# Identification of low temperature resistant embryonic stages for improving seed production in Muga silkworm, *Antheraea assama*, Westwood for cold preservation of eggs

THESIS SUBMITTED

TO

THE UNIVERSITY OF NORTH BENGAL

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN ZOOLOGY

By

SAMIRAN GHOSH

Supervisor
Dr. Nilay Ray

DEPARTMENT OF ZOOLOGY

UNIVERSITY OF NORTH BENGAL

RAJA RAMMOHANPUR, DARJEELING

April 2018

**DEDICATED TO**

**Those patiently expecting my Thesis**

URKUND

## Urkund Analysis Result

| | |
|---|---|
| Analysed Document: | Samiran_Zoology(1).pdf (D37684300) |
| Submitted: | 4/18/2018 7:48:00 AM |
| Submitted By: | nbuplg@gmail.com |
| Significance: | 1 % |

Sources included in the report:

http://docplayer.net/48042321-45-minutes-0-75-hours-1-hour-30-minutes-1-5-hours-4-hours-20-minutes-hours.html

Instances where selected sources appear:

4

Samiran Ghosh

Associate Professor
P.G. Dept. of Zoology
Hooghly Mohsin College (Gov
Chinsura, Hooghly, W.B

Dr. Nilay Ray, WBES.
Associate Professor of Zoology
Post Graduate Department of Zoology
*Hooghly Mohsin College*
Chinsuarh, Hooghly

Chinsurah – 7125101
Hooghly, West Bengal, India.
Phone: +91 033 26802252 (Office)
+919830634564
FAX: +91 033 26810544
Email: hooghlymohsincollege@gmail.com (Office)
watchnilay@gmail.com
URL: www.hooghlymohsincollege.org

*Government of West Bengal*

Date:

# CERTIFICATE

This is to certify that the thesis titled '**Identification of low temperature resistant embryonic stages for improving seed production in Muga silkworm, Antheraea assama, Westwood for cold preservation of eggs**' embodies the records of original investigation carried out by Mr. Samiran Ghosh, M.Sc under my supervision. Mr. Ghosh worked on this topic for about ten years.

Mr. Ghosh has fulfilled the requirements of the University of North Bengal for submission of his thesis. I am pleased to forward this thesis for submission to the University of North Bengal for consideration for the award of the degree of **Doctor of Philosophy** (Ph.D.) in Science (Zoology).

Nilay Ray 25/4/18

(NILAY RAY)

# ACKNOWLEDGEMENT

Within a fortnight after joining in Education Service, I approached him for guidance to prepare a UGC Minor Research Project. That Christmas vacation, both of us spent to shape that project. After a year back sanction was reached. But over the year we tried to isolate embryo in various ways. Finally 'Hot water method' was standardized and we were fascinated to look at those embryo preparations in light microscope. Then cold shock experiments were designed and again our results were conclusive. Then he had shared the idea of metabolic shift during refrigeration. Then also many mysteries of muga embryogenesis were gathered in Laboratory note books. He is my guide Dr. Nilay Ray. His intellect and vision are inevitable spirit behind decade long progression. I am lucky indeed to enjoy independence which I have enjoyed under his guidance. Thank you 'Sir' for everything through which I have progressed.

We spent many festive evening for the care during rearing. I was fascinated when they collect plants, care worms with such affection that reflects their dedication. They are no one but my laboratory colleagues, Dr Indrajit Biswas, Dr. Uddalok Bhattacharya and Dr. Supriya Biswas. Thank you 'Masters' for yours supports.

Dr. Abhijit Dutta, erstwhile Presidency College and Prof. R. Bhar, Jadavpur University extended their support to explore microscopic studies. Dr. Narayan Biswas and Dr S.N. Bagchi, Central Silk Board, provided their support as and when it was required.

I like to express my gratitude to all faculty members of Department of Zoology, University of North Bengal to provide this opportunity. I do acknowledge University Grants Commission for their financial support.

At last my'Family', whatever nonsense I am doing always, they extend their sensible support for that. Thank you' Family'.

Samiran Ghosh.

# CONTENTS

**CHAPTER IV**



**CHAPTER V**



**CHAPTER VI**



**CHAPTER VII**

# 1. INTRODUCTION

## 1.1 Brief history of Sericulture

Cultural, political and economic history of mankind, for last 5000 years, is intermingled with the voyage of silk. Archeological evidences dating dyed silk gauze from 3600 BC and complex woven design from 2400 BC, establish the period of the origin of sericulture. According to Chinese legends silk was created by Empress Xi Lung, wife of Yellow Emperor (2678-2598 BC). Methods for domestication of silkworm became highly developed during Shang dynasty (1750-1100 BC). *Bombyx mori* had originated from *B. mandarina* through the domestication of different wild races within a short time. During Han period (200BC to AD200), large state of silk weaving workshop had appeared. The technology of cocoon cooking did not however penetrate the west until $6^{th}$ century AD. In the $3^{rd}$ and $4^{th}$ centuries BC silk had reached India. Chinese silk goods reached the Mediterranean at least the $2^{nd}$ century BC, but only became common in the $4^{th}$ century. Along with Chinese silk Romans received silk from Asia Minor, Syria and Wild silks from India (Hutcher & Battery, 2011).

Although finished silk goods were traded widely from China, sericulture spread much later; by Chinese peasants to Korea from where it reached Japan and also was smuggled to Persia, not started producing silk by AD500. The silkworm had been introduced into Byzantium in about AD553 and the emperor Justinian embarked on enforcing a silk monopoly. From this origin sericulture spread via Arabian Gulf to Egypt between 800-900AD and along the coast of North Africa by the Moors to Spain and Portugal and from Greece during mid $12^{th}$ century to Sicily and Italy and later in the $15^{th}$ century to France (Hutcher & Battery,2011).

So, first silk seen in Europe, was from wild silkworms as mentioned by Aristotle and Pliny - from an industry, which had started in at least $4^{th}$ century BC, entered on the Mediterranean islands of Cos. A cast of cocoon was reported from excavation of settlements of Santorini, a Mediterranean island destroyed about 14 th century BC and identified as *Pachypasa otus*. This species probably gives rise to Cos silk and continued to be used to produce silk into $19^{th}$ century (Hutcher & Battery, 2011).

A variety of moths were used for wild silk production in India before *B. mori* was introduced. *Antheraea paphia* (Saturnidae) produces Tusseh silk, which accounts for

7% of world wide wild silk production. *Antheraea pernyi* and *A. yamamai*, respectively Chinese and Japanese Oak silk moth had also been introduced into North America and Europe, have become naturalized. Eri silk moth (*Samia cynthia*) is native to China and was introduced to many parts of Europe and USA for silk production (Tazima,1984).

*A.assama* has been used in India to avoid religious taboos associated with the destruction of life. The silk worm spins a cocoon with a build in exit hole, so that the adult can emerge without damage the silk thread (Hutcher & Battery,2011).

**1.2 Present state of Indian Sericulture**

So Sericulture has a long tradition with human. Global silk production had increased from 139100.02 MT to 192692.45MT in 2016. In 2010 China, India, Thailand and Brazil accounted for 99.95% of silk production of which China contributes 82.2%, India contributes 16.6%, Thailand contributes 0.59% and Brazil contributes 0.56% (Berda, Jr. & Pereira, 2013). India is the only country produces known four varieties of silk including mulberry, Eri, Tasar and Muga. Mulberry is the largest industry accounting 76% of the entire silk production. According to International Sericulture Commission website, almost 76 million people across 51,000 villages operating 328627 handlooms and 45,867 power looms with 8,14,616 weavers are the key components of Indian Sericulture. From 2010 to2016 India has the second largest production of 30348 MT followed by Uzbekistan (1256MT), Thailand (712 MT), Brazil (650 MT), Vietnam (523 MT), North Korea (365MT) and Iran (125MT).China is the largest producer of silk (158400MT in 2016) over last decade.

Indian sericulture industry is growing steadily from 23060MT silk production with 2240 crore (In Rs) export earning and 7.56 million employments in 2011-12 to 32000MT silk production, 3172 croe (in Rs) export earning and 9.24 million employments. But import for raw silk had also decreased from 7338 MT in 2009 to 2010 to 3489 MT in 2014-15. But a decade ahead that import amount was steady (*i.e.* 7948 MT in 2004-05 to 7338 MT in 2009-10). Though sericulture is a labor intensive industry, employment generation is also increased from 5.95 million people in 2005-06 to 9.25 million in 2016-17. Indian sericulture industry exports 360 million USD of which 70% is comprised of natural silk yarn and fabrics, 13% made up and 26% garments. But domestic demand, now, stands at 28800 MT, compared to 23679 MT annually (Kumaresan & Qadri, 2012).

Chiefly bivoltine raw silk production has increased from 1200MT in 2009-10 to 3870 MT in 2014-15 but stability of production is yet questionable. Kumaresen and Qadri, 2012 discussed in detail, comparing sericulture profile, from1996-97 to 2009-10. Annual growth of Mulberry sericulture was 2.10% whereas 7.49% growth was recorded in Non mulberry section, of which highest growth was attained by Eri sericulture (78%). But Muga sericulture grew at the rate of 4.32% to reach 105 MT during 2009-2010 from 73 MT, during 1996-97. But now mulberry silks have higher instability in their production compared to non mulberry silks.

In 2009-10, Karnataka had 44.67% followed by Andhra Pradesh (31.38%), West Bengal with (11.43%) and Tamilnadu (7.56%) were the major producer in raw silk production. In India, Karnataka (44.67%), Andhra Pradesh (19.80%) and West Bengal (6.78%) are major land user of mulberry sericulture too. Raw silk production is also increased from 26.69kg/ha in 1981-82 to 88.78kg/ha in 2009-10 at a compound growth rate of 4.9% per. anum. In mulberry sericulture the area was increased steadily from 179,896 ha in 1981-82 to 2 80,651 ha in 1996-97, but after sharp decline it was 183,733ha in 2009-10.

### 1.3 Muga culture in focus

#### 1.3.1 State of Muga culture in India

In the perspective of rural poverty and unemployment in terai zone of West Bengal having low productive agriculture and industrial backwardness, the sericulture in general and muga culture in particular may have special reference for the economic up lift of rural people. Muga culture is an agro based small scale industry of North East India (42642 families) and around 34316 families from Assam are directly engaged in this culture. Three districts of West Bengal, viz Cooch Behar, Jalpaiguri, and in Darjeeling, Kalimpong have included into commercial production of Muga. Sikkim, Bageswar in Uttaranchal, Chintapalli in Andhrapradesh and Hosangabad in Madhya Pradesh have been reported rearing of Muga silkworm, but its commercial exploitation is yet to take off (Jayprakash et. al. 2012).

Muga silkworm, *Antheraea assama*, West wood (Lepideptera: Saturnidae) is multivoltine and eggs are non-diapause type, therefore muga culture requires continuous multiplication of the species. This polyphagous insect is feeding out door on

trees of wide range of host plant, Som, *Persia bombycina*, (Kost) and Soalu, *Litsaea monopelata* (Rox.) are considered primary food plants. Silk worm feeding on Som produces yarn of good quality while that feeding on Soalu produces better fecundity. Owing to distinct host plant preference by muga silkworm, lower Assam extending from Kamrup to Kokrajhar, Nalbari, Barpeta that abound in Soalu, was considered as seed zone while rest of Assam that abounds in Som was considered as commercial zone. Silkworm seed are produced by the farmers from lower Assam and reared in Upper Assam during the commercial crop Jethua (May-June) and Kotia (October-November).

### 1.3.2. Constraints of Egg supply in Muga culture

Out of 9241 hectares of existing muga plantation available all over north eastern states, Assam, alone posses around 6755 hectares and commercial rearing of muga silkworm for production of silk is confined around upper Brahmaputra Valley (Ranjan and Hajarika,2012). Though commercial crop rearing periods are May-June and October-November, but muga silkworm reared out door 5-6 times in a year exposing to intermittent climatic fluctuation particularly during seed crop production. The adverse climatic condition as very high temperature and humidity, heavy and continuous rainfall storm etc during seed crop rearing to supply required quantity of quality seed for subsequent commercial rearing which on turn becomes uncertain, leading to low production of seed (Sahu *et. al*, 1998). Estimating from the available plantation, about 1.5 core dlf are required annually. However presently around 60-30 lakh dlfs are produced leaving 20-40% of the plantations are unutilized (Joyprakash *et. al*,2012). Raw silk production statistics showed that over 50 years or so production of muga has increased only 2 times as against 21 times in mulberry (Das,2000). Last ten years, production of muga raw silk is fluctuating in between 100-124MT. This is a gloomy state of growth in muga silk production (Joyprakash *et. al.,* 2012).According to the Annual report of Central silk Board, in 2014-15, 4.5% rise in production of muga silk was reported (158MT).

Success of a crop is directly dependent on quality silkworm seed that is characterized by a brood which is completely free from disease, has more quality of viable egg, given uniform hatching and ensures crop stability (Choudhury & Sahu, 2000). Seed production under Government monitoring agencies are able to fulfill only around 10%

of the demand, balance of 90% is produced by the farmers who do not follow any seed testing protocol, which on turn adversely affect the success of the crop.

### 1.3.3 Refrigeration of egg is an alternative for quality seed supply in Muga culture

For maintaining quality dlfs, a 4 tier system of quality seed production was adopted by Muga Silkworm Seed Organization (MSSO), Central Silk Board, Guwahati. Presently two P4 units in Tura (Meghalaya) and Mandipahar (Meghalaya) and P3 units are in Hahim and Narayanpur (Assam), Adokgiri, Nongpoh and Rompara (Meghalaya) and Jia (Arunachal Pradesh). Therefore refrigeration schedule for proper transportation of seed supply to the farmers' field is a necessity. Season specific cropping schedule was standardized by MSSO linking the two commercial crops along with the nature of seed.

As the muga seeds are non diapauses type, low temperature preservation of seeds to arrest the developmental process for some time to skip the adverse environmental condition may overcome the constraint. An egg incubation schedule has been formulated for obtaining uniform hatching, where 4 days of eggs are incubated in BOD incubator at 26±1.5$^{o}$ C and 85±3% relative humidity using Potassium Chloride solution. Under normal condition, the incubation period in muga silkworm eggs is 8 days, in summer and 11-12 days in winter. This short period of incubation hinders long distance transportation. A schedule has been developed by MSSO where 4 days eggs are preserved at 12.5$^{o}$C for 9 days to prolong the embryonic period by 22 days (Rajan & Hajarika, 2012) Development of egg preservation technique for muga silk worm eggs of different embryonic ages have been kept at low temperature (5$^{o}$C) by following some intermediate steps of preservation for different durations (10days to 20 days with 10 days interval to detect suitable embryonic stages for long term preservation (Rajan and Hajarika,2012). This is why, low temperature tolerant embryonic stages and standardization and improvement of preservation technology are the primary necessity.

It, therefore, requires detailed analysis of embryogenesis in order to identify low temperature tolerant embryonic stages and subsequent standardization and improvement of low temperature egg preservation technology to ensure sufficient seed supply to the commercial rearing to make muga culture more remunerative.

### 1.3.4 Identification of low temperature resistant embryonic stages for muga seed technology

For the success of the sericulture industry, proper supply of silk worm egg is essential. The hatching period of eggs must coincide with the availability of suitable leaves as well as environmental conditions. Therefore, the hatching of the larvae has to be controlled, accelerated or postponed by artificial treatment under refrigerated condition (Upadhyay and Pandey, 2000). In the life cycle of the silkworm, refrigeration is usually done during all developmental stages, viz., egg, larva, cocoon and moth, but the cold treatment is to be restricted to any one of the developmental stages of the silkworm to avoid deleterious effect (Jolly, 1983). Hiratsuka (1975) opined that the eggs after oviposition can be preserved for 20 day without affecting the rearing characteristics. Moreover an increase in chilling duration beyond 20 days results in decrease in hatching percentage of non hibernating eggs (Narayanaswamy and Govindan, 1987). According to Yuyin (2000), for silkworm the safety time for cold inhibition is 3-5 days and at $5^0$ C, with an intermediate temperature of 10-$13^0$ C for 2-3 hours. Researches on cold preservation of mulberry silkworm egg have been tried successfully because of the availability of detailed information on embryonic development on *Bombyx mori* in diapausing and non-diapausing eggs (Dutta, 1988; Otsuki, 1997; Tazima1978). Moreover, biochemical findings to identify the cold resistant stages are also available in *Bombyx mori* (Sakano *et al*, 2004).

Developmental processes including differentiation and organogenesis in muga silkworm (*Antheraea assama,* Ww) egg is a continuous programme because these are non-diapause eggs and are laid by multi-voltine muga silk worm and larva hatch on day 7 after lay. Efforts for cold preservation as well as diapause induction in muga silkworm is not fruitful yet as because success of cold preservation of egg or diapause induction solely depends on the developmental events and needs studies on detailed embryogenesis. No complete information is available on embryogenesis of Muga silkworm eggs, except some preliminary works by Singha*et al.* (1998), and identification of some developmental stages by Ghosh and Ray (2006). And regarding cold preservation, very little information is there on muga silkworm egg. On the basis of preliminary study, Ghosh and Ray (2005) opined that preservation period becomes shorter as the age of the egg progress-a maximum of 15 days for 48 hours and a maximum of 12 days for 96 hour eggs. When 48 hours eggs need to be preserved, 15

days preservation at 8ºC shows 18 days delayed hatching with high hatching percentage and at 6ºC hatching is delayed by 15 days, also with high hatching percentage. Hatching can be delayed for 2 weeks in 72 hours eggs when preserved for 15 days at 6ºC. Delayed hatching (9-13days) with satisfactory hatching percentage can be obtained for 96 hours eggs when preserved for maximum of 12 days at 4ºC. So, it can be assumed that low temperature stress on Muga silk worm can be exploited to control hatching period as and when needed.

This situation needs a detailed study on embryogenesis including biochemical clues and subsequent standardization and improvement of cold preservation technique which can mitigate the problem of seed supply in suitable environmental condition. The present research design has been drawn accordingly with the following objectives:

1. To identify embryonic developmental events chronologically from post fertilization stage to chitinizaton stage with a view to observe the detailed embryogenesis.

2. To identify low temperature resistant embryonic stage(s) with a view to improve cold preservation technology of seed.

3. To determine the optimum low temperature used as cold stress to the identified low temperature resistant embryonic stage(s) for successful low temperature preservation.

4. To determine the optimum period of low temperature stress to the identified embryonic stages, so that the differentiation period can be delayed to ensure seed supply at desired quantity- when required.

5. To investigate the biochemical changes in embryonic developmental events during normal differentiation as well as during low temperature stress as a comparative analysis with an objective to study the changes of embryonic differentiation in normal and stressed condition.

# 2. REVIEW OF LITERATURE

## 2.1. Embryonic development in Muga Silkworm, *A. asama* under normal condition

Johannsen and Butt (1941) have reviewed embryology of Insects and Myriapods. Later Jura (1972) has documented the development of Apterygote insects. Anderson (1972) also review an account of embryonic development in Hemimetabolus insects. The evolutionary significance of insect development in relation to Annelids and other Arthropods is emphasized in detail by Anderson (1973). In the review by Pflugfelder (1958), Kranse (1957, 1958), Scidiel (1961), Connce (1961, 1973) and Kiifun (1971), physiological aspects of embryology are discussed.

In the eggs of most insects there is distinction between the anterior and posterior poles which bears definite relation to the position of the future embryo. The nucleus resides in the central part of the yolk in the unfertilized egg. During division of nucleus, polar bodies are formed and reabsorbed. Then it is enclosed towards the periphery of the egg. But after fertilization the zygote nucleus moves inward and divides into daughter nucleus (Richards and Davis, 1977).

Oogenesis is associated with a process of cellular morphogenesis (Waddington, 1967) as well as transmission of genetic information and energy stores. Changes in internal and external construction of insect egg explicate the process of oogenesis (Anderson, 1972, Connce, 1973) and also isolate genetic factors in egg from Drosophila (King.1970) and *Bombyx mori* (Tazima, 1964). In polytrophic *Bombyx mori* oocyte, follicles constitute (Legay, 1974) two synchronous event during development. During fast phase incredible extent of growths (3,000-10,000 times) are considered.

### 2.1.1 Cleavage and Blastoderm Formation

After division of zygote nucleus, cleavage nuclei develop and are covered by stellate mass of protoplasm. Significant number of cleavage cell assembled to drift to the periphery of the egg and fused with periplasm and to form continuous cellular layer of blastoderm. Future germ cells in insect are derived from posterior blastoderm cell. A columner cell layer on the ventral part of the egg derived from blastoderm. Those cleavage cells remain in the yolk, form primary yolk cell or vitellophages, which

become augmented by secondary yolk cell derived by immigration of cell from the blastoderm. Function of the yolk cell is to liquefy the yolk (Miya,2003).

Differentiation of germ cells in silkworm takes place just after certain cleavage nuclei enter into the pole-plasm regions of periplasm (Miya 1953) as observed in the Diptera, some species of Coleoptera and Hymenoptera. Germinal cytoplasm, oösome appear in the posterior pole of the egg in dipteral and some other insects (Johannsen and Butt,1941), where as analogous region of the silk worm seems to be placed at the ventral side of egg (Miya,1950).

The cleavage nuclei migrate into the peripheral layer shows proliferation with the subsequent formation of cell wall. The germ band is continuously contiguous in normal embryo with normal differentiation of germ cell (Miya, 2003).

In a definite region in the posterior pole of Dipterans along with some coleopteran and hymenoptera eggs, cleavage nuclei differentiate into germ cells. This region is clearly distinguishable by the existence of peculiar chromophile granules or the special affinity for basic dyes. It is designated as germinal cytoplasm (Miya, 2003).

The germinal cytoplasm is found to exist in the silkworm; it corresponds to the presumptive genital region observed at the blastoderm stage. There is no evidence between the extent of the germinal cytoplasm and the number of germ cells. The proliferation of the primodial germ cell may probably go ahead the completion of the blastoderm. (Miya, 1958)

No morphological variation is vivid soon after formation of germ cell (16hours after egg deposition) to gonad forming embryo. Multiplication stage of germ cell may appear either during early gonad formation or blastokinesis. Free germ cells are also distinct between mesodermal cells in body cavity (Miya, 2003).

### 2.1.2. Differentiation of Blastoderm

Embryonic primordium is formed from blastoderm and embryonic ectoderm to cover yolk surface partially. The embryonic primordium gives rise to most of the tissue and organ.

Various ‘presumtive areas’ of differentiatiated blastoderm can therefore be marked on fate map which show that embryonic primordial comprises five main regions (Miya, 2003).

1. A narrow mid ventral band of presumptive mesodermal cell , at each end of which lie
2. The small presumptive areas of anterior and posterior mid gut
3. In front of mid gut area presumptive stomodeum appear and in behind proctodaeum develop.

4. Broad head lobes are formed after joining a pair of lateral ventral branch of presumptive ectoderm with anterior part of presumptive stomodaeum.

Among Pterygotes, secondary dorsal organ present from the serosa.

There is an essential similarity in the process of blastoderm formation in different species of insects ( Anderson, 1962). Within 6-24 hours of embryogenesis blastoderm formation has completed at 27˚C in *Bombyx* sp. Then cleavage furrow proceeds further from egg surface into periplasm, where series of nuclei are arranged in row (Huettner, 1923; Mahowald, 1963; Fullilove & Jacobson, 1971; Sanders, 1975). Iwsaki (1931) opined similarity in the process of blastoderm formation between *Bombyx* spand *Drosophila* sp. But Takeuse et al.( 1980) and Keino and Takesue (1982) describes the differences between the blastoderm formation in *Drosophila* sp and *Bombyx* sp.

First, very thin periplasm in *Drosophila* sp becomes thicker which does not appear in *Bombyx* sp (Turner & Mahowald, 1976). Second, No typical syncitial blastoderm is formed. Third, typical cleavage furrow is absent during transformation of cleavage nuclei into blastoderm. Plasma membrane outside periplasm does not invaginate. Cleavage nuclei push plasma membrane around each nuclei. Blastoderm cells get its cytoplasm as an extension of periplasm around nuclei at $Yg_2$ area. Taakesue et al. (1980) confirm that the width of peripheral layer does not change during blastoderm formation in *Bombyx* eggs. The surface structure of the fertilized eggs of the silkworm, under goes series of changes during early embryogenesis which can be seen more clearly by SEM. During cleavage nuclei migrate to the egg surface and finally form the

blastoderm cells. So mode of blastoderm formation *Bombyx* and *Drosophila* are different (Miya, 2003).

In *Bombyx*, whole surface of the silkworm egg is covered with an array of finger like microprojections up to 4 hours of oviposition. Then they gradually disappear with appearance of ruffle like microprojections. Cleavage nuclei do not arrive at the periplasm until 9 hours (Takesue,1982). In *Bombyx* microvilli reappeared while blastoderm cells are being formed. The reappearance of microvilli concomitant with blastoderm cleavage (Takesue, 1980).It has been suggested that microvilli at the leading edge of the cleavage furrow, probably holds for blastoderm cell formation. In insects, early development of embryo requires a great increase in production of new membrane to accommodate cytokinesis of large number of cells. No cleavage furrow is formed (Miya, 2003).

### 2.1.3 Fate Map

*Bombyx mori* is a good subject for embryological studies owing to its well documented genetics and its wide spread research (Tazima, 1978). Accurate fate maps are an essential precondition for interpreting correctly the result of various types of developmental experiment. The establishment of *Bombyx* fate map attempted by two methods micro-cantery (Kuwana and Takami, 1957) and genetic mosaics (Katsuki et al.1980).Local cantery map by Kuwana and Takami (1957) shows presumptive regions for the procephalon, gnathal segments, thorax and abdomen. But mosaic method yields only primordia in the egg.

There are two important differences between *Drosophila* and *Bombyx* fate map are 1) presumptive regions for head structures and gnathal segments are mapped in *Bombyx*, but not in *Drosophila*; 2) In *Bombyx* the presumptive regions for the abdominal segments (specially for the posterior several segments) are more narrowly spaced in *Drosophila* (Katsuki et al.1980).

However most features, the *Bombyx* egg belongs in the same category as the *Drosophila* egg, the long germ type (Sander, 1976) it has meroistic oogenesis; presumptive germ anlage occupies most of the length of the egg and duration of embryogenesis is relatively short. But unique in *Bombyx* egg is its ability to fate map all

the larval segments at the fertilization stage suggests that all the abdominal are determined during the same time period as the rest of the germ band.

### 2.1.4 The germ band and gastrulation

The germ band arises by growth and differentiation of the embryonic primodium. It is elongated or oval in shape and usually single layered, but during development it turns into two layerd structure. Gastral groove emerge with proliferation and later sinks, commonly known as mid ventral groove. Mesodermal structure and mid gut rudiments are derived from inner layer of gastral groove. Embryonic ectoderm and stomodeal and proctodeal rudiments are derived from outer layer of gastral groove. .(Görg, 1959; Stribel, 1960; Roonwal, 1936-37; Lonvet, 1964; Goss, 1952-53; Vllamqun, 1964; Amy,1961)

Due to continuous nuclear and cell division, shape and size of embryo is changing till initiation of gastrulation. Germ analogue become distinct just before the starting of gastrulation (Nagy et al.1994).

### 2.1.5 Extra embryonic membrane

Germ bands remain covered by extra embryonic membrane over yolk cell. Growth of germ bands accelerates sinking into yolk. In lepideptera extra embryonic ectoderm cells covers the embryonic primordium forming serosa, then the edges of germ band move downwards and proliferate to form amnion and finally yolk invades the amino serosal space (Christensen,1945, Anderson and Wood,1968).

### 2.1.6 Blastokinesis

Lepidepteran blastokinesis is an unique kind (Reed and Day, 1966; Anderson and Wood, 1968). Aamniotic and serosal membrane are associated with extra embryonic development in insects. Extra embryonic membranes are associated with blastokinetic movement. Blastokinesis are advanced in hemimetabolus insects. In holometabolus insects some of the events of blastokinesis are followed (Larinink, 1997). Blastokinesis involves morphogenetic movrmrnts, ie. Movements associated with membrane formation, anatrepsis and ketatrepsis and dorsal closure prior to their demise. During anatrepsis, the germ band has immersed in yolk. Second stage involved elongation and segmentation germ band and limb rudiment development. It ends with rapture of extra

embryonic membrane and replacement of embryo into its normal position over ventral surface and anterior pole of egg (Panfilio, 2008).

### 2.1.7 Mesoderm formation

In the silk worm, as reported by Takami (1946), the presumptive mesodermal region is determined at an early stage. Cells in the region seem to become mesodermal cells through the invagination process of the primitive groove. In silk worm differentiation of mesodermal cells are influenced by regional effects of the ectodermal layer (Miya, 1960).

### 2.1.8 Dorsal closure

As the embryo develops it grows round the yolk and the dorsal and non-embryonic portion of the former blastoderm becomes more and more restricted. The final closure of the embryo and the fate of the extra embryonic membranes exhibit important differences among various insects which may be classified into four distinct types. It is necessary to distinguish between definitive closure of the embryo which is accomplished by mid dorsal junction of the upward growing ectoderm of each side and the provisional dorsal closure, which may precede this and brought about by extra embryonic membranes (Tazima, 1964).

a. Involution through the formation of dorsal aminoserosal sac: the two envelope rapture and with the upward growth of embryo, their contracted remains become carried on the dorsal side of the yolk. Here they form secondary dorsal organ. Ultimately, secondary dorsal organ under goes dissolution and the embryonic ectoderm completes the dorsal closure.

b. Involution of the amnion with retention of serosa: The amnion raptures ventrally and grows round the yolk so as to enclose it dorsally, becoming at the same time separated from the serosa. With upward growth of the embryo the amnion become compressed into a small dorsal tract- dorsal organ. Dorsal organ integrates in the yolk with the dorsal closure of the embryo. The serosa persists until late stage as a complete membrane applied to the inner aspect of the chorion.

c. Involution of the serosa with retention of the embryo: The serosa alone raptures and contracts to form the dorsal organ, which becomes absorbed in the yolk. The amnion afterwards grows over this area, so as to entirely enclose the egg and persist until the time of hatching.

d. Maintenance of amnion and serosa: Amnion continues to grow to cover yolk. It is separated from yolk. In lepideptera a quantity of yolk is retained between these two envelopes and serves as first food of young larva (Miya,2003).

### 2.1.9 Segmentation

Metameric segments along the anterior posterior axis are a fundamental feature of the arthropod body plan, which includes a diversity of segmentation mechanism (Damen, 2007; Liu, 2012). Insect embryonic segmentation can be classified in to two phases, i.e. long and short germ types. During large germ type segmentation, segmental patterning with large embryonic rudiments appears in syncitium. In short germ type embryonic rudiments and anterior segments only appear and other segments develop posterior growth zone (Nakao, 2012; Nakao, 2010).

It is hypothesized as long germ type has evolved from short germ type and and that shift is realized through anterior acquisition centre (Davis and Patel, 2002, Liu and Kufman, 2005; Lynch et al. 2006; Peel et al. 2005). But Nakao, 2012, opined that anterior system becomes important as insects evolve from short germ to long germ types. *Bombyx mori* Orthodenticle (Bm-otd) and Caudal (Bm-cad) gene expression confirmed that. Mechanism of segmentation in *Bombyx mori* involved the expression of even-skipped(eve), engrailed (en), caudal(cad), wnt 1/wingless (wg) during short germ type segmentation (Nakao,2010). Pair rule, eve stripes and segmental, en and wnt1/wg stripes are expressed over embryo (Nagy and Carrol, 1994; Dearden and Akam, 2001; Kopf et al.2004; Miyawaki et al., 2004; Angelini and Kaufman,2005; Shinmyo et al. 2005) in *Bombyx*. Notch signaling is involved in appendage development in *Bombyx*, but not Groucho (Gro) dependent Pair rule process (Liu, 2012).

### 2.1.10 Larval Legs

The adult body pattern in insects either can be developed from imaginal disc or from juvenile instars (Carroll et al. 2001). *B. mori* larva has three pairs of thoracic legs and five pairs of abdominal legs. Abdominal legs are lost during larval to pupal

metamorphosis (Singh et al. 2007; Gopinathan et al.1997; Suzuki and Palopoli, 2001; Ueno et al. 2005). There is a standing debate whether larval legs or imaginal disc, is the source for adult legs. Kellog and Bodenstein (1904) have opined for imaginal disc as source. In*B. mori* legs have developed from larval appendages (Singh et. al, 2007). 'Distalless' and 'extradentricle' have expression in leg primordial. Homeotic gene Abd-B suppresses lepiodepteran proleg development in posterior abdomen in *B. mori* (Tomita and Kikuchi, 2009).

Allometry yields familiarity between mouse and elephant. In *Bombyx* allometric relationship for metabolism both across all developmental stages and within each stage would not reflect conventional scaling coefficient ($b \neq 0.75$) (Myer and Burggren, 2010). Higher scaling in *Bombyx*is likely correlated with changes over all mitochondrial density rather than specific changes in body proportion of tissues with higher intrinsic metabolic intensity. Silk producing insects have rapid embryonic development as well as larval development.

Origin of egg stage in animal development is now questionable from the perspective of developmental and evolutionary narratives. It is rightly questioned 'Why can taxa with in a given phylum exhibit different egg type, pass through common intermediate morphology i.e. "phylotypic stage'? (Newman, 2011) Arthropods are distinct with this diversity. Aim of the present study is to reveal the 'hourglass' events of embryogenesis of one of the unique silk producing insect, *Antheraea assama*, Ww which do not show diapause. Genome wide microarray (Akitamo et al. 2017) and Transcriptome analysis (Chen et al. 2017) reveal the variation between diapause and nondiapause eggs in Bombyx. Events of embryonic hourglass can be divided into 'Dynamical patterning module' (DPMs) and 'Egg patterning processes' (EPPs). There is probability that different body plans had their origin in self organizing physical process in ancient cluster of cell. Egg is a 'set of independent evolutionary innovation' spins through developmental networks. Events during embryogenesis are multi-cellular patterning, set with in initial and boundary conditions by DPMs, where as phylotypic body plans are determined by EPPs and only focus on embryonic hour glass puzzle (Newman, 2011).

## 2.2 Embryonic development in muga silkworm *A. asama* in cold stressed condition

Insects adapted for diverse environment, having limited ability to regulate body temperature (Bale and Hayward, 2010). Strategies adopted for thermally stressful environments are behavioral avoidance, like migration and seasonal changes in cold tolerance. Freeze tolerance and freeze avoidance are the key ways adopting overwintering through synthesis of ice nucleating agents, cryo-protectants, anti freeze proteins and modification of membrane lipid composition. Overwintering also invite a hypo metabolic state called diapause in temperate and colder climates (Delinger, 1986). In *Bombyx* sp. colder climate initiate diapause during embryogenesis (Delinger, 2002).

Short term 'chilling' of insect eggs is utilized to lengthen the embryonic period without compromising quality of egg. Periods of low oxygen may induce delay in embryogenesis (Chino, 1957). Snobe et al. (1979) show that in diapause silk worm eggs, due to reduction of oxygen permeability of egg membranes, hypoxia is introduced to lower rate of metabolism and polyol accumulation. Extra embryonic regions are more sensitive for freezing than the embryo (Imanishi et al. 1966). In *A. assama* body temperature of $5^{th}$ instar larvae is determined by environmental temperature and solar radiation (Bordoli & Hazarika, 1994)

Immediate, accumulative and latent effects are characterized in House fly, *Musca domestica* as chilling injury. Chilling has immediate effect as injury depending on age of embryo and latent effect will work on post embryonic stage of development (Felton and Sumner, 1995). Hypoxia causes lowering of metabolic rate (Hochachka et al., 1996). In *Antheraea* cold preservation induce 'Quiescence' (Thangavelu, 1985). Again in silk worm Upadhyaya and Pandey, 2000 suggest that short term egg refrigeration is affected by low cocoon weight.

## 2.3 Low temperature stress on eggs for cold preservation of Muga silk worm *A. asama*

The pattern of development of egg is governed by the egg structure (Boswell and Mahowald, 1988) as described by different arthropods (Engelmann, 1970; Jura, 1972; Balinsky, 1981). Toyma (1902) studies in detail the embryogenesis of silk worm

(*Bombyx mori*). Since then search for suitable stage for refrigeration is continued till date.

In 1969, Takami reviewed on embryogenesis of *Bombyx mori*. Nakada(1932) and Takami & Kitojawa (1960), have described in detail of morphological changes during development. Stages in embryo plays crucial role in success after cold preservation (Salt, 1961; Rockstain, 1974; Sander et al.1985 and Sonobe et al.1986). Recent studies have widened the dimension like physiology, biochemistry and metabolic activity and also transmission of diapause (Yaginuma et al.1990; Yamashita and Yagunima, 1991).

Earlier studies confirm (Andrewatha and Birch, 1954, Watters, 1967 and Howe, 1967) that early embryonic stages are sensitive for refrigeration temperature. Studies on *Bombyx mori* indicated that eggs incubated for 1.5 days at room temperature are suitable for long term preservation (Dutta et al. 1972). Oak tasar egg (*Antheraea proylei*) can be stored for 30 days without affecting rearing (Ilohal et al. 1987). Vemenanda et al. (2004) has claimed to store the same for 17 days in refrigeration in *Bombyx*. But Pandey et al.(1992) has reported 10 days refrigeration does not affect hatchability where as 1%, 3% and 15.5% embryonic death have recorded when stored at 7and 11±2˚C for 20, 30 and 40days respectively where as 6.46% and 9.8% embryonic death is recorded when stored for 30 and 40 days at 5±2˚C. Ranna et al. (2002) reported that the age of eggs and period of refrigeration have significant effect on hatching. Tayede et al. 1987 identifies that eggs or embryos do not develop beyond 5.5˚C. When *Samia cynthia ricini* eggs are utilized for refrigeration at 0˚C for 15 days and storage at 5-10˚C for 5-10 days record highest effective rate of rearing (Nagina & Nageshchandra, 1988). In *P. ricini*, Viswakarma(1992-93) has reported that 3 days old egg in summer and 5 days old egg in winter are cold resistant. Govindan et al., 1980 mentioned about adverse effect of refrigeration beyond 5 days on hatching.

## 2.4 Low temperature stress for improved schedule of cold preservation technique in Muga culture

Muga seed cocoon (*Antheraea assama*) when preserves for 5 to 50 days at 4±1˚C (Sengupta and Singh, 1974) gradual reduction of hatching is reported after 40 days. How preservation of muga seed cocoon at low temperature effect, is studied by Subba Rao and Choudhury, 1976. Delay in moth emergence is reported by Choudhury (1981 and 2006) when preserved at 2.5 to 5˚C for above 75 days in winter and 30 days in

summer. Three months preservation at 5˚C and 10˚C at 2590 m altitude, better results are obtained by Thangavelu et al. (1985). Refrigeration of muga seed cocoon at 5˚ to 12˚C for 10-20 days reveal better moth emergence, pairing, fecundity and hatchability, as confirmed by several other studies also (Choudhury et al.1982, Choudhury et al.2010 and Bora et al. 1990 and Bora 2006).

Moth emergence can be delayed 60-80 days instead of 30 days in control as and when cocoons are conserved at 8±1˚C(Khanikor and Dutta,1997). At 8±1˚C preservation, autumn cocoons delayed for 60-120 days, late autumn cocoons are delayed 40-45 days to 80-100 days and spring cocoons are delayed for 14-18 days to 30-42 days (Khanikor and Dutta, 1998a). Sengupta et al. (1995) has reported that 10 days old cocoon are preserved at 10˚C for 45 days without affecting reproduction. Low temperature resistant stage are longest embryonic stages appeared at 36 hours of oviposition during May and 114-126 hours of oviposition during November (Singha et al. 1998).

But when 5±1˚C, 7±1˚Cand 9±1˚C low temperature regime are utilized for 30, 40, 45, 50, 60, 65, 70 and 75 days preservation, moth emergence, pairing, fecundity and hatchability are declined. Delay in moth emergence is reported up to 60-120 days against 28-30 days in control. This strategy is utilized to synchronize between seed crop and commercial crops (Khanikor and Dutta, 1998b). Ghosh and Ray (2005) reported that 15 days cocoon preservation may delay moth emergence for 10 days, adult moth preservation for 5days and eggs after 24 hour lying for 21 days.

### 2.5 Biochemistry of embryonic development of Muga silk worm *A. assama*

#### 2.5.1Biochemistry of embryonic development of Muga silk worm *A. assama* in normal condition

In an insect egg, proteins are stored in yolk granule (Agrell and Landquiest, 1974, Raikhel and Dhadialla, 1992), carbohydrates as glycogen granules (Gutzeit et al.1994, Yamashita and Hasegawa, 1985) and lipids in oocyte cytoplasm (Vanet et al. 1995). During embryogenesis, proteins are utilized as amino acid source; carbohydrates and lipids are as sources for energy (McGregor and Loughton, 1974; Steele, 1981; Beenakkers et al.1981a).

Carbohydrate

Quality eggs play pivotal role in the success of sericulture. Proper embryonic development is important for egg production. Embryogenesis is divided into two phases, differentiation and organogenesis. Soon after laying, differentiation starts and continues for four days in *Bombyx* and the organogenesis continues till hatching. These two phases have distinct energy metabolism pathway for diapause and non diapause silk worm egg (Pounuvel et al. 2010). As the metabolic activities are intense during different stages of embryogenesis, it requires constant energy for growth, differentiation and maintenance off the cells in living condition.

Storage forms of carbohydrate changes according to the developmental stage of eggs and glucose become to be higher in late stage (Okazaki and Yamashita, 1981). Initially glycolytic carbon flows into sorbitol over first two days. Anaerobic carbohydrate catabolic root synthesize protectents (eg. Sorbitol or Glycerol). If non diapause, encounter unfavorable environmental stress, initial root of anaerobic metabolism is abundant by the third day in order to allow metabolic rate and growth to accelerate. The egg contain high level of glycogen initially, about 92 hour after oviposition and then decline (Sakano et al. 2004). Glycogen consumption is increases substantially within 3-4 day, NAD-SDH (NAD- Sorbitol dehydrogenase), on day 3, quickly employs sorbitol to form fructose. The catabolism of sorbitol produce NADH, can be funneled into mitochondrial electron transport system to derive ATP synthesis (Storey and Storey, 1990; Storey and Storey, 1991; Yaginuma et al.1990a).

Protein

Initially morphology and physiology of embryogenesis is studied in detail (reviews by Johansen and Butt, 1941) and Hagan, 1957, rather than biochemistry of insect development (McGregor and Lughton, 1973). Indira (1963) and Chen and Brigel (1963) reported on amino acid and protein metabolism during embryonic development. Naturally free amino acid increases (Colombo et al. 1961, 1962) with concomitant increase in peptide levels. After oviposition until day 3, total soluble protein decreases, it goes up again soon after that. Then levelling out of protein amount is in the final days of embryonic development. During early days of embryonic development rise in amino acid pool utilizes in protein synthesis after blastokinesis forms (Chen and Briegel,1965

and Colombo et al.1961) Similar phenomenon is also reported in*Spheroderma molestum* (Indira,1963).

During embryogenesis, proteins among three macromolecules (i.e. protein, carbohydrate and lipid) contribute most to catalyze chemical reaction during differentiation as structural components and also enzymes (Nace, 1970). Probability of pivotal role of proteins during embryogenesis (Waddington, 1962), protein synthesis and morphogenesis are closely linked events (Brachet, 1957. 1962; Casperson, 1950).

In *Bombyx mori* egg contains 20% proteins (Otuski et al. 1997). During diapause protein contents increases from 0 hours and continues till $5^{th}$ day and after that the steady state is maintained.

Cholesterol

Phospholipids and cholesterols are well known constituents of tissue lipids (Dukes, 1955). Ecdysone, the moulting hormone is synthesized from cholesterol (Karlson, 1963; Karlson and Hoffmeister, 1963). But insects are incapable of synthesizing cholesterol (Levinson, 1964). Cholesterol and β- sitosterol are present in *Bombyx.* In *B. mori*, cholesterol is formed from conversion of β- sitosterol in dietary mulberry leaves (Ikekawa et al. 1966). Cholesterol synthesis or selective retention is highest during embryonic development (Saito et al. 1963). *B. mori* egg contains 12% of wet weight and 27% of dry weight as lipid (Yamahama et al. 2008). Non diapause egg has more cholesterol content than diapause egg. During embryogenesis cholesterol is also a source of energy other than glycogen (Karmavav and Nan, 1969).

DNA

DNA content in non diapause eggs increases through the progression of embryogenesis (Kurata et al. 1980). Even in diapause eggs DNA level remain constant until 400 days after oviposition. Due to high or low temperature stress no significant variation in thymidine incorporation reflects limited effect on DNA synthesis (Park and Yoshitake, 1969). Thymidine incorporation happen only brain and sub-oesophageal ganglia after blastokinesis. But in diapause *Bombyx* egg DNA content increased more than 10 times during the first day and remained at that level as long as diapaiuse maintained (Furusawa et al.1985). Sudden increase in DNA content up to 3 days after oviposition

may be due to vigorous cell division needed for the formation of diapause egg (Otsuki et al.1978, Furusawa et al.1985).

NAD-SDH

Various metabolic events have been characterized during embryonic development of non diapause silk worm eggs incubated at 25˚C. The eggs contain high levels of glycogen until about 92 hours after oviposition, but then amount of glycogen declines until hatching (Yamashita, 1965).Corresponding to the appearance of glycogen phosphorylase activity ( Yamashita, 1975), on day 2 sorbitol levels increase but then decrease rapidly on day 3 (Furuswa and Yang,1987). The appearance of sorbitol correlates with a sharp increase in activity of NAD- sorbitol dehydrogenase (NAD-SDH) (Yaginuma and Yamashita, 1979; Yaginuma et al.1990 b). Trehalase level increases after 2-3 days but then decrease as hatching approaches and trehalse activity increases around day 6 ( Yamashita 1965).

In non mulberry sericulture research work in this regard is scanty. Singha et al. (1987, 1989) reported changes in concentration of free amino acids in the developing embryo of *Antheraea pyroli*. Pant and Sharma (1976) observes glycogen is the main source of energy during embryogenesis in *A. mylitta* and Krishnappa et al. (2001) records changes in the levels of carbohydrate, lipid and moisture content during embryogenesis in *Samia cynthia ricni*. In muga silk worm egg protein and carbohydrate quantities are characterized (Ghosh and Ray, 2005). In a comprehensive study between pebrine infected and healthy embryo of *A. assama* quantitizes the dynamic pattern of protein and carbohydrate during embryogenesis (Choudhuri et al. 2013). Another study compares carbohydrate, protein, lipid concentration in oocyte and eggs during different rearing season to standardize the quality of egg (Choudhury et al. 2013). But detail biochemical study during embryonic development only reveals stages of protein utilizations and synthesis during normal and stress regime.

### 2.5.2 Biochemistry of metabolic shift during cold temperature stress of Muga silk worm *A. assama*

Differentiation and organogenesis are the two stages occurring during embryogenesis of *Bombyx mori* and *Antheraea assama*. Soon after oviposition differentiation continues upto 4days. After 4 days organogenesis starts and continues until hatching. Different

level of energy metabolism is required for these two stages (Yaginuma and Yamashita, 1999). In nondiapause eggs development continues for 9.5 days when incubated at 25˚C (Niimi, Yamashita and Yaginuma, 1993). During diapuuse initiation glycogen is utilized in to sorbitol and glycerol is utilized and during diapause termination by chilling at 5˚C also. These polyols have been thought to function as cryoprotectants to stabilize subcellular structure and function (Sujuki et al.1983; Storey and Storey,1988; Yamashita and Yaginuma,1991) and are recently proposed to function as arresting factor of embryonic development (Horie et al. 2000). Diapause is maintained as long as eggs are incubated at 25˚C, but diapuse is broken when eggs are exposed to 5˚C for about two months. NAD-SDH (Sorbitol dehydrogenase) enhanced more to metabolize sorbitol and glycerol kinase has been characterized to metabolize glycerol, during diapause breaking (Kihara et al.2009). Occurrence of BM Sdh also correlated with two developmental phases, growth of embryo and the formation of larval tissues. In the first phase an increase in the amount of the transcripts for BM Sdh resulted from embryonic cells rather than yolk cells. In the second phase transcripts is abandoned in fat body cells of pharate larva.

NAD SDH

NAD SDH is accumulated in *Bombyx mori* diapause eggs, as incubated at 5˚C (Niimi and Yaginuma, 1992). In non diapause *Bombyx* egg during first phase, the amount of transcripts for BmSDH ( *Bombyx* homolog of mammalian sorbitol dehydrogenase) from embryonic cell increases. In second phase abundant transcript is recorded in fat body cell of pharate larva (Niimi et al.1993). During embryonic diapause, termination induced by 5˚C incubation, extra cellular signal regulated kinase (ERK) activates and regulates sorbitol-glycogen conversion (Fujiwara, 2006). During termination of diapause glycogen is synthesized from sorbitol and utilized for embryogenesis (Yaginuma and Yamashita, 1978). At 5˚C, NAD-SDH controls this conversion (Yaginuma and Yamashita, 1979; Yaginuma et al.1990), but on incubation at around 0˚C do not show stimulation of NAD SDH activity, in *Bombyx* diapause eggs, where a high concentration of sorbitol is maintained (Yaginuma et al.1990). Sorbitol dehydrogenase gene (SDH.2) do not expressed till 18 hours and 24 hours in non diapause eggs. But diapause induced eggs reveals a lower expression of gene until 48 hours after oviposition while higher expression is observed in non diapause eggs except at 48 hours. After cloning of Sdh gene, it is evident that Sdh.1 activity is dependent on

5˚C acclimatization of diapause egg leading increase in SDH activity, than SDH 2ab (Rubio et al. 2011). General hypothesis regarding evolution of SDH gene include initial duplication for SDH1 and SDH2 and further duplication of SDH2 into SDH2a and SDH2b gene (Rubio et al.2011).

Trehalose

Trehalose, non reducing disaccharides of glucose is principal sugar circulating in the haemolymph of most insects. Two glycolytic intermediates *i.e.* glucose-1-phosphate and glucose-6-phosphate are condensed to form trehalose. Trehalose acts as 1) Energy store; 2) a cryo-protectant; 3) a protein stabiliser during osmotic and thermal stress and 4) a component of feedback mechanism regulating feeding behaviour and nutrient intake.

Temperature dependent activation of glycogen phosphorylase and synthase in fat body of *Philosamia cynthia*, may be responsible for temperature dependent inter conversion between glycogen and trehalose (Hayakawa and Chino, 1982). At 8˚C fat body glycogen is converted into haemolymph trehalose where as reverse reaction take place when pupae are returned to a higher temperature (20-25˚C). Fat body glycogen phosphorylase is converted to phosphorylase when pupae are transferred from 5˚C to 25˚C and active form decreases gradually. Synthatase activity remains low when pupae are returned from 25˚C to 2˚C. Temperature change does not affect phosphorylase activity but synthase activity increases when the pupae are exposed to a higher temperature. Nondiapause pupae of *P. cynthia ricini,* no significant accumulation of trehalose after 2˚C exposure for long period has reported (Hayakawa and Chino, 1981). Phosphofructokinase regulates glycerol and trehalose after polyol accumulation and glycerol 3 phosphate dehydrogenase regulate glycerol formation (Hayakawa and Chino, 1982). Cold temperature (5-15˚C) retard NAD SDH activity and sorbitol accumulates in egg (Toshibu et al. 1990). When NAD SDH activity appears then sorbitol is converted into glycogen. In the diapause eggs of *Bombyx mori,* NAD SDH plays pivotal role for sorbitol degradation during termination. Up to 2 days after oviposition NAD SDH remains low both in diapause and nondiapause eggs. After that it is maintained low in diapause egg, but has increased in non diapause egg. Soon before diapause break down NAD SDH activity is increased in diapause eggs (Yaginuma and Yamashita, 1979).

In presence of diapause hormone $C^{14}$ trehalose converts into glycogen (Yamashita and Hasegawa, 1976). Bombyxin induce hypotrehalosamia by promoting hydrolysis of

haemolymph trahalose to glucose and there by faciliting its transport into tissues. In addition Bombyxin reduce the glycogen content in the fat body and concurrently raise the percentage of active glycogen phosphorylase in tissues (Satake et al. 1997).

Oxidative Stress

As a when the rate of generation of oxygen radicals are more than the rate of their decomposition, oxidative stress introduces in living organisms (Sies, 1986). 1-3% or more of oxygen for respiratory process is converted to reactive oxygen species (ROS) by univalent reduction of oxygen (Kodri et al. 2015). Enhanced production of ROS with simultaneous impairment for scavenging system leads to oxidative stress. Stress situation can be attained through chemicals, physical and physiological stressor and that may upset functional homeostasis. Dynamic equilibrium between ROS production and removal are essential. But when balance shifts for oxidants, oxidative stress is introduced.

Three major ROS system are evident in insects i.e. (a) Superoxide radicals ($O_2^{-\cdot}$); (b) Hydroxyl radicals ($^{\cdot}$ OH) and (c) Hydrogen peroxide ($H_2O_2$). Super oxide ion radicals are spilled from 'leaky' mitochondrial respiratory chain and then super oxide dismutase converts that into $H_2O_2$ and easily diffuses through plasma membrane. Another source is via Xanthine oxidase. It is postulated that former arises from reduced components of the respiratory chain that build up during ischemic phase and undergo auto-oxidation when oxygen is rapidly reintroduced during reperfusion phase (Marcelo, et al.1998; Hochachoka, et al.1996; Ruuge, 1991). Xanthine oxidase is fuelled during reperfusion by the buildup of its substrates (xanthine and hypoxanthine) during ischemia. Hypoxanthine is formed as evidence for ATP catabolism (Mercelo,et al.1998).

Xanthine oxidase

In *Bombyx*, super oxide anion, hydrogen peroxide and hydroxyl free radical are the witness for oxygen consumption in aerobic cell. Oxidation of hypoxanthine and xanthine to produce superoxide anion $H_2O_2$, are reflected through Xanthne oxidase. Super oxide anion is converted to $H_2O_2$ by super oxide dismutase (SOD) (Zhao & Shi, 2010). Catalase (CAT) and Ascorbate peroxidise eliminate $H_2O_2$ in insect (Bolter and Chefurka, 1990; Yamato et al. 2005).

$H_2O_2$ content in diapause and non diapause eggs open a new line of investigation into the physiology of diapause. $H_2O_2$ belongs to reactive oxygen species (ROS) known as

oxidants that can react with various cellular targets thereby causing cell damage or even cell death. Significant increase of $H_2O_2$ coinciding with the decline of hatchability observe with the non diapause eggs for more than 30days of the 5˚C chilling may be related to the oxidative damage caused by $H_2O_2$,. Diapause animals are generally resistant to wide range of environmental stress including radiation, temperature extremes, chemical carcinogen and mutagen. Resistance to oxidative processes has been most commonly measured parameters (Stuart and Brown, 2016) compared to non diapause egg. Diapause eggs contain higher $H_2O_2$, higher Xanthine oxidase (XO) and lower Catalase (CAT) during the 5˚C chilling.

NADH Peroxidase

Antioxidant enzymes and small antioxidant molecules perform effective response against oxidants in insects. Super oxide dismutase (SOD), Catalase, Glutathione transferase and Glutathione reductase are candidate enzymes in insects (Felton and Sumners, 1995). Due to lack of glutathione peroxidise effect, Catalase (CAT) solely perform the job of oxidant removal in insects (Orr and Sohal 1992; Shoal et al.1993). Other enzymes like ascorbic peroxidase (Mathews et al.1977), dehydro ascorbic acid reductase (Sumners and Felton, 1993), Chorion peroxidise (Han, Li and Li, 2000a) have been reported. In *Ades aegypti*, NADH is oxidised for paroxidase activity.(Hau et al. 2000b) . NADH Peroxidase is structurally similar to glutathione reductase (GR). The charge transfer thiolate in GR is structurally equivalent to the redox active cystine in NADH Peroxidase ( Rebecca, and Palfey, 2010). The activity of thioredoxin reductase (TrXR) is detected in ovaries of *Bombyx mori,* but not in eggs while neither ovaries nor eggs show glutathione peroxidise (Zhao et al. 2014). Zhao and Shi, 2010 also reports that diapause egg also contain higher $H_2O_2$, higher XO and lower CAT compare to non-diapause egg during 5˚C chilling, $H_2O_2$ and catalase expression in silkworms eggs are involved in diapause initiation and termination (Sima, et al 2011).

Low temperature also promotes ROS and lead to OS (Lalouette, et al. 2011) damage and a worm recovery period activated the antioxidant system allowing repair of cold induced damage. When insects are cooled sufficiently they suffer an initial loss of neuromuscular function (Chill coma) (Overqaard and MacMillan, 2017). The adaptation and acclimation responses that allow some insects to tolerate low temperature are multifactorial and involve several physiological and biochemical adjustments. Even parental exposure to an abnormal environment during germ cell

maturation affected glycolysis and subsequent fertilization in *Bombyx mori* (Tao, et al, 2015). Glycometabolic shift through obstructed nicotinamide adenine nucleotide ($NAD^+$) regeneration and in active TCA, leading to accumulation of large amounts of pyruvic acid and lactic acid may be responsible for parental transcript legacy.

# 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= ($\Delta$ $A_{340nm}$/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 $\Delta A_{340}$/min using the maximum linear rate for both the test and blank.

Calculations:

Units/mg enzyme = [ $\Delta A_{340}$/ min Test -$\Delta A_{340}$/ 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 in$A_{290}$ for approximately 5 minutes. Obtain the $\Delta A_{290}$/minute using the linear rate for both the test and blank.

Calculations:

Units/ml Enzyme: [($\Delta A_{290}$/min Test-$\Delta A_{290}$/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.

# 4. RESULTS

## 4.1 Embryonic development of Muga silkworm, *A.assama*

### 4.1.1 Detailed embryonic development of Muga silkworm, *A.assama* under normal condition

#### 4.1.1.1 Embryonic development at 6 hours

##### 4.1.1.1.1 Embryonic development at 6 hours after oviposition under Microscope

###### 4.1.1.1.1.1 Embryonic development at 6 hours after oviposition under Stereo Binocular Microscope

After 6 hours of egg deposition cleavage nuclei were migrated to the surface of the egg and arranged spherically (Plate1A) starting a stage of de-lamination.

###### 4.1.1.1.1.2 Embryonic development at 6 hours after oviposition under Scanning Electron Microscope

The sphere had swelled at the micropyle end and at least at opposite end of egg (Plate 2A).

##### 4.1.1.1.2 Embryonic development after 6 hours incubation through histological sections

These one cell layered epithelium was differentiated into blastoderm (Plate 5A).

#### 4.1.1.2 Embryonic development at 12 hours

##### 4.1.1.2.1 Embryonic development at 12 hours after oviposition under Microscope

###### 4.1.1.2.1.1 Embryonic development at 12 hours after oviposition under Stereo Binocular Microscope

After 12 hours of egg deposition, blastomeres were rapidly increased to develop blastoderm which was very thin in the dorsal site (Plate1B) and thick at the ventral site.

#### 4.1.1.2.1.2 Embryonic development at 12 hours after oviposition under Scanning Electron Microscope

Ventral plate was emerged through condensation, both from the cephalic and caudal end (Plate 2B). Microvilli like projections were evident on blastoderm.

#### 4.1.1.2.2 Embryonic development after 12 hours incubation through histological sections

Basement membrane had separated blastomeres from vitellophages, accumulated through endocytosis activity. Germ cells were appeared in the posterior pole outside blastoderm. On yolk mass outer yolk cells were distinct (Plate 5B).

Soon after formation of blastoderm, gradual invagination inside egg was initated. Amniotic fold appeared with inner and outer layer which was transformed later into chorion and amnion, respectively. Serosa having flattened cell, formation was completed by this time. Amnion covers germ band (Plate 5C). Constriction to ventral plate gives rise to germ band (Plate2B, Plate 5D). Germ band already under gone gastrulation and differentiate into ectoderm. Also pseudo-startified lanceolate cells were evident.

### 4.1.1.3 Embryonic development at 24 hours

#### 4.1.1.3.1 Embryonic development at 24 hours after oviposition under Microscope

#### 4.1.1.3.1.1 Embryonic development at 24 hours after oviposition under Stereo Binocular Microscope

After 24 hour, muga silk worm egg germ band had appeared with proctocephalon and protocrom (Plate 1C).

#### 4.1.1.3.1.2 Embryonic development at 24 hours after oviposition under Scanning Electron Microscope

Soon after attaining ‘daruma’ stage, primitive groove appeared along the median line of the germ band surface, which was narrow at the centre and wider at two edges (Plate 2C).

#### 4.1.1.3.2 Embryonic development after 24 hours incubation through histological sections

This stage had resembled with ‘kokeshi’ of *Bombyx* sp. Segmentation was initiated during this stage.

### 4.1.1.4 Embryonic development at 48 hours

#### 4.1.1.4.1 Embryonic development at 48 hours after oviposition under Microscope

##### 4.1.1.4.1.1 Embryonic development at 48 hours after oviposition under Stereo Binocular Microscope

After 48 hours, germ band had appeared around the egg periphery (Plate 1D).

#### 4.1.1.4.1.2 Embryonic development at 48 hours after oviposition under Scanning Electron Microscope

Distinct germ band had covered the yolk over almost entire length of the egg (Plate 2D).

#### 4.1.1.4.2 Embryonic development after 48 hours incubation through histological sections

After 48 hours, amniotic membrane was extended along the whole length of embryo by elongated flattened cells. The ectoderm had evolved multi-stratified layer with elongated cells (Plate 6, A &B). Fluid filled amniotic cavity became distinct. Serosa had covered just under the chorion surrounding embryo, amnion and yolk and presence of two membranes were called synapomorphy.

### 4.1.1.5 Embryonic development at 72 hours

#### 4.1.1.5.1 Embryonic development at 72 hours after oviposition under Microscope

##### 4.1.1.5.1.1 Embryonic development at 72hours after oviposition under Stereo Binocular Microscope

As development continued 72 hours embryo had started blastokinesis soon after formation of germ band (Plate 1 E).

#### 4.1.1.5.1.2 Embryonic development at 72hours after oviposition under Scanning Electron Microscope

Blastokinesis had entailed early entry and later exit of embryo from yolk as vivid in Plate 2 E.

#### 4.1.1.5.2 Embryonic development after 72 hours incubation through histological sections

Blastokinesis had two types of movements *i.e.* anatrepsis (upward) and ketatrepsis (down ward) were shown in Plate 6C&D. During anatrepsis, entry of tissues into yolk was evident. Embryo extends in length and segmentation and appendage formation initiated. The ectoderm was crossed by transverse furrows, limited different segments and differentiated polygonal neuroblast with clear edges and neurotic projections. Neural groove also appeared during this stage (Plate6 D). Protuberance had appeared in thoracic segment. At the later stage protuberance appeared in thoracic segments also and continued to appear in abdominal segments.

### 4.1.1.6 Embryonic development at 96 hours

#### 4.1.1.6.1 Embryonic development at 96 hours after oviposition under Microscope

#### 4.1.1.6.1.1 Embryonic development at 96 hours after oviposition under Stereo Binocular Microscope

In 96 hours embryo (Plate 1 F) katatrepsis was initiated as evasion or outward movement of both embryo and amnion. After blastokinesis egg again had emerged on the surface of egg.

#### 4.1.1.6.1.2 Embryonic development at 96hours after oviposition under Scanning Electron Microscope

During progressive emergence head and antennae, the legs and lastly abdomen was released from yolk (Plate 2F&3B).

#### 4.1.1.6.2 Embryonic development after 96 hours incubation through histological sections

A back flip or 180˚ revolution of embryo was observed (Plate 6 E & F). A pair of labial protuberance became distinct in front of head fold. Stomodeum and proctodeum had appeared and gradually become tubular.

In the thoracic region rudiments of appendages had appeared and in cephalic region, formed by the beginning of stomodaeum. The differentiation of labrum, occurred in protocephalon, over which yolk was present.

In the 96 hours egg, the caudal area was surrounded by the amino proctodeal cavity bounded by amnion and then invaginate to originate proctodaeum, along with coelomic cavities, behind which germ cells are clustered. In the proctocephalon there were two coelomic cavities. Amniotic cavity had enlarged and the amnion surrounding the embryo and yolk. Serosa had secreted a distinct culticular layer. Here segmentation was more advanced. In the protocorm, the buds of the gnathal appendages, of the three pairs of the legs and of the ten abdominal segments were evident. In the first abdominal segment there was pleuropodium of conical shaped and small structure. In the following seven segments and in the tenth there were proleg buds. The ventral nerve cord was well defined.

### 4.1.1.7 Embryonic development at 120 hours

#### 4.1.1.7.1 Embryonic development at 120 hours after oviposition under Microscope

##### 4.1.1.7.1.1 Embryonic development at 120 hours after oviposition under Stereo Binocular Microscope

During 120 hours embryo (Plate1G), length of embryo had covered almost whole length of the embryo and both end came in contact which was called dorsal closure.

#### 4.1.1.7.1.2 Embryonic development at 120 hours after oviposition under Scanning Electron Microscope

Dorsal closure was distinctly vivid after utilization of yolk along with extra embryonic membranes (Plate 2G).

#### 4.1.1.7.2 Embryonic development after 120 hours incubation through histological sections

Soon after dorsal closure involution was started to move embryo from ventral side to dorsal side (Plate7A). Vertical turning of posterior abdominal segment had placed the abdominal region in a straight line. Then abdominal region reached towards anterior region at the level of prothorax. Fore gut and hind gut had differentiated from anterior and posterior ectodermal invagination respectively (Plate7B, C, D).

### 4.1.1.8 Embryonic development at 6 hours

#### 4.1.1.8.1 Embryonic development at 6 hours after oviposition under Microscope

##### 4.1.1.8.1.1 Embryonic development at 6 hours after oviposition under Stereo Binocular Microscope

After 144 hours (Plate1H), head capsule formation was completed and mouth parts became mature.

#### 4.1.1.8.1.2 Embryonic development at 6 hours after oviposition under Scanning Electron Microscope

Three segmented antennae with antennal setae, mandibles and labrum were well developed.

#### 4.1.1.8.2 Embryonic development after 6 hours incubation through histological sections

Tips of labrum and labium became segmented (Plate7E, F). Thoracic legs became segmented with claws at distal end. Rudiments of setae had developed on body surface.

### 4.1.1.9 Embryonic development at 168 hours

#### 4.1.1.9.1 Embryonic development at 168 hours after oviposition under Microscope

##### 4.1.1.9.1.1 Embryonic development at 168 hours after oviposition under Stereo Binocular Microscope

Entire body of 168 hours embryo (Plate 1I) had covered with strong setae and embryonic moult was occurred in this stage.

#### 4.1.1.9.1.2 Embryonic development at 168 hours after oviposition under Scanning Electron Microscope

Caudal horns appeared in this stage. Mandibles became sclerotised and pigmented at the distal end. Larval eyes (i.e. Ocelli) appear as six brown spot on either side of head. The spiracles were clearly visible on the sides of body. Head capsule and mouth appendages were sclerotised and well pigmented. Rectal sac became distinct (Plate 4 A, B,C,D).

#### 4.1.1.9.2 Embryonic development after 168 hours incubation through histological sections

The amnion and serosa disappeared by fragmentation. Embryo had ingested the embryonic membranes and sensitive for adverse environmental condition. Entire body of embryo became sclerotised.

### 4.1.2. Effect of low temperature stress on embryonic development:

#### 4.1.2.1 Effect of low temperature stress on 12 hours embryo

After 3days refrigeration, 12 hours of eggs, embryo became larger in size. Germ band became thick (Plate11A&B).

#### 4.1.2.2 Effect of low temperature stress on 24 hours embryo

After 3days refrigeration 24 hour muga silk worm eggs became larger, covering the entire length of the egg and were showing resemblance with 48 hour embryos (Plate11C&D).

#### 4.1.2.3 Effect of low temperature stress on 48 hours embryo

After 3days refrigeration 48 hours egg was continued to grow in slower rate and were showing resemblance with 96hours embryo (Plate E&F).

#### 4.1.2.4 Effect of low temperature stress on 72 hr embryo

As development continues slowly 72 hours embryo after 3 days refrigeration had shown resemblance with 120 hr embryo (Plate G&H). However electron microscopic studies and histological studies confirm similar stage specific characteristics.

### 4.2 Effect of temperature stress on different embryonic stages

To identify low temperature resistant embryonic stages from 24 hours to 144 hours embryo in eleven treatments (*viz.* 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 hours) under four temperature shocks (4, 6, 8, 10±1˚C) for ten days were taken for hatching percentage and incubation periods. Data thus obtained were recorded and analyzed statistically.

#### 4.2.1 Effect of temperature stress on different embryonic stages on hatching percentage

#### 4.2.1.1. Effect of temperature stress at 4±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (90.85%) followed non-significantly by 36 hours (89.85%), 48 hours (89.69%), 60 hours (89.37%), 72 hours (89.36%) and control (82%) and significantly by 84 hours (67.23%) , 96 hours (59.94%), 108 hours (57.96%) having non significant variation between 84 hours to 108 hours and others. Lowest hatching was observed at 144 hours(44.29%) having non significant variation with 120 hours (66.46%) and 132 hours (45.34%) (Table 1)

**Table1.** Effect of low temperature stress at 4±1˚C for different embryonic stages on hatching percentage

| Embryonic Stage | Hatching percentage |
|---|---|
| 24hours | 90.85±0.2 |
| 36hours | 89.85±0.22 |
| 48hours | 89.69±0.24 |
| 60hours | 89.37±0.62 |
| 72hours | 89.36±0.47 |
| 84hours | 67.23±0.34 |
| 96hours | 59.94±0.37 |
| 108hours | 57.96±0.50 |
| 120hours | 46.46±0.12 |
| 132hours | 45.34±0.99 |
| 144hours | 44.29±0.43 |
| Control | 82.00±0.05 |
| CD at 5% | 9.52 |

### 4.2.1.2. Effect of temperature stress at 6±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (89.79%) followed non significantly by 36hours (89.75%), 48 hours (89.58% ), 60 hours (89.53%), 72 hours (88.93%) and control (82%) and significantly by 84 hours (59.96%) and 96 hours (46.93%) having non significant variation between the two. Lowest hatching percentage was recorded at 144 hours (18.67%) increased non significantly at 132 hours (22.36%) and 120 hours (25.12%) (Table2).

**Table2.** Effect of low temperature stress at 6±1˚C for different embryonic stages on hatching percentage

| Embryonic Stage | Hatching percentage |
|---|---|
| 24hours | 89.79±0.189 |
| 36hours | 89.75±0.34 |
| 48hours | 89.58±0.55 |
| 60hours | 89.53±0.46 |
| 72hours | 88.93±0.26 |
| 84hours | 59.96±0.78 |
| 96hours | 46.93±0.53 |
| 108hours | 39.74±0.07 |
| 120hours | 25.124±0.1 |
| 132hours | 22.36±0.56 |
| 144hours | 18.676±0.91 |
| Control | 82.00±0.05 |
| CD at 5% | 18.73 |

### 4.2.1.3. Effect of temperature stress at 8±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (89.58%) followed by non significant changes by 36 hours (89.71%), 48 hours (77.74%) and control (82%) and significantly by 60 hours (61.70%), 72 hours (43.59%), 84 hours (39.32%) having non significant variation between them and followed significantly by 96 hours (32.04%). No hatching was recorded at108hours, 120hours, 132hours and 144hours (Table 3).

**Table3.** Effect of low temperature stress at 8±1˚C for different embryonic stages on hatching percentage

| Embryonic Stage | Hatching percentage |
|---|---|
| 24hours | 89.58±0.37 |
| 36hours | 89.71±0.07 |
| 48hours | 77.74±0.64 |
| 60hours | 61.70±0.14 |
| 72hours | 43.59±0.19 |
| 84hours | 39.32±0.44 |
| 96hours | 32.04±0.88 |
| 108hours | 0.0000 |
| 120hours | 0.0000 |
| 132hours | 0.0000 |
| 144hours | 0.000 |
| Control | 82.00±0.05 |
| CD at 5% | 24.47 |

### 4.2.1.4. Effect of temperature stress at 10±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (83.51%) followed non significantly by 36 hours (79.41%), 48 hours (64.76%) and control (82%) and followed significantly by 60 hours (45.95%), 72 hours (39.94%) having non significant variation between the two. (Table 4)

**Table 4.** Effect of low temperature stress at 10±1˚C for different embryonic stages on hatching percentage

.

| Embryonic Stage | Hatching percentage |
|---|---|
| 24 hours | 83.51±0.76 |
| 36 hours | 79.41±0.81 |
| 48 hours | 64.76±0.73 |
| 60 hours | 45.95±0.66 |
| 72 hours | 39.94±0.28 |
| 84 hours | 0.0000 |
| 96 hours | 0.0000 |
| 108 hours | 0.0000 |
| 120 hours | 0.0000 |
| 132 hours | 0.0000 |
| 144 hours | 0.000 |
| Control | 82.00±0.05 |
| CD at 5% | 22.51 |

#### 4.2.1.5. Combined effect of different temperature stresses and different embryonic stages on hatching percentage

24 hours embryo at 4±1˚C showed highest hatching percentage (90.85%) having non significant variation with the same embryonic stage at 6±1˚C(89.79%) and 8±1˚C(89.58%); 36 hours embryonic stages at 4±1˚C(89.85%), 6±1˚C(89.75%), 8±1˚C(89.71%); 48 hours embryonic stages at 4±1˚C(89.69%), 6±1˚C(89.58%), 60 hours at 4±1˚C (89.37%),6±1˚C (89.53%) and 72 hours embryonic stages at 4±1˚C(89.36%), 6±1˚C (88.93%) and significantly by 24 hours embryo at 10±1˚C (83.51%) and by others (Table 5).

So it was observed that control treatments and the embryonic stages up to 72 hours when treated with 4±1˚ and 6±1˚, temperature stress showed non significant variation in hatching percentage. As the temperature shock decreased to 8±1˚C or 10±1˚ the hatching percentage was also decreased but still close to control up to 48 hours of embryonic stage.

Moreover no hatching from 84 hours onwards embryonic ages was found at 10±1˚C from 108 hour embryonic stage at 8±1˚C (Table 5).

**Table 5.** Combined effect of different low temperature stresses for different embryonic stages on hatching percentage

| | 4±1˚C | 6±1˚C | 8±1˚C | 10±1˚C |
|---|---|---|---|---|
| 24h | 90.85±0.2 | 89.79±0.19 | 89.58±0.37 | 83.51±0.76 |
| 36h | 89.85±0.22 | 89.75±0.34 | 89.71±0.07 | 79.41±0.81 |
| 48h | 89.69±0.24 | 89.58±0.55 | 77.74±0.64 | 64.76±0.73 |
| 60h | 89.37±0.62 | 89.53±0.46 | 61.70±0.14 | 45.95±0.66 |
| 72h | 89.36±0.47 | 88.93±0.26 | 43.59±0.19 | 39.94±0.28 |
| 84h | 67.23±0.34 | 59.96±0.78 | 39.32±0.44 | 0.0000 |
| 96h | 59.94±0.37 | 46.93±0.53 | 32.04±0.88 | 0.0000 |
| 108h | 57.96±0.50 | 39.74±0.07 | 0.0000 | 0.0000 |
| 120h | 46.46±0.12 | 25.124±0.1 | 0.0000 | 0.0000 |
| 132h | 45.34±0.99 | 22.36±0.56 | 0.0000 | 0.0000 |
| 144h | 44.29±0.43 | 18.676±0.91 | 0.0000 | 0.0000 |
| CD at 5% | 3.07 | | | |

### 4.2.2 Effect of different temperature stress on incubation period of different embryonic stages

#### 4.2.2.1 Effect of temperature stress at 4±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 108 hours (18.72 days) non significantly followed by 120 hours (18.66 days), 132 hours (18.62 days) and144 hours (18.60 days) and followed significantly by 60 hours (18.17 days), 48 hours (18.16 days), 72 hours (18.14 days),84 hours (18.04 days) and 96 hours (18 days). Lowest incubation period was observed from 24 hours (16.96 days) followed significantly by 36 hours (17.98 days). Incubation period was significantly higher in all the treatments over control (7days) (Table 6).

**Table 6.** Effect of low temperature stress at 4±1˚C for different embryonic stages on incubation period (days)

| Embryonic Stage | Incubation Period(days) |
|---|---|
| 24h | 16.96±0.31 |
| 36h | 17.58±0.09 |
| 48h | 18.16±0.54 |
| 60h | 18.17±0.56 |
| 72h | 18.14±0.62 |
| 84h | 18.04±0.62 |
| 96h | 18.00±0.67 |
| 108h | 18.72±0.75 |
| 120h | 18.66±0.81 |
| 132h | 18.62±0.87 |
| 144h | 18.60±0.95 |
| Control | 7±0.03 |
| CD at 5% | 0.31 |

#### 4.2.2.2 Effect of temperature stress at 6±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 48 hours (18.14 days) non significantly followed by 72 hours (18.13 days), 84 hours (17.86 days), 60 hours (17.75 days), 96 hours (17.43 days), 36 hours (17.42 days) and 24 hours (17.08 days). Lowest incubation period was observed at 144hours (11.02 days) and non significantly followed by 132hours (11.03 days) and 120hours (12.83 days) embryo. Incubation period was significantly higher in all the treatments over control (7days) (Table 7).

**Table 7.** Effect of low temperature stress at 6±1˚C for different embryonic stages on incubation period (days)

| Embryonic Stage | Incubation Period (days) |
|---|---|
| 24h | 17.08±0.35 |
| 36h | 17.42±0.59 |
| 48h | 18.14±0.97 |
| 60h | 17.75±0.20 |
| 72h | 18.13±0.52 |
| 84h | 17.86±0.34 |
| 96h | 17.43±0.25 |
| 108h | 16.06±0.26 |
| 120h | 12.83±0.94 |
| 132h | 11.03±0.35 |
| 144h | 11.02±0.36 |
| CD at 5% | 1.50 |

### 4.2.2.3 Effect of temperature stress at 8±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 24 hours (16.95days), non significantly followed by 60 hours (16.92 days), 48 hours (16.77days), 36 hours (16.55days), 72 hours (15.21days), 84 hours (15.12 days) and 96 hours (15.04 days) and significantly by control (7days). As no hatching was observed from 108 hours, zero incubation days recorded. Incubation period was significantly higher in all the treatments over control (7days) till 96 hours(Table 8).

**Table 8.** Effect of low temperature stress at 8±1˚C for different embryonic stages on Incubation period (days)

| Embryonic Stage | Incubation Period ( days) |
|---|---|
| 24h | 16.95±0.09 |
| 36h | 16.55±0.5 |
| 48h | 16.77±0.36 |
| 60h | 16.92±0.62 |
| 72h | 15.21±0.88 |
| 84h | 15.12±0.92 |
| 96h | 15.04±0.97 |
| 108h | 0 |
| 120h | 0 |
| 132h | 0 |
| 144h | 0 |
| Control | 7±0.03 |
| CD at 5% | 4.81 |

#### 4.2.2.4 Effect of temperature stress at 10±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 24 hours (16.93 days) non significantly followed by 36 hours (16.48 days), 48 hours and 60 hours (16.39 days) and 72 hours (15.01 days). As no hatching was observed from 84 hours, zero incubation days were recorded. Incubation period was significantly higher in all the treatments over control (7days) till 72 hours(Table 9)

**Table 9.**Effect of low temperature stress at 10±1˚C for different embryonic stages on incubation period (days)

| Embryonic Stage | Incubation Period (days) |
|---|---|
| 24h | 16.93±0.05 |
| 36h | 16.48±0.87 |
| 48h | 16.39±0.07 |
| 60h | 16.39±0.34 |
| 72h | 15.01±0.04 |
| 84h | 0 |
| 96h | 0 |
| 108h | 0 |
| 120h | 0 |
| 132h | 0 |
| 144h | 0 |
| Control | 7±0.03 |
| CD at 5% | 5.09 |

#### 4.2.2.5 Combined effect of different temperature stresses and different embryonic stages on incubation period

It revealed that highest incubation period was observed at 4±1˚C low temperature stress, by108 hours (18.72 days), non significantly followed by 4±1˚C x 120 hours (18.66 days), 4±1˚C x 132 hours (18.62 days) and 4±1˚C x 144 hours (18.60 days) and significantly by 4±1˚C x 60hours (18.17 days), 4±1˚C x 48hours (18.16 days), 4±1˚C x 72 hours (18.14 days), 4±1˚C x 84 hours (18.04 days), 6±1˚C x 48 hours (18.14 days) 6±1˚C x 72 hours (18.13 days) having non significant variation among them. Lowest incubation period was observed from 6±1˚C x 144 hours (11.02 days) followed non significantly by 6±1˚C x 132 hours (11.03 days) (Table 10).

**Table 10.** Combined effect of different low temperature stress for different embryonic stages on incubation period (days)

| | 4±1˚C | 6±1˚C | 8±1˚C | 10±1˚C |
|---|---|---|---|---|
| 24h | 16.96±0.31 | 17.08±0.35 | 16.95±0.09 | 16.93±0.05 |
| 36h | 17.58±0.09 | 17.42±0.59 | 16.55±0.5 | 16.48±0.87 |
| 48h | 18.16±0.54 | 18.14±0.97 | 16.77±0.36 | 16.39±0.07 |
| 60h | 18.17±0.56 | 17.75±0.20 | 16.92±0.62 | 16.39±0.34 |
| 72h | 18.14±0.62 | 18.13±0.52 | 15.21±0.88 | 15.01±0.04 |
| 84h | 18.04±0.62 | 17.86±0.34 | 15.12±0.92 | 0 |
| 96h | 18.00±0.67 | 17.43±0.25 | 15.04±0.97 | 0 |
| 108h | 18.72±0.75 | 16.06±0.26 | 0 | 0 |
| 120h | 18.66±0.81 | 12.83±0.94 | 0 | 0 |
| 132h | 18.62±0.87 | 11.03±0.35 | 0 | 0 |
| 144h | 18.60±0.95 | 11.02±0.36 | 0 | 0 |
| CD at 5% | 0.23 | | | |

Finally it can be said that embryonic stages up to 72 hours showed better hatching percentage (90.85 to 88.93%) over control (82%) though the variations were non significant for both low temperature stresses 4±1˚C and 6±1˚C. Incubation period however, was found highest in late embryonic ages only in 4±1˚C, but for 6±1˚C up to 96 hours the incubation periods were longer. But if the embryonic age were considered to be deleted from the actual ten days shock (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7days respectively for 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144hours), then early ages up to 96 hours showed higher incubation periods.

For higher levels of temperature shock that is 8±1˚C and 10±1˚C up to 36 hours old embryo showed higher hatching percentage than control though the variations were non significant. For these two temperatures incubation period were found longer up to 72 hours. For 8±1˚C up to 96 hours can be considered as after that no hatching was observed (Table 10a). Regression analysis also confirmed present findings (Plate 12).

**Table 10a.** Combined effect of different temperature stress and embryonic age on hatching percentage and incubation period

| | 4±1˚C | | 6±1˚C | | 8±1˚C | | 10±1˚C | |
|---|---|---|---|---|---|---|---|---|
| | H | I | H | I | H | I | H | I |
| 24h | 90.85±0.2 | 16.96±0.31 | 89.79±0.19 | 17.08±0.35 | 89.58±0.37 | 16.95±0.09 | 83.51±0.76 | 16.93±0.05 |
| 36h | 89.85±0.22 | 17.58±0.09 | 89.75±0.34 | 17.42±0.59 | 89.71±0.07 | 16.55±0.5 | 79.41±0.81 | 16.48±0.87 |
| 48h | 89.69±0.24 | 18.16±0.54 | 89.58±0.55 | 18.14±0.97 | 77.74±0.64 | 16.77±0.36 | 64.76±0.73 | 16.39±0.07 |
| 60h | 89.37±0.62 | 18.17±0.56 | 89.53±0.46 | 17.75±0.20 | 61.70±0.14 | 17.25±0.62 | 45.95±0.66 | 16.39±0.34 |
| 72h | 89.36±0.47 | 18.14±0.62 | 88.93±0.26 | 18.13±0.52 | 43.59±0.19 | 15.21±0.88 | 39.94±0.28 | 15.01±0.04 |
| 84h | 67.23±0.34 | 18.04±0.62 | 59.96±0.78 | 17.86±0.34 | 39.32±0.44 | 15.12±0.92 | 0 | 0 |
| 96h | 59.94±0.37 | 18.00±0.67 | 46.93±0.53 | 17.43±0.25 | 32.04±0.88 | 15.04±0.97 | 0 | 0 |
| 108h | 57.96±0.50 | 18.72±0.75 | 39.74±0.07 | 16.06±0.26 | 0 | 0 | 0 | 0 |
| 120h | 46.46±0.12 | 18.66±0.81 | 25.124±0.1 | 12.83±0.94 | 0 | 0 | 0 | 0 |
| 132h | 45.34±0.99 | 18.62±0.87 | 22.36±0.56 | 11.03±0.35 | 0 | 0 | 0 | 0 |
| 144h | 44.29±0.43 | 18.60±0.95 | 18.676±0.91 | 11.02±0.36 | 0 | 0 | 0 | 0 |

So it can be concluded that embryonic stages of 24, 36, 48, 60, 72, 84 and 96 hours can be identified as low temperature resistant embryonic stages, more precisely,up to 96 hours embryonic stages can be explored for improvement of cold preservation technology of seed.

### 4.3 Effect of different low temperature stress in identified low temperature resistant embryonic stages

The identified low temperature resistant stages namely 24, 36,48, 60, 72, 84 and 96 hours old embryonic stages were given low temperature stresses from 4±1˚C to 10±1˚C with 1˚C interval that is 4±1˚C, 5±1˚C, 6±1˚C, 7±1˚C, 8±1˚C, 9±1˚C and 10±1˚C to determine the optimum low temperature for cold stress for successful low temperature preservation basedon hatching percent and incubation days. Data thus obtained were recorded and analyzed statistically.

#### 4.3.1. Effect of different low temperature stress in identified low temperature resistant embryonic stages on hatching percentage

##### 4.3.1.1. Effect of different low temperature stress in 24 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (90.85%), followed non significantly by 5±1˚C (90.32%), 6±1˚C (89.79%), 7±1˚C (89.69%), 8±1˚C (89.58%) and 9±1˚C (89.54%) and significantly by 10±1˚C (83.51%). Experimental treatments till 10±1˚C (83.51%) had shown better hatching percentage than hatching percent in normal condition (82%) ( Table 11).

**Table 11.** Effect of different low temperature stress on hatching percentage for 24 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 90.85±0.02 |
| 5±1˚C | 90.32±0.06 |
| 6±1˚C | 89.79±0.19 |
| 7±1˚C | 89.69±0.33 |
| 8±1˚C | 89.58±0.37 |
| 9±1˚C | 89.54±0.03 |
| 10±1˚C | 83.51±0.76 |
| CD at 5% | 2.69 |

#### 4.3.1.2. Effect of different low temperature stress in 36 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.85%), followed non significantly by 5±1˚C (89.79%), 6±1˚C (89.75%), 7±1˚C (88.23%) and 8±1˚C (87.71%) and significantly by 9±1˚C (82.56%). Lowest hatching percentage was recorded at 10±1˚C (79.41%) having non significant variation with9±1˚C. Temperature stress up to 8±1˚C showed better performance than normal condition (82%). (Table 12)

**Table 12**. Effect of different low temperature stress on hatching percentage for 36 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 89.85±0.22 |
| 5±1˚C | 89.79±0.17 |
| 6±1˚C | 89.75±0.34 |
| 7±1˚C | 88.23±0.05 |
| 8±1˚C | 87.71±0.07 |
| 9±1˚C | 82.56±0.67 |
| 10±1˚C | 79.41±0.81 |
| CD at 5% | 3.18 |

#### 4.3.1.3 Effect of different low temperature stress in 48 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.69%), followed non significantly by 5±1˚C (89.64%) and 6±1˚C (89.58%) and significantly by 7±1˚C (78.72%) and others. Lowest hatching percentage was recorded at 10±1˚C (64.76%) followed significantly by 9±1˚C (72.25%). Temperature stress up to 6±1˚C showed better performance than normal condition (82%).(Table 13)

**Table 13**. Effect of different low temperature stress on hatching percentage for 48 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 89.69±0.24 |
| 5±1˚C | 89.64±0.44 |
| 6±1˚C | 89.58±0.55 |
| 7±1˚C | 78.72±0.12 |
| 8±1˚C | 77.74±0.64 |
| 9±1˚C | 72.25±0.07 |
| 10±1˚C | 64.76±0.73 |
| CD at 5% | 6.91 |

#### 4.3.1.4 Effect of different low temperature stress in 60 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 5±1˚C (89.59%), followed non significantly by 6±1˚C (89.53%) and 4±1˚C (88.37%) and significantly by 7±1˚C (71.62%) and others. Lowest hatching percentage was recorded at 10±1˚C (45.95%) followed non significantly by 9±1˚C (54.83%) and significantly by others. Temperature stress up to 6±1˚C showed better performance than normal condition (82%). (Table 14)

**Table 14**. Effect of different low temperature stress on hatching percentage for 60 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 88.37±0.62 |
| 5±1˚C | 89.59±0.38 |
| 6±1˚C | 89.53±0.46 |
| 7±1˚C | 71.62±0.62 |
| 8±1˚C | 61.70±0.14 |
| 9±1˚C | 54.83±0.31 |
| 10±1˚C | 45.95±0.66 |
| CD at 5% | 12.93 |

#### 4.3.1.5 Effect of different low temperature stress in 72 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.36%), followed non significantly by 5±1˚C (89.14%) and 6±1˚C (88.93%) and significantly by 7±1˚C (60.48%) and others. Lowest hatching percentage was recorded at 10±1˚C (39.94%) followed non significantly by 9±1˚C (41.77%) and 8±1˚C (43.59%). Temperature stress up to 6±1˚C showed better performance than normal condition (82%). (Table 15)

**Table15.**Effect of different low temperature stress on hatching percentage for 72 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 89.36±0.47 |
| 5±1˚C | 89.14±0.09 |
| 6±1˚C | 88.93±0.26 |
| 7±1˚C | 60.48±0.51 |
| 8±1˚C | 43.59±0.19 |
| 9±1˚C | 41.77±0.16 |
| 10±1˚C | 39.94±0.28 |
| CD at 5% | 16.39 |

#### 4.3.1.6 Effect of different low temperature stress in 84 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (67.23%), followed non significantly by 5±1˚C (66.6%), 6±1˚C (59.96%) and 7±1˚C (50.64%) and significantly decreased at 8±1˚C (39.32) and 9±1˚C (29.66%). No hatching was recorded at 10±1˚C. All temperature stresses showed poor performances than normal condition (82%). (Table 16)

**Table 16**. Effect of different low temperature stress on hatching percentage for 84 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 67.23±0.34 |
| 5±1˚C | 66.6±0.51 |
| 6±1˚C | 59.96±0.78 |
| 7±1˚C | 50.64±0.07 |
| 8±1˚C | 39.32±0.44 |
| 9±1˚C | 29.66±0.11 |
| 10±1˚C | 0 |
| CD at 5% | 18.75 |

#### 4.3.1.7 Effect of different low temperature stress in 96 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (59.94%), followed non significantly by 5±1˚C (59.43%) and 6±1˚C (46.93%) and significantly by 7±1˚C (39.49%) and 8±1˚C (32.04%). No hatching was recorded at 9±1˚C and 10±1˚C. All temperature stresses showed poor performances than normal condition (82%). (Table 17)

**Table 17**.Effect of different low temperature stress on hatching percentage for 96 hours embryonic stage

| Temperature | Hatching percentage |
|---|---|
| 4±1˚C | 59.94±0.37 |
| 5±1˚C | 59.43±0.06 |
| 6±1˚C | 46.93±0.53 |
| 7±1˚C | 39.49±0.56 |
| 8±1˚C | 32.04±0.88 |
| 9±1˚C | 0 |
| 10±1˚C | 0 |
| CD at 5% | 19.6 |

#### 4.3.1.8Combined effect of different low temperature stress on identified low temperature resistant embryonic stages on hatching percentage

Highest hatching % was observedup to9±1˚C for 24 hours, up to 7±1˚C for 36hours and up to 6±1˚C for 48 to 72 hours of embryonic stages having non significant variation among them (90.85 to 88.23%). 36 hours embryonic stage having 8±1˚C temperature stress showed hatching percentage of 87.71% while at 9±1˚C this stage show 82.56% hatching. All these hatching percentages are higher than or at per hatching in normal condition (82%) (Table 18).

**Table 18.** Combined effect of different low temperature stresses on hatching percentage for identified low temperature resistant embryonic stages

| | 4±1˚C | 5±1˚C | 6±1˚C | 7±1˚C | 8±1˚C | 9±1˚C | 10±1˚C |
|---|---|---|---|---|---|---|---|
| 24h | 90.85±0.2 | 90.32±0.06 | 89.79±0.189 | 89.69±0.33 | 89.58±0.37 | 89.54±0.03 | 83.51±0.76 |
| 36h | 89.85±0.22 | 89.79±0.17 | 89.75±0.34 | 88.23±0.05 | 87.71±0.07 | 82.56±0.67 | 79.41±0.81 |
| 48h | 89.69±0.24 | 89.64±0.44 | 89.58±0.55 | 78.72±0.12 | 77.74±0.64 | 72.25±0.07 | 64.76±0.73 |
| 60h | 89.37±0.62 | 89.59±0.38 | 89.53±0.46 | 71.62±0.62 | 61.70±0.14 | 54.83±0.31 | 45.95±0.66 |
| 72h | 89.36±0.47 | 89.14±0.09 | 88.93±0.26 | 60.48±0.51 | 43.59±0.19 | 41.77±0.16 | 39.94±0.28 |
| 84h | 67.23±0.34 | 66.6±0.51 | 59.96±0.78 | 50.64±0.07 | 39.32±0.44 | 29.66±0.11 | 0 |
| 96h | 59.94±0.37 | 59.43±0.06 | 46.93±0.53 | 39.49±0.56 | 32.04±0.88 | 0 | 0 |
| CD at 5% | 2.36 | | | | | | |

### 4.3.2. Effect of different low temperature stress in identified low temperature resistant embryonic stages on incubation period

#### 4.3.2.1 Effect of different low temperature stress in 24 hours embryonic stage on incubation period

Highest incubation period was observed at 6±1˚C (17.08 days) followed significantly by 5±1˚C (17.02 days), 7±1˚C (16.97 days), and 4±1˚C (16.96 days) and others. Lowest incubation period was observed from 10±1˚C (16.93 days). (Table 19)

**Table 19.**Effect of different low temperature stress on incubation period (Days) for 24 hours embryonic stage

| Temperature | Incubation Period ( Days) |
|---|---|
| 4±1˚C | 16.96±0.31 |
| 5±1˚C | 17.02±0.17 |
| 6±1˚C | 17.08±0.35 |
| 7±1˚C | 16.97±0.03 |
| 8±1˚C | 16.95±0.09 |
| 9±1˚C | 16.94±0.51 |
| 10±1˚C | 16.93±0.05 |
| CD at 5% | 0.04 |

#### 4.3.2.2 Effect of different low temperature stress in 36 hour embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (17.58 days), followed non significantly by 6±1˚C (17.42 days) and 5±1˚C (17.03 days) and followed significantly by 7±1˚C (16.92 days) and others. Lowest incubation period was observed at 10±1˚C (16.48 days) having non significant variation with 9±1˚C (16.59 days), 8±1˚C (16.55 days) and7±1˚C (16.92 days). (Table 20)

**Table 20.** Effect of different low temperature stress on incubation period (Days) for 36 hours embryonic stage

| Temperature | Incubation Period (Days) |
|---|---|
| 4±1˚C | 17.58±0.09 |
| 5±1˚C | 17.03±0.23 |
| 6±1˚C | 17.42±0.59 |
| 7±1˚C | 16.92±0.44 |
| 8±1˚C | 16.55±0.5 |
| 9±1˚C | 16.59±0.81 |
| 10±1˚C | 16.48±0.87 |
| CD at 5% | 0.56 |

#### 4.3.2.3 Effect of different low temperature stress in 48 hours embryonic stages on incubation period

Highest incubation period was observed at 5±1˚C (18.17 days), followed non significantly by 4±1˚C (18.16 days), 6±1˚C (18.14 days) and significantly by 7±1˚C (16.90 days) and others. Lowest incubation period was observed at 10±1˚C (16.39 days) having non significant variation with 9±1˚C (16.54 days), 8±1˚C (16.77 days) and 7±1˚C (16.90 days). (Table 21)

**Table 21.** Effect of different low temperature stress on incubation period (Days) for 48 hours embryonic stage

| Temperature | Incubation Period (Days) |
|---|---|
| 4±1˚C | 18.16±0.54 |
| 5±1˚C | 18.17±0.15 |
| 6±1˚C | 18.14±0.97 |
| 7±1˚C | 16.90±0.15 |
| 8±1˚C | 16.77±0.36 |
| 9±1˚C | 16.54±0.11 |
| 10±1˚C | 16.39±0.07 |
| CD at 5% | 0.71 |

#### 4.3.2.4 Effect of different low temperature stress in 60 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.17 days), followed non significantly by 6±1˚C (17.75 days), 5±1˚C (17.57 days) and followed significantly by 8±1˚C (16.92 days) and others. Lowest incubation period was observed at 10±1˚C (16.39 days) having non significant variation with 9±1˚C (16.82 days), 8±1˚C (16.92 days) and 7±1˚C (16.89 days).(Table 22)

**Table 22.** Effect of different low temperature stress on incubation period (Days) for 60 hours embryonic stage

| Temperature | Incubation Period (Days) |
|---|---|
| 4±1˚C | 18.17±0.56 |
| 5±1˚C | 17.57±0.67 |
| 6±1˚C | 17.75±0.20 |
| 7±1˚C | 16.89±0.74 |
| 8±1˚C | 16.92±0.62 |
| 9±1˚C | 16.82±0.45 |
| 10±1˚C | 16.39±0.34 |
| CD at 5% | 0.64 |

#### 4.3.2.5 Effect of different low temperature stress in 72 hours embryonic stages on incubation period

Highest incubation period was observed at 5±1˚C (18.18 days), followed non significantly by 4±1˚C (18.14 days), 6±1˚C (18.13 days) and significantly by 7±1˚C(16.53 days) and others. Lowest incubation period was observed at 10±1˚C (15.01 days) having non significant variation with 9±1˚C (15.16 days), 8±1˚C (15.21 days). (Table 23)

**Table 23.** Effect of different low temperature stress on incubation period (Days) for 72 hours embryonic stage

| Temperature | Incubation Period (Days) |
|---|---|
| 4±1˚C | 18.14±0.62 |
| 5±1˚C | 18.18±0.91 |
| 6±1˚C | 18.13±0.52 |
| 7±1˚C | 16.53±0.47 |
| 8±1˚C | 15.21±0.88 |
| 9±1˚C | 15.16±0.75 |
| 10±1˚C | 15.01±0.04 |
| CD at 5% | 1.27 |

### 4.3.2.6 Effect of different low temperature stress in 84 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.04 days) followed non significantly by 6±1˚C (17.86 days) and 5±1˚C (17.65 days) and significantly by7±1˚C (16.09 days) and others. Lowest incubation period was observed at 9±1˚C (13.46 days) At 10±1˚C no hatching was observed. (Table 24)

**Table 24.** Effect of different low temperature stress on incubation period (Days) for 84 hours embryonic stage

| Temperature | Incubation Period (Days) |
|---|---|
| 4±1˚C | 18.04±0.62 |
| 5±1˚C | 17.65±0.01 |
| 6±1˚C | 17.86±0.34 |
| 7±1˚C | 16.09±0.13 |
| 8±1˚C | 15.12±0.92 |
| 9±1˚C | 13.46±0.21 |
| 10±1˚C | 0 |
| CD at 5% | 1.46 |

### 4.3.2.7 Effect of different low temperature stress in 96 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.00 days) followed non significantly by 5±1˚C (17.46 days) and 6±1˚C (17.43 days) and significantly by 7±1˚C (15.66 days) and 8±1˚C (15.04 days). No hatching was observed at 9±1˚C and 10±1˚C (Table 25)

**Table 25.** Effect of different low temperature stress on incubation period (Days) for 96 hours embryonic stage

| Temperature | Incubation Period(Days) |
|---|---|
| 4±1˚C | 18.00±0.67 |
| 5±1˚C | 17.46±0.09 |
| 6±1˚C | 17.43±0.25 |
| 7±1˚C | 15.66±0.15 |
| 8±1˚C | 15.04±0.97 |
| 9±1˚C | 0 |
| 10±1˚C | 0 |
| CD at 5% | 1.13 |

### 4.3.2.8 Combined effect of different low temperature stress on identified low temperature resistant embryonic stages on incubation period

Highest incubation period was observed from 4±1˚C temperature stress on 48 hours to 96 hours embryo (18.0 to 18.17 days); 48 and 72 hours at both 5±1˚C and 6±1˚C temperature stress (18.13 to 18.18 days) followed significantly by 60 hours (16.96 days) and 84 hours(17.86 days) embryo at 6±1˚C and also significantly with 60 hours (17.57 days) and 84 hours (17.65 days) egg at 5±1˚C and 36 hours embryo at 4±1˚C(17.58 days) having non significant variation among them. More over from 7±1˚C to higher range, performance were poor and even at 10±1˚C, hatching was not observed beyond 72 hours.(Table 34)

**Table 26.** Combined effect of different low temperature stresses on incubation period for identified low temperature resistant embryonic stages

| | 4±1˚C | 5±1˚C | 6±1˚C | 7±1˚C | 8±1˚C | 9±1˚C | 10±1˚C |
|---|---|---|---|---|---|---|---|
| 24h | 16.96±0.31 | 17.02±0.17 | 17.08±0.35 | 16.99±0.03 | 16.95±0.09 | 16.94±0.51 | 16.93±0.05 |
| 36h | 17.58±0.09 | 17.03±0.23 | 17.42±0.59 | 16.97±0.44 | 16.55±0.5 | 16.59±0.81 | 16.48±0.87 |
| 48h | 18.16±0.54 | 18.17±0.15 | 18.14±0.97 | 16.94±0.15 | 16.77±0.36 | 16.54±0.11 | 16.39±0.07 |
| 60h | 18.17±0.56 | 17.57±0.67 | 16.96±0.20 | 16.93±0.74 | 16.92±0.62 | 16.42±0.45 | 16.39±0.34 |
| 72h | 18.14±0.62 | 18.18±0.07 | 18.13±0.52 | 16.53±0.47 | 15.21±0.88 | 15.16±0.75 | 15.01±0.04 |
| 84h | 18.04±0.62 | 17.65±0.01 | 17.86±0.34 | 16.09±0.13 | 15.12±0.92 | 13.46±0.21 | 0 |
| 96h | 18.00±0.67 | 17.46±0.16 | 17.43±0.25 | 15.66±0.15 | 15.04±0.97 | 0 | 0 |
| CD at 5% | 0.19 | | | | | | |

Again it was found that embryonic stages up to 72 hours performed better when the temperature stress was high **(**4±1˚C to 6±1˚C) when compared to normal condition. Though, early embryonic stages could survive well even in 10±1˚C also. More over it was observed that 60% hatching could be done up to 96 hour when treated with very low cold shock.

Similarly incubation days become shorter in older embryos with the increase of temperature. However, very early embryos showed longer incubation days even in 10±1˚C (Table 27). Regression analysis also confirmed present findings (Plate 13).

**Table 27.** Combined effect of different temperature stress and identified low temperature resistant embryonic age on hatching percentage and incubation period

| | 4±1˚C | | 5±1˚C | | 6±1˚C | | 7±1˚C | | 8±1˚C | | 9±1˚C | | 10±1˚C | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | H | I | H | I | H | I | H | I | H | I | H | I | H | I |
| 24h | 90.85 ±0.2 | 17.18 ±0.31 | 90.32 ±0.06 | 17.26 ±0.17 | 89.79± 0.189 | 17.33 ±0.35 | 95.69 ±0.33 | 16.79 ±0.03 | 99.58 ±0.37 | 16.25 ±0.09 | 93.54 ±0.03 | 17.59 ±0.51 | 93.51 ±0.76 | 18.93 ±0.05 |
| 36h | 89.85 ±0.22 | 17.58 ±0.09 | 89.3± 0.17 | 17.03 ±0.23 | 84.75± 0.34 | 16.48 ±0.59 | 88.23 ±0.05 | 17.52 ±0.44 | 89.71 ±0.07 | 18.55 ±0.5 | 82.56 ±0.67 | 17.99 ±0.81 | 79.41 ±0.81 | 17.42 ±0.87 |
| 48h | 89.58 ±0.24 | 18.22 ±0.54 | 89.64 ±0.44 | 17.31 ±0.55 | 79.69± 0.55 | 16.39 ±0.97 | 78.72 ±0.12 | 17.3± 0.15 | 77.74 ±0.64 | 18.20 ±0.36 | 72.25 ±0.07 | 17.49 ±0.11 | 64.76 ±0.73 | 16.77 ±0.07 |
| 60h | 88.37 ±0.62 | 18.17 ±0.56 | 88.95 ±0.38 | 17.57 ±0.67 | 79.53± 0.46 | 16.96 ±0.20 | 71.62 ±0.62 | 17.1± 0.74 | 61.70 ±0.14 | 17.25 ±0.62 | 54.83 ±0.31 | 16.82 ±0.45 | 45.95 ±0.66 | 16.39 ±0.34 |
| 72h | 86.93 ±0.26 | 18.13 ±0.62 | 86.14 ±0.09 | 17.84 ±0.91 | 77.36± 0.47 | 17.54 ±0.52 | 60.48 ±0.51 | 16.53 ±0.47 | 43.59 ±0.19 | 15.51 ±0.88 | 41.77 ±0.16 | 15.26 ±0.75 | 39.94 ±0.28 | 15.01 ±0.04 |
| 84h | 67.23 ±0.34 | 18.44 ±0.62 | 66.6± 0.51 | 17.65 ±0.01 | 59.96± 0.78 | 16.86 ±0.34 | 50.64 ±0.07 | 16.09 ±0.13 | 39.32 ±0.44 | 15.32 ±0.92 | 29.66 ±0.11 | 13.46 ±0.21 | 0 | 0 |
| 96h | 59.94 ±0.37 | 18.76 ±0.67 | 59.43 ±0.06 | 17.47 ±0.09 | 46.93± 0.53 | 16.18 ±0.25 | 39.49 ±0.56 | 15.66 ±0.15 | 32.04 ±0.88 | 15.14 ±0.97 | 0 | 0 | 0 | 0 |

So from overall results, up to 96 hours of embryo could be identified as low temperature resistant embryonic stages excluding the in between hours (36, 60 and 84 hours) of respective day old embryo having non significant variation for better handling and easy identification during large quantity preservation. . And at the same time, low temperatures with in 4±1˚C and 6±1˚C could be applied for determination of optimum low temperature stress period.

### 4.4. Effect of preservation periods after low temperature stress to the identified embryonic stages

24 , 48, 72 and 96 hours embryonic stages were given 4˚, 5˚ & 6±1˚C temperature stress at different preservation periods namely 3 days, 7 days, 10 days, 15 days and 21 days to determine the optimum periods of low temperature stress. Days delay means the period from egg laying to hatching excluding the preservation days. Data thus obtained were analyzed statistically.

### 4.4.1 Effect of preservation period after low temperature stress to the identified embryonic stages on hatching percentage

#### 4.4.1.1 Effect of preservation period for 3 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.92%) having non-significant variation with all the treatments except the hatching percentage of 96 hours old egg at 5˚ & 6±1˚C. (Table 28)

**Table 28.** Effect of 3 days preservation on hatching percentage of identified embryonic stages at low temperatures

| 3d | 4±1˚C | 5±1˚C | 6±1˚C |
|---|---|---|---|
| 24h | 90.92±0.05 | 90.88±0.25 | 90.83±0.14 |
| 48h | 90.65±0.41 | 90.66±0.04 | 90.7±0.64 |
| 72h | 90.63±0.09 | 90.6±0.23 | 90.58±0.85 |
| 96h | 90.54±0.35 | 90.48±0.15 | 90.4±0.41 |
| CD at.5% | 0.407 | | |

### 4.4.1.2 Effect of preservation period for 7 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.9%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 7 days preservation showed lower hatching percentage, significantly being lowest from the embryo at 6±1˚C temperature stress.. (Table 29)

**Table 29**. Effect of 7 days preservation on hatching percentage of identified embryonic stages at low temperatures

| 7 d | 4±1˚C | 5±1˚C | 6±1˚C |
|---|---|---|---|
| 24h | 90.9±0.14 | 90.79±0.17 | 90.68±0.04 |
| 48h | 90.56±0.96 | 90.56±0.55 | 90.56±0.18 |
| 72h | 90.54±0.98 | 90.53±0.55 | 90.50±0.11 |
| 96h | 80.16±0.23 | 77.51±0.37 | 73.36± 0.45 |
| CD at 5% | 3.37 | | |

#### 4.4.1.3 Effect of preservation period for 10 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.85%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 10 days preservation showed significantly poor hatching percentage, lowest being from the embryo at 6±1˚C (43.93%) temperature stress (Table 30)

**Table 30.** Effect of 10 days preservation on hatching percentage of identified embryonic stages at low temperatures

| 10 d | 4±1˚C | 5±1˚C | 6±1˚C |
|---|---|---|---|
| 24h | 90.85±0.2 | 90.32±0.06 | 89.79±0.189 |
| 48h | 89.69±0.24 | 89.64±0.44 | 89.58± 0.55 |
| 72h | 89.36±0.47 | 89.14±0.09 | 88.93± 0.26 |
| 96h | 59.94±0.37 | 59.43±0.06 | 46.93± 0.53 |
| CD at 5% | 3.81 | | |

#### 4.4.1.4 Effect of preservation period for 15 days on hatching percentage

Highest hatching percentage was recorded on 48 hours embryonic stage at 4±1˚C (89.66%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 15 days preservation showed significantly very poor hatching percentage, lowest being from the embryo at 6±1˚C (19.46%) temperature stress (Table 31)

**Table 31.** Effect of 15 days preservation on hatching percentage of identified embryonic stages at low temperatures

| 15 d | 4±1˚C | 5±1˚C | 6±1˚C |
|---|---|---|---|
| 24h | 89.54±0.61 | 89.58±0.35 | 89.63±0.8 |
| 48h | 89.66±0.55 | 89.45±0.22 | 89.39±0.29 |
| 72h | 88.76±0.76 | 88.69±0.03 | 88.63±0.59 |
| 96h | 19.93±0.6 | 19.51±0.02 | 19.46±0.44 |
| CD at 5% | 5.51 | | |

#### 4.4.1.5 Effect of preservation period for 21 days on hatching percentage

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (55.05%) having non-significant variation with 5±1˚C (53.15%) and 6±1˚C (50.1%) and 48 hour embryo at 4±1˚C (49.8%) and significantly by others up to 72 hours old embryo. As no larva hatched, hatching percentage was zero from 96 hour embryo irrespective of temperature stress. (Table 32)

**Table 32.** Effect of 21days preservation on hatching percentage of identified embryonic stages at low temperatures

| 21d | 4±1˚C | 5±1˚C | 6±1˚C |
|---|---|---|---|
| 24h | 55.05±0.11 | 53.15±0.43 | 50.1±0.49 |
| 48h | 49.8±0.59 | 28.18±0.15 | 15.16±0.21 |
| 72h | 39.93±0.52 | 22.07±0.7 | 12.1±0.07 |
| 96h | 0 | 0 | 0 |
| CD at 5% | 11.13 | | |

#### 4.4.1.6 Combined effect of preservation period, temperature stress and embryonic age on hatching percentage

Highest hatching percentage was observed from 24 hour and 48 hour old embryo when preserved for 3, 7, 10 and 15 days; from 72 hours old embryo for 3,7 and 10 days and also from 96 hours old embryo for 3 days irrespective of temperature stress among 4±1˚C to 6±1˚C ( 88.93 to 90.9%) significantly followed by 72 hours when preserved for 15 days irrespective of temperatures (88.62 to 88.7%). 96 hours old embryo when preserved for 7 days showed better performance also.(73.30 to 80.10%). Any embryonic age from 24 to 96 hours when preserved for 21 days irrespective of any temperature showed very poor performance even no hatching from 96 hours embryo. (Table 33)

**Table 33.** Combined effect of preservation period, temperature stress and embryonic age on hatching percentage

| Preservation Day | 3d | | | 7d | | | 10d | | | 15d | | | 21d | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4±1 ˚C | 5±1 ˚C | 6±1 ˚C | 4±1 ˚C | 5±1 ˚C | 6±1 ˚C | 4±1˚ C | 5±1˚ C | 6±1˚ C | 4±1 ˚C | 5±1 ˚C | 6±1 ˚C | 4±1 ˚C | 5±1 ˚C | 6±1 ˚C |
| 24h | 90.92±0.05 | 90.88±0.25 | 90.83±0.14 | 90.9±0.14 | 90.79±0.17 | 90.68±0.04 | 90.85±0.2 | 90.32±0.06 | 89.79±0.189 | 89.54±0.61 | 89.58±0.35 | 89.63±0.8 | 55.05±0.11 | 53.15±0.43 | 50.1±0.49 |
| 48h | 90.65±0.41 | 90.66±0.04 | 90.7±0.64 | 90.56±0.96 | 90.56±0.55 | 90.56±0.18 | 89.69±0.24 | 89.64±0.44 | 89.58± 0.55 | 89.66±0.55 | 89.45±0.22 | 89 .39± 0.29 | 49.8±0.59 | 28.18±0.15 | 15.16±0.21 |
| 72h | 90.63±0.09 | 90.6±0.23 | 90.58±0.85 | 90.54±0.98 | 90.53±0.55 | 90.50±0.11 | 89.36±0.47 | 89.14±0.09 | 88.93± 0.26 | 88.76±0.76 | 88.69±0.03 | 88.63±0.59 | 39.93±0.52 | 22.07±0.7 | 12.1±0.07 |
| 96h | 90.54±0.35 | 90.48±0.15 | 90.4±0.41 | 80.16±0.23 | 77.51±0.37 | 73.36± 0.45 | 59.94±0.37 | 59.43±0.06 | 46.93± 0.53 | 19.93±0.6 | 19.51±0.02 | 19.46±0.44 | 0 | 0 | 0 |
| CD at 5% | 1.972 | | | | | | | | | | | | | | |

### 4.4.2. Effect of preservation period after low temperature stresses to the identified embryonic stages on hatching days delay

#### 4.4.2.1 Effect of preservation period for 3 days

Longest days delay of hatching was observedby 48 hours embryo irrespective of temperature stress (7.16 to 7.23 days) followed significantly by 24 hours embryo irrespective of temperature stress and by 72 hour embryo at 6±1˚C (7.06 to 7.13 days) followed significantly by others. Shortest days delay was observed from 96 hours embryo (6.32 to 6.40 days) (Table 34)

**Table 34.** Effect of 3 days preservation on hatching days delay of identified embryonic stages at low temperatures

| Preservation Day | 3d | | |
|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 7.06±0.02 | 7.08±0.05 | 7.13±0.03 |
| 48h | 7.16±0.07 | 7.22±0.33 | 7.23±0.26 |
| 72h | 7.01±0.05 | 7.09±0.09 | 7.03±0.25 |
| 96h | 6.32±0.11 | 6.37±0.01 | 6.4±0.15 |
| CD at 5% | 0.07 | | |

### 4.4.2.2 Effect of preservation period for 7 days

Longest days delay of hatching was observed by 48 hours embryo at 4±1˚C (8.13 days) followed non significantly by 24 hours at any temperature (8 to 8.1 days), 72 hour at any temperatures (7.99 to 8.06 days) and 48 hours at 6±1˚C (7.93 days). Significantly shorter days delay was recorded from 96 hours embryo.(Table 35)

**Table 35.** Effect of 7 days preservation on hatching days delay of identified embryonic stages at low temperatures

| Preservation Day | 7d | | |
|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 8±0.01 | 8.05±0.04 | 8.1±0.01 |
| 48h | 8.13±0.05 | 7.85±0.19 | 7.93±0.09 |
| 72h | 7.99±0.02 | 8.01±0.41 | 8.06±0.16 |
| 96h | 6.5±0.33 | 6.45±0.05 | 6.40±0.01 |
| CD at 5% | 0.25 | | |

#### 4.4.2.3 Effect of preservation period for 10 days

Longest days delay of hatching was observed by 24 hours and 48 hours embryo irrespective of temperature stress (8.96 to 9.17 days). Significantly followed by 72 hours (8.13 to 8.18 days) and 96 hours (6.43 to 7.0 days) having non significant variation between temperature stress. (Table 36)

**Table 36.** Effect of 10 days preservation on hatching days delay of identified embryonic stages at low temperatures

| Preservation Day | 10d | | |
|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 8.96±0.01 | 9.02±0.31 | 9.08±0.21 |
| 48h | 9.16±0.04 | 9.17±0.15 | 9.14±0.01 |
| 72h | 8.14±0.01 | 8.18±0.07 | 8.13±0.1 |
| 96h | 7.00±0.41 | 6.46±0.16 | 6.43±0.17 |
| CD at 5% | 0.28 | | |

#### 4.4.2.4 Effect of preservation period for 15 days

Longest days delay of hatching was observed by 24 hours and 48 hours embryo irrespective of temperature stress, 72 hours and 96 hours at 4±1˚C (3.1 to 3.96 days) and significantly by others. (Table 37)

**Table 37.** Effect of 15 days preservation on hatching days delay of identified embryonic stages at low temperatures

| Preservation Day | 15d | | |
|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 3.96±0.09 | 3.95±0.15 | 3.96±0.05 |
| 48h | 3.1±0.06 | 3.28±0.07 | 3.96±0.01 |
| 72h | 3.13±0.05 | 3.08±0.04 | 3.03±0.05 |
| 96h | 3.16±0.07 | 3.09±0.08 | 3.03±0.07 |
| CD at 5% | 0.86 | | |

#### 4.4.2.5 Effect of preservation period for 21 days

96 hours embryo showed no hatching. Among the 24 hours and 72 hours, 24 hours old embryo showed 2.08 days delay at 5±1˚C followed significantly by 6±1˚C (2.03 days) and 4±1˚C (1.96 days). Other treatments showed delay less than one day. (Table 38)

**Table 38.** Effect of 21 days preservation on hatching days delay of identified embryonic stages at low temperatures

| Preservation Day | 21d | | |
|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 1.96±0.01 | 2.08±0.01 | 2.03±0.06 |
| 48h | 0.64±0.05 | 0.69±0.04 | 0.63±0.01 |
| 72h | 0.34±0.06 | 0.37±0.15 | 0.35±0.02 |
| 96h | 0 | 0 | 0 |
| CD at 5% | 0.2 | | |

#### 4.4.2.6 Combined effect of preservation period, temperature stress and embryonic age on days delay of hatching

Longest days delay was observed 9.17 days (48 hours embryo at 5±1˚C preserved for 10 days) followed by non significantly by 9.16 days (48 hours embryo at 4±1˚C for 10 days), 9.14 days (48 hours embryo at 6±1˚C for 10 days), 9.08 days (24 hours embryo at 6±1˚C for 10 days), 9.02 days (24 hours embryo at 5±1˚C for 10 days) and significantly followed by 8.96 days (24 hours embryo at 4±1˚C for 10 days), and then non significantly by 8.18 days (72 hours embryo at 5±1˚C for 10 days), 8.14 days(72 hours embryo at 4±1˚C for 10 days), 8.13 days (72 hours embryo at 6±1˚C for 10 days), 8.13 days (48 hours embryo at 4±1˚C for 7 days) , 8.1 days (24 hours embryo at 6±1˚C for 6 days) ,8.06 days(72 hours embryo at 6±1˚C for 7 days),8.05 days (24 hours embryo at 5±1˚C for 7 days) and significantly by 8.01 days (72 hours embryo at 5±1˚C for 7 days), 8 days (24 hours embryo at 4±1˚C for 7 days),7.99 days (72 hours embryo at 4±1˚C for 7 days). (Table 39)

**Table 39.** Combined effect of preservation period, temperature stress and embryonic age on days delay of hatching

| P. Day | 3d | | | 7d | | | 10d | | | 15d | | | 21d | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4±1˚C | 5±1˚C | 6±1˚C | 4±1˚C | 5±1˚C | 6±1˚C | 4±1˚C | 5±1˚C | 6±1˚C | 4±1˚C | 5±1˚C | 6±1˚C | 4±1˚C | 5±1˚C | 6±1˚C |
| 24h | 7.06±0.02 | 7.08±0.05 | 7.13±0.03 | 8±0.01 | 8.05±0.04 | 8.1±0.01 | 8.96±0.01 | 9.02±0.31 | 9.08±0.21 | 3.96±0.09 | 3.95±0.15 | 3.96±0.05 | 1.96±0.01 | 2.08±0.01 | 2.03±0.06 |
| 48h | 7.16±0.07 | 7.22±0.33 | 7.23±0.26 | 8.13±0.05 | 7.85±0.19 | 7.93±0.09 | 9.16±0.04 | 9.17±0.15 | 9.14±0.01 | 3.1±0.06 | 3.28±0.07 | 3.96±0.01 | 0.64±0.05 | 0.69±0.04 | 0.63±0.01 |
| 72h | 7.1±0.05 | 7.09±0.09 | 7.03±0.25 | 7.99±0.02 | 8.01±0.41 | 8.06±0.16 | 8.14±0.01 | 8.18±0.0 7 | 8.13±0.1 | 3.13±0.05 | 3.08±0.04 | 3.03±0.05 | 0.34±0.06 | 0.37±0.15 | 0.35±0.02 |
| 96h | 6.32±0.11 | 6.37±0.01 | 6.4±0.15 | 6.5±0.33 | 6.45±0.05 | 6.40±0.01 | 7.00±0.41 | 6.46±0.16 | 6.43±0.17 | 3.16±0.07 | 3.09±0.08 | 3.03±0.07 | 0 | 0 | 0 |
| CD at 5% | | | | | | | 0.155 | | | | | | | | |

**Table 40** Combined effect of different preservation period on different temperature stress and identified low temperature resistant embryonic age on hatching percentage and days delay of hatching

| | 3 | | | | | | 7 | | | | | | 10 | | | | | | 15 | | | | | | 21 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4±1˚C | | 5±1˚C | | 6±1˚C | | 4±1˚C | | 5±1˚C | | 6±1˚C | | 4±1˚C | | 5±1˚C | | 6±1˚C | | 4±1˚C | | 5±1˚C | | 6±1˚C | | 4±1˚C | | 5±1˚C | | 6±1˚C | |
| | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D | H | D |
| 24h | 90.92± 0.05 | 7.06± 0.02 | 90.88± 0.25 | 7.08± 0.05 | 90.83± 0.14 | 7.13± 0.03 | 90.9±0 .14 | 8±0.0 1 | 90.79± 0.17 | 8.05± 0.04 | 90.68± 0.04 | 8.1±0. 01 | 90.85± 0.2 | 8.96± 0.01 | 90.32± 0.06 | 9.02± 0.31 | 89.79± 0.189 | 9.08± 0.21 | 89.54± 0.61 | 3.96± 0.09 | 89.58± 0.35 | 3.95± 0.15 | 89.63± 0.8 | 3.96± 0.05 | 55.05± 0.11 | 1.96± 0.01 | 53.15± 0.43 | 2.08± 0.01 | 50.1±0 .49 | 2.03± 0.06 |
| 48h | 90.65± 0.41 | 7.16± 0.07 | 90.66± 0.04 | 7.22± 0.33 | 90.7±0 .64 | 7.23± 0.26 | 90.56± 0.96 | 8.13± 0.05 | 90.56± 0.55 | 7.85± 0.19 | 90.56± 0.18 | 7.93± 0.09 | 89.69± 0.24 | 9.16± 0.04 | 89.64± 0.44 | 9.17± 0.15 | 89.58± 0.55 | 9.14± 0.01 | 89.66± 0.55 | 3.1±0. 06 | 89.45± 0.22 | 3.28± 0.07 | 89 .39±0. 29 | 3.96± 0.01 | 49.8±0 .59 | 0.64± 0.05 | 28.18± 0.15 | 0.69± 0.04 | 15.16± 0.21 | 0.63± 0.01 |
| 72h | 90.63± 0.09 | 7.1±0. 05 | 90.6±0 .23 | 7.09± 0.09 | 90.58± 0.85 | 7.03± 0.25 | 90.54± 0.98 | 7.99± 0.02 | 90.53± 0.55 | 8.01± 0.41 | 90.50± 0.11 | 8.06± 0.16 | 89.36± 0.47 | 8.14± 0.01 | 89.14± 0.09 | 8.18± 0.0 7 | 88.93± 0.26 | 8.13± 0.1 | 88.76± 0.76 | 3.13± 0.05 | 88.69± 0.03 | 3.08± 0.04 | 88.63± 0.59 | 3.03± 0.05 | 39.93± 0.52 | 0.34± 0.06 | 22.07± 0.7 | 0.37± 0.15 | 12.1±0 .07 | 0.35± 0.02 |
| 96h | 90.54± 0.35 | 6.32± 0.11 | 90.48± 0.15 | 6.37± 0.01 | 90.4±0 .41 | 6.4±0. 15 | 80.16± 0.23 | 6.5±0. 33 | 77.51± 0.37 | 6.45± 0.05 | 73.36± 0.45 | 6.40± 0.01 | 59.94± 0.37 | 7.00± 0.41 | 59.43± 0.06 | 6.46± 0.16 | 46.93± 0.53 | 6.43± 0.17 | 19.93± 0.6 | 3.16± 0.07 | 19.51± 0.02 | 3.09± 0.08 | 19.46± 0.44 | 3.03± 0.07 | 0 | 0 | 0 | 0 | 0 | 0 |

It was observed that up to 15 days preservation early embryonic stages showed good hatching percentage up to 15 days, but the days delayed regarding hatching was very short (nearly 3 days). More over 96 hours embryo showed good hatching percentage when preserved for three days, but the days delayed was nearly one day short than up to 72 hours. So it can be concluded that up to ten days of preservation were found suitable for hatching percentage to ensure desired quantity of seed when required up to a delay of 9 days (Table 40). Regression analysis also confirmed present findings (Plate 15 and 16)

So it can be concluded that early embryos up to 72 hours can be preserved for up to 10 days at any cold shock with 4±1˚C to 6±1˚C, so that the differentiation period can be delayed to ensure seed supply at desired quantity when required. More over performances of 96 hour embryo can also meet the demand of seed supply to some extent.

## 4.5 Biochemical changes in embryonic developmental events of *A. assama*

### 4.5.1 Biochemical changes in embryonic developmental events during normal differentiation of *A. assama*

#### 4.5.1.1 Biochemical changes in carbohydrate content in embryonic developmental events during normal differentiation of*A. assama*

ANOVA analysis confirmed that the changes in carbohydrate content during embryonic development are significant ($P<0.05$). Carbohydrate content was decreased from 24 hr (33.98±0.009) to 72 hr (25.76±0.005) old egg significantly, then increased on 96 hr (27.64±0.019) old and again decreased significantly till before hatching after 168 hr (13.32±0.009). (Table 41)

**Table 41.** Biochemical changes in carbohydrate content in embryonic developmental events during normal differentiation of *A. assama*

| Embryo(hr) | Carbohydrate (mg/g) |
|---|---|
| 24hr | 33.98±0.009 |
| 48 hr | 29.56±0.004 |
| 72hr | 25.76±0.005 |
| 96hr | 27.64±0.019 |
| 120hr | 25.16±0.19 |
| 144hr | 23.26±0.006 |
| 168hr | 13.32±0.009 |
| CD at 5% | 1.63 |

### 4.5.1.2. Biochemical changes in protein content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in protein content during embryonic development are non significant (P>0.05). Protein content was decreased from 24 hr (163.74±0.035) to 72 hr (132.53±0.02) significantly and from 72 hr (132.53±0.02) to 96 hr (127.25 ±0.025) non significantly and then to 120 hr (121.17±0.04) old eggs, significantly. Again, it was increased on 144 hr (175.08±0.03) old eggs significantly and then decreased significantly till before hatching after 168 hr (112.17±0.058). (Table 42)

**Table 42.** Biochemical changes in protein content in embryonic developmental events during normal differentiation of *A. assam*

| Embryo (hr) | Protein (mg/g) |
|---|---|
| 24hr | 163.74±0.035 |
| 48 hr | 142.63±0.015 |
| 72hr | 132.53±0.02 |
| 96hr | 127.25 ±0.025 |
| 120hr | 121.17±0.04 |
| 144hr | 175.08±0.03 |
| 168hr | 112.17±0.058 |
| CD at 5% | 5.836 |

### 4.5.1.3 Biochemical changes in cholesterol content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in cholesterol content during embryonic development are significant (P<0.05). Cholesterol content was increased from 24 hr (105.88±0.027) to 48 hr (111.76±0.021) old egg non significantly, then increased up to 96 hr (253.56±0.86) old significantly and again decreased non significantly till before hatching after 168 hr (108.82±0.02). (Table 43)

**Table 43.** Biochemical changes in cholesterol content in embryonic developmental events during normal differentiation of *A. assama*

| Embryo (hr) | Cholesterol (mg%) |
|---|---|
| 24hr | 105.88±0.027 |
| 48 hr | 111.76±0.021 |
| 72hr | 188.23±0.009 |
| 96hr | 253.56±0.86 |
| 120hr | 200.61±0.019 |
| 144hr | 129.76±0.02 |
| 168hr | 108.82±0.02 |
| CD at 5% | 64.64 |

### 4.5.1.4 Biochemical changes in DNA content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in DNA content during embryonic development are significant ($P<0.05$). DNA content was increased from 24 hr (0.55±0.02) to 120 hr (2.45±0.025) old egg significantly, then increased up to 144 hr (2.60±0.045) old non significantly and finally increased significantly till before hatching after 168 hr (3.05±0.025). (Table 44)

**Table 44.** Biochemical changes in DNA content in embryonic developmental events during normal differentiation of *A. assama*

| Embryo(hr) | DNA (mg/ml) |
|---|---|
| 24hr | 0.55±0.02 |
| 48 hr | 1.25±0.03 |
| 72hr | 1.95±0.015 |
| 96hr | 2.15±0.035 |
| 120hr | 2.45±0.025 |
| 144hr | 2.60±0.045 |
| 168hr | 3.05±0.025 |
| CD at 5% | 0.217 |

### 4.5.1.5 Biochemical changes in Threhalose content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in Trehalose content during embryonic development are significant (P<0.05). Trehalose content was initially decreased from 24 hr (2.32±0.0125) to 48 hr (0.78±0.0118) old egg significantly, then increased up to 120 hr (59.13±0.002) old significantly and again decreased significantly till before hatching after 168 hr (12.45±0.058). (Table 45)

**Table 45.** Biochemical changes in Threhalose content in embryonic developmental events during normal differentiation of *A. assama*

| Embryo (hr) | Trehalose(mg/ml) |
|---|---|
| 24hr | 2.32±0.0125 |
| 48 hr | 0.78±0.0118 |
| 72hr | 14.78±0.008 |
| 96hr | 17.12±0.005 |
| 120hr | 59.13±0.002 |
| 144hr | 16.34±0.017 |
| 168hr | 12.45±0.058 |
| CD at 5% | 4.996 |

### 4.5.1.6 Biochemical changes in NAD-SDH in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in NAD-SDH content during embryonic development are significant (P<0.05). NAD-SDH content was initially increased from 24 hr (0.19±0.004) to 72 hr (3.13 ±0.021) old egg significantly, then decreased up to 120 hr (0.42 ±0.015) old significantly and again increased significantly by 144 hr (2.04±0.001) and finally decreased by 168 hr (1.98± 0.01). (Table 46)

**Table 46.** Biochemical changes in NAD-SDH in embryonic developmental events during normal differentiation of *A. assama*

| Embryo(Hr) | NAD-SDH(Ux$10^{-3}$/ml) |
|---|---|
| 24hr | 0.19±0.004 |
| 48 hr | 1.92±0.055 |
| 72hr | 3.13 ±0.021 |
| 96hr | 2.73 ±0.025 |
| 120hr | 0.42 ±0.015 |
| 144hr | 2.04±0.001 |
| 168hr | 1.98± 0.01 |
| CD at 5% | 0.279 |

### 4.5.1.7 Biochemical changes in NADPH-Peroxidase in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in NADPH-Peroxidase content during embryonic development are non significant (P>0.05). NADPH-Peroxidasecontent was initially increased from 24 hr (0.0395 ± 0.0005) to 48 hr (0.0450±0.0001) old egg significantly, then decreased up to 96 hours egg (0.0142±0.0002) significantly and again increased in 120 hr (0.0517±0.0674) old significantly and then decreased non significantly till before hatching after 168 hr (0.0244±0.0004). (Table 47)

**Table 47. B**iochemical changes in NADPH-Peroxidase in embryonic developmental events during normal differentiation of *A. assama*

| Embryo age (h) | Peroxidase in x$10^{-3}$ U/mg |
|---|---|
| 24h | 0.0395 ± 0.0005 |
| 48h | 0.0450±0.0001 |
| 72h | 0.0335±0.0004 |
| 96h | 0.0142±0.0002 |
| 120h | 0.0517±0.0674 |
| 144h | 0.0487±0.0002 |
| 168h | 0.0244±0.0004 |
| CD at 5% | 0.0035 |

#### 4.5.1.8 Biochemical changes in XO in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in XO content during embryonic development are significant ($P<0.05$). XO content was initially increased from 24 hr (0.059±0.003) to 48 hr (0.197±0.002) old egg significantly, then increased up to 120 hr (1.102±0.004) old significantly and decreased non significantly (1.219±0.009) but again increased before hatching after 168 hr (1.552±0.019). (Table 48)

**Table 48.** Biochemical changes in XO in embryonic developmental events during normal differentiation of *A. assama*

| Embryo age | XO in x10-3U/ml |
|---|---|
| 24h | 0.059±0.003 |
| 48h | 0.197±0.002 |
| 72h | 0.118±0.004 |
| 96h | 0.373±0.009 |
| 120h | 1.102±0.004 |
| 144h | 1.219±0.009 |
| 168h | 1.552±0.019 |
| CD at 5% | 0.156 |

### 4.5.2 Biochemical changes in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

#### 4.5.2.1 Biochemical changes in carbohydrate content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

##### 4.5.2.1.1 Biochemical changes in carbohydrate content in different identified embryonic stages

**24 Hours**

In case of 24 hr eggs there was significant decrease in carbohydrate level after 3 (35.81±0.05), 7 (20.27±0.09) and 10 (6.25±0.03) days preservation. There was

significant increase of carbohydrate level than control after 3days (35.81±0.05) preservation and significant decrease after 7 (20.27±0.09) and 10 days (6.25±0.03) preservation. (Table 49)

**48 Hours**

In case of 48 hr eggs there was significant decrease in carbohydrate level after 3(27.28±0.06), 7 (13.72±0.11) and 10 days (12.45±0.17) preservation. There was significant decrease of carbohydrate level than control after 3 (27.28±0.06), 7 (13.72±0.11) and 10 days (12.45±0.17) preservation. (Table 49)

**72 Hours**

In case of 72 hr eggs there was significant decrease in carbohydrate level after 3 (10.88±0.15), 7 (12.46±0.13) and 10 days (12.45±0.05) preservation. There was significant decrease after 3 (10.88±0.15), 7 (12.46±0.13) and 10 days (12.45±0.05) preservation. (Table 49)

**96 Hours**

In case of 96 hr eggs there was non significant increase of carbohydrate level between 3 (21.8±0.05) and 7 days (20.26±0.07) preservation but significant decrease was reported after 10 days (14.8±0.02) preservation. There was significant decrease of carbohydrate level than control after 3 (21.8±0.05), 7 (20.26±0.07) and 10 days (14.8±0.02) preservation. (Table 49)

**4.5.2.1.2 Biochemical changes in carbohydrate content in different identified embryonic stages at different cold preservation periods**

**After 3Days**

After 3 days preservation, carbohydrate level decreased in 48 hr (27.28±0.06), 72 hr (10.88±0.15) and 96 hr (21.8±0.05) than 24 hr (35.81±0.05) level significantly. (Table 49)

**After 7 Days**

After 7 days preservation, carbohydrate level decreased significantly in 48 (13.72±0.11) and 72 hr (12.46±0.13) and non significantly increased in 96 hr (20.26±0.07) than 24 hr (20.27±0.09) . (Tabl 49)

**After 10 Days**

After 10 days preservation, carbohydrate level increased in 48 (12.45±0.17), 72(12.45±0.05) and 96 hr (14.8±0.02) significantly, than 24 hr (6.25±0.03). (Table 49)

But at normal development carbohydrate level decreased significantly by 48 hr (29.56±0.04), 72 hr (25.76±0.05) and non significantly increased in 96 hr (27.64±0.19). (Table 49)

**Table 49.** Biochemicalchanges in carbohydrate content (mg/g) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 35.81±0.05 | 27.28±0.06 | 10.88±0.15 | 21.8±0.05 |
| 7 | 20.27±0.09 | 13.72±0.11 | 12.46±0.13 | 20.26±0.07 |
| 10 | 6.25±0.03 | 12.45±0.17 | 12.45±0.05 | 14.8±0.02 |
| Control | 33.98±0.09 | 29.56±0.04 | 25.76±0.05 | 27.64±0.19 |
| CD at 5% | 2.02 | | | |

**4.5.2.2 Biochemical changes in protein content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama***

**4.5.2.2.1 Biochemical changes in protein content in different identified embryonic stages**

**24 Hours**

In case of 24 hr eggs there was significant increase in protein level after 7 (265.63±0.21) days and 10 days (253.52±0.04) preservation than after 3 days

(155.65±0.05) preservation. There was significant increase of protein level than control after 7 (265.63±0.21) and 10 days (253.52±0.04) preservation, but non significant decrease after 3days (155.65±0.05) preservation. (Table 50)

**48 Hours**

In case of 48 hr eggs there was significant increase in protein level after 7 (280.05±0.31) and 10 days (233.5±0.11) preservation than 3 days (154.68±0.06) preservation. There was significant increase of protein level than control after 7 (280.05±0.31) and 10 days (233.5±0.11) preservation and non significant increase was recorded after 3 days (154.68±0.06) preservation. (Table 50)

**72 Hours**

In case of 72 hr eggs there was significant increase in protein level after 7 (273.52±0.07) and 10 days (248.29±0.06) preservation than 3 days (155.2±0.11) preservation. There was significant increase of protein level than control after 7 (273.52±0.07) and 10 days (248.29±0.06) preservation and non significant increase was recorded after 3 days (155.2±0.11) preservation. (Table 50)

**96 Hours**

In case of 96 hr eggs there was significant increase in protein level after 7 (242.52±0.07) and 10 days (237.8±0.14) preservation than 3 days (154.63±0.05) preservation. There was significant increase of protein level than control after 3 (154.63±0.05), 7 (242.52±0.07) and 10 days (237.8±0.14) preservation. (Table 50)

**4.5.2.2.2Biochemical changes in protein content in different identified embryonic stages at different cold preservation periods**

**After 3 Days**

After 3 days preservation, non significant change in protein level was evident in 48 (154.68±0.06), 72 (155.2±0.11) and 96 hr (154.63±0.05) egg than 24 hr (155.65± 0.05) egg. (Table 50)

**After 7 Days**

After 7 days preservation, non significant change in protein level was evident in 48 (280.05±0.31), 72 (273.52±0.07) and 96 hr (242.52±0.07) egg than 24 hr (265.63±0.21) egg. (Table 50)

**After 10 Days**

After 10 days preservation, non significant change in protein level was evident in 48 (233.5±0.11), 72 (248.29±0.06) and 96 hr (237.8±0.14) egg than 24 hr (253.52±0.04) egg. (Table50)

But at normal development protein level increased non significantly from 24 hr (163.74±0.035), 48 hr (142.6±0.015), 72 hr (132.53±0.02) and 96 hr (127.25±0.025). (Table 50)

**Table 50.** Biochemical changes in protein content (mg/g) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 155.65±0.05 | 154.68±0.06 | 155.2±0.11 | 154.63±0.05 |
| 7 | 265.63±0.21 | 280.05±0.31 | 273.52±0.07 | 242.52±0.07 |
| 10 | 253.52±0.04 | 233.5±0.11 | 248.29±0.06 | 237.8±0.14 |
| Control | 163.74±0.035 | 142.6±0.015 | 132.53±0.02 | 127.25±0.025 |
| CD at 5% | 30.427 | | | |

#### 4.5.2.3 Biochemical changes in cholesterol content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

##### 4.5.2.3.1 Biochemical changes in cholesterol content in different identified embryonic stages

**24 Hours**

In case of 24 hr eggs there was non significant decrease in cholesterol level after 3(75.90±0.05) and 7 (73.19±0.10) and 10 days (64.33±0.05) preservation. There was significant decrease of cholesterol level than control after 3(75.90±0.05) , 7 (73.19±0.10)and 10 days (64.33±0.05) preservation. (Table 51)

**48 Hours**

In case of 48 hr eggs there was no significant decrease in cholesterol level after 3(76.57±0.07), 7(71.75±0.04) and 10 days (60.82±0.08) preservation. There was significant decrease of cholesterol level than control after 3 (76.57±0.07), 7 (71.75±0.04) and 10 days (60.82±0.08) preservation. (Table 51)

**72 Hours**

In case of 72 hr eggs there was no significant decrease in cholesterol level after 3(70.12±0.08), 7 (69.03±0.02) and 10 days (59.07±0.03) preservation. There was significant decrease of cholesterol level than control after 3(70.12±0.08), 7(69.03±0.02) and 10 days (59.07±0.03) preservation. (Table 51)

**96 Hours**

In case of 96 hr eggs there was no significant decrease in cholesterol level after 3 (72.68±0.06), 7 (65.75±0.04) and 10 days (57.01±0.09 ) preservation. There was significant decrease of cholesterol level than control after 3 (72.68±0.06), 7(65.75±0.04) and 10 days (57.01±0.09) preservation. (Table 51)

##### 4.5.2.3.2Biochemical changes in cholesterol content in different identified embryonic stages at different cold preservation periods

**After 3 Days**

After 3 days preservation, non significant change in cholesterol level was evident in 48 (76.57±0.07), 72(70.12±0.08) and 96 hr (72.68±0.06) egg than 24 hr (75.90±0.05) egg. (Table 51)

**After 7 Days**

After 7 days preservation, non significant change in cholesterol level was evident in 48 (71.75±0.04), 72 (69.03±0.02) and 96 hr (65.75±0.04) egg than 24 hr (73.19±0.10) egg. (Table 51)

**After 10 Day**

After 10 days preservation, non significant change in protein level was evident in 48 (60.82±0.08), 72(59.07±0.03) and 96 hr (57.01±0.09) egg than 24 hr (64.33±0.05) egg. (Table 51)

But during normal development, cholesterol level increased non significantly from 24 hr (105.88±0.028) to 48 hr (111.76±0.022), but significantly by 72 hr (188.23±0.010) and 96 hr (253.56±0.87). (Table 51)

**Table 51.**Biochemical changes in cholesterol content (mg %) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 75.90±0.05 | 76.57±0.07 | 70.12±0.08 | 72.68±0.06 |
| 7 | 73.19±0.10 | 71.75±0.04 | 69.03±0.02 | 65.75±0.04 |
| 10 | 64.33±0.05 | 60.82±0.08 | 59.07±0.03 | 57.01±0.09 |
| Control | 105.88±0.028 | 111.76±0.022 | 188.23±0.010 | 253.56±0.87 |
| CD at 5% | 9.08 | | | |

#### 4.5.2.4 Biochemical changes in DNA content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

##### 4.5.2.4.1 Biochemical changes in DNA content in different identified embryonic stages

**24 Hours**

In case of 24 hr eggs there was significant increase in DNA level after 7 (1.97±0.08) and 10 days (3.01±0.05) preservation than 3 days (0.051±0.01) preservation. There was significant decrease of DNA level than control after 3 days (0.051±0.01) preservation, but significantly increased after 7 (1.97±0.08) and 10 (3.01±0.05) days preservation. (Table 52)

**48 Hours**

In case of 48 hr eggs there was significant increase in DNA level after 7 (2.17±0.09) and 10 days (3.01±0.05) preservation than 3 days (0.073±0.03) preservation. There was significant decrease of DNA level than control after 3 days (0.073±0.03) preservation, but significantly increased after 7 (2.17±0.09) and 10 days (3.01±0.05) preservation. (Table 52)

**72 Hours**

In case of 72 hr eggs there was significant increase in DNA level after 7 (2.44±0.09) and 10 days (3.18±0.17) preservation than 3days (0.099±0.07) preservation. There was significant decrease of DNA level than control after 3 days (0.099±0.07) preservation, but significantly increased after 7 (2.44±0.09) and 10 days (3.18±0.17) preservation. (Table 52)

**96 Hours**

In case of 96 hr eggs there was significant increase in DNA level after 7 (2.61±0.02) and 10 days (3.26±0.06) preservation than 3 days (0.127±0.01) preservation. There was significant decrease of DNA level than control after 3days (0.127±0.01) preservation, but significantly increased after 7 (2.61±0.02) and 10 days (3.26±0.06) preservation. (Table 52)

**4.5.2.4.2 Biochemical changes in DNA content in different identified embryonic stages at different cold preservation periods**

**After 3Days**

After 3 days preservation, non significant change in DNA level was evident in 48 (0.073±0.03), 72 (0.099±0.07) and 96 hr (0.127±0.01) egg than 24 hr (0.051±0.01) eggs. (Table 52)

**After 7 Days**

After 7 days preservation, non significant change in DNA level was evident between 24 hr (1.97±0.08) and 48 hr (2.17±0.09) old eggs, but significant increase was noticed between 72(2.44±0.09) and 96 hr (2.61±0.02) eggs. (Table 52)

**After 10 Days**

After 10 days preservation, non significant change in DNA level was evident in 48(3.17±0.21), 72 (3.18±0.17) and 96 hr (3.26±0.06) egg than 24 hr (3.01±0.05) egg. (Table 52)

But during normal development, DNA level was increased significantly from 24 (0.55±0.02) hr to 48(1.25±0.03) hr and 72 (1.95±0.015) hr but non significantly between 72(1.95±0.015) hr and 96 (2.15±0.35) hr. (Table 52)

**Table 52.** Biochemical changes in DNA content (mg/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 0.051±0.01 | 0.073±0.03 | 0.099±0.07 | 0.127±0.01 |
| 7 | 1.97±0.08 | 2.17±0.09 | 2.44±0.09 | 2.61±0.02 |
| 10 | 3.01±0.05 | 3.17±0.21 | 3.18±0.17 | 3.26±0.06 |
| Control | 0.55±0.02 | 1.25±0.03 | 1.95±0.015 | 2.15±0.35 |
| CD at 5% | 0.328 | | | |

#### 4.5.2.5 Biochemical changes in trehalose content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

##### 4.5.2.5.1 Biochemical changes in trehalose content in different identified embryonic stages

**24 Hours**

In case of 24 hr eggs, there was significant decrease, in trehalose level after 3 (35.77±0.04), 7 (6.25±0.05) and 10 days (20.24±0.06) preservation. There was significant increase of trehalose level than control after 3 (35.77±0.04) and 10 days (20.24±0.06) preservation and non significantly changed after 7days (6.25±0.05) preservation. (Table 53)

**48 Hours**

In case of 48 hr eggs there was significant decrease in trehalose level after 7(12.43±0.02) and 10 days (17.72±0.04) preservation than 3 days (28.78±0.04) preservation. There was significant increase of trehalose level than control after 3 (28.78±0.04), 7 (12.43±0.02) and 10 days (17.72±0.04) preservation. (Table 53)

**72 Hours**

In case of 72 hr eggs there was non significant increase in trehalose level after 10 days (16.04±0.08) preservation than 3 days (10.82±0.01) preservation but significant increase was reported after 7 days (53.68±0.05) preservation. There was significant increase of trehalose level than control after 7 days (53.68±0.05) preservation and non significantly changed after 3 (10.82±0.01) and 10days (16.04±0.08) preservation. (Table 53)

**96 Hours**

In case of 96 hr eggs there was non significant change in trehalose level after 3 (20.25±0.04), 7 (21.74±0.04) and 10 days (14.78±0.04) preservation. There was non significant increase of trehalose level than control after 3 (20.25±0.04), 7 (21.74±0.04) and 10days (14.78±0.04) preservation. (Table 53)

**4.5.2.5.2Biochemical changes in trehalose content in different identified embryonic stages at different cold preservation periods**

**After 3Days**

After 3 days preservation, significant change in trehalose level was evident in 48 (28.78±0.04), 72 (10.82±0.01) and 96 hr (20.25±0.04) egg than 24 hr (35.77±0.04) eggs. (Table 53)

**After 7 Days**

After 7 days preservation, significant change in trehalose level was evident in 48 (12.43±0.02), 72 (53.68±0.05) and 96 hr (21.74±0.04) egg than 24 hr (6.25±0.05) eggs. (Table 53)

**After 10 Days**

After 10 days preservation, non significant change in trehalose level was evident in 48 (17.72±0.04), 72 (16.04±0.08) and 96 hr (14.78±0.04) egg than 24 hr (20.24±0.06) eggs. (Table 55)

But during normal development, trehalose level was decreased non significantly from 24 hr (0.778±0.02) to 48hr (0.778±0.02), but significantly increased from 48 hr (0.778±0.02) to 72 hr (14.782±0.02) and 96 hr (17.12±0.006). (Table 53)

**Table 53.**Biochemical Changes in trehalose (mg/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 35.77±0.04 | 28.78±0.04 | 10.82±0.01 | 20.25±0.04 |
| 7 | 6.25±0.05 | 12.43±0.02 | 53.68±0.05 | 21.74±0.04 |
| 10 | 20.24±0.06 | 17.72±0.04 | 16.04±0.08 | 14.78±0.04 |
| Control | 2.34±0.005 | 0.778±0.02 | 14.782±0.02 | 17.12±0.006 |
| CD at 5% | 6.345 | | | |

**4.5.2.6 Biochemical changes in NAD-SDH content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama***

**4.5.26.1 Biochemical changes in NAD-SDH content in different identified embryonic stages**

**24 Hours**

In case of 24 hr eggs there was no significant increase in NAD-SDH level after 3(2.53±0.05) and 7 days (2.6±0.11) preservation but significantly decreased after 10 days (0.692±0.05) preservation. There was significant increase of NAD-SDH level than control after 3(2.53±0.05), 7(2.6±0.11) and 10 days (0.692±0.05) preservation. (Table 54)

**48 Hours**

In case of 48 hr eggs there was no significant increase in NAD-SDH level after 3(2.05±0.06) and 7 days (2.18±0.07) preservation but significantly decreased after 10 days (1.05±0.02) preservation. There was significant increase of NAD-SDH level than control after 3(2.05±0.06), 7(2.18±0.07) and 10 days (1.05±0.02) preservation. (Table 54)

**72 Hours**

In case of 72 hr eggs there was significant increase in NAD-SDH level after 3 (3.06±0.09) and 7 days (5.26±0.03) preservation but significantly decreased after 10 days (1.37±0.02) preservation. There was significant increase of NAD-SDH level than control after 3 (3.06±0.09), 7 (5.26±0.03) and 10 days (1.37±0.02) preservation. (Table 54)

**96 Hours**

In case of 96 hr eggs there was no significant increase in NAD-SDH level after 3 (0.87±0.14) and 7 days (2.88±0.03) preservation but significantly decreased after 10 days (3.36±0.02) preservation. There was significant increase of NAD-SDH level than control after 3 (0.87±0.14) , 7 (2.88±0.03) and 10 days (3.36±0.02) preservation. (Table 54)

**4.5.2.6.2Biochemical changes in NAD-SDH content in different identified embryonic stages at different cold preservation periods**

**After 3Days**

After 3 days preservation, NAD-SDH level decreased in 48 hr (2.05±0.06) , non significantly, then increased by 72 hr (3.06±0.09) significantly and also decreased by 96 hr (0.87±0.14) significantly. (Table 54)

**After 7 Days**

After 7 days preservation, NAD-SDH level decreased in 48 hr (2.18±0.07), non significantly, then increased by 72 hr (5.26±0.03) old significantly and also decreased by 96 hr (2.88±0.03) significantly. (Table 54)

**After 10 Day**

After 10 days preservation, NAD-SDH level decreased in 48 hr (1.05±0.02) non significantly, then increased by 72 hr (1.37±0.02) significantly and also decreased by 96 hr (3.36±0.02) significantly. (Table 54)

But during normal development, NAD-SDH level increased significantly by 24hr (0.19±0.004), 48 hr (1.03±0.04) , 72 hr (1.39±0.001) and 96 hr (3.32±0.002) . (Table 54)

**Table 54.**Biochemical changes in NAD-SDH content (Ux$10^{-3}$/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 2.53±0.05 | 2.05±0.06 | 3.06±0.09 | 0.87±0.14 |
| 7 | 2.6±0.11 | 2.18±0.07 | 5.26±0.03 | 2.88±0.03 |
| 10 | 0.692±0.05 | 1.05±0.02 | 1.37±0.02 | 3.36±0.02 |
| Control | 0.19±0.004 | 1.03±0.04 | 1.39±0.001 | 3.32±0.002 |
| CD at 5% | 0.219 | | | |

### 4.5.2.7 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

#### 4.5.2.7.1 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages

**24 Hours**

In case of 24 hr eggs there was significant increase in NADPH-Peroxidase level after 7 days (0.100±0.04) preservation but, significantly decreased after 10 days (0.033±0.05) preservation than 3 days (0.0126±0.02) preservation. There was significant decrease of NADPH-Peroxidase level than control after 3 days (0.0126±0.02) preservation, but significantly increased after 7 days (0.100±0.04) preservation and non significantly increased after 10 days (0.033±0.05) preservation. (Table 55)

**48 Hours**

In case of 48 hr eggs there was significant decrease in NADPH-Peroxidase level after 7 days (0.059±0.005) preservation and significantly increased after 10 days (0.458±0.008) preservation than 3 days (0.199±0.007) preservation. There was significant increase of NADPH-Peroxidase level than control after 3 days (0.199±0.007) preservation, but non significantly decreased after 7 days (0.059±0.005) preservation and significantly increased after 10 days (0.458±0.008) preservation. (Table 55)

**72 Hours**

In case of 72 hr eggs there was significant increase in NADPH-Peroxidase level after 7 days (0.1003±0.002) preservation but, significantly decreased after 10 days (0.062±0.005) preservation than 3 days (0.018±0.007) preservation. There was significant decrease of NADPH-Peroxidase level than control after 3 days (0.018±0.007) preservation, but significantly increased after 7 days (0.1003±0.002) and 10 days (0.062±0.005) preservation. (Table 55)

**96 Hours**

In case of 96 hr eggs there was significant decrease in NADPH-Peroxidase level after 7 days (0.085±0.004) preservation and significantly increased after 10 days (0.212±0.03) preservation than 3 days (0.184±0.003) preservation. There was significant increase of

NADPH-Peroxidase level than control after 3(0.184±0.003), 7(0.085±0.004) and 10 days (0.212±0.03) preservation. (Table 55)

**4.5.2.7.2 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages at different cold preservation periods**

**After 3Days**

After 3 days preservation, NADPH-Peroxidase level increased by 48 hr (0.199±0.007) significantly, then decreased by 72 hr (0.018±0.007) significantly and also increased by 96 hr (0.184±0.003) significantly. (Table 55)

**After 7 Days**

After 7 days preservation, NADPH-Peroxidase level decreased in 48 hr (0.059±0.005) , significantly, then increased by 72 (0.1003±0.002) hr old significantly and also decreased by 96 hr (0.085±0.004) significantly. (Table 55)

**After 10 Day**

After 10 days preservation, NADPH-Peroxidase level increased in 48 hr (0.458±0.008) significantly, then decreased by 72 hr (0.062±0.005) significantly and also increased by 96 hr (0.212±0.03) significantly. (Table 55)

But during normal development, NADPH-Peroxidase level maintained non significant variation by 24 hr (0.04±0.05),48 hr (0.045±0.04) and 72 hr (0.034±0.04) but decreased significantly by 96 hr (0.014±0.04). (Table 55)

**Table 55.**Biochemical changes in NADPH-Peroxidase content (Ux$10^{-3}$/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*.

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 0.0126±0.02 | 0.199±0.007 | 0.018±0.007 | 0.184±0.003 |
| 7 | 0.100±0.04 | 0.059±0.005 | 0.1003±0.002 | 0.085±0.004 |
| 10 | 0.033±0.05 | 0.458±0.008 | 0.062±0.005 | 0.212±0.03 |
| Control | 0.04±0.05 | 0.045±0.04 | 0.034±0.04 | 0.014±0.04 |
| CD at 5% | 0.019 | | | |

### 4.5.2.8Biochemical changes in XO content in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

#### 4.5.2.1 Biochemical changes in XO content in different identified embryonic stage

**24 Hours**

In case of 24 hr eggs there was significant decrease in XO level after 7 days (0.39±0.09) preservation and non significantly increased after 10 days (0.42±0.01) preservation than 3 days (0.55±0.03) preservation. There was significant increase of XO level than control after 3 (0.55±0.03), 7(0.39±0.09) and 10 days (0.42±0.01) preservation. (Table 56)

**48 Hours**

In case of 48 hr eggs there was significant decrease in XO level after 7 days (0.28±0.01) preservation, but significantly increased after 10 days (0.39±0.01) preservation than 3 days (0.52±0.07) preservation. There was significant increase of XO level than control after 3(0.52±0.07),7 (0.28±0.01) and 10 days (0.39±0.01) preservation. (Table 56)

**72 Hours**

In case of 72 hr eggs there was significant decrease in XO level after 7 days (0.14±0.02) preservation and significantly increased after 10 days (0.35±0.05) preservation than 3 days(1.21±0.05) preservation. There was significant increase of XO level than control after 3 (1.21±0.05), 7 (0.14±0.02) and 10 days (0.35±0.05) preservation. (Table 56)

**96 Hours**

In case of 96 hr eggs there was significant increase in XO level after 7 (0.47±0.03) and 10 days (0.67±0.05) preservation than 3 days (0.22±0.04) preservation. There was significant decrease of XO level than control after 3 days (0.22±0.04) but increased after 7(0.47±0.03) and 10 days (0.67±0.05) preservation significantly. (Table 56)

**4.5.2.2Biochemical changes in** XO **content in different identified embryonic stage at different cold preservation periods**

**After 3Days**

After 3 days preservation, XO level decreased by 48 hr (0.52±0.07) non significantly, then increased by 72 hr (1.21±0.05) significantly and also decreased by 96 hr (0.22±0.04) significantly. (Table 56)

**After 7 Days**

After 7 days preservation, XO level decreased by 48 (0.28±0.01) and 72 hr (0.14±0.02) significantly and then increased by 96 hr (0.47±0.03) significantly. (Table 56)

**After 10 Day**

After 10 days preservation, XO level decreased by 48 hr (0.39±0.01) non significantly, then decreased by 72 hr (0.35±0.05) significantly and also increased by 96 hr (0.67±0.05) significantly. (Table 56)

But during normal development, XO level significantly increased by 24 hr (0.06±0.02) and 48 hr (0.20±0.01), then decreased by 72 hr (0.12±0.01) but again increased significantly by 96 hr (0.37±0.08). (Table 56)

**Table 56.**Biochemical changes in XO content ($Ux10^{-3}$/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

| Preservation Days | 24h | 48h | 72h | 96h |
|---|---|---|---|---|
| 3 | 0.55±0.03 | 0.52±0.07 | 1.21±0.05 | 0.22±0.04 |
| 7 | 0.39±0.09 | 0.28±0.01 | 0.14±0.02 | 0.47±0.03 |
| 10 | 0.42±0.01 | 0.39±0.01 | 0.35±0.05 | 0.67±0.05 |
| Control | 0.06±0.02 | 0.20±0.01 | 0.12±0.01 | 0.37±0.08 |
| CD at 5% | 0.046 | | | |

# 5. DISCUSSION

## 5.1 Embryonic development of Muga silkworm, *A.assama*

Success of sericulture depend on quality of silkworm egg preservation techniques are developed for providing suitable treatment during embryonic development in *Bombyx* and other wild silk moths also.

Tricohmiroff (1879), first has studied embryogenesis of silkworm, to characterize blastoderm formation, differentiation of endoderm and development of various organs. In Japan, studies on silkworm embryogenesis are initiated by Toyama (1896). He describe all major steps of embryogenesis *i.e.* Egg architecture, blastoderm and germ band formation, winter diapauses and also period of embryogenesis and formation of organ. Later Ikada (1910, 1912) examine embryogenesis of blastoderm to diapauses stage1. Soon after establishment of strategic base for strategies of embryogenesis, researchers emphasize on a) easy recognition of developmental stages for practical purposes and b) reexamination with necessary correction of each described phenomenon during embryogenesis.

Takami (1969) divided developmental stages of silk worm into following six stages *i.e.* 1) Prediapause, 2) Diapause, 3) Hibernation, 4) Critical Stage, 5) Formation of organ and 6) Completion of larva. The prediapause includes seven stages, *i.e.* fertilization, cleavage, germanlage formation, yolk cleavage, pyriform shaped stage, kokeshi (China spoon like stage) and chemical spatula like stage. Two stages of diapauses and four stages of hibernating period is recognized. Organ formation stage is divided into 10 stages, *i.e.* 1) appearance of labral appendages, 2) shortening stages, 3) Cephalo-thoracic segmentation; 4) Blastokinesis; 5) Completion of blastokinesis, 6) appearance of trichogen cell, 7) appearance of setae and appearance of tracheal taenidia. The completion of larva includes five stages *i.e.* 1) Head pigmentation-I, 2) Head pigmentation-II, 3) Body pigmentation-I and 4) Body pigmentation –II and hatching. Ohtsuki (1979) reclassify the scheme into 30 stages (Yamashita and Yaginuma, 1991).

Among genus *Antheraea*, *A. yamami* embryonic development is rapid than *Bombyx* and *A. pernyi* (Baba et al.1997). In case of *Antheraea assama* earlier report on embryogenesis is reported by Singha et al. (1998), Ghosh and Ray, (2006) and Goswami et al. (2013). Total 168 hours embryogenesis has reported where as in *A.*

*yamami* it is of 240 hours. Present study also confirms that the 168 hrs long episodes of embryogenesis are rapid indeed with similar pattern of developmental schemes as *Bombyx* or *A. yamami*.

Various changes during development is a continuous phenomenon and really difficult to differentiate. Embryonic development in *Bombyx* is chronologically described as KO, OTSU, HEI etc. On the other hand numerical expression considering physiological and developmental milieu is also described (Ohtsuki, 1979, Miya, 2003). None of the earlier studies on *Antheraea assama* describe in detail of the developmental milestones as described in *Bombyx,* present study is planned to look inside the developmental scheme of *A. assama* considering age of egg as only reference at 20˚C incubation.

In *Bombyx* cleavage nuclei penetrate into the superficial layer of the periplasm and enveloped by oolemma to produce blastoderm cell (Takesue et al.1977, 1980). Takeuse, 1982 also reported array in of finger like microprojections upto 4 hrs of oviposition. After, their disappearance, ruffle like microprojections appeared. Reappearance of microvilli is concomitant with blastoderm cleavage (Takeuse, 1980). In *A. assama* microvilli are distinct until 12 hrs of oviposition (Plate 5D).

Takami (1969) classify embryogenesis after blastoderm completion into germ band appearance, yolk cleavage, pyriform shaped stage, kokeshi shaped stage and chemical spatula shaped stage. But Ohtsuki (1979) described into germ band formation, protocephanlon differentiation, spoon shaped stage and telson differentiation. In muga silkworm during embryogenesis also germanlage differentiate into germ band. Secondary vitelline membrane separates germanlage from the inner yolk system. Extra embryonic origin cells gradually flattened and extended to the ventral side of germanlage. This membrane is slightly sink inwards to form another cellular membrane which may cover the whole egg surface (Plate 6 AB). This is called Serosa. Primordial amnion cells appear at the germ band periphery.

Soon after that germ band decreases in width but enlarge anterior and posterior side. Anterior germ band region noticed bilateral exclusion of aggregates of cytoplasamic materials in the anterior germ band region (Plate 6A). This phenomenon leads to differentiation of Protocephalon and protocorm that immediately appears after wards. Periphery of early germ band turn flat due to primordial amnion cells and expand under the germ band.

Though the germ band is decreasing in width as well as extending anterior and posterior and proctocephalon and protocorm emerge distinctly. Now germ band attains pyriform shape. Eggs incubated at 20˚C reaches this stage with in 30 hrs as *Bombyx* (Miya, 2003). Primordial amnion cell form continuous amnion at the ventral side of the germ band. Soon after that, germ band also covered by serosa and amnion. Continued from the previous stage germ band starts to sink into yolk system. Cephalic and caudal ends curve inwards. Spaces between germband and yolk cells are full of liquid material supposed to contain glycogen granules and proteins.

After distinct emergence of proctocephalon and protocorm, 'spoon shaped embryo' has appeared. After 36 hour incubation at 20˚C, diapauses eggs reached at this stage (Miya,2003), but non diapauses eggs reach Telson differentiation stage. Distinct features of this stage is deeply curved proctocephalon and protocorm (Plate 6C). Germ band elongates and invagination due to gastrulation generates ectoderm and mesoderm. Due to gastrulation longitudinal furrows is formed by elongation (Plate 6D). Primitive body segments appeared due to mesoderm segmentation and posterior end of embryo, recognized as Telson becomes distinct (Plate 6E).

Events of development are more or less similar to *Bombyx* embryogenesis as described by Miya, (2003) with major changes in time frame. Soon after 72 hour stage, *A.assama* embryogenesis becomes rapid.

Upward of cells along the ventral line and midline of germ band, is called gastral goove. The inner layer of cells of the gastral groove is called mesoderm. At this level embryo sinks into the inner parts of yolk mass. In the center of egg spaces without yolk cell can be observed. Now embryo starts extension and neural groove appeared due to invagination of mesoderm. At this stage gnathal, thoracic, abdominal segments appeared. Labrum appears as callus like process at the front end of proctocephalon. After that spiracular invagination develop (Plate 6D).

Like *Bombyx* shortening event did not appear. But gnathal segments unite to form head and thorax. During initial development embryo is ventral side facing. Through a sigmoid movement from the caudal end, embryo changes its position and moves to the dorsal side. This is called blastokinesis. This process is divided into three steps, *i.e.* a) early stage, b) middle stage and c) final stage of blastokinesis, where in middle stage sigmoid appearance is distinct and finally revolution is complete in final stage.

After blastokinesis and growth of embryonic ectoderm over the dorsal portion and end up with to enclose the yolk within the embryo and this is called ‘dorsal closure’. Dorsal integument formation is completed except for part of thorax (Plate7ABC).

Next small masses of Trichogen cells are going to produce setae formed on the embryo surface (Plate 10AB). Trichogen cells are large cells that secreates the long tapering hair of the insect bristle. A smaller Tormogen cell forms the circular chitinous socket around the base of the bristle. Soon after that taenidia are formed within the trachea, tracheal system is visible from outside. The formation of larval organ is completed.

After that pigmentation process starts from head region to a brown color. After completion of head pigmentation other body parts start to be pigmented (Plate1 HI). After completion of body parts eats and brake down chorion in the micropylar region to hatch.

Organ formation:

Brain

The nervous system differentiates into four principal part; a) the brain or supraoesophageal ganglia, situated in the head above esophagus, (Plate7A, B, C) b) the suboesophageal ganglia also situated in the head, below the esophagus, c) ventral nerve cord running ventrally through thorax and abdomen swollen into paired ganglia at the posterior body parts and d) sympathetic nerve system innervating mouth and esophagus.

Early development of brain starts from neuroblast distributed through large area (Plate6 E, F). The proctocerbrum sometimes called the ganglion of first head segment forms the fore most and greater portion of brain and follows the deuterocerebrum, ganglion of second head segment and send nerves to antennae and hind most tritocerebrum, the ganglion of the third or intercalary segment, from which the nerve cord to sympathetic ganglion is sent off.

Gut

Three segments of guts at first develop separately then complete alimentary canal is developed (Ogawa, 2004). When plugs of cells at the end of foregut, each end of mid gut and end of hind gut die and three gut segments are unite (Plate 8C). As the anterior

and posterior mid gut primordial come together they enclose remaining yolk sac within mid gut. In the 120 hours stage alimentary canal is differentiated into three parts *i.e.* fore gut, mid gut and hind gut (Plate7A). Large amount of yolk remains in the mid gut lumen (Plate 8A). Magnified image shows Columnar cell and goblet cell from the mid gut epithelium (Plate 8B). In 144 hours stage mid gut reduced its width without thick yolks. Majority of yolk substances may be absorbed by the mid gut cell after digestion, leaving only part in alimentary canal.

Malphigian tubule

Develop as evagination of anterior proctodeum and mark the junction between mid gut and hind gut (Plate9A). Malphigian tubules make first appearance as minute buds from lateral and ventro-lateral sides of the anterior region of hind gut (Plate9B). Gradually the length of the bud increased (Plate9C).

Anterior wall of tubules are continuous with the wall of midgut and posterior end with hind gut. A thin membrane stretches across the anterior end of proctodaeum, making posterior end of mid gut and is called met-enteric membrane (Hagan,1951).

The region of union of posterior wall of tubules and proctodaeum differentiate from mid gut as well as from the proctodaeum, called posterior interstitial ring and later transformed to proctodaeal valve (Srivastava and Bahadur, 1961). Tubules lie anteriorly to the ring (Plate 9D).

Basal part of each lobule finally fused, thus forming a single vesicle from which both tubules of a pair appear to arise. Later length of each tubule has increased and become slightly twisted in the form of an inverted S. At last vesicles become coiled to form a few loops and Malphigian tubule developed.

**Embryonic development of Muga silkworm, *A.assama* during low temperature stress**

Many animals have evolved to survive seasonally recurring adverse conditions through slowing down of metabolism, reduction or elimination of cell division and also cessation of morphological development characterized as ‘resting stage’( Denlinger,2002). Present study also confirmed developmental delay in 24hours, 48 hours and 72 hours egg is vivid even after 3 days refrigeration (Plate 11 A-H). Beyond

72 hours eggs occasional emergence is a familiar event during cold preservation. Though *A. assama* egg is a nondiapause type egg, only refrigeration shock will induce delay in development and there is no definite 'resting stage' as in pea aphids (Denlinger and Lee, 2010). Thangavelu (1985) reports about the possibility to induce 'quiescence' through low temperature stress in *A. assama*, but embryonic age specific dormant stages become distinct through present study.

**5.2 Effect of temperature stress on different embryonic stages**

Insects adapted for diverse environment, having limited ability to regulate body temperature (Bale and Hayward, 2010). Strategies adopted for thermally stressful environments are behavioural avoidance, like migration and seasonal changes in cold tolerance. Freeze tolerance and freeze avoidance are the key ways adopting overwintering through synthesis of ice nucleating agents, cryo-protectants, anti freeze proteins and modification of membrane lipid composition. Overwintering also invite a hypo metabolic state called diapause in temperate and colder climates (Denlinger, 1986). In *Bombyx* sp. colder climate initiate diapause during embryogenesis (Denlinger, 2002). Short term 'chilling' of insect eggs is utilized to lengthen the embryonic period without compromising quality of egg. Periods of low oxygen may induce delay in embryogenesis (Storey and Storey, 1988).

Eggs are considered as the key factor for sericulture industry as only the quality egg can ensure a good harvest of healthy crop. The embryonic developmental stage in silk worm is very susceptible to environmental conditions like temperature, humidity and rainfall which can greatly influence the seed.

To combat with the environmental adversity cold preservation of larva, cocoon and seed is one of the fundamental techniques. Stages of embryo plays crucial role for the success of cold preservation. The resistant embryo to cold storage and its sensitivity varies according to the developmental stages. And hence determination of stages of embryo is most important (Yamashita, 1965). In mulberry silk worm earlier studies also confirm that early embryonic stages are sensitive for refrigeration(Chino, 1957)

In the present study all the embryonic ages in muga silk worm having an incubation period of 7 days or 168 hours to hatch are taken under consideration, *viz*. 24, 36, 48, 60, 72, 84, 96, 108,120, 132 and 144hours for identification of low temperature resistant

embryonic stages to improve cold preservation technology of seed. For identification of embryonic stages two lower level temperature stresses (4±1˚C and 6±1˚C) and two higher levels of temperature stresses (8±1˚C or 10±1˚C) are given and seeds are preserved for 10 days.

The embryonic stages up to 72 hours shows better hatching percentage (90.85% to 88.93%) over control (82%) though the variations are non significant for both low temperature stresses 4±1˚C and 6±1˚C. Incubation period, however, is found highest in late embryonic ages only in 4±1˚C, but for 6±1˚C up to 96 hours the incubation periods are longer. But if the embryonic age were considered to be deleted from the actual ten days shock, then early ages up to 96 hours shows higher incubation periods.

For higher levels of temperature shock, *i.e*, 8±1˚C and 10±1˚C, up to 36 hours old embryo shows higher hatching percentage than control though the variations are non significant. For these two temperatures incubation period are found longer up to 72 hours. For 8±1˚C, up to 96 hours can be considered as after those eggs do not hatch. (Plate 12)

So, observation shows that control treatments and the embryonic stages up to 72 hours when treated with 4±1˚C and 6±1˚C temperature stress have non significant variation in hatching percentage. As the temperature shock decreases to 8±1˚C or 10±1˚C, the hatching percentage also decreases but still close to control up to 48 hours of embryonic stage. Moreover, there is no hatching from 84 hours onwards embryonic ages at 10±1˚C and from 108 hour embryonic stage at 8±1˚C. Incubation period is longer in higher level of temperature stresses and in early ages of embryo.

According to Yaginuma (1990), young age multi-voltine mulberry silkworms egg can tolerate low cold temperature but not advanced embryos. The present findings have clear conformity with Yaginuma(1990). Dutta et al. 2012 on eri silk worm have similar findings that 36-40 hours old age groups can be preserved for maximum days without any adverse effect. Fresh and one day old egg of *A. mylitta* also shows maximum increase of the incubation period (Nayek and Dash, 1989). Moreover, in muga silk worm also, Singha et al. (1998) identify 36 hours of embryonic stage as longest low temperature resistant stage.

So it can be concluded that embryonic stages of 24, 36, 48, 60, 72, 84 and 96 hours can be identified as low temperature resistant embryonic stages *i.e*, up to 96 hours embryonic stages can be explored for further studies with a variety of cold shock range for final selection of low temperature resistant embryonic stages for improvement of cold preservation technology of seed.

### 5.3 Effect of different low temperature stress in identified low temperature resistant embryonic stages

Refrigeration of muga seed cocoon at 5˚ to 12˚C for 10-20 days reveal better moth emergence, pairing, fecundity and hatchability, as confirmed by several other studies also (Choudhury 1981, Choudhury et al.2012 and Bora 2006, Bora et al. 1990 and Bora et al.1992). Moth emergence can be delayed 60-80 days instead of 30 days in control as and when cocoons are conserved at 8±1˚C(Khanikor and Dutta,1997). At 8±1˚C preservation, autumn cocoons delayed for 60-120 days, late autumn cocoons are delayed 40-45 days to 80-100 days and spring cocoons are delayed for 14-18 days to 30-42 days (Khanikor and Dutta, 1998).

But adverse climatic condition can prevail just before the egg hatching. As muga silk worm rearing is an outdoor technique, these adverse climatic condition affect severely at farmers' point. In this situation, not the cocoon but the egg preservation to delay the hatching for atleast one or two week can handle the demand of farmers. Toyma (1896, 1902) studies in detail the embryogenesis of silk worm (*Bombyx mori*), since then search for suitable stage for refrigeration is continued till date.

Short term 'chilling' of insect eggs is utilized to lengthen the embryonic period without compromising quality of egg. Periods of low oxygen may induce delay in embryogenesis (Storey,1982). Sonobe et al. (1986) show that in diapause silk worm eggs, due to reduction of oxygen permeability of egg membranes, hypoxia is introduced to lower rate of metabolism and polyol accumulation. Extra embryonic regions are more sensitive for freezing than the embryo (Imanishi et al. 1996).

Keeping this earlier findings under consideration the present study identifies the embryonic stages for cold shock (24 hour to 96 hours old egg). Now the optimum low temperature should be determined as cold stress from a wide range of temperature shock from 4±1˚C to 10±1˚C with an interval of 1˚C in the present study.

Highest hatching percentage is observedup to9±1˚C for 24 hours, up to 7±1˚C for 36 hours and up to 6±1˚C for 48 to 72 hours of embryonic stages having non significant variation among them (90.85% to 88.23%), 36 hours embryonic stage having 8±1˚C temperature stress shows hatching percentage of 87.71% while at 9±1˚C, this stage show 82.56% hatching. All these hatching percentages are higher than or at per hatching in normal condition (82%). So,24 hours and 36 hours old embryo can withstand 4±1˚C to 9±1˚C and 48 hours to 72 hours old embryo can tolerate 4±1˚C to 6±1˚C regarding better hatching percentage.(Plate 13)

Now, highest incubation period is observed from 4±1˚C temperature stress on 48 hours to 96 hours embryo (18.0 to 18.17 days); 48 and 72 hours at both 5±1˚C and 6±1˚C temperature stress (18.13 to 18.18 days) followed significantly by 60 hours (17.57 days) and 84 hours (17.65 days) embryo at 6±1˚C and also significantly with 60(17.57 days) and 84 hours (17.65 days) egg at 5±1˚C and 36 hours embryo at 4±1˚C(17.58 days) having non significant variation among them. More over from 7±1˚C to higher range, performance is poor and even at 10±1˚C, hatching is not observed beyond 72 hours. (Plate 13)

Though the first day egg (24hours and 36 hours) can tolerate up to 9 ±1˚C for quality hatching, the incubation days are not satisfactory from the preservation standpoint as incubation days become shorter in older embryos with the increase of temperature.

And again, embryonic stages up to 72 hours perform better when the temperature stresses are high **(**4±1˚C to 6±1˚C) compared to normal condition. More over it is observed that 60% hatching can be done up to 96 hour when treated with very low temperature cold shock.

So from overall results, up to 96 hours of embryo can be identified as low temperature resistant embryonic stages excluding the in between hours (36, 60 and 84 hours) of respective day old embryo having non- significant variation, for better handling and easy identification during large quantity preservation.And at the same time, low temperatures up to 6±1˚C are optimum for low temperature stress

These findings have clear conformity with the findings of Pandey et al. (1992) who identify 5±2˚C and 7±2˚C as cold shock in oak tasar. Tayede et al. (1987) identifies 5.5˚C as maximum cold shock in multivoltine *Bombyx mori* , Nagina and

Nageshchandra (1988) identifies 5-10˚C in *Philosamia ricini* and Khanikar and Dutta, 2006 identifies 5 to 9˚C as low temperature regime in*A. assama*.

### 5.4. Effect of preservation periods after low temperature stress to the identified embryonic stage

Safe period for cold storage of silk worm eggs depend upon the stage of embryo( Yamashita and Yaginuma,1991) . Many workers have attempted refrigeration of non diapauses egg to postpond hatching (Vemananda Reddy et al.2004, Kumareshan et al. 2004 ). In the present study the stage of the embryo has been identified which are 24 hours to 96 hours. Temperature stress from 4˚C to 6 ˚C is found optimum for cold stress. Now determination of optimum period of low temperature stress to these identified embryonic stages has been studied. The optimum period of preservation from 3 days to 3 weeks( 3 days,7 days, 10 days, 15 days and 21 days) has been taken under consideration to find out the delay of hatching period to combat adverse climatic condition as well as leaf supply. From the farmers point of view favourable condition both in the form of weather and quality leaf when persist desired quantity seed should be supplied for successful muga culture.

Keeping these problems under consideration the present study shows that highest hatching percentage is observed from 24 hour and 48 hour old embryo when preserved for 3, 7, 10 and 15 days; from 72 hours old embryo for 3,7 and 10 days and also from 96 hours old embryo for 3 days irrespective of temperature stress among 4±1˚C to 6±1˚C ( 88.93 to 90.9%) significantly followed by 72 hours when preserved for 15 days irrespective of temperatures (88.62 to 88.7%). 96 hours old embryo when preserved for 7 days shows better performance also (73.30 to 80.10%). Any embryonic age from 24 to 96 hours when preserved for 21 days irrespective of any temperature show very poor performance even no hatching from 96 hours embryo.(Plate 14)

Longest days delay is observed 9.17 days (48 hours embryo at 5±1˚C preserved for 10 days) followed non significantly by 9.16 days (48 hours embryo at 4±1˚C for 10 days), 9.14 days (48 hours embryo at 6±1˚C for 10 days), 9.08 days (24 hours embryo at 6±1˚C for 10 days), 9.02 days (24 hours embryo at 5±1˚C for 10 days) and followed significantly by 8.96 days (24 hours embryo at 4±1˚C for 10 days), and then non significantly by 8.18 days (72 hours embryo at 5±1˚C for 10 days), 8.14 days(72 hours embryo at 4±1˚C for 10 days), 8.13 days (72 hours embryo at 6±1˚C for 10 days),

8.13 days (48 hours embryo at 4±1˚C for 7 days) , 8.1 days (24 hours embryo at 6±1˚C for 6 days) ,8.06 days (72 hours embryo at 6±1˚C for 7 days),8.05 days (24 hours embryo at 5±1˚C for 7 days) and significantly by 8.01 days (72 hours embryo at 5±1˚C for 7 days), 8 days (24 hours embryo at 4±1˚C for 7 days), 7.99 days (72 hours embryo at 4±1˚C for 7 days. (Plate 15)

So, it is observed that up to 15 days preservation, early embryonic stages show good hatching percentage, but the days delayed is very short (nearly 3 days) for 15 days preservation. More over 96 hours embryo shows good hatching percentage when preserved for three days, though the days delayed is nearly one day short than up to 72 hours when preserved for 3 days. So it can be said that up to ten days of preservation is suitable for hatching percentage to ensure desired quantity of seed when required up to a delay of 9 days.

Thangavelu et al. (1985), in *A. assama* observe 7 days of normal incubation period can be extended through 10 days of cold preservation. Present investigation also confirms that in muga silkworm 7 days of normal incubation period can be extended up to a maximum of 9 days of cold preservation. So nearly three weeks (19 days) delay of the hatching can be done which are crucial to the farmers point of view to meet the adverse climatic conditions and leaf requirements. Up to 72 hours of egg at 4±1˚C to 6±1˚C are very much sensitive to cold stress. Similar findings are also present in *Bombyx mori* non diapausing egg (Dutta et al. 1972),in Bivoltine (Vemananda Reddy et al.2004), in *Samia cynthia ricini* (Nagina and Nagesh Chandra 1988) in *Samia ricini (*Sarkar et. al.2012) and in *A.assama* (Ghosh and Ray, 2005)

Nevertheless, 96 hours embryo, *i.e* after 4 days of egg laying if preserved for 3 days, can extend the incubation period for about 7 days which can be utilized in case of sudden adverse climatic condition.

Finally it can be concluded that embryonic stages up to 72 hours (in extreme conditions up to 96 hours) can be utilized for low temperature cold preservation for 10 days(in extreme conditions 3 days or 7 days) at any temperature within 4±1˚C and 6±1˚C with an ultimate objective to supply the desired quantity of quality seed (with higher hatching percentage) to the farmers for successful muga culture in particular and sericulture in general.

Probable causes for better performance with in 6±1˚C by early stage embryo as well as developmental delays to with stand temperature stress can be biochemically justified.

### 5.5 Biochemical changes in embryonic developmental events of *A. assama*

#### 5.5.1 Biochemical changes in embryonic developmental events during normal differentiation of *A. assama*

**Carbohydrate:**

During *A. assama* embryogenesis carbohydrate content decreases from 24 hr old embryo to 72 hr hours old embryo significantly, then increases on 96 hr old embryo and again decreased significantly till before hatching after 168 hr of incubation (Fig 1). Gradual depletion of carbohydrate content acts as utilization for embryogenic process, metabolism and chitin synthesis in *Philosamia ricini* (Pant and Nautiyal, 1974, Krishnappa et al. 2001). Present study we have also shown that in case of *A. assama* gradual decrease of carbohydrate content following anaerobic route in egg from33.98± 0.009mg/g to 25.75±0.006 mg/g. soon after aerobic path way initiated to meet extra demand of energy for histogenesis and carbohydrate content is increased to 27.64± 0.019 mg/g and gradually decreased to 13.23± 0.009 mg/g before hatching after utilizing most of stored carbohydrates Carbohydrate utilization profile during embryogenesis is reported in in *Samia cynthia ricini* ( Krishnappa et al. 2001) and *A. mylitta* ( Sinha et al.1991).

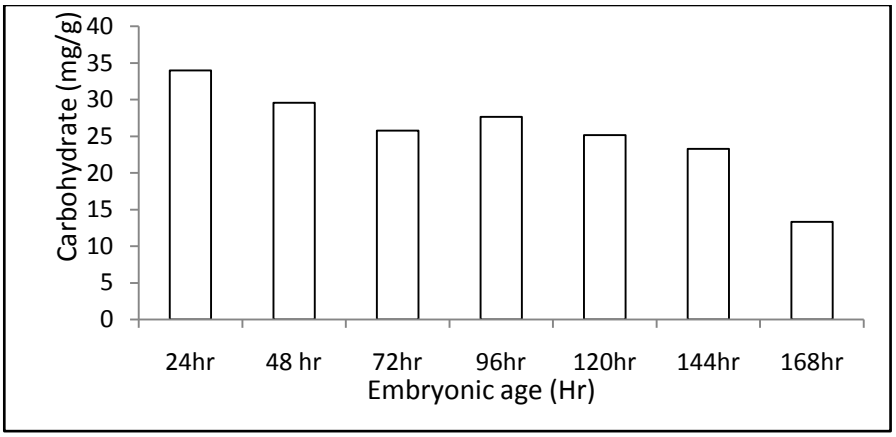



**Fig1.** Showing changes in carbohydrate contents during normal differentiation of *A. assama*

**Protein**

During embryogenesis of *A. assama* protein content decreases from 24 hr old embryo to 72 hr old embryo significantly and from 72 hr (132.53±0.02) to 96 hr (127.25 ±0.025) non significantly and then to 120 hr (121.17±0.04) old eggs significantly. Again, it increases on 144 hr old eggs significantly and then decreased significantly till before hatching after 168 hr of incubation (Fig 2). In non diapauses egg and artificially diapauses terminated eggs decline in protein content is much earlier than diapauses egg (Moorthy et al.2007). Initial total protein concentration in *Philosamia ricini* egg declines during early embryogenesis, rises again (on day 6) hatching depicting intensive tissue transformation during early embryogenesis (Pant and Nautiyal, 1974) *A. assama.*

In mature egg of most insects vitelline contributes 80-90% of total protein content and is utilized during embryogenesis. Histological and embryological studies on *A.assama* embryogenesis also confirms that utilization (Plate I & V).

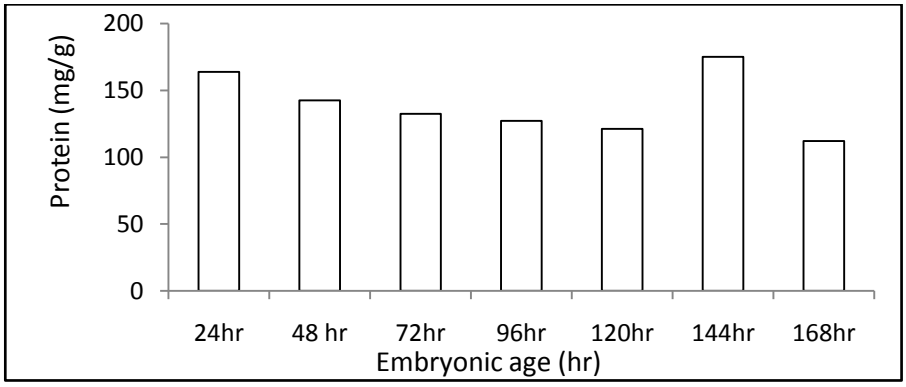



**Fig 2.** Showing changes in protein contents during normal differentiation of *A. assama*

**Cholesterol:**

During embryogenesis of*A. assama*cholesterol content increases from 24 hr old embryo to 48 hr old embryo non significantly, then increases up to 96 hr old embryo significantly and again decreased non significantly till before hatching (168 hr) (Fig 3 ). Non-diapause eggs contain more cholesterol compared to diapauses eggs (Sonobe et al. 1999; Makka and Sonobe, 2000). In diapauses egg bulk of ecdysteroids exists as conjugated form (phosphoric esters) but in non diapauses eggs free forms coexist with conjugated forms (Ohnishi et al.1977; Mizuno et al.1981). Sonobe et al. (1997) reported that in non diapause eggs, Ecdysteroid (E) and 20E sharply increase from the second

day (late gastrula) to the 4th day (organogenesis). Egg ecdysteroids are metabolized in different ways in diapauses and non diapauses eggs. Continuous supply of ecdysteroid 20E may be required to induce embryonic development ( Makka et al.2002).

In *B. mori* eggs are capable for synthesizing 20 E from cholesterol via ketodiol and is synthesized in yolk cell (Snobe and Yamada, 2004). In nondiapause egg ketodiol is metabolized to 20E, which is not formed in diapauses egg. Hydroxylation at C20 of E is catalyzed by Ecdysone 20 hydroxylase (E 20OH ase) is a rate limiting step in the formation of 20E from ketodiol in *B.mori* egg. In non diapauses egg, increase in the activities of both E20OHase and Ecdysteroid phosphate phosphatase (EPPase). EPPase catalyzes the dephosphorylation of ecdysteroid phosphates.

In non diapause *Bombyx* egg first and second layers of embryonic cuticles are formed when labral lobe differentiates (approx. 72 hours) and head and thorax appear (approx. 96 hours) respectively( Takei and Nagashima,1975; Otshuki et al. 1976). The first and second layers of embryonic cuticles formed during marked upsurge of free ecdysteroid including 20E which begins to increase at the gastrula stage and peaks at the blastokinesis ( Yamada and Sonobe, 2003)

In *A. assama*also during embryogenesis, cholesterol depletion is highest within 24 hours probably for the supply of precursors for ecdysteroid synthesis. Rise in cholesterol content in the later part of the embryogenesis may be due to complete utilization of yolk before hatching (Fig 3). In 96 hours embryo cuticles are evident (Plate 6C). After 72 hour, cholesterol content is also in peak (Fig 3) to provide precursor for necessary ecdysteroid for embryonic molting (Plate 6D).This findings have clear conformity with that of other silkworm.

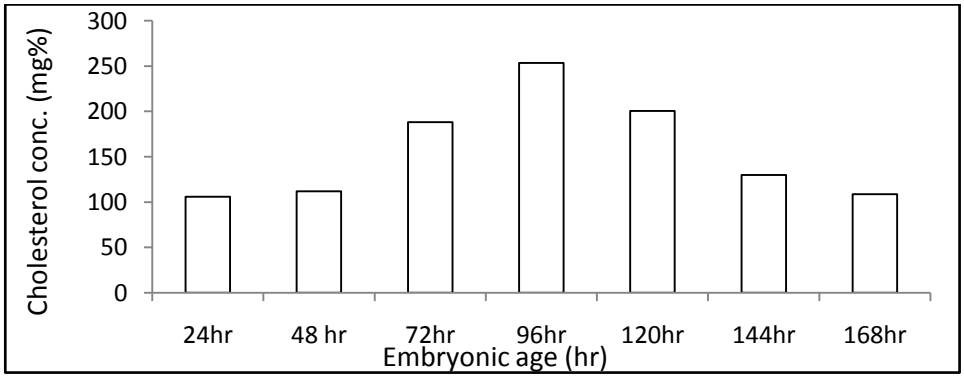



**Fig 3**showing changes in cholesterol contents during normal differentiation of *A. assama*

**DNA**

In a nondiapause egg within 24 hours of oviposition DNA content increases in *Bombyx* ( Furusawa et al. 1985). In present study also *A. assama* DNA content is increases from 24 hr old embryo to 120 hr old embryo significantly, then increases up to 144 hr old non significantly and finally increased significantly till before hatching (168 hr) (Fig 4). There is a sudden decrease of pyruvate content of egg from day 1 to day 3 and is continued the trend (Choudhury,1998). There is a probability for utilization of pyruvate in DNA synthesis initially. From day 2 onwards increase in NAD- SDH activity confirms the increase in fructose as well as NADPH in egg (Table ). In *Bombyx* also level of Phosphofruct kinase activities reported during early embryogenesis (Sakano et al.2004). Pentose phosphate pathway is hypothesized as an alternate route for carbohydrate catabolism (Storey, 1982). During early anaerobic phase of embryogenesis, fructose 5 phosphate may be converted to ribose 5 phosphate and then ribose is pulled into pathways for synthesis of deoxyribonucleotides. Thioredoxin, a cofactor is also involved in reduction of ribonucleotides utilizing available NADPH (Storey and Storey, 2012). Detail study may establish this hexose monophosphate shunt during early embryogenesis

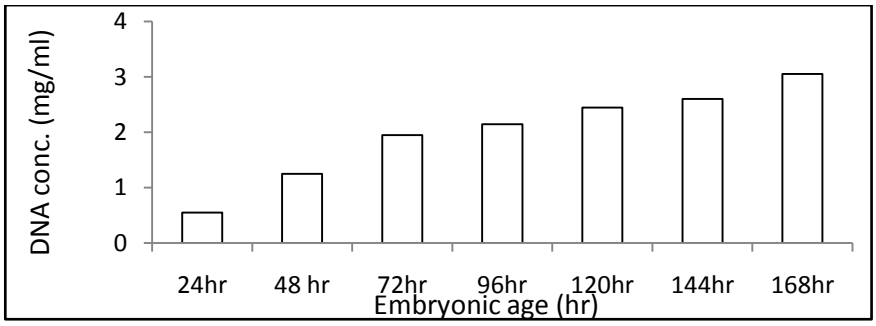



**Fig 4** showing changes in DNA contents during normal differentiation of *A. assama*

**Trehalose**

During embryogenesis of*A. assama* trehalose content initially decreases from 24 hr old embryo to 48 hr old embryoegg significantly, then increased up to 120 hr old significantly and again decreased significantly till before hatching (168 hr).(Fig 5) Trehalose is also another form to store energy in egg (Hasegawa and Yamashita, 1965) and also involved in organogenesis (Becker et al.1996). In *Philosamia ricini* egg, Singh and Singh (1980) reported trehalose peak on day 2 (after laying) and a larger peak on

day 7 (two days before emergence) as well as increment in glucose level and decrease in trehalose during peaks. Again in *P. ricini*trehalose peak is also observed on day 3 and day 5 only (Choudhury,1998). It is evident now that trehalose is the energy currency to sustain in the second phase of the embryonic development.

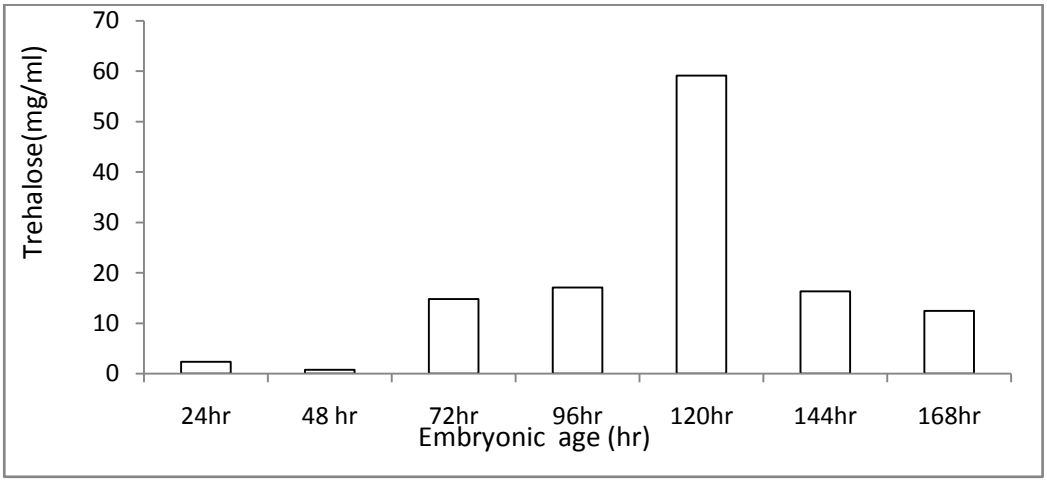



Fig 5 showing changes in trehalose contents during normal differentiation of *A. assama*

**NAD-SDH**

During embryogenesis of*A. assama*NAD-SDH content initially increases from 24 hr old embryo to 72 hr old embryo significantly, then decreases up to 120 hr old embryo significantly and again increases significantly by 144 hr old embryo (2.04±0.001) and finally decreases by 168 hr. (Fig 6).

The shifting pattern of carbohydrate metabolism during embryonic development in non diapauses egg is reported (Sakamo, 2004). In terms of carbohydrate substrate (glycogen) and sugar product (Sorbitol, glucose, fructose, trehalose and glycerol) at last three phases are distinct during embryogenesis; (1) an initial temporary accumulation of sorbitol and (2) synthesis of terhalose both in phase 1 and phase 2 followed by (3) glycolysis and trehalose degradation accompanied by elevated activities of PFK, PK and terhalase phase 2 (Sakamo,2004).

In *Bombyx,* sorbitol accumulate over first two days of embryonic life. For first 2 days glycolytic carbon flows into sorbitols. NAD-SDH converts sorbitol into fructose without using ATP. Cofactor $NAD^+$ converts into NADH is utilized for ATP synthesis through mitochondrial electron transport system (Storey and Storey, 1990; 1991; Yaginuma et al.1990a).

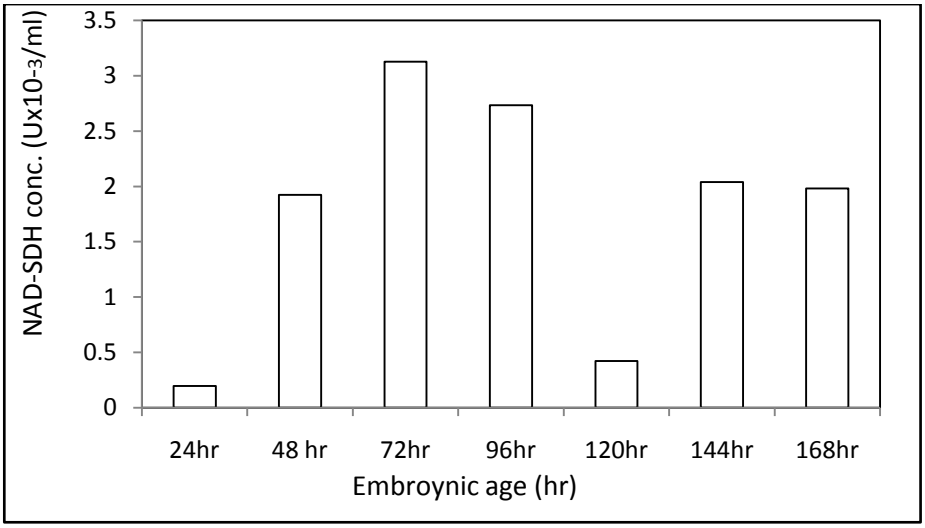



**Fig 6**showing changes in trehalose contents during normal differentiation of *A. assama*

**NADPH- Peroxidase**

During *A. assama* embryogenesis NADPH-Peroxidasecontent is initially increased from 24 hr old embryo to 48 hr old embryo significantly, then decrease up to 96 hours embryo significantly and again increase in 120 hr old embryo significantly and then decrease non significantly till before hatching after (168 hr) (Fig 7).Super oxide dismutase (SOD), Catalase, Glutathione transferase and Glutathione reductase are candidate enzymes in insects (Felton and Sumners, 1995). Due to lack of glutathione peroxidise effect, catalase (CAT) solely perform the job of oxidant removal in insects (Shoal et al.1993). NADH Peroxidase is structurally similar to glutathione reductase (GR). The charge transfer thiolate in GR is structurally equivalent to the redox active cystine in NADH Peroxidase ( Rebecca and Palfey, 2010). The activity of thioredoxin reductase (TrXR) is detected in ovaries of *Bombyx mori*, but not in eggs while neither ovaries nor eggs show glutathione peroxidise (Zhao et al. 2014). NADH Peroxidase play active role during both crucial phases of embryogenesis.

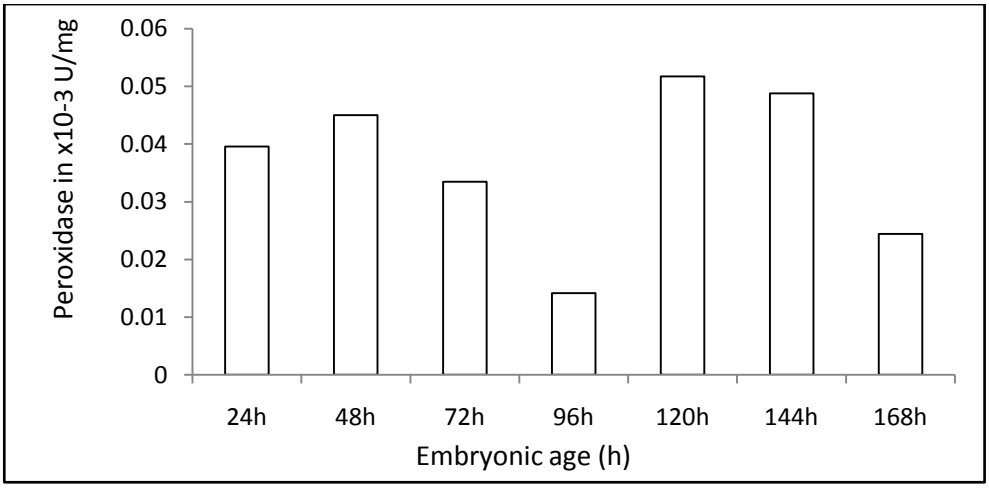



**Fig7**showing changes in NADH Peroxidase contents during normal differentiation of *A. assama*

**Xanthine Oxidase**

During *A. assama* embryogenesis Xanthine Oxidase (XO) content is initially increases from 24 hr old embryo to 48 hr old embryo significantly, then increases up to 120 hr old embryo significantly and also increased non significantly till hatching (168 hr). In *Bombyx*, super oxide anion, hydrogen peroxide and hydroxyl free radical are the witness for oxygen consumption in aerobic cell. Oxidation of hypoxanthine and xanthine to produce superoxide anion $H_2O_2$, are reflected through Xanthne oxidase. Super oxide anion is converted to $H_2O_2$ by super oxide dismutase (SOD) (Zhao & Shi, 2010). Like *Bombyx*, in *A. assama*during embryogenesis XO activities increase initially on 24 hours and sharply increase from 96 hours onwards and reached highest on 168 hours reflecting ATP catabolism during embryo development (Fig. 8).

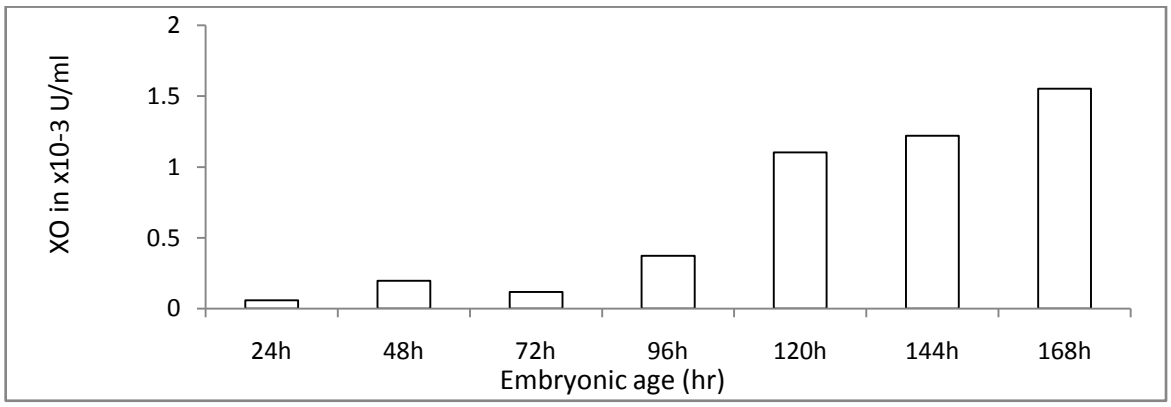



**Fig 8**showing changes in XO contents during normal differentiation of *A. assama*

### 5.5.2 Biochemical changes in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

In *A.assama*, decrease in carbohydrate level is significant during low temperature preservation . Non significant change is observed in carbohydrate content after 3 days 'short chill' in 24 and 48 hr old eggs, but in 72 and 96 hr old eggs have decreased significantly. Up to 72 hr eggs significant decrease in carbohydrate contents are observed during different preservation period. But increase in carbohydrate content is observed only in 96 hr old eggs. However 7 and 10 days low temperature preservation cannot induce significant change in carbohydrate contents between 48 and 72 hr eggs. It may be evident that they have similar cold sensing capacity as well as metabolic strategy.

Soon after 3 days preservation non significant change in protein level is evident, it reflects that all identified embryonic stages can with stand cold shock. But after 7 and 10 days preservation protein contents have increased significantly. It can be said that proteins are synthesized in response to cold shock for longer periods.

Significant decrease in cholesterol content after 3 days short chill is reported in, but during control embryogenesis, continuous increase in cholesterol content is observed, but after low temperature stress cholesterol contentdecreases non significantly. Again non significant decrease is noticed after 7 and 10 days low temperature stress. Low temperature stress may induce delay, utilizing stored energy for cellular maintenance; a 'quiescence' state can be attained by developing embryo.

Soon after 3 days 'short chill' DNA quantities are decreased in identified cold resistance embryonic stages indicating hindered cellular proliferation immediately. But after 7 and 10 days low temperature preservation DNA contents are gradually increased. After withstanding cold shock precursors for nucleotide biosynthesis are increased through converting fructose into ribose through hexose mono phosphate shunt.

Low temperature preservation induced biochemical studies reveal presence of innate mechanism to with stand stress (Lee Jr. 2010). Metabolic shift to utilize carbohydrate as energy source during delayed development is reflected through studies on trehalose and sorbitol dehydrogenase activity. Changes in enzyme quantity and cold with standing

proteins (Storey and Storey, 2010, 2012) may contribute to protein profile during stress experiment. Cholesterol may act as source of energy as well as precursors for endocrine requirements, necessary for delayed development. Initial carbohydrate utilization may deplete precursors for DNA synthesis, but metabolic shift may lead to mono phosphate shunt after resisting low temperature stress, can induce higher DNA synthesis after prolong preservation. But detailed study on the key players of two independent strategy, *i.e.* metabolic shift and oxidative stress resistance, can reflect innate mechanism and possible linkage between the pathways (Denlinger et al. 2010).

Trehalose is most dependable candidate as temperature sensor. Increase in trehalose content to withstand cold stress is observed in 24 hr, 48hr and 96 hr embryo of *A assama* ( Plate 17). After 3 and 7 days stress significant change in trehalose content is observed, but after 10 days stress non significant change in trehalose content reflect the possibility of mechanism to withstand. 72 hr embryo is mostly engaged in developmental milieu initially, soon after it is ready to flux trehalose after 7 days preservation.

In *A. assama* initial plan for embryonic development is continued even during cold storage. So 24 hr embryo utilized energy through conversion of sorbitol to fructose until day 7 of refrigeration. In 72 hour stage NAD SDH level significantly increased after 3, 7 and 10 days preservation demands its cold resistance capacity. After that embryonic development attained a state to compensate energy as a response to cold stress. 48hr and 72 hr eggs also follow the same path, utilizing energy for minimal development and then reduction of enzyme activity recorded. (Plate 16).

After termination of diapauses , sorbitol is utilized as glycogen during embryogenesis (Yaginuma and Yamashita,1978). Chilling at 5˚C induced NAD SDH to convert sorbitol (Yaginuma and Yamashita, 1979). SDH mRNA expressed in diapauses eggs after chilling at 5˚Cfor 40-50 days (Niimi et al. 1993a)confirm the present findings on *A.assama*.

From the present study it is revealed that there is no cessation of developmental plan rather delay is possible during cold storage of nondiapause silkworm *A.assama*. Glycometabolic shift during cold storage maintain a balance between development program and stress management through fine co-ordination. Resistance of diapauses eggs to the 5˚C chilling is significantly higher compared to nondiapause eggs as the

hatchability increased in diapauses egg but decreased in non diapauses eggs after more than 30days of 5˚C chilling(Sakano,2004).

$H_2O_2$ play an importantrole in diapause initiation in *Bombyx* (Zhao et al. 2000) compared to non diapause eggs lower content of $H_2O_2$ and peak of catalase gene expression are observed in diapause eggs during diapause initiation. Significant increase in $H_2O_2$ and the marked suppression of catalase gene expression are observed when diapause initiation is prevented with hydrochloric acid. Even diapauses initiation can also be prevented with exogenous $H_2O_2$.

Main candidate for antioxidant enzyme system is catalase in silkworm. Zhao and Shi (2010) also reports that diapause egg also contain higher $H_2O_2$, higher XO and lower CAT compared to non-diapause egg during 5˚C chilling, $H_2O_2$ and catalase expression in silkworms eggs are involved in diapause initiation and termination. NADH Peroxidase is an alternative antioxidant may involve in glycometabolic shift. 48hr and 96 hr embryo passed through the crucial transitions of embryogenesis where higher NADH Peroxidase activity is essential as alternate antioxidant system along with catalase. Reduction of NADPH to $NAD^+$ siphoned NADPH generated by NAD SDH and another way NAD can be utilized by NAD SDH to break sorbitol into fructose. Enhancement of enzyme activity after 7 day in 24 hr and 72 hr embryo also establishes link between metabolism during embryogenesis and management of stress. Soon after refrigeration in 24 and 48 hours egg, NADH Peroxidase content have increased significantly, reflect its necessity to with stand oxidative stress during pre blastokinesis stages. In post blastokinetic stages significant increase in enzyme level is noticed only after 7 and 10 days preservation, where also embryo meet oxidative stress after low temperature preservation.( Plate 19).

In A. assama egg due to cold shock oxidative stress is evident in early embryogenesis (i.e. 24, 48 and 72 hr) and Xanthine oxidase (XO) activity is increased to withstand the stress, and then decreased after 10 days preservation and homeostatic level is maintained. But 96 hr embryo can withstand initial chill, but due to continuous embryonic development, enhanced metabolic requirement and continued oxidative stress may lead to the enhancement of XO enzyme activity. Initial cold shock (3days) of eggs up to 72 hour , sudden significant rise in XO level confirm the idea that it is a definite candidate to monitor oxidative stress in embryo.(Plate 18).

Finally regression analysis also confirms that the changes in activity of NAD SDH, Trehalose, Xanthine oxidase and NADPH Peroxidase during refrigeration for different periods (3, 7 and 10 days) and different embryonic stages are correlated significantly (Plate16, 17, 18 and 19). It establishes presence of inherent homeostatic mechanism to withstand stress induced metabolic shift as well as continuation of developmental plan with an inevitable delay.

# 6. SUMMARY

Cultural, political and economic history of mankind, for last 5000 years, is intermingled with the voyage of silk. So Sericulture has a long tradition with human. Indian sericulture industry is growing steadily from 23060MT silk production with 2240 crore (In Rs) export earning and 7.56 million employments in 2011-12 to 32000MT silk production, 3172 croe (in Rs) export earning and 9.24 million employments. But domestic demand, now, stands at 28800 MT, compared to 23679 MT annual production. Annual growth of Mulberry sericulture is 2.10% whereas 7.49% growth is recorded in Non mulberry section.

In the perspective of rural poverty and unemployment in terai zone of West Bengal having low productive agriculture and industrial backwardness, the sericulture in general and muga culture in particular may have special reference for the economic up lift of rural people. Muga culture is an agro based small scale industry of North East India (42642 families) and around 34316 families from Assam are directly engaged in this culture. Muga silkworm, *Antheraea assama*, West wood (Lepideptera: Saturnidae) is multivoltine and eggs are non-diapause type, therefore muga culture requires continuous multiplication of the species. This polyphagous insect is feeding out door on trees of wide range of host plant, Som, *Persia bombycina*, (Kost) and Soalu, *Litsaea monopelata* (Rox.) are considered primary food plants. Silk worm feeding on Som produces yarn of good quality while that feeding on Soalu produces better fecundity.

The adverse climatic condition as very high temperature and humidity, heavy and continuous rainfall storm etc during seed crop rearing to supply required quantity of quality seed for subsequent commercial rearing which on turn becomes uncertain, leading to low production of seed. Estimating from the available plantation, about 1.5 core dlf are required annually. However presently around 60-30 lakh dlfs are produced leaving 20-40% of the plantations are unutilized. Raw silk production statistics show that over 50 years or so production of muga has increased only 2 times as against 21 times in mulberry. Last ten years, production of muga raw silk is fluctuating in between 100-124MT. This is a gloomy state of growth in muga silk production. According to the Annual report of Central silk Board, in 2014-15, 4.5% rise in production of muga silk is reported (158MT).

As the muga seeds are non diapause type, low temperature preservation of seeds to arrest the developmental process for some time to skip the adverse environmental condition may overcome the constraint. Development of egg preservation technique for muga silk worm eggs of different embryonic ages have been kept at low temperature (5$^{o}$C) by following some intermediate steps of preservation for different durations (10days to 20 days with 10 days interval to detect suitable embryonic stages for long term preservation. Developmental processes including differentiation and organogenesis in muga silkworm (*Antheraea assama*, Ww) egg is a continuous programme because these are non-diapause eggs and are laid by multi-voltine muga silk worm and larva hatch on day 7 after lay.

Developmental stages of silk worm are classified into following six stages *i.e.* 1) Prediapause, 2) Diapause, 3) Hibernation, 4) Critical Stage, 5) Formation of organ and 6) Completion of larva. The prediapause includes seven stages, *i.e.* fertilization, cleavage, germanlage formation, yolk cleavage, pyriform shaped stage, kokeshi (China spoon like stage) and chemical spatula like stage. Two stages of diapause and four stages of hibernating period is recognized. Organ formation stage is divided into 10 stages, *i.e.* 1) appearance of labral appendages, 2) shortening stages, 3) Cephalo-thoracic segmentation; 4) Blastokinesis; 5) Completion of blastokinesis, 6) appearance of trichogen cell, 7) appearance of setae and appearance of tracheal taenidia. The completion of larva includes five stages *i.e.* 1) Head pigmentation-I, 2) Head pigmentation-II, 3) Body pigmentation-I and 4) Body pigmentation –II and hatching. Present study also confirms that the 168 hrs long episodes of embryogenesis are rapid indeed with similar pattern of developmental schemes as *Bombyx* or *A. yamami*. After 6 hours of egg deposition cleavage nuclei are migrated to the surface of the egg and arranged spherically starting a stage of de-lamination.In *A. assama* microvilli are distinct until 12 hrs of oviposition.

**1.** After 6 hours of egg diposition cleavage nuclei migrate to the surface of the egg and arrange spherically starting a stage of delamination. The sphere swell at the micropyle end and at least at opposite end. These one cell layered epithelium differentiated into blastoderm. After 12 hours of egg deposition rapid increase of blastomeres develop blastoderm which exceedingly thin in the dorsal site and thick at the ventral site. Condensation both from the cephalic and caudal end develop ventral plate. Microvilli like projections are evident on blastoderm. Basement membrane separates blastomeres

from vitellophages accumulated through endocytosis activity. Germ cells are appeared in the posterior pole outside blastoderm. On yolk mass outer yolk cells are distinct. Soon after formation of blastoderm, gradual invagination inside egg starts. Amniotic fold appears with inner and outer layer which transform later chorion and amnion respectively. Serosa consisting flattened cell, formation is completed by this times. Amnion covers germband. Constriction to ventral plate gives rise to germ band. Germband already under gone gastrulation and differentiate into ectoderm and also pseudo-startified lanceolate cells are evident. After 24 hour muga silk worm egg germ band appeared with proctocephalon and protocrom. Soon after attaining'daruma' stage, primitive groove appeared along the median line of the germ band surface, which is narrow at the center and wider at two edges. This stage resemble with 'kokeshi' of Bombyx sp. Segmentation is initated during this stage. After 48 hours amniotic membrane extended along the whole length of embryo by elongated flattened cells. The ectoderm has evolved multistartified layer with elongated cells. Fluid filled amniotic cavity become distinct. Serosa lies just under the chorion surrounding embryo, amnion and yolk and presence of two membranes are called synapomorphy. As development continues 72 hours embryo starts blastokinesis soon after formation of germ band. Blastokinesis entails early entry and later exit of embryo from yolk. Blastokinesis has two types of movement.ie. anatrepsis (upward) and ketatrepsis (down ward). During anatrepsis invagination of tissues into yolk is evident. Embryo extends in length and segmentation and appendage formation initiated. The ectoderm is crossed by transverse furrows limitating the different segments and differentiate polygonal neuroblast with clear edges and neurotic projections. Neural groove also has appeared during this stage. Protuberance is appeared in thoracic segment. At the later stage protuberance is appeared in thoracic segment. At the later stage protuberances is also appeared in abdominal segment. In 96 hours embryo katatrepsis has initiated as eversion or outward movement of both embryo and amnion. A back flip or 180˚ revolution of embryo is observed. A pair of labial protuberance becomes distinct in front of head fold. Stomodeum and proctodeum has appeared and gradually become tubular. In the 96 hours egg, the caudal area is surrounded by the amino proctodeal cavity bounded by amnion and the invagination will originate proctodaeum, along with coelomic cavities behind which germ cells are clustered. In the proctocephalon there are two coelomic cavities. Amniotic cavity has enlarged and the amnion surrounds the embryo and yolk. Serosa has secreted a distinct culticular layer. The segmentation is more advanced.

During progressive emergence head and antennae, the legs and lastly abdomen released from yolk. In the thoracic region rudiments of appendages appear and in cephalic region formed by beginning of stomodaeum. The differentiation of labrum occurs in protocephalon , over which yolk is still present. In the protocorm, the buds of the gnathal appendages, of the three pairs of the legs and of the ten abdominal segments are evident. In the first abdominal segment there is pleuropodium, conical shaped and small. In the following seven segments and in the tenth there are proleg buds. The ventral nerve cord is well defined. During 120 hours embryo, length of embryo covers almost whole length of the embryo and both end come in contact which is called dorsal closure. Soon after dorsal closure involution starts to move embryo from ventral side to dorsal side. Vertical turning of posterior abdominal segment put the abdominal region in a straight line. Then abdominal region reached towards anterior region at the level of prothorax. Fore gut and hind gut differentiate from anterior and posterior ectodermal invagination respectively. After 144 hours, head capsule formation is completed and mouth parts become mature. Three segmented antennae with antennal setae, mandibles and labrum are well developed. Tips of labrum and labium become segmented. Thoracic legs become segmented with claws at distal end. Rudiments of setae develop on body surface. Entire body of 168 hours embryo covered with strong setae and embryonic moult is occurred in this stage. Caudal horns appeared in this stage. Mandibles become sclerotised and pigmented at the distal end. Larval eye *(i.e.* Ocelli) appears as six brown spot on either side of head. The spiracles are clearly visible on the sides of body. Head capsule and mouth appendages are sclerotised and well pigmented. The amnion and serosa disappear by fragmentation. Embryo ingests the embryonic membranes and sensitive for adverse environmental condition. Entire body of embryo become sclerotised.

During embryogenesis organ formations have started. The nervous system differentiates into four principal part; a) the brain or supraoesophageal ganglia, situated in the head above esophagus, b) the suboesophageal ganglia also is situated in the head, below the esophagus, c) ventral nerve cord running ventrally through thorax and abdomen, has swollen into paired ganglia at the posterior body parts and d) sympathetic nerve system innervating mouