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## NOTE

**Heat shock proteins of the oyster parasite *Perkinsus marinus***C. T. Tirard<sup>1</sup>, R. M. Grossfeld<sup>1,\*</sup>, A. K. Volety<sup>2</sup>, F.-L. E. Chu<sup>2</sup><sup>1</sup> Department of Zoology and Animal Physiology Program, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, North Carolina 27695-7617, USA<sup>2</sup> Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, Virginia 23062, USA

**ABSTRACT:** The susceptibility of the eastern oyster *Crassostrea virginica* to infection by the protozoan parasite *Perkinsus marinus* is influenced by temperature. Because of the crucial roles of heat shock proteins in cellular thermal tolerance and in host-parasite adaptations in other species, we compared the *in vitro* heat shock responses of cultured *P. marinus* and of oyster hemocytes. The parasite and host heat shock proteins were different in size and in immunochemical specificity. In addition, the thermal threshold for inducing the response was higher for *P. marinus* acclimated to the same temperature as the oysters. The results suggest that *P. marinus* is likely to employ heat shock proteins as part of its adaptive survival repertoire and that it may be able to function normally under conditions of hyperthermia that evoke an emergency physiological response from the oyster defense cells. Furthermore, they indicate that it is feasible to investigate the host's and parasite's adaptive responses to each other, since their individual responses are readily distinguishable.

**KEY WORDS:** Parasite · *Perkinsus marinus* · Oysters · *Crassostrea virginica* · Hemocytes · Stress proteins · Hyperthermia

The progressive decrease in oyster *Crassostrea virginica* harvests along much of the eastern seaboard of the United States during the last 3 decades has been attributed primarily to mortality associated with 2 protozoan parasites, *Perkinsus marinus* and *Haplosporidium nelsoni* (Andrews 1988). The impact of these pathogens is determined to a large extent by ambient salinity and temperature (Haskin & Ford 1982, Chu & LaPeyre 1993, Chu et al. 1993, Ragone & Burreson 1993). In laboratory and field studies on intact oysters, the prevalence and intensity of infection by *P. marinus* were shown to be greatest at a salinity of about 26 to 32 ppt and a temperature of about 20 to 28°C and least at low salinity and temperature.

The effect of temperature and salinity could reflect a differential adaptability of the host and parasite. For

example, *in vitro* studies with isolated oyster hemocytes have indicated an influence of these factors on the defense-related functions of spreading time, rate of movement, and foreign particle binding (Fisher 1988, Fisher & Tamplin 1988, Fisher et al. 1992). On the other hand, the effect of temperature and salinity could reflect a differential sensitivity of the host and parasite. For example, *in vitro* studies with *Haplosporidium nelsoni* (Ford & Haskin 1988) or *Perkinsus marinus* (Calvo et al. 1994) have indicated that these parasites may be more vulnerable than the oyster cells to an osmotic shock. In particular, after exposure to a salinity of about 4 ppt, the parasites are less viable than the hemocytes. This may account in large part for the low incidence of infection of oysters in low salinity environments.

These studies lead to the conclusion that the outcome of the pathogenic challenge will be determined by factors that affect the innate ability of the host and parasite to respond to their common environment. Since a period of *Perkinsus marinus* development is spent within the host's hemocytes or other cells (Andrews 1988), the outcome of the interaction also may be dependent on the response of this parasite and the hemocytes to each other.

Based upon studies of vertebrate cells and their natural parasites, the induced synthesis of stress proteins ('SPs') is a biochemical mechanism that is likely to be important for the responses of oyster defense cells and of *Perkinsus marinus* to both environmental and biological challenges. These highly conserved proteins are essential for housekeeping and adaptive functions in virtually all animal cells (Lindquist & Craig 1988, Welch et al. 1989, Angelidis et al. 1991, Lathigra et al. 1991, Li et al. 1991, Nover 1991, Polla 1991, Solomon et al. 1991, Morimoto 1993, Young et al. 1993). Vertebrate immune cells and microorganisms, including protozoan parasites, exhibit similar SP responses

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to heat and other ambient shocks (Lathigra et al. 1991, Nover 1991, Polla 1991, Shinnick 1991, Murray & Young 1992, DeNagel & Pierce 1993). In parasites, induction of SPs, for example by exposure to the elevated temperature of a new host, is believed to be an important determinant of virulence towards the host (Lathigra et al. 1991). It is also considered to be an adaptation that allows the parasite to survive and differentiate within the confines of a potentially threatening host cell environment (Lathigra et al. 1991, Murray & Young 1992). On the other hand, because SPs are the dominant antigens of a broad array of microbes, they trigger reactive responses of vertebrate immune cells that protect the host (Shinnick 1991, Healy et al. 1992, Murray & Young 1992, Kantengwa & Polla 1993, Young et al. 1993). In fact, it is the capacity of macrophages and lymphocytes to recognize microbial SPs and to respond to them utilizing their own SP response that is believed to insure the outcome of the interaction in favor of a healthy host (Ciavarra & Simeone 1990, Donati et al. 1991, Polla 1991, Healy et al. 1992, Murray & Young 1992, Kantengwa & Polla 1993, Young et al. 1993).

