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RESEARCH ARTICLE

Thermal Stress Induced Lipidogenic Changes in The Testis and Ovaries of The Silkworm Hybrids and Their Economic Parameters

Niveditha S Rao, Abhilash H. K. and Jagadeesh Kumar T. S.

ABSTRACT

In the present era Silkworm *Bombyx mori* L, a very important economical insect and backbone of the silk industry, is fully reliant on humans for its life cycle. In India, the tropical climate prevails in most of the sericulture belt, where temperature goes beyond the ambient during summer, adversely affecting the silkworm rearing relating to qualitative and quantitative traits. In order to investigate the environmental stress, effect of Thermal Stress (TS) on silkworm, *Bombyx mori* organs and its quantitative traits, an experiment was conducted using different breeds, PM × CSR2 (Crossbreed) and FC1 × FC2 (Double hybrid) in male and female larvae. Further, 5th instar silkworm male and female larvae were exposed to 35±1˚C temperature and the lipidogenic consistent changes in testis and ovaries was observed compared to unexposed batches, which was noticed higher temperature on lipid content of testis and ovaries was reduced. With respect to quantitative traits, larvae exhibit the differentiation in male and female silkworm. It has been noticed that, female silkworm larvae showed comparatively increased trend rather than male individuals as well as hybrids exhibited upward trend response to the higher temperature when compared to crossbreed. This finding provide a better understanding of cellular protective mechanisms against environmental stress (high temperature) and gives knowledge about effect and significance difference between the lipidogenic and quantitative changes in male and female larvae of different breeds.

Keywords: *Bombyx mori*, Thermal Stress, hybrid, crossbreed & lipidogenic.

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INTRODUCTION

The silkworm, *Bombyx mori is* an important economically beneficial insect for production of raw silk. Recently, it has shown to be a good model for biological science due to its excellent biological characteristics, such as easy of rearing large body and availability of genomic information (Mita *et al.,*2004; Xia *et al.,* 2004). The silkworm strains of temperate countries like Japan, China etc. are bivoltine, which are characterized by longer larval duration with high silk content and superior silk quality. However, they are highly susceptible to tropical climatic conditions and less tolerant to higher temperature, Temperature is physical factor plays a major role in growth and productivity of the silkworm, as silkworm is poikilothermic organism (Benchamin and Jolly, 1986). There is an ample literature showing good quality cocoons are produced within a temperature range of 20-27°C and level above these makes the cocoon quality worse (Krishnaswami *et al.,* 1978). The effect of temperatures higher than 30°C on silkworm larvae was reported earlier by Takeuchi *et al.,* (1964) and Ohi and Yamashita (1977). India is a tropical country where air temperature in the summer can range between 35-40°C in the day and 28-35°C at night. These fluctuations in the temperature have an adverse effect on the survival and pupation of silkworms, especially the bivoltine breeds incurring heavy loss to the sericulture industry.

Many silkworm characteristics are not only controlled by genes but also influenced by environmental factors such as temperature (Watanabe, 1918, 1919, 1924, 1928 and Kogure, 1933). However, in order to introduce bivoltine races in a country with a tropical climate, it is necessary to maintain stability of silkworm breeds in cocoon crop production under a higher temperature environment (Suresh Kumar *et al.,* 2002).

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The environment is dynamic and hence brings about profound changes in physical and biotic factors dominating the expression of commercial characters in silkworm (Kobayashi *et al.,* 1986). An earlier report suggests that, environmental stresses diminish antioxidant status and cause oxidative stress in Lepidoptera (Grubor-Lajsic, 1997). Living organism need mechanisms regulating reactive oxygen species (ROS) such as oxygen peroxide and superoxide anion.

In Antarctica midge *Belgica Antarctica,* oxidative stress was observed on exposer to heat stress and gene encoding catalase which was also elevated in response to dehydration (Lopez-Martinez *et al*., 2008). ROS is harmful to living organisms because ROS tend to give oxidative damage to proteins, nucleic acids and lipids (Hermeslima and Zenteno-Savin, 2002).

High temperature affects nearly all biological processes including the rates of biochemical and physiological reactions (Hazel, 1995; Willmer *et al.,* 2004) and it eventually can affect the quality and quantity of cocoon crop in silkworm. Several reports (Ueda and Lizuka, (1962); Shirota, (1992) Tazima and Ohnuma, (1995) demonstrated the silkworms were more sensitive to high temperature during 4th and 5th stages for selection of thermo tolerant silkworm breeds, the 5th instar proved to be the best stage for purpose of screening and evaluation of robust and thermo tolerant bivoltine breeds of the silkworm *Bombyx mori* L.

Temperature is one of the most important environmental factors that induce physiological changes in organisms. Previous studies suggested that, thermal stress may diminish the antioxidant state and cause oxidative stress (An & Choi 2010), such oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and a biological systems ability to readily detoxify the reactive intermediates, or to easily repair the resulting damage (Rahman *et al.,* 2006). Heat stress signal transduction pathways and defense mechanisms are intimately associated with ROS (Pnueli *et al.,* 2003), whereas, low temperature stress induces H_2O_2 accumulation in cells (O'kane *et al.,* 1996).

MATERIALS AND METHODS

The present experimental programme is designed as per the standard rearing method suggested by Krishnaswami (1978) and the silkworm layings namely, PM × CSR_2 and FC_1 × FC_2 breeds were released and incubated at NSSO Mysuru for the progressive embryonic developmental period till the day of completion of pinhead stage and the hatched larvae brought to the department of studies in sericulture science in the loose egg box without disturbing the silkworm larvae from cold storage NSSO Mysore.

To conduct the silkworm rearing under ambient temperature of 25°C± 1 and relative humidity of 80% is maintained in rearing house. The healthy silkworm larvae were fed with the S_{36} variety till the completion of 3rd instar and during 4th and 5th instar silkworms fed with V_1 variety of mulberry *(Morus alba*) leaves till the completion of spinning period with two feeding schedules.

The experimental batches of silkworm breeds are divided into two groups such as control batches (T_0) at ambient temperature 28°C of and the imposed temperature of 35°C in BOD incubator (T_1) with 3 different replications of 100 larvae each during 5th instar.

Temperature treatments:

The ambient temperature (28±1°C) was maintained as a control and whereas the imposed temperature where the air temperature during summer is 35-40°C in the day time. Imposed temperature of 35±1°C was tested using a Biological Oxygen Demand (BOD) incubator.

The temperature stressed larvae were exposed to 15×15 min period at a high temperature (35±1°C) separately followed by a 15min ''rest'' period at 28±1°C.

Control batches of the silkworm were maintained at a constant 28±1°C. The exposing duration to thermal stress was started from the 1st to 5th day of 5th instar. Appropriate plastic boxes (25×18×7 cm in size) with a net lid were made and used to transfer silkworm larvae from the rearing house to the BOD. After imposed temperature treatment, the silkworm larvae were transferred to the standard rearing condition at 28±1°C. All experiment silkworms were not fed during incubation under higher temperature and fed fresh mulberry leaves 15 min after the of BOD incubation

Silkworms exposed to 35 ± 1° C in a BOD incubator during experimentation

twice per day, the humidity in the BOD was adjusted to the rearing house humidity of 75±2% using wet foam pad around the silkworm in rearing trays.

Tissue preparations:

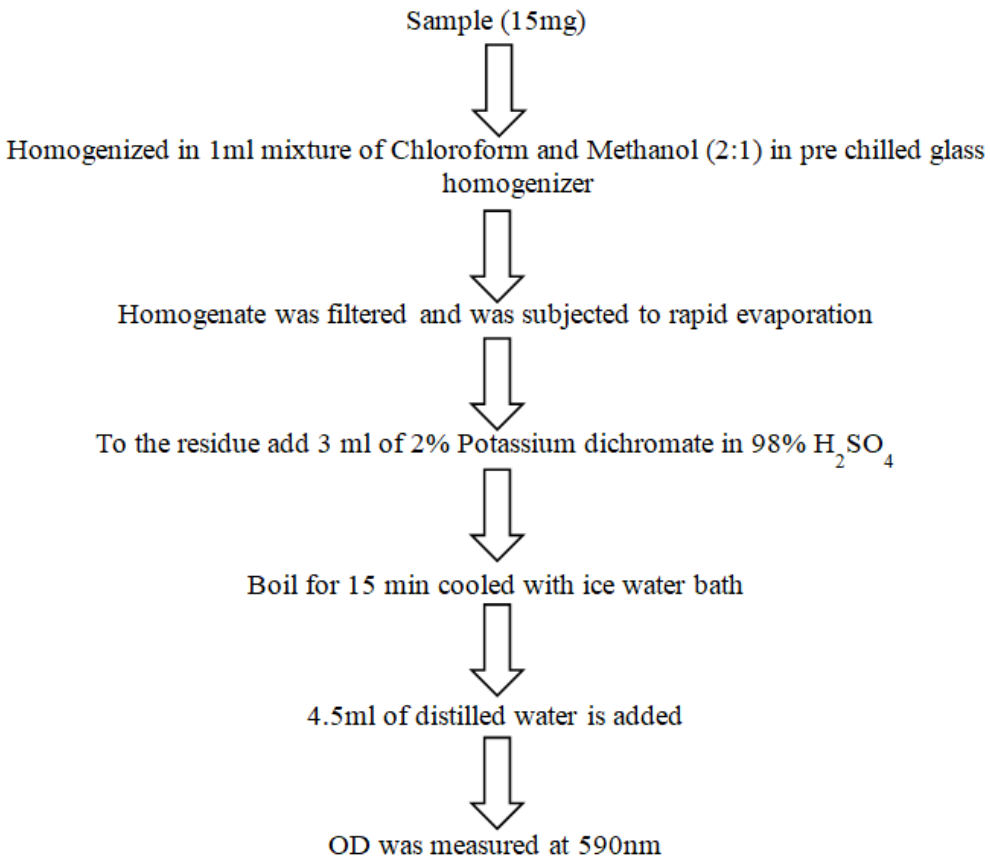
The tissue preparations were made from the first day of 1st instar to 5th day of 5th instar in both control and higher of 35±1° C temperature treated batches using the method of Jagadeesh Kumar (2010). To prepare the tissues for lipid extraction, the reproductive organs namely testis and ovaries as a samples were collected by sampling 4-5 silkworm larvae by random selection in each replication. The homogenate were collected and stored at -20°C until utilized for experimentation.

Lipid content by semi-micro method of Bragdon (1951) modified by Pande *et al.*, (1963)

Lipid content of samples was estimated by semi-micro method of Bragdon (1951) modified by Pande *et al*., (1963) the active tissues namely, testis and ovaries of the silkworm PM × CSR_2 and FC_1 × FC_2 breeds were used for estimation. 15mg of chilled sample was homogenized in 1ml mixture of chloroform and methanol (2:1 V: V) in pre-chilled glass homogenizer. The homogenate was filtered and was subjected to rapid evaporation. To the residue, 3ml of 2% potassium dichromate in 98% sulphuric acid (w/v) was added and boiled in water bath exactly for 15 minutes. The test tubes were cooled in ice water bath for 15minutes, 4.5 ml of distilled water was added and the mixture was cooled again. The colour intensity was read at 590nm with a suitable blank and lipid levels are expressed in mg of lipid per gram wet weight of tissue.

Assessment of quantitative traits:

The performance of all genotypes had chosen for the present study by analyzing eight economic characters namely, larval weight, cocoon weight, shell weight, shell ratio, filament length, filament weight, denier and renditta using following formulae.



Flow Chart of The Experimentation

Larval weight

The average weight of 10 larvae was taken after the last feeding, just before beginning spinning cocoon.

Cocoon weight

The average weight of 10 cocoons was taken after removing the useless floss on the cocoons (Deflossing).

Shell weight

The average weights of 10 cocoon shells were taken separately after removing the pupae.

Shell percentage

$$\frac{\text{Weight of the shell}}{\text{Weight of the cocoon}} \times 100$$

Filament length

The total length of silk filament from a single cocoon was reeled using an epprouvette. (The mean value of 10 observations was considered).

Filament weight

The total weight in grams of silk filament of a single cocoon was estimated. (The mean value of 10 observations was considered).

Denier

$$\frac{\text{Filament weight}}{\text{Filament length}} \times 9000$$

Renditta

$$\frac{\text{Weight of cocoon}}{\text{Weight of silk reeled from the same cocoon}}$$

Statistical analysis of the data

The experimental data are collected with reference to lipidogenic changes in testis and ovaries and economic parameters of the silkworm were analyzed statistically for test of significance using Fisher's method of analysis of variance (ANOVA).

RESULTS

The present investigation was programmed to observe the temperature induced changes in the lipid level of testis and ovaries of male and female silkworm larvae of PM × CSR_2 and FC_1 × FC_2 breeds chosen for the study.

The lipids are high yielding energy active biomolecules encountered to the metabolic interconversion during each and every stages of developmental period compared to other biomolecules such as protein, glycogen etc. lipids plays a key role not only for the metabolic inter conversion but also the structural constructive purpose of cells and differentiation provides the mobilization of converted energy into different cell signaling pathway keeping in view that the lipidogenic changes in testis of male larvae and ovaries of female larvae were recorded after exposure to the 35˚C in a controlled conditions of BOD incubator during 5th instar 1st day to 5th day it is an active growth period for the most of the energy transformation mobilization and utilization for complete biosynthesis of silk protein hence forth the 5th instar silkworm are utilized in the present approach.

The day to day changes in the lipid content of testis and ovary was found to be noticed a consistent level of order of increase was observed both in testis and ovary of silkworm PM × CSR_2. It is true that, the temperature of 35˚C plays a role as physical stimuli and activates the biosynthesis pathway for lipogenesis in fat body tissues musculature and also the male and female reproductive organ are the energy transferred during the course of the events of spermatogenesis and oogenesis.

The effect of temperature mainly accomplished the total observational changes (Table 1, figure 1) on 1st day (1.94) in testis and (0.47) in ovary consequently the level of changes indicated a drastic increase on 3rd day (15.29) in testis and (10.03) in ovary followed by 4th day relatively very low in the increase compared to control whereas the last day of 5th instar a drastic decline in the female about (-23.97) as an uncommon observational changes noticed day before spinning under imposed thermal stress condition compared to control batches of male and female larvae of PM × CSR_2.

Similarly in FC_1 × FC_2 (Table 2, Figure 2) also utilized for the lipidogenic changes under imposed temperature condition both in testis and ovary (during 5th instar male and female silkworm larvae the changes observed on 1st day is about (0.37) in testis (0.63) in a ovary showed a positive trend on 3rd day (8.33) and (14.51), on 5th day (11.26) and (0.09) in testis and ovary respectively.

Whereas, the second and 4th day a negative relationship coordinated in male and female silkworm larvae of FC_1 × FC_2 depicted that, (-8.86) and (2.90) on 2nd day (-2.73) (-3.18) on 4th day both in testis and ovaries respectively. It is an ontogenic relationship observed an alternate day of changes due to the acute thermal stress induced the active release of glycerides, triglycerides and diglycerides for the utilization during the course of the larval, pupal transformation. Hence most of the energy is acquainted with the development process as the pupal stage is non

feeding period mostly restricted for biosynthesis of target protein molecule necessary to facilitate the post transcriptional events. To execute the biosynthetic pathway of protein synthesis the PM × CSR_2 and FC_1×FC_2 two productive silkworm robust potential breeds sensitive to the temperature regimes can trigger a biological system to attain resistance to the thermal stress can implies an organismic changes in the manifestation and expression of qualitative an d quantitative parameters and better performance as a result of food consumption incurred during the course of the imposed stress condition in selected silkworm hybrids under BOD incubator.

The larval weight of PM × CSR_2 and FC_1 × FC_2 (Table 3, 4; Figure 3, 4) to different temperature regime of 35˚C under controlled conditions attributed to play a key role in the improvement of larval weight in both male and female of both the hybrids day to day increase is up to 5th day and slightly declined on 6th and 7th day in male individuals whereas, in the female the quantum of changes limited to 4th day as higher of (8.99) and slowly decreased towards the day of spinning period.

The same pattern of changes was observed in FC_1 × FC_2 silkworm breed could attribute slow steady changes but relatively low in both male and female silkworm larva of FC_1 × FC_2 as a result of which till the day of spinning period the influence of temperature is favorable towards the larval weight and vulnerable to the PM × CSR_2 compared to FC_1 × FC_2.

Besides, the effect of temperature on lipidogenic content and the larval weight of the two selected robust hybrids and the economic parameters of PM×CSR_2 (Table 5 & 6) under imposed thermal stress condition revealed a marginal decrease in all the eight economic parameters such as cocoon weight, pupal weight, shell weight, shell ratio, filament length, filament weight, Denier and renditta. Both in male and female batches subjected to temperature treatment separately except the percent changes in the female individuals of PM × CSR_2 in relation to shell weight and shell ratio was about (1.06) (2.26) respectively on the other hand FC_1 × FC_2 (Table 7, 8) also revealed a same pattern and negative trend in manifestation of all the eight economic parameter considering the 35˚C is a critical temperature the expression and manifestation of cocoon character might not be more favorable to improve the productive traits the cocoon weight (-2.21), pupal weight (-3.67), shell weight (-0.88), shell ratio (1.34), filament length (-3.07), filament weight (-1.47), Denier (1.66) and renditta (-0.76) in male on the other hand the percent changes in female population of FC_1 × FC_2 exhibits (-0.076) in cocoon weight, pupal weight (-0.89), (-0.72) in shell weight, (0.04) in shell ratio, (-1.49) in filament length, (-2.85) filament weight, (-1.38) in Denier and finally (2.31) in renditta were recorded after the exposure to 35˚C during 5th instar. Thus the present findings of project work carried out in relation to the influence of higher temperature on the lipidogenic changes in testis and ovaries during 5th instar and economic parameters of PM × CSR_2 and FC_1 × FC_2.

With the aim of examine the effect of thermal stress, the images of silkworm larvae were captured on 5th instar before spinning and negligible changes were observed between control and thermal treated batches of PM × CSR2 and FC1 × FC2 of male and female (Figures 5 and 6). As well, after spinning, silkworm cocoons were selected randomly and captured to observe the difference in the physical properties. There are no significant changes noticed among the control and treated batches of both hybrids in male and female (Figures 7 and 8). Thus the experiment clearly elucidates the thermal stress on silkworm larvae and cocoons did not show the significant difference between the control and treated batches of both hybrids.

Furthermore, present investigation pointed out that the effect of temperature on economical characters between PM × CSR2 and FC1 × FC2 of male and female, the resistance for high temperature was observed more in females of both hybrids with respect to cocoon weight, pupal weight and renditta. Whereas, equal resistance was reported in both male and females with respect to shell weight, filament length, filament weight and denier. Then, males with with respect to shell ratio shown better resistance compared to females of both hybrids (Figure 9A, 9B – 16A, 16B). Thus, the graphical representation indicates the difference in economical characters, which is directly proportional to temperature.

DISCUSSION

The lipids are the most active components of biological system can assist the large number of cellular and sub cellular energy dependent functional changes attributed for aerobic and anaerobic respiration as a result the cell to cell, organ to organ, tissue to tissue and inter transformation of energy dependent molecules to facilitate the inter connectivity of glucose, proteins, amino acids and nucleic acid metabolism. It is a noteworthy that, lipids are high energy yielding structural integrity accomplishes the most vulnerable dynamic functions. Lipids composed of derivatives of glycerides, steroids etc. During the constructive process of physiological status of the cells

Table 1: Changes in lipid content of testis and ovary of PM×CSR_2 exposed to 35˚C±1˚C temperature during fifth instar. (mg/gm of tissue)

Days	*1*		*2*		*3*		*4*		*5*	
	Testis	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*
Control	0.206 ± 0.01	0.211 ± 0.00	0.249 ± 0.01	0.243 ± 0.01	1.275 ± 0.60	1.515 ± 0.40	1.152 ± 0.01	1.164 ± 0.01	1.026 ± 0.02	1.331 ± 0.60
35°C	0.210 ± 0.01	0.212 ± 0.01	0.249 ± 0.02	0.244 ± 0.00	1.470 ± 0.45	1.667 ± 0.05	1.155 ± 0.01	1.202 ± 0.00	1.025 ± 0.00	1.012 ± 0.01
Percent Change	(1.94)*	(0.47)*	(0.00)*	(0.41)*	(15.29)**	(10.03)**	(0.26)*	(3.26)*	(-0.10)*	(-23.97)*

*-Non-significant, **-Significant

Table 2: Changes in lipid content of testis and ovary of FC_1×FC_2 exposed to 35˚C±1˚Ctemperature during fifth instar. (mg/gm of tissue)

Days	*1*		*2*		*3*		*4*		*5*	
	Testis	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*	*Testis*	*Ovary*
Control	0.268 ± 0.01	0.315 ± 0.01	0.316 ± 0.02	0.345 ± 0.02	1.404 ± 0.00	1.126 ± 0.60	1.247 ± 0.06	1.227 ± 0.05	1.012 ± 0.01	1.083 ± 0.05
35°C	0.269 ± 0.02	0.317 ± 0.01	0.288 ± 0.04	0.335 ± 0.04	1.521 ± 0.03	1.286 ± 0.04	1.213 ± 0.13	1.188 ± 0.05	1.126 ± 0.00	1.084 ± 0.01
Percent Change	(0.37)*	(0.63)*	(-8.86)*	(-2.90)*	(8.33)*	(14.21)**	(-2.73)*	(-3.18)*	(11.26)**	(0.09)*

*-Non-significant, **-Significant

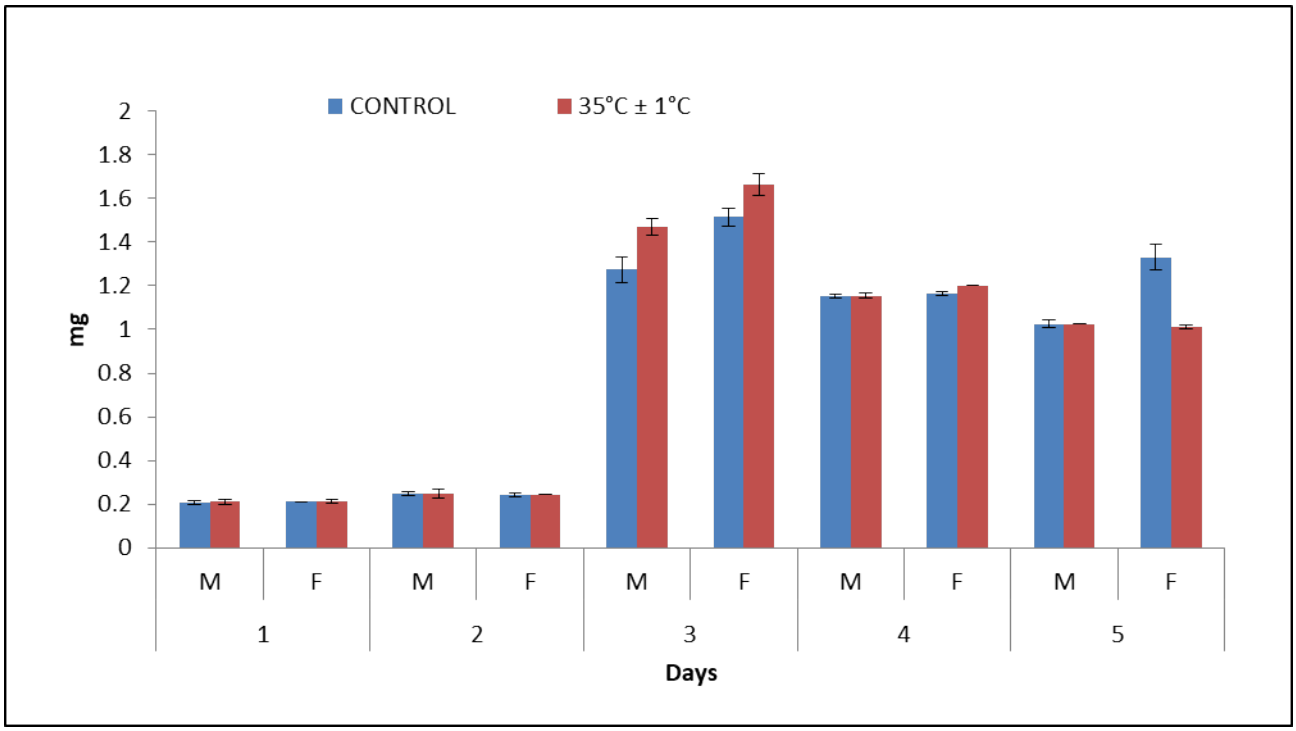


Figure 1: Effect of temperature on lipid content in testis and ovaries of fifth instar silkworm PM × CSR_2.

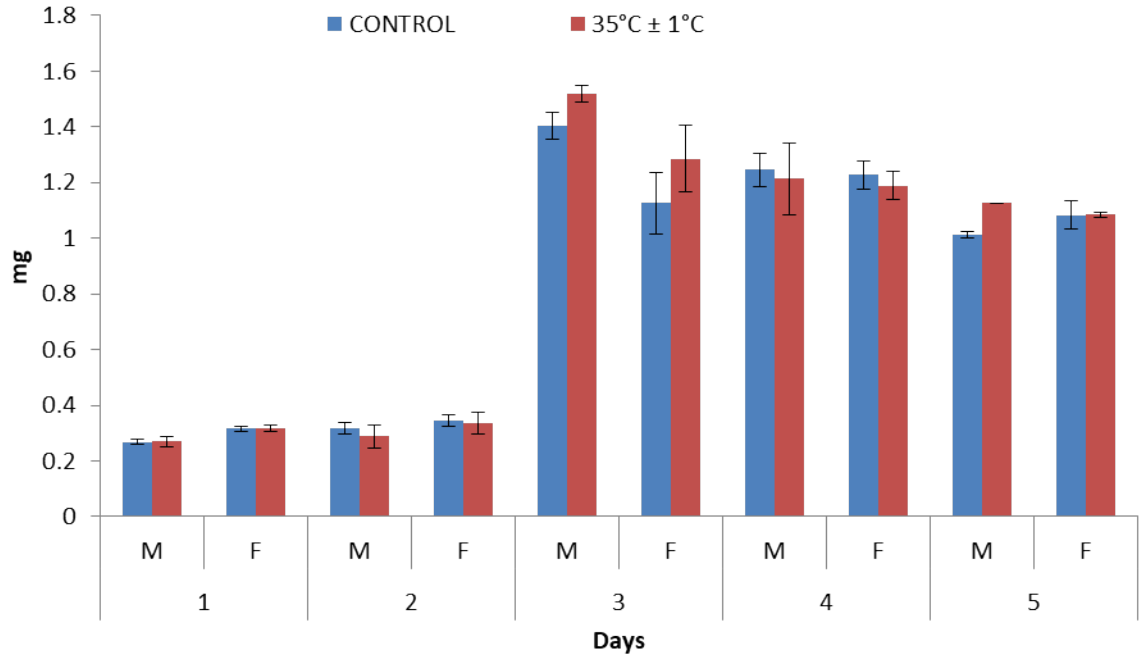


Figure 2: Effect of temperature on lipid content in testis and ovaries of fifth instar silkworm $FC_1 \times FC_2$.

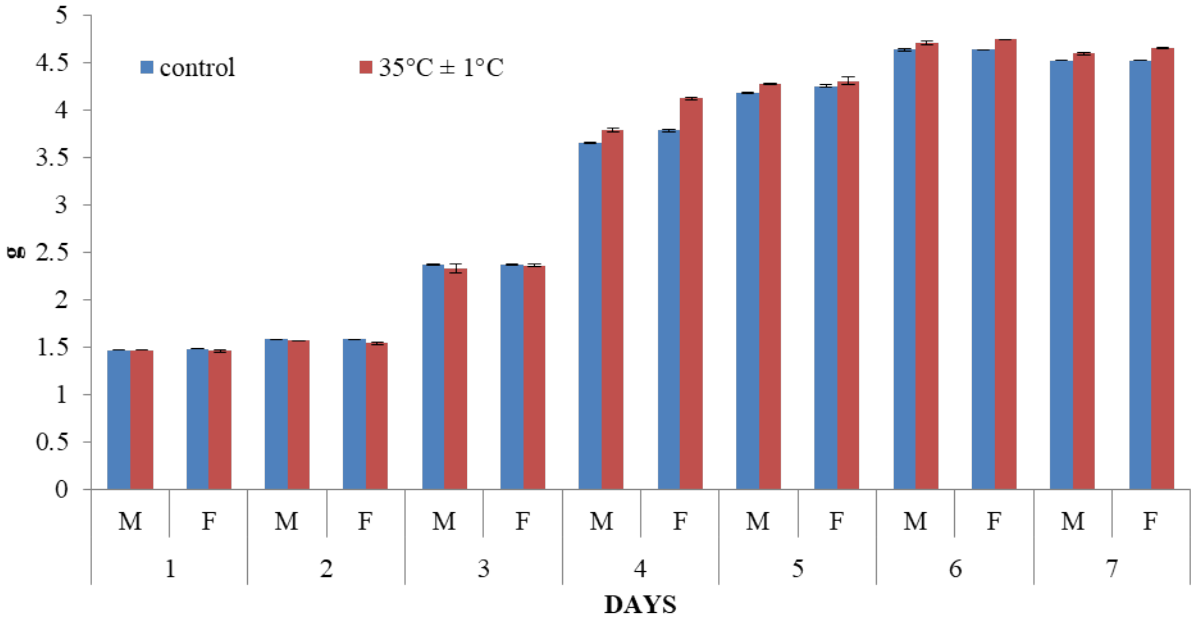


Figure 3: Effect of temperature on larval growth of $PM \times CSR_2$ during fifth instar

Table 3: Changes in larval weight of PM × CSR_2 exposed to 35˚±1˚C temperature during fifth instar.

	1		*2*		*3*		*4*		*5*		*6*		*7*	
	Male	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
Control	1.47 ± 0.00	1.48 ± 0.00	1.58 ± 0.00	1.58 ± 0.00	2.37 ± 0.01	2.37 ± 0.01	3.65 ± 0.01	3.78 ± 0.01	4.18 ± 0.01	4.25 ± 0.01	4.63 ± 0.01	4.63 ± 0.00	4.52 ± 0.00	4.52 ± 0.01
35°±1°C	1.47 ± 0.00	1.46 ± 0.01	1.57 ± 0.00	1.54 ± 0.01	2.33 ± 0.05	2.36 ± 0.01	3.78 ± 0.02	4.12 ± 0.01	4.27 ± 0.01	4.30 ± 0.04	4.71 ± 0.02	4.74 ± 0.00	4.59 ± 0.01	4.65 ± 0.01
Percent change	(0.00)*	(1.35)*	(0.63)*	(2.53)*	(1.69*	(0.42)*	(3.56)*	(8.99)*	(2.15)*	(1.18)*	(1.73)*	(2.38)*	(1.55)*	(2.88)*

*-Non-significant, **-Significant

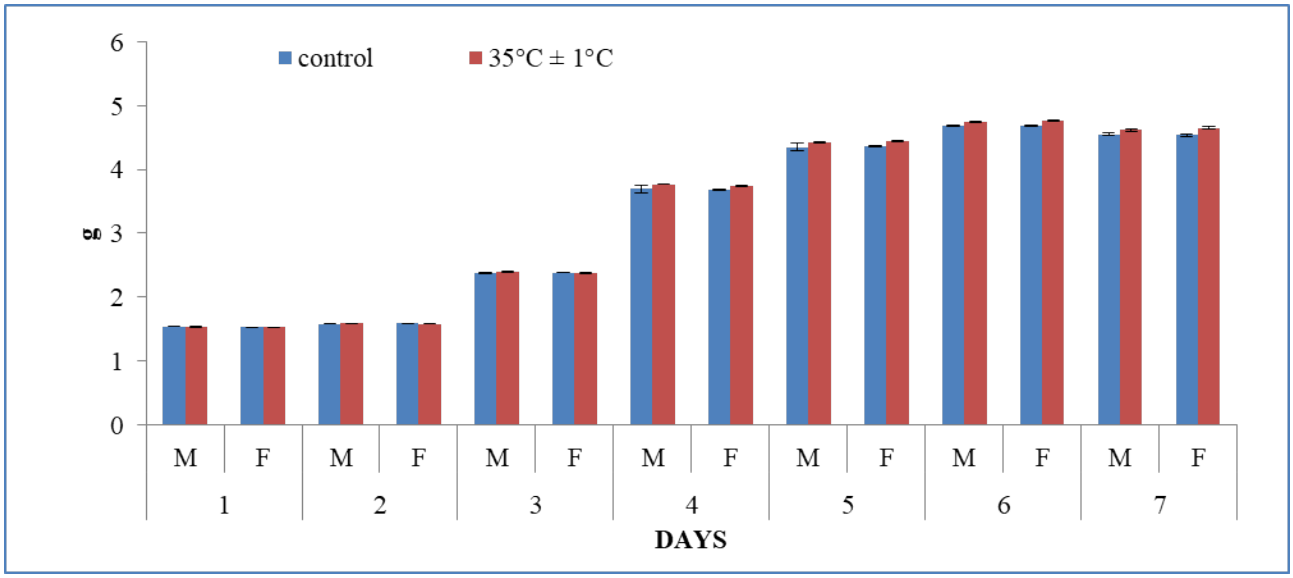
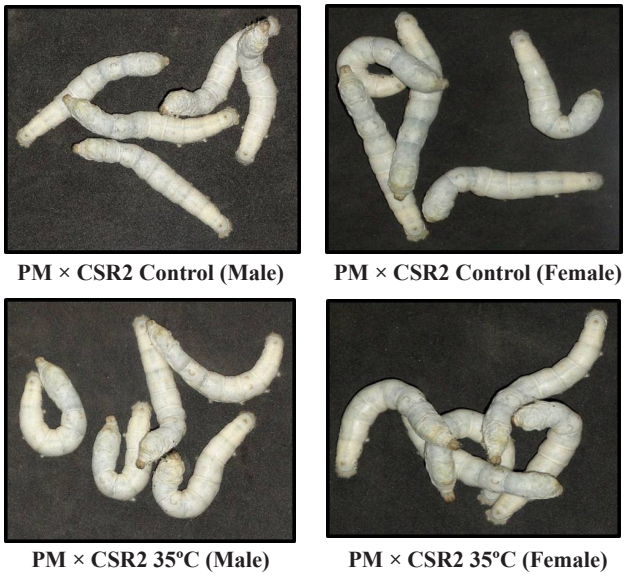


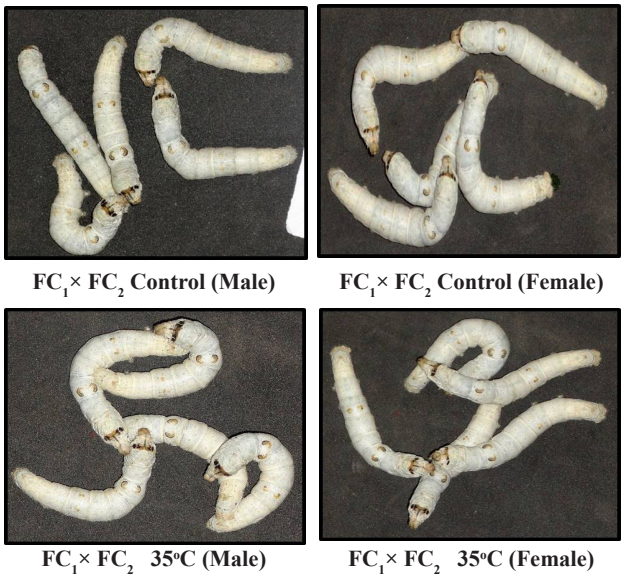
Figure 4: Effect of temperature on larval growth of the FC_1 × FC_2 during fifth instar.



PM × CSR2 Control (Male) PM × CSR2 Control (Female)

PM × CSR2 35ºC (Male) PM × CSR2 35ºC (Female)

Figure 5: Fifth instar silkworm larvae of PM × CSR2 control and temperature imposed silkworm.



FC_1× FC_2 Control (Male) FC_1× FC_2 Control (Female)

FC_1× FC_2 35ºC (Male) FC_1× FC_2 35ºC (Female)

Figure 6: Fifth instar larvae of control and temperature imposed silkworms of FC_1 × FC_2.

Table 4: Changes in larval weight of $FC_1 \times FC_2$ exposed to 35 ± 1˚C temperature during fifth instar.

Treatment	*1*		*2*		*3*		*4*		*5*		*6*		*7*	
	Male	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
Control	1.54 ± 0.00	1.53 ± 0.00	1.58 ± 0.00	1.59 ± 0.00	2.38 ± 0.01	2.38 ± 0.00	3.70 ± 0.06	3.68 ± 0.01	4.35 ± 0.06	4.36 ± 0.01	4.68 ± 0.01	4.68 ± 0.01	4.55 ± 0.02	4.54 ± 0.02
35°C±1°C	1.54 ± 0.01	1.53 ± 0.00	1.59 ± 0.00	1.58 ± 0.00	2.39 ± 0.01	2.38 ± 0.01	3.79 ± 0.00	3.75 ± 0.01	4.42 ± 0.01	4.44 ± 0.01	4.74 ± 0.01	4.76 ± 0.01	4.62 ± 0.02	4.65 ± 0.02
Percent change	(0.00)*	(0.00)*	(0.63)*	(-0.63)*	(0.42)*	(0.00)*	(2.43)*	(1.90)*	(1.61)*	(1.83)*	(1.28)*	(1.71)*	(1.54)*	(2.42)*

*-Non-significant, **-Significant

Table 5: Economic parameters of male PM × CSR_2 exposed to 35 ± 1˚C temperature during fifth instar.

Treatment	*Cocoon weight (g)*	*Pupal weight (g)*	*Shell weight (g)*	*Shell ratio (%)*	*Filament length (m)*	*Filament weight (g)*	*Denier*	*Renditta*
Control	1.73 ± 0.01	1.36 ± 0.02	0.34 ± 0.04	19.57 ± 2.39	882.33 ± 4.50	0.30 ± 0.01	3.06 ± 0.03	5.77 ± 0.12
35˚±1˚c	1.65 ± 0.01	1.32 ± 0.01	0.33 ± 0.01	20.01 ± 0.60	794.00 ± 10.44	0.25 ± 0.00	2.87 ± 0.02	6.52 ± 0.11
Percent change	(-4.51)*	(-3.09)*	(-2.36)*	(2.21)*	(-10.01)*	(-15.56)*	(-6.15)*	(13.06)*

*-Non-significant, **-Significant

Table 6: Economic parameters of female PM × CSR_2 exposed to 35 ± 1˚C temperature during fifth instar

Treatment	*Cocoon weight (g)*	*Pupal weight (g)*	*Shell weight (g)*	*Shell ratio (%)*	*Filament length (m)*	*Filament weight (g)*	*Denier*	*Renditta*
Control	1.93 ± 0.01	1.60 ± 0.01	0.31 ± 0.01	16.34 ± 0.37	855.33 ± 11.68	0.26 ± 0.01	2.76 ± 0.04	7.33 ± 0.15
35°±1°C	1.90 ± 0.01	1.58 ± 0.01	0.32 ± 0.02	16.71 ± 0.93	839.67 ± 5.03	0.26 ± 0.00	2.79 ± 0.02	7.30 ± 0.10
Percent change	(-1.18)*	(-1.17)*	(1.06)*	(2.26)*	(-1.83)*	(-0.76)*	(1.09)*	(-0.44)*

*-Non-significant, **-Significant

Table 7: Economic parameters of male $FC_1 \times FC_2$ exposed to 35˚±1˚C temperature during fifth instar.

Treatment	*Cocoon weight (g)*	*Pupal weight (g)*	*Shell weight (g)*	*Shell ratio (%)*	*Filament length (m)*	*Filament weight (g)*	*Denier*	*Renditta*
Control	1.907 ± 0.03	1.44 ± 0.03	0.46 ± 0.00	24.01 ± 0.45	1129 ± 3.61	0.36 ± 0.01	2.89 ± 0.04	5.24 ± 0.16
35˚±1˚C	1.86 ± 0.01	1.38 ± 0.01	0.45 ± 0.00	24.34 ± 0.15	1094 ± 7.02	0.36 ± 0.01	2.94 ± 0.05	5.20 ± 0.08
Percent Change	(-2.21)*	(-3.67)*	(-0.88)*	(1.34)*	(-3.07)*	(-1.47)*	(1.66)*	(-0.76)*

*-Non-significant, **-Significant

Table 8: Economic parameters of female $FC_1 \times FC_2$ exposed to 35 ± 1˚C temperature during fifth instar.

Treatment	*Cocoon weight (g)*	*Pupal weight (g)*	*Shell weight (g)*	*Shell Ratio (%)*	*Filament length (m)*	*Filament weight (g)*	*Denier*	*Renditta*
Control	2.27 ± 0.01	1.81 ± 0.01	0.46 ± 0.00	20.26 ± 0.13	1139 ± 17.56	0.37 ± 0.01	2.96 ± 0.02	6.06 ± 0.09
35˚±1˚C	2.25 ± 0.01	1.79 ± 0.01	0.46 ± 0.00	20.27 ± 0.08	1122 ± 7.09	0.36 ± 0.01	2.92 ± 0.01	6.91 ± 0.05
Percent change	(-0.76)*	(-0.89)*	(-0.72)*	(0.04)*	(-1.49)*	(-2.85)*	(-1.38)*	(2.31)*

*-Non-significant, **-Significant

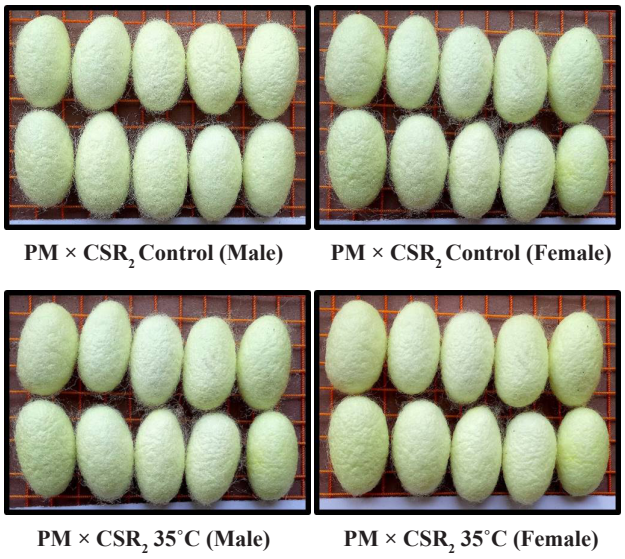


Figure 7: Cocoons of control and temperature imposed of PM × CSR_2.

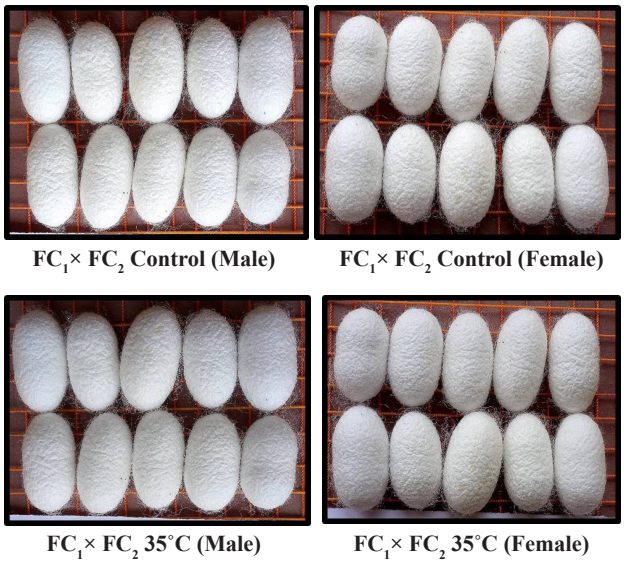
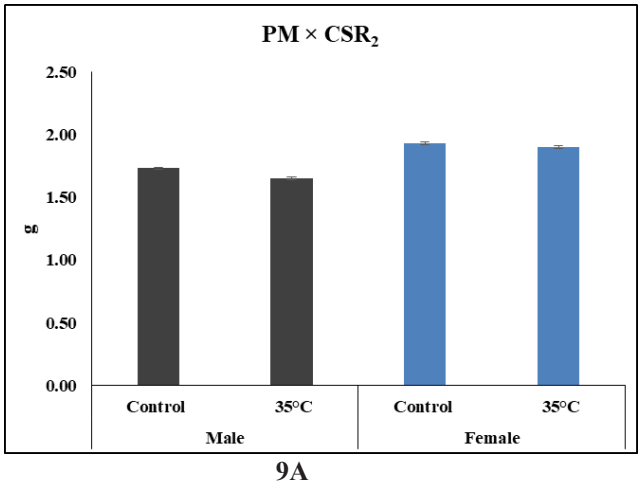
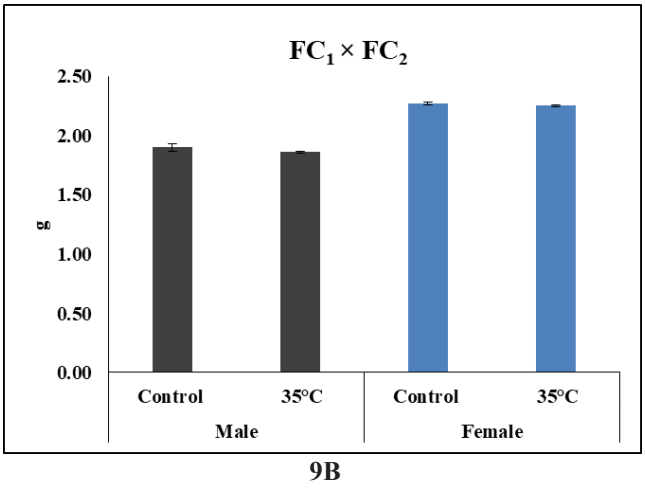


Figure 8: Cocoons of control and temperature imposed of $FC_1 \times FC_2$.

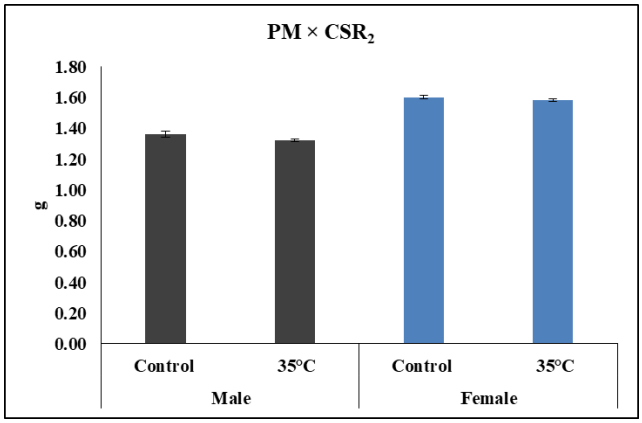


9A

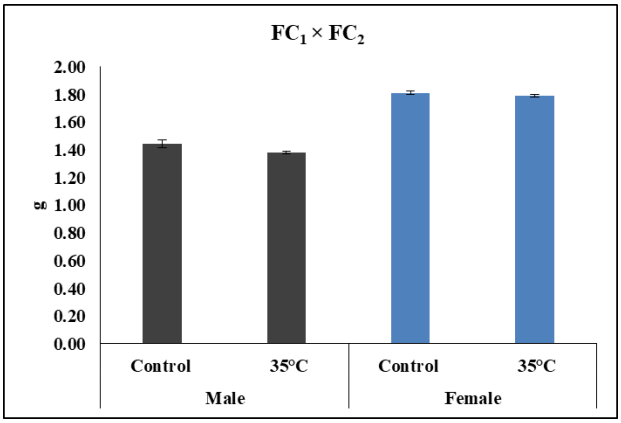


9B

Figure 9A & 9B: Effect of temperature on cocoon weight of PM × CSR_2 and $FC_1 \times FC_2$.

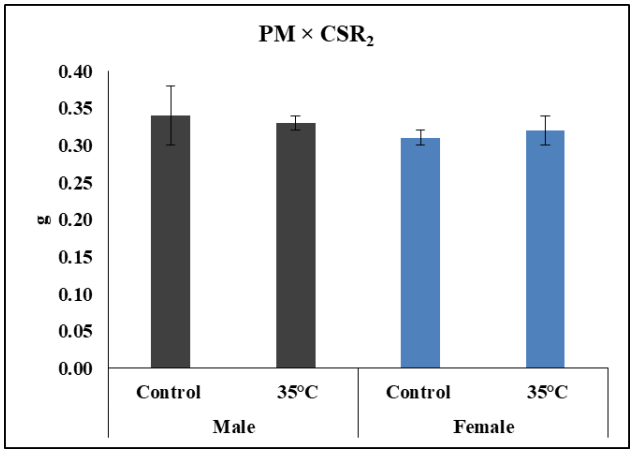


10A

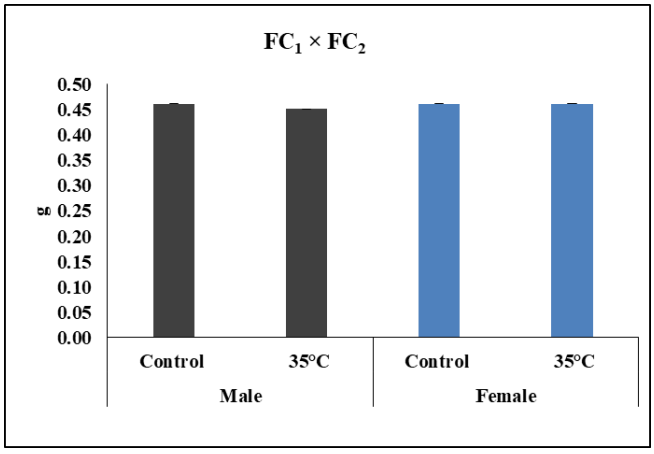


10B

Figure 10A & 10B: Effect of temperature on pupal weight of PM × CSR_2 and FC_1 × FC_2

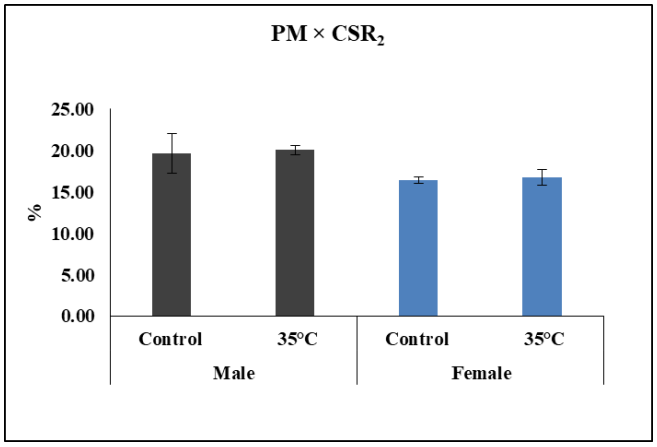


11A

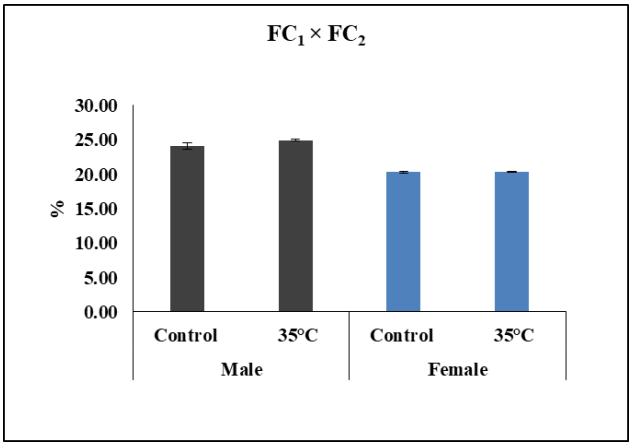


11B

Figure 11A & 11B: Effect of temperature on shell weight of PM × CSR_2 and FC_1 × FC_2

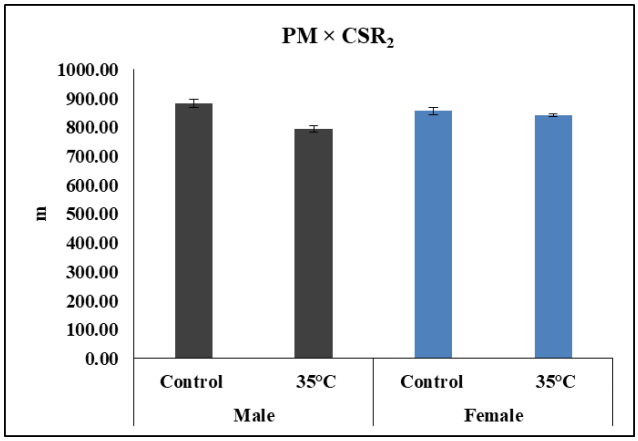


12A

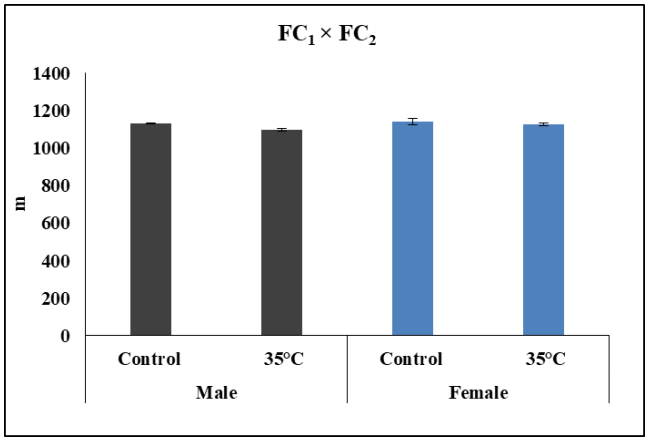


12B

Figure 12A & 12B: Effect of temperature on shell ratio of PM × CSR_2 and FC_1 × FC_2

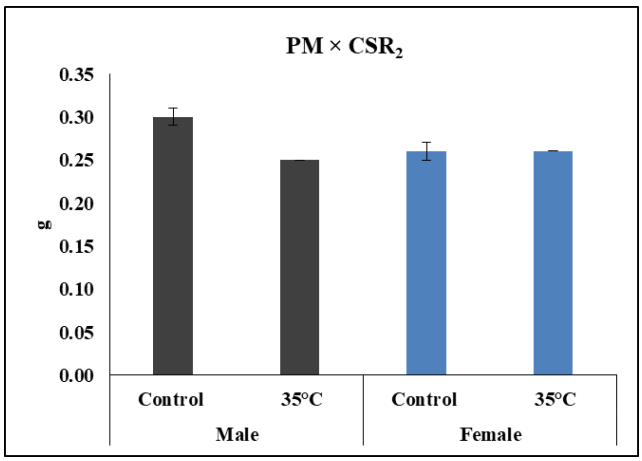


13A

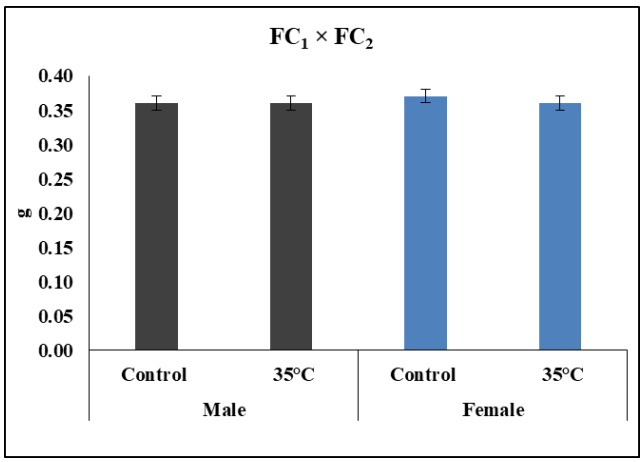


13B

Figure 13A & 13B: Effect of temperature on filament length of PM × CSR_2 and FC_1 × FC_2

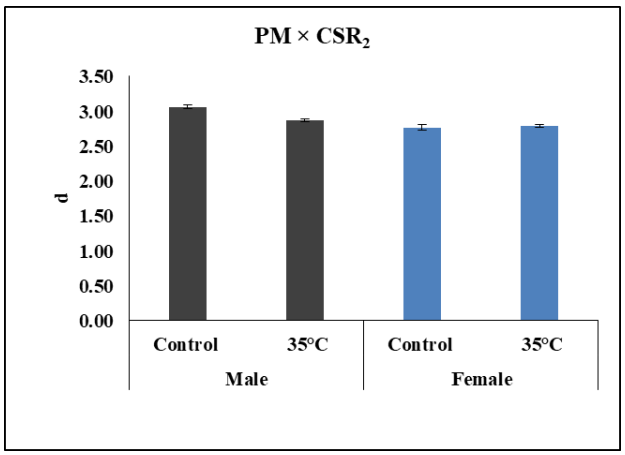


14A

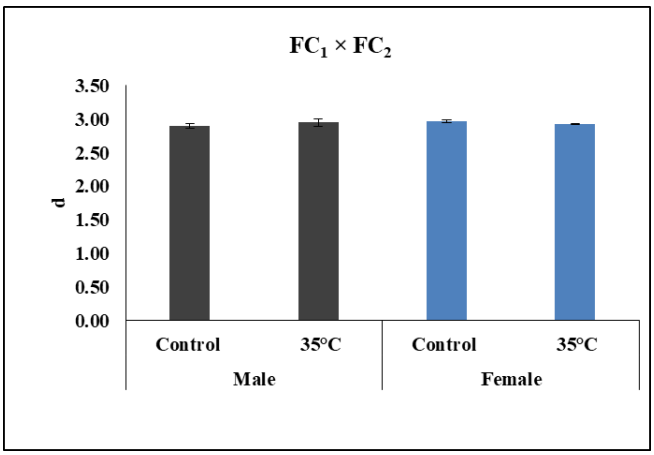


14B

Figure 14A & 14B: Effect of temperature on filament weight of PM × CSR_2 and FC_1 × FC_2

