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# Thermo-Tolerance of Silkworm *Bombyx Mori*: the Role of HSP19.9 and HSP20.8 Genes in Mitigating Thermal Stress

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**Abstract**

Silkworms are the sole producers of silk, relying exclusively on mulberry leaves for nourishment. Climatic variations during their feeding phase can lead to thermal stress, which negatively impacts the vitality of the silkworms that directly related to the silk production on a global scale. Therefore, it is crucial to choose a resilient hybrid that can adapt to these environmental shifts, with the selection process focused on its capacity to withstand increasing temperatures. The study's findings demonstrated that *B. mori* larvae exhibited a remarkable increase in the activation of the genes*HSP-19.9, and20.8*responsible for heat shock protein, rising by approximately 294%, and 339%after five hours of heat exposure at 40ºC during the second day of the fourth larval instar. Furthermore, the degree of expression of the *HSP-20.8 gene* was notably higher than that of the other gene *HSP-19.9*.

**Keywords:** Silkworm, Heat shock protein, Thermal stress.

## 1. Introduction:

Changes in the climate affect everyone on the planet. With temperatures rising to 40–45°C throughout the raising seasons, Egypt's agricultural has suffered as a result of the changes. The typical activities of many insects are at risk due to the rising temperatures caused by the global climate. Exposure to elevated heat can change the metabolic processes and the makeup of the gut microbiota, as well as affect the insectimmune, maturation, improvement, and nutrition assimilation [1, 2]. For example, increasing temperature might change the equilibrium of *LAB* in Drosophila's hind gut, which would ultimately contribute to shorter lives. [3]. A major economically important insect that generates silk is the silkworm, *B. mori.* [4]. According to [5], silkworms are extremely environmentally conscious, especially elevated temperatures that speed up physiological processes. For silkworms, thermality is essential since it influences their economic, pathogenic, and genetic characteristics as well as their disease resistance [6]. According to [5], later instars thrive at elevated temperatures, which shorten their larval phase and accelerate their development., whereas early instars can withstand high temperatures. Temperature increases throughout the feeding phase have an effect on the productivity and cause the larvae to react differently to these circumstances. Variations in temperature have an impact on the physical traits of silkworms [7].

Control humidity and temperature to guarantee sustainable cocoon production [8]. Altering the *BmGrpE* gene, which plays a crucial role in the *Hsp* transcription factorcan boost the silkworm's resistance to high temperatures [9]. The mechanism behind silkworms' resistance to high temperatures, however, is unknown. Some breeds are more resistant to biotic stressors, like as temperature, by nature. According to [10], certain silkworm breeds, such as the Nistari,strain of *B. mori*, can tolerate temperature extremes of up to 32°C. Their pupation percentages are over 80% in unfavorable summer seasons compared to 94% in favorable seasons (Spring -Autumn). *B. mori* is harmed by rising temperatures; for instance, Nistari eggs lose their ability to reproduce at 43°C [11]. *B. mori*$CSR_{2\ x}$ PM died at 40°C and missing the oviposition at 30°C when maintained in a lab environment at those temperatures[12]. Research by [13] indicates that when *B. mori* experiences temperatures over 35°C, the thermality leads to oxidative damage, as observed in both Polyvoltine and Bivoltine varieties. The environment in which *B. mori* grows has a significant impact on its general biology and life cycle, impacting the absorption of nutrients and reducing the lifetime of larvae [14, 15]. From archaea to developed animal, all species have substantially conserved *Hsps.* By assisting freshly generated proteins in folding and overcoming stress-induced denaturation of other proteins, these molecular chaperones help protect organisms from external pressures. The cell triggers a strong defensive reaction by generating different kinds of Stress proteins, (Hsp70, 90, 100, and 60), that belong to heat shock protein family. This mechanism underscores the remarkable resilience of our bodies as they work to manage challenging circumstances. [16, 17, 18].

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Additional Hsp genes, that consists of(hsp90, 70, 27.4, 25.4, 23.7, 21.4, 20.8, 20.4, 20.1, and 19.9) have been shown to be present in *B. mori* [19, 20, 21].

Proteomic analyses of the fatty tissues of silkworms that are resilient to heat and those that are sensitive to it have indicated a connection between *hsp70* and *sHsp* and silkworm therm-tolerance. Furthermore, qPCR findings from [22] show that *hsp19.9* and *hsp20.4* are over expressed as a result of heat shock. The analysis of RNA sequencing identified the genes that were differently showed in the fat tissues of *Bombyx mori* genderthat exposed to prolonged high temperatures. Our research demonstrated that Heat shock Protein genes are controlled in both silkworm gender, although *hsp*25.4 expression is reduced in the male fat body.

Wang[19] discuss how silkworms subjected to long time of thermality showed increasing in the midgut levels of *hsp*90, 70, 40-3, 23.7, and 19.9. The thermo-resistant silkworm strain exhibited elevate of *hsp*23.7, and 19.9 mRNA compared to the thermo-susceptible strain. Although earlier studies have shown that the expression of *HSPs* is crucial for silkworms' ability to endure high temperatures, but it remains unclear if these heat shock proteins also influence how silkworms respond to low temperatures [9; 23]. Silkworm larvae have been exposed to low, regular, and extremely high temperatures during this investigationbefore undergoing RNA-seq analysis. The research concentrated on three underexplored areas: how silkworms respond to low temperatures, identifying DEGs common to both decrease and increase temperature treatments, and exploring the potential link between silkworm diseases and high temperatures.

