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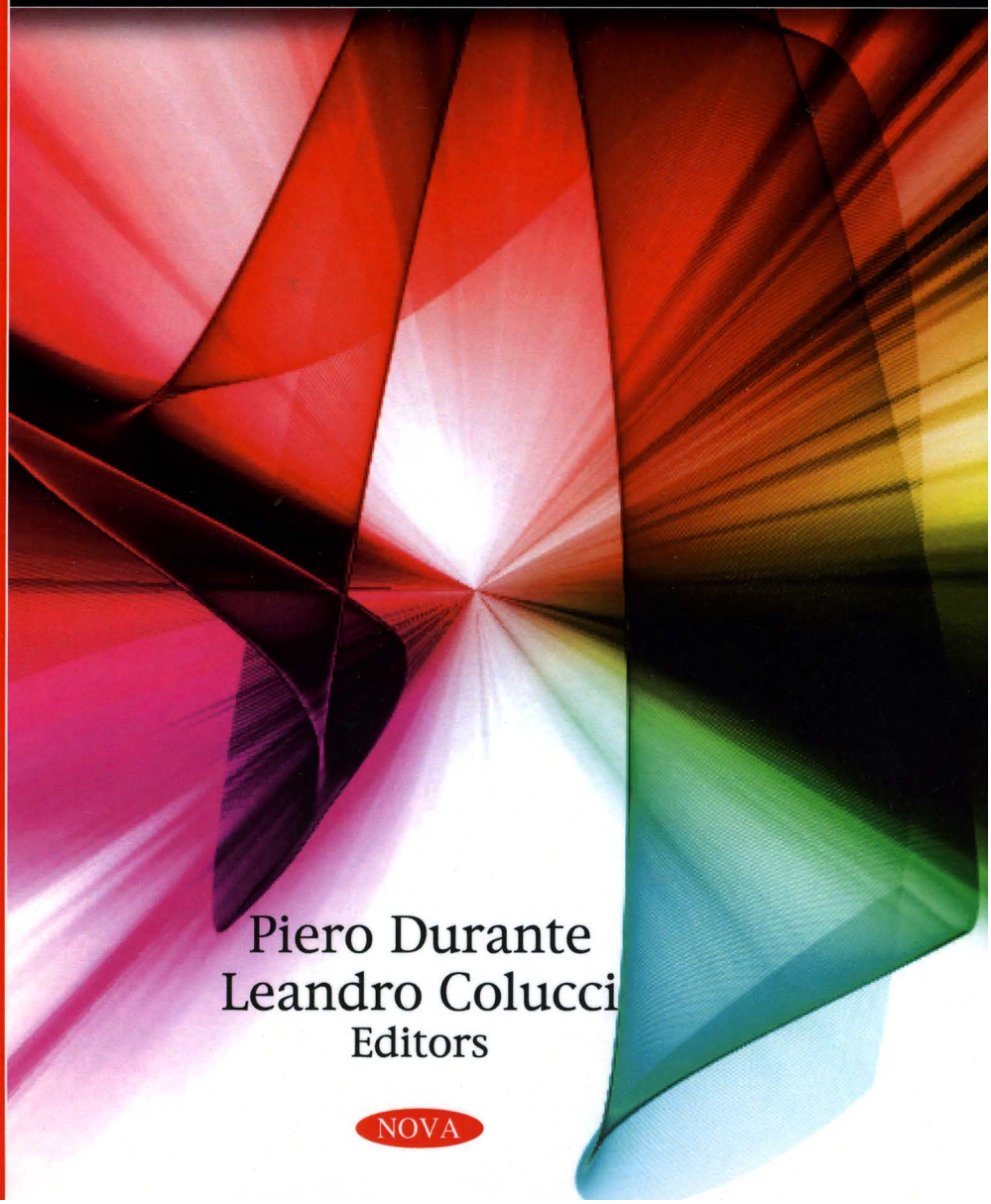

Piero Durante
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Editors

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HANDBOOK OF MOLECULAR CHAPERONES: ROLES, STRUCTURES AND MECHANISMS

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AND
LEANDRO COLUCCI
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Chapter VIII

Free-Living Protists as a Model for Studying Heat Shock Proteins in the Cell

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Abstract

Heat shock proteins (HSP) are closely involved in response of organisms to adverse natural and anthropogenic factors. Within one of the recent directions of HSP studies, attempts are made to uncover mechanisms underlying the organisms' adaptation to various stresses. Promising model objects for this research are protists - lower eukaryotes that are at the same time a cell and a fully fledged organism.

In this paper, we present some results of our studies on HSP70 level in intact cells of several free-living protists and on the characteristics of its dynamics in the cells in response to the changes in natural environmental factors, salinity and temperature. The protists chosen for the study, the amoebae and the ciliates, possess an essentially different organization of the cell and belong to the most phylogenetically distant groups.

In many cases, a high constitutive level of HSP70 was recorded in intact cells under normal (non-stressful) conditions. It may be considered as a universal pre-adaptation of these protists to possible drastic environmental changes.

The strains of Amoeba proteus and several related species were very similar as to the level of HSP70 and the position of the stained zone on the blots, despite differences in geographic provenance, temperature conditions in natural habitats the strain was isolated from, and the strain age. Species of other genera of freshwater lobose amoebae studied, though close to Amoeba, differed considerably in the HSP70 level.

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Out of the seven strains of the facultative parasites Acanthamoeba, only two had a noticeable constitutive level of HSP70. Differences in the constitutive level of HSP70 in the cells of different acanthamoebae strains may reflect their potential pathogenicity.

The ciliates used in the study represented various ecological groups different as to their attitude to environmental salinity. They were shown to employ various strategies of the chaperone system response to increasing and decreasing salinity of the medium. Constitutive level of HSP70 in their cells correlated with the degree of the salinity tolerance.

Introduction

Living organisms respond to various stresses by activation of their defensive mechanisms. A basic defensive mechanism is the synthesis of stress proteins, including the heat shock proteins (HSP). They are represented by a number of families, differing in molecular weight, nucleotide sequences of the encoding genes, and functions. The best studied HSP are those with the molecular weight of 70 kDa (HSP70). HSP70 are highly conservative proteins with a low species-specificity and a recognized chaperone activity. They are present in the cytoplasm of animals and plants, including unicellular ones, in both inducible and constitutive forms [Lindquist, 1986; Feder & Hofmann, 1999; Margulis & Guzhova, 2000, 2009].

A high HSP70 level in cells under normal, that is, non-stressful conditions may indicate a better adaptive potential of the organism. HSP70 level in the cell was shown to correlate positively with the temperature conditions in the environment [Lyashko et al., 1994]. For poikilotherms, this regularity was elevated to the rank of a general rule: the higher the environmental temperature, the higher the level of HSP70-like proteins in the organisms' cells [Ulmasov et al., 1992].

One of the recent directions in HSP-related research is the study of their role in adaptation of organisms to stresses [Feder & Hofmann, 1999; Evgen'ev et al., 2005; Garbuz et al., 2008]. In these studies, the stress is usually represented by temperature, and the objects, by multicellular organisms, cultures of their tissues, or prokaryotic microorganisms.

In this paper, we would like to draw the researchers' attention to protists as promising objects for the study of adaptive mechanisms at the biochemical and the molecular level. The name "protists" is currently applied to diverse lower eukaryotes that have in common a simple cellular organization: they are mostly unicellular and sometimes multicellular but without true tissues [Whittaker, 1969; Corliss, 1984; Cavalier-Smith, 1999]. Their main merit as model objects for experimental research resides in the fact that a protist is at the same time a cell and an independent fully fledged organism. Therefore, the protists' response to, for instance, a stress is not mediated by the specific features of cell functioning in different tissues, and thus can be considered as a simplified model of the multicellular animals' response. Incidentally, HSP level in the cells of various tissues of an organism subjected to a salinity stress may be considerably different [Deane & Woo, 2004].

