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Research Article

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ABSTRACT

Thermotolerance is a temporary resistance against lethal temperatures. Induction of heat shock proteins (HSP), mainly HSP70, has been correlated to this response. Our research demonstrated the pre-exposure to sublethal temperatures of 30, 33 and 36 °C increased the survival of *Biomphalaria glabrata* to lethal temperature of 42 °C. This animal is a freshwater snail recognized as a suitable organism for experimental research. Additionally, it was observed an induction of HSP70 expression in digestive gland in all temperatures tested but not in ovotestis and head/foot tissues. Despite the higher expression of HSP70 had been detected at 30 °C, the animals exposed to 33 °C presented the highest survival. Our results indicate the increase of HSP70 is involved in the resistance to lethal temperature in *B. glabrata* as well other proteins.

Keywords: Thermotolerance; *Biomphalaria glabrata*; HSP70.

HSP: Heat Shock Protein; HSP70: Heat Shock Protein of 70 kDa; *B. glabrata*: *Biomphalaria glabrata*; *S. mansoni*: *Schistosoma mansoni*.

INTRODUCTION

Thermotolerance is a phenomenon in which an initial, nonlethal heat induces temporary resistance against subsequent lethal heat shock. Induction of heat shock proteins (HSP) has been positively correlated to this response (Lindquist, 1986; Lindquist and Craig, 1988).

Experiments with a pre-exposure to heat, followed by a shift to an even higher temperature, resulted in the ability to organisms to withstand the latter temperature which would otherwise be lethal. This approach has been performed in both cultured cells (Anderson *et al*., 1988) and a wide variety of organisms, including bacteria (Boutibonnes et al., 1992), fungus (Ferreira et al., 2005), insects (Carretero et al., 1991; Kalosaka et al., 2009), plants (Ahn et al., 2004), sea urchin (Sconzo et al., 1986) and molluscs (Brun et al., 2009; Jackson et al., 2011). The thermotolerance was positively correlated with the amount of heat shock proteins produced, mainly HSP70 which is the most studied family of heat shock proteins.

The HSP70 family represents one of the most highly conserved classes of the heat shock proteins with molecular weights in the range of 68 to 75 kDa. Among the various isoforms, HSP70 are often the prominent proteins to be expressed following environmental assaults. The HSP70 family is present in subcellular compartments and primarily binds to target proteins to modulate protein folding, in this way these proteins prevent the formation of aggregates, and also aid the correct intracellular localization of proteins. Under adverse environmental conditions, the high levels of HSP70 assure protection to the cells from proteotoxicity, repairing damaged proteins (Powers and Workman, 2007).

Biomphalaria glabrata (Say, 1818), a freshwater mollusc, is the intermediate host of *Schistosoma mansoni* (Sambon, 1907), the trematode that causes schistosomiasis (DeJong et al., 2001; Knight et al., 2000) .

Its genetic uniformity in addition to physiological characteristics, background knowledge of biological properties and ease of experimental manipulation contributes for it employment in experimental protocols (Estevam et al., 2006; Grazeffe et al., 2008; Nakano et al., 2003; Tallarico et al., 2004).

B. glabrata cope with large variations in environmental temperature. The optimal range for survival is 20 to 30 ºC; at 15 ºC, these snails become numb, below 10 ºC, they stop feeding and laying eggs, and no longer survive at 5 ºC for 4 to 5 days. At 40 ºC, survival reaches 4 hours, but at 50 ºC, no more than few minutes (Rey, 1991).

In this work, we investigated the effects of pre-exposure to sublethal temperatures in survival at lethal temperatures and in HSP70 expression in *B. glabrata*.

MATERIAL AND METHODS

Animals

A pigmented wild-type strain of *B. glabrata* (obtained from Barreiro, Minas Gerais, Brazil) has been reared for several generations in controlled conditions. The animals, *S. mansoni* negative, were maintained in plastic aquaria with filtered, dechlorinated and aerated water, pH around 7.0, at room temperature (25 ºC ±2 ºC), and daily fed with fresh lettuce (*Lactuca sativa*) and fish ration once a week.

A population of 105 sexually mature snails, 4-6 months old, and with a shell diameter of 14.5 ±1.5 mm were used. They were distributed in three groups of 35 individuals each: 15 individuals were maintained around 25 ºC as control (10 to survival experiment and 5 to analysis of HSP70) and 20 individuals were exposed to sublethal temperatures (10 to survival experiment and 10 to analysis of HSP70).

Exposure to heat

The animals were maintained in BOD climatic chamber at 30 (30.1±0.5), 33 (33.1±0.2) and 36 (35.9±0.1) ºC, for five days in plastic aquaria with filtered water in a volume of 50 mL per snail, pH around 7.0, and fed with fresh lettuce.

After the fifth day, the snails submitted to sublethal temperatures and the control were exposed to the lethal temperature of 42 ºC (Rey, 1991). The snails were observed each hour, and the death confirmed by the absence of heart beating using stereoscopy.

Analysis of HSP70

Digestive gland, ovotestis and head/foot tissues were dissected, and analyzed as pools for each experimental group. The tissue extracts were prepared according to Todeschini et al. (2007), using RIPA Buffer plus protease inhibitors; after centrifugation at 14000*g*, the protein content of supernatants was estimated using NanoDrop spectrophotometer. The tissue extracts were electrophoresed on 10% discontinuous sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) using Bio-Rad® minigel apparatus. Separated proteins were transferred onto nitrocellulose membranes (0.45 µm) using Mini Trans-Blot cell (Bio-Rad®). The HSP70 was detected using anti-HSP70 from *Tripanossoma brucei* policlonal antibody, produced in rabbit and kindly provided by Dr. James D. Bangs from the University of Wisconsin Madison. Blots were blocked with PBS-milk (5%); then incubated for 1 hour with anti-HSP70 antibody (1:1000 in PBS-milk), and washed with PBS-milk; finally incubated with goat anti-rabbit horseradish peroxidase conjugated antibody (1:2000 in PBS-milk) for 1 hour and washed again. The visualization was done with Revelator kit (Pierce®) and photographic film.

Bands were analyzed using the free software ImageJ (Abramhoff et al., 2004; Rasband, 2012).

Statistical analysis

For the analysis of survival data, it was considered the Weibull model for interval censored data, described in Sun (2006). The interval censored data is a consequence of the experimental design used here: in each group, we observed the number of deaths per hour, not the exact moment of each death. The statistical analysis was performed using two softwares: WinBUGS (Lunn et al., 2000) and R (R Development Core Team, 2010). The paradigm of the statistical analysis is Bayesian with non-informative prior distributions (Ghoshet al., 2006). For the parameter estimation, it was considered the posterior means and the Credible Intervals (CI) that are the Bayesian version of Confidence intervals. For testing hypotheses, it was used the Full Bayesian Significance Test described in Pereira et al. (2008). The evidence value used for the test procedure is the Bayesian version of the usual p-value: p- and e-values measure the significance of the hypotheses. In our case, we test possible differences among the survival functions involved.

The Weibull survival model can be presented as follow. Let *T* the random variable representing the time to death of one subject, a snail. If *t* is any possible time of death, we write:

$$\Pr\{T > t\} = \exp\left\{-\left(\frac{t}{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3}\right)^{\gamma}\right\}$$

The variables X_1, X_2, X_3 only indicate the group the observation in evaluation belongs:

a. $(X_1,X_2,X_3) = (0,0,0)$ indicates that the observation belongs to the control group;
b. $(X_1,X_2,X_3) = (1,0,0)$ indicates that the observation belongs to the 30 °C group
c. $(X_1,X_2,X_3) = (0,1,0)$ indicates that the observation belongs to the 33 °C group; and
d. $(X_1,X_2,X_3) = (0,0,1)$ indicates that the observation belongs to the 36 °C group.

The parameters β_0, β_1, β_2, β_3 are positive real numbers that indicates the influence of the groups. Test the differences among groups correspond to test differences among these parameters. If all groups respond in the same way we could consider that the only parameter different of zero would be β_0. Testing the difference of 30 and 33 °C would correspond to test if $\beta_1 = \beta_2$, and so on. The parameter γ adjusts the quality of the model fitting; its interpretation is not of relevance for our purpose.

RESULTS AND DISCUSSION

Survival

After the pre-exposure of snails to three different sublethal temperatures, the molluscs were challenged to lethal temperature. Table 1 summarizes the raw data of survival of the snails after the exposure to 42 °C. After 3 hours, less than 50% snails were alive in the control group, and after 5 hours at 42 °C, no survivor was observed. Pre-exposure to heat extended the survival of snails to 5 hours in the group exposed to the temperature of 30 °C group; to 9 hours in the 33 °C group and to 7 hours in the 36 °C group.

Table 1. Number of surviving snails after exposure to the lethal temperature of 42 °C.

Hours after the exposure	Control group (%)	Group pre-exposed to 30 °C (%)	Group pre-exposed to 33 °C (%)	Group pre-exposed to 36 °C (%)
0	30 (100)	10 (100)	10 (100)	10 (100)
1	30 (100)	10 (100)	10 (100)	10 (100)
2	25 (83)	10 (100)	10 (100)	10 (100)
3	13 (43)	9 (90)	10 (100)	10 (100)
4	1 (10)	5 (50)	8 (80)	10 (100)
5	0	2 (20)	7 (70)	7 (70)
6		0	6 (60)	5 (50)
7			5 (50)	1 (10)
8			4 (40)	0
9			2 (20)	
10			0	

Table 2 shows the Weibull model parameter values and the significance difference from zero. Table 3 presents the estimation of the expected survival time. Figure 1 shows the survival function of all groups.

Table 2. Point and interval estimation for the parameters.

Parameter	Mean	SD	CI 95%	ev(H_0)
γ	4.5074	0.5204	(3.501;5.534)	--
β_0	3.0999	0.1448	(2.821;3.387)	--
β_1	1.3876	0.3843	(0.668;2.151)	≈ 0.005
β_2	4.7164	0.6216	(3.580;5.981)	≈ 0
β_3	3.0990	0.4968	(2.117; 4.068)	≈ 0

SD: posterior standard deviation; CI: credible interval.

Table 3. Point and interval estimation for posterior expectation of survival time by group.

Group	Mean	SD	CI 95%	CI 99%
Control	2.8283	0.1358	(2.547; 3.080)	(2.489; 3.205)
Pre-exposedto 30 °C	4.0943	0.3287	(3.439; 4.729)	(3.292; 5.012)
Pre-exposedto 33 °C	7.1317	0.5598	(6.005; 8.195)	(5.827; 8.832)
Pre-exposedto 36 °C	5.6557	0.4331	(4.798; 6.492)	(4.648; 6.873)