## 2. Material and methods:

The experiment was conducted at two different locations: the Sericulture Research Department of the Plants Protection Institute, Agricultural Research Center, Giza, and the Microbial Genetics Department of the Biotechnology Institute, National Research Center, Giza.

### 2.1. Preparation of stock culture

Imported silkworm seeds ($F_1$ X $X_7$)from China were nurtured till hatching at 27 ± 1°C and 80 ± 5% relative humidity. [24] claims that hygienic conditions were used during the rearing process. Newly hatched silkworms are raised in the spring at temperatures between 29 and 32 °C and relative humidity levels between 80 and 90%. From hatch to spin, silkworms were fed on clean mulberry leaves.

### 2.2. Experimental Design

Around 900 larvae were separated and divided into three groups, each comprising 300 larvae on the first day of the 4$^{th}$ larval instar. The treatments administered were as follows: Treatment (T1) - The larvae were exposed to a temperature of 40 ℃ for 5 hours in an incubator, after which they were returned to room temperature until they commenced spinning. Control (T2) - The larvae were kept at room temperature until they began to spin. The untreated group also consisted of three groups, each containing 300 larvae.

### 2.3. Heat shock protein (*Hsp*) genes expression in *Bombyx mori*

**2.3.1.** Total RNA isolation

Using the industry-standard TRIzol® Reagent extraction procedure (Invitrogen, Germany), total RNA was extracted from each insect tissue. Finally, 1 milliliter of TRIzol® Reagent was used for every 50–100 mg of tissue to homogenize the samples. The homogenized material was then allowed to remain it at room temperature for fifteen minutes. Chloroform was added in 0.2 milliliters for every milliliter of TRIzol® Reagent. After 15 seconds of high-speed vortexing, the samples were allowed to remain at room temperature about 3 min.. The samples were vortexed at high speed for 15 seconds and then left at room temperature for three minutes. Following this, the samples were centrifuged at 12,000 x g for 15 minutes at a temperature of 4 °C. After being incubated for 10 minutes at 15 to 30 °C, the samples were put in the centerfuge for 10 min at 4 °C at a maximum of 12,000 x g. The precipitated RNA, which was often undetectable, formed a gel-like pellet on the tube's side and bottom prior to centrifugation. The supernatant was subsequently removed. A volume of 1 milliliter of 75% ethanol was utilized to wash the RNA pellet. The samples were vortexed together and then put in the centrifuge for five minutes at 4 °C, reaching a maximum speed of 7,500 x g. Following the removal of the supernatant, the RNA pellet was permitted to air dry for ten minutes, in accordance with references [25, 26].

#### 2.3.2. Reverse transcription (RT) reaction

The whole Poly(A)+ RNA extracted from insect tissues was converted into cDNA in a 20 µl volume using the Revert AidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). A master mix (MM) solution was enriched with five micrograms of total RNA as described in [26]. The reaction was terminated by heating to 99°C for five minutes. The reaction tubes holding the RT preparations were flash-cooled in an ice chamber prior to being utilized for Real Time Polymerase Chain Reaction (RT-PCR) DNA amplification.

**2.3.3. Polymerase Chain Reaction in Real Time (RT-PCR)**

The StepOneTM Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the insects' cDNA copy number. As stated in [29]. Table 1 lists the exact primer sequences for the genes that were utilized.

**Table 1:** Primer sequences used to quantify HSP gene expression levels in real-time quantitative PCR experiments.

| **Gene variant** | **Pre-sequencing (5' to 3')** | **Genetic Database (accession no)** |
|---|---|---|
| ***GAPDH*** | F: CACTTGGAGGGAGGTGCTAA<br>R: AGCAGTTGTGGCATGAACAG | NM_001043921.1 |
| **A** | F: ATGTCGTTGATTCCGTGGTT<br>R: TTAAGCCTTGTCGCCGTTGG | NM_001043519.2 |
| **B** | F: ATGTCGTTGCTACCATATTTC<br>R: CTACTTTTCCTTCTCGTTGGC | NM_001098324.1 |

HSP: Heat shock protein genes. A: *HSP19.9* B:*HSP20.8*

**2.3.4. Statistical analysis**

Finney's [28] description of probit analysis was applied to obtain the LC50 data. The collected data was evaluated using Duncan's test ($P < 0.05$) and one-way analysis of variance (ANOVA). All biochemical and molecular genetic data were presented as means ± SEM. The data analysis was conducted using version 0.26 of the (SPSS) for Windows.

**3. Results**

**3.1. Variations in the gene expression (*HSP-19.9, and 20.8*) *genes*in the hybridtissues following the subjecting to temperature.**

The evidence indicated, the levels of the expression of HSPG (HSP-19.9, and*HSP 20.8*) increased about 294% and 339% repectively, level of the*B. mori* larvae in their fourth instar were exposed to heat for five hours at 40ºC. compared to control *B. mori* larvae, these increases were statistically significant ($P<0.05$). Additionally, thetranscriptionlevel of the *HSP-20.8 gene*was notably higher than that of the *HSP-19.9 gene,* as seen in Figures (1 and 2). these findings summerized that the production of both, HSP-19.9, and*HSP 20.8* genes in *B. mori.*

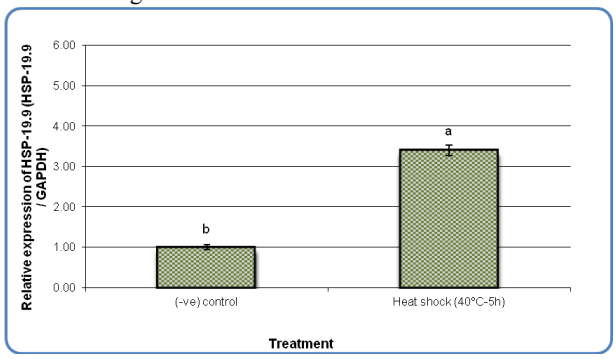


**Fig. 1:**Following five hours of heat treatment at 40ºC, *B. mori* tissues exhibit altered expression of the HSP-19.9 *gene*. The mean ± standard deviation is used to illustrate the data. The mean values for tissues with divergentuppercase letters differed significantly ($P<0.05$).

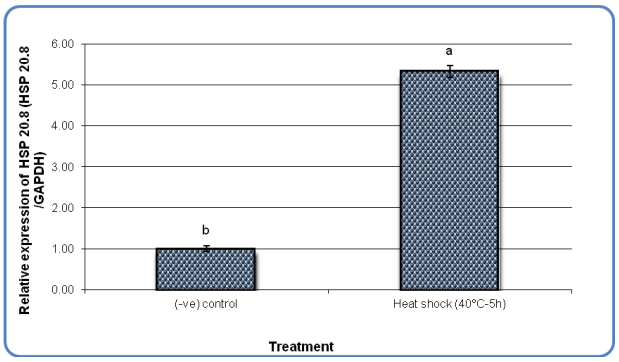


**Fig. 2:** Following five hours of heat treatment at 40ºC, the tissues of *B. mori* exhibit altered expression of the HSP-20.8 *gene*. The mean ± standard deviation is used to illustrate the data. The mean values for tissues with unlike superscript letters differed considerably (P<0.05).

**4. Discussion**

The results demonstrated that larvae exposed to heat for five hours at 40ºC had higher expression degree of the *HSP-19.9 and HSP-20.8 genes* than control *B. mori* larvae. Furthermore, *HSP-20.8* gene expression intensities were significantly increasedover the heat shock protein (HSP-19.9). Our observations align with those of [29], they discovered that the expression levels of Heat Shock Protein geneswere significantly higher in thermally stressed tissue and progressively decreased throughout the repair period. The *HSP* gene showed the highest expression in fat tissue, followed by the midgut and the silk gland.The qPCR analysis revealed that *Nistari* exhibited transcript levels of *sHsp20.4* and *sHsp20.1*, as well as *Hsp70*, that were 9.7 times higher than those of *CSR2*.Similarly, comparing$SK_4C$ to $CSR_2$a 1.5-fold increase in *Hsp70* expression and a 3.5-fold increase in *sHsp20.4* and *sHsp20.1*expression. The expression levels of *Hsps* during heat shock were associated with the percentage the pupa duration at elevated temperatures for each of the three breeds.

It has been discovered that the heat shock protein 70 family is present throughout the silkworm genome. Using recognized naming conventions and evolutionary studies, four HSP70s and five HSC70s were grouped.

Heat shock proteins, on the other hand, were more conservative and constitutively generated in a range of silkworm larvae tissues. The levels of *HSP70-1*, *HSP70-2*, and *HSP70-3*expression were increased by frustrations related to high temperatures (37 °C and 42 °C) and low temperatures (2°C), with the greatest induction taking place at 4147.3, 607.1, and 1987.3 times, respectively. Curiously, the thermality also slightly increased the expression of *HSC70-1*, *HSC70-4*, and *HSC70-5* in the midgut and/or fattytissues. Moreover,the expression of HSP70-1 was increased by the insecticides dichlorvos and phoxim, whereas the majority of HSC70 genes showed reduced activity.The findings indicated that stress-inducible variants play a more crucial role in adapting to various stressors compared to HSC70s [30].

**5. Conclusion**

This research focuses on the issue of global climate change, specifically in Egypt, and its impact on silkworm strains with varying susceptibility to thermal effects. These effects influence all biological processes, directly affecting silk production. Therefore, it was essential to conduct genetic research to understand how silkworms regulate their heat shock proteins (*HSPs*) and to identify the key factors enabling their tolerance to rising temperatures during the feeding season. The findings of this study support the recommendation of a hybrid that can better adapt to these critical conditions

**6. Conflicts of interest**

"There are no conflicts to declare".

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