Despite the attractiveness of protists as research objects, their HSP70 are rather poorly studied in comparison to those of bacteria and multicellular animals. Moreover, most of the information available concerns HSP70 in protists parasitizing warm-blooded hosts [for

example see: Requena et al., 1992; Field et al., 2000; Perez-Serrano et al., 2000; Bakatselou et al., 2003; Varadharajan et al., 2004, etc.]. Free-living protists have been paid little attention on this point. There are just a few studies of HSP70 in ciliates [for example see: McMullin & Hallberg, 1987; La Terza et al., 2001, 2004; Smurov et al, 2007; Podlipaeva et al, 2008], flagellates [Drzymalla et al., 1996; Barque et al., 2000], and amoebae [Kalinina et al., 1988; Podlipaeva, 2001; Podlipaeva et al., 2006].

The functions of HSP70 in unicellular organisms are usually investigated in the context of their adaptation to the changes in the environmental temperature. However, HSP and, in particular, HSP70 are involved in adaptation to various adverse factors. In this respect, a very interesting topic is the adaptation of protists to the changes in the water salinity. Salinity, alongside with temperature, is a key factor in the evolution of aquatic organisms. The dependence of the protists' chaperone system upon salinity may be detected in experiments recording the constitutive level of HSP70, its expenditure, and its induction in response to the salinity stress under constant temperature conditions in protists from various ecological groups. Comparative analysis of such data obtained from evolutionary distant protists would reveal the universal characteristics underlying the chaperone system's functioning. Moreover, the regularities thus discovered might also be expected to be true of the multicellular organisms.

In this paper, we report the results of our studies on HSP70 level in intact cells of free-living protists and on the characteristics of HSP70 induction in the cells in response to the changes in salinity and temperature. The protists chosen for the study, the amoebae and the ciliates, belong to phylogenetically distant groups with an essentially different organization of the cell.

Material and Methods

Organisms and Cultures

Lobose Amoebae

Freshwater lobose amoebae used in the study were represented by 15 strains (clones) of different provenance (Table 1) taken from the Collection of the Laboratory of Cytology of Unicellular Organisms (Institute for Cytology of the Russian Academy of Sciences). The strains belonged to the species *Amoeba proteus, A. amazonas, A. borokensis, "A. indica", Amoeba* sp. and *Trichamoeba* sp. The amoebae were cultivated in the modified Prescott and Carrier solution according to the standard procedure [Prescott & Carrier, 1964] at room temperature. They were fed on *Tetrahymena pyriformis* ciliates (strain GL).

Acanthamoebae

Seven strains of amphizoic (species capable of living as free-living or as parasites) acanthamoebae *(Acanthamoeba* spp.) used in the study were obtained from the collection of the Parasitology Institute of the Czech Republic Academy of Sciences (from Prof. I. Dykova) (Table 2). Strains 4465, 4337, 3668 [Dykova et al., 1999], 4774, 4628 and 4690 [I. Dykova, unpublished data] were isolated in the Czech Republic from the internal organs of roach *(Rutilis rutilis),* pikeperch *(Esox lucius)* and perch *(Perca fluviatilis).* Strains P121 and P128

were isolated in Peru in 2005 from the gills of fish *Calophysus macropterus* and *Amblydoras hancockii* (I. Dykova, unpublished data). All the strains were cultured under laboratory conditions [Dykova et al., 1999].

Table 1. Free-Living Lobose Amoebae Species and Strains Studied

NN	Species and Strains	About the Strain
1	*Amoeba proteus* Da	Received from Southampton University (UK) in 1970, isolated from Floral Park (New York, USA), not later than 1950.
2	*Amoeba proteus* B	Received from Budapest (Hungary) in 1959, there from King's College (London, UK).
3	*Amoeba proteus* tD	Received from King's College (London, UK) in 1963, initially referred to separate species *A. discoides* (for systematic status see: Jeon & Lorch, 1973; Page, 1988).
4	*Amoeba proteus* tP	Received from Southampton University (UK) in 1975.
5	*Amoeba proteus* Val	Established in 1989, isolated from Lake Sys'jarvi, Valaam Archipelago (North-Western Russia).
6	*Amoeba proteus* Kan	Established in 1989, isolated from Lake Kanevskoe, Valaam Archipelago (North-Western Russia).
7	*Amoeba proteus* Binucl	Received not later than 1977, origination is obscure.
8	*Amoeba proteus* Lesch	Received not later than 1977, origination is obscure, initially referred to separate species *A. lescherae* (for systematic status see: Page, 1988; Sopina, 2000).
9	*Amoeba proteus* Neapol	Established in 2005, isolated from Naples region (Italy).
10	*Amoeba proteus* Obsc	Date of receiving and strain origination are obscure.
11	*Amoeba amazonas* Amaz	Received from D.M. Prescott in 1969, isolated from Amazon River in Brazil, initially referred to *A. proteus* (for systematic status see: Friz, 1992).
12	*Amoeba indica* Ind	Received from CCAP (Cambridge, UK) in 1985, isolated from local pound in Bombay (India) in 1971, initially referred to separate species, but formal species description is obscure (for systematic status see: Page, 1988; Friz, 1992; Сопина, 2000).
13	*Amoeba borokensis* Bor	Established in 1974, isolated from local pond in Borok (Yaroslavl' region, Russia), initially referred to *A. proteus* (for systematic status see: Kalinina et al, 1986).
14	*Amoeba* sp. Belomor	Established in 1986, isolated from freshwater lake, (White Sea region, North-Western Russia), presumably not a member of the genus *Amoeba*.
15	*Trichamoeba* sp. As 102	Established in 1971, isolated from small river in Azerbaijan, initially referred to *A. proteus,* but considerably differs from this species (Sopina, 2000) and provisionally referred to *Trichamoeba* sp. (Ivanova et al., 2004).

Table 2. Amphizoic Strains of *Acanthamoeba* Studied

NN	Strains	Place and date of isolation	Notes
1	4465	Vltava River, South Bohemia, 1990	The perch (*Perca fluviatilis*), brain
2	3668	Skalice River, Central Bohemia, 1990	The catfish (*Silurus glanus*), spleen
3	4690	Czech Republic, 1991	The roach (*Rutilis rutilis*), brain
4	4774	Czech Republic, 1991	The pike (*Exox lucius*), kidney
5	4628	Czech Republic, 1991	The perch (*Perca fluviatilis*), brain
6	P121	Peru, 2004	The catfish (*Calophysus macropterus*), gills
7	P128	Peru, 2004	The catfish (*Amblydaras hancockii*), gills

Free-living acanthamoebae were represented by strain Am61, obtained from the Laboratory of Soil Cryology (Institute for Physical-Chemical and Biological Problems of Soil Sciences of the Russian Academy of Sciences, Puschino). This strain was isolated from the upper soil horizon of the tundra in the Eastern Arctic Sector (Russia). Accumulative cultures were established on the agarized Prescott and James medium in the field [Page, 1988]; in the laboratory, individual cysts were extracted and used to obtain monocultures. The amoebae were cloned and maintained in the laboratory in Petri dishes at room temperature.

The cysts of ancient acanthamoebae were extracted from samples of late Pleistocene and Holocene permafrost and soil buried in it in the Eastern Arctic Sector (Russia), the age of the samples being 32-35 thousand years [Shatilovich et al., 2005]. Viable cysts were found in accumulative cultures obtained after de-freezing the samples according to the previously described procedure [Shatilovich et al., 2005]. Trophozoites of the excysted amoebae were established in an independent culture (strain Am8) maintained in Petri dishes on the agarized Prescott and James medium under a liquid layer (Cerophyl-Prescott infusion) at room temperature [Page, 1988]; the Petri dishes were stored in a sterile box. Morphological characters of the cysts and the trophozoites correspond fully to the diagnosis of the genus *Acanthamoeba* [A. Goodkov, unpublished data].

