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Effect of heat shock on embryonic development and its impact on commercial traits of silkworm *Bombyx mori* L.

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ABSTRACT: The early stage of an organism – embryonic stage, architects all the post embryonic developments which are governed by genetic and environmental conditions, but the effect of hot events during that stage remain obscure in the silkworm *Bombyx mori* L. Thus, APM1; a multivoltine parental breed of a ruling CB and APHO1 silkworm breed developed through induction of thermotolerance, and APHO1 breed to examine the impact of heat shock (HS) on the embryo and resultant larvae. Different developmental stages of embryo were exposed to varied HS temperatures for 2 h followed by a 2 h recovery period. After HS the eggs and the resultant larvae were reared under normal environmental conditions. Interestingly, 45^{0}C although determined as lethal temperature yielded vibrant larvae. Whereas APM1 and APHO1 eggs heat shocked at 35^{0}C exhibited increased hatching (91.66 and 69.33%), larval weight (1.72 and 3.33 g), effective rate of rearing (72.39 and 81.93%), cocoon weight (1.01 and1.6 g), shell weight (0.12 and 0.29 g), shell ratio (13.11 and 20.52%) and pupal weights (0.87 and1.29g) when compared to control APM1 and APHO1. Besides increased total protein content, expression of 205 kDa, 90 kDa and 70 kDa heat shock proteins and the glycogen content was found more on day - 3 compared to day - 2 in the embryos of APM1 and APHO1 which eventually declined as the embryonic development proceeded to hatching. This work shows that APM1 and APHO1 eggs had shown profound response to HS temperatures exhibiting varied acquired thermotolerance to overcome fluctuating environmental condition.

KEY WORDS: Strains, APM1, APHO1, thermotolerance, protein, glycogen changes

INTRODUCTION

The sericulture industry has contributed significantly to the economic development of many countries due to the commercial importance of silk in the textile world as silkworms are easy to rear under domestication. Thus, the silkworm, *Bombyx mori* was not only exploited over a long period for cocoon

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production but also widely used in basic research, biotechnology and as a molecular model insect. In tropical countries like India, to achieve sustainable sericulture, silkworm strains that withstand high temperature and frequent fluctuations are in need. But, the domesticated silkworm, *B. mori,* is highly sensitive to the fluctuating environmental conditions during different stages of development due to poikilothermic nature. However, sensitivity varies among different silkworm races, strains, and breeds. Comparatively, bivoltine silkworm breeds have enough potential for the production of superior cocoons qualitatively and quantitatively over multivoltines but cannot thrive well in adverse or fluctuating environmental conditions. In India, the temperature and humidity vary from season to season, region to region, while fluctuation occur between dawn to dusk. Sericulturists experience a great risk in rearing silkworms. The fluctuated environment leads to improper growth of embryo, poor hatching, weak larvae, inferior cocoons and ultimately production of low-grade silk. Even, few hours of elevated temperature ~40° C and above in rearing house causes significant damage in commercial and biological traits of silkworm *B. mori* (Manjunatha *et al*., 2005 and Vasudha *et al*., 2006).

The effect of temperature on the development of *B. mori* has been studied extensively both in larval and embryonic stages but not much information is available on the impact of HS on hatching of the embryo which is an index of embryonic development (Manjunatha *et al*., 2005, 2008). In the field of sericulture, breeders agree that, the development of thermotolerant bivoltine breeds which are suitable for high temperature environment and yet productive by following conventional breeding strategy is a difficult task. Resistance to high temperature has been recognized as a heritable characteristic in silkworm and the possibility for the temperature tolerant silkworm races were suggested by "Kato" as early as 1989. Therefore, means other than the conventional breeding methods are to be adopted to attain the goal. With the aid of modern biotechnological tools, it may be possible to quantify the factors responsible for the expression of temperature tolerance. It was found recently that supplementation of spermidine in micromolar concentrations helps in thermotolerance (Anugata et al, 2022). The increased thermotolerance in spermidine supplemented silkworms was due to elevated levels of caldopentamine (Anugata et al, 2023). However, the mulberry silkworm is one of the most thermal-sensitive organisms. Intensive and careful domestication over centuries has apparently deprived this taxon of opportunities to acquire thermo-tolerance.

The terms heat shock and thermotolerance, acclimation and hardening are commonly used to describe the changes in an organism living state caused by external environments and treatments (Bowler, 2005; Loeschchke and Soresen, 2005; Langerspetz, 2006). Unfortunately, the usage of these terms in silkworm research has not been well defined and requires systematic study to draw a line between them. Comparatively, heat shock response among different silkworm races/strains of the polyvoltine, bivoltine and univoltine varies significantly and thermotolerance increases as the larval development proceeds (Vasudha *et al*., 2006; Manjunatha *et al*., 2010; Raju *et al.*, 2018; Shou-Min Fang *et al.*, 2021).

It is well known that improper management of light, temperature and humidity during embryonic developments affects the characters in successive generations. The effect of temperature on the development of silkworm larvae has been studied extensively, but there is a need to study the effect of temperature on the growth and development of silkworm embryos. Very few reports are available on the impact of heat shock on hatching, biochemical composition, biological and commercial traits that pre-determine the embryonic stage.

With this backdrop, the current investigation was undertaken using APM1 and APHO1 silkworm breeds, to evaluate the effect of HS on embryonic development of *B. mori* eggs which decides the post embryonic development, protein expression and commercial traits. Results showed that embryos exposed to HS during embryonic stages acquired varied thermos- tolerance.

MATERIALS AND METHODS

The newly emerged moths of silkworm, *B. mori* strains, APM1 and APHO1 (both for heat shock treatment and for control batch) were drawn from the germplasm of the APSSRDI for the preparation of loose eggs towards present investigation. Fertile and unfertilized eggs were separated by brine treatment. The fertile eggs thus collected were incubated in a natural room environment without using any instruments to maintain optimum temperature and relative humidity.

HS incubation was performed from day-2 after oviposition till blue egg stage at 24 hr intervals. About 40 eggs in each replication were exposed to HS temperatures of 35, 40 and 45°C in the water bath for 2 h followed by 2 h recovery period at room temperature. A control batch in three replications were maintained at room temperature that ranged from 28 to 31°C with relative humidity of 56 – 67 per cent. After heat shock all the eggs including control batch were preserved in the plastic tray that was covered with paraffin paper and wet foam pads until blue egg stage. They were transferred to the black box for 24 h, and the next day all the eggs were exposed to light for hatching.

The eggs of HS treatment along with control batches were brushed separately in triplicates and reared until spinning under natural environmental conditions with temperature fluctuations from 26 to 31°C and relative humidity of 56 to 84 per cent (Vasudha *et al.,* 2006).

Analysis of biochemical, biological, and commercial traits: Sensitivity to varied HS temperatures was measured based on per cent hatching as an index of embryonic development. Protein was extracted and quantified by using biophotometer (Lowry *et al*., 1951). The SDS-PAGE was performed as stated by Weber and Osborn (1969) with necessary changes as suggested by Vasudha *et al*. (2006). Carbohydrate (glycogen) was estimated by Anthrone method following the earlier protocol (Sadasivan *et al.,* 2009). Effective rate of rearing (ERR), larval weight, cocoon weight, pupal weight, shell weight and shell ratio were recorded for analyzing the commercial traits.

Data were statistically analyzed using statistical software package (IBM-SPSS) version 23. Kolmogorov – Smirnov test was applied to check the data distribution. Variables were normally distributed. Two-way ANOVA was utilized to study the effect of time, temperature, and their interaction on the studied variables. Least significant difference (LSD, at $p < 0.05$) was used to check for significant differences among the studied groups. Multiple linear regression analysis was utilized to illustrate the relationship between time, temperature, species, and the studied parameters.

RESULTS

Changes in the protein profile due to heat shock at the embryonic stage of *B. mori:*

Quantitative changes: The APM1 and APHO1 eggs exhibited significant variations in the protein content after HS treatment. In APM1, high protein content was seen in day - 6 eggs HS at 35 °C, while in control it was low. Comparatively, the protein content found increased in day - 2, -3, and -4 eggs HS at 35 °C; day - 2, -4, and -7 eggs HS at 40 °C; day - 2, -3, -4 and -6 eggs HS at 45 °C, whereas it was declined in other days of respective eggs HS in 35, 40 and 45 °C (Fig. 1).

The bivoltine silkworm strain APHO1 was also responding to HS at 35 °C with increased protein content at day - 4, -3 and -8 respectively compared to control. The protein content in the eggs exposed to HS at 40 and 45°C was found to increase on day -3, -4, -5, -7 and -8 and day -4, -6 and -8 respectively. Whereas the protein content decreased in other batches of eggs HS at 35, 40 and 45°C compared to control (Fig. 1).

Qualitative changes: Further, to check if there are qualitative changes, the protein isolated from the embryos of the breeds APM1 and APHO1 under control and high temperature conditions was separated on SDS-PAGE gel. Protein profile of day - 2 showed a total of 18-19 protein bands in APM1 and APHO1 whereas on day - 6, 16-17 bands are seen. The 4 significant protein bands referable to vitellin-L, egg specific protein, vitellin-H, and 30kDa protein are similar between the

strains but differ in intensity, higher intensity was seen in 45°C treated embryos. A couple of extra protein groups were seen on day - 6 which may be a mark of the improvement of new organs in the egg. A protein band of 205kDa was found overexpressed in both the strains on day 2, but 18 kDa and 19 kDa proteins were found degrading in both the strains on day - 6 treated embryos. In addition, 90kDa and 70 kDa heat shock proteins were also found more expressed in APM1 in comparison to APHO1 but the rate of expression intensity differed (Fig. 2). Results showed several qualitative changes in embryos after HS.

Changes in the glycogen content due to heat shock at embryonic stage of *B. mori.:*

Glycogen content in embryos of APM1 and APHO1 found declined from day - 3 till hatching both in control and HS batches. In APM1, the highest glycogen content was noticed in day - 3 old embryos which decreased until hatching in control batch of eggs. However, the content of glycogen was more on day - 3 eggs of all the HS induced eggs, but their quantity declined as the embryonic development proceeded. Interestingly, the quantum of glycogen was found variable either increased or decreased in the different developmental stages of embryos exposed to different HS temperatures. Accordingly, the glycogen content found increased in all the HS induced eggs at 35, 40 and 45°C in day 5 old embryos compared to control (Fig. 3).

The glycogen content in APHO1 was also found to be increased on day - 7 eggs subjected to HS at 35, 40 and 45°C compared to control. Whereas in HS induced eggs of different age group the glycogen content was less compared to control.

