3 MATERIALS AND METHOD

All experiments were done in the Muga Research Laboratory, Department of Zoology, Achrya B.N. Seal College, Cooch Behar, West Bengal. Cooch Behar district (26˚57 ′40′′N and 26˚ 32′ 20′′ N latitude and 88˚ 47′ 44′′ E and 89˚ 54′ 35′′ E longitude) was in Terai region (25˚ 57′N and 27˚N latitude and 88˚25′ E and 89˚54′ E longitude)of West Bengal, neighbouring to Kokrajhar and Dhubri district of Assam. Cooch Behar district was situated 43 m above mean sea level. It was also included into sub tropical climatic zone with minimum temperature of 11.19-30.24˚C and maximum of 20.54- 34.24˚C. Relative humidity at 8.30 AM during March- July was recorded 58-89% and at 5.30 PM, was 48-81%. Warm as well as humid climatic condition of the region is suitable for muga culture.

3.1 Embryonic development of Muga Silk worm

Healthy cocoon was collected from Extension Centre, Regional Muga Research Station, Central Silk Board, Coochbehar. After moth emergence, coupling was done and eggs were collected for incubation in 25±1˚C and 75% humidity. Eggs were collected from incubator after 24 hour, 48 hour, 72 hour, 96 hour, 120 hour and 144 hour for study.

3.1.1 Hot water method for Embryo preparation

Hot water method was modified in the following way for microscopic preparation. For removal of hard covering over eggs were treated in 2% Potassium hydroxide solution. Then eggs were boiled in hot water (80^{o}C) for 3 minutes in water bath to solidify embryo. Different hour eggs were maintained in same temperature, then fixed eggs were preserved in chromoformalin and heat at 80^{o}C and eggs were kept in dark till the temperature become normal. After overnight preservation eggs were passed through graded alcohol. Then only embryo was dissected out with pointed needle and forceps and stained in Borax carmine. Embryo of different stages was on served in Stereo Binocular microscope and photograph was taken for detailed study.

3.1.2 Preparation for Scanning Electron microscopy (SEM)

For removal of hard covering over eggs were treated in 2% Potassium hydroxide solution. Then eggs were boiled in hot water (80^{o}C) for 3 minutes in water bath to solidify embryo. Different hour eggs were maintained in same temperature, then fixed

in 2.5% Gluteraldehyde-0.1 m Phosphate Buffer (pH7.5) for 2 hours and washed in 0.1 m Phosphate Buffer (pH7.5). The specimens were dehydrated by graded series of alcohol and transferred to the absolute alcohol. Then the specimens were dried in vacuum evaporator and were plated with Gold and observed in SEM (Jeol) (Keino and Takesue, 1982).

3.1.3 Preparation for histology

Eggs of different ages after hot water method dehydrated by serial transfer through graded ethanol solutions and embedded in Paraffin. Sagittal or frontal sections of 5µm were cut with a rotary microtome (Leica RM 2125). Sections were stained with haematoxylin and eosin and mounted with DPX mountant.

The serial sections were observed with Olympus light microscope equipped with digital camera to observe the events of embryonic development (Martini et al. 2011).

3.2 Effect of temperature stress on different embryonic stages

Eggs were incubated in BOD incubator for 10 days as 10 days refrigeration did not affect hatachibility (Pandey et al. 1992) at 4±1˚C, 6±1˚C, 8±1˚C and 10±1˚C for hatching percentage and incubation period (including respective embryonic age) for the study of low temperature preservation. Also a batch of eggs was allowed to hatch in normal conditions as control to measure the effect on hatchability and incubation period.

3.3 Effect of different low temperature stress in identified low temperature resistant embryonic stages

Eggs were incubated in BOD incubator for the study of the effect of low temperature preservation at 4±1˚C, 5±1˚C, 6±1˚C, 7±1˚C, 8±1˚C, 9±1˚C and 10±1˚C incubating 24, 36, 48, 60, 72, 84, 96 hours egg 10 days, at 4±1˚C, 5±1˚C, 6±1˚C, 7±1˚C, 8±1˚C, 9±1˚C and 10±1˚C for hatching percentage and incubation period (including respective embryonic age) . Also a batch of eggs was allowed to hatch in normal conditions as control to measure the effect on hatchability and incubation period.

3.4 Effect of preservation periods after low temperature stress to the identified embryonic stage

Eggs were collected from first day laying for the study of the effect of low temperature preservation at 4±1˚C, 5±1˚C and 6±1˚C in BOD incubator, incubating 24, 48, 72 and 96 hours egg for 3,7,10,15 and 21 days, at 4±1˚C, 5±1˚C and 6±1˚C for hatching percentage and days delay of hatching. Days delay means the period from egg laying to hatching excluding the preservation days.Also a batch of eggs was allowed to hatch in normal conditions as control to measure the effect on hatchability and incubation period.

3.5 Biochemistry of embryonic development of muga silk worm *A. assama*

3.5.1 Biochemistry of embryonic development of muga silk worm *A. assama* in normal condition

Fertilized eggs laid by ten healthy female moths of *A. assama* wit in 24 hours of oviposition were pooled together and incubated at 25±1˚C. From that lot, sample of 25 eggs each were drawn randomly from the pool after 24 hour interval till hatching for the estimation of carbohydrate, protein, cholesterol, DNA, trehalose, NAD Sorbitol dehydrogenase (NAD Sdh), NADH Peroxidase and Xanthine oxidase (XO).

Eggs of definite age were kept at 0˚C for 30 minutes to chill them. 10% (w/v) homogenate of eggs were prepared in ice cold double distilled de ionized water with a homogenizer kept immersed in crushed ice. The homogenate was filtered through a double layered muslin cloth and centrifuged at 6200 rpm at 5˚C for 15 min. The supernatant was used for biochemical assay.

3.5.1.1 Estimation of total Carbohydrate content

The supernatant homogenate cooled at room temperature (10-15˚C) for about 30 min, and then supernatant was used for estimation of carbohydrate by the method described by Travelyan and Harrison, 1952.

Reagents required:

Anthrone reagent (0.2%w/v): 0.2g anthrone was dissolved in 100ml of sulphuric acid (85% w/v) with stirring and was prepared before use.

Sulphuric acid (85%w/v): to 15ml glass distilled water 85ml concentrated H_2SO_4 (pre cooled, AR, sp.gr.1.84) was slowly added, mixed well and cooled in ice.

Standard Glucose solution: 100 mg glucose dissolved in 100ml water.

Working standard (50µg/0.1ml): 5ml of stock solution diluted to 10ml of distilled water.

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007

3.5.1.2 Estimation of total Protein

Protein was estimated by Folin- Ciocalteau's method as modified by Lowry et.al.1951.

Reagents: a) Sodium hydroxide(0.1N) 0.4g sodium hydroxide dissolved in 100ml water.

b) 2% Sodium carbonate (Solution A) 2g Sodium carbonate dissolved in 100ml sodium hydroxide (0.1N)

c) 0.5% Coopper Sulphate in 1%potassium sodium terterate (Solution B): 1g potassium sodium terterate dissolved in 100 ml distilled water and 0.5g Copper sulphate added to the solution.

d) Solution C : to 100ml solution A, 2ml of solution B added.

e) Folin-phenol reagent (1:1) : Folin –phenol reagent (2N) diluted with distilled water.

f)Bovine Serum Albumin(BSA) stock solution: 5mg BSA dissolved in 10ml 0.1 N NaOH

g) Working Standard (50µg BSA/1ml): 1ml stock solution diluted to 10 ml with 0.1N NaOH.

Experimental Procedure: The tissue homogenate centrifuged at 6200 rpm and added volume of 10% TCA precipitate the protein, then kept at low temperature (10-15˚C) for about 30 min. This was centrifuged at 6200 rpm and residue dissolved in appropriate volume of 1N NaOH to dissolve the precipated protein and used for estimation of protein.

Suitable aliquots were pipette out in a series of tubes and volume made up to 1ml with sodium hydroxide (0.1N). To each tube solution C (5ml) was added, mixed well and allowed to stand at room temperature for 10 min. Folin- phenol reagent (0.5ml) was added and the contents of the tubes mixed well and allowed to stand for 30 min. at room temperature. The blue colour developed was measured at 650nm. A reagent blank and standard solution was also run simultaneously.

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007

3.5.1.3 Estimation of Cholesterol

Cholesterol was estimated by the method described by Boyer (2006).

Reagents:

Cholesterol aqueous standard I: (200mg/100ml) in water containing stabilizers and Sodium azide as preservative.

Cholesterol aqueous standard II: this contain cholesterol (50mg/100ml) in water solution containing stabilizers and Sodium azide as preservative.

LDL precipitating Reagent: the solution contain Phosphotungstate, magnesium ion and sodium azide.

Cholesterol assay solution: the stock reagent contains pancreatic cholesterol esterase, microbial cholesteroloxidase, horseperoxidase, 4-aminoantipyrine and phenol.

Experimental procedure:

3 ml cuvettes are required for pipette in following way.

Reagents	Test	Blank	Standard
Water	-	0.02ml	-
Cholesterol standard I	-	-	0.2ml
Sample	0.02ml	-	-
Cholesterol Enzyme Reagent	1.0ml	1.0ml	1.0ml
Saline Water	2.0ml	2.0ml	1.0ml

Incubate at 37˚C water bath for 10-15 min. observes at A_{510nm} within 30 min after removal from water bath.

Measurement of Cholesterol:

Following equation was utilized for calculation of cholesterol concentration.

Total Cholesterol concentration (mg/100ml) = $[A_{510(S)}-A_{510(C)} / A_C] \times C_S$

A_C: Absorbance of diluted serum at 510nm

$C_{S:}$ Concentration of Standard Cholesterol (C_S) in 200mg/100ml

3.5.1.4 Isolation and estimate of DNA:

Reagents: a) SSC (0.14M solution of NaCl containing 0.02M Sodium citrate, pH7.4)

b) 2M NaCl solution

c) Ethanol (Ice cold)

Experimental Procedure: Suspend the egg in SSC and homogenize in cold homogenizer. Centrifuge at 3000 rpm in cold centrifuge for 10 minutes. Precipitate was homogenized and supernatant was discarded and then again centrifuged at 3000rpm for 10min. then sediment was dissolved in 2M NaCl. Centrifuge at 10000 rpm for 10 min. then the supernatant was slowly added to the ice cold ethanol of twice of the volume .Fibrous white DNA precipitates, collected and again dissolved in SSC in cold.

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007

Estimation of DNA

DNA was isolated and estimated by the methods described by Plummer (1998).

Reagents: a) DNA Standard (Calf thymusDNA;100µg/ml)

b) 1% Diphenyl amine in 100ml glacial acetic acid

c) Concentrated H_2SO_4: 2.5ml

Experimental procedure: Add 5ml of reagent mix well and heat in a boiling water bath for 10 minutes. After cooling measure absorbance at A_{595nm}.

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007

3.5.1.5 Estimation of Trehalose

Tissue homogenates were deproteinized with 70% ethanol (1:2). Repeat at low temperature (10-15˚C) for 15-20 min. Then centrifuged at 6200rpm and clear supernatant was employed for determination of terhalose. Trehalose was estimated by the method of Wyatt and Kalf (1957).

Reagents: a) Sulphuric acid(0.1N): To ice cold distilled water 0.277 ml conc.H_2SO_4 (AR, Sp. Gr. 1.84) added slowly and volume made up to 100 ml with water.

b) Sodium hydroxide (6N): 24g sodium hydroxide was dissolved in distilled water , mix well and cooled in ice bath.

c) Sulphuric acid (85%w/v): to 15ml glass distilled water 85ml concentrated H_2SO_4 (pre cooled, AR, sp.gr.1.84) was slowly added, mixed well and cooled in ice.

d) Anthrone reagent (0.2% w/v): 0.2g anthrone was dissolved in 100ml of sulphuric acid (85% w/v) with stirring and was prepared before use.

e) Standard Trehalose solution (100µg/ml): 10mg trehalose was dissolved in 100ml distilled water and store at 10˚C.

Experimental procedure: Suitable aliquots (0.5-1.0ml) of supernatant were pipette into a series of glass tubes and evaporated to dryness in a boiling water bath. The residues was redissolved in 0.1N Sulphuric acid (2ml), the tubes were capped with aluminium foil and heated for 10 min. in boiling water bath the solution was then made alkaline by addition of 6N NaOH and heated at 100˚C for 10 min to destroy all other sugars. The samples are then chilled at 0˚C and 5ml of Anthrone reagent (0.2 w/v) was added slowly and mixed well. The tubes were allowed to stand for for 30 min at room temperature and intensity of colour developed was measured at 590nm. A reagent blank and standard solution of trehalose ware also runs simultaneously.

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007 and regression analysis was done in IBM SPSS Statistics 20 software

3.5.1.6 Estimation of NAD Sorbitol dehydrogenase

NAD Sorbitol dehydrogenase was estimated by the method described by Gerlach and Hiby, 1974.

Reagents: a) 0.1M Tris HCl Buffer (pH 8.8)

b) 0.2M Sorbitol

c) 0.15mM NAD^+

d) 20mM $MgSO_4$

Experimental Procedure: Prepare all reagents and Pipette (in ml) the following reagents into suitable cuvettes.

Reagents	Test	Blank
0.1M Tris HCl Buffer (pH 8.8)	2.35	2.35
0.2M Sorbitol	0.50	0.50
0.15mM NAD^+	0.05	0.05
20mM $MgSO_4$		
Mix by inversion and equilibrate to 25˚C. Monitor the A_{340nm} until constant, using a suitable thermo stated spectrophotometer. Then add		
Enzyme solution	0.10	-
BSA	-	0.10

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 min. Obtain the A_{340nm}/minute using maximum liner rate for both test and blank.

Units/ ml enzyme= (Δ A_{340nm}/min Test - A_{340nm}/min Blank)(3)(df) / (6.22) (0.1)

Total volume(in ml) of assay= 3ml

Dilution factor=df

Milli-molar excitation co-efficient of NAD^+ at 340nm=6.22

Volume (in ml) of enzyme =0.1

One unit will convert 1.0µ mole sorbitol into D-fructose per minute at pH7.5 at 25˚C.

Statistical analysis:

Descriptive statistical analysis was done in MS Excel software and regression analysis was done in IBM SPSS Statistics 20 software.

3.5.1.7 Estimation of NADH Peroxidase (Dolin, M I, 1957)

NADH Peroxidase was estimated by the method described by Dolin, 1957.

Reagents required:

a)100mM Sodium Acetate Buffer with 0.3mM Ethylenediaminetetraacetic acid, pH 5.4 at 25˚C

Adjust pH with 1M HCL or 1M NaOH

b)0.11% (V/V) Hydrogen Peroxide Solution:

Prepare 100 ml in deionized water using Hydrogen Peroxide , 30% (V/V)

c)1000 mM Tris Solution

Prepare 10 ml in deionized water using Trizma Base.

d) 23.4 mM β Nicotinamide Adenine Dinucleotide , Reduced form.

Add one drop per ml of solution c to neutralize the solution.

e) NADH Peroxidase Enzyme solution

Experimental procedure:

Pipette (in ml) the following reagents in the cuvettes:

	Test	Blank
Reagent a(Buffer)	2.57	2.57
Reagent b ($H_2O_{2)}$	0.36	0.36
Deionized water	-----	0.10
Mix by inversion and equilibrate to 25˚C. Then add		
Reagent d (β NADH)	0.02	0.02
Reagent e (enzyme solution)	0.10	-----

Immediately mix by inversion and monitor the decrease in A_{340} for approximately 5minutes.

Obtain ΔA_{340}/min using the maximum linear rate for both the test and blank.

Calculations:

Units/mg enzyme = [ΔA_{340}/ min Test -ΔA_{340}/ min Blank] / (6.22) (mg enzyme/ml RM)

6.22 = milimolar extinction coefficient of β NADH at 340nm

RM = Reaction mix

Statistical analysis: Descriptive statistical analysis was done in MS Excel 2007 and regression analysis was done in IBM SPSS Statistics 20 software.

3.5.1.8 Estimation of Xanthine oxidase

Xanthine oxidase was estimated by the method described by Bergmeyer, Gawehn and Grassl (1974).

Reagents:

a) 50mM Potassium Phosphate Buffer, pH 7.5 at 25˚C

b) 0.15mM Xanthine Solution

c) Xanthine oxidase enzyme solution

Experimental procedure:

Pipette (in ml) the following reagents into suitable quartz cuvettes:

Reagent	Test	Blank
Reagent a (Buffer)	1.90	1.90
Reagent b (Xanthine)	1.00	1.00
Deionized water	-----	0.10
Mix by inversion and equilibrate to 25˚C. Monitor the A_{290} until constant. Then add		
Reagent c (Enzyme solution)	0.10	------

Immediately mix by inversion and record the increase inA_{290} for approximately 5 minutes. Obtain the ΔA_{290}/minute using the linear rate for both the test and blank.

Calculations:

Units/ml Enzyme: [(ΔA_{290}/min Test-ΔA_{290}/min Blank)x(3)x(df)]/ (12.2)x (0.1)

3= total volume (in ml) of assay

df= dilution factor

12.2= Milimolar extinction coefficient of Uric acid at 290nm

0.1= volume 9in ml) of enzyme used

Statistical analysis:

Descriptive statistical analysis was conducted utilizing MS Excel 2007software and Regression analysis was conducted utilizing IBM SPSS Statistics 20 software.

3.3.2 Biochemistry of metabolic shift during cold temperature stress of muga silk worm

Fertilized eggs laid by ten healthy female moths of *A. assama* within 24 hours of oviposition were pooled together and incubated at 25±0.5˚C. From that lot, sample of 100 eggs each were drawn randomly from the pool after 24 hour interval till 96 hours for refrigeration at 5±1˚C for 3days, 7 days and 10 days. Soon after refrigeration period 25 eggs are collected for the estimation of carbohydrate, protein, cholesterol, DNA, trehalose, NAD Sorbitol dehydrogenase (NAD Sdh), NADH Peroxidase and Xanthine oxidase (XO). Control was maintained at 25±1˚C.

Eggs of definite age were kept at 0˚C for 30 minutes to chill them. 10% (w/v) homogenate of eggs were prepared in ice cold double distilled de ionized water with a homogenizer kept immersed in crushed ice. The homogenate was filtered through a double layered muslin cloth and centrifuged at 6200rpm at 5˚C for 15 min. The supernatant was used for biochemical assay.

Methods for estimation of carbohydrate, protein, cholesterol, DNA, trehalose, NAD Sorbitol dehydrogenase (NAD Sdh), NADH Peroxidase and Xanthine oxidase (XO) were described earlier section.