Ciliates

The ciliates used in the study were two *Paramecium* species, *P. jenningsi* (strain SRI-10) and *P. nephridiatum* (strain SR98-1), both from the culture collection of the Laboratory of

Invertebrate Zoology (the Biological Research Institute of the St. Petersburg State University, Russia), and *Tetrahymena pyriformis* (amicronucleate strain GL) from the culture collection of the Laboratory of Cytology of Unicellular Organisms (Institute for Cytology of the Russian Academy of Sciences). The ciliates were cultured according to the standard procedures: on the lettuce medium inoculated with *Klebsiella aerogenes* [Sonnebom, 1970] at room temperature.

Temperature Studies

Lobose Amoebae

Amoebae *Amoeba proteus,* strain Val normally cultivated at 20 °C, were treated at 37 °C during lh. The portions of cells were separated from the major culture volume immediately after the treatment (0h) and after some time, different in different experiments. Every portion, as well as the control, untreated amoebae, was precipitated in a low speed (1000 rpm) centrifuge and homogenized in buffer for extraction according to the previously described procedures [Podlipaeva, 2001; Plekhanov et al., 2006].

Acanthamoebae

Cells of strain Am61 cultured at room temperature were heat shocked at 40 °C for 1 h or cold shocked at 4 °C for 1 h.

Cells of the ancient strain Am8 cultured at room temperature were heat shocked at 40 °C for 1 h or cold shocked at 8 °C for 1 h.

Immediately after the shock, the acanthamoebae together with the medium were collected into 2 ml tubes and centrifuged for 5 min at 12 000 rpm. After centrifugation, the precipitated cells were stored for some time in the refrigerator at -20 °C and then treated further as previously described [Podlipaeva et al., 2006, 2008].

Ciliates

Strains of the ciliates *(P. jenningsi, P. nephridiatum* and *T. pyriformis)* cultivated both in freshwater and in salty water medium, were heat shocked (at 37 °C for 1 h) in order to confirm that the polypeptides revealed in salinity shocks experiments indeed belonged to HSP70.

Salinity Studies

Four ecological groups of protists, differing in their attitude to salinity, occur in aquatic habitats [Smurov & Fokin, 2001; Kudryavtseva et al., 2007]. The first group, steno-freshwater species, comprises protists that can live under salinity not exceeding 2.5-3 ‰. The second group comprises protists that can live in the salinity range from 0 to 6-8 ‰ (freshwater species). Species of the third group, meta-freshwater species, can live in more salty water, up to 12-16 ‰. The fourth group, truly euryhaline species, comprises protists that can tolerate direct transfer from salty seawater to freshwater.

The procedures used for determining the salinity tolerance ranges of the protists studied and the design of experiments on the impact of environmental salinity changes are discussed in detail elsewhere [Smurov & Fokin, 2001; Plekhanov et al., 2006; Smurov et al., 2007]. Below we present only brief schemes of the experiments.

P. jenningsi and A. proteus Val (Steno-Freshwater Species)

The upper limits of the salinity tolerance of the individuals of these species acclimated to freshwater conditions (0 ‰) were 3.25 ‰ for *P. jenningsi* and 2.5 ‰ for *A. proteus* strain Val. For the individuals acclimated to 2 ‰, the upper limits of salinity tolerance were 6 ‰ and 3.5 ‰, respectively.

Cells grown in freshwater medium (0 ‰) were placed for 2 h in a medium with 2 ‰ salinity. The cultures acclimated to 2 ‰ salinity were placed in fresh water also for 2 h. Protists cultivated in the media with the initial salinity values were used as control; the culture density was the same in the control and in the experiments.

Tetrahymena pyriformis (Meta-Freshwater)

The ciliates were acclimated to fresh water (0 ‰), to 2 ‰ salinity and to 10 ‰ salinity. The upper limits of their salinity tolerance were, respectively, 8 ‰, 12 ‰ and 15 ‰. Cells acclimated to 10 ‰ salinity tolerated direct transfer into fresh water (0 ‰), and could later adapt to it.

Some of the *T. pyriformis* cells were placed for 1 h into water with a different salinity: the cells acclimated to 2 ‰ were placed in 10 ‰, and the cells acclimated to 10 ‰, into 2 ‰. Then the ciliates were returned to the medium with the initial salinity. For control, ciliates were placed for the same time into water with the salinity usual for them.

Paramecium nephridiatum (Euryhaline)

Ciliates were acclimated to freshwater medium (0 ‰) and to the 10 ‰ medium. The upper limit of the tolerance range for the cells acclimated to fresh water was set at 20 ‰.

Some of the *P. nephridiatum* cells acclimated to fresh water (0 ‰) were placed for 1 h into water with a 10 ‰ salinity; some of the cells acclimated to 10 ‰ were placed for 1 h into fresh water (0 ‰); then in both cases the cells were returned to the medium with the initial salinity. These cells were considered as treated by salinity shock. For control, ciliates were placed into water with the salinity usual for them for the same time. Some of the cells were subjected to an analogous impact with the following difference: after the transfer into water with a different salinity, the cells were not returned to the medium with the initial salinity after an hour but were left in the water with a different salinity until the end of the experiment (up to 24 h). These cells were considered as subjected to adaptation.

Electrophoresis and Western Blotting

Samples of cells were prepared for SDS-electrophoresis [Laemmli, 1970] as previously described [Podlipaeva, 2001; Plekhanov et al., 2006]. Electrophoresis was immediately followed by electroblotting [Towbin et al., 1979] conducted overnight at 6V. HSP were

revealed after treatment of nitrocellulose by monoclonal antibodies SPA 822 against HSP70 (Stressgen Technologies, Canada), the antibodies being specific both to the constitutive and the inducible form of HSP70. Binding zones of proteins and anto-HSP70 antibodies were stained on nitrocellulose by means of the secondary biotin-conjugated antibodies conjugated with alkaline phosphatase (Sigma Chemical Company) as a result of enzymatic reaction. The molecular weight of the polypeptides revealed was determined using High Range Rainbow Molecular Weight Markers of 14-220 kDa (Amersham Biosciences, England) and bovine serum albumin (66 kDa).

Results and Discussion

Lobose Amoebae

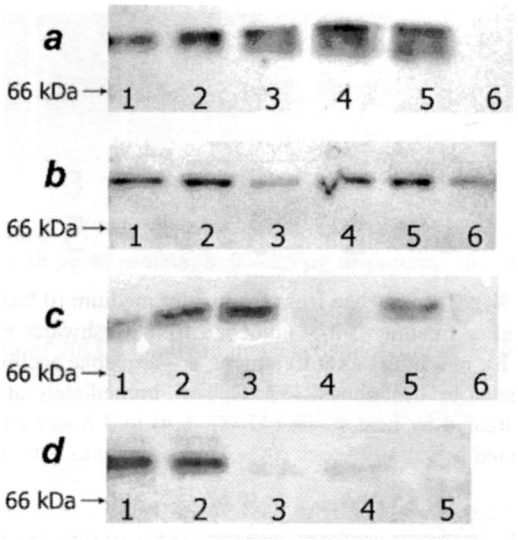
Constitutive Level of HSP70 in Cells

Almost all the lobose amoebae studied possessed constitutive HSP70, which was revealed at blots at rather low loads, 5-7 ug (Figure 1 a, b, d) and 11-12 ug (Figure 1 c) of total protein per starting gel. The *Amoeba proteus* strains and the related *Amoeba* species *(A. amazonas, "A. indica"* and *A. borokensis)* were very similar as to the level of HSP70 and the position of the stained zone on blots, which corresponded approximately to a polypeptide of 70-72 kDa. Only the "youngest" strain of the collection - the Neapol strain, isolated from nature in 2005 - had a somewhat lower level of HSP70 (Figure 1 b, *lane* 3). At the same time, in order to secure any noticeable staining of the 70 kDa zone in the amoebae of strain Belomor, the total protein load on the starting gel had to be increased first up to 12 ug (Figure 1 c, *lane* 4), and then up to 15 ug (Figure 1 d, lane 4). The weakly stained zone revealed occupied on the blot a slightly lower position than the stained zone of the other *Amoeba* strains studied. Finally, in strain AS 102 (tentatively assigned to the genus *Trichamoeba,* see Table 1) no zone corresponding to 70 kDa could be revealed at all. At present, we cannot be sure whether the absence of this zone was associated with an insufficient protein load on the starting gel or with a characteristic feature of HSP of this strain. In the latter case, it may be supposed according to preliminary data, that the HSP of this strain is represented only by heavy isoforms about 97 kDa.

