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HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology

Martin E. Feder
Department of Organismal Biology and Anatomy and Committee on Evolutionary Biology, University of Chicago, 1027 East 57th Street, Chicago, Illinois 60637; e-mail: m-feder@uchicago.edu

Gretchen E. Hofmann
Department of Biology, University of New Mexico, Albuquerque, NM 87131; e-mail: ghofmann@unm.edu

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ABSTRACT

Molecular chaperones, including the heat-shock proteins (Hsps), are a ubiquitous feature of cells in which these proteins cope with stress-induced denaturation of other proteins. Hsps have received the most attention in model organisms undergoing experimental stress in the laboratory, and the function of Hsps at the molecular and cellular level is becoming well understood in this context. A complementary focus is now emerging on the Hsps of both model and nonmodel organisms undergoing stress in nature, on the roles of Hsps in the stress physiology of whole multicellular eukaryotes and the tissues and organs they comprise, and on the ecological and evolutionary correlates of variation in Hsps and the genes that encode them. This focus discloses that (*a*) expression of Hsps can occur in nature, (*b*) all species have *hsp* genes but they vary in the patterns of their expression, (*c*) Hsp expression can be correlated with resistance to stress, and (*d*) species' thresholds for Hsp expression are correlated with levels of stress that they naturally undergo. These conclusions are now well established and may require little additional confirmation; many significant questions remain

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unanswered concerning both the mechanisms of Hsp-mediated stress tolerance at the organismal level and the evolutionary mechanisms that have diversified the *hsp* genes.

INTRODUCTION

Although heat-shock proteins (Hsps) first achieved notoriety as gene products whose expression is induced by heat and other stresses (1, 2), discoveries of the past decade have shifted the focus of research to understanding the roles of Hsps as molecular chaperones (3–5). As a result, Hsps, their close relatives, their molecular partners, and many newly discovered proteins are now known to play diverse roles, even in unstressed cells, in successful folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins (6); failure of these activities is thought to underlie numerous and important human diseases (7). Nonetheless, many of the questions of the past either remain unanswered, awaiting the development of appropriate experimental tools, or can now be revisited with new insights gleaned from the emerging understanding of molecular chaperones. This review seeks to facilitate the examination or re-examination of Hsps as responses to natural stress in diverse organisms inhabiting environments outside the laboratory, the function of Hsps in tolerance of natural stresses, and ecological and evolutionary variation in the heat-shock system. The review sequentially considers (*a*) the principal implications of laboratory-based studies for ecological and evolutionary research on Hsps, (*b*) expression of Hsps in nature, (*c*) covariation of Hsp expression with environmental and biological gradients of stress intensity, (*d*) the consequences of Hsp expression for fitness, and (*e*) evolutionary variation in Hsps and the genes that encode them. The primary objective of this review is to redirect the focus of evolutionary and ecological research on Hsps beyond the conclusions that are now well-established and onto the many important questions that remain unanswered.

STATE OF THE LITERATURE

Established Conclusions

The relevant literature on Hsps and molecular chaperones is huge, now comprising more than 12,000 references. Even a review of the relevant reviews is difficult. For this reason, we begin by describing several well established conclusions and cite a few of the many excellent recent reviews at diverse levels of sophistication (4–6, 8–11).

The genes encoding Hsps (*hsps*) are highly conserved and occur in every species in which they have been sought. Many of these genes and their products can be assigned to families on the basis of sequence homology and typical molecular weight (6): *hsp110*, *hsp100*, *hsp90*, *hsp70*, *hsp60*, *hsp40*, *hsp10*, and small *hsp* families. Gething (6) recognizes 7 additional families and 12 genes/proteins for which families have not yet been described. In eukaryotes, many families comprise multiple members that differ in inducibility, intracellular localization, and function.

Hsps function as molecular chaperones; i.e. they interact with other proteins and, in so doing, minimize the probability that these other proteins will interact inappropriately with one another. Hsps recognize and bind to other proteins when these other proteins are in non-native conformations, whether due to protein-denaturing stress or because the peptides they comprise have not yet been fully synthesized, folded, assembled, or localized to an appropriate cellular compartment. Binding and/or release of these other proteins is often regulated by association with and/or hydrolysis of nucleotides. Typically, Hsps function as oligomers, if not as complexes of several different chaperones, co-chaperones, and/or nucleotide exchange factors. Interaction with chaperones is variously responsible for (*a*) maintaining Hsps' partner proteins in a folding-competent, folded, or unfolded state; (*b*) organellar localization, import, and/or export; (*c*) minimizing the aggregation of non-native proteins; and (*d*) targeting non-native or aggregated proteins for degradation and removal from the cell. Presumably, the last two functions are most important in coping with environmental stress.

Not all Hsps are stress-inducible, but those that are respond to a variety of stresses, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances. Activation of various intracellular signaling pathways results in Hsp expression. All known stresses, if sufficiently intense, induce Hsp expression. Accordingly, Hsps are equally well termed stress proteins, and their expression is termed the stress response. A common aspect of these inducing stresses is that they result in proteins having non-native conformations (12), which is consistent with the function of Hsps as molecular chaperones.

Implications of the Published Literature for Ecological and Evolutionary Studies of Hsps

Space limitations necessitate that we choose among numerous equally valuable references in preparing this review. To present both the breadth and depth of research relevant to the evolutionary and ecological physiology of the heat-shock response, we have compiled a near-comprehensive bibliography of

that literature, which is available electronically (13) on the World Wide Web in the Supplemental Materials Section of the main Annual Reviews site (http://www.AnnualReviews.org).

A first implication of this massive literature is that the Hsp field has long ago concluded its exploratory phase. Showing that an as-yet-unexamined species expresses Hsps in response to heat or other stresses no longer has any particular novelty.

Second, much of the work on Hsps outside the laboratory or in nonmodel organisms was undertaken before the molecular diversity of Hsps and their function as molecular chaperones was obvious. In the interim, the experimental tools for examining Hsps and the standards for such examinations have both advanced considerably. As a result, much of the earlier work on evolutionary and ecological physiology of Hsps regrettably either does not withstand current scrutiny or contributes little to issues of current interest. Several issues are obvious:

1. Many of the apparently singular Hsps of previous years, often detected by one-dimensional electrophoresis and autoradiography, are now known to represent entire families of Hsps, often with (*a*) discrete distributions within the cell (e.g. cytoplasmic-nuclear, mitochondrial, chloroplast, or endoplasmic reticulum), (*b*) different degrees of inducibility (constitutively expressed, constitutively expressed but increasing during or after stress, exclusively inducible), (*c*) differing kinetics of induction and removal from the cell, and (*d*) differing tissue specificity. Representing this diversity as a single Hsp or two Hsps ("constitutive" and "inducible") through use of nonspecific probes or lysates of whole organisms and organs can obscure phenomena of great significance (e.g. compare Refs. 14 and 15 with 16). This problem is sometimes remediable only with great difficulty. Often, highly specific probes are available only for standard model organisms, particularly at the level of proteins, and great care must be taken in applying these probes to non-standard organisms (17).

2. Inducible stress tolerance is increasingly understood to result from numerous molecular mechanisms, of which Hsps are collectively only one. Other mechanisms include synthesis of osmotic stress protectants such as polyols and trehalose, modifications of the saturation of cell membrane lipids (homeoviscous adaptation), compensatory expression of isozymes or allozymes of significant enzymes, metabolic arrest, radical scavengers (superoxide dismutase, glutathione system, cytochrome P450), and so on. Accordingly, the unambiguous attribution of stress tolerance to Hsps in general or to any specific Hsp requires more than correlative evidence (18–21). Increasingly, proof resulting from genetic or direct experimental manipulation is

becoming the standard for establishing the functional or evolutionary significance of Hsps. Again, this rising standard is often met only with great difficulty in ecological and evolutionary physiological studies, for many of the techniques for genetic and experimental manipulation are not readily applicable to the more ecologically and evolutionarily interesting species.

HSP-INDUCING STRESS IN NATURE AND NATURAL INDUCTION OF HSPS

Depending on their geographic locale, organisms in nature risk exposure to temperatures ranging from -100° to more than 100°C, and comparable extremes of chemical and gas concentration, food and water availability, hydrostatic pressure, radiation, and toxic substances of human origin. Seemingly, Hsp expression should be a common occurrence in nature. In reality, however, movement and/or other behaviors may often enable organisms to avoid Hsp-inducing stress in nature by exploiting equable microhabitats in otherwise stressful environments (22–24). Also, biochemical specializations other than Hsps may stabilize many organisms (or particular stages of their life cycles) so that environmental extremes are not particularly stressful.

Even equable environments can contain Hsp-inducing microhabitats, and even mild stresses can induce Hsps when multiple stresses act in combination. For these reasons, we can assume neither the presence nor the absence of Hsp expression in nature; for that matter, we still do not know in any comprehensive sense whether wild organisms routinely, occasionally, or seldom express inducible Hsps. A growing body of evidence, however, establishes that at least in some circumstances and taxa, Hsp induction is not solely a laboratory phenomenon.

One caution in evaluating the subsequent account is that organisms in nature seldom undergo only one stress at a time. For example, an insect larva undergoing natural heat stress in a rotting fruit may simultaneously experience intense ultraviolet radiation, desiccation, and diverse alcohols and aldehydes, among other stresses. This situation differs from that in most laboratory experimentation, which involves one or a few stresses and makes attribution of Hsp expression to a particular stress in nature more complicated.

Aquatic Temperature Stress

Due to the physical characteristics of water, the aquatic environment can be extremely stressful to its inhabitants. In general, the high specific heat and thermal conductivity of water ensure that the majority of aquatic organisms will have body temperatures equivalent to that of their surroundings. Furthermore, the relative thermal homogeneity of aquatic environments can frustrate behavioral

avoidance of thermal extremes. Some aquatic ectotherms nonetheless inhabit thermally equable habitats or waters with enough thermal diversity to enable behavioral thermoregulation; our focus is on those species that do not.

In the aquatic environment, habitual exposure to Hsp-inducing thermal stress may be most common in sessile organisms that occur in shallow, stagnant water (e.g. ponds, tidepools, swamps, tidal flats) or in the intertidal zone. Corals, for example, routinely undergo thermal stress that results in bleaching, during which the corals' endosymbionts die. Even modest increases in water temperature of 1-2°C can bleach corals; these temperatures also induce Hsp expression in several species (25, 26). Marine intertidal invertebrates undergo even larger increases in body temperatures during tidal emersion (27–30). For example, during aerial exposure intertidal mussels' body temperatures exceed seawater temperatures by more than 20°C (31), resulting in Hsp expression (29). A similar phenomenon occurs in encysted brine shrimp (*Artemia*) embryos (32, 33). Even relatively mobile aquatic ectotherms such as fish may undergo heat shock in nature (34, 35). For example, gobiid fishes of the genus *Gillichthys* can become trapped in shallow water, which is heated by the sun. Summer-acclimatized fish have higher levels of Hsp90 in brain tissue than do winter-acclimatized fish (34). In addition, the threshold Hsp induction temperature for one species, *G. mirabilis*, is significantly higher in summer than in winter. These data suggest that seasonal variation in water temperature can alter the heat-shock response. More exotic venues for aquatic thermal stress include thermal effluents of power plants, hydrothermal vents, and thermal hot springs, in which temepratures can exceed 100°C (see *Hsps of Archaea*).

Terrestrial Temperature Stress

Unlike aquatic environments, terrestrial environments often offer diverse heat sources and sinks and retreats that organisms can exploit to avoid thermal stress. Thus, natural thermal stress and accompanying Hsp expression in terrestrial environments typically involve limitations in mitigating thermal extremes by movement and conflicts between thermoregulation and other needs. Salamanders, for example, which ordinarily maintain cool temperatures in nature, can inadvertently retreat beneath small sunlit rocks that become warm enough to induce *hsp70* mRNA expression (36); by abandoning these rocks to find cooler retreats they may risk immediate desiccation or even warmer temperatures.

The least equivocal case for routine exposure to Hsp-inducing temperature stress is for plants, which cannot change location except as seeds or pollen and can be limited in their ability to adjust heat exchange with the environment (37). Thus, plants in nature can become extremely hot (38). By inference, the entire range of plant heat-shock responses (8, 39) should manifest themselves in nature. Indeed, a small number of case studies document natural Hsp expression

(40–45), which can be greatest at times of day or in regions of an individual plant at which temperatures are highest (46). Plant species can differ dramatically, however, in both the magnitude and diversity of the particular Hsps that are expressed during days with especially warm weather (41). Plants should also be prone to natural cold stress (47), which ought to induce expression of Hsps (48–51).

Not surprisingly, therefore, many of the cases of natural thermal stress in animals on land involve animals that live inside or on plants (e.g. 52). *Drosophila* larvae and pupae encounter temperatures exceeding 40°C if the necrotic fruit they infest is in the sun, and express Hsp70 in response (53, 54). Presumably, other animals that cannot escape or offset intense solar heat loads will also express Hsps in nature; this hypothesis awaits systematic study. A unique case concerns desert ants, *Cataglyphis*, which voluntarily undergo body temperatures of >50°C, presumably to escape predators (55). The concentration of Hsp70 family members increases in this species before it naturally encounters high temperatures, as if in anticipation (56).

Terrestrial vertebrates are often especially effective in escaping heat stress, but both they and invertebrates are occasionally hyperthermic during intense physical activity, fever, or to conserve water. In birds and mammals, such hyperthermia activates HSF (the heat-shock transcription factor) and increases the level of Hscs (Hsp cognates, constitutively-expressed Hsps) and Hsps (57–59). Natural hypothermia of animals can be far more conspicuous than natural heat stress, involving diapause, overwintering in exposed sites, hibernation, and sometimes outright freezing. Diverse insects express Hsps in response to cold shock or during overwintering in diapause, although the identity of these Hsps, their tissue specificity, and their developmental regulation vary greatly (60–64). Some euthermic rodents express 70-kDa Hsps in response to cold ambient temperatures, possibly in tandem with nonshivering thermogenesis (65), and ground squirrels (*Spermophilus*) increase Hsp70 family members and ubiquitin-protein conjugates during hibernation (66).

In summary, laboratory studies of the heat-shock response often have proceeded far in advance of fieldwork that establishes an ecological context for their interpretation. Documentation of both natural thermal stress and Hsp expression in nature can provide this context, and a small but growing number of field studies demonstrate that such documentation is feasible.

Inducing Stresses Other than Temperature

Virtually every nonthermal stress can induce Hsps (10, 67). Rarely, however, are these nonthermal stresses ecologically relevant; the literature in this area typically focuses on chemical stressors, and the corresponding data are essentially pharmacological. Even when the stress in question is ecologically

relevant, few studies of multicellular eukaryotes examine it in the field or in intact tissues and organisms. Some exceptional work, however, concerns plants and brine-shrimp (*Artemia*). The resurrection plant, a desert species, expresses Hsps in vegetative tissues during water stress; this expression is thought to contribute to desiccation tolerance (68). Similarly, rice seedlings express two proteins in the Hsp90 family upon exposure to water stress and elevated salinity (69). Embryos of the brine shrimp, one of the most hypoxia-tolerant metazoans, contain large quantities of p26, a molecular chaperone hypothesized to stabilize proteins during long bouts of anaerobic dormancy (see *Development*). Clearly, additional evolutionary physiological research in this area is sorely needed.

A recurrent theme is that thermal stress and these alternative stressors often result in different patterns of Hsp expression, indicating a diversity of regulatory mechanisms. Examples include variation in the expression of Hsp70 and ubiquitin in the *Drosophila* central nervous system under anoxia (70), and in protein expression during osmotic shock in isolated fish gill cells (71).

Bioindicators

Owing to its responsiveness to diverse forms of stress, the heat-shock response has undergone widespread application in biomonitoring and environmental toxicology (72–75). In many cases, Hsps are especially useful biomarkers because their induction is much more sensitive to stress than traditional indices such as growth inhibition. The use of Hsps as biomarkers is most widespread in aquatic toxicology. Most of the literature demonstrates elevated Hsp levels or induction of Hsps under laboratory conditions and then proposes Hsps as a potential indicator of pollutants or toxins in the environment. For example, exposing freshwater sponges to pollutants extracted from river water elevates Hsp70 levels, which increase still further when thermal stress is also imposed (76). Additional examples of Hsp expression in aquatic toxicology concern rotifers, (77), marine sponges (78), amphipods (79), polychaetes (80), mollusks (81–84), and fish (85–87). Other applications purposefully deploy organisms in potentially polluted aquatic systems as biosensors (88, 89).

In the terrestrial environment, where heavy metal contamination and pesticide or herbicide accumulation can be critical problems, common soil organisms such as invertebrates (90) are useful Hsp-biomonitors of toxicants. For example, centipedes (*Lithobius*) collected from near a smelter had higher Hsp70 levels than those collected from unpolluted areas (91). Potentially, combinations of heavy metals can induce such distinctive patterns of Hsp expression in soil nematodes that these patterns can become diagnostic fingerprints for specific toxicants in soils (92).

Some aspects of the stress response, however, present problems for the use of Hsps as biomarkers in environmental toxicology. Because so many different

stresses induce Hsps, investigators may be unable to attribute changes in Hsp expression to any particular environmental stress. Organisms in the field often undergo multiple stresses simultaneously, the interaction of which can yield significant Hsp expression even when no single monitored toxicant is at harmful levels. Conversely, Hsps induced by another stress can enhance tolerance of a toxicant whose presence is being monitored. Laboratory studies support the difficulty of teasing apart environmental factors and attributing Hsp induction to a single stressor. For example, freshwater sponges exhibit greater tolerance of pollutants following a sublethal heat stress (76). Among the vertebrates, diseased fish have elevated levels of Hsps in their tissues, and disease-related expression may interfere with the use of Hsps as a biomarker (93). Thus, because numerous factors can induce Hsp expression and stress tolerance, the utility of Hsps as biomarkers of environmental toxins may be limited.

ENVIRONMENTAL AND BIOLOGICAL CORRELATES OF THE HEAT-SHOCK RESPONSE AND HEAT-SHOCK PROTEINS

Many investigators view correlations of organismal traits (e.g. Hsp expression) and environmental or biological variables (e.g. level of environmental stress, developmental stage, distinctive role in a parasitic or symbiotic relationship with another species) as prima facie evidence of biological adaptation, and thus have actively sought such correlations in terms of the heat-shock response. While the probative value of such evidence in establishing adaptation has met with skepticism (94), in this section we consider the evidence for such correlations, whatever their meaning.

Variation in the Stress Response Along Environmental Gradients of Stress

To understand how Hsps result in stress tolerance at the organismal level, many investigators have characterized the stress response along gradients that occur in nature. One central question is whether organisms from environments with little stress have a different or reduced stress response compared with organisms from environments with much stress. Little and much stress might correspond to the center and edge of a species' range, low versus high elevation, xeric and hot versus mesic and cool climate, temperate versus tropical/polar latitude, low versus high intertidal, and so on. In general, the resulting data support a correlation among Hsp expression, stress tolerance, and gradients of environmental stress. These gradients have received uneven attention, however, and their study has yielded mixed results. Comparative studies across many degrees of latitude have not produced the same results as studies of gradients on smaller scales

(e.g. diurnal or microclimatic variation in stress). Currently, not a single study has examined the stress response over the entire geographical distribution of a species; thus, whether species at the extremes of their distributions have an augmented heat-shock response is yet to be determined.

The majority of multi-species comparative studies focus on three aspects of the stress response: the minimum (threshold) and maximum temperatures at which Hsps are expressed and/or are present in cells, Hsp concentrations in cells, and the diversity of the specific Hsps that are expressed. Except for the work on threshold and maximum temperatures, much of this literature is a hodgepodge of disconnected studies that are seldom comparable because of methodological differences and permit few conclusions other than that species vary in the details of their stress response. Whether this variation has environmental correlates is uncertain. A rare and exemplary exception is the work of Bosch and colleagues on species of *Hydra* (95, 96); below we discuss this and other similar work.

In general, the threshold temperature for Hsp induction is correlated with the typical temperatures at which species live, with thermophilic species having a higher threshold than psychrophilic species. For example, a relatively cold-water, northern species of mussel (*Mytilus trossulus*) has a lower threshold for Hsp70 expession than its congener, *M. galloprovincialis*, a warm-water species with a more southern distribution (97). Limpet species that occur in the upper regions of the intertidal (*Lottia digitalis* and *Lottia pelta*) induce Hsps at 3–5°C higher than the threshold for limpets that occur lower in the intertidal (*Tectura scutum*) (AL Haag & GE Hofmann, unpublished data). Subtidal species of the marine snail *Tegula* exhibit much the same pattern (98). Aggregate expression of Hsp70 family members (17) occurs at 3–4°C higher in *Drosophila melanogaster* than in *D. ambigua*, a fruit fly of Palearctic origin (56). The same study reports a similar pattern for the desert ant *Cataglyphis* and *Formica polyctena*, a red wood ant from a temperate climate. One remarkable outcome of the *Cataglyphis* study is that Hsp synthesis in the desert ants continues at temperatures up to 45°C, whereas temperatures above 39°C inhibit Hsp synthesis in the temperate species. A similar pattern (although not as extreme) is evident for desert and non-desert *Drosophila* (22). These results suggest that translation itself may have an upper thermal maximum that varies among species adapted to different temperature environments.

