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Changes in the embryonic protein profile and hatching as a response to thermal stress in the Eri silkworm, *Samia cynthia ricini*

Punyavathi, Koushik Hullahalli Kumar, Sentimenla Moatemjen, Likhith Gowda Mahadevegowda, Manjunatha Hosaholalu Boregowda*

Department of Studies in Sericulture, Proteomics and Genomics Laboratory, University of Mysore, Mysore, India.

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ABSTRACT

The biological architecture determining the post-embryonic development and traits is well programmed during the process of morphogenetic movements and organogenesis in the embryo. However, changes in the environmental temperature for a few hours, which is uncommon, affecting the embryo development, protein expression, and hatching of larvae in the Eri silkworm (*Samia cynthia ricini*) remain enigmatic. Hence, for the first time, the eggs of new Eri silkworm breed C2 were exposed to heat shock (HS) temperature of 35°C, 40°C, and 45°C for 2 hours not only to measure heat sensitivity but also to uncover differential expression of proteins in a different age of the embryo. Interestingly, the quantum of protein not only increased but also a differential expression of 70, 60, 45, 36, and 30 kDa proteins was obvious due to induction of HS. The induction of HS has shown a significant impact on embryonic development wherein 45°C is found to be lethal as none of the eggs hatched. On other hand, an improvement in the hatching was observed in the eggs HS at 40°C, which could be due to the expression of HS proteins (HSPs). Taken together, we suggest that hatching of the embryo is one of the key traits to determine tolerance potential of the silkworm strains/breeds to heat stress by expressing HSPs. Therefore, this strategy shall be followed for development of a new Eri silkworm breed with better acquired tolerance to high temperature suitable for tropics.

1. INTRODUCTION

The Indian silk industry comprises all the four economically important trading varieties—mulberry, tasar, muga, and eri, while the Eri belongs to the family Saturniidae and the order Lepidoptera. Interestingly, next to the mulberry silkworm, *Bombyx mori*, the only completely domesticated silkworm is the Eri silkworm (Donovan, *Samia cynthia recini*), which shares 19.40% of the total silk production—35,261 MT, while mulberry silk accounts for 71.50%, Tasar 8.44%, and Muga 0.66% [1], and play a significant role in the textile industry of the world. In India, Assam and its adjoining foot hills are essentially the original habitat of Eri silkworm, still it has been cultivated in Arunachal Pradesh, Nagaland, Meghalaya, Manipur, and Mizoram. Furthermore, it is spreading to different non-traditional states like Andhra Pradesh, Tamil Nadu, Karnataka, Gujarat, Jharkhand, and Chhattisgarh. Thus, growing of Eri silkworms not only provides substantial income as a subsidiary occupation but also acts as an income enhancer which gains much significance in the sericulture industry as an important socio-economic factor. Since most of the farmers still rear Eri silkworms in a traditional manner, there is tremendous scope for innovation and intervention of technology for improvement of Eri cocoon production [2].

Towards this, Eri silkworm breeds with high potential and productivity are in need and are developing using two wild silkworm breeds—*S. cynthia* and *Samia canningi*, besides, domesticated *Samia ricini* (Donovan). Currently, *S. ricini* is the only Vanya silk that commercially cultivated multivoltine species of the Eri silkworm, which can be reared throughout the year by feeding castor (*Ricinus communis*), kesseru (*Heteropanax fragrans Roxb.)*, borpat (*Ailanthus grandis Prain*), and tapioca (*Manihot esculenta Crantz*) leaves. However, until now, the research work carried out for the genetic improvement of this economically

*Corresponding Author
Manjunatha Hosaholalu Boregowda, Department of Studies in Sericulture, Proteomics and Genomics Laboratory, University of Mysore, Mysore, India. E-mail: manjunathahb @ gmail.com

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important silkworm, especially with regard to higher fecundity and survival is inadequate. Therefore, there is a need to initiate a systematic breeding programme for the genetic improvement of Eri silkworm (*S. ricini*), particularly for introducing traits of economic importance such as higher fecundity and survival, tolerance to biotic and abiotic stress, and higher productivity. Towards this, improved breeds/hybrids are liable to eradicate the prevailing constraints in commercial Ericulture.

One of the limiting factors affecting the Eri silkworm rearing is low survival and yield during high temperature, especially in a hot climate as in summer. However, a new Eri silkworm hybrid—C2 developed for commercial exploitation following conventional breeding strategy [3] also exhibits a substantial mortality due to adverse climatic conditions [4]. Whereas, some of the ecoraces viz SaKKU1 is known to have tolerance to high temperatures of 42°C ± 1°C [5]. But due to Global warming and seasonal variations, Eri silkworm rearing is forced to undertake even at the temperature of 35°C, while larval growth not only gets affected but also consequently infected by the pathogens causing heavy cocoon crop loss. In addition, environmental fluctuation occurs between dawns to dusk, it affects, not only rearing but also alters embryonic growth, hatchability, and larval vigour [6]. As a fact, the elevated temperature of 40°C and above for a few hours in the rearing house considerably damage the biological and commercial traits of the silkworm, *B. mori* [7,8]. So far, no such research has been undertaken in Eri silkworm breeding, a systematic investigation is warranted to exploit breed potential that can enhance acquired tolerance to harsh climatic conditions.

Towards this, unlike *B. mori*, the genetic potential of Eri silkworm to acquire tolerance to hot climatic conditions by expressing gene/genes has not been studied in detail either in the larval stage or egg stage. Under these circumstances, to overcome unfavourable stress conditions that are induced by oxidative stress, nutritional deficiency, UV radiation, chemicals, harmful microorganisms, and ischemia reperfusion injury [9], an organism produces defence protein/proteins—Heat Shock Proteins (HSPs). The HSPs play an important role in stabilizing biochemical processes in cells and other protein protection [10]. Keeping the significance of HS response in a biological system and no such research has been carried out in the Eri silkworm, *S. cynthia ricini*, the present investigation was carried out to assess the effect of HS on embryonic development that determines the post embryonic and biological traits.

2. MATERIALS AND METHODS

2.1. Materials

The eggs of new Eri silkworm breed C2 developed through hybridization of two potential parents SRI-018 (Genung) and SRI-001 (Borduar) were procured from Eri Silkworm Seed Production Centre, Central Silk Board, Hosur, Tamil Nadu for the present study.

2.2. Methods

2.2.1. Maintenance of eggs

The Eri silkworm eggs were maintained in the laboratory at 25°C to 26°C and relative humidity of 80% to 85% until hatching.

2.2.2. Induction of HS

HS was induced to eggs from day-3 (after oviposition) till hatching at 24 hours intervals. About 20 eggs in each replication—three replications for each treatment—were exposed to HS temperatures of 35°C, 40°C, and 45°C in the water bath for 2 hours followed by 2 hours recovery period at room temperature. A control batch in three replications was concurrently maintained at room temperature.

2.2.3. Extraction and quantitative estimation of protein

For extraction of protein, five eggs from control and HS treatment groups were collected separately. All these eggs in a group were homogenized separately in the extraction buffer containing phenylmethylsulfonyl fluoride and dithiothreitol and then centrifuged at 4°C at 4,000 rpm for 15 minutes. The resultant supernatant was collected independently for quantitative and qualitative analysis of protein. Quantitative estimation of protein in the samples derived from both HS induced and control eggs were estimated directly by using Biophotometer (Eppendorf, Germany) at 260 nm.

2.2.4. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed as described by Weber and Osborn [11] with necessary modifications [8]. Briefly, the two glass plates and combs were cleaned thoroughly and then assembled (Clever, UK). 12% separating and 5% stacking gel was prepared for resolving the proteins. 20 µl of protein sample derived from each treatment was mixed with 20 µl of sample buffer [4% SDS, 50 mMTris-HCl (pH 6.8), 10% β-mercaptoethanol, 20% glycerol and 0.1% Bromophenol blue] and loaded into a lane, while protein molecular marker was loaded into a separate lane. After electrophoresis, the gel was stained in the Coomassie brilliant blue R-250 for 2–3 hours followed by destaining to detect protein bands. The gel images were captured using a gel documentation unit (Cleaver, UK) and subjected for densitometri analysis. The molecular weight of protein bands was analyzed employing TotalLab 1D software.

2.2.5. Embryonic response to heat sensitivity

Sensitivity to varied HS temperature was measured based on the percent of hatching as an index of embryonic development and an average hatching percentage was determined.

2.2.6. Data analysis

All the data derived from three replications of different treatments were used to draw mean values along with standard deviation and significant variations employing one way analysis of variance using SPSS software.

3. RESULTS

3.1. Proteome Profile of the Eri Silkworm Eggs

A total of 14 to 15 discrete protein bands with rf values of 0.035 to 0.85 were obvious in day-3 to day-8 normal and HS induced

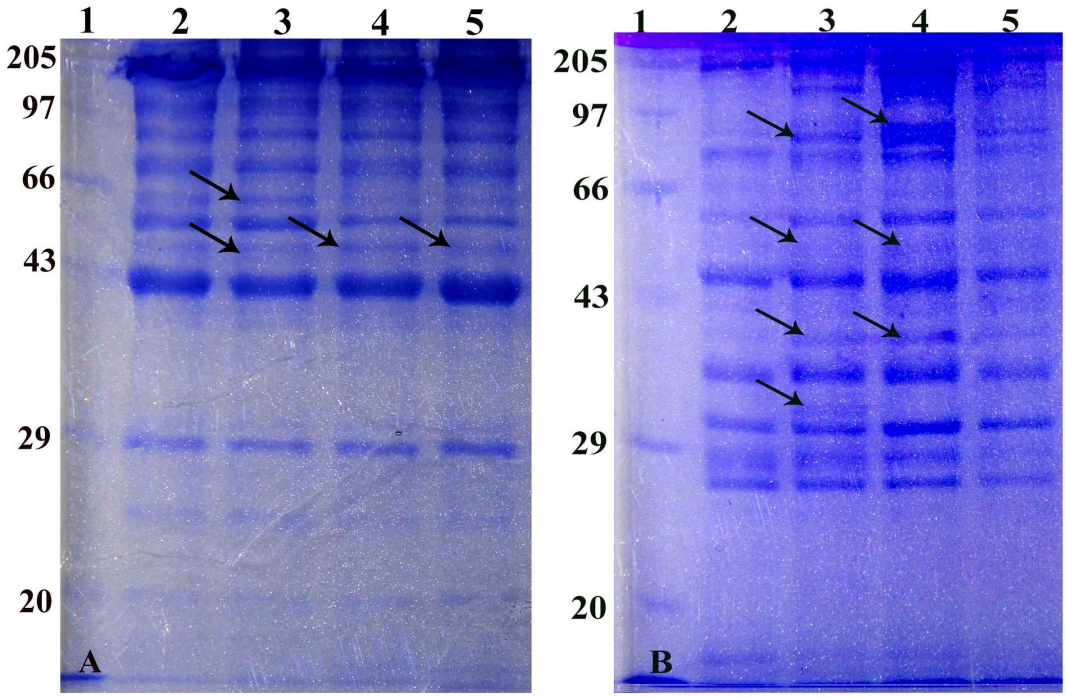

Figure 1: Changes in the protein as influenced by HS in the embryo of Eri silkworm breed C2. (A: day-5, B: day-7. Lane 1—molecular marker, 2—control, 3—35°C, 4—40°C, and 5—45°C. Arrows indicate expressed HSPs).

eggs of Eri silkworm (Fig. 1). Of which, while the molecular mass of 60 and 45 kDa proteins were found over-expressed in day-5 eggs HS at 35°C, a 45 kDa protein over-expressed in the eggs HS at 40°C and 45°C (Fig. 1). Concomitantly, the expression of 70, 60, 36, and 30 kDa proteins was higher in day-7 eggs after HS at 35°C, whereas 70, 60, and 36 kDa proteins were found over-expressed in the eggs HS at 40°C with different rf values (Table 1). The densitometry analysis revealed differential expression of varied HSPs at different HS temperatures (Fig. 2). Interestingly, few proteins were found degraded in the eggs exposed to HS at 45°C. Comparatively, relative abundance and intensity of normal proteins and synthesis or over-expression of HSPs differ distinctly among different days of eggs.

3.2. Quantitative Changes in the Total Protein Content of Embryo

A discrete variation in the quantum of protein in day-3 till day-8 eggs of C2 exposed to varied HS temperature (35°C, 40°C, and 45°C) was observed (Fig. 3). Among different days of the embryos, a high amount of protein content measuring 46.66 µg/µl was recorded in the day-6 eggs HS at 40°C as against 8.41 µg/µl in control batches, while the protein content was declined to 36.05 µg/µl in the batches HS at 35°C and 45.08 µg/µl in the batches HS at 45°C. Concurrently, the day-4 eggs have protein content 26.82 µg/µl as highest in the batches HS at 40°C as against 10.34 µg/µl in control. Interestingly on day-5, the highest amount of 35.88 µg/µl protein content was recorded from the batches HS at 40°C but it was declined measuring 10.69 µg/µl in the eggs HS at 45°C. Interestingly, the amount of protein content was gradually increased till day-6 and decreased thereafter. Subsequently, on day-7, the highest amount of 22.11 µg/µl protein content was noticed in the eggs HS at 40°C, which was found to decline in the eggs HS at 45°C (9.94 µg/µl). On day-8, the total protein content was found to decline in all the HS treatment groups of eggs compared to control (10.53 µg/µl).

3.3. Determination of Heat Sensitivity on the Embryonic Development

The eggs from day-3 to day-8 exposed to varied HS temperatures of 35°C, 40°C, and 45°C exhibit discrete variations in hatching as an index of embryonic development (Fig. 4). Interestingly, the highest hatching 99.33% was noticed in the day-5 eggs HS at 40ºC and the percent of improvement in hatching recorded was 8.75% compared to control. From day-3 to day-7, hatching percentage was higher in the group exposed to HS at 40ºC (98.33%, 93.67%, 99.33%, 96.33%, and 96.67%, respectively) compare to 35ºC (91.67%, 90.67%, 91.67%, 97, 94%, and 95.67%, respectively). Zero percent hatching was recorded on day-8 HS at 40°C and 45°C besides none of the eggs were hatched in all the batches exposed to HS at 45°C as evident from shrunken chorion shell (Fig. 5D). Comparatively, increased hatching of embryos was recorded at 40°C followed by 35°C as against control which is statistically significant at $p < 0.05$.

4. DISCUSSION

The application of HS principles and technique has a greater value in the Ericulture industry as has been aptly demonstrated in the mulberry silkworm, *B. mori* developing potential breeds with

Table 1: Densitometry analysis of differentially expressed proteins due to thermal stress in the Eri silkworm (breed C2) eggs.

HS Temperature (°C)	Day	kDa	Volume	Peak height	Area	Rf
35	5	60	88048	157.60	560	0.246
		45	85402	152.90	560	0.330
	7	70	70818	128.18	552	0.148
		60	63747	115.39	552	0.316
		36	64771	117.78	552	0.452
		30	64870	117.49	552	0.561
40	5	45	85494	162.66	524	0.334
	7	70	72005	140.38	512	0.146
		60	61839	120.86	512	0.318
		36	71451	139.57	512	0.37
45	5	45	85173	164.55	516	0.327