To sum up, most of the freshwater lobose amoebae strains studied possess a rather high HSP70 level under non-stress conditions. This characteristic did not correlate with any individual features of the strains, such as geographic provenance, temperature conditions in natural habitats the strain was isolated from, and the strain age (i.e. the time of maintenance in the laboratory). These results contradict the rule about a positive correlation between the temperature conditions in the habitat and the constitutive level of HSP70 in the cells of poikilotherms [Ulmasov et al., 1992].

There were no noticeable differences in the constitutive level of HSP70 in the cells of the *Amoeba proteus* strains and the related *Amoeba* species and in the distribution of the HSP isoforms revealed by anti-HSP70 antibodies. At the same time, the cells of the Belomor strain, traditionally denoted in the Collection as *Amoeba* sp. (Table 1), differed from all the strains and species of the *"proteus"* group in having a very low level of HSP70. Belomor

strain also differs from the other *Amoeba* strains in isozyme spectres of some enzymes, and probably does not belong to the genus *Amoeba* at all [Friz, 1992; Sopina, 2000]. Strain AS 102, in which we failed to reveal any 70 kDa zone, is also not an *Amoeba* [Sopina, 2000] but a *Trichamoeba* species [Ivanova et al., 2004]. Interestingly, though all the lobose amoebae strains in the collection are cultured and fed under the same conditions, strains Belomor and AS 102 are the most unstable and, one may say, capricious, demanding more frequent transfers and changes of the medium. One may suppose that such "petulance" reflects the connection between the low constitutive level of HSP70 and low adaptive capacities. One may further speculate that the differences in the level and the molecular characteristics of stress proteins are expressed in the lobose amoebae at the genus level.



a: *I* —*A. proteus* strain B, 2 — *A. proteus* strain Val, 3 — *A. proteus* strain Da, *A* - *A. amazonas* strain Amaz, 5 — *A. indica* strain Ind, 6 - *Amoeba* sp. strain Belomor (5-7 pg of protein at each lane);
b: *I* -*A. proteus* strain Val, 2 - *A. proteus* strain tP, *3* -*A. proteus* strain Neapol, 4 - *A. proteus* strain Obsc, 5 - *A. proteus* strain Lesch, 6 - *A. proteus* strain Binucl (7 pg of protein at each lane);
c: *I* — *A. proteus* strain Da, *2* — *A. proteus* strain Kan, 3 - *A. proteus* strain tD, 4 — *Amoeba* sp. strain Belomor, 5 - *A. borokensis* strain Bor, 6 - *Trichamoeba* sp. strain AS102 (11-12 pg of protein at each lane);
d: *I* - *A. proteus* strain Da, 2 - *A. proteus* strain B, 3 and 4 - *Amoeba* sp. strain Belomor, 5 - *Trichamoeba* sp. strain AS 102 (lanes 1-3 and 5 — 7 pg of protein, lane 4 — 15 pg of protein).

Figure 1. Heat shock protein of 70kDa family in intact cells of freshwater lobose amoebae of various strains.

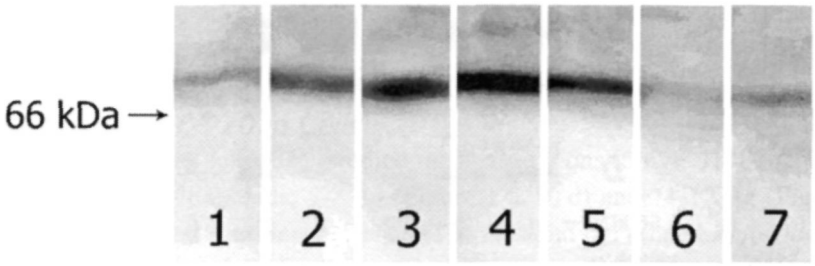
Salinity and Thermal Stresses

Cells of *A. proteus* strain Val were treated with thermal and salinity stresses in order to determine the dynamics of changes in the level of HSP70.

When the salinity of the medium increased from 0 to 2 ‰, HSP70 concentration increased markedly 3 h after the stress (Figure 2, *lane* 3). Thermal stress in the amoebae grown in freshwater medium resulted in an increase in the level of the same antigen as in the case of salinity shock (Figure 2, *lane* 2). The amoebae acclimated to 2 ‰ had a higher constitutive level of HSP70 (Figure 2, *lane* 4) than those adapted to fresh water (Figure 2,

lane 1). An increased HSP70 level at 2 ‰ salinity (the value close to the limit of this strain's tolerance range, 2.5 ‰) confirms that this salinity value is damaging for this strain. Additional stresses - transfer of cells acclimated to 2 ‰ into freshwater medium (Figure 2, *lane* 7) and heat shock (Figure 2, *lanes* 5 and 6) - result in a decrease in the HSP70 level.

The amoebae react to an increase in environmental temperature first by a decrease and then by an increase in the HSP70 concentration. The peak in the expenditure appears to fall on the time 3 h after the shock, after which the HSP70 level in the cells increases. The dynamics of expenditure-synthesis of HSP70 after salinity shock in the amoebae of this strain is similar to this dynamics after heat shock. Transfer of cells acclimated to 2 ‰ into freshwater medium results, similarly to the heat shock, in the protein expenditure.



1 - intact amoebae cells (0 ‰); 2 - amoebae from freshwater medium (0 ‰), treated by heat shock (37 °C, 1 h), in 3 hours after treatment; 3 - amoebae from freshwater medium (0 ‰), treated by salinity shock (2 ‰, *2* h), in 3 hours after treatment; 4 - amoebae acclimated to 2 ‰; 5 - amoebae acclimated to 2 ‰, treated by heat shock (37 °C, 1 ч), immediately after treatment; 6 - amoebae acclimated to к 2 ‰, treated by heat shock (37 °C, 1 ч) in 3 hours after treatment; 7 - amoebae acclimated to 2 ‰, treated by salinity shock (0 ‰, 2 h), in 3 hours after treatment.

Figure 2. Heat shock protein of 70 kDa family in the cells of freshwater lobose amoeba *Amoeba proteus* strain Val, cultured in media of different salinity, and treated by salinity and heat shocks.

Acanthamoebae

Constitutive Level of HSP70 in the Cells and Thermal Stresses

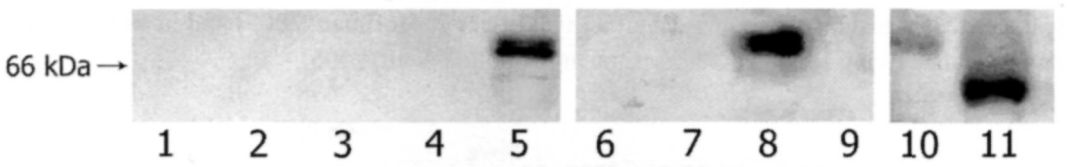
Out of the seven strains of the amphizoic acanthamoebae studied, only two strains - 4465 and 4628 - were shown to possess constitutive HP70 (Figure 3). In both these strains, the position of the stained zone on the blots was similar to that of the HSP70 zone in the *Amoeba* strains studied. Unfortunately, scarcity of the material prevented us from performing experiments with further increase in the load on the starting gel, which could reveal a stained zone in the acanthamoebae of the other amphizoic strains.

