasting lethal temperature responses (Fig.

197 1). Overall, *S. yirgalemense* displayed greater survival at higher temperatures than *H. zealandica*

198 (simplified Wald $z = -4.48$, $p < 0.001$) (Table 1). For lower lethal temperatures, this pattern was reversed:

199 *H. zealandica* had higher survival at low temperatures than *S. yirgalemense* (simplified Wald $z = 7.99$, p

200 < 0.001) (Table 1). These differences are also reflected in thelethal temperature values predicted by the

201 generalized linear models (Table 2). The predicted curves for survival of the two species show distinct

202 and contrasting responses to upper and lower lethal temperatures (Fig. 1).

203 For both species, acclimation of thermal limits in both species resulted in both negative and positive

204 responses to experimental temperatures as seen in both lower and higher survival limits at different

205 temperatures (Fig. 2). There was considerable variation between acclimation treatments, and the overall

206 effect of acclimation and the interaction with temperature was non-significant (Table 3). For *H.*

207 *zealandica* ULT performance was increased by the 30°C acclimation treatment, especially at the 39°C test

208 temperature, although this was not significant. There was no increase in survival at 41°C test temperatures

209 from any of the acclimation treatments. For *S. yirgalemense*, the 30°C acclimation increased survival at

210 41°C and even slightly at 43°C, although this again was not significant.

211 For lower lethal temperatures, *H. zealandica* individuals acclimated at 5°C have decreased performance

212 until the experimental temperatures dropped below -12.5°C, when this acclimation treatment gave

213 increased survival down to -15°C (significantly so from 20 and 30°C acclimations; $\chi^2{}_1 = 6.0$, $p = 0.014$

214 and $\chi^2{}_1 = 6.4$, $p = 0.011$, respectively). Acclimations treatments of 5 and 30°C were significantly different

215 from one another for *H. zealandica* ($\chi^2{}_1 = 6.2$, $p = 0.013$) and this was driven by the differences at -7 and

216   -13°C ($\chi^2_1 = 8.3$, $p = 0.004$ and $\chi^2_1 = 6.2$, $p = 0.011$, respectively), displaying both decreased and

217   increased survival, respectively. This pattern was also observed for the 5 and 20°C acclimation

218   treatments, at the -7°C test temperature the 5°C acclimation treatment displayed significantly decreased

219   survival from the 20 and ($\chi^2_1 = 5.0$, $p = 0.025$) and then atthe -13°C test temperature, the 5°C

220   acclimation treatment again gave significantly increased survival over the 20°C treatment ($\chi^2_1 = 6.0$, $p =$

221   0.014) While there was insufficient data to test for acclimation and LLT for *S. yirgalemense*, there

222   appears to be a well-defined threshold of survival around -5°C, with temperatures below this resulting in

223   complete mortality and acclimation treatments unable to elicit a shift this threshold.

224   In the replication of acclimation experiments we observed some significant differences between

225   experiments (Supplementary Material 1). Particularly for *H. zealandica* ULTs, the second cohort

226   displayed significantly higher survival at 37°C ($\chi^2_1 = 25.6$, $p = $ <0.001; Supplementary Material 1).

227   Likewise, at 41°C for *S. yirgalemense*, the first cohort was significantly different from the second ($\chi^2_1 = $

228   5.3, $p = $ <0.021) and the acclimation response was also more evident in the first experiments

229   (Supplementary Material 1). For *H. zealandica* LLTs the cohorts were significantly different at -5°C($\chi^2_1 = $

230   9.4, $p = 0.002$), -7°C ($\chi^2_1 = 4.3$, $p = 0.39$) and -13°C ($\chi^2_1 = 10.9$, $p = $<0.001). While these differences in

231   response between the experimental cohorts were significant, the pattern of interaction between

232   acclimation and temperature was largely preserved within both cohorts and on the combined data of these

233   experiments (Supplementary Material 1).

234   **Discussion**

235   Our results here demonstrate contrasting thermal tolerance profiles for two EPN species and support

236   findings that thermal tolerance varies widely across different EPN species (e.g. Morton & García-del-

237   Pino, 2009; Shapiro-Ilan *et al.*, 2014). The two contrasting thermal tolerance curves were obtained on

238   isolates that have been kept under the same laboratory conditions for many generations (Malan *et al.*,

239   2006; 2011) and thus the differences observed here are likely to be due to fixed genetic differences rather

240   than recent environmental influences (i.e. acclimatization). However, inadvertent directional selection

241   during laboratory propagation may have influenced the estimates of thermal tolerance for these species

242   (Grewal *et al.*, 1996). The two species are also from separate monogeneric families within the Rhabditida

243   and have thus have had disparate evolutionary histories. It has been proposed that *Heterorhabditis* species

244   are endemic to warmer climates, whilst *Steinernema* species more common in temperate regions (see

245   Grewal *et al.*, 1994), however our estimates of tolerances from the laboratory-propagated lines did not

246   reflect this. Further studies on field collected EPNs are therefore required to examine if patterns of

247   thermal resistance are consistently different between the two families in line with biogeographical

248   hypotheses.