SD: posterior standard deviation; CI: high credible interval

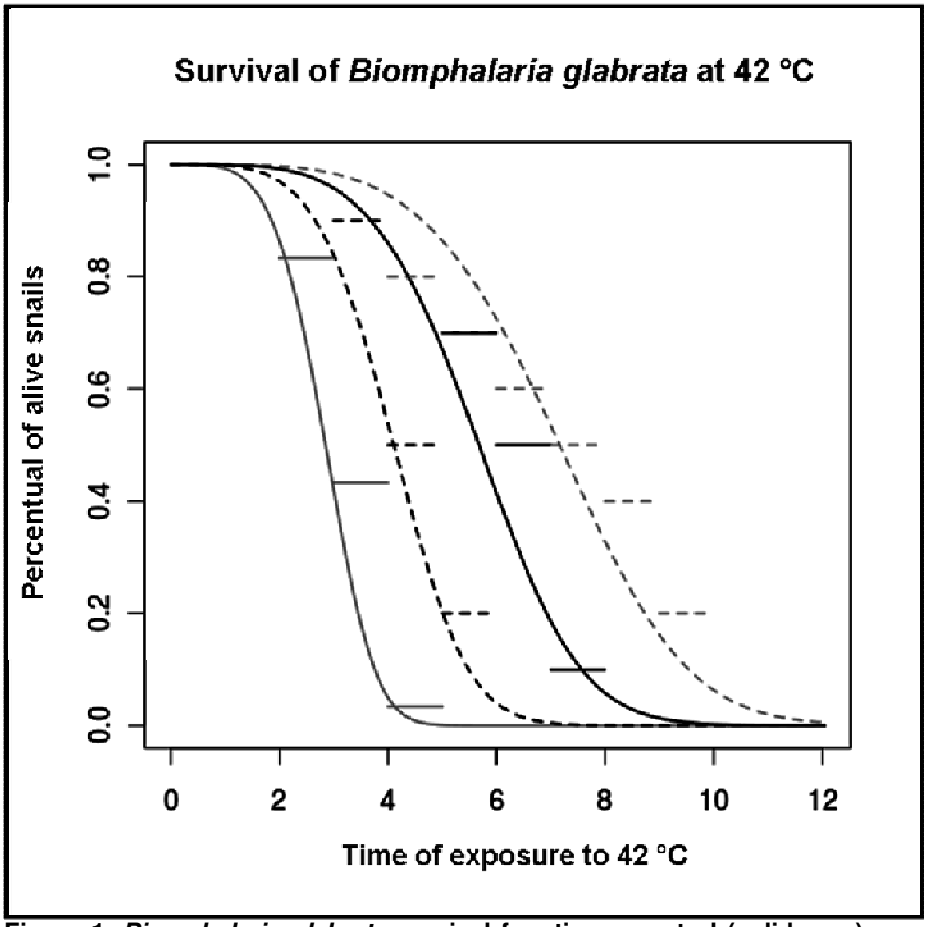

Figure 1. ***Biomphalaria glabrata*** **survival functions: control (solid grey); pre-exposed to 30 °C (dashed black); pre-exposed to 33 °C (dashed grey); pre-exposed to 36 °C (solid black). Horizontal lines represent the observed survival percentage for each time interval.**

Represent by E_0, E_1, E_2 and E_3 the posterior expected survival times for all groups: control, pre-exposed to 30 °C, pre-exposed to 33 °C, and pre-exposed to 36 °C, respectively. We have that the posterior probability of ordered expected survival time is $Pr(E_0<E_1<E_3<E_2)= 0.982$. This result indicates that there must exist differences between groups and that the pre-exposed to 33 °C (and control) has the biggest (lowest) expected survival time.

Our survival experiments confirmed the observation of Rey (1991) about the lethal temperature, as we observed no survivors after 5 hours at 42 °C in the control group. Our results indicated that the pre-exposure to sublethal temperatures induced a protective biological response against the effects of heat at lethal temperature, allowing an extended survival of the animals. This observation confirms that the thermotolerance is a ubiquitous reaction and an answer of ectotherms to adapt to thermal changes of environment.

HSP70 analysis

The Western blotting revelation system results in emission of luminescence proportional to the concentration of protein. The amount of protein is directly proportional to the impression in the photographic film , which was

evaluated by ImageJ software. This software analyzes the image based on the RGB color model detecting 256 shades of gray, therefore, the analyzed data range from 0 to 255, where the lowest value is attributed to black and the highest attributed to white.

In figure 2, it is shown pools of 10 animals of each pre-exposed group. From all tissues tested, the digestive gland showed the highest intensity of HSP70 band and among them 30 °C was the most, followed by 33 and 36 °C groups.

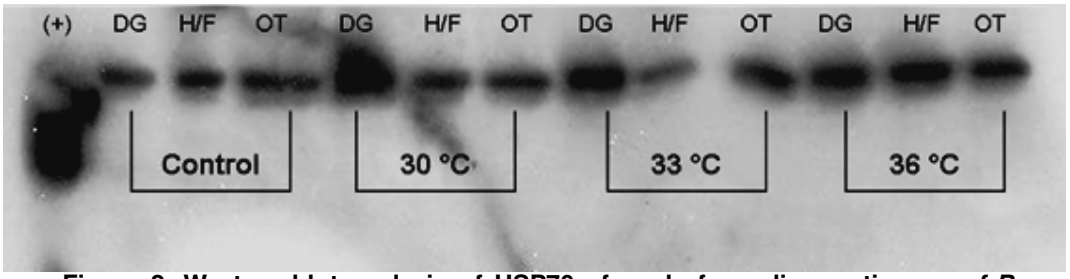

Figure 2. Western blot analysis of HSP70 of pools from diverse tissues *of B. glabrata* after exposure to sublethal temperatures. (+): Epimastigota extract (positive control for the antibody). DG: Digestive gland. H/F: Head/Foot. OT: Ovotestis. The snails of Control were maintained at 25 °C.

On the other hand, ovotestis showed no variation in the HSP70 expression in all temperatures analyzed. For head/foot tissue, it was observed a decrease of HSP70 with the heat in relation to control group, except for 36 °C pre-induced group, which expression of HSP70 was higher than the control. We detect one isoform of HSP70 that is constitutive and can be induced after heat.

The graph and table in figure 3 show the inverse values of shades of gray to associate greater values as darker or higher expression**.**

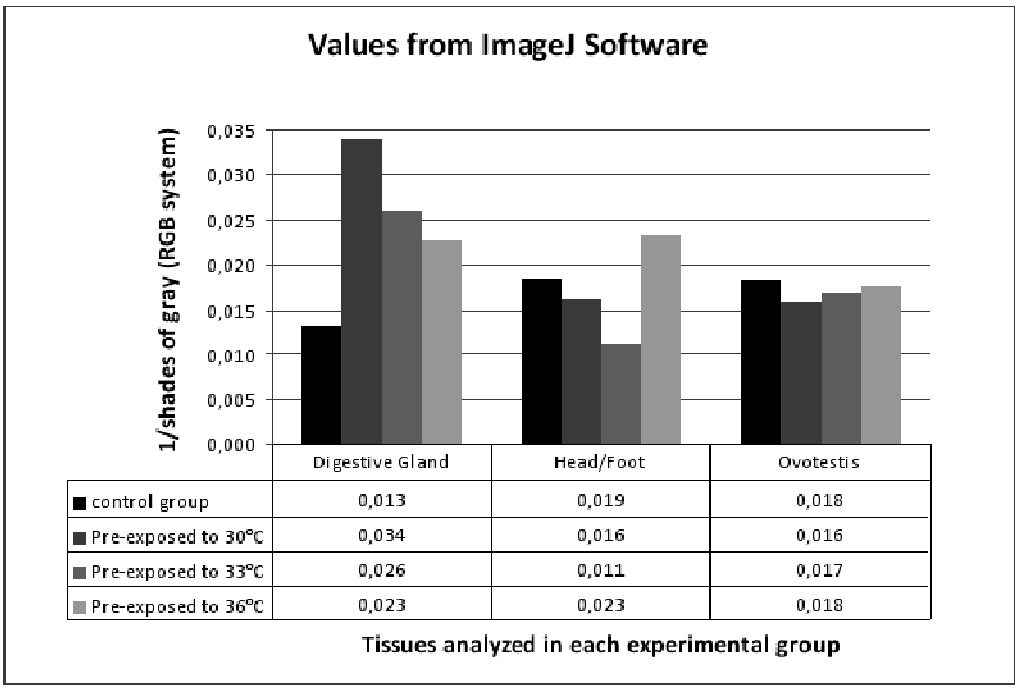

Figure 3. ImageJ software analysis of HSP70 bands.

The constitutive expression of HSP70 seems to be higher in ovotestis and head/foot than in digestive gland. The HSP70 response to heat is different in each tissue analyzed, but digestive gland presented the best association with induction of HSP70 in the groups pre-exposed to sublethal temperatures.

It was used pool of animals to minimize the individual variation observed in HSP70 expression of *B. glabrata*. These variations among tissues and among individuals were also observed in fish (Webb and Gagnon, 2009) and oyster (Piano et al., 2004). The exposure of *B. glabrata* to sublethal temperatures protected them to lethal temperatures and the HSP70 seems to be involved in this protection. But our results showed that the level

of induced HSP70 are not tightly linked to the degree of resistance to lethal temperature indicating that there may be more than this protein involved in the thermotolerance response of *B. glabrata*.

Although we found a higher expression of HSP70 in digestive glands, Piano et al. (2004) have not found induction of HSP69 mRNA in the same tissue of the oyster *Ostrea edulis* after exposure to thermal stress. The highest response of digestive gland in *B. glabrata* may be due to its role in digestion, accumulation and detoxification of all particles ingested by the snail (Ruppert, Fox and Barnes, 2005).

Notwithstanding our results pointed the digestive gland as the most suitable tissue to studies of HSP70; other groups did not found the same results in other mollusc species. Piano et al. (2004) suggest that the oyster digestive glands are poorly affected by heat and that HSP70 is a constitutively expressed chaperone in this tissue. Jackson et al. (2011) reported similar responses to sublethal heat shock in digestive gland and gill of *Crassostrea gigas* with increased levels of the 70 kDa family.

Our results suggest that there is a single form of HSP70, which is constitutively expressed and induced by heating. Similarly, Encomio and Chu (2007) showed that heat stress increased the expression of the constitutive 69 kDa isoform in *Crassostrea virginica*. On the other hand, Jackson et al. (2011) observed two constitutive forms, 72 and 77 kDa, and an inducible of 69 kDa in the oyster *C. gigas* and *C. virginica*.

Among aquatic molluscs, both bivalves and snails are considered excellent objects in monitoring the presence and effects of substances representing danger for living in aquatic ecosystems. They can be applied in both passive and active monitoring (Salánki et al., 2003). Many authors described the mollusc *B. glabrata* as a good model for environmental monitoring (Estevam et al., 2006; Nakano et al., 2003; Tallarico et al., 2004). In addition, the heat shock proteins can be used as an indicator for generalized stress response and, in particular, as a tool to evaluate the level of toxicity (Gupta et al., 2010). The protein HSP70 seems to be a good biomarker for environmental contamination, with safeguards because of the variability of basal levels of HSP70 in organisms and/or tissues from the same organism and the response to a variety of stress stimuli. After all, their evaluation may provide good indication of stressful situations in the environment, leading to deeper and more specific investigations to identify sources of aggression to the organism.

We claim the *B. glabrata* as a bioindicator because it responds to thermal stress with an increase of HSP70 expression, and also it is easy to collect in the field, to keep in the laboratory, and it responds rapidly to the stimulus. In addition, the digestive gland of *B. glabrata*, besides being one of the most exposed organs to environmental contaminants, is very simple to dissect, easy to homogenate and provide huge amounts of protein permitting diverse tests. Further studies on the physiology of the mollusc *B. glabrata*, and its responses to a large range of compounds could provide subsidies to the establishment of this snail as indicator of environmental stress.

Concluding, in this study, the occurrence of thermotolerance in *B. glabrata* was demonstrated, and this response might be related to the induction of the HSP70 protein, although not solely.

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