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Heat shock enhances thermotolerance of infective juvenile insect-parasitic nematodes *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae)

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Abstract. Insect-parasitic nematodes possess many of the attributes of ideal biological control agents, but intolerance to extreme temperatures can restrict their use. We examined whether heat-shock treatments could improve nematode survival and infectivity at temperatures that normally inhibit their activity (35 and 40 °C). Nematodes exposed to a sub-lethal temperature (35 °C) for 3 h with a latency period of 1–2 h at 25 °C killed insects at 35 and 40 °C. Correlative evidence was obtained between increased thermotolerance and the synthesis of 70-kDa heat-shock proteins (hsps). These results provide the first evidence of hsp synthesis in the development of thermotolerance and biological activity in the non-feeding, developmentally arrested, infective juvenile nematodes.
Key words. Heat-shock proteins; insect-parasitic nematodes; thermotolerance; infectivity.

Environmental concern about chemical insecticides serves as a strong impetus for the development of biological control agents[1]. Among biological control agents, insect-parasitic nematodes in the families Steinernematidae and Heterorhabditidae possess tremendous potential as alternative to chemicals[2]. These nematodes possess attributes such as broad host range, high virulence and cost-effective mass production. Consequently, apart from *Bacillus thuringiensis*-based products, nematodes are the most widely used biological control agents in the world[3].

Infective juvenile nematodes locate and penetrate insect hosts within 4–6 h when placed in close proximity[4], usually entering the host through natural body openings. Upon entry into the hemocoel, the nematodes release symbiotic bacteria carried within their intestines. The bacteria multiply rapidly, killing the insect within 24–48 h by septicemia[5]. Although insect-parasitic nematodes provide acceptable control of insect pests in protected environments (e.g. glasshouse crops and mushrooms), reliable, predictable results are often more difficult to achieve in the field[6]. Temperature is the most important environmental factor affecting nematode motility, survival, infectivity, development and reproduction[7]. The temperature activity range of insect parasitic nematodes is narrow, and the infectivity and persistence of most species are significantly reduced above 30 °C[7,8]. If the survival of nematodes at warmer temperatures can be enhanced, even for several hours, the nematodes may be more effectively used against insect pests in warmer climates.

Heat-shock treatment at sub-lethal temperatures leads to a transient resistant state by the organism against subsequent heat stress[9]. This phenomenon is known as thermotolerance and has generally been shown in tissues or growing stages of eucaryotes and procaryotes. Heat and other environmental stress factors have been shown to induce the synthesis of heat-shock proteins (hsps) in cells ranging from yeast to mammalian systems[10]. The duration of heat-shock treatment and the amount of heat-shock proteins produced are positively correlated[11–13]. We tested whether preconditioning of infective juvenile nematodes at sub-lethal temperatures (heat shock) can be used to enhance their thermotolerance and efficacy at otherwise lethal temperatures. We also evaluated whether enhanced thermotolerance is related to the de novo synthesis of heat-shock proteins in developmentally arrested, non-feeding, infective juvenile nematodes.

Materials and methods

Infective juvenile *H. bacteriophora* (HP 88) were reared in last-instar wax moth *Galleria mellonella* L. larvae at 25 °C. Nematodes used for the experiments were less than one week old and were held in water at 25 °C. The survival of *H. bacteriophora* at different temperatures was assessed by transferring nematodes to preheated water at 30, 35, 40, 45 or 50 °C. Survival was recorded at 0.25, 0.5, 1, 2, 4, 8, 16, 24 and 36 h. Time to kill 50% of the nematodes was calculated using probit analysis.

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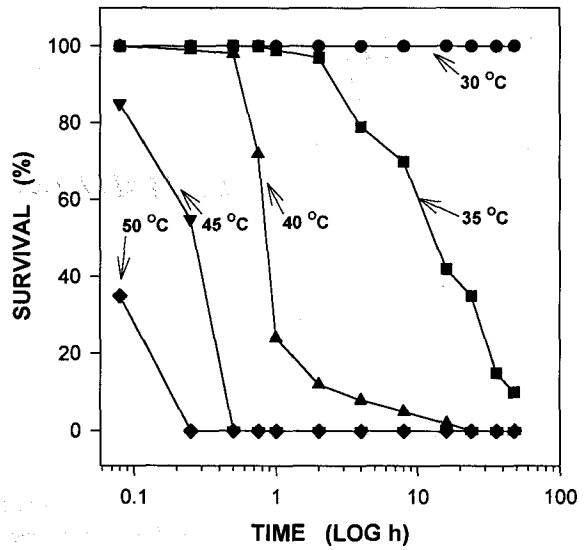

Figure 1. Survival of infective juvenile *Heterorhabditis bacteriophora* at 30 (●), 35 (■), 40 (▲), 45 (▼) and 50 °C (◆).

For assessment of thermotolerance, we chose 35 °C as the preconditioning temperature due to moderate impact on nematode survival. Preconditioning was carried out in glass test tubes containing 3 ml of water and 30,000 infective juveniles. After preconditioning for 2, 3 or 4 h at 35 °C, the nematodes were held at 25 °C for 0, 1, 2, 3 or 4 h before their survival was monitored at 40 °C for 6 h. The preconditioned nematodes with an optimum conditioning period of 3 h at 35 °C and 2 h at 25 °C were exposed to *G. mellonella* larvae in 9-cm petri dishes lined with filter paper at the rate of 1000 infective juvenile with 10 larvae as described by Grewal[4] and incubated at 25, 30, 35 and 40 °C. The experiment was repeated three times with three replicates. Insect hosts were collected from the filter paper, washed to remove the nematodes on the cuticle and placed at 25 °C for further development. After 36–48 h, host mortality was recorded. The number of nematodes established was determined after dissection at 72 h. Data were analysed by analysis of variance.

Synthesis of heat-shock proteins was examined using an in vitro translation system (Promega). Nematodes were exposed to 35 °C for 3 h with 2-h latency to induce heat-shock proteins. Untreated nematodes were held at 25 °C. Nematodes were concentrated under vacuum and ground to a paste in the presence of liquid nitrogen and guanidine thiocyanate. Phenol/chloroform was used to separate the aqueous phase, and isopropanol was used to precipitate the RNA. The in vitro translation reaction mixture consisted of rabbit reticulate lysate, ribonuclease inhibitor, amino acid mixture without methionine, [^{35}S] methionine and nematode RNA. These mixtures were incubated at 30 °C for 60 min. The reaction was arrested by adding gel-loading buffer and heating for 5 min at 100 °C. Equal amounts of proteins (30 µg/lane) were loaded onto each lane with a molecular weight marker in one lane. A constant current of 45 mA was applied to separate the proteins. After 4 h, the gel was dried and autoradiographed.

To characterise the proteins, Western blot analysis was carried out. Equal amounts of protein, 30 µg/lane, were loaded on 10% SDS-ploy acrylamide minigels (Bio-Rad). After electrophoresis for 90 min at 40 mA, the proteins were transferred to nitrocellulose using a Bio-Rad blot-transfer system. Bovine serum albumin (4% BSA) in TRIS-buffered saline (TBS) was used to block the reaction for 1 h at room temperature or overnight at 4 °C; the blot was incubated with primary antibody at