Antarctic organisms represent a special case of psychrophily because the temperatures they experience are both extremely cold and extremely stable. In combination, do these conditions result in the evolutionary loss of a heat-shock response? In the subtidal alga, *Plocamium cartilagineum*, heat-inducible *hsp70* and *ubiquitin* transcription still occur, although the threshold is a spectacularly cold 5°C (99). Antarctic yeast species express Hsps at much lower temperatures than does *Saccharomyces*, and at least one species lacks inducible

thermotolerance (100, 101). In Antarctic fish, the picture is not as clear. Although a broadly cross-reactive anti-Hsp70 antibody can detect isoforms of Hsp70 in various tissues of the fish *Trematomus bernacchii*, heat shock temperatures from 6 to 10°C do not induce additional Hsp70 accumulation (GE Hofmann, unpublished data). A member of the *hsp70* gene family is present in two Antarctic fish species, *T. bernacchii* and *Notothenia coriiceps* (AC Whitmer & GE Hofmann, personal communication), and has been sequenced in Antarctic fish species (102). At the other extreme, some hyperthermophilic Archaea require temperatures in excess of 100°C to induce Hsp expression (see *Hsps of Archaea*).

Thermal stress gradients can be seasonal as well as geographic. In some cases, both Hsp expression and thermotolerance increase during warm seasons. The intertidal mussel *Mytilus californianus* displays significantly different Hsp induction profiles in summer than in winter, and summer-acclimatized mussels induce Hsps at a threshold temperature that is 6°C higher than the threshold in winter-acclimatized mussels (103). However, whether the accentuated Hsp expression in mussels in summer results in greater thermotolerance at the organismal level is unknown. Fish (34, 35) and intertidal invertebrates (31) also vary seasonally in Hsp expression.

In addition to work we cite elsewhere, other studies examine geographical gradients in fish (104, 105, 105a), maize (106), reptiles (107), and *Drosophila* (108); intertidal gradients in limpets (27); diurnal temperature change in *Drosophila* (109) and intertidal mussels (29); diurnal variation in spruce trees (46); and seasonal variation in insects (110).

One issue for future consideration is whether Hsps in general are specialized to function at higher temperatures than other proteins (especially enzymes), and whether homologous Hsps of species from various thermal environments have corresponding variation in thermostability of function (111). For example, that an Hsp's resistance to thermal denaturation varies according to the thermal niche of the species that expresses it has been demonstrated for only a single Hsp, alpha-crystallin (112). Another issue is how differing thresholds of Hsp expression have evolved, whether through mutations in HSF, general thermostability of proteins and cells (113), or some other mechanism (114).

The Parasitic Environment

The roles of Hsps in host-parasite interactions have received considerable attention from both clinical and biological perspectives, with the majority of the research in two general categories. First, from the perspective of the host, Hsps expressed by invading parasites are potent antigens that elicit an immune response (115–117); parasites' Hsps are thus potentially useful in generating vaccines (118). From the perspective of the parasite, the synthesis of Hsps is a

cellular defense mechanism that enables the parasite to live in different thermal environments throughout its life-cycle (119). Parasites that infect mammalian and avian hosts can undergo profound changes in temperature during the transition to these hosts (with internal temperatures of 37°C or above) from ectotherm hosts or free-living stages. Induction of Hsps commonly accompanies this transition. Numerous studies have demonstrated developmentally regulated expression of Hsps in parasites; expression differs throughout the life-cycle both quantitatively and in the types of Hsps that temperature change induces. For example, mRNA transcripts for *hsp70* and *hsp83* homologues increase up to 100-fold as *Trypanosoma brucei* leaves the tsetse fly and enters a mammalian host (120). Aquatic snails release cercariae of the parasitic helminth, *Schistosoma mansoni*, into freshwater; cercariae penetrate human skin and develop into adult worms, eventually causing liver cirrhosis. The cercariae express two heat-inducible proteins that are not present in other stages (121).

Parasites that have an insect as the invertebrate vector have received much attention with regard to the developmentally regulated expression of Hsps. Examples include parasitic nematodes (122); cestode parasites (123); the malarial organism *Plasmodium* (124); *Borrelia burgdorferi*, the etiological agent of Lyme disease (125); the protist *Leishmania* (126); *Trypanosoma cruzi* (127); and *Theileria* (128).

Some parasite life cycles do not involve an animal vector; a free-living stage of the parasite occurs in water or soil and enters the host. In several cases, induction of Hsps accompanies the transition from the environment into the host. In the fungal parasite *Histoplasma capsulatum*, the temperature shift upon infection of a mammalian host cues both the transformation from a mycelial form to a budding yeast morphology and the expression of Hsps (129, 130). *Eimeria*, an intestinal parasite of numerous animals, expresses Hsp90 during infective life stages. *Eimeria* parasites are particularly interesting because this genus infects diverse hosts with correspondingly diverse body temperatures (e.g. marine fish, poultry, and cattle). However, specificity of infection is high at the species level, e.g. cattle are the exclusive host for *Eimeria bovis* (131). Whether the heat-shock response of *Eimeria* co-evolved with its speciation into these hosts is an open question.

Finally, the heat stress that infective life cycle stages of parasites experience is as diverse as their hosts. In nature, parasites of ectotherms can encounter dramatic shifts in temperature when their hosts' body temperature varies, as has been reported for parasites of reptiles, fish, and intertidal organisms (132, 133).

Symbiosis

Just as Hsps may play an important role in parasitism, in which one species maintains a close but antagonistic relationship with others, they also function

in symbiosis, in which interspecific relationships can be equally close but not adversarial. Perhaps the most general example of this point concerns mitochondria and chloroplasts, which evolved from endosymbionts that colonized other cells early in the history of life. These organelles often require proteins that are encoded in the nuclear genome and synthesized by the host cell, and hence must be imported into the organelle. Hsps play diverse roles in this process in mitochondria. A cytoplasmic Hsp70 family member maintains peptides in an unfolded conformation, which enables the peptides to pass through pores in the mitochondrial membrane; a mitochondrial Hsp70 is part of the protein machinery that imports the peptide; and the Hsp60/Hsp10 apparatus participates in the folding of the imported protein (134). Several groups of primitive eukaryotes contain still other endosymbiotically derived organelles, the hydrogenosome and the nucleomorph, whose Hsps share a characteristic sequence with those of mitochondria and proteobacteria (135–138). The Hsp sequence similarities have been used to suggest that hydrogenosomes may derive from mitochondria, share a common origin with mitochondria, or represent independent colonizations of early eukaryotic cells (135–137, 139).

Aside from endosymbiotically derived organelles, the best-studied symbioses concern bacterial endosymbionts that infect insects, including aphids, flies, ants, and cockroaches. Aphids, for example, harbor the bacterium *Buchnera* in specialized cells (bacteriocytes) within a distinctive structure in the body cavity, the bacteriome (140). The bacteria express a protein, symbionin, at especially high levels, and this protein is a member of the GroEL (Hsp60) family. Other bacterial chaperones, including GroES (Hsp10) and DnaK (Hsp70), are also present at high levels (140). A similar phenomenon is evident in tsetse flies (141).

