The molecular chaperone Hsp70 from the thermotolerant Diptera species differs from the *Drosophila* paralog in its thermostability and higher refolding capacity at extreme temperatures

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Abstract

Previously, we demonstrated that species of the Stratiomyidae family exhibit higher tolerance to thermal stress in comparison with that of many representatives of Diptera, including *Drosophila* species. We hypothesized that species of this group inherited the specific structures of their chaperones from an ancestor of the Stratiomyidae family, and this enabled the descendants to colonize various extreme habitats. To explore this possibility, we cloned and expressed in *Escherichia coli* copies of the *Hsp70* genes from *Stratiomys singularior*, a typical eurythermal species, and *Drosophila melanogaster*, for comparison. To investigate the thermal sensitivity of the chaperone function of the inducible 70-kDa heat shock proteins from these species, we used an in vitro refolding luciferase assay. We demonstrated that under conditions of elevated temperature, *S. singularior* Hsp70 exhibited higher reactivation activity in comparison with *D. melanogaster* Hsp70 and even human Hsp70. Similarly, *S. singularior* Hsp70 was significantly more thermostable and showed in vitro refolding activity after preheating at higher temperatures than *D. melanogaster* paralog. Thermally induced unfolding experiments using differential scanning calorimetry indicated that Hsp70 from both Diptera species is formed by two domains with different thermal stabilities and that the ATP-binding domain of *S. singularior* is stable at temperatures 4 degrees higher than that of the *D. melanogaster* paralog. To the best of our knowledge, this study represents the first report that provides direct experimental data indicating that the evolutionary history of a species may result in adaptive changes in the structures of chaperones to enable them to elicit protective functions at extreme environments.

Keywords *Drosophila melanogaster* · *Stratiomys singularior* · Hsp70 · Luciferase · Refolding · Thermostability

Introduction

An understanding of how various defensive mechanisms, such as heat shock response (HSR), evolved in natural populations will provide a way to evaluate how organisms withstand the threat of various environmental stressors, including local temperature elevation and global warming. The analysis of a broad spectrum of organisms from different taxa, including insects, lizards, amphipods, and mammals, performed during the last three decades enabled us to reveal several basic features of HSR common to most of the studied organisms (Ulmasov et al. 1992; Garbuz et al. 2008; Bedulina et al. 2013; Evgen'ev et al. 2014). An ecology-based, large-scale analysis with the goal of demonstrating the protective role of Hsps, especially Hsp70, specifically in thermally adapted non-model organisms was initiated in the 1980s by several groups, including our group (Evgen'ev et al. 1987; Feder and

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Hofmann 1999; Tomanek and Somero 1999). Numerous studies of various species have demonstrated that Hsp genes and, in particular, members of the Hsp70 family are exceptionally conserved. Thus, the human chaperone HSP70 is 73% identical to *Drosophila* and 47% identical to *Escherichia coli* DnaK (Hunt and Morimoto 1985).

The molecular basis of Hsp70 chaperoning activity stems from the ability of this protein to bind misfolded or denatured proteins and hence prevent their aggregation, which is harmful for cells (Hartl et al., 2011; Nillegoda et al., 2015; Fernandez-Fernandez & Valpuesta, 2018). The ability of Hsp70 proteins to bind various misfolded proteins is due to their structure, comprising the N-terminal ATP-binding domain (NBD) and C-terminal peptide-binding region (PBD). Hsp70 molecular chaperones are highly conserved ATPases that use the energy of ATP binding and hydrolysis to regulate their interactions with the hydrophobic regions of unfolded proteins (Fuertes et al., 2004; Dores-Silva et al., 2015; Xu, 2018). The interaction of damaged proteins with Hsp70 leads to their stabilization in the unfolded state and thus prevents aggregation (Nollen & Morimoto, 2002; Fernandez-Fernandez & Valpuesta, 2018).

To compare HSR in various Diptera species isolated from thermally contrasting areas, we investigated several species of the Stratiomyidae family that are commonly known as “soldier flies.” Stratiomyidae larvae of different species are known to inhabit extreme semi-aquatic conditions with temperatures ranging from 4 to 50 °C and locations that vary from hot volcanic springs to cold streams (Rozkosny, 1997; Brammer & Dohlen, 2007). In contrast to *Drosophila* species, in which the level of Hsp70 is hardly detectable under normal physiological conditions and dramatically increased after heat shock (HS), in the studied Stratiomyidae species, we detected a high constitutive level of inducible Hsp70 under normal temperature conditions, while temperature elevation was able to induce only a moderate increase in Hsp70 concentration (Garbuz et al., 2008; Zatsepina et al., 2016).

In the present study, we investigated inducible Hsp70 in *Stratiomys singularior*, a very common and widely distributed transpalearctic eurythermal species. We collected specimens of this species in the Crimea, where *S. singularior* larvae are abundant in shallow pools saturated with H₂S that have temperatures of up to 40–42 °C in summer and mineralization of approximately 400 g/L (Golubkov et al., 2007).

Several genes belonging to the Hsp70 family were previously cloned from this species and sequenced (Garbuz, Yushenova, Zatsepina, Przhiboro, Bettencourt, & Evgen'ev, 2011). For *S. singularior*, LT50 for 30 min of heat shock is 45.5 °C, while the critical temperature is approximately 47 °C (Garbuz et al., 2008). In most wild type *Drosophila melanogaster* strains, these values are 40 °C and 41 °C, respectively (Zatsepina et al., 2001). Characteristically, the lifetime of induced Hsp70 in Stratiomyidae species exceeds that

of *D. melanogaster* several-fold (Zatsepina et al., 2016). Furthermore, the magnitude of thermotolerance observed in the studied Stratiomyidae species exceeds that of most insect species, with *Bombyx mori* and the desert ant (*Cataglyphis*) being prominent exceptions (Evgen'ev et al., 1987; Gehring & Wehner, 1995). A demonstrated comparative uniformity in thermotolerance in terms of LT50 and critical temperatures is observed among the studied Stratiomyidae species despite their quite different natural thermal environments (Garbuz et al., 2008).

Based on these data, we cannot state that the exceptionally high thermotolerance exhibited by all studied Stratiomyidae species represents local genetic adaptation to elevated temperatures achieved by natural selection. Our preferred hypothesis to explain the adaptation of all the studied Stratiomyidae species to extreme conditions is that high thermotolerance may be a characteristic feature of the ancestor of the whole Stratiomyidae family that enabled the descendants to successfully colonize various inhospitable environments (Zatsepina et al., 2016). There are phylogenetic and paleontological data in support of this hypothesis, suggesting that Mesozoic Stratiomyidae were already preadapted to colonize different extreme habitats, and such colonization occurred repeatedly for different genera in this taxon (Rozkosny, 1997; Whalley & Jarzembowski, 1985).

The characteristic differences in the sequences and/or structures of heat shock proteins present in the Stratiomyidae species may account for the uniformity in the thermotolerance exhibited by Stratiomyidae species dwelling in different thermal environments. To verify this possibility, we cloned and expressed genes encoding inducible members of the *hsp70* family from *D. melanogaster* and *S. singularior* in *E. coli*. The purified Hsp70 proteins from these species were compared with regard to the thermostability of both of their domains and their ability to exert chaperone functions by using in vitro luciferase refolding assay.

Materials and methods

The generation of the expression constructs and protein purification

Since the inducible *hsp70* genes from both Diptera species do not contain introns, we used genomic DNA isolated from adult *D. melanogaster* and *S. singularior* specimens to clone the paralogous genes, *Hsp70Aa* and *Hsp70S1*, respectively. Sequences of both genes are given in the GenBank under accession numbers NM_169441 (*D. melanogaster*) and HQ184404 (*S. singularior*). The genes were amplified by PCR primers containing a *Nde*I site at the 5'-end and a *Xho*I site at the 3'-end. The following primers were used for amplification:

D. melanogaster forward CTCACACCATATGCCTGCTA TTGGAATCGA; reverse ATTAGACTCTCTCGAGTTAG TCGACCTCCTCGA

S. singularior forward AAGAAAAGTTGACATATGCC TGCAGTAGGA; reverse CTGTGACCTCGAGTTAATC TACTTCCTCAACTGTCGGA

For the expression of the proteins, a commercial pET-14b plasmid containing a 6His-tag at the N-terminus of the synthesized protein was used.

Recombinant proteins containing 6His-tags at the N-terminus were expressed in *E. coli* and purified essentially as described earlier (Bobkova et al., 2015). As a control, we used previously generated human recombinant Hsp70 expressed in *E. coli*. The *E. coli* BL21 strain was used for protein expression. The cells were grown in SOB medium to an OD₆₀₀ of 0.6–0.8 at 37 °C, and protein expression was induced by adding 0.5 mM IPTG. After this, the cells were grown for another 3 h at 37 °C and harvested by centrifugation at 4000g. The obtained 6His-tagged Hsp70 protein from both species was purified using Ni-NTA resin columns according to the manufacturer's instructions (QIAGEN, Ni-NTA Superflow BioRobot Hand Book). The final removal of LPS contamination from the isolated proteins was performed by double passage through polymyxin B-agarose columns (Sigma). The Hsp70 protein content was determined using a modified Bradford protocol, and the purity was confirmed by PAGE followed by staining with Coomassie Blue (Figure S1).

The purity of the resulting Hsp70 preparations from both species was higher than 95%, and according to a standard LAL test, the endotoxin levels were less than 0.1 U/mL.

Luciferase assay and thermal stability

To obtain lysates for the luciferase assays enriched with co-chaperones from the DNAJ and BAG families, we used the human lung cancer cell line A549, which was obtained from the Collection of Cell Cultures of the Institute of Cytology (St. Petersburg, Russia). To minimize the effect of intracellular endogenous Hsp70, the cells were transduced with a lentiviral vector bearing anti-HSP70 shRNA. The resulting cell line, A549-HSP-, was obtained by selecting transduced cells in puromycin-containing medium. The downregulation of Hsp70 expression was confirmed by western blotting (Meshalkina et al., 2016). Refolding assays were performed using a modified protocol from Thulasiraman & Matts (1998). Recombinant firefly luciferase (Promega) in refolding buffer (25 mM Tricine, 50 mM KCl, 5 mM MgCl₂, and 2 mM ATP) at a 2.3 μM concentration was diluted 100-fold in denaturing buffer (25 mM Tricine, 50 mM KCl, 5 mM MgCl₂, and 5 mM DTT). The thermal denaturation was performed at 43 °C using a thermoshaker until the loss of 99% of luciferase activity was observed. The thermally denatured luciferase was diluted 100-fold in refolding buffer, which contained A549 or A549-

HSP70 cell lysate (500 μg/mL), 10 mM creatine phosphate, 16 units/mL creatine phosphokinase, and aliquots of the investigated chaperones (or BSA as a negative control). To assess the thermal stability of the Hsp70 proteins from the two Diptera species, the preparations were incubated at elevated temperatures ranging from 40 to 49 °C for 20 min. To monitor the thermal stability of the compared Hsp70 preparations of different origins, the proteins were pre-incubated at different temperatures ranging from 40 to 49 °C for 30 min, and the preheated proteins were assayed for reactivation capacity. To monitor the luciferase reactivation kinetics, 5 μL of refolding mix was added to a 45-μL aliquot of Bright-GLO Luciferase Assay reagent (Promega, USA). Luciferase activity was measured in a Fluorophot Charity luminometer (Nanospecter, Russia). The reactivation capacity was calculated as a ratio between the signals obtained from the activity of the experimental and the control intact luciferases.

Differential scanning calorimetry

Microcalorimetric measurements of the Hsp70 proteins from both Diptera species were carried out with a VP-DSC microcalorimeter (MicroCal, USA) at a heating rate of 1 K/min with 15 μM protein solutions, as described elsewhere (Mitkevich et al., 2003). The curves were corrected for the instrumental baseline obtained by heating the solvent used for the protein solutions. The effect of 5 mM ATP on the Hsp70 structure was also investigated using thermally induced unfolding. To analyze the functions of the excess heat capacity (C_p), the Origin-differential scanning calorimetry (DSC) software package was used. Computer deconvolution of the melting curves was performed under the assumption that a melting curve is an algebraic sum of peaks corresponding to cooperative one-step transitions. The accuracy of the calorimetric enthalpy (ΔH) was ± 10% and that of the denaturation temperature (T_d) was ± 0.2 °C. PBS containing 0.35 potassium chloride, pH 7.4, and 5 mM MgCl₂ was used as a buffer.

Statistical analysis

The experimental results of the refolding assays were statistically processed using the Statistica software (Version 10; Statsoft)^{a,b}. Four-way ANOVA analysis with repeated measurements with three replicates for the raw data from the luciferase reporter assay was used. The origin of the protein, its concentration, and the time and conditions of the sample preparation were taken into account. The ANOVA design was limited to these three interacting factors. Post hoc analysis with an LSD test was used to evaluate the refolding efficacy of the different proteins under specific experimental conditions.

Results

Monitoring of the refolding activity of the Hsp70 preparations under standard conditions

In the first stage, it was necessary to compare the refolding activity of the Hsp70 preparations isolated from the two Diptera species and that of the control human Hsp70 chaperone protein at normal temperature (25 °C). The technique that was used is routinely used to demonstrate the protein refolding activity of purified molecular chaperones of different origins (Zippay et al., 2004). In the current study, which was conducted under normal temperature conditions, all three preparations of Hsp70 isolated from *S. singularior*, *D. melanogaster*, and humans exhibited similar chaperone activity and were able to partially reactivate thermally denatured luciferase with time- and concentration-dependent kinetics, as expected. Notably, in this series of experiments, *D. melanogaster* Hsp70 showed slightly increased refolding activity compared with the two other preparations (human and *S. singularior*) under standard experimental conditions (Fig. 1a). See statistical analysis (Tables 1, 2, and 3).

Thermal sensitivity of protein folding by Hsp70 preparations of different origins throughout the range of elevated temperatures

To compare the thermal sensitivity of protein folding of the three Hsp70 preparations, we used two complementary approaches for exploring ecologically relevant temperatures (40 °C) for both Diptera species. In the first series of experiments, the Hsp70 preparations were pre-incubated at 40 °C for 30 min, and their refolding activity was subsequently determined at 25 °C (Fig. 1b). In the second independent series of experiments, the measurements of luciferase reactivation by the different Hsp70 preparations were conducted at 40 °C at 30–60-min intervals (Fig. 1c). The analysis of both series of experiments enabled us to conclude that under conditions of elevated temperature (40 °C), the *S. singularior* Hsp70 preparation exhibited higher activity in comparison with both of the other preparations at all time intervals and at both concentrations in the refolding assays (Fig. 1b, c; Fig. 2; Figure S2; Table S1).

We used four-way ANOVA with repeated measurements with three replications in analysis of the raw data from the luciferase reporter assay. The origin of the protein, its concentration, and the time and conditions of the sample preparation were taken into account. The ANOVA design was limited to these three factors for the analysis

of the interaction. The results of refolding assay data analysis are presented in Table S1.

The statistical analysis confirmed (four-way ANOVA and Fisher LSD test) that the refolding activity level of the *S. singularior* chaperone was significantly higher at 40 °C (Table S1 and S2). Similar results were obtained in another independent series of experiments where we used lysates obtained from cells in which the synthesis of endogenous Hsp70 was inhibited (knock-down line) and that had higher concentrations of the compared chaperones (Figure S2; Table S3).

In the next step, we compared the refolding capacities of different Hsp70 preparations pre-incubated for 20 min at temperatures ranging from 40 to 49 °C, which includes extreme, ecologically non-relevant temperatures for *D. melanogaster* but relevant temperatures for *S. singularior*. The luciferase assays demonstrated that the *in vitro* chaperone activity of the preheated *D. melanogaster* Hsp70 started to dramatically decline at temperatures of 45 °C and higher, while a similar decline in *S. singularior* Hsp70 activity occurred only after treatment at 47 °C, which is the temperature that many Stratiomyidae species encounter in nature (Garbuz et al., 2008) but is lethal for *D. melanogaster* flies and larvae. The refolding activity of the *S. singularior* Hsp70 preheated at up to 49 °C was dramatically decreased and became equal to that of *D. melanogaster* (Fig. 3), since both proteins apparently became inactivated at this temperature.

Our *in vitro* assays clearly demonstrated that *S. singularior* Hsp70 exhibited higher refolding activity at elevated temperatures in comparison with that of the *D. melanogaster* paralog and was characterized by significantly higher thermostability in terms of its chaperone function (Fig. 3). To deepen our knowledge of the structure-function relationships of the Hsp70 chaperones from the two Diptera species, we produced both recombinant proteins in a soluble and functional state and monitored the thermal stability of these proteins in solution using scanning calorimetry.

Thermal stability of HSP70 chaperones of different origins

To monitor the thermal stability of the Hsp70 chaperones from *D. melanogaster* and *S. singularior* in solution, we used differential scanning calorimetry (DSC). The stability was estimated depending on the presence of ATP in the solution. Since Diptera Hsp70 proteins, like other Hsp70 proteins, are formed by two domains, the N-terminal ATPase domain (NBD) and the C-terminal peptide-binding domain (PBD), the temperature dependence of the partial molar heat capacity of both *S. singularior* Hsp70 and *D. melanogaster* HSP70 exhibited two peaks,

Fig. 1 Time course measurements of luciferase reactivation by inducible Hsp70 chaperones of different origins (Dm-*D. melanogaster*; Ss-*S. singularior*; Hu-human) under different temperature conditions. Luciferase was thermally denatured and then added to assay tubes containing refolding buffer, 2 mM ATP, and different concentrations of chaperones in a final volume of 100 μ L. At 30–60-min intervals, aliquots of the samples were added to 50 μ L of LAR, and luciferase activity was measured as described in the “Materials and methods” section at 25 °C. Refolding activity was expressed as a relative activity ratio between the experimental signal and the signal from control experiments, which used luciferase that was not denatured. At each time point, the values in the columns represent the mean $\pm$ SD of three replicates. A- luciferase refolding capacity of the three Hsp70 preparations at 25 °C measured at 30–60 min intervals. B- luciferase refolding capacity of the three Hsp70 preparations pretreated at 40 °C for 30 min then measured at 25 °C at 30–60-min intervals. C- luciferase refolding capacity of the three Hsp70 preparations monitored at an elevated temperature (40 °C) at 30–60-min intervals

as expected (Fig. 4). The T_d values of the first peak for both proteins were practically the same (Table 4), while the T_d of the second peak of *S. singularior* Hsp70 was 6 degrees higher than that of the *D. melanogaster* paralog. It was also demonstrated that denaturation was sensitive to the presence of ATP in the solution. Thus, upon complex formation with ATP, the T_d of the first calorimetric

peak increased by 10.2 °C for *S. singularior* Hsp70 and by 4.5 °C for *D. melanogaster* HSP70, while the T_d of the second peak did not change for either proteins (Table 1, Fig. 4). This suggests that the first peak corresponded to the melting of the ATP-binding domain and that the formation of a complex with ATP stabilized this domain. The significantly higher thermal stability of *S. singularior*

Table 1 Fisher LSD test results for the factors “protein” and “conditions” interaction

Cell No.	Conditions	Protein	{1} 0.188	{2} 0.169	{3} 0.174	{4} 0.182	{5} <i>0.191</i>	{6} 0.174	{7} 0.149	{8} <i>0.169</i>	{9} 0.145
1	Normal	A		<i>0.0008</i>	<i>0.0037</i>	0.1631	0.2949	<i>0.0036</i>	<i>0.0000</i>	<i>0.0007</i>	<i>0.0000</i>
2	Normal	S3	<i>0.0008</i>		0.2737	<i>0.0061</i>	<i>0.0002</i>	0.2788	<i>0.0004</i>	0.8935	<i>0.0001</i>
3	Normal	2	<i>0.0037</i>	0.2737		<i>0.0362</i>	<i>0.0009</i>	0.9897	<i>0.0001</i>	0.2255	<i>0.0000</i>
4	Pre-heat 40 °C	A	0.1631	<i>0.0061</i>	<i>0.0362</i>		<i>0.0290</i>	<i>0.0355</i>	<i>0.0000</i>	<i>0.0050</i>	<i>0.0000</i>
5	Pre-heat 40 °C	S3	0.2949	<i>0.0002</i>	<i>0.0009</i>	<i>0.0290</i>		<i>0.0008</i>	<i>0.0000</i>	<i>0.0002</i>	<i>0.0000</i>
6	Pre-heat 40 °C	2	<i>0.0036</i>	0.2788	0.9897	<i>0.0355</i>	<i>0.0008</i>		<i>0.0001</i>	0.2298	<i>0.0000</i>
7	Refolding 40 °C	A	<i>0.0000</i>	<i>0.0004</i>	<i>0.0001</i>	<i>0.0000</i>	<i>0.0000</i>	<i>0.0001</i>		<i>0.0004</i>	0.3162
8	Refolding 40 °C	S3	<i>0.0007</i>	0.8935	0.2255	<i>0.0050</i>	<i>0.0002</i>	0.2298	<i>0.0004</i>		<i>0.0001</i>
9	Refolding 40 °C	2	<i>0.0000</i>	<i>0.0001</i>	<i>0.0000</i>	<i>0.0000</i>	<i>0.0000</i>	<i>0.0000</i>	0.3162	<i>0.0001</i>	

Significant differences are in italics. The refolding parameters acquire maximal values in the case of *S. singularior* Hsp70 and significantly exceeded the other preparations in this respect at elevated temperatures (Tab. S1). The figures depict dynamics of refolding activity of each preparation studied depending on the temperature of refolding assay per se and whether Hsp70 preparation was preheated at 40 °C

Hsp70 corroborated the results of the luciferase assay, demonstrating that this protein has increased chaperone activity compared with that of the *D. melanogaster* paralog at all elevated temperatures tested (Fig. 3).

Discussion

From the very beginning of the broad-scale investigations conducted into the role of Hsps in adaptation to extreme and

highly variable environments, members of the Hsp70 family have represented the main focus of such studies (Feder & Hofmann, 1999; Evgen'ev et al., 2014). Thus, many attempts have been made to correlate the synthesis of Hsp70 with adaptation to thermally fluctuating or extreme ecological niches. The investigation of various species belonging to different classes has demonstrated that, in general, organisms inhabiting extreme zones or regions with fluctuating thermal conditions usually express high levels of Hsp70 and other Hsps under normal conditions that enable them to withstand

Table 2 Effects of the protein origin factors and experimental conditions on refolding. Four-way ANOVA with repeated measurements with three replications

Effect	SS	Degree of freedom	MS	F	p
Conditions	<i>0.023455</i>	2	<i>0.011727</i>	<i>109.27</i>	<i>0.000002</i>
Protein	<i>0.004432</i>	2	<i>0.002216</i>	<i>20.64</i>	<i>0.000694</i>
Time	<i>0.942651</i>	2	<i>0.471326</i>	<i>4391.45</i>	<i>0.000000</i>
μg_mL_Hsp70	<i>0.315856</i>	1	<i>0.315856</i>	<i>2942.91</i>	<i>0.000000</i>
Conditions*protein	<i>0.007453</i>	4	<i>0.001863</i>	<i>17.36</i>	<i>0.000522</i>
Conditions*time	<i>0.014520</i>	4	<i>0.003630</i>	<i>33.82</i>	<i>0.000046</i>
Protein*time	0.000574	4	0.000143	1.34	0.335948
Conditions*μg_mL_Hsp70	<i>0.016818</i>	2	<i>0.008409</i>	<i>78.35</i>	<i>0.000006</i>
Protein*μg_mL_Hsp70	0.000274	2	0.000137	1.28	0.330133
Time*μg_mL_Hsp70	<i>0.061976</i>	2	<i>0.030988</i>	<i>288.72</i>	<i>0.000000</i>
Conditions*protein*time	0.001742	8	0.000218	2.03	0.168412
Conditions*protein*μg_mL_Hsp70	0.000420	4	0.000105	0.98	0.470363
Conditions*time*μg_mL_Hsp70	<i>0.010854</i>	4	<i>0.002713</i>	<i>25.28</i>	<i>0.000136</i>
Protein*time*μg_mL_Hsp70	0.000248	4	0.000062	0.58	0.687632
Error	0.000859	8	0.000107		

The origin of hsp70 proteins per se and its interaction with probes processing and preparation has significant influence on refolding efficacy ($p < 0.001$). The impact of other interactions of the proteins origin with reaction time and proteins concentration does not differ from the sum of impacts of each of interacting factors ($p > 0.05$). Significant differences are in italics

Table 3 The differences in the refolding activity of the compared Hsp70 chaperones monitored at 40 °C at 1- and 2-h intervals. (Fisher LSD test results for the factors “protein” and “conditions” interaction)

Protein	Time	{1} 13.667	{2} 22.933	{3} 17.100	{4} 26.333	{5} 15.233	{6} 24.533
1	A 1 h		<i>0.0000</i>	<i>0.0289</i>	<i>0.0000</i>	<i>0.2796</i>	<i>0.0000</i>
2	A 2 h	<i>0.0000</i>		<i>0.0012</i>	<i>0.0302</i>	<i>0.0001</i>	<i>0.2700</i>
3	S 1 h	<i>0.0289</i>	<i>0.0012</i>		<i>0.0000</i>	<i>0.2022</i>	<i>0.0002</i>
4	S 2 h	<i>0.0000</i>	<i>0.0302</i>	<i>0.0000</i>		<i>0.0000</i>	<i>0.2177</i>
5	C 1 h	<i>0.2796</i>	<i>0.0001</i>	<i>0.2022</i>	<i>0.0000</i>		<i>0.0000</i>
6	C 2 h	<i>0.0000</i>	<i>0.2700</i>	<i>0.0002</i>	<i>0.2177</i>	<i>0.0000</i>	

Significant differences are in italics

unfavorable environments (Evgen'ev et al., 2014). Furthermore, the dependence of the induced expression of certain Hsp70 family members on the severity of heat shock has been demonstrated by several groups, suggesting differences in the protective efficacy and likely structures of individual Hsp70 family members present in the same species (Ulmasov et al., 1992; Tomanek & Somero, 1999).

Therefore, one may assume that some presumptively adaptive changes as a result of the evolutionary history of the species may be present in the sequences of *hsp70* genes. Such changes at the sequence level may affect the inducibility, level of expression, and stability of *hsp70* mRNA and Hsp70 protein and, hence, contribute to the heat shock tolerance of an organism. It is noteworthy that although there are data on the presumptive adaptive role of several SNPs detected in the human HSPA1A gene and the higher thermotolerance of

different haplotypes described in several other species groups (Singh et al., 2006; Basiricò et al., 2011; Yang et al., 2014; Marada et al., 2016), all these data are based on association and correlation analysis, and Hsp70 proteins from related species or populations from different thermal environments, to the best of our knowledge, were never directly compared in terms of their thermostability parameters and chaperone function. With this in mind, we cloned and expressed inducible *hsp70* genes from two Diptera species in *E. coli* (*D. melanogaster* and *S. singularior*) that differ drastically in their responses to HS and their basic thermotolerance (Garbuz et al., 2011). Notably, in our previous investigation, we introduced the *S. singularior* Hsp70 gene into a *D. melanogaster* strain devoid of any endogenous Hsp70 genes via P-mediated transformation (Shilova et al., 2018). Thermotolerance experiments demonstrated that the larvae of this transgenic strain that contained a single *S. singularior* Hsp70 gene exhibited significantly higher thermotolerance in comparison with another transgenic strain that contained one copy of the endogenous *D. melanogaster* Hsp70 gene. These results imply that the structure of *S. singularior* Hsp70 itself plays an important role in responses to HS in general.

Although correlations between amino acid usage and environmental temperatures in prokaryotes are well documented, the understanding of the mechanisms involved in thermal adaptation remains incomplete (Nakashima, Fukuchi, & Nishikawa, 2003; Venev & Zeldovich, 2018). Limited information about the amino acid residues that contribute to thermoresistance in eukaryotic organisms, which was obtained predominantly in thermophilic fungi, is available (van

Fig. 2. The dependence of the refolding activity of individual Hsp70 preparations on preheating (40 °C) and the temperature of assay. The least squares means of refolding are given for experiments conducted at different temperatures and concentrations; the vertical bars denote 0.95 confidence intervals. Current effect “conditions*protein” interaction: $F(\text{Bobkova et al., 2015; Evgen'ev et al., 1987}) = 17.359, p = 0.00052$. The most effective refolding of protein S3 was exhibited in sample preparation mode “pre-Heat 40 °C” in an assay performed at 40 °C.

Fig. 3 Time course measurements of denatured luciferase reactivation by Hsp70 chaperones of different origins (Dm-*D. melanogaster*; Ss-*S. singularior*; Hu-human) preheated at temperatures ranging from 40 to 49 °C. Luciferase was thermally denatured and then added to assay tubes containing refolding buffer and 2 mM ATP at a final volume of 100 μ L. At 30–60-min intervals, aliquots of the samples were added to 50 μ L of LAR, and luciferase activity was measured as described in the “Materials and methods” section. At each time point, the values in the columns represent the mean $\pm$ S.D. of three replicates. Asterisks indicate significant differences between refolding activities exhibited by the compared chaperones at different temperatures. *** $p < 0.001$, ** $p < 0.01$

Noort et al., 2013). It was shown by several groups that the stabilization of protein tertiary structures to prevent heat denaturation in various thermoresistant species was usually achieved by means of specific amino acid substitutions. These substitutions, which are associated with thermophily,

result in the formation of additional intramolecular links in the vicinity of the active center, leading to the stabilization of the protein structure (Fields, 2014; Van Noort et al., 2013).

Therefore, it is possible that structural differences occurring in Hsp70s isolated from organisms from different thermal

Fig. 4 The thermally induced unfolding of the *D. melanogaster* (a, c) and *S. singularior* (b, d) Hsp70 molecular chaperones in the absence of ATP (a and b) and in the presence of ATP (c and d). The curves depict the temperature dependence of the excess heat capacity (Cp) of the proteins and represent their deconvolution as two well-defined transitions (red lines). The origin of the Hsp70 proteins *per se* and their interactions during probe processing and preparation have a significant influence on their refolding efficacy ($p < 0.001$). The impact of the other interactions of the proteins in terms of reaction time and protein concentration did not differ from the sum of the impacts of each of the interacting factors ($p > 0.05$).

Table 4 Thermal denaturation parameters of *D. melanogaster* (Dm) and *S. singularior* (Ss) Hsp70 chaperones and their complexes with ATP

Sample	T_{d1}^a (°C)	ΔH_1^b (kcal/mol)	T_{d2}^a (°C)	ΔH_2^b (kcal/mol)
Dm	41.9	71	48.3	59
Dm-ATP	46.4	64	48.8	69
Ss	42.2	68	54.5	58
Ss-ATP	52.4	67	54.4	56

^a T_{d1} and T_{d2} denaturation temperature of first and second calorimetric peak, correspondingly

^b ΔH_1 and ΔH_2 calorimetric enthalpy of first and second peak, correspondingly

environments may represent one of the possible adaptations occurring in the evolutionary history of a species. It is of note that this ideology considering Hsps stability as a possible factor in acquired thermotolerance was suggested in pioneer works of Hightower's group (Leung et al., 1996). In the present investigation, we compared the thermostability and refolding efficiency of inducible Hsp70 chaperones isolated from moderately and highly thermotolerant Diptera species. The analyses performed demonstrated that recombinant *S. singularior* Hsp70 displayed higher thermostability than the paralogous *D. melanogaster* protein, as revealed by the functional in vitro luciferase refolding assay and DSC method (Figs. 3 and 4).

Overall, the in vitro data show that Hsp70 chaperones from both Diptera species are able to bind and temporarily stabilize the activity of thermally denatured luciferase at temperature ranges that correlate with the temperatures encountered by the compared species (moderate for *D. melanogaster* and extreme for Stratiomyidae species) (Rozkosny, 1997). Translated into an ecological context, these results suggest that the compared inducible members of the Hsp70 family probably have activity that is similarly thermally sensitive in vivo. It is noteworthy that similar studies performed using luciferase refolding assays and Hsc70 from eurythermal goby fish demonstrated that while the protein displayed chaperone function in vitro, in contrast to the findings of our studies, it was relatively thermally insensitive throughout the range of experimental temperatures (Zippay et al., 2004). This may be explained by the constitutive nature of goby protein expression; in our studies, we used inducible members of the Hsp70 family.

Although our luciferase refolding assays did not include native co-chaperones, we observed refolding activity using lysates from mammalian cells, as was done in other studies. It was shown that the expression of the C-terminal peptide-binding domain as well as the full-size Hsp70 protein protects mouse cells from stress (Li et al., 1992). While numerous studies demonstrated the in vitro refolding activity of molecular chaperones, including Hsp70, very few of them considered the action of the chaperones in question with respect to the ecological or evolutionary relevance of the temperature

range explored (Zippay et al., 2004). Furthermore, in most studies, the chaperones of phylogenetically very distant organisms (e.g., yeast and humans) have been compared (Schumacher, Hansen, Freeman, Alnemri, Litwack, & Toft, 1996). In our investigation, we compared Hsp70 chaperones from Diptera species exhibiting strikingly different thermotolerance and HSRs. The luciferase reactivation assays that explored Hsp70 preparations preheated at temperatures ranging from 40 to 49 °C demonstrated that the in vitro chaperone activity of *D. melanogaster* Hsp70 started to dramatically decrease during pretreatments at and above 45 °C, which is much higher than the lethal temperature of *D. melanogaster*. Characteristically, a similar decline in *S. singularior* Hsp70 refolding activity takes place only after pretreatment at 47 °C, which is a temperature that many Stratiomyidae species may encounter in nature (47 °C) and is inevitably lethal for *D. melanogaster* flies and larvae.

In the next stage, we used a biophysical method (DSC) to monitor the melting kinetics of the studied proteins in solution by considering that global protein stability may be correlated with evolutionary adaptations to temperature. Overall, this analysis indicated that the two functional domains, the ATPase moiety and the substrate protein binding region, of the Hsp70 chaperones from both of the Diptera species studied showed different sensitivities to thermal denaturation and formed two distinct peaks (Fig. 4). Similarly, in *Plasmodium falciparum*, Hsp70 also contains two domains, and fluorescence and circular dichroism studies demonstrated that the N-terminal/nucleotide-binding domain (NBD) was relatively unstable in isolation, while the C-terminal domain functioned as a stabilization domain (Misra & Ramachandran, 2009).

The DSC analysis demonstrated that the ATP-binding domain of *D. melanogaster* shows significantly lower thermal stability than that of *S. singularior* (Fig. 4, Table 1). These data fully corroborated our in vitro analysis of the refolding properties of these chaperones and represent a rare example in which the characteristic molecular features of chaperones have been demonstrated in an evolutionary and ecological context.

We are well aware that Hsp70 members are not the only adaptogenes responsible for stress resistance. Thus, the enhanced expression of small Hsps, particularly Hsp22, increased the resistance of flies to heat shock and other forms of stress (Morrow et al., 2016). It is also known that representatives of different groups of Hsps, including the Hsp70 family, function in cells in cooperation with other Hsps and multiple cofactors (Hartl et al., 2011; Xu 2018). It is noteworthy that Hsps are not the only protective molecules that provide heat resistance at the cellular and/or organismal level; trehalose in yeast is a prominent example of another type of protective molecule (Hottiger et al., 1987). Furthermore, post-translational modifications of Hsps may also play an important role in thermotolerance. Thus, variations in glycosylation

patterns observed in camel and human HSP70 probably play an important role in the more efficient proteostasis and higher thermotolerance observed in camelids (Hoter et al., 2018). Similarly, a cytosolic ATPase (OLA1) was shown to stabilize mammalian HSP70, leading to upregulation of HSP70 as well as increased survival during heat shock (Mao et al., 2013).

In our previous studies, we compared Hsp70 from Stratiomyidae species with paralogs from several highly thermotolerant organisms, including *Bombyx mori*, *Locusta migratoria*, and *Drosophila mojavensis* (Garbuz et al., 2011). Although the analysis enabled us to determine the amino acid residues specific to Hsps from Stratiomyidae species and other highly thermotolerant organisms, including mammals, it would be premature to speculate on any functional significance of these amino acids because such speculations would require the inclusion of many more Hsp70 sequences in the analysis and mutagenesis of candidate SNPs. These mutation sites may be useful as molecular genetic markers to assist selection for heat tolerance.

Our study should contribute to the understanding of the molecular mechanisms underlying thermo-adaptation in various organisms and aid in the development of new versions of proteins resistant to temperature elevation and other forms of stress.

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Author contributions G.D. and M.E. designed the study. G.D. and D.A. cloned Hsp70 and expressed in *E. coli*. S.D. and M.B. conducted refolding assays. K.A. analyzed the data and performed statistics. M.V. conducted thermostability analysis of the proteins by DSC method.

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