249   While the overall effects of acclimation were complex, both species exhibited some improved survival

250   after being acclimated at 30°C for high temperature assays, and consequently, do not support evidence of

251   a direct trade-off between plastic and basal ULT. Other EPN studies have identified that acute acclimation

252   provides improved thermal performance for some species (e.g. *S. carpocase* and *S. feltiae*; Jagdale &

253   Grewal, 2003) but not others (e.g. *S. ribrave*; Jagdale & Grewal, 2003), indicating that trade-offs and

254   mechanisms may exist in EPNs. While there is currently little information on trade offs between basal and

255   induced thermal limits in EPN systems, previous studies of *Drosophila* species have identified trade-offs

256   between basal low-temperature tolerance and acute low-temperature plasticity, but at high temperatures

257   increased basal tolerance was accompanied by increased plasticity (Nyamukondiwa *et al*., 2011).

258   However, thermal acclimation may provide other costs and benefits to performance, such as increased

259   resistance to desiccation or reduced fecundity or longevity, which were not the focus of investigation here

260   (Kleynhans *et al*., 2014; Terblanche, 2014). While our period of 24 hours for acclimation may be

261   considered quite short, short-term acute responses to temperature regimes have been documented in

262   EPNs. For instance, a few hours at 35°C has improved both *H. bacteriophora* survival and infectivity at

263   40°C (Selvan *et al*., 1996), one to two days acclimation has improved thermal tolerance in *S. carpocase*

264   and *S. feltiae* (Jagdale & Grewal, 2003), and an overnight freezing treatment prior to LLT characterization

265   has demonstrated improved survival in *S. feltiae* (Ali & Wharton, 2013). As we were examining

266   responses of previously uncharacterized species, we selected a time frame for acclimation that should not

267   overtly stress the organism prior to treatment, but be sufficient to elicit a response. The onset and reversal

268   of acclimation responses are not well understood even in more well-examined groups such as insects

269   (Weldon *et al.,* 2011), thus future tests should explore these responses further in EPNs the range of

270   conditions that elicit acclimation responses and the persistence thereof.

271   Unlike most EPN species, which are freeze tolerant, *H. zealandica* has been shown to exhibit a freeze

272   avoidance strategy, enabled through a protective sheath (Wharton & Surrey, 1994), and cold acclimation

273   has been found to decrease freezing survival in *H. zealandica* (Surrey, 1996). We also found evidence for

274   acclimation to decrease survival at some low temperatures on the isolate used: however, at the lowest

275   temperatures the 5°C acclimation treatment actually increased survival. In addition to the presence or

276   absence of a protective sheath which greatly influences *H. zealandica* survival at low temperatures

277   (Wharton & Surrey, 1994), species that undergo anhydrobiosis require that dehydration is actually

278   possible through differences in water vapour pressure between the surrounding ice and bodily fluids

279   (Holmstrup *et al*. 2002). Anhydrobiosis has evolved as a freeze avoidance strategy in addition to

280   supercooling in many soil invertebrates (Holmstrup *et al*. 2002), and as the IJs were in an aqueous

281   solution, such protective dehydration could probably not occur in the liquid medium we used for our

282   assays, which may have influenced survival results for low temperatures tested here. Variation in results

283   may also arise from differences in how acclimation and freezing are implemented into experiments, as

284   substantial variation in results of acclimation experiments appear to be common for EPNs (Ali &

285   Wharton, 2013). Differences from previous studies may also be related to our thermal stage design and

286   reliance on freezing to occur spontaneously, as opposed to samples being seeded (inoculated) with ice

287   crystals in other studies (e.g. Ali & Wharton, 2013). Ali & Wharton (2013) also suggest that the duration

288   for which IJs are exposed to the temperature of ice nucleation has implications on survival of freeze-

289   tolerant species such as *S. feltiae*. Experimental setup and freeze avoidance strategies are thus both likely

290   to introduce further variation and have implications for species comparisons, especially for species where

291   cryoprotective dehydration needs to occur (Brown & Gaugler, 1998; Ali & Wharton, 2013). This is

292   something that should be considered in broader comparisons of cold tolerance across EPN species.

293   The differences between the cohorts in the replication of our acclimation experiments could be due to a

294   number of factors. Given that nematodes are propagated under controlled conditions, the most likely

295   sources of variation are perhaps differences in age of the nematodes on a given experimental day and/or

296   variation in host health and nutritional content, e.g. lipid composition (Grunder & Jurg, 2005) which may

297   have a marked effect on plasticity and basal tolerance, especially if linked to IJ ability to synthesise heat

298   shock proteins and cryoprotectants such as trehalose and glycerol (Grewal & Jagdale, 2002; Jagdale *et al*.,

299   2005). Observer bias is also unlikely as a consistent directional influence since observers were well

300   trained and produced inconsistent differences/similarities in direction of effects on the same treatment

301   (see e.g. discussions in Terblanche *et al*., 2011; Castaneda *et al*., 2012; Blackburn *et al*., 2014). The

302   cultures are maintained in a highly stable environment so environmental differences in terms of

303   temperature and humidity are unlikely. And while we only conducted experiments on IJs that were less

304   than 2 weeks old, the age of the IJ EPNs prior to experiment should be further investigated. We attempted

305   to use all EPNs at the same time, but our experiment setup is limited to one stage and thus we are required

306   to run our experiments in series, spanning over two weeks to complete them all. Time post emergence

307   may be an important factor in thermal stress resistance.

308   Thermal tolerance of EPN species plays a key role in components of its life history, including mobility of

309   the IJ, infection, development and reproduction (Grewal *et al*., 1994; Morton & García-del-Pino, 2009;

310   Salame *et al.,* 2012; Shapiro-Ilan *et al*., 2014). These results encourage for more EPN species to be

311   characterized using these methods to better understand thermal tolerance in these nematodes and how this

312   relates to biogeographical patterns as well as the evolution of thermal resistance in this group.

313   **Acknowledgements**

314   The authors thank Elizabeth Louw for assisting with experiments. Md Habibullah Bahar and C. Jaco Klok

315   provided useful comments during review of this manuscript.

316   **References**

317

318   Ali, F., & Wharton, D. A. (2013). Cold tolerance abilities of two entomopathogenic nematodes,

319   *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology*, *66*(1), 24-29.

320   Ali, F., & Wharton, D. A. (2014). Intracellular freezing in the infective juveniles of *Steinernema feltiae*:

321   an entomopathogenic nematode. *PloS one*, *9*(4), e94179.

322   Angilletta, M. J. (2009). *Thermal adaptation: a theoretical and empirical synthesis*. Oxford University

323   Press.

324   Blackburn, S., van Heerwaarden, B., Kellermann, V., & Sgrò, C. M. (2014). Evolutionary capacity of

325   upper thermal limits: beyond single trait assessments.*The Journal of Experimental Biology*, *217*(11),

326   1918-1924.

327   Brown, I. M., & Gaugler, R. (1996). Cold tolerance of steinernematid and heterorhabditid nematodes.

328   *Journal of thermal biology*, *21*(2), 115-121.

329   Brown, I. M., & Gaugler, R. (1998). Survival of steinernematid nematodes exposed to freezing. *Journal*

330   *of thermal biology*, *23*(2), 75-80.

331   Calosi, P., Bilton, D. T., & Spicer, J. I. (2008). Thermal tolerance, acclimatory capacity and vulnerability

332   to global climate change. *Biology Letters*, *4*(1), 99-102.

333   Castaneda, L. E., Calabria, G., Betancourt, L. A., Rezende, E. L., & Santos, M. (2012). Measurement

334   error in heat tolerance assays. *Journal of Thermal Biology*, *37*(6), 432-437.

335   Chown, S. L., & Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary

336   contexts. *Advances in insect physiology*, *33*, 50-152.

337   De Waal, J. Y., Malan, A. P., & Addison, M. F. (2011). Efficacy of entomopathogenic nematodes

338   (Rhabditida: Heterorhabditidae and Steinernematidae) against codling moth, *Cydia pomonella*

339   (Lepidoptera: Tortricidae) in temperate regions. *Biocontrol Science and Technology*, *21*(10), 1161-1176.

340   Faulkner, K. T., Clusella-Trullas, S., Peck, L. S., & Chown, S. L. (2014). Lack of coherence in the

341   warming responses of marine crustaceans. *Functional Ecology*, *28*(4), 895-903.

342   Gaugler, R., Lewis, E., & Stuart, R. J. (1997). Ecology in the service of biological control: the case of

343   entomopathogenic nematodes. *Oecologia*,*109*(4), 483-489.

344   Gilchrist, G. W. (1995). Specialists and generalists in changing environments. I. Fitness landscapes of

345   thermal sensitivity. *American Naturalist*, 252-270.

346   Grewal, P. S., & Jagdale, G. B. (2002). Enhanced trehalose accumulation and desiccation survival of

347   entomopathogenic nematodes through cold preacclimation. *Biocontrol Science and Technology*, *12*(5),

348   533-545.

349   Grewal, P. S., Selvan, S., & Gaugler, R. (1994). Thermal adaptation of entomopathogenic nematodes:

350   niche breadth for infection, establishment, and reproduction. *Journal of Thermal Biology*, *19*(4), 245-253.

351   Grewal, P. S., Gaugler, R., & Shupe, C. (1996). Rapid changes in thermal sensitivity of

352   entomopathogenic nematodes in response to selection at temperature extremes. *Journal of invertebrate*

353   *pathology*, *68*(1), 65-73.

354   Griffin, C.T., Boemare, N.E. & Lewis, E.E., (2005). Biology and behaviour. In: Grewal, P.S., Ehlers, R.-

355   U. & Shapiro-Ilan, D.I. (eds). Nematodes as biocontrol agents. CABI Publishing, Wallingford, UK. pp.

356   47 – 75.

357   Grunder, J.M. & Jung, K. (2005). Quality control of entomopathogenic nematodes: proceedings of

358   workshops and meetings held in Merelbeke (Belgium), Maynooth (Ireland), Vienna (Austria) and

359   Wageningen (the Netherlands), 1999-2001. Wädeswil, Switzerland.

360   Hill, M. P., Chown, S. L., & Hoffmann, A. A. (2013). A predicted niche shift corresponds with increased

361   thermal resistance in an invasive mite, *Halotydeus destructor*. *Global Ecology and Biogeography*, *22*(8),

362   942-951.

363   Hoffmann, A. A., Chown, S. L., & Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial

364   ectotherms: how constrained are they?. *Functional Ecology*,*27*(4), 934-949.

365   Holmstrup, M., Bayley, M., & Ramløv, H. (2002). Supercool or dehydrate? An experimental analysis of

366   overwintering strategies in small permeable arctic invertebrates. *Proceedings of the National Academy of*

367   *Sciences*, 99(8), 5716-5720.

368 Hunt, D.J. (2007). Overview of taxonomy and systematics. In: Nguyen, K,B. & Hunt, D.J. (eds).

369 Entomopathogenic nematodes: Systematics, phylogeny and bacterial symbionts. Brill Leiden-Boston, The

370 Netherlands. pp. 27-57.

371 Jagdale, G. B., & Gordon, R. (1998). Effect of propagation temperatures on temperature tolerances of

372 entomopathogenic nematodes. *Fundamental and applied nematology*, *21*(2), 177-184.

373 Jagdale, G. B., & Grewal, P. S. (2003). Acclimation of entomopathogenic nematodes to novel

374 temperatures: trehalose accumulation and the acquisition of thermotolerance. *International Journal for*

375 *Parasitology*, 33(2), 145-152.

376 Jagdale, G. B., Grewal, P. S., & Salminen, S. O. (2005). Both heat-shock and cold-shock influence

377 trehalose metabolism in an entomopathogenic nematode. *Journal of Parasitology*, 91(5), 988-994.

378 Kleynhans, E., Mitchell, K. A., Conlong, D. E., & Terblanche, J. S. (2014). Evolved variation in cold

379 tolerance among populations of Eldana saccharina (Lepidoptera: Pyralidae) in South Africa. *Journal of*

380 *Evolutionary Biology*, *27*(6), 1149-1159.

381 Lacey, L.A., & Georgis, R. (2012). Entomopathogenic nematodes for control of insect pest above and

382 below ground with comments on commercial production. *Journal of Nematology* 44 (2), 218-225.

383 Le Vieux, P. D., & Malan, A. P. (2013). The potential use of entomopathogenic nematodes to control

384 *Planococcus ficus* (Signoret)(Hemiptera: Pseudococcidae). *South African Journal of Enolology and*

385 *Viticulture* 34 (1), 296-306.

386 Malan, A. P., Nguyen, K. B., & Addison, M. F. (2006). Entomopathogenic nematodes (Steinernematidae

387 and Heterorhabditidae) from the southwestern parts of South Africa. *African Plant Protection,* 12, 65-69.

388 Malan, A. P., Knoetze, R., & Moore, S. D. (2011). Isolation and identification of entomopathogenic

389 nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth.

390 *Journal of invertebrate pathology*, *108*(2), 115-125.

391 Mekéte, T., Gaugler, R., Nguyen, K. B., Mandefro, W., & Tessera, M. (2005). Biogeography of

392 entomopathogenic nematodes in Ethiopia. *Nematropica*, *35*(1), 31-36.

393 Morton, A., & García-del-Pino, F. (2009). Ecological characterization of entomopathogenic nematodes

394 isolated in stone fruit orchard soils of Mediterranean areas. *Journal of Invertebrate Pathology*, 102(3),

395 203-213.

396   Navon, A., & Ascher, K. R. S. (Eds.). (2000). *Bioassays of entomopathogenic microbes and nematodes*.
397   Cabi.

398   Nyamukondiwa, C., & Terblanche, J. S. (2010). Within generation variation of critical thermal limits in
399   adult Mediterranean and Natal fruit flies *Ceratitis capitata* and *Ceratitis rosa*: thermal history affects
400   short-term responses to temperature. *Physiological Entomology*, *35*(3), 255-264.

401   Nyamukondiwa, C., Terblanche, J. S., Marshall, K. E., & Sinclair, B. J. (2011). Basal cold but not heat
402   tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). *Journal of*
403   *evolutionary biology*, *24*(9), 1927-1938.

404   Nguyen, K. B., Tesfamariam, M., Gozel, U., Gaugler, R., & Adams, B. J. (2004). *Steinernema*
405   *yirgalemense* n. sp. (Rhabditida: Steinernematidae) from Ethiopia. *Nematology*, *6*(6), 839-856.

406   Ogiga, I. R., & Estey, R. H. (1974). The use of Meldola Blue and Nile Blue A, for distinguishing dead
407   from living nematodes. *Nematologica*, *20*(3), 271-276.

408   Perry, R. N., & Wharton, D. A. (Eds.). (2011). *Molecular and physiological basis of nematode survival*.
409   CABI.

410   R Core Team (2014). R: A language and environment for statistical computing. R Foundation for
411   Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

412   Salame, L., Glazer, I., Miqaia, N., & Chkhubianishvili, T. (2010). Characterization of populations of
413   entomopathogenic nematodes isolated at diverse sites across Israel. *Phytoparasitica*, *38*(1), 39-52.

414   Selvan, S., Grewal, P. S., Leustek, T., & Gaugler, R. (1996). Heat shock enhances thermotolerance of
415   infective juvenile insect-parasitic nematodes *Heterorhabditis bacteriophora* (Rhabditida:
416   Heterorhabditidae). *Experientia*, 52(7), 727-730.

417   Shapiro, D. I., Glazer, I., & Segal, D. (1996). Trait stability and fitness of the heat tolerant
418   entomopathogenic nematode *Heterorhabditis bacteriophora* IS5 strain. *Biological Control*, *6*(2), 238-244.

419   Shapiro-Ilan, D. I., Brown, I., & Lewis, E. E. (2014). Freezing and desiccation tolerance in
420   entomopathogenic nematodes: Diversity and correlation of traits. *Journal of nematology*, *46*(1), 27-34.

421   Stenekamp, D. (2011). Optimization of a mass-rearing system to produce codling moth, *Cydia pomonella*,
422   for a Sterile Insect Release programme in South Africa. PhD dissertation, Faculty of AgriSciences,
423   Stellenbosch University, Stellenbosch, 125 pp.

424   Surrey, M. R. (1996). Effect of rearing method and cool temperature acclimation on the cold tolerance of
425   *Heterorhabditis zealandica* infective juveniles (Nematoda: Heterorhabditidae). *CryoLetters, 17,* 313-320.

426   Terblanche, J. S. (2014). Physiological performance of field-released insects.*Current Opinion in Insect*
427   *Science*, *4*, 60-66.

428   Terblanche, J. S., Hoffmann, A. A., Mitchell, K. A., Rako, L., le Roux, P. C., & Chown, S. L. (2011).
429   Ecologically relevant measures of tolerance to potentially lethal temperatures. *The Journal of*
430   *experimental biology*, *214*(22), 3713-3725.

431   Van Niekerk, S., & Malan, A. P. (2012). Potential of South African entomopathogenic nematodes
432   (Heterorhabditidae and Steinernematidae) for control of the citrus mealybug, *Planococcus citri*
433   (Pseudococcidae). *Journal of Invertebrate Pathology*, *111*(2), 166-174.

434   Weldon, C. W., Terblanche, J. S., & Chown, S. L. (2011). Time-course for attainment and reversal of
435   acclimation to constant temperature in two *Ceratitis* species. *Journal of Thermal Biology*, 36(8), 479-485.

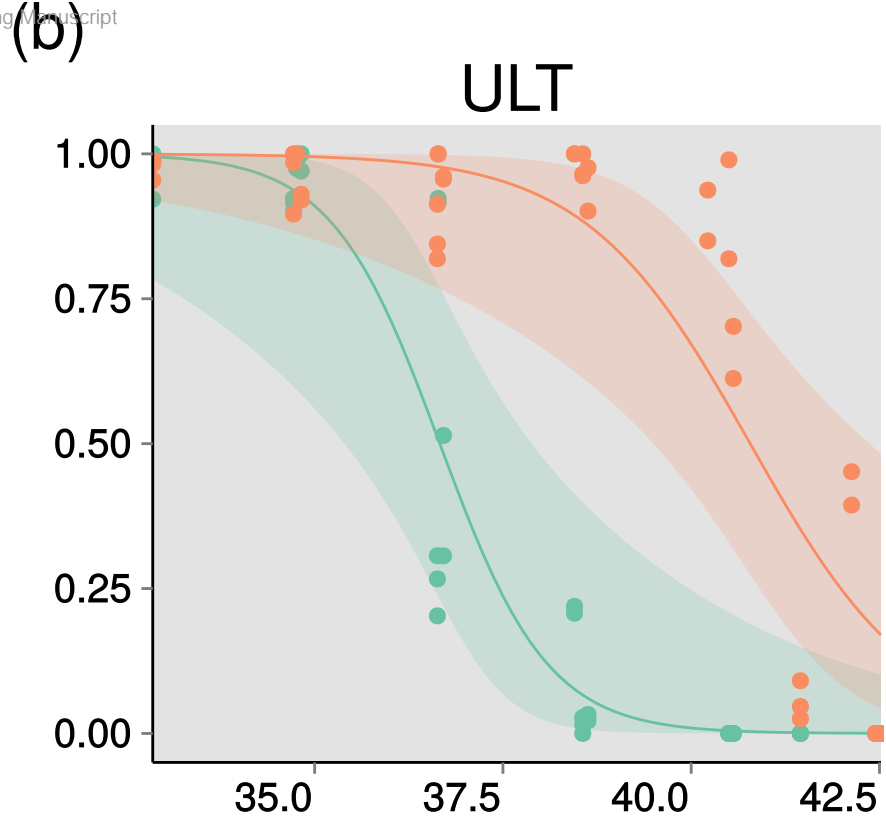
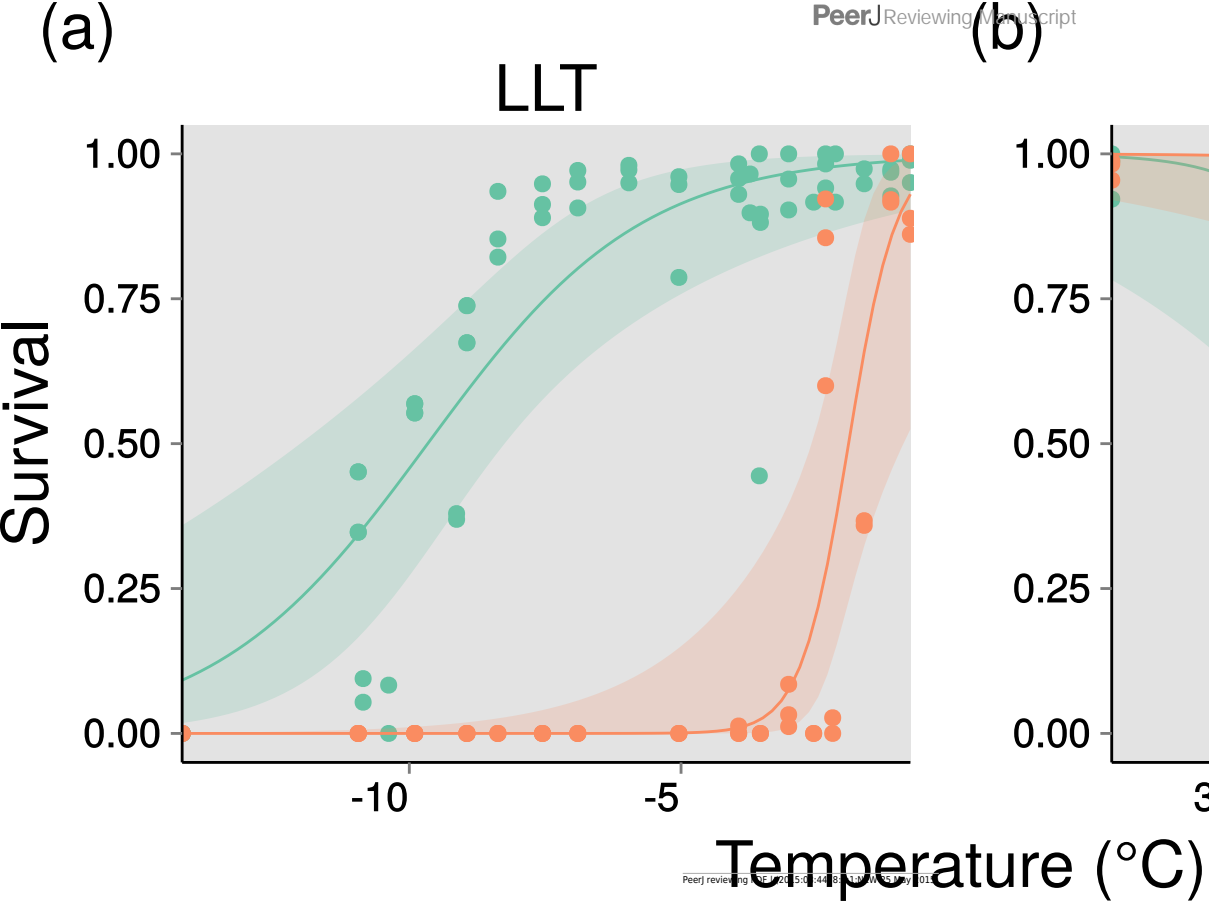
436   Wharton, D. A. (2011). Cold tolerance. *Molecular and physiological basis of nematode survival. CABI*
437   *Publishing, Wallingford*, 182-204.