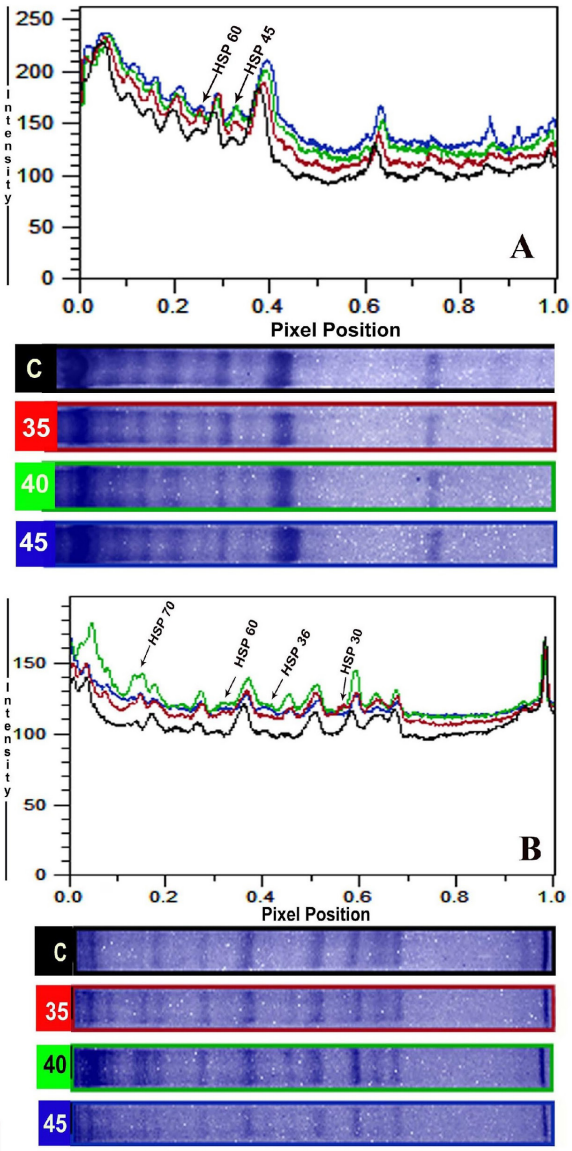

Figure 2: Changes in the protein profile and densitometric analysis as influenced by HS in the embryos of Eri silkworm breed C2 (A: day-5, B: day-7, and C: control).

greater acquired tolerance to a higher temperature and fluctuating environmental conditions [12,13]. However, this phenomenon has not been fully explored and unequivocally beneficial to the Eri silkworm, which is the only completely domesticated silkworm next to *B. mori*. Thus, in the present investigation, for the first time, we explored the feasibility of employing this novel strategy not only in the analysis of Eri silkworm breed for their tolerance level to harsh temperature but also in the development of new Eri silkworm breeds, which can minimize the temperature-dependent crop loss towards sustainable Ericulture industry in India.

Not being exceptional, compared to mulberry silkworm (*B. mori*), the growth and development of Eri silkworms are also greatly affected by high temperature, especially in a hot climate during summer that resulted in low survival and yield [3]. To fill this gap, some effort has been made to develop high temperature tolerant eco-races by directional selection at high temperature 42°C ± 1°C and low humidity 50% ± 5% and demonstrated that this trait is heritable. Hence, we have considered the embryonic stage for the first time to enhance this heritable trait by activating the gene/s that encode for the synthesis of protein/s (HSPs) as this phenomenon is well studied in *B. mori* [13] unlike Eri silkworm. Because, HSPs are not only acts as molecular chaperons but *hsp90* and *hsp70* expressing conjointly and consistently in all the instars female and male larvae of *B. mori* are also play a vital role in growth and development of silkworm larvae [12]. Besides, HSP70 is known to play a vital role in heat tolerance as a molecular chaperon by chaperoning unfolded proteins and protecting organisms from extreme temperature [14]. Such detail studies are warranted in Eri silkworm employing advanced proteomic tools and techniques—two-dimensional electrophoresis, Immunoblot assay, mass spectrometry, and quantitative polymerase chain reaction as has been aptly followed to understand the functional complex of HSPs in *B. mori* [12].

Notably, a drastic variation was observed after Eri eggs were exposed to HS at 45°C with the highest content and rate of HSP expression on day-6. The variable quantum of protein recorded from different groups is due to the environmental fluctuation encountered by the normal eggs, but stability of the protein content in the HS induced eggs endorse molecular chaperon activity of HSPs as has been reported in the HS induced eggs

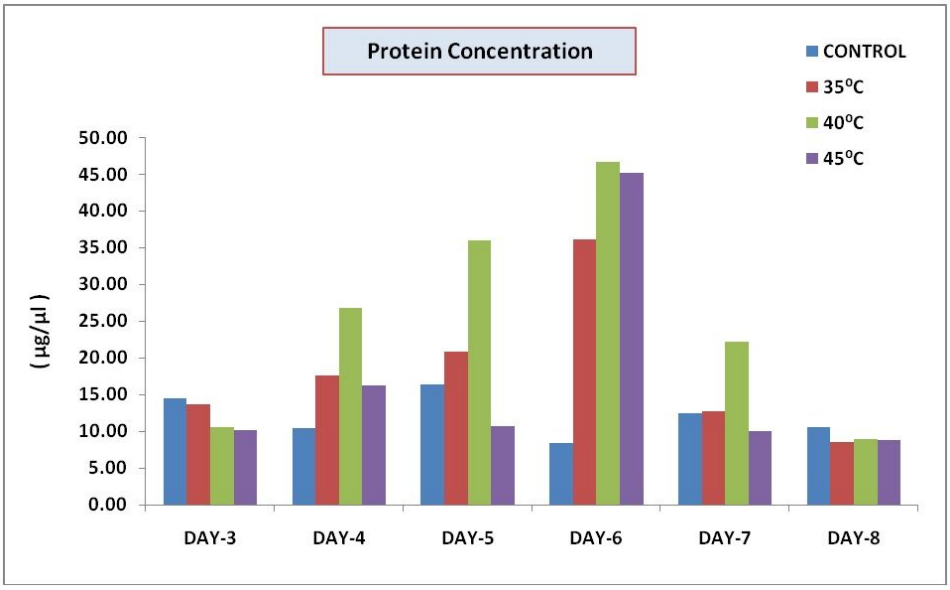

Figure 3: Changes in the total protein content due to thermal stress during embryonic development in the Eri silkworm breed C2.

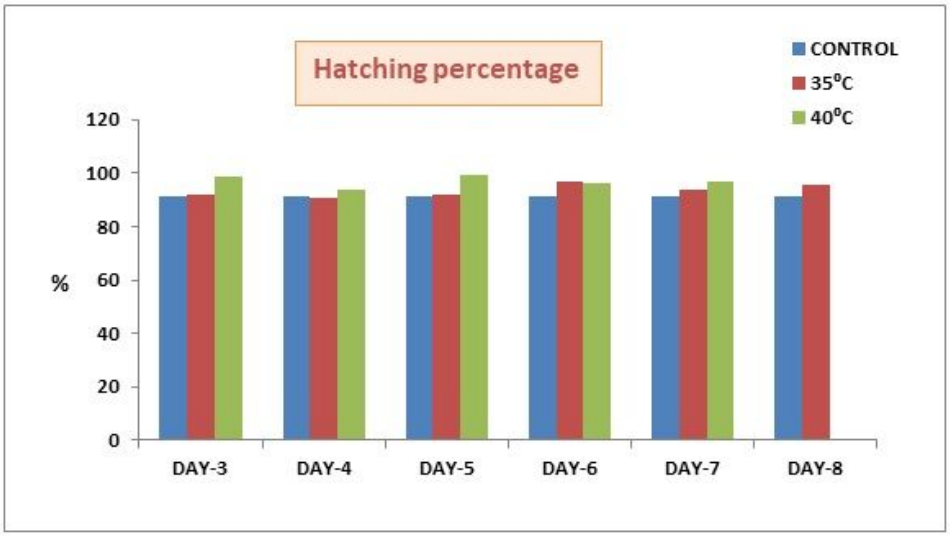

Figure 4: Changes in the percent of hatching due to thermal stress during embryonic development in the Eri silkworm breed C2.

of *B. mori* [15]. Further, expression of different sets of HSPs with molecular mass 70, 60, 45, 36, and 30 kDa revealed that HS temperature of 35°C and 40°C has induced a quite beneficial effect on embryonic development by enhancing the percent of hatching over its non-HS induced Eri eggs. Among different sets of HSPs expressed in the eggs of Eri silk moth, expression of HSP70 is important as it is involved in growth and development as has been reported from the mulberry silkworm, *B. mori* [12] and malaria parasite, *Plasmodium falciparum* [16]. Whereas, small HSPs with molecular mass of 45, 36, and 30 kDa observed in the present study might be involved in preventing irreversible protein aggregation [17] and refolding [18] at the time of thermal stress. As a result, percent of hatching is higher in HS induced batches than the non-HS batches. Interestingly, HSP45 (a family of HSP40) has also significantly facilitated the Eri embryo to adapt to thermal stress as has been reported in the fruit flies [19]. As BmHsp27.4 has an important role in its response to induced thermal stress *B. mori* [20], expression of 30 kDa HSP in Eri silkworm has significant impact on hatching. However, expression of different sets of HSPs, which includes, 60, 45, 36, and 30 kDa proteins collectively supporting the embryo to overcome thermal stress, which is unique report from Eri silkworm, as has been reported in the fifth instar larvae of *B. mori* [8]. Towards this, Li *et al.* [21] also suggest that higher

Figure 5: Effect of HS on the embryonic development of Eri silkworm breed C2 (A—control, B—35°C, C—40°C, and D—45°C).

levels of these HSPs expression play important roles in providing resistance to high temperature stress in different varieties of silkworm. Concomitantly, despite HS treatment during embryogenesis explicit a significant variation in the hatchability, cent per cent mortality was obvious at 45°C due to death of an embryo, which was inferred based on the shrunken chorion shell as one of the strong pieces of evidence. Similar findings were also reported from both poly- and bi-voltine silkworm strains of *B. mori* [7,13]. These salient findings revealed that 45°C is also lethal for the embryonic development in the Eri silkworm as in the case of *B. mori*, which is a unique feature of the study.

By and large, the molecular mechanism of differentially expressed HSPs at different developmental stages of the life cycle and sex is still remained obscure in Eri silkworm. Interestingly, the expression of HSPs has not been observed in the eggs of *Drosophila* when subjected to HS treatment [22], but differential expression of HSPs was so obvious in the eggs of Eri silkworm used in the present study. This can be further correlated with the embryonic sensitivity against HS that varies with different organisms even between two species—*Bombyx* and *Samia*, but affects the hatchability significantly as recorded in the present study. Embryonic death noticed in all the groups of Eri silkworm eggs due to HS at 45°C is with the fact that denaturation of proteins that are essential for the growth and development of the embryo and did not protect their unfolding by the synthesis of HSPs [7]. Moreover, recently, a phenomenon on the principle and hypothesis of the HS response states that optimal level of HSP expression is beneficial for embryonic development and survival; massive expression of HSPs has a negative physiological effect leading to the death of an organism as a fact of autophagy [23]. But, how this autophagy operates during HSPs expression/degradation coupled with recovery of cellular machinery for the biosynthesis of silk protein in *B. mori* larvae is unclear [12], while the same hypothesis needs to be explored even in Eri silkworm. Thus, the findings of the present study not only provide a better understanding of HSPs that cumulatively play a significant role in the survival of embryos and/or larvae under thermal stress condition, but also helps in the integrative management of the pest *Glyphodes pyloalis* [24] as Eri silkworm is one of the pests of Castor plant, which belongs to the order Lepidoptera that comprises most of the agricultural pests.

5. CONCLUSION

The findings of the present study unraveled a significant impact of thermal stress (HS) on embryonic development with altered protein profile and hatching. While a mild HS at 35°C or 40°C at a specific stage of embryonic development induces expression of HSPs facilitating the embryo to overcome the fluctuation in the environmental conditions and exhibit higher percent of hatching than the eggs are not exposed to thermal stress (control). HS temperature of 45°C is found lethal as none of the eggs were hatched. Thus, we strongly propose that as the hatching of the embryo is one of the key traits to determine tolerance level to thermal stress in any of the silkworm strains/breeds, this strategy shall be followed for the development of new Eri silkworm breeds with better acquired tolerance to high temperature.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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