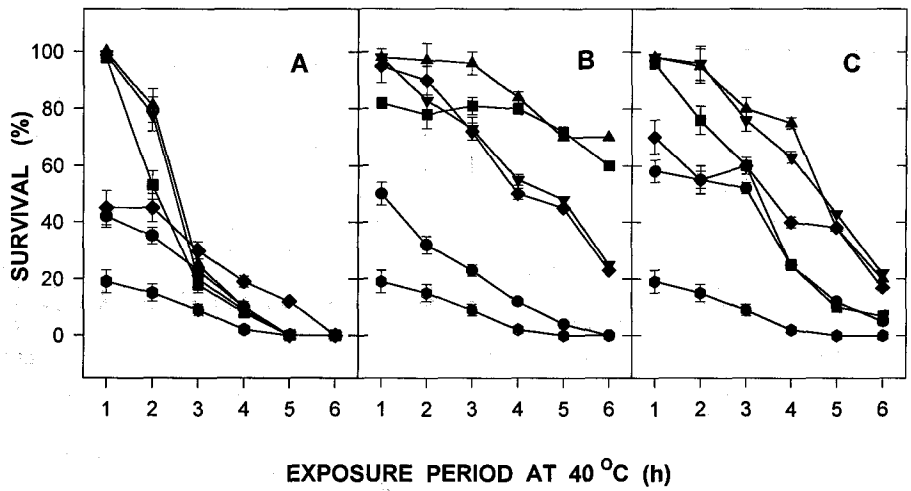

Figure 2. Nematode survival at 40 °C following different preconditioning regimes. Nematodes were exposed to 35 °C for 2 (*A*), 3 (*B*) and 4 h (*C*) and evaluated for thermotolerance at 40 °C after 0 (●), 1 (■), 2 (▲), 3 (▼) and 4 h (◆) latency period at 25 °C. Untreated nematodes (●) were held at 25 °C.

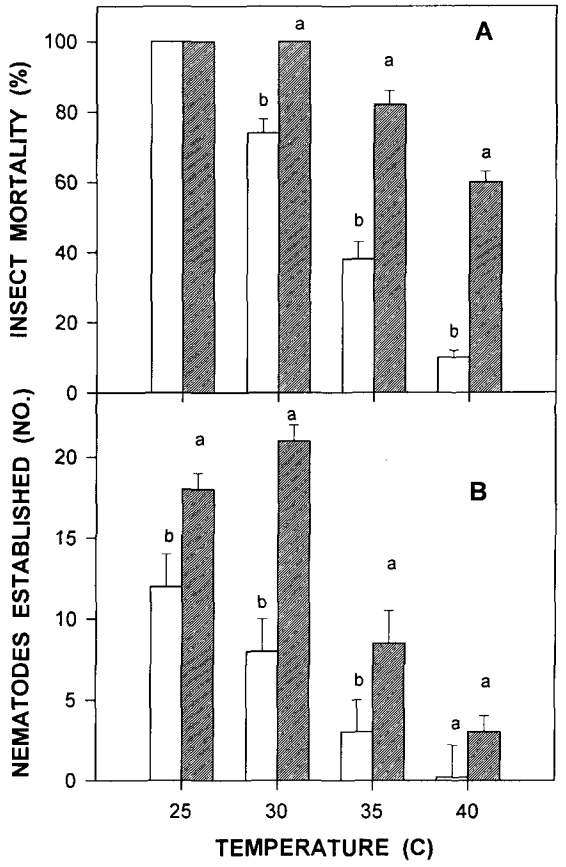

Figure 3. Effects of preconditioning treatment on infectivity (*A*) and number of nematodes established (*B*) in last instar *Galleria mellonella* larvae. Control nematodes (open bar) were kept at 25 °C. Preconditioned nematodes (striped bar) were held at 35 °C for 3 h with 2 h latency period at 25 °C. Bars with the same letters were not significantly different from each other ($p = 0.05$).

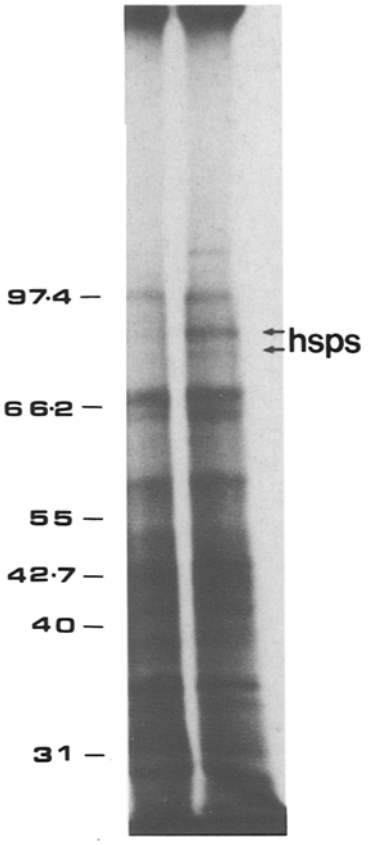

Figure 4. Autoradiography of proteins from infective juvenile *Heterorhabditis bacteriophora* after preconditioning treatment (lane A) (35 °C for 3 h with 2 h latency period at 25 °C) and untreated nematodes (lane B). Molecular sizes are indicated at left in kilodaltons.