Using the vertebrate studies as a model, we have been investigating the response of oyster hemocytes to ambient stresses as a means of understanding how these cells adapt to potential threats to their survival. In a previous publication (Tirard et al. 1995), we reported that an acute heat shock of oyster hemocytes *in vitro* induced the synthesis and accumulation of the class of stress proteins known as heat shock proteins ('HSPs'), some of which may also be induced by a variety of other stresses (Lindquist & Craig 1988, Welch et al. 1989, Nover 1991). In this communication, we report that a similar *in vitro* heat stress to *Perkinsus marinus* induced a comparable set of HSPs.

**Materials and methods.** *Perkinsus marinus* merozoites/meronts were cultured at 26°C in 25 cm<sup>2</sup> flasks containing 10 ml of modified DMEM:Ham's F-12 nutrient mixture (Gauthier & Vasta 1993). The cultured cells varied between 3 and 20 µm in size and consisted of about 95% merozoites/meronts and a few pre-zoosporangia stage cells (<5% of the total). The various life stages of the parasite, representing about 5 to 12 × 10<sup>6</sup> particles, were collected together by centrifugation of 1 ml medium at 25 × g for 10 min. The supernatant was removed immediately from the loosely compacted pellet to minimize loss of cells. The parasites were resuspended in 1 ml Medium 199 adjusted to 25 ppt salinity, for treatment at 25, 37, 42, or

46°C for 1 h. Then, they were pelleted and resuspended in another 1 ml modified Medium 199 for a 2 h recovery at 25°C, after which they were incubated at 25°C and 25 ppt salinity with 1 ml Earles Balanced Salt Solution containing radioactive methionine and cystine. The procedures for cell incubation and lysis, quantitation of incorporated amino acid, and for detection of radioactive proteins by SDS (sodium dodecyl sulfate) polyacrylamide gel autoradiography were identical to those reported in another publication (Tirard et al. 1995). At least 95% of the cells were judged to be viable by their lack of staining with trypan blue at the start of the experiment and after the recovery period.

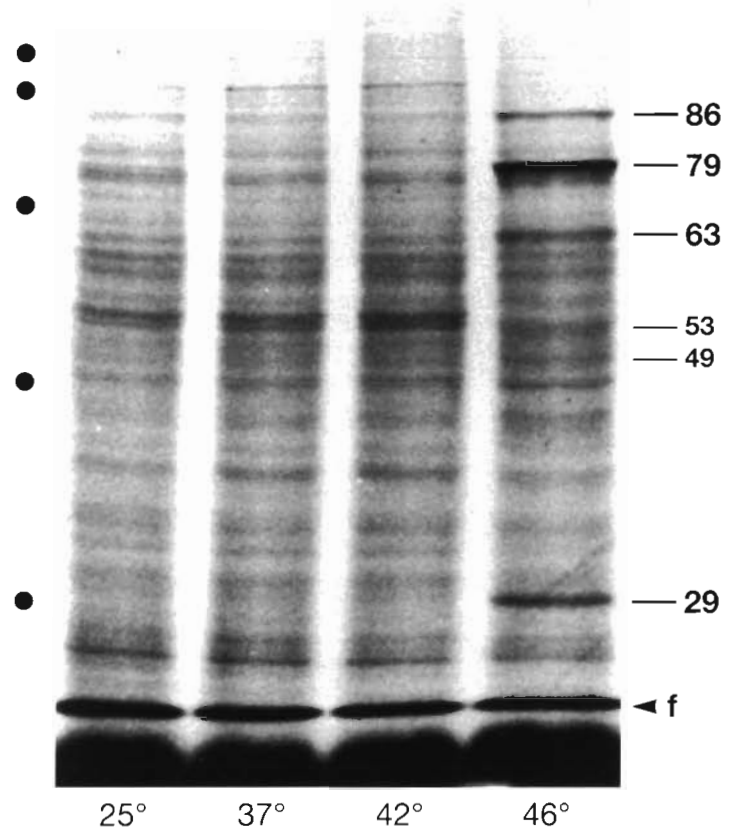


Fig. 1 *Perkinsus marinus*. Effect of temperature on parasite protein synthesis. Samples of cultured *P. marinus* were incubated *in vitro* at 25, 37, 42, or 46°C for 1 h. Afterwards, they were incubated for 2 h at 25°C, and then for 1 h at 25°C with radioactive amino acid. Gel lanes were loaded with equal amounts of protein radioactivity. The major heat-induced radioactive proteins are denoted by solid lines at the right of the gel autoradiograph, next to calculated estimates of their molecular masses (in kDa). Solid circles at the left indicate, from the top down, the corresponding locations of non-radioactive protein standards of 116, 97.4, 66, 45, and 29 kDa. The arrow and letter f near the bottom right of the gel in each figure indicate the location of the dye front

**Results and discussion.** When the parasites were incubated at the control temperature of 25°C, significant amounts of large and medium-size radioactive proteins were produced. Most of the radioactive amino acid was incorporated into small polypeptides that migrated at or just ahead of the dye front at the bottom of the gel (Fig. 1), where most of the Coomassie Blue-stained tissue protein was located. These results suggest either

that the cells normally produce an abundance of such polypeptides or that the incubation or culture conditions were not optimal for complete maturation of the cells and of their protein synthetic machinery. When the temperature was raised to 37 or 42°C, there was no change in amino acid incorporation or in the pattern of protein synthesis. However, parasites incubated at 46°C for 1 h incorporated only one-third as much tracer

into protein as the control samples and expressed a characteristic heat shock response involving synthesis of HSPs of various sizes. The most prominent parasite HSPs had molecular masses of about 86, 79, 63, and 29 kDa (Fig. 1). Careful examination of the autoradiographs revealed that none of these bands corresponded in size to radioactive proteins present in the samples at lower temperatures. Thus, like the 'inducible' HSPs of other species, their synthesis was preferentially activated by an appropriate stress and likely to contribute significantly to cellular adaptation to such stress (Welch et al. 1989). These HSPs are similar in size to those induced by a less severe heat stress of oyster hemocytes *in vitro*. The most prominent hemocyte HSPs had molecular masses of about 85, 70, 37, 34, and 32 kDa (Tirard et al. 1995).

The parasite and host heat shock responses were clearly distinguishable in 5 respects (Fig. 2). First, the HSPs were different in size. Second, the thermal threshold for inducing the response by 1 h *in vitro* was higher for *Perkinsus marinus* than for oyster hemocytes, requiring more than 42°C for the former but only 39 to 41°C for the latter in these and previous experiments. This was true whether the parasite had been held previously for 1 wk or more at 25 or 21°C. Third, in Western blots the parasite HSPs did not significantly bind a monoclonal antibody (clone 3a3; Affinity BioReagents, Neshanic, NJ, USA) that recognizes 2 HSP70 isoforms in oyster hemocytes (Tirard et al. 1995). Fourth, unless subjected to a very severe heat shock, the oyster hemocytes produced little if any radioactive polypeptide smaller than the bromophenol blue tracking dye at 2 h whereas most of the label incorporated by parasites at that time

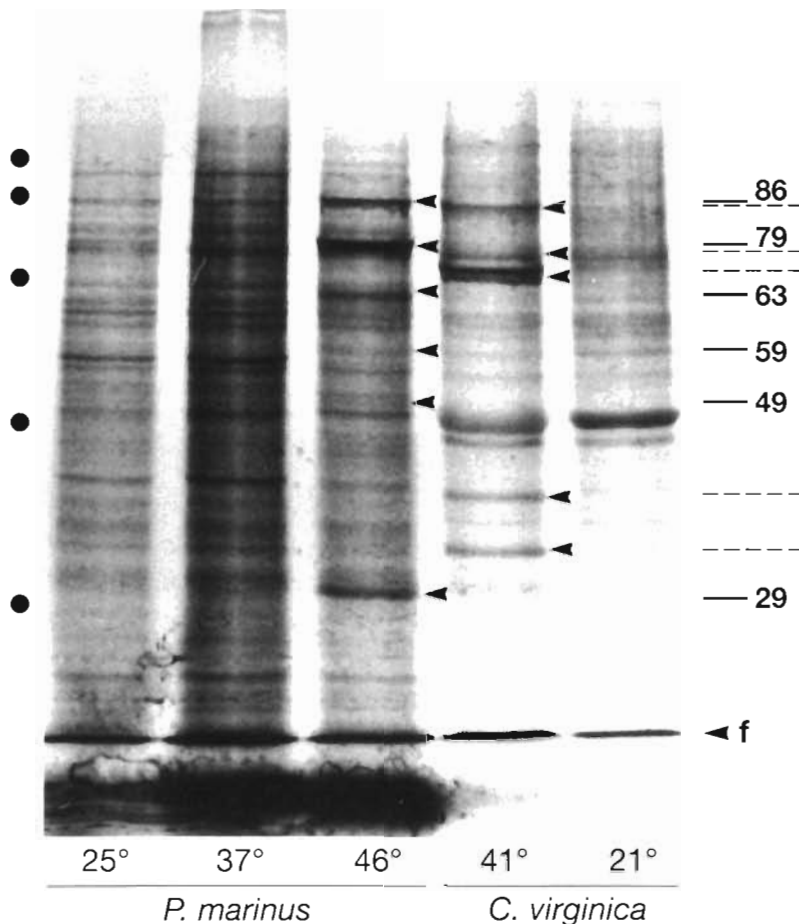


Fig. 2. *Perkinsus marinus* and *Crassostrea virginica*. Comparative heat shock responses of parasites and oyster hemocytes. Samples of cultured *P. marinus* or of *C. virginica* hemocytes were incubated separately *in vitro* for 1 h at the control temperature (25 or 21°C, respectively) or at an elevated temperature. Afterwards, they were incubated for 2 h at the control temperature, and then for 1 h at the control temperature with radioactive amino acid. Samples from the 2 species were arranged in opposite order on the gel to facilitate comparison of the respective heat shock responses. The major heat-induced radioactive proteins of the parasite at 46°C are denoted by thick solid lines and estimated molecular masses (in kDa) at the right of the gel autoradiograph, with corresponding arrowheads to the right of the 46°C lane. The 37°C sample has the same protein profile as the 25°C sample but all of its bands are proportionally darker because more protein radioactivity was inadvertently applied to this lane. The major heat-induced radioactive proteins of the oyster cells at 41°C are denoted by thin broken lines at the right of the autoradiograph, with corresponding arrowheads to the right of the 41°C lane. Solid circles at the left indicate, from the top down, the corresponding locations of non-radioactive protein standards of 116, 97.4, 66, 45, and 29 kDa

migrated ahead of the dye front. Fifth, the induced expression of HSPs in oyster hemocytes was accompanied by a relative decrease in synthesis of an actin-like protein of about 45 kDa (Fig. 2 and Tirard et al. 1995), whereas no comparable decrease was observed for the cultured parasites.

There are 3 important theoretical and practical implications of these results. First, since HSPs are known to be crucial determinants of cellular stress tolerance in a broad array of single-celled and multicellular animals (Angelidis et al. 1991, Li et al. 1991, Nover 1991, Solomon et al. 1991), the results suggest that *Perkinsus marinus* may employ HSPs as part of its adaptive survival repertoire. Second, the higher thermal threshold for eliciting the parasite's response suggests that it may be able to function normally under some conditions of hyperthermia that evoke an emergency physiological response from the oyster defense cells. This may afford the parasite an advantage in its interaction with cells of a potential molluscan host. Third, the results indicate that it is feasible to investigate the host's and parasite's adaptive responses to each other in a mixed culture or *in vivo*, since their individual responses are readily distinguishable.

Because we employed a severe acute hyperthermic shock to demonstrate inducible HSP expression in the parasite and oyster cells, it remains uncertain what relevance the results might have to host-parasite adaptations under environmental conditions that occur more commonly in the natural habitat. Within the range of distribution of *Crassostrea virginica*, water temperatures occasionally warm to 37°C (Galtsof 1964, Paynter & Dimichele 1990). Furthermore, oysters in the intertidal zones of the U.S. southern coastal regions often are exposed to the sun at low tide, at which time their core temperatures may reach 46 to 49°C for up to 2 to 3 h (Galtsof 1964, Kenny et al. 1990). Bodily functions may be maintained if exposure to temperatures as high as 42°C is not too prolonged. These are sufficiently severe hyperthermic stresses to activate oyster hemocyte HSP synthesis *in vitro* or *in vivo* (Tirard & Grossfeld unpubl. results) and possibly *Perkinsus marinus* HSP synthesis as well. Under such extreme conditions, HSPs may contribute to host and parasite adaptations that impact survival of each and the intensity of infection. Might HSPs also be essential for adaptation of these organisms under less severe environmental conditions more characteristically observed in the wild? We suggest that there exists a precedent to expect that they are. The heat shock response of mammalian immune cells is sensitized to heat both by simultaneous exposure to other stressors and by chemical changes occurring in other cell types (Ciavarrá & Simeone 1990). Furthermore, the cellular response to hyperthermia is dependent on the combined influence

of intensity and duration of exposure. Thus, a milder heat stress administered over a longer time may have an effect equivalent to a more severe heat stress occurring more transiently if both conditions are sufficient to denature proteins (DiDomenico et al. 1982). These observations suggest that it will be important to examine the responses of oyster hemocytes and tissues to acute and chronic thermal stresses *in vivo*, particularly as they occur during other natural stresses and in field situations.

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