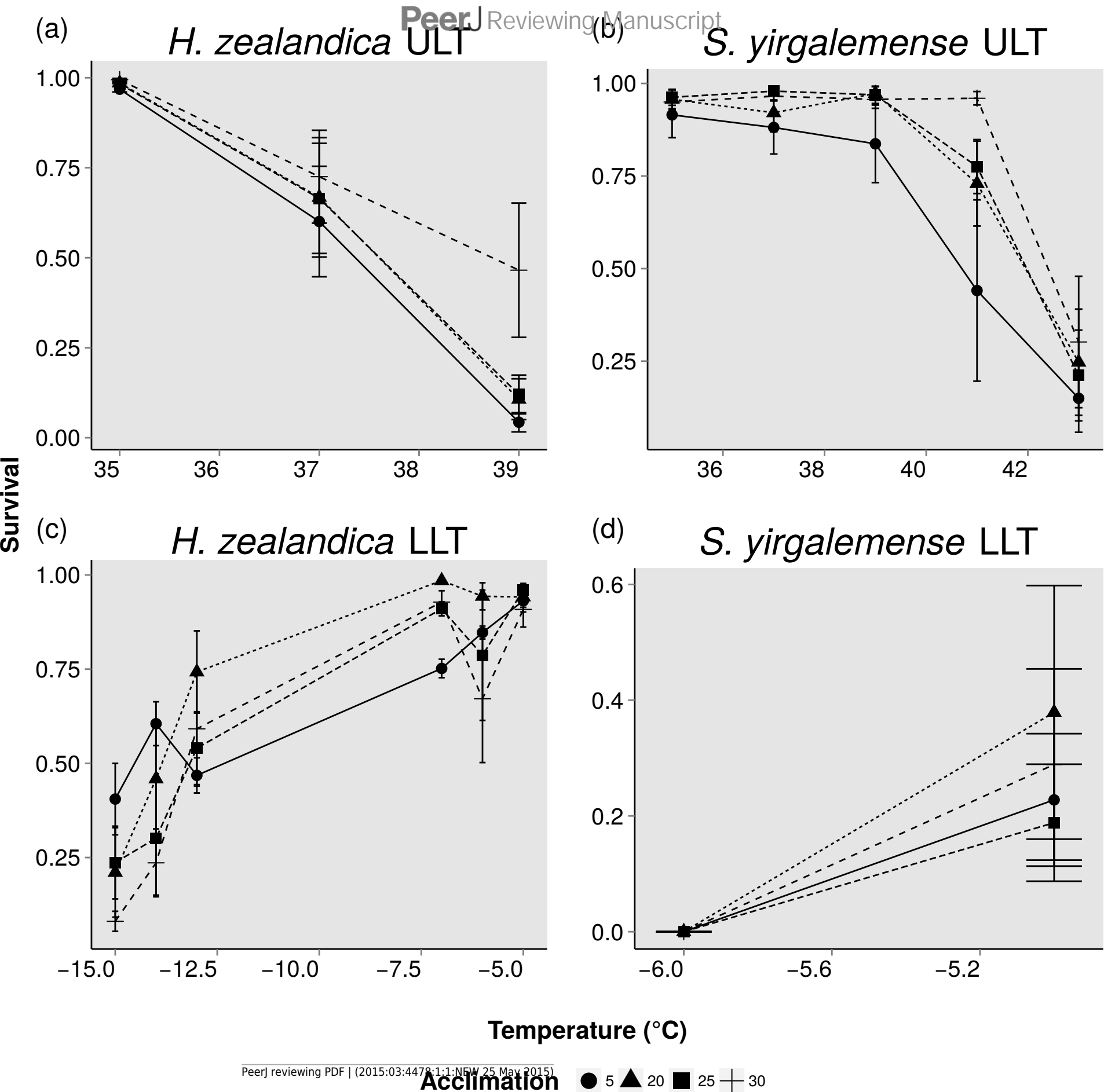
438   Wharton, D. A., & Surrey, M. R. (1994). Cold tolerance mechanisms of the infective larvae of the insect
439   parasitic nematode, *Heterorhabditis zealandica* Poinar. *CryoLetters, 15,* 353-360.

440   White, G.F. (1927) A method for obtaining infective nematode larvae from cultures. *Science*, 66, 302-
441   303.

442

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.* See Table 1 for model summaries.

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, Survival measured between 0 (complete mortality) and 1 (complete survival). **(a)** Upper lethal temperatures and acclimation for *Heterorhabditis zealandica.* **(b)** Upper lethal temperatures and acclimation for *Steinernema yirgalemense* **(c)** Lower lethal temperatures and acclimation for *Heterorhabditis zealandica.* **(d)** Lower lethal temperatures and acclimation for *Steinernema yirgalemense.*

Generalised Linear Model summary for the effect of temperature on upper (ULT) and lower lethal temperature (LLT) limits of *Heterorhabditis zealandica and Steinernema yirgalemense.*

[a] residual deviance = 45.51, df = 60 [b] residual deviance = 52.66, df = 48 [c] residual deviance = 31.26, df = 35 [d] residual deviance = 23.76, df = 38

	Effect	df	Estimate	SE	z	P
LLT *H. zealandica*[a]	Intercept	1	4.94	0.58	8.58	<0.001
	Temperature	1	0.51	0.64	7.94	<0.001
S. yirgalemense[b]	Intercept	1	4.35	0.75	5.81	<0.001
	Temperature	1	2.25	0.34	6.56	<0.001
ULT *H. zealandica*[c]	Intercept	1	51.13	6.65	7.69	<0.001
	Temperature	1	-1.39	0.18	-7.7	<0.001
S. yirgalemense[d]	Intercept	1	37.22	8.02	4.64	<0.001
	Temperature	1	-0.91	0.2	-4.63	<0.001

[a] residual deviance = 45.51, df = 60
[b] residual deviance = 52.66, df = 48
[c] residual deviance = 31.26, df = 35
[d] residual deviance = 23.76, df = 38

Generalised Linear Model / logit model predictions for 10, 50 and 90% survival of the population (i.e. LT_{90}, LT_{50}, LT_{10}) of *Heterorhabditis zealandica and Steinernema yirgalemense* at upper and lower temperatures.

Upper Lethal Temperatures (°C)

% Survival	H. zealandica		S. yirgalemense	
	LT	SE	LT	SE
10	38.2	0.3	43.2	0.7
50	36.7	0.2	40.8	0.3
90	35.1	0.2	38.4	0.6

Lower Lethal Temperatures (°C)

% Survival	H. zealandica		S. yirgalemense	
	LT	SE	LT	SE
10	-14.0	0.8	-3.3	0.1
50	-9.7	0.3	-2.4	0.0
90	-5.4	0.5	-1.6	0.1

Wald's χ^2 test with categorical acclimation and set temperature variables for both Lower Lethal Temperature (LLT) and Upper Lethal Temperature (ULT).

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.

ULT

H. zealandica

	χ^2	df	P
Temp	15.0	2	<0.001
Acclimation	0.34	3	0.95
Acclimation x Temp	4.5	6	0.61

S. yirgalemense

	χ^2	df	P
Temp	26.1	4	<0.001
Acclimation	0.51	3	0.92
Acclimation x Temp	4.8	11	0.94

LLT

H. zealandica

	χ^2	df	P
Temp	42.9	6	<0.001
Acclimation	6.8	3	0.08
Acclimation x Temp	23.0	15	0.08