Contrary to the freshwater lobose amoebae, in *Acanthamoeba* differences in the HSP70 level were expressed within the genus; they may even be confined to strains within a species. The constitutive level of HSP70 is known to correlate positively with the degree of pathogenicity of the species and/or strain of acanthamoebae and with its higher tolerance of stresses, including thermal stresses [Perez-Serrano et al., 2000]. Though all the amphizoic acanthamoebae strains in the present study were isolated from various internal organs of fish, they are not obligatory but facultative parasites [Dykova et al., 1999]. The differences in the

constitutive HSP70 level between the acanthamoebae strains studied may also reflect their potential pathogenicity.

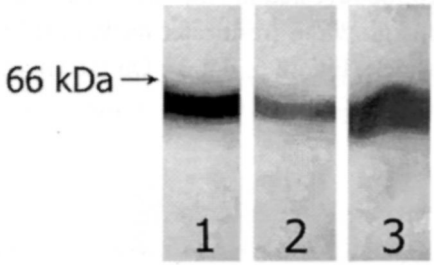
Western blotting of trophozoites of the ancient *Acanthamoeba* strain Am8 revealed HSP in unstressed amoebae, in heat shocked amoebae and in cold shocked amoebae Figure 4. The stained binding zones with antibodies against HSP70 were situated in all the three cases somewhat lower than the molecular weight marker of 66 kDa (see also Figure 3 *lane* 11). Therefore, the protein found had a molecular weight of about 60 kDa and belonged to the family of HSP70.

Noteworthy, ancient acanthamoebae had a very high constitutive level of HSP (Figure 4, *lane* 3). The expenditure of the stress protein was somewhat less after cold shock (Figure 4, *lane* 1) than after heat shock (Figure 4, *lane 2).*



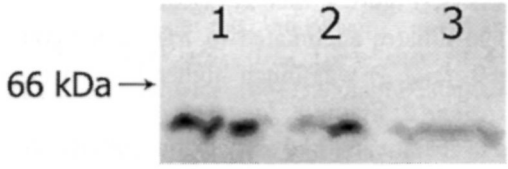
1 - 4690, 2 - P121, 3- P128, 4 - 4774, 5 - 4628, 6 - 3668, 7 - P128, 8 - 4628 , 9 - 4774, 10 - 4465, 11 - Am8 (lanes 1-7-35 |ig of protein, 8, 9 - 25 ug of protein, 10-40 ng of protein, 11-20 ug of protein).

Figure 3. Heat shock protein of 70kDa family in intact cells *of Acanthamoeba* ssp. of various strains.



1 - cells after cold shock (8 °C), 2 - cells after heat shock (40 °C), 3 - intact cells.

Figure 4. Heat shock protein of 70kDa family in intact and stressed cells of ancient tundra *Acanthamoeba* sp. strain Am8.



1 - intact cells, 2 - cells after heat shock (40 °C), 3 - cells after cold shock (4 °C).

Figure 5. Heat shock protein of 70kDa family in intact and stressed cells of contemporary tundra *Acanthamoeba* sp . strain Am61.

Contemporary acanthamoebae from tundra (strain Am61) had a high constitutive level of HSP70 (Figure 5, *lane* 1). The cells of this strain were heat shocked at 40°C and cold

shocked at 4 °C. The expenditure of HSP70 was observed in both cases, but was higher after cold shock (Figure 5, *lane* 3) than after heat shock (Figure 5, *lane 2).* Noteworthy, the stained zone was situated on the blot somewhat lower than the molecular weight marker of 66 kDa. Thus, both ancient (Am8) and contemporary (Am61) strains of acanthamoebae from tundra had a high constitutive level of HSP70 and did not demonstrate typical heat shock response (HSR), that is, the induction of HSP70 in response to the shock. Their HSP, revealed by anti-HSP70 antibodies, were situated on the blot at the same position(lower than 66 kDa), differing to the amphizoic acanthamoebae (strains 4465 and 4628) and the freshwater lobose amoebae, whose HSP had a molecular weight of about 70 kDa. In both strains of acanthamoebae from tundra, HSP70 was expended after heat and cold shock, but in strain Am8 the expenditure was greater after heat shock, while in Am61 it was greater after cold shock. Lack of HSR may be associated with the initially high constitutive level of HSP70, as is the case, for instance, in some Antarctic fish [Place & Hoffman, 2005] and in embryos and larvae of the "living fossil", the horseshoe crab [Botton et al., 2006].

Ciliates, Salinity and Thermal Stresses

Paramecium jenningsi

In the total protein extract of the steno-freshwater ciliate P. jenningsi an antigen cross-reacting with anti-HSP70 antibodies was revealed. Its position on the blot was the same as that of the analogous polypeptide revealed in A. proteus. As shown by dot-blotting, the level of HSP70 in P. jenningsi cells grown in freshwater medium (Figure 6, dot 1) was very low, but increased after salinity shock (Figure 6, dot 3). On the contrary, ciliates acclimated to 2 ‰ had a high constitutive level of HSP70 (Figure 6, dot 2), which decreased after salinity shock (Figure 6, dot 4).

Paramecium nephridiatum

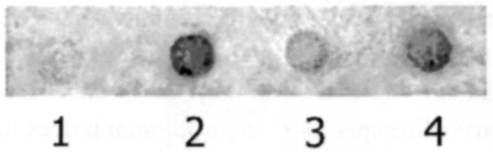
A polypeptide antigen (about 70 kDa) was revealed by western blotting in the total protein extract of *P. nephridiatum* cells, both in intact (control) cells after prolonged acclimation to freshwater medium or salty water medium, and in salinity shocked cells (Figure 7-9). Besides, *P. nephridiatum* cells acclimated to 10 ‰ were shown to possess an additional polypeptide with a molecular weight of about 60 kDa, which also cross-reacted with antibodies against HSP70 (Figure 7, *lane* 2; Figure 8, *lane* 2; Figure 9, *lane* 2).

The level of HSP70 in ciliates acclimated to freshwater conditions (Figure 7, *lane* 1; Figure 8, *lane* 1, Figure 9, *lane* 3) was much higher than in ciliates acclimated to 10 ‰ (Figure 9, *lane 2;* Figure 7, *lane* 2; Figure 8, *lane 2).*

In ciliates acclimated to fresh water and salty water, salinity shock elicited an asymmetric response as to the expenditure-synthesis of HSP70. The HSP70 concentration in the cells transferred from the 10 ‰ medium into fresh water (Figure 7, *lane* 3) was much higher than that in the cells transferred from fresh water into salty water (Figure 7, *lane* 4), the time after transfer being equal.

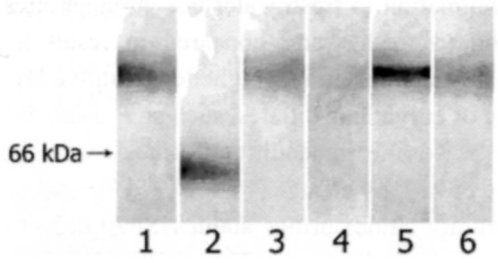
The level of 70 kDa protein after transfer from 10 ‰ medium to fresh water (10 —> 0 ‰) was higher than that in the control (Figure 7, *lanes 2,* 3; Figure 8, *lanes 2,* 3). After transfer

from fresh to salty water (0 —> 10 ‰), the HSP70 staining zone was weak, the protein concentration being much less than in the control (Figure 7, *lanes* 1, 4).



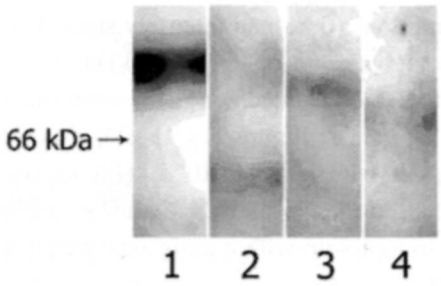
1 - intact cells from 0 ‰, 2 - cells acclimated to 2 ‰, 3 - cells from 0 ‰ treated by salinity stress (2 ‰), 4 - cells acclimated to 2 ‰ treated by salinity stress (0 ‰).