The function and significance of these high Hsp levels is enigmatic. The Hsps apparently are not a response of the endosymbionts to a novel (and therefore stressful) host environment, as the *Buchnera*/aphid symbiosis is 150–250 million years old. The endosymbionts, however, have been evolving at an especially high rate; thus, the elevated molecular chaperones could be compensating for decreased protein stability due to the accumulation of numerous amino acid substitutions (142, 143). Nonetheless, the bacteria themselves can mount a strong heat-shock response when their host undergoes stress (144). Other relevant symbioses include X-bacteria in the symbiosomes of *Amoeba* (e.g. 145), *Bradyrhizobium* and *Rhizobium* in the root nodules of nitrogen-fixing plants (146), and the zooxanthellae component of corals (26, 30). A recurrent theme is that the endosymbionts modify the amount and/or diversity of Hsps present in the symbiosis. In some cases, this modification is thought to contribute to the maintenance of the endosymbionts within the host, and in others to the augmentation of the heat-shock response of the symbiosis as a whole. Finally,

Wolbachia, a bacterial endosymbiont that infects millions of arthropod species, both interferes with the mating of infected and uninfected hosts and can alter their constitutive expression of Hsp70 and Hsp90 family members (147). Simulated natural heat stress can diminish this reproductive interference, possibly by overriding the symbiont's effect on the host Hsps.

Development

Many species exhibit characteristic and distinctive patterns of Hsp expression (or nonexpression) during the various stages of development, including gametogenesis, embryogenesis, and metamorphosis (e.g. 148–151). These patterns are often consistent with enhanced stress resistance in developmental stages that encounter unusual levels of environmental stress or during circumstances such as dormancy and diapause (see below). In other cases, developmental programs of Hsp expression ensue even in the absence of any obvious environmental stress. One common pattern is that one or more Hsps are not expressed in the initial phases of embryogenesis (152–156) or late in gametogenesis (157–161), possibly because Hsps can be harmful to developing cells (see *Deleterious Aspects of Hsps*). Parental provision of Hsps or *hsp* mRNAs can sometimes override gametic or embryonic absence of Hsp expression (162, 163); in other cases this absence presumably poses a significant problem for continued development in the face of stress (164). Stress not only can kill vulnerable developmental stages outright, but also can produce lasting damage to surviving organisms, such as the phenocopying of genetic defects; Hsps may minimize such defects (165, 166).

Adaptational analyses of the developmental expression of Hsps are diverse. Some plant seeds presumably must endure extremes of heat, desiccation, and other stresses before germinating, and some must germinate under especially challenging conditions. However, although seeds clearly undergo developmentally regulated expression of Hsps and embryos can express Hsps in response to environmental stress (167–169), few investigators have considered whether these patterns of expression are amplified or modified in species and ecotypes that naturally encounter especially challenging stress regimes (68, 170). Our state of knowledge is similar for fungal spores, which express particular Hsps in a developmentally regulated program (e.g. 171, 172). Several interesting case studies are available for animals, although a general pattern is yet to emerge. In the most spectacular example, encysted brine-shrimp (*Artemia*) embryos undergo developmental arrest, in which they may survive for years without environmental water or oxygen. The encysted embryos accumulate enormous concentrations (15% of total protein) of a small Hsp (173–177) and trehalose (178), and suppress ubiquitination of damaged proteins (179). Non-adult *D. melanogaster* infest necrotic fruit, which can become extremely warm if it is sunlit (53, 54); this species mounts a massive heat-shock response, which

is greatest in the developmental stages that presumably undergo the most exposure to natural heat stress (18, 19, 180). Other flies overwinter while at a particular developmental stage, and undergo considerable Hsp expression in response to cold (60–63). Later in development, ubiquitin may assist in the degeneration of flight muscles that are no longer needed after nuptial flights of insects (181). Finally, the temperature threshold for expression of Hsps may itself undergo modification; e.g. the threshold decreases in mammalian testis, in which gametogenesis normally occurs at lower temperatures than in the core of the body (182, 183).

Aging and Senescence

As mammals age, damage to proteins progressively accumulates, and both the ability to express Hsps (e.g. Hsp or *hsp* mRNA levels after a standard exposure to heat or other stress) and stress tolerance deteriorate (184, 185). Moreover, individual Hsps can become less able to mitigate the effects of stress on proteins as mammals age (186). In ecological and evolutionary terms, whether similar Hsp-aging relationships are important or even evident in wild organisms is unknown, although these relationships occur in diverse species in the laboratory: *Drosophila* (187, 188), nematodes (189–191), and *Daphnia* (192).

These findings have provoked great interest in how Hsps potentially affect senescence and lifespan. A unifying hypothesis in the foregoing work is that protein damage, due primarily to oxidation/free radical activity, gradually accumulates during the life of a cell or organism and can lead to death if unabated; Hsps and other molecular stress responses ordinarily can mitigate this damage to some extent, and the decreasing expression of Hsps with age therefore contributes to mortality. If this hypothesis is correct, then treatments that both reduce damage to protein and increase Hsp expression (e.g. heat shock) should prolong life. In nematodes (*Caenorhabditis elegans*), some single-gene mutations that increase lifespan are associated with increased thermotolerance, but through as-yet-undescribed mechanisms (189–191). In *Drosophila*, heat shock extends lifespan (193), and this extension is enhanced in flies transformed with additional copies of the *hsp70* gene (194). Nutrient deprivation can also extend life in rodents, presumably by reducing the metabolic rate and consequently, oxidative damage to proteins; starvation, however, variously increases, decreases, or has no effect on Hsp expression (195–199).

FITNESS CONSEQUENCES OF HSP EXPRESSION

General Issues and Beneficial Aspects of Hsps

Understanding the consequences of variation in Hsps and the stress response for Darwinian fitness requires a detailed appreciation of the mechanisms by which Hsps mitigate the impact of stress on individuals in natural populations. These

mechanisms are becoming well understood at the level of model proteins with which Hsps can interact, but are progressively less well understood at the level of the cell, tissue, organ, and whole organism. At the level of the model protein, various stresses clearly either directly or indirectly result in conformational change, and Hsps typically promote the reacquisition or maintenance of the native structure and function by minimizing the tendency of non-native proteins to interact inappropriately (200, 201). In cells, stress-induced conformational change, protein aggregation, and molecular chaperoning of model proteins are also well established (200–202), and many cellular components differ in stress-tolerant and stress-intolerant cells (67).

Two primary issues impede the linkage of variation in these well-established mechanisms and phenomena to variation in the fitness of individual complex multicellular eukaryotes. First, is the variation in sensitivity to stress among cells, cell types, tissues, organs, and organisms attributable to a small number of critical lesions, especially sensitive targets of stress and functions of specific Hsps in protecting or repairing these lesions/targets? Or is variation in sensitivity to stress an aggregate function of a widespread and diverse impact of stress on cellular structures, with Hsps mitigating multiple lesions in diverse ways (21)? The former alternative may be more analytically tractable than the latter. Second, given that cells and organisms may have multiple Hsps in each Hsp family, multiple Hsp families, and multiple non-Hsp mechanisms of stress mitigation, how can we unambiguously establish the contribution or importance of any particular Hsp, Hsp family, or mechanism in the complex cell, tissue, organ, or organism? Much of the published literature on the functional consequences of Hsp expression for whole organisms or the cells they comprise runs afoul of these issues. Literally thousands of studies report correlations between Hsp expression, diverse biological functions in the face of stress, and stress tolerance, but these typically conclude that their findings are at best consistent with a role of one or more Hsps in stress tolerance. Evaluating the roles of single factors in complex systems is an ongoing challenge in most areas of the biological sciences, and the heat-shock field largely has not yet deployed counterparts of the solutions that other fields have developed.

One conspicuous and major exception includes techniques and approaches, primarily drawn from molecular biology and genetics, that allow the manipulation of individual Hsps or specific genes that encode them. In rare instances, a species naturally may have an unusual genetic system (203) or a diminished suite of Hsps (95, 204) that accomplishes the same end; also, several chemical compounds may specifically inhibit one or more Hsps (e.g. 205, 206). The general implication of the resulting work (Table 1) is that, even in whole organisms or the cells they comprise, variation in single Hsps can be consequential for fitness. Some specific implications are as follows: Individual Hsps can have

Table 1 Phenotypes of multicellular eukaryotes, and the cells and tissues that they comprise, for which Hsps are necessary and/or sufficient[a]

Protein	Phenotype
Hsp10	Cellular: tolerance of ischemia (no phenotype) (308); tolerance of ischemia when co-expressed with Hsp60 (308)
Hsp27	Cellular: resistance to chemotherapeutic drugs (309); resistance to hydrogen peroxide (310, 311); resistance to hydrogen peroxide (no phenotype) (312); resistance to ultraviolet radiation (no phenotype) (312); resistance of tumor cells to monocytes (311); sensitivity to lymphokine-activated killer cells (no phenotype) (311); tolerance of hyperthermia (312–316); resistance to tumor necrosis factor (317) (310); tolerance of ischemia (318); resistance of actin polymers to cytochalasin (314); accelerated nuclear protein aggregation (319); accelerated decline of thermal radiosensitization (319)
Crystallin	Cellular: tolerance of hyperthermia (320, 321); tolerance of ischemia (318); resistance to tumor necrosis factor (310); resistance to hydrogen peroxide (310)
Hsp60	Cellular: tolerance of hyperthermia (no phenotype) (322, 323); tolerance of ischemia (no phenotype) (308, 322, 323); tolerance of ischemia when co-expressed with Hsp10 (308)
Hsp65	Cellular: tumor regression (324); loss of tumorigenicity (325) Tissue/organ: regression of malignant tumors (324)
Hsp70	Cellular: tolerance of hyperthermia (326) (316, 322, 323, 327–342); tolerance of ischemia/hypoxia (322, 323, 340, 343–345); recovery from translational and transcriptional inhibition following heat shock (335); regulation of heat-shock response (331, 346, 347); tolerance of endotoxin (348); reduced protein denaturation upon heat exposure (349); tumorigenicity (350); cell proliferation (351, 164); resistance to hydrogen peroxide (311); resistance of tumor cells to monocytes (311); sensitivity to lymphokine-activated killer cells (no phenotype) (311); escape from drug-induced cell cycle arrest (352); protein glycosylation (353); tolerance of ultraviolet radiation (354); apoptosis (351, 355, 356); resistance to apoptosis (no phenotype) (328, 329) Tissue/organ: recovery of contractility after ischemia (345, 357–359); reduction in myocardial infarct size (345, 359); reduction of hyperthermic damage to midgut (221); resistance of heart to ischemic injury (357–359); resistance of hippocampus to ischemic injury (360) Organismal: tolerance of hyperthermia (18–20, 109, 156, 203, 221, 224, 278, 361); growth and development (222); regulation of heat-shock response (361); persistence in nature (no phenotype) (277)
Hsc70	Organismal: tolerance of hyperthermia (203)
Hsp72	Cellular: apoptosis (no phenotype) (362); protection against heat-induced nuclear protein aggregation (319); protection against hypoxia (363); protection against thermal radiosensitization (319) Tissue/organ: reduction in myocardial infarct size (364)
Grp78	Cellular: protein secretion (229–231)

(*Continued*)

Table 1 (*Continued*)

Protein	Phenotype
Hsp90	Cellular: tolerance of hyperthermia (322, 323, 328, 329, 340); tolerance of ischemia (no phenotype) (322, 323, 340); apoptosis (362); apoptosis (no phenotype) (328, 329); cell proliferation and cell cycle control (365); glucocorticoid receptor function (205)
Hsp100	Organismal: host infection in *Leishmania* (126)
Hsp101	Cellular: tolerance of hyperthermia (366)
Many Hsps	Cellular: recovery of cell proliferation after heat shock (367); recovery from chromosome damage after heat shock (367–369); tolerance of hyperthermia (370, 371); tolerance of ischemia (336)
HSF	Organismal: oogenesis and development (372); thermotolerance (372, 373)

[a]In all cited work, specific Hsps have undergone experimental or natural manipulation.

pleiotropic effects, interacting with multiple systems in diverse ways. Findings from manipulations of individual Hsps usually (but not always) are consistent with the outcome of correlative studies (see above). Finally, despite the huge body of work on Hsps and the growing use of manipulative techniques, we have remarkably little physiological insight into exactly how the activity of Hsps culminates in the enhanced stress tolerance of multicellular eukaryotes and the cells and tissues that they comprise.

Interestingly, one clear conclusion that correlative studies have yielded is that Hsps cannot account for the entirety of inducible stress tolerance (207–217). Indeed, some component of inducible stress tolerance may be unrelated to protein synthesis in general (214, 218, 219).

Deleterious Aspects of Hsps

The many advantages of the heat-shock response suggest that natural selection should maximize the expression of Hsps. By contrast, the genes encoding Hsps have not undergone unlimited amplification in the genome, and the Hsps themselves are subject to strict autoregulation by multiple molecular mechanisms (220). These contrary findings suggest that Hsps can have both positive and negative impacts on fitness, and that natural selection may have acted to balance these impacts in setting the level of Hsp expression. For example, while small to moderate increases in Hsp70 levels enhance inducible thermotolerance in *Drosophila*, large increases in Hsp70 levels actually decrease thermotolerance (221); evolution thus may favor an intermediate level of Hsp70. A common theme in related work is that high levels of Hsps may be especially detrimental to cells or developmental stages in which cell growth and division proceed at high rates. *Drosophila* larvae transformed with extra copies of the *hsp70* gene have greater larva-to-adult mortality and slower development than do control

larvae; these strain differences are proportional to the number of Hsp-inducing heat shocks administered to the larvae (222). Larvae naturally varying in Hsp70 expression display a similar pattern (223). *Drosophila* cells engineered to express Hsp70 constitutively at first grow more slowly than control cells, but subsequently resume control growth rates once the Hsp70 is sequestered from the cytoplasm (164); indeed, *Drosophila* embryos remove Hsp70 from their cells rapidly after heat shock (224). A yeast strain that cannot express Hsp104 grows faster than its wild-type counterpart on some media (171). More generally, most animal species that have been studied do not mount a heat-shock response during early stages of embryogenesis (see *Development*), when protein synthesis may be especially intense.

These negative effects may have at least two nonexclusive explanations (222, 225, 226): First, Hsps at high concentration could be toxic, directly interfere with ongoing processes in the cell, or otherwise alter function to the detriment of the cell (220). Second, the synthesis and degradation of Hsps could consume an intolerably large fraction of a cell's or organism's nutrient and energy stores, and/or occupy so large a fraction of the synthetic/catabolic apparatus that the processing of other essential biomolecules suffers (226–228). Consistent with the first explanation, cellular sequestration of Hsp70 is correlated with the resumption of proliferation in cells constitutively expressing this protein (164). Also, overexpression of an Hsp70 family member inhibits protein secretion and reduction increases secretion in mammalian cells in culture (229–231); excess amounts of another Hsp70 family member can promote protein aggregation in vitro (M Borrelli & J Lepock, personal communication); and Hsp70 can perturb the normal structures of nascent polypeptides (232).

Tests of the second explanation have manipulated the costs of or resources for Hsp expression. Growth of corn in nitrogen-rich soil increases the synthesis of Hsps in response to a standard heat shock (233); in plants grown in nitrogen-poor soil, other proteins may be catabolized to supply amino acids for synthesis of Hsps (234). These findings suggest that Hsp synthesis can be nitrogen-limited in plants. Starvation reduces the expression of Hsp 70 family members in mice (195). In *Drosophila* larvae, by contrast, co-expression of β-galactosidase and Hsps has no greater cost for growth and development than does expression of Hsps alone (225). Further study of this apparent trade-off of the benefits and disadvantages of Hsp expression, moreover, has the potential to link evolutionary and mechanistic views of this problem that heretofore have been separate (222, 225).

MICROEVOLUTIONARY VARIATION IN HSPS

Hsps are routinely touted as adaptations that arose and are maintained via natural selection for stress resistance. Origin and maintenance of a trait by selection

require that it vary within populations, and that this intra-population variation have a genetic basis and affect the Darwinian fitness of individuals. Here we ask whether Hsps, the genes that encode them, and the factors that modify their expression display such patterns of variation and undergo stabilizing or directional selection in response to environmental stress.

First, not all intrapopulation and intraspecific variation results from genetic differences. For example, seasonal acclimatization and temperature acclimation in the laboratory can alter the minimum temperature at which *Gillichthys*, a gobiid fish, expresses an Hsp90 family member (34, 235). Seasonal acclimatization likewise affects Hsp70 levels in mussels (*Mytilus*) (103), and routine culture temperature affects the magnitude and temporal pattern of Hsp expression in HeLa cells (236). Such changes may stem from alterations in the cellular environment that modify the activation of HSF (113, 182, 236, 237). These changes, however, are not universal; laboratory thermal acclimation does not alter the thermal sensitivity of Hsp expression in fish hepatocytes in culture (238), *Drosophila* larvae (19), and mussels (103).

Even when acclimation and seasonal change are controlled, however, individuals within a population or species may vary in Hsp expression and/or the genes that determine it. Relevant research has examined this issue on two levels: direct sequence variation and restriction fragment length polymorphisms (RFLPs) (240). The sequence of *hsp70* varies among strains of the parasite *Trypanosoma* (241, 242) and the nematode *C. elegans* (243), and among conspecifics for some but not all of the mammalian *hsp70* family members (240), as does that of the 3′ untranslated region of *hsp27* in normotensive and hypertensive rats (244). RFLPs consistent with intraspecific variation either in the *hsp* genes or flanking regions are detectable in the *hsp60* and *hsp70* of the spirochete *Borrelia* (245), in multiple *hsp70* family members of mammals (240, 246, 247), and in several plant species (248–250). One putative instance of intraspecific variation in *hsp* copy number concerns *D. melanogaster*, in which at least five nearly identical copies of *hsp70* occur at two chromosomal loci. At locus 87A7, two copies are arranged as an inverted repeat (251, 252); at 87C1, two copies flank a region containing at least one additional copy (252, 253) plus numerous α/β repeats, which encode heat-inducible mRNAs of no proven function (254–257). Up to five additional *hsp70* copies have been reported, with copy number varying among strains and time of year (253, 255, 258–261). However, these reports either cannot exclude that such variation is actually in intergenic regions or that the reports are for *Drosophila* cells in culture or mutagenized laboratory strains rather than wild or even wild-type strains. The organization of the two chromosomal loci reportedly varies among natural populations (253). A less equivocal instance of evolutionary change in gene copy number concerns *Arabidopsis*, in which ecotypes vary in the number of ubiquitin-encoding repeats (262). For all

of the foregoing reports, the functional significance of intraspecific variation awaits elucidation or direct verification.

Hsp expression also exhibits genetic variation among individuals of a species; often, this variation is correlated with stress resistance (248, 250, 263–271). For example, isofemale lines of *Drosophila* founded from a single wild population differ more than twofold in Hsp70 expression; this variation is correlated with thermotolerance and is heritable (180, 223). Similarly, in the pathogenic fungus *Histoplasma*, naturally temperature-insensitive strains express more *hsp70* mRNA and do so at lower temperatures than in a temperature-sensitive strain (272). In humans, fibroblasts isolated from desert-dwelling Turkmen express more Hsps and have greater thermotolerance than fibroblasts from residents of more equable climates (273); presumably, however, these peoples do not differ in body temperature.

Given that the patterns of variation necessary for natural selection occur within species, that selection can alter Hsp expression is not surprising. Laboratory evolution at high temperatures paradoxically lowers Hsp70 expression and inducible thermotolerance in *Drosophila* (19, 274), and selection for resistance to hyperthermic paralysis alters both the *hsp68* promoter and the *hsr-omega* locus in *Drosophila* (254, 275). Additional findings relevant to natural selection and its underlying genetic basis come from closely related species, some so similar that they hybridize. In the fish species *Poeciliopsis monacha* and *P. lucida*, an unusual genetic system permits the generation of hemiclonal lines in which the paternal genome varies against a constant maternal genome. Hemiclonal thermotolerance was most strongly related to Hsc70 and only secondarily to Hsp70 levels, in a pattern consistent with straightforward Mendelian inheritance of parental genotypes and adaptation to the local thermal environment. By contrast, the heat-shock response of interspecific hybrids of tomato (*Lycopersicon*) is not intermediate to the parental responses (276). Non-hybridizing congeners often exhibit a correlation among the actual or inferred incidence of thermal stress in their environment, heat-shock response, and stress tolerance. Such data are now available for diverse animals (see *Variation in the Stress Response Along Environmental Gradients of Stress*).

A particular problem with such species comparisons is that the interpretation of the observed patterns is readily confounded by phylogenetic and statistical issues. A more general problem with both laboratory evolution and species comparisons is that they describe only a supposed correlation of the heat-shock genes/proteins of interest with evolution and seldom can establish the importance of the genes/proteins of interest to evolutionary process and outcome (19, 20). Study of free-ranging organisms (277) with *hsp* transgenes (e.g. 109, 278) may contribute much to resolving these problems.

THE EVOLUTIONARY HISTORY OF HSPS AND THE GENES THAT ENCODE THEM

Hsps are among the most ancient and highly conserved of all proteins. Homologues of Hsps occur in every species in which they have been sought, and in all kingdoms of living things. Thus, Hsps represent a remarkable example of molecular "descent with modification" at the levels of gene sequence, genomic organization, regulation of gene expression, and protein structure and function. So clear are the patterns of descent and modifications that they can be used to establish the evolutionary origins and the phylogenetic affinities of the major groups of organisms.