1:1000 dilution in 15 ml of 1% BSA/TBS for 2 h. Th blot was washed with four changes of 0.1% Tween/TB (TTBS) over 1 h, rinsed with TBS and incubated wit secondary goat anti-mouse antibody at a 1:1000 dilution in 1% BSA/TBS for 1 h. After washing with four changes of TTBS over 1 h and rinsing with 0.5 M TRIS (pH 7.2), the blot was developed in 0.5 M TRIS and 0.0025 M diaminobenzidine with hydrogen peroxide. Antibodies were obtained from StreesGen (Vancouver, Canada) and goat anti-mouse secondary antibody, labelled with horseradish peroxide, was obtained from Sigma (St. Louis, MO).

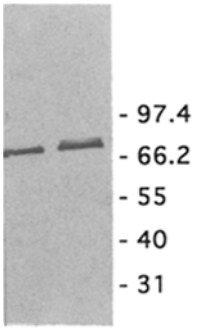

Figure 5. Monoclonal antibody specific to constitutive and induced forms of heat-shock proteins (hsps) reacted to hsps of *Heterorhabditis bacteriophora*. Preconditioned nematodes (lane A) expressed both forms of hsps, whereas untreated nematodes (lane B) showed only the constitutive form.

Results and discussion

More than 90% of infective juvenile *H. bacteriophora* were inactivated upon exposure to 40 °C within two hours (fig. 1). Time to kill 50% of nematodes (LT_{50}) was 5, 10, 40 and 300 min at 50, 45, 40 and 35 °C respectively. Preconditioning of *Drosophila*[14] and cynobacteria[15] at moderate temperature increases survival during subsequent exposure to stress; therefore, we chose 35 °C as a preconditioning temperature for *H. bacteriophora*. The duration of exposures at 35 °C and subsequent incubation at 25 °C affected nematode survival at 40 °C (fig. 2). Best results were obtained with 3-h preconditioning at 35 °C with a subsequent latency

period of 2 h at 25 °C. This preconditioning regime extended survival of nematodes from less than 1 to 6 h at 40 °C.

Infectivity (i.e., insect mortality) of preconditioned nematodes was significantly higher than the untreated nematodes at 30, 35, and 40 °C (fig. 3). The number of nematodes established in insect cadavers also increased at 25, 30, 35 °C following preconditioning treatment. These results support our hypothesis and demonstrate that preconditioning of nematodes at sub-lethal temperatures enhances their thermotolerance and infectivity potential at otherwise lethal warm temperatures.

We evaluated the changes in the electrophoretic pattern of proteins in preconditioned and untreated nematodes. When nematodes were exposed to 35 °C, comparison of the densitometric volume of protein synthesis changed significantly ('*t*' test, $p = 0.02$). A 5 major polypeptide of molecular mass of 70 kDa was detected in the preconditioned nematodes (fig. 4). The 70-kDa hsp gene is highly conserved, and enhanced thermotolerance is often correlated with the presence of this protein[16,17]. Studies on polymerase chain reaction (PCR) amplification of genomic DNA and the use of hsp70 probes indicate the abundance of hsp70 genes in the insect parasitic nematodes (S. Hashmi, pers. comm.)

Characterisation of nematode proteins with antibodies for constitutive and induced forms of hsps provided further support to the hypothesis that heat-shock proteins were synthesised during preconditioning treatment. These studies showed that preconditioned nematodes possessed both constitutive and induced forms of 70-kDa hsps, whereas the untreated nematodes only had constitutive forms. This was seen as an overlapping band of 70 kDa (fig. 5). Although synthesis of heat-shock proteins and development of thermotolerance has been demonstrated in the nematode *Caenorhabditis elegans*[18,19], this is the first demonstration of de novo synthesis of hsps in a non-feeding, developmentally arrested, juvenile stage of insect-parasitic nematodes.

The transient enhanced thermotolerance of insect-parasitic nematodes may be of significant applied value. We suggest that enhanced thermotolerance should improve nematode performance in high-temperature environments.

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