Figure 6. Heat shock protein of 70 kDa family in the cells of freshwater ciliate *Paramecium jenningsi* strain SRI, cultured in the media of different salinities and treated by salinity stresses (Dot-Blotting).



1 - intact cells acclimated to freshwater medium (0 ‰); 2 - cells acclimated to 10 ‰; 3 - cells acclimated to l0 ‰, 2 h after salinity stress (0 ‰, l h - "shock"); 4 - cells acclimated to freshwater medium (0‰), 2 h after salinity stress (10 ‰, 1 h - "shock"); 5 - ciliates, acclimated to the freshwater medium (0 ‰), 3 h after remaining in the stress salinity of 10 ‰ ("adaptation"); 6 - cells acclimated to 10 ‰, 3 h after remaining in the stress freshwater (0 ‰) medium ("adaptation").

Figure 7. Heat shock protein of 70kDa family in intact cells of euryhaline ciliate *Paramecium nephridiatum* and after two types of stresses - "shock" and "adaptation" (part 1).



1 - intact cells acclimated to the freshwater medium (0 ‰); 2 - cells acclimated to 10 ‰; 3 - cells acclimated to 10 ‰, 2 h after salinity stress (0 ‰, 1 h - "shock"); 4 -cells acclimated to 10 ‰, 3 h after remaining in the stress freshwater (0 ‰) medium ("adaptation").

Figure 8. Heat shock protein of 70kDa family in intact cells of euryhaline ciliate *Paramecium nephridiatum* and after two types of stresses - "shock" and "adaptation" (part 2).

Adaptation to a new salinity (that is, a prolonged variant of the salinity shock) in the euryhaline ciliate *P. nephridiatum* resulted in HSP70 induction in experiments with both directions of salinity changes (Figure 7, *lanes* 1, 5; Figure 8, *lanes 2,* 4).

The protein with a molecular weight of about 60 kDa was present in a considerable quantity, higher than that of the protein of about 70 kDa, in intact *P. nephridiatum* cells

acclimated to 10 ‰ (Figure 7, *lane* 2; Figure 8, *lane 2;* Figure 9, *lane* 2) and was almost absent in intact cells acclimated to fresh water (Figure 7, *lane* 1; Figure 8, *lane* 1; Figure 9, *lane* 3).

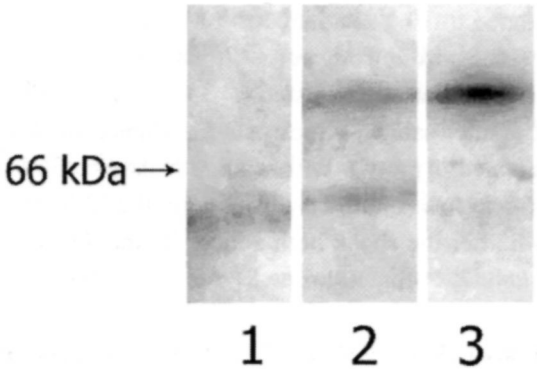
To sum up, steno-freshwater and euryhaline ciliates have different strategies of the chaperone system's response to increasing and decreasing salinity. In the steno-freshwater species *P. jenningsi,* acclimation results in an increase in the constitutive level of HSP70 in the cell, whereas in the euryhaline species *P. nephridiatum* it does not result in any change in the HSP70 level. Decreasing salinity of the medium is accompanied in euryhaline organisms by an increased level of HSP70 in the cell: in *P. nephridiatum* individuals acclimated to fresh water (0 ‰) the level of HSP70 was much higher than in *P. nephridiatum* individuals acclimated to salty water (10 ‰*)* (Figure 7-9). These results indicate that for euryhaline paramecia a prolonged acclimation to fresh water is a stronger stress than acclimation to 10 ‰. Prolonged acclimation to fresh water appeared to result in the activation of their chaperone system, which was manifested in a higher constitutive level of HSP70.

The chaperone system of euryhaline ciliates, such as *P. nephridiatum,* is characterized by an asymmetric response as to the expenditure-synthesis of HSP70 after salinity shock depending on the direction of salinity change (Figure 7, 9).

Adaptation to new salinity values brings about HSP70 induction in *P. nephridiatum* in the case of salinity changes in both directions. This observation agrees with the reports that in some instances only a prolonged stress elicits HSP70 induction. For example, in the ciliate *Moneuplotes crassus* the level of HSP70 was the same as in intact cells after a short heat shock (5 min) but increased after a prolonged heat shock, reaching the maximum after 180 min [Ullmann et al., 2004].

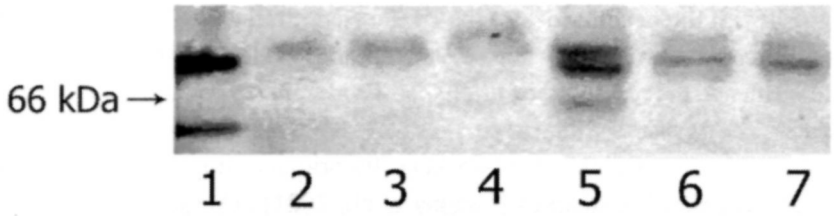
Tetrahymena pyriformis

In the meta-freshwater ciliate *T. pyriformis* two zones were revealed in control cells cultivated under optimal conditions (0 ‰): an intensely stained zone with a molecular weight of about 70 kDa (72 kDa) and a zone of about 60 kDa (Figure 10, *lane* 1). In intact *T. pyriformis* cells acclimated to 2 ‰, a stained zone of a somewhat larger molecular weight (73 kDa) was revealed (Figure 10, *lane 2;* Figure 11, *lane* 1), which remained almost unchanged 2, 4 and 24 hour after the salinity shock (1 h at 10 ‰) (Figure 10, *lanes* 3, 4; Figure 11, *lanes* 2-4). Besides, both in the control and in the "24 h after shock" sample there was a weakly stained zone corresponding to a protein with a molecular weight of about 65 kDa (Figure 10, *lanes* 2; Figure 11, *lanes* 1, 4).



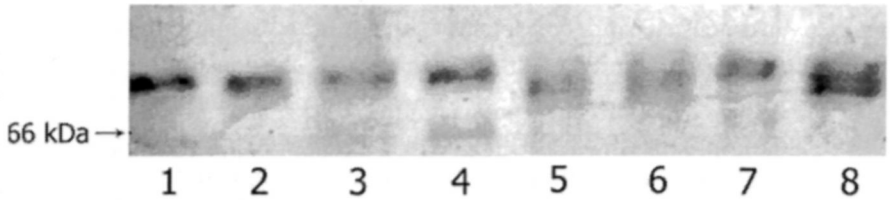
1 - cells acclimated to 10 ‰, after heat shock (37 °C, 20 min); 2 - cells acclimated to 10 ‰; 3 - intact cells acclimated to freshwater medium (0 ‰).

Figure 9. Heat shock protein of 70 kDa family in the cells of euryhaline ciliate *Paramecium nephridiatum*, acclimated to 10 and 0 ‰, and treated by heat shock.



1 - intact cells acclimated to freshwater (0 ‰) medium; 2 - cells acclimated to 2 ‰; 3 - cells acclimated to 2 ‰ in 2 hours after salinity shock (10 ‰, 1 h); 4 - cells acclimated to 2 ‰ in 24 hours after salinity shock (10 ‰, 1 h); 5 - cells acclimated to 10 ‰ ; 6 - ciliates acclimated to 10 ‰, in 2 hours after salinity shock (2 ‰, 1 h); 7 - cells acclimated to 10 ‰, in 24 hours after salinity shock (2 ‰, 1 h).

Figure 10. Heat shock protein of 70 kDa family in intact cells of freshwater ciliate *Tetrahymena pyriformis* and after different periods of salinity stresses treatment (part 1).