Hsps of Archaea and Exceptional Prokaryotes

The Archaea or archaebacteria are the most extremophilic and most primitive organisms. The heat-shock response of extremophilic Archaea and nonarchaeal extremophiles occurs at remarkably high temperatures (279), e.g. 88°C in *Sulfolobus* (279) and >100°C in the hyperthermophilic species designated ES4, a heterotrophic sulfur reducer isolated from a deep-sea hydrothermal vent (280). The archaeal genome encodes homologues of most Hsps represented in other prokaryotes and eukaryotes (279), as well as their consensus promoter sequences (281). Notably, the archaeal Hsp60 homologues assemble into a dual ring-like structure, termed a rosettasome or thermosome, that resembles the structure that the chaperones GroEL and GroES form in bacteria (279, 282, 283). The archaeal structures have ATPase activity and can bind denatured proteins (282). At least some Archaea and Eubacteria differ in the number of monomers that comprise these structures (284). Surprisingly, the archaeal Hsp60s (e.g. TF55 of *Sulfolobus*) most closely resemble not a bacterial homologue, but the eukaryotic protein TCP1, which assembles into the t-complex polypeptide 1 ring complex (TRiC) in the cytosol (285, 286). Previously, Hsp60 homologues were thought to be absent from the eukaryotic cytosol. A growing body of evidence suggests that TCP1/TRiC and GroEL/GroES play comparable roles in their respective organisms and cellular compartments (287–289). Meanwhile, Trent (290) has suggested that the primary function of TF55 may be cytoskeletal, with molecular chaperoning a secondary or derived function.

Genes encoding Hsps are present even in the smallest of genomes. These include the genomes of mycoplasmas (291) and the nucleomorph, the vestigial nucleus of a phototrophic eukaryotic endosymbiont in cryptomonad algae (138). The section on symbiosis (see above) reviews the distribution of Hsps in various other organelles of endosymbiotic origin. Apparently, the problem of protein folding is ancient and ubiquitous, necessitating molecular chaperones in these diverse cases.

Large-Scale Evolution of hsp Genes

The extraordinarily conserved nature of *hsp* genes (292) has facilitated their cloning, sequencing, and comparison in diverse organisms; their evolution is now becoming understood in detail. Gupta and colleagues have undertaken the most extensive surveys of *hsp* sequences, with a particular focus on organisms deemed critical to understanding the relationships of major taxa (292–297). The interpretations resulting from these comparisons relate to hypotheses about (*a*) the origin of eukaryotic cells, the eukaryotic nucleus, and endoplasmic reticulum (292, 296); (*b*) polyphyletic versus monophyletic origin of the major bacterial groups (292–294); and (*c*) the validity of the three-domain (Archaea, bacteria, and eukaryotes) dogma (292, 294). Whereas these interpretations are controversial, if not revolutionary, and therefore have not received universal acceptance, they nonetheless clearly illustrate how comparative analyses of *hsp* genes may address fundamental issues in evolutionary history.

On a less grand scale, *hsp* gene families represent superb case studies of how one or a small number of primitive genes can diversify to encode a suite of compartment- and function-specific proteins. One of many examples is *dnaK*, a single gene in Archaea and bacteria that has become the complex multigene *hsp70* families of *Saccharomyces* (298), *Drosophila* (253, 299), and *Homo* (300). Another example concerns the small Hsps, which evolution recruited to become a major component of the lens of the eye: alpha crystallin (301). A growing body of work examines the discrete evolutionary events by which these changes may have occurred, including gene duplication/conversion events (302), retrotransposition, horizontal exchange of genomes, and others. New technologies promise to advance this work exponentially.

Discrete Examinations of Molecular Evolution

Ideally, a complete study of the evolutionary physiology of Hsps might examine how the following co-evolve as populations or how closely related species enter environments in which they face novel stresses: sequence (both coding and non-coding) of the gene(s) for a particular Hsp, regulation of *hsp* gene expression, the role and importance of the Hsp in stress resistance, and the intensities and durations of stress that the populations and species actually face. Much of the evolutionary physiological investigation of Hsps fails to attain this admittedly ambitious goal for one or more reasons: (*a*) The species under study are too distantly related to reconstruct the functional, environmental, and genetic events during their divergence; (*b*) molecular biology, manipulative genetics, physiology, and environmental assessment are not all possible for the species in question; or (*c*) the breadth of the techniques and approaches necessary to perform such research is too daunting for a single research program. Two case studies exemplify both how this goal could be approached and how far the field has yet to go to attain it.

The coelenterate *Hydra oligactis* and several of its congeners are the only multicellular eukaryotes reported not to express Hsps in response to heat shock and other stresses. Other congeners (e.g. *Hydra attenuata* and *H. magnipapillata*) have a well-developed stress response; these and other data for putative ancestors of *Hydra* suggest an evolutionary loss of Hsp expression in *H. oligactis* (95, 96). Physiologically and ecologically, *H. oligactis* is deficient in inducible stress tolerance and disappears from certain habitats in nature during periods of stress (95). Subsequent work suggests that, at least for Hsp70, the loss has occurred due to mutations that affect the stability of *hsp70* mRNA, as *H. oligactis* has an *hsp70* gene and expresses a heat-inducible *hsp70* mRNA in quantities similar to that in the heat-tolerant *H. magnipapillata* (96).

Dipteran insects and their ancestors have undergone an evolutionary proliferation of *hsp70* genes. Mosquitoes and *Drosophila* share a distinctly arranged duplication of the inducible *hsp70* gene (303), suggesting that this proliferation predates the original diversification of the Diptera. Within the genus *Drosophila*, all groups other than the *melanogaster* subgroup of species apparently retain the primitive copy number of two (108, 304, 305). Within the *melanogaster* subgroup, all species examined to date have four copies except for *D. melanogaster* (253), which has at least five *hsp70* copies in its haploid genome (see Microevolutionary Variation in Hsps). Curiously, *D. melanogaster* expresses no Hsp100 family members, which are critical for thermotolerance in other organisms (204, 306). The proliferation of *hsp70* copies is correlated with the ecological and biogeographic distribution of *Drosophila* species (19). Whereas most *Drosophila* species have small geographic ranges or narrow ecological niches, two of the *melanogaster* subgroup species (*simulans* and *melanogaster*) have cosmopolitan distributions, and a third (*yakuba*) is ecologically diverse throughout sub-Saharan Africa.

CONCLUSION

Ecological and evolutionary physiological analysis of heat-shock proteins may be nearing the end of its initial descriptive phase. Although accounts of spectacular levels of Hsp-mediated stress resistance and exceptional consequences of Hsp expression will continue to be newsworthy, the major patterns of Hsp expression in multicellular eukaryotes are becoming so obvious that additional descriptive work is becoming increasingly difficult to justify. Clearly, however, major questions remain unanswered. How the activities of Hsps at the molecular level culminate in organismal stress tolerance and how the *hsp* genes, their regulation, the function of the proteins they encode, and the environments faced by the organisms in which they occur all co-evolve are but two of the

unresolved issues reviewed here. The perspective of evolutionary physiology can make significant contributions to the resolution of these and other issues. By placing results in actual environmental contexts, by assessing phenotypes of Hsps in the context of whole multicellular organisms, and by characterizing extant and historical variation in Hsps in natural populations and taxa, evolutionary physiologists can complement and extend a spectacular area of research that has been largely restricted to the molecular/cellular levels in the laboratory. By the same token, insights and techniques that laboratory-based investigators provide promise to continue to revolutionize the ecological and evolutionary study of Hsps. These approaches are both logical partners and necessary complements to one another (22, 307). Our understanding of Hsps has much to gain from the continued if not expanded synergy of these approaches.

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