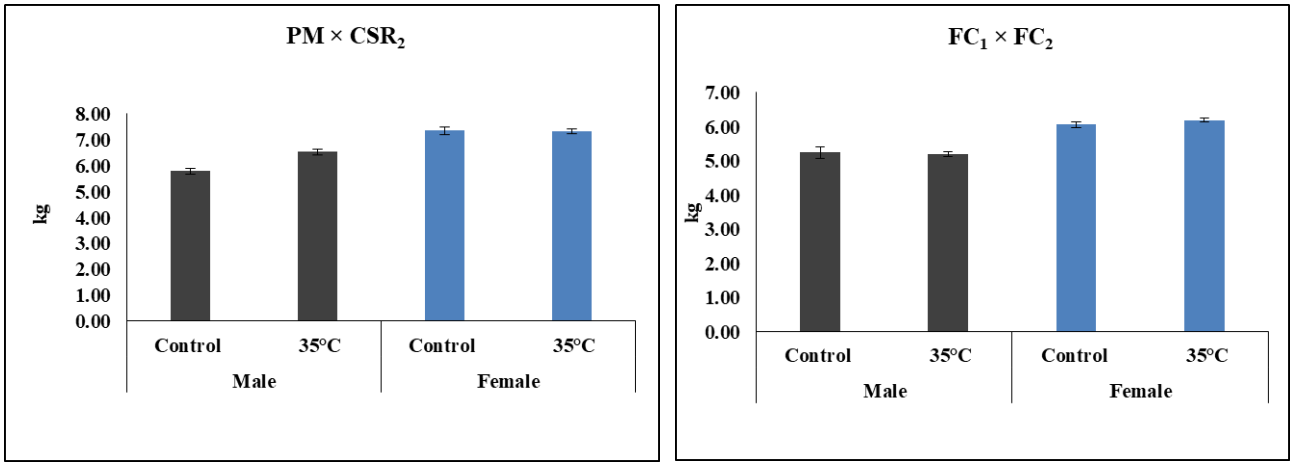


15A



15B

Figure 15A & 15B: Effect of temperature on Denier of PM × CSR_2 and FC_1 × FC_2



16A 16B

Figure 16A & 16B: Effect of temperature on Renditta of PM × CSR_2 and FC_1 × FC_2

depends on the dissociation of the glycerides or metabolism of glycerides. It is a quite large event incurred in the biological organisms especially in liver, muscles, neurons, adipose tissue, fat body tissue etc.

Various classes of lipids such as phospholipids, glycolipids and spingolipids are the combination also responsible for turn over the production of steroids, corticoids and hormones regulating the most important structural function during β oxidation of fatty acids.

In silkworms, the biosynthesis of neuron secretion are the most unique components carrying the informational signal from one to another to regulate the various intra and inter cellular energy converting and energy utilization path way in day to day development of organisms and developmental transformation acquainted with a complete transformation and mobilization into different stages of development in a life cycle.

Fat body is an active tissue stored all the essential energy dependent biomolecules as a reservoir especially for the lipid derivatives. It can transit in each and every day for developmental nourishment hence forth, the testis and ovaries are the two organismic entity observed during the fifth instar which facilitates the reproductive structural changes for the synthesis of gametes and consequent development in all sexually reproducing organisms including mulberry silkworm,*Bombyx mori,* however to understand the thermal stress induced changes in the lipid level confined to fifth instar silkworm of two potential breeds namely PM × CSR_2 and FC_1 × FC_2 used in the present investigation.

The ISSR-PCR technique shown to be a reliable and reproducible in molecular biology for comparative and evolutionary genetical distance studies in the silkworm *Bombyx mori* L. ISSR and SSRs, due to their abundance and dispersal in the genome, have been preferred to study the relationship between closely related populations (Deshpande *et al.,* 2001). The present ISSR profile is highly reproducible, consistent and informative of all the primers used. The reproducibility of ISSR assay lies in the principle of ISSR-PCR. The ISSR assay is based on the use of primers which are not arbitrary, but designed a prior to anchor to anonymous simple sequence repeat (SSR) loci (Zietkiewicz *et al.,* 1994; Wolfe *et al.,* 1998) and are long and repetitive in nature. The primers require a stringent annealing temperature and due to low primer-template mismatch the ISSR-PCR yields highly reproducible patterns. The present assay has given more emphasis and an excellent consistent and reproducible profile.

The ISSR assay has yielded multiple products with differential molecular weight that, ranges from 200-3000 bp. The present study has proven the efficiency of ISSR-PCR in generating high level of polymorphism in bivoltine silkworm breeds. Genetic diversity and differentiation among populations of the Indian Eri silkworm, *Samia Cynthia ricini* revealed by ISSR markers (Vijayan *et al.,* 2006).Genetic differentiation induced by artificial selection through four continuous generations in an inbred population of the silkworm, revealed by RAPD and ISSR marker systems (Appukuttan *et al.,* 2005). The high variability in the number of the band products amplified by all the primers

in the present investigation and suggests the hyper variable nature and type of simple sequence repeats in the genomes of different breeds. The great number of distinct products amplified by 5 selected primers were recorded and high amount of polymorphic bands revealed that the ubiquitous and hypervariable nature of ISSR markers and it suggests that the applicability of ISSR-PCR in genome fingerprinting at the interspecies level (Zietkiewicz *et al.,* 1994).

The result shows that, the performance of ISSR primers varies across the texa which reflects different relative frequencies of microsatellite motifs in the genome of different silkworm breeds and hybrids. In the present investigation, the genetic differentiation induced by artificial selection through seven continuous generations in the silkworm inbreed populations could be a consequence of random drift, which causes changes in gene frequencies reflected in changed generation means (Falconer and Mackay 1996).

The higher degree of polymorphism generated by ISSR primers in the selection of lines denotes the abundance and variation of SSRs among them (Russel *et al.,* 1997). Continuous selection and inbreeding could have induced homozygous state of recessive gene (Strunnikov 1995). That could be accredited to the selection of the allelic variants that influence the phenotypic expression of characters (Chani *et al.,* 2002). Such a distortion of loci (RFLPs and RAPDs) was observed in barley (Graner *et al.,* 1991, Manninen 2000), potato (Rivard *et al.,* 1996) and in microspore derived rice (Xu *et al.,* 1997). Stop codons are AT-rich and it can be presumed that mutation of pressure plays a significant role in the occurrence and distribution of stop codons (Xia *et al.,*2003).

Variation in the number of the bands per primer per sample (different groups of breeds and hybrids at different conditions) among all the samples indicated that, the SSRs are relatively abundant and hyper variable and reflects the type of repeats in the genomes of the present study. Present results suggest that inter-SSR-PCR can be used to identify the presence of repeated elements targeted by the different primers used and to evaluate their distribution within different genomes (Zietkiewicz *et al.,* 1994). Perusal of dendrograms in the present ISSR assay shows that, in the case of selected bivoltine silkworm breeds during seven generations exposure to stressful condition at 40±1°C, the breeds with dumbell shaped cocoon (NB_4D_2 and CSR_4) clustered in the same ladder, obviously. It can be explained due to magnitude of genetical similarity in a developmental and evolutionary duration through an adaptability procedure by selection at 40±1°C temperature. As well as, all temperature regimes CSR_2 and KA breeds were identified at the same cluster.

Study of silkworm hybrids in relation to dendrograms shows the different clustering of the same hybrids prepared from two different groups of the parents control groups and improved groups. Further, the change in the gene frequency of the same parents in relation to be thermo tolerant after seven generations artificial selections under undesirable and stressful temperature condition was confirmed on the basis of results obtained. Based on the clustering of stress based improved hybrids shown lesser variation in clusters it can be suggested that, similarity in the gene frequency becomes more in the breeds and accordingly in their F_1 hybrids after artificial selection of parents through several generation under imposed higher temperature regimes.

Temperature is one of the most important environmental variables that induce physiological change in organisms (Jia *et al.,* 2011). Arthropods have evolved a series of diverse behavioral and physiological strategies to avoid temperature impairments, such as seeking shelter, changing the fluidity of cell membranes, and accumulating sugars, polyols, antifreeze proteins and amino acids (Storey 1997; Wang and Kang, 2005). Previous studies indicated that, antioxidant enzymes and heat shock proteins are associated in response to higher temperature and other stresses in nature in a wide range of organisms (Feder and Hofmann 1999; Martindale and Holbrook 2002; Sørensen *et al.,* 2003). The predatory mite *N. cucumeris* is a poikilothermic organism and its body temperature is highly affected by the ambient temperature. In this study we showed that LPO and antioxidant enzymes (CAT, SOD, GSTs, POX and T-AOC) in *N. cucumeris* changed significantly under various thermal stresses. The mites used in the current study were mass-reared under optimal conditions for reproduction and development and they had never been subjected to thermal stress. Commercially available mites are thought to be inherently less cold hardy and to have a lower capacity to acclimate to low temperatures than wild ones (Morewood, 1993).

Temperature is one of the most important environmental variables that affect invertebrates (Bale *et al.,* 2002). To understand the oxidative stress induced in *B. dorsalis* by environmental temperature changes, an index of oxidative stress (LPO), as well as the activity of antioxidant enzymes (CAT, GSTs, POX, and SOD) and T-AOC were measured. A major oxidation product of per oxidized polyunsaturated fatty acids, MDA, has been used to determine the degree of LPO and as a biological marker of oxidative stress (Rio *et al.*, 2005). MDA concentrations

in *B. dorsalis* were significantly increased compared to the control when exposed either to heat or cold shock. Our results clearly demonstrated that in *B. dorsalis* thermal stress was accompanied by lipid per oxidation and other responses to oxidative stress, similar to other animals (An & Choi 2010; Yang *et al.,* 2010). Thus we found that in response to heat stress, MDA concentration significantly increased both with temperatures ascending above 27°C and temperature descending below 27°C to reach its maximum value at 5°C indicating that this highly unfavorable condition causes much LPO product to be generated under much lower temperatures, e.g. 0°C or -2.5°C, the fly's metabolism may be diverted along other pathways such as protein oxidation or DNA damage (Nordberg and Arnér, 2001) rather than only increased lipid per oxidation in response to extremely adverse conditions.