1 - intact cells acclimated to 2 ‰; 2 - cells acclimated to 2 ‰ in 2 hours after salinity shock (10 ‰, 1 h); 3 - cells acclimated to 2 ‰ in 4 hours after salinity shock (10 ‰, 1 h); 4 - cells acclimated to 2 ‰ in 24 hours after salinity shock (10 ‰, 1 h); 5 - cells acclimated to 10 ‰; 6 - cells acclimated to 10 ‰, in 2 hours after salinity shock (2 ‰, 1 h); 7 - cells acclimated to 10 ‰, in 4 hours after salinity shock (2 ‰, 1 h); 8 - cells acclimated to 10 ‰, in 24 hours after salinity shock (2 ‰, 1 h).

Figure 11. Heat shock protein of 70 kDa family in intact cells of freshwater ciliate *Tetrahymena pyriformis* and after different periods of salinity stresses treatment (part 2).

T. pyriformis acclimated to 10 ‰ had a constitutive HSP with a molecular weight of 72 kDa, the same as in the ciliates from fresh water (Figure 10, *lanes* 1,5; Figure 11, *lane* 5) and somewhat lower than in the ciliates acclimated to 2 ‰ (Figure 11, *lanes* 1, 5). The level of this protein decreased 2 h after salinity shock at 2 ‰ (Figure 10, *lane* 6; Figure 11, *lane* 6). After 4 h, the blot contained a protein with a slightly higher molecular weight (73 kDa), which substituted the protein found in the control and in the "2 h after shock" sample (Figure 11, *lane* 7). Finally, 24 h after the shock both zones, 72 and 73 kDa, were distinctly stained on the blot (Figure 10, *lane* 7; Figure 11, *lane* 8). No protein with a molecular weight about 65 kDa was found.

We failed to reveal any induction of HSP70 synthesis in *T. pyriformis* cells after salinity shocks in both directions of salinity change (Figure 10, 11). On the contrary, there was a noticeable expenditure of the 72 kDa protein, which substituted the 65 kDa protein 4 h after the shock. The levels of HSP70 in the cells acclimated to different salinities were similar, contrary to, for instance, the euryhaline *P. nephridiatum.* However, such a distribution of HSP in *T. pyriformis* may be associated not only with the ecological characteristics of this species but also with the peculiarities of the strain used. Strain GL is an amicronucleate one; it has been cultivated continuously for several decades under stable laboratory conditions, which could influence the reactivity of its chaperone system.

Freshwater *Tetrahymena* ciliates are known to possess a heat shock protein, called HSP90, with a molecular weight of 82-85 kDa. Its primary structure does not have a high homology with HSP70 of vertebrates [Frankel et al., 2001]. The protein found in our study has common antigen determinants with HSP70 of vertebrates, a fact that may indicate a considerable homology in primary structure.

As noted above, there are several ecological groups of aquatic protists different as to their attitude to environmental salinity [Smurov & Fokin, 2001; Kudryavtseva et al., 2007]. Similar groups are known in multicellular animals, for instance, mollusks and crustaceans [Aladin, 1996]. Since the emergence of such groups is an outcome of a long evolution, it may be suggested that they possess certain characteristic features of the chaperone system's functioning. Indeed, steno-freshwater and euryhaline protists have different strategies of the chaperone system response to an increase or a decrease in the salinity. In freshwater species, acclimation to an increased salinity results in an increase in the constitutive level of HSP70 in the cell, whereas in euryhaline species it does not change the constitutive level of HSP70. Euryhaline organisms respond to a decreased salinity by an increase in the HSP70 level in the cell. Therefore, in the process of acclimation these ciliates turn out to be, in a way, pre-adapted to abrupt changes in the environmental salinity and respond to them by using the pool of HSP accumulated in the cell. The meta-freshwater species *T. pyriformis,* intermediate as to its ecological characteristics, demonstrates a weak response to salinity changes. The level of HSP70 in *T. pyriformis* cells changes little, i.e. the chaperone system of this species is less reactive than in steno-freshwater or euryhaline species. To note, the chaperone system of the *Paramecium* species studied is more mobile than that of *Tetrahymena pyriformis* in response to both a prolonged salinity acclimation and a short stress.

Constitutive level of HSP70 in the cells of the ciliates studied correlates with the degree of their salinity tolerance. In the steno-freshwater species *P. jenningsi,* this level was lower in the fresh than in salty water. In the euryhaline ciliate *P. nephridiatum,* this level was higher

in fresh than in salty water, while in the meta-freshwater *T. pyriformis* it remained stable in both media. The constitutive level of HSP70 in the cells of these ciliates may reflect, in some respect, the conditions in the habitat of their origin: freshwater bodies in the case of *P. jenningsi,* freshwater or brackish-water bodies in the case of *T. pyriformis,* brackish-water estuaries or seas in the case of P. *nephridiatum.* Differences in salinity tolerance limits of the species studied suggest that, even though all of them may live in fresh water, they are pre-adapted to salinity changes within these limits.

Our results show that steno-freshwater and euryhaline ciliates possess a pronounced HSR, whereas the ciliate with intermediate adaptive capacities *(T. pyriformis)* lacks a pronounced HSR but has an initially high constitutive HSP level. An initially high level of HSP70 appears to correlate with the lack of HSR also in the tundra strains of acanthamoebae. These strains were isolated from habitats with extreme fluctuations of the environmental factors, first of all, temperature. So, a high constitutive level of HSP70 and a lack of HSR allow the species to exist under harsh conditions. However, in order to be able to colonize new habitats (e.g. salty water for freshwater species and vice versa) the presence of HSR is essential.

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References

Aladin, N. V. (1996). *Salinity adaptations in Ostracoda and Branchiopoda: Proceedings of the Zoological Institute, Vol. 265.* St. Petersburg (Russia): Zoological Institute RAS.

Bakatselou, C, Beste, D., Kadri, A. O., Somanath, S. & Clarka, C G (2003). Analysis of genes of mitochondrial origin in the genus Entamoeba. *J. Eukar. Microbiol., 50,* 210-214.

Barque, J. P., Schedler, P., Floch, E. & Bonaly, J. (2000). In Euglena gracilis, a heat-shock protein related to hsc73 is constitutive and stress inducible. *Arch. Biochem. Biophys., 378,* 1-5.

Botton, M. L., Pogorzelska, M., Smoral, L., Shehata, A. & Hamilton, M. G (2006). Thermal biology of horseshoe crab embryos and larvae: A role for heat shock proteins. *Journal of Experimental Marine Biology and Ecology, 336,* 65-73.

Cavalier-Smith, T. (1999). Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. *J. Eukaryot. Microbiol, 46,* 347-366.

Corliss, J. O. (1984). The kingdom Protista and its 45 phyla. *BioSystems, 17,* 87-126.

Deane, E. E. & Woo, Norman Y. S. (2004). Differential gene expression associated with euryhalinity in sea bream (Sparus sarba). *Am. J. Physiol. Regul. Integr. Comp. Physiol, 287,* 1054-1063.

Drzymalla, C, Schroda, M. & Beck, C F. (1996). Light- inducible gene HSP70B encodes a chloroplast-localized heat shock protein in Chlamydomonas reinhardtii. *Plant Mol. Biol., 31,* 1185-1194.

Dykova, I., Lom, J., Schroeder-Diedrich, J. M., Booton, G C & Byers, T J. (1999). Acanthamoeba strains isolated from organs of freshwater fishes. *J. Parasitol., 85,* 1106-1113.

Evgen'ev, M. B., Garbuz, D. G. & Zatsepina, O. G (2005). Heat shock proteins: functions and role in adaptation to hyperthermia. *Ontogenez, 36,* 265-273.

Feder, M. E. & Hofmann, G E. (1999). Heat-shock proteins molecular chaperones, and the stress response: evolutionary and ecological physiology. *Ann. Rev. Physiol., 61,* 243-282.

