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# Expression profile of heat shock response factors during hookworm larval activation and parasitic development

Verena Gelmedin[1], Angela Delaney[2], Lucas Jennelle[3], John M. Hawdon*

*Research Center for Neglected Diseases of Poverty, Department of Microbiology, Immunology and Tropical Medicine, George Washington University Medical Center, Washington, District of Columbia, United States*

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ABSTRACT

When organisms are exposed to an increase in temperature, they undergo a heat shock response (HSR) regulated by the transcription factor heat shock factor 1 (HSF-1). The heat shock response includes the rapid changes in gene expression initiated by binding of HSF-1 to response elements in the promoters of heat shock genes. Heat shock proteins function as molecular chaperones to protect proteins during periods of elevated temperature and other stress. During infection, hookworm infective third stage larvae (L3) undergo a temperature shift from ambient to host temperature. This increased temperature is required for the resumption of feeding and activation of L3, but whether this increase initiates a heat shock response is unknown. To investigate the role of the heat shock in hookworm L3 activation and parasitic development, we identified and characterized the expression profile of several components of the heat shock response in the hookworm *Ancylostoma caninum*. We cloned DNAs encoding an *hsp70* family member (*Aca-hsp-1*) and an *hsp90* family member (*Aca-daf-21*). Exposure to a heat shock of 42 °C for one hour caused significant up-regulation of both genes, which slowly returned to near baseline levels following one hour attenuation at 22 °C. Neither gene was up-regulated in response to host temperature (37 °C). Conversely, levels of *hsf-1* remained unchanged during heat shock, but increased in response to incubation at 37 °C. During activation, both *hsp-1* and *daf-21* are down regulated early, although *daf-21* levels increase significantly in non-activated control larvae after 12 h, and slightly in activated larvae by 24 h incubation. The heat shock response modulators celastrol and KNK437 were tested for their effects on gene expression during heat shock and activation. Pre-incubation with celastrol, an HSP90 inhibitor that promotes heat shock gene expression, slightly up-regulated expression of both *hsp-1* and *daf-21* during heat shock. KNK437, an inhibitor of heat shock protein expression, slightly down regulated both genes under similar conditions. Both modulators inhibited activation-associated feeding, but neither had an effect on *hsp-1* levels in activated L3 at 16 h. Both celastrol and KNK437 prevent the up-regulation of *daf-21* and *hsf-1* seen in non-activated control larvae during activation, and significantly down regulated expression of the HSF-1 negative regulator *Aca-hsb-1* in activated larvae. Expression levels of heat shock response factors were examined in developing *Ancylostoma ceylanicum* larvae recovered from infected hosts and found to differ significantly from the expression profile of activated L3, suggesting that feeding during *in vitro* activation is regulated differently than parasitic development. Our results indicate that a classical heat shock response is not induced at host temperature and is suppressed during larval recovery and parasitic development in the host, but a partial heat shock response is induced after extended incubation at host temperature in the absence of a developmental signal, possibly to protect against heat stress.



*Abbreviations:* iL3, third stage larva; IIS, insulin/insulin growth factor-like signalling; HSF, heat shock factor; HSR, heat shock response; HSP, heat shock protein; HSB, heat shock binding protein; p.i., post-infection.

* Corresponding author at: The George Washington University Medical Center, Ross Hall 519, 2300 Eye St. NW, Washington, D.C. 20037, United States. Fax: +1 202 994 2913.
*E-mail address:* jhawdon@gwu.edu (J.M. Hawdon).
[1] Present address: Biomedizinisches Forschungszentrum Seltersweg, Institute for Parasitology, Justus Liebig University, Giessen, Germany.
[2] Present address: Program in Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, United States.
[3] Present address: Beirne Carter Center for Immunology Research, University of Virginia, Charlottesville, VA, United States.

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## 1. Introduction

Soil-transmitted nematodes infect about 1.3 billion people worldwide with the morbidity predominantly attributed to roundworms, whipworms, and hookworms [1]. Hookworms of the species *Ancylostoma duodenale* and *Necator americanus* infect more than 700 million people worldwide annually, causing malnutrition and iron-deficiency anaemia leading to diminished cognitive abilities and underdevelopment in children [2,3]. In addition to children, pregnant women and the elderly are most vulnerable for infections [1,4]. Infections occur primarily through contact with infective larvae in contaminated soil, which penetrate the skin and develop in the small intestines to the reproductive, feeding adult stage that causes blood loss. These soil-dwelling infective third stage larvae (iL3) are non-feeding and developmentally arrested. During invasion of a permissive host, stimulating cues re-initiate feeding and development. In addition, the transition to a parasitic life is accompanied by evasion of the host's defences including the release of excretory/ secretory products and a shift from aerobic towards anaerobic energy metabolism. The signals that initiate development and the associated molecular mechanisms during the transition to parasitism are poorly understood [5]. Current treatments for hookworm disease fail to prevent re-infections, resistance to chemotherapy is beginning to emerge, and efforts to develop a vaccine have been unsuccessful [6,7]. Improved understanding of the underlying mechanisms involved in the resumption of development is critical to development of new control strategies.

One of the initial events upon entry of a larva into a host is thought to be the resumption of feeding. This process can be mimicked *in vitro* by low pH treatment followed by incubation at host temperature in the presence of serum and glutathione. All three stimuli are required to reinitiate feeding *in vitro* [8–13], but development from the L3 to the fourth larval stage (L4) only occurs in a suitable host. If iL3 enter a non-suitable host, feeding can be reinitiated, but the larvae remain in a non-developing, dormant stage (paratenesis). The "package of signals" which initiates development must therefore differ from that which initiates feeding.

Recent studies investigating the obligate non-developing iL3 stage of parasitic nematodes suggest that developmental arrest is regulated by a similar mechanism as the non-developing, facultative dauer stage of the free-living nematode *Caenorhabditis elegans* [14–17]. In *C. elegans*, the forkhead transcription factor DAF-16/FOXO is one of the key regulators of dauer, and is negatively regulated by the insulin/insulin growth factor-like signalling (IIS) pathway [18,19]. Besides DAF-16, dauer formation requires the transcription factor heat shock factor-1 (HSF-1) as shown in dauer constitutive mutants and wild type animals [20,21]. Furthermore, the heat shock response (HSR) requires DAF-16 activity, and nuclear export of DAF-16 during recovery from heat shock involves HSF-1 and HSP70/HSP-1 [22].

Hookworms encounter temperature fluctuations as free-living larval stages, but also experience a temperature shift from ambient to that of their endothermic host during infection. The elevated temperature of the host is required for *in vitro* activation of hookworm L3 [8], and is likely a component of the host-specific signal package that initiates development. This, together with the interaction of DAF-16 and HSF-1 in the dauer pathway, suggests a potential role for HSF-1 and HSR factors in the hookworm infectious process.

The HSR consists of four main components, which act in a highly conserved mechanism. The accumulation of unfolded proteins as a consequence of elevated temperature leads to a rapid increase of cytoprotecting proteins in order to prevent cell damage. The synthesis of these chaperones, called heat shock proteins (HSP), is regulated by HSF-1. In the absence of stress, the inactive monomeric form of HSF-1 is bound by HSP-70 and other chaperones in the cytoplasm. Accumulation of denatured proteins recruits HSP-90 and HSP-70 from the complex and allows HSF-1 to translocate into the nucleus, where it assembles into a homotrimer and is phosphorylated at activating serine residues. In its activated state, HSF-1 binds to heat shock response elements in the promoter regions of heat shock associated genes and leads to their expression. Negative feedback loops repress heat shock associated gene expression. In addition to HSP-70, HSF-1 is also negatively regulated by heat shock binding protein 1 (HSB-1). During attenuation, or recovery from heat shock, HSB-1 and HSP-70 bind to the HSF-1 trimer causing its dissociation from DNA and disassembly into the inactive state [21,23]. In addition to regulating the HSR, HSF-1 and HSPs are required for normal development [20,24–26], with the monomeric form of HSF regulating genes involved in development [27].

To date, the HSR has not been described for hookworms. We recently analysed the function of *Aca*-HSB-1 [28], and herein investigate the expression of other main regulators of the HSR, HSF-1, HSP-1/HSP-70, and DAF-21/HSP-90 during heat shock. We compare the HS expression profile to the profile of *in vitro* activated L3 as well as developing parasitic larvae. Furthermore, we investigate the role of heat shock on activation by determining the influence of known HSR modulators on the expression of HSR factors.

## 2. Material and methods

### 2.1. Ethics statement

All experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the George Washington University Medical Center Institutional Animal Care and Use Committee (protocol number: A147).

### 2.2. Parasites

For heat shock and *in vitro* larval activation experiments, the Baltimore strain of *A. caninum* (US National Parasite Collection No. 1000655) was maintained and harvested from dog feces as described elsewhere [28]. An Indian strain of *A. ceylanicum* (USNPC No. 102954) was maintained in Syrian hamsters [29] and dogs. Developing parasitic larval stages were obtained from three *A. ceylanicum* infected hamsters per time point and separated into different developmental stages by visual examination under a dissecting scope. *A. ceylanicum* adults were recovered 12 d post infection (p.i.).

### 2.3. In vitro *activation and heat shock experiments of iL3*

*In vitro* activation of iL3 and heat shock experiments were conducted as published [28]. For activation, iL3 were decontaminated in 0.12 N HCl for 30 mins at 22 °C followed by 3 washes in $RPMI_{1640}$ tissue culture medium supplemented with 25 mM HEPES (pH 7.0) and antibiotics (RPMI-c). Decontaminated iL3 (5000) were incubated in 0.5 ml of RPMI-c in individual wells of 24-well tissue culture plates at 37 °C, 5% $CO_2$ for 24 h. Larvae were activated by the inclusion of 10% (v/v) of a <10 kDa ultrafiltrate of canine serum and 15 mM *S*-methyl-glutathione (GSM, Sigma Chemical, St. Louis, MO), whereas non-activated L3 were incubated in RPMI-c only. To determine the percentage feeding (*i.e.*, activation), an aliquot of L3 were incubated as above in fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA, Sigma Chemical) for 2 h, washed in PBS, transferred to a microscope slide and examined under ultraviolet illumination as described previously [10]. Larvae that had ingested the FITC-BSA were counted and expressed as a percentage of the total number counted. Only experiments in which a minimum of