CAT, GSTs, POX and SOD are important antioxidant defense enzymes and they act in a coordinated manner to counteract oxidative stress generated by high concentrations of ROS inside the cell. Among these antioxidant enzymes in insects, CAT has been considered to be solely responsible for the scavenging H_2O_2, because insects are deficient in selenium-dependent glutathione peroxidase, which is a scavenger present in other organisms (Ahmad & Pardini 1990; Sohal *et al.,* 1990). However, CAT removes H_2O_2 only at high cellular concentrations and is inefficient at removing H_2O_2 at low concentrations (Ahmad *et al.,* 1991). Although CAT activity was too low to detect in citrus red mite exposed to thermal stress (Yang *et al.,* 2010), it was significantly increased in the fat body of the silkworm (*Bombyx mori* L.) 5th instars exposed to thermal stress (Nabizadeh & Kumar, 2011) and in the cotton bollworm, *Helicoverpa armigera* (Hübner), exposed to UV light irradiation (Meng *et al.,* 2009). In this study oriental fruit fly CAT activity increased significantly as temperatures deviated from the control of 27°C. The over-expression of CAT under heat or cold shock resulted in the enzymes enhanced removal of H_2O_2 and hence its prevention of damage by oxidative stress.

GSTs have been reported as a biomarker for assessing the environmental impact of organic xenobiotics that generate oxidative stress (Monteiro *et al.,* 2006). However, when exposed to thermal stress for 3 hr, oriental fruit fly GST activity increased significantly only at the highest temperatures (37.5 and 40°C). With longer exposures GST activity also increased significantly at lower temperatures (-5°C and -2.5°C for 6 hr) compared to the control (27°C). This may suggest that GST is involved in the inactivation of accumulated toxic lipid per oxidation products and acute temperature-induced oxidative stress, such as occurs in the Asian citrus psyllid, Diaphorina citri Kuwayama, Hemiptera, Psyllidae (Marutani-Hert *et al.,* 2010) and gold fish, *Carassiusauratus* L., Cypriniformes: Cyprinidae (Lushchak & Bagnyukova, 2006). It also had an obvious time-dependent effect as in citrus red mite, Panonychuscitri (McGregor), Acari, Tetranychidae (Yang *et al.,* 2010).

Besides GST, insects have POX activities (Mathews *et al.,* 1997). POX breaks down H_2O_2 (Lee *et al.,* 2005). In this study, POX activities decreased significantly under thermal stress compared to the control. POX activity was highest at 27°C, i.e., about 30 Umg-1 protein, but the bar graphs also show that at no temperature or duration did POX decline below 20 U mg-1 protein. Thus despite declining by as much as one-third even under the most adverse conditions, POX maintains a substantial protectant against ROS under all conditions. However, in many animals such as gold fish (Lushchak & Bagnyukova, 2006) and honey bee, *Apis mellifera* L., Hymenoptera: Apidae (Corona & Robinson 2006), POX significantly increased under thermal stress. But POX of citrus red mite exposed to thermal stress did not change significantly (Yang *et al.,* 2010).The functions of POX in response to thermal stress deserves further investigation. SOD is an important constituent of cellular defense against oxidative stress and is among the most potent antioxidants known in nature (Bafana *et al.,* 2011). It plays an important role in reducing the high level of superoxide radical induced by low or high temperatures (Celino *et al.,* 2011). In this study, SOD activities under thermal stress significantly increased compared with control. This suggests that SOD was induced by temperature changes and thus protected the flies from thermal stress. Such a case was reported in 1969 (McCord & Fridovich 1969a, b). Both SOD and CAT directly scavenge ROS. SOD removes O_2 - through the process of dismutation to O_2, H_2O_2 and then H_2O_2 is sequentially reduced to H_2O and O_2 by CAT (Kashiwagi *et al.,* 1997). However, because in our results, CAT was present at higher levels than SOD, it would indicate that, under thermal stress, H_2O_2 was being produced directly by other processes.

As a tool to assess redox status and as a representative measure of the total antioxidant capacity existing in the organism, the T-AOC assay has been widely used (Ghiselli *et al.,* 2000; Meng *et al.,* 2009; Sashidhara *et al.,* 2011). T-AOC of *B. dorsalis* exposed to thermal stress did not change significantly compared to the control except when stressed at 40°C for 3 hr. This may suggest that TAOC level was not significantly modified. Besides antioxidant enzymes, some non-enzymatic substances, such as trehalose (Mahmud *et al.,* 2010) and α-tocopherol (Kaur *et al.,* 2009),

can play a role in antioxidant stress. Heat shock protein can cooperate with antioxidant enzymes to deal with ROS damage (Zhou *et al.,* 2010). The result in this study may suggest that oriental fruit flies have evolved other defense mechanisms against thermal stress allowing the organisms to survive. In conclusion, we have confirmed that thermal stress disturbs the redox balance in *B. dorsalis,* which leads to oxidative stress. This indicates that thermal stress damages oriental fruit flies, and is a major potential factor generating oxidative stress products. To counter this stress, antioxidant enzymes provide antioxidant defense and protection. POX activity and T-AOC play minor roles in scavenging deleterious LPO. Nevertheless, significantly enhanced CAT, GST and SOD activities in response to thermal stress are likely a defense mechanism against oxidative damage due to the accumulation of ROS. These processes may be mirrored in the fly's physiological adaptations. However, prolonged exposure to heat or cold shock resulted in decreased activities of CAT, GST and SOD, accompanied by impaired antioxidant capacity and high levels of oxidative stress.

The silkworms like other living organisms, face different environmental conditions during its lifecycle in which the intensity of dominant factors and their combination vary to different degrees causing reversible or irreversible changes in the metabolism. These changes cause survival or death of an organism. The observation of pupal mortality in the present study was due to the high temperature stress was imminent. The differential mortality among the ecoraces may be due to the genetic capacity to sustain high temperature stress. Laria considered as wild ecorace of tropical tasar silkworm has sustained less damage compared to the semi-domesticated Daba and this may be due to higher level of tolerance to different biotic and abiotic stress in wild ecoraces. Also, there was sexual dimorphism in the expression of tolerance to the stress. Higher mortality in male population is attributed to higher the metabolic stress corresponding to lower fat composition as stored energy in male pupae compared to females. The protein levels in the haemolymph increased significantly in response to higher temperature due to high metabolic rate and synthesis of new proteins by the tissues and released into haemolymph.

In contrast, the protein level in the fat body was decreased in the treated sets this is because of increased stress and organism struggle for the survival where, supplementation of heat shock proteins and carbohydrates synthesized by fat metabolism which transported to the haemolymph (Joy and Gopinathan, 1995). This also confirms that proteins are not a source of energy in the stress condition but are involved in the modulation of the silkworm physiological activity to protect from temperature stress. Similarly, the total carbohydrate levels in the haemolymph was increased in the high temperature exposed sets and converse to the decreased levels in the fat body this is in concomitant with the earlier works (Joy and Gopinathan, 1995; Zhao, 1997). Carbohydrates are essential components for the energy demand during stress condition. The biomolecules such as glycerol, sorbitol and other polyols acts as thermo protectants (Chen and Haddad, 2004; Shamitha and Rao, 2008) are synthesize in the tissues and release into the haemolymph. Other commonly reported compounds are trehalose, glucose, fructose and mannitol, these compounds are believed to protect organisms during adverse conditions like temperature stresses (Forcella *et al.,* 2007). Decrease in the carbohydrate level in the fat body may indicate the possibility of utilization of fat body during non-feeding stage of the silkworm as a source to meet the emergent energy needs as well as their utilization in the production of some new proteins/biomolecules to cope up with the temperature stress.

It has been reported that, the increase in the total haemocyte count in response to high temperature and also cause a physiological damages in the haemocytes (Pandey *et al*., 2010). Similar results were recorded in the present study, about four time increase in the haemocyte count observed in treated sets of both ecoraces studied. However, higher counts were with wild ecorace Laria compared to semi-domesticated Daba. Fecundity is considered as one of the most desired quantitative traits of commercial importance in silkworm. The genotype-environment interaction has highly significant influence on the fecundity of the silkworms, extreme temperatures adversely affects the total egg productivity of mother moth (Singh and Kumar, 2008; Kumar *et al.,* 2008; Srivastava *et al.,* 2001). The significantly reduced fecundity due to higher temperature stress in the present study would be due the diversification of protein metabolism and energy to synthesize new proteins in the haemolymph to support the physiological mechanism to tolerate high temperature stress condition. The differential performance of the Daba and Laria with reference to fecundity attributed to the genetic endowment of the two ecoraces. A synonymous effect was also observed in case of fertility rate of eggs with differential impact in Laria and *Daba ecoraces*.

Terrestrial insects are often by exposed to constant high ambient temperatures in their natural environment. Even domesticated economic insects like the mulberry

silkworm, *Bombyx mori* L. are exposed to extremes of ambient temperatures as they are distributed across different latitudes. Exposures to the prevailing higher or lower ambient temperatures lead to the development of voltinism in *Bombyx mori* L. Bivoltine races with embryonic diapause evolved in temperature climates as multivoltine races are reared in large quantity in tropical Indian subcontinent in all seasons of the year. As the two voltine races are adapted to contrasting temperature regimes, significant differences in their adaptation to high temperature in summer season are noticed. The traditional multivoltine race, Pure Mysore (PM) is well acclimated to high temperature as it has been bred and reared continuously for over 200 years in tropical Indian climates. NB_4D_2 evolved as a line from a double hybrid cross of Japanese parents [(koko) × (seihaku) × (N_{124}) × (J_{124})] is considered as a temperature tolerant bivoltine race since its introduction in the year 1972 CSR_2.

An inbreed line derived from a cross between Japanese parents (Shurei × Shogestu) spins oval cocoons which is Chinese character in bivoltine silkworm races. CSR_2 is less temperature tolerant bivoltine than NB_4D_2 and introduced to tropical climates more recently in 1998. The period of exposure to hot climates thus play a more significant role than the thermal history of parents in high temperature adaptation of race. A significant difference in the larval and pupal mortality among the three races was observed during rearing in summer seasons of the year. There are practically no studies on the role of the factors responsible for the differences in the temperature tolerance observed in the three races.

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