Field, J., Van Dellen, K., Ghosh, S. K. & Samuelson J. (2000). Responses of Entamoeba invadens to heat shock and encystation are related. *J. Eukar. Microbiol., 47,* 511-514.

Frankel, J., Williams, N. F, Nelsen, E. M. & Keeling, P. J. (2001). An evaluation of Hsp90 as a mediator of cortical patterning in Tetrahymena. *J. Eukar. Microbiol., 48,* 147-160.

Friz C. T. (1992). Taxonomic analysis of seven species of family Amoebidae by isozymic characterization of electrophoretic patterns and the descriptions of a new genus and new species: Metamoeba n. gen., *Amoeba amazonas n. sp. Arch. Protistenk., 142,* 29-40.

Garbuz, D. G, Zatsepina, O. G, Przhiboro, A. A., Yushenova, I., Guzhova, I. V. & Evgen'ev, M. B. (2008). Larvae of related Diptera species from thermally contrasting habitats exhibit continuous up-regulation of heat shock proteins and high thermotolerance. *Molecular ecology, 17,* 4763-4777.

Ivanova, L. O., Podlipaeva, Yu. I. & Goodkov, A. V. (2004). New data on the old cell cultures from the collection of strains of free-living freshwater amoebae. *Mol. Biol. of the Cell, 15 (Suppl.),* 503a.

Jeon, K. W & Lorch, I. J. (1973). Strain specificity in Amoeba proteus. In: *K. W. Jeon (Ed.). The Biology of Amoeba* (pp. 549-568). New York: Acad. Press.

Kalinina, L. V, Afon'kin, S. Yu., Gromov, D. B., Khrebtukova, I. A. & Page F. C (1986). Amoeba borokensis n. sp., a rapidly dividing organism especially suitable for experimental purposes. *Arch. Protistenh., 132,* 343-361.

Kalinina, L. V, Khrebtukova, I. A., Podgornaya, O. L., Wasik, A. & Sikora, J. (1988). Heat shock proteins in Amoeba. *Eur. J. Protistol., 24,* 64-68.

Kudryavtseva, A. V, Smurov, A. O. & Goodkov, A. V. (2007). Salinity tolerance of lobose amoebae and ciliates. *Protistology, 5,* 46.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227,* 680-685.

La Terza, A., Papa, G, Miceli, C & Luporini, P. (2001). Divergence between two antarctic species of the ciliate Euplotes, E. focardii and E. nobilii, in the expression of heat-shock protein 70 genes. *Mol. Ecol., 10,* 1061-1067.

La Terza, A., Miceli, C & Luporini, P. (2004). The gene for the heat-shock protein 70 of Euplotes focardii, an Antarctic psychrophilic ciliate. *Antarctic Science, 16,* 23-28

Lindquist, S. (1986). The heat-shock response. *Ann. Rev. Biochem., 55,* 1151—1191.

Lyashko, V. N., Vikulova, V. K., Chernicov, V. G, Ivanov, V. I., Ulmasov, Kh. A., Zatsepina, O. G. & Evgen'ev, M. B. (1994). Comparison of the heat shock response in ethnically and ecologically different human populations. *Proc. Natl. Acad. Sci. USA, 91,* 12492-12495.

Margulis, B. A. & Guzhova, I. V. (2000). Stress proteins in eukaryotic cells. *Tsitologiya, 42,* 323-342.

Margulis, B. A. & Guzhova, I. V. (2009). Dual role of chaperones in the response of a cell and of a whole organism to stress. *Tsitologiya, 51,* 219-228.

McMullin, Th. W. & Hallberg, R. L. (1987). A normal mitochondrial protein is selectively synthesized and accumulated during heat shock in Tetrahymena thermophila. *Mol. Cell Biol.,* 7, 4414-4423.

Page, F. C. (1988). A new key to Freshwater and Soil Gymnamoebae. *Ambleside: Freshwater Biol. Ass.*

Perez-Serrano, J., Martinez, J., Perez, B., Bernadina, W. E. & Rodriguez-Caabeiro, F. (2000). In vitro shock response to different stressors in free living and pathogenic Acanthamoeba. *Intern. J. Parasitol., 30,* 829-835.

Place, S. P. & Hofmann, G E. (2005). Constitutive expression of stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. *Polar Biol., 28,* 261-267.

Plekhanov, A. Yu., Smurov, A. O., Podlipaeva, Yu. I., Ivanova, L. O. & Goodkov A. V. (2006). Heat shock proteins of freshwater protists and their involvement in adaptation to changes in salinity of environment. *Tsitologiya, 48,* 350-354.

Podlipaeva, Y. I. (2001). Heat shock protein of 70 kDa in Amoeba proteus. *Protistology, 2,* 123-129.

Podlipaeva, Ju. I., Shmakova, L. A., Gilichinski, D. A. & Goodkov, A. V (2006). Heat shock protein of hsp70 family revealed in some contemporary freshwater amoebids and in Acanthamoeba sp. excysted from cysts isolated from permafrost samples. *Tsitologiya, 48,* 691-694.

Podlipaeva, Yu. I., Smurov, A. O. & Goodkov, A. V. (2008). Expression of heat-shock protein 70 kDa in Tetrahymena pyriformis during cell adaptation to salinity changes in the medium. *Cell and Tissue Biology, 2,* 373-5.

Prescott, D. M. & Carrier, R. F. (1964). Experimental procedures and cultural methods for Euplotes eurystomus and Amoeba proteus. In: *D. M. Prescott (Ed.), Methods in cell physiology* (pp. 85-95). New York - London: Acad. Press.

Requena, J. M., Jimee-Ruiz, H., Soto, M., Assiego, R., Santaren, J. F., Lopez, N. C, Pataroyo, M. E. & Alonso C (1992). Regulation of HSP70 expression in Trypanosoma cruzi by temperature and groing phase. *Mol. Biochem. Parasitol., 53,* 201-211.

Shatilovich, A. V, Shmakova, L. A., Gubin, S. V, Goodkov, A. V. & Gilichinsky D. A. (2005). Viable protozoa in late Pleistocene and Holocene permafrost sediments. *Doklady Biological Sciences, 401,* 136-138.

Smurov, A. O. & Fokin, S. I. (2001). Use of salinity tolerance data for investigation of phylogeny of Paramecium (Ciliophora, Peniculia). *Protistology, 2,* 130-138.

Smurov, A. O., Podlipaeva, Ju. I. & Goodkov, A.V. (2007). Heat shock protein of the Hsp70 family in the euryhaline ciliate Paramecium nephridiatum and its role in adaptation to the salinity changes. *Cell and Tissue Biology, 1,* 244-247.

Sonneborn, T. M. (1970). Methods in Paramecium research. Meth. *Cell Physiol, 4,* 241-339.

Sopina, V. A. (2000). Electrophoretic forms of glucose-6-phosphate dehydrogenase, acid phosphatase and esterase in Amoeba species amoebas. *Tsitologiya, 42,* 1134-1143.

Towbin, H., Staeheln, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA, 76,* 4350-4354.

Ullmann, J., Reidt, U., & Klein, A. (2004). The expression of the HSP70 gene in Moneuplotes crassus is controlled by a two-step process at the transcript level. *J. Eukar. Microbiol., 51,* 344-350.

Ulmasov, Kh. A., Shammakov, S., Karaev, K. & Evgen'ev M. (1992). Heat shock proteins and thermoresistance in lizards. *Proc. Natl. Acad. Sci. USA, 89,* 1666-1670.

Varadharajan, S., Chandrashekar, B. K. S., Rangarajan, P. N. & Govindarajan, P. (2004). Localization of ferrochelatase in Plasmodium falciparum. *Biochem. J., 384,* 429-436.

Whittaker, R. H. (1969). New concepts of kingdoms of organisms. Evolutionary relations are better represented by new classifications than by the traditional two kingdoms. *Science, 163,* 150-160.