Determination of sensitivity to heat shock at the embryonic stage of *B. mori.:*

The hatching of eggs of polyvoltine-APM1 and bivoltine-APHO1 silkworm strains was determined as it is an important index to determine the sensitivity towards HS treatment. The results state that, the hatching of eggs of polyvoltine-APM1 and bivoltine-APHO1 silkworm strains increased on day - 5, -6 and -7 at 35°C HS but it declined at 40°C HS on all the days.

Concomitantly, the bivoltine silkworm strain APHO1 also showed positive response to HS at 35°C with increased hatching against control. In addition, the eggs exposed to HS at 40°C HS was also increased hatching on day - 3 and -6, respectively, while other days eggs were shown reduced hatching compared to control (Fig. 4), which is statistically significant at $P< 0.01$. More interestingly, delayed hatching was recorded from the eggs subjected for HS at 45°C and quite a few larvae hatched later recovered well.

Changes in the larval growth due to heat shock at embryonic stage:

The larval growth as influenced by HS at different embryonic stages was measured based on their weight from day - 2 till blue egg stage. Interestingly, increased weight was observed not only in the larvae derived from day - 6 embryo HS at 35°C but also at 40°C compared to control batch which is significant at $p < 0.01$. Correspondingly, larvae survived after HS of day - 2 till day - 6.

In case of APHO1, an average weight of the larvae obtained from the embryos HS at 35°C. Increase in weight was recorded in the larvae derived from day - 2 embryo HS at 35°C compared to control which is significant at $P < 0.01$. The larvae which survived after HS of embryos on day - 3 at 45°C exhibited better growth compared to control (Figs. 5, 6).

Changes in the Effective Rate of Rearing (ERR) due to Heat shock at embryonic stage:

The ERR denotes for the larvae succeeding to spin cocoons. Eventually, the silkworm larvae derived from HS induced eggs of APM1 and APHO1 were reared under natural environmental conditions prevailed in the rearing house. Interestingly, 2 to 34 per cent of improvement in ERR was recorded in the population derived from different age group of APM1 eggs HS at 35 40 and 45^0C with highest ERR 34.39 per cent in day - 5 eggs HS at 40^0C (Fig. 7).

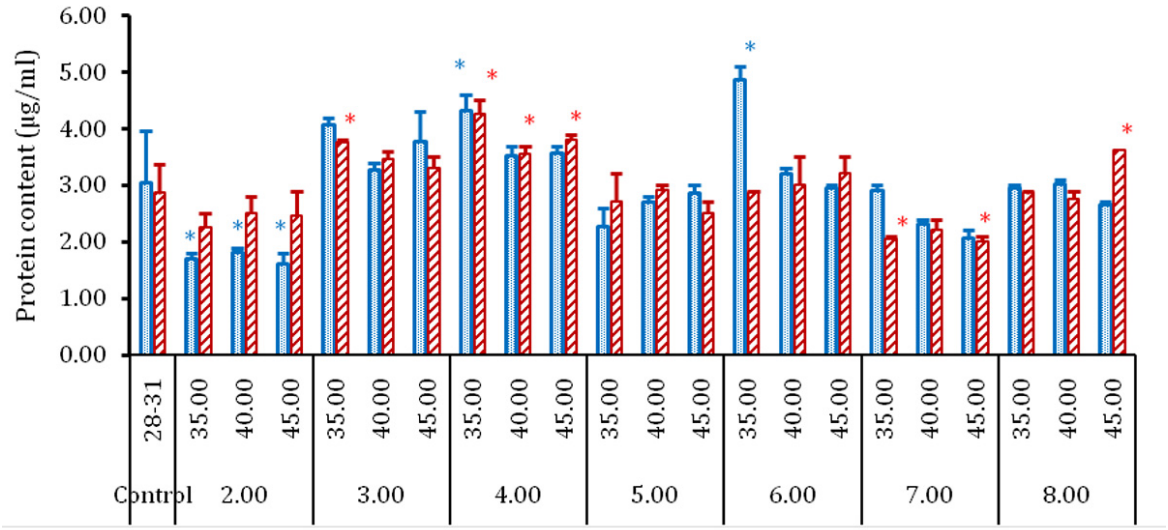

Fig. 1 The protein content (µg/ml) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4,5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

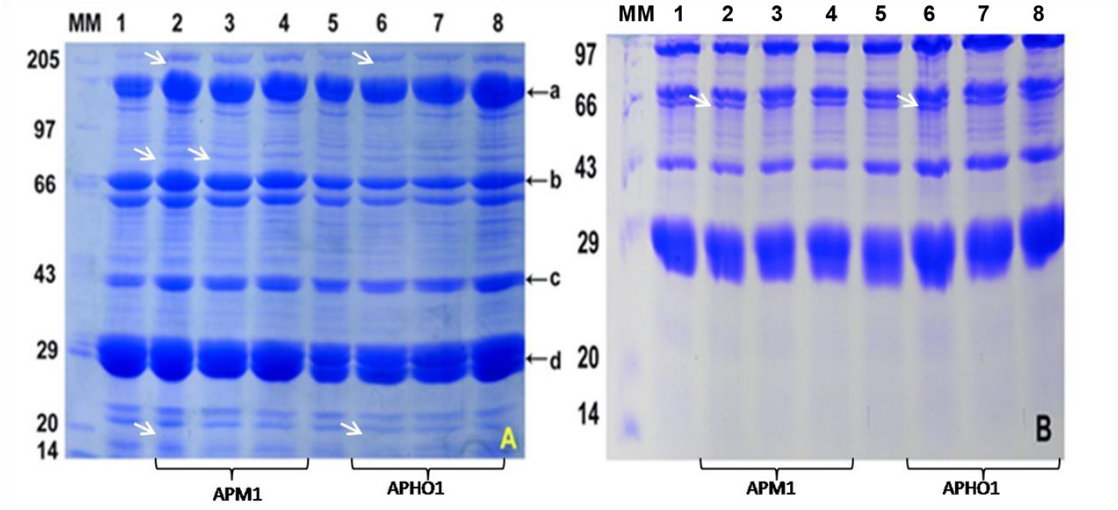

Fig. 2 Differential expression of protein profile of silkworm egg of APM1(Lanes 1 to 4: Lane 1 – Control, Lane 2 – 35°C, Lane 3 – 40°C , Lane 4 - 45°C) and APHO1(Lanes 5 to 8: Lane 5 – Control, Lane 6 – 35°C, Lane 7 – 40°C, Lane 8 - 45°C). Gel A-Day2 and B-Day6 -Arrows show the difference in expression of proteins compared to control. Numbers shown at the top of the lanes represent different days.
Band a – vitellin-H; Band b-Egg specific protein; Band c-vitellin-L; Band d-30kDa proteins

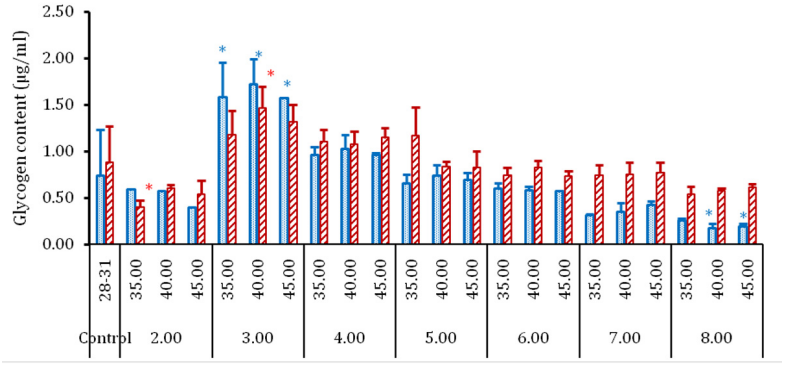

Fig 3. The glycogen content (µg/ml) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4,5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

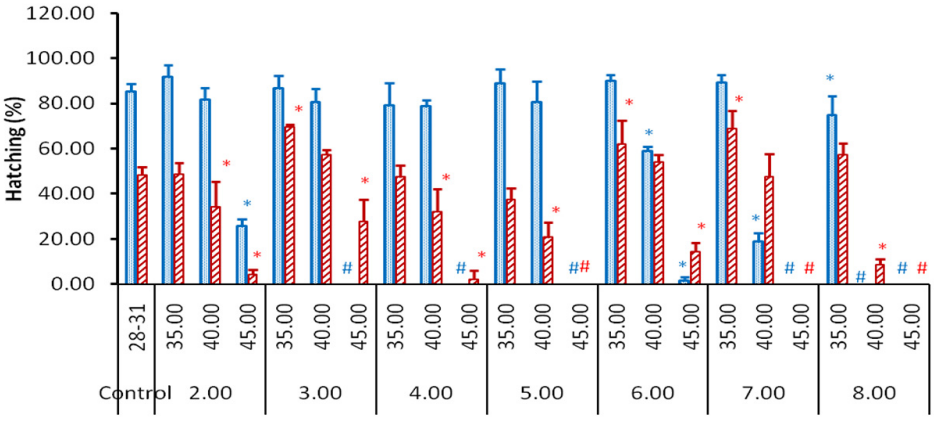

Fig 4. The hatching (%) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4, 5, 6, 7 and 8). (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

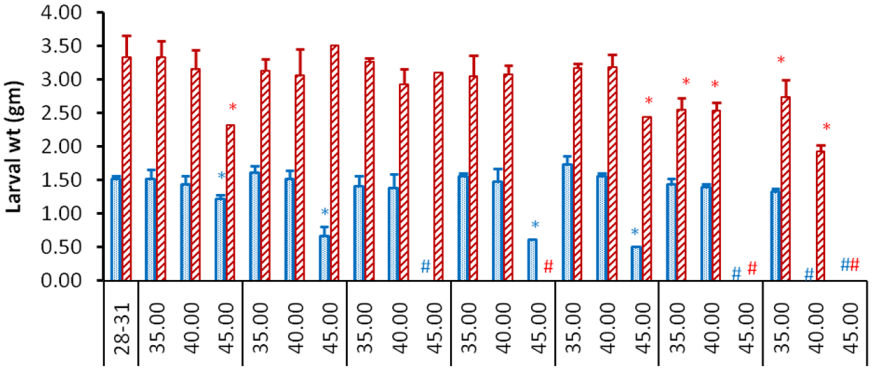

Fig 5. The larval weight (g) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (1, 2, 3, 4, 5, 6, 7 and 8 days). Blue bar represents APM1 and Red bar represents APHO1 breed. Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

ERR was severely affected due to HS at embryonic stage of APHO1, wherein good cocoons although produced but comparatively lower yield than control (81.63%). More interestingly, larvae derived from the eggs of APM1 and APHO1 HS at 45^0C recovered well and spun good cocoons and their ERR although appears to be low in comparison with control but found quite significant.

Cocoon weight: Weight of the cocoon spun by the APM1 silkworm larvae derived from HS induced embryos on day - 2 and - 4 at 35, 40 and 45°C was found significantly increased compared to control. The highest weight of the cocoon was observed in day - 4 embryo HS at 35°C. An average weight of cocoon was observed that corresponds to day - 2, 3, 4, 5, 6, 7 and 8 embryos HS at 40°C respectively that significant at P <0.01. Comparatively, the larvae survived upon HS of eggs at 45°C spun the cocoon which showed increased weight against control. (Fig. 8)

Concomitantly, the bivoltine silkworm strain APHO1 was also showed positive response to HS at 35°C with increased cocoon weight on day - 3 embryo subjected to HS against control More interestingly, larvae derived from the day - 3 eggs HS at 45^0C recovered well and spun good cocoon.

Shell Weight: The cocoon shell weight also unequivocally affected as that of cocoon weight due to fluctuated environmental conditions in the rearing house. As a result, shell weight in control was 0.11g and 0.31g in APM1and APHO1 respectively. However, larvae survived after HS at 45^0C during embryonic stage were spun the cocoons with an average shell weight in APM1and APHO1 by having marginal difference against their respective control batches (Fig. 9).

Cocoon shell ratio: The cocoon shell ratio was also correspondingly affected as that of cocoon and shell weight due to HS at embryonic stage both in APM1 and APHO1 silkworm strains. The cocoon

Table 1. Multiple linear regression (stepwise); explanatory variables entered were time (days), temperature (°C) and breed type to explain variations in the hatching percentage, larval weight, ERR, protein and glycogen levels

Dependent variable	Explanatory variables	â-coefficient			$t_{statistic}$	P-value
		Unstandardized	SE	Standardized		
Protein level	Temperature	-0.025	0.017	-0.133	-1.486	0.140
	Time	-0.015	0.034	-0.039	-0.438	0.662
	breed	-0.012	0.135	-0.008	-0.087	0.931
Glycogen content	Temperature	0.000	0.007	-0.005	-0.060	0.952
	Time	-0.092	0.015	**-0.470**	-6.039	0.000
	breed	0.145	0.061	0.185	2.382	0.019
Hatching (%)	**Temperature**	-6.53	0.384	**-0.796**	-16.99	0.000
	breed	-15.85	3.14	-0.236	-5.05	0.000
	Time	-3.51	0.784	-0.209	-4.47	0.000
Larval weight (gm)	breed	1.43	0.112	**0.629**	12.76	0.000
	Temperature	-0.124	0.014	-0.447	-9.06	0.000
	Time	-0.188	0.028	-0.330	-6.69	0.000
ERR	**Temperature**	-4.23	0.494	**-0.571**	-8.56	0.000
	Time	-5.41	1.00	-0.357	-5.36	0.000
	breed	7.023	4.00	0.116	1.754	0.082

â: represents regression coefficient with the negative signs indicating inverse association between the studied variables

shell ratio highest in the population derived from the eggs of APM1 HS at 35, 40 and 45°C respectively, which is significant at P <0.01. Concomitantly, no improvement was recorded in 35 and 40°C but at 45°C the increased shell ratio was recorded against control in the population derived from the day - 6 embryos of APHO1, which is significant at P <0.01. Obviously, induction of HS at 35, 40 and 45°C during different days of embryonic development affected the cocoon shell ratio both in APM1 and APHO1 of *B. mori* (Fig.10).

Pupal weight: Weight of the pupa, as an index of its growth, showed highest weight in the population derived the embryos of HS at 35 and 40°C on day - 7 and day - 2 respectively, which is significant at P <0.01. More importantly, the APM1 and APHO1 silkworm embryo HS at 45°C on day - 2 and - 3 were also exhibited marginal difference in weight by their respective controls (Fig. 11)

Tables 1 and 2 clarify the results of stepwise multiple linear regression analysis applied to identify the explanatory variables associated with the changes in the studied dependent variables. The strongest predictor of cocoon weight, shell ratio, and pupal weight was the temperature. There was a negative association between temperature and the change in these variables. However, each change in shell weight was highly associated with breed type. Thus, the shell weight in APHO1 was 0.132 higher than in APM1.There were strong negative associations of temperature with both the percentage of hatching and ERR. Moreover, the larval weight was higher in APHO1 than APM1 by 1.43. On the other hand, no association was observed between the protein levels and any of the explanatory variables. Time was the strongest predictor for change in the glycogen content. As each increase in time by one day, there was a decrease by 0.092 in the glycogen content.

DISCUSSION

The sericulture industry has contributed significantly to the economic development of many countries due to the commercial importance of silk in the Textile World and easy to rear silkworms under domestication. Due to continuous domestication, *B. mori* larvae lost their tolerance to high temperature and resistance to diseases. Thus, heavy loss of cocoon crop has been experienced by the farmers during critical climatic conditions. In view of this commercial importance, as well as to evaluate thermotolerance based on biochemical constituents that associated with it.

We have selected popular parental silkworm strains APM1 and APHO1 to examine the impact of HS during embryonic development, which determine the post embryonic development and cocoon characteristics for the first time as most of the studies were confined to either egg and/or larval stages (Manjunatha *et al.,* 2010). Since, it is well known that temperature plays a significant role on growth and productivity of silkworm (Sujatha *et al.,* 2001; Howrelia, 2011) a correlation study was carried out between APM1 and APHO1 in relation to heat shock response of embryos, growth and development of silkworm larvae and cocoon characteristics. It is evident from the earlier studies also that induction of HS for an hour at temperature ranging from 35 to 45°C and above has great impact on embryonic development (Manjunatha *et al*., 2005) in terms of hatching but its influence on post embryonic stages was not studied. Further, early embryonic stages until end of blastokinesis were found to be sensitive -to HS compared to late embryonic stages. In support of this, Coulon and Mathelin (1991) also opined that the initiation phase (from 72 to 120 h) is more sensitive to stress than deep phase.

Towards this, while eggs of APM1 and APHO1 subjected for HS at 35, 40 and 45°C for 2 h with 2 h recovery from day - 2 to blue egg stage (day - 8 or 9) at 24h intervals exhibited changes in the per cent of hatching revealing 45°C as lethal temperature for both the silkworm strains. The highest of 91.6 per cent hatching while noticed in day - 2 eggs of APM1 subjected for HS at 35°C, APHO1 was also showed positive response at same HS temperature with an improvement of 43.4 per cent against control (48.33%) denoting that exposure of silkworm eggs to mild HS temperature of 35°C induces highest hatching than control. Interestingly, weight of larvae derived from the eggs

Fig. 6 Effect of heat shock during embryonic development in relation to larval growth of *Bombyx mori* strain
Left side - (i) APM1 and right side - (ii) APHO1: A-Control, B-35, C-40 and D-45ºC

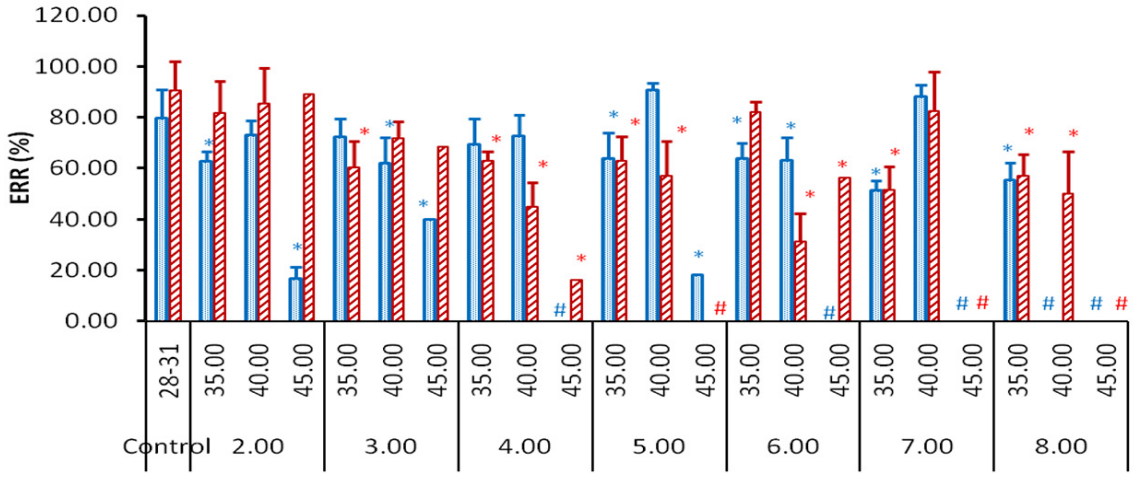

Fig. 7 ERR (%) of two species APM1 and APHO1, under different temperatures (35, 40 and 45ºC) and after different time intervals (day - 1, 2, 3, 4, 5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

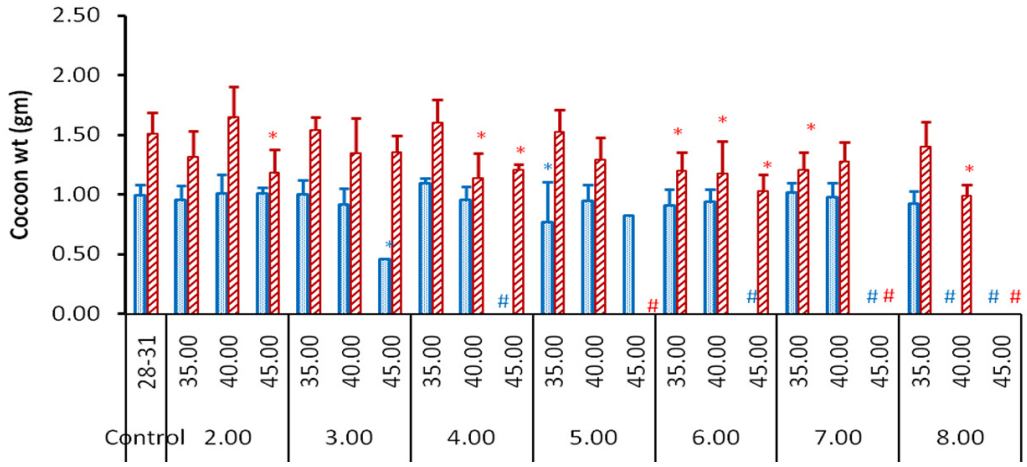

Fig. 8 Cocoon weight (g) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (1, 2, 3, 4, 5, 6, 7 and 8 days) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

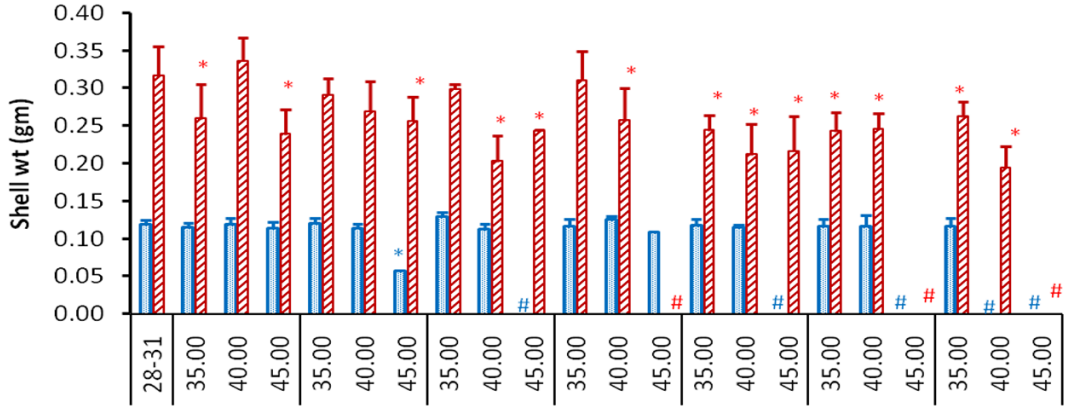

Fig. 9 The shell weight (g) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4, 5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

of APM1 HS at 35 and 40°C showed better growth with 22.63 and 12.31 than APHO1 against their respective controls. Whereas larval weight declined in HS induced embryos at 45°C compared to control indicating the lethal effect of high temperature on embryonic development that inturn produced either dead or weak larvae. Notably, some of the larvae hatched out after HS at 45°C although showed slow growth by taking longer duration but recovered well and grow either equal or much better than control exhibiting their acquired thermotolerance and emerged as healthy moth. Thus, the phenomenon of heat shock/thermotolerance that expressed in the survivals upon HS could be a potent breeding material for development of silkworm strains with acquired thermotolerance for tropics.

In APM1, 4.8 µg ml^{-1} (high) of protein content was seen in day - 6 eggs HS at 35°C, which is found to be decline in other days of eggs HS at 35, 40 and

Table 2. Multiple linear regression (stepwise); explanatory variables entered were time (days), temperature (°C) and breed type to explain variations in the pupal weight, shell ratio, shell weight, and cocoon weight

Dependent variable	Explanatory variables	ß-coefficient			$t_{statisti}$	P-value
		Unstandardized	SE	Standardized		
Cocoon weight (gm)	Temperature	-0.068	0.005	-0.538	-13.28	0.000
	breed	0.411	0.042	0.401	9.89	0.000
	Time	-0.097	0.010	-0.378	-9.32	0.000
Shell weight (gm)	breed	0.132	0.007	0.643	18.32	0.000
	Temperature	-0.011	0.001	-0.428	-12.19	0.000
	Time	-0.016	0.002	-0.311	-8.849	0.000
Shell ratio (%)	Temperature	-1.082	0.089	-0.554	-12.11	0.000
	breed	4.826	0.730	0.302	6.61	0.000
	time	-1.163	0.182	-0.291	-6.37	0.000
Pupal weight (gm)	Temperature	-0.056	0.009	-0.536	-12.83	0.000
	Time	-0.084	0.004	-0.393	-9.41	0.000
	breed	0.299	0.036	0.352	8.42	0.000

ß:represents regression coefficient with the negative signs indicating inverse association between the studied variables

45 °C than that of control (3.9 µg ml^{-1}). Similarly, the bivoltine silkworm strain APHO1 was also responding to HS at 35, 40 and 45°C with increased protein content ranging from 2.05 (day-7) to 4.25µg ml^{-1} (day-4), 2.25 (day-7) to 3.55µg/ml (day-4) and 2.05 (day-7) to 3.80 µg ml^{-1} (day-4) compared to control (2.7 µg ml^{-1}) indicating varied effect of HS on different embryo stages of *B. mori.* On the other hand, the protein content was also found to decline up to -29.31 (45°C) and -16.6 (35°C) in APM1 and APHO1 indicating the sensitivity to HS that facilitates unfolding of proteins in the embryos of APM1 and APHO1.

Furthermore, the increased quantity of protein observed in the present study is due to synthesis or over expression of 205 kDa, 90 kDa and 70 kDa heat shock proteins both in the APM1 and APHO1 HS at 35, 40 and 45°C compared to control. The expression of HSPs might involve in protecting the silkworm embryos from the fluctuated environmental condition prevailed during incubation period that resulted in increased percent of hatching compared to control as has been observed in the HS induced larvae (Manjunatha *et al*., 2010; Shabir and Manjunatha 2010; Vasuhdha *et al.*, 2006; Shou-Min Fang *et al.*, 2021).

Interestingly, the glycogen content was found more on day - 3 compared to day - 2 in the embryos of APM1 and APHO1 eventually declined as the embryonic development proceeds until hatching indicating their utilization as a source of energy. This was also observed in the embryos of NB4D2 and PM silkworm strains (Manjunatha *et al*., 2008), since carbohydrates and proteins play a vital role in the development, morphogenesis and intermediary metabolic pathway of insects (Wyatt *et al*., 1978). However, the glycogen content found variable in all the HS induced eggs while it increased in 35°C (0.65 mg ml^{-1}), 40°C (0.73 mg ml^{-1}) and 45°C (0.69 mg ml^{-1}) HS induced day-5 eggs of PM compared to control (0.63 mg ml^{-1}). The biochemical process in increased content of glycogen in the HS induced embryos is unclear and offers detailed investigation. Meanwhile, the glycogen content was found to decrease compared to control, which can be attributed that the HS induced embryos might have utilized more energy to overcome the thermal stress and might be insufficient to facilitate normal

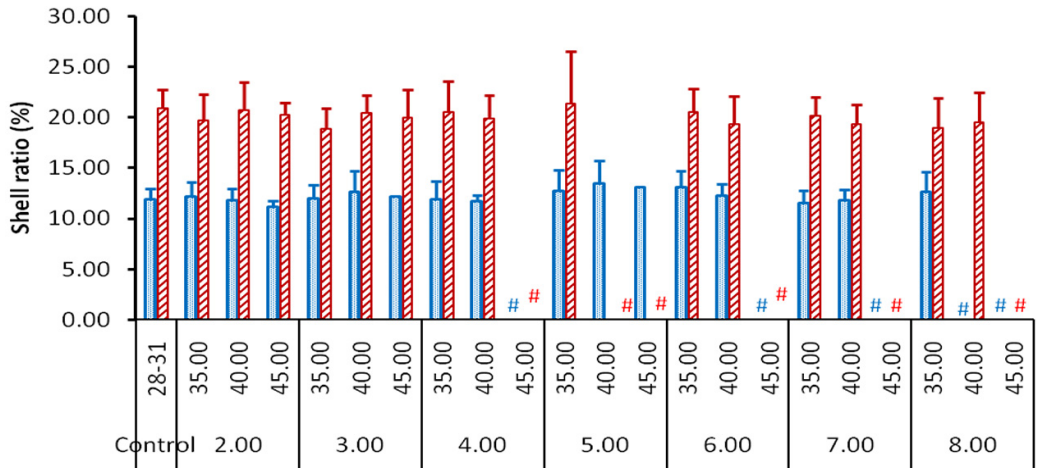

Fig. 10 The shell ratio (%) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4, 5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality. *: represent significant ($p<0.05$) difference, as compared to the corresponding control

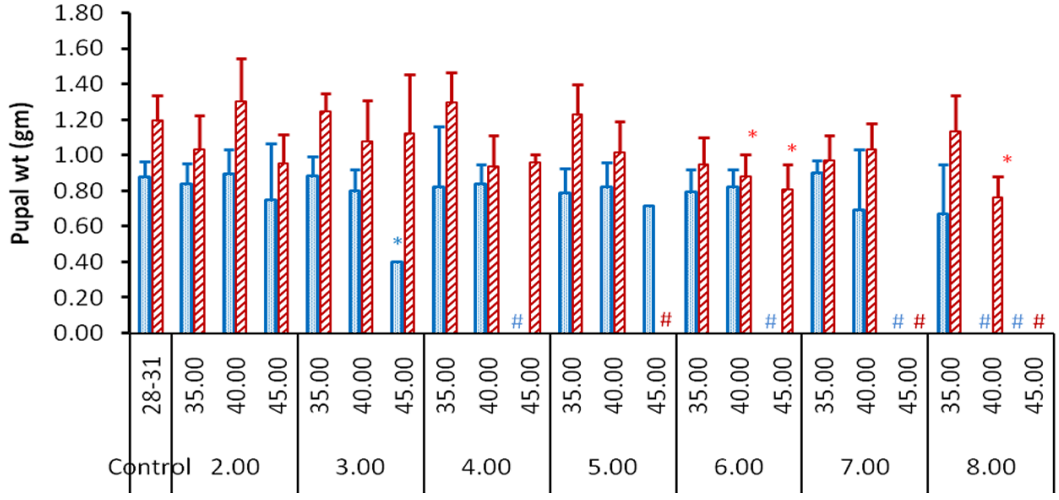

Fig. 11 The pupal weight (g) of two species APM1 and APHO1, under different temperatures (35, 40 and 45°C) and after different time intervals (day - 1, 2, 3, 4,5, 6, 7 and 8) (Blue bar represents APM1 and Red bar represents APHO1 breed). Each bar represents mean ± standard error of mean. #: represents mortality *: represent significant ($p<0.05$) difference, as compared to the corresponding control

hatching that resulted in embryonic death within the egg or at the time hatching or after hatching or embryos develop as weak larvae. Thus, it is suggested that since the silkworm embryos are highly sensitive to fluctuating environmental conditions they should be preserved under optimum conditions or even 2 h of thermal stress above threshold cause embryonic death or weak larvae which intern might affect other post embryonic developments and cocoon traits.

Based on these findings we conclude that eggs of the APM1 and APHO1 when exposed to critical temperature even for 2 h affect the hatching, altered

the protein and glycogen content, larval weight, ERR, cocoon weight, shell weight and pupal weight. However, mild HS either at 35 or 40°C at specific stage of the egg might facilitate the embryo to exhibit acquired tolerance to fluctuated environmental conditions and produce good quality cocoons. In addition, induction of HS at 45°C might produce thermotolerant silkworm strains or which can be used as potent parental breeding materials for development of thermotolerant silkworm strains for commercial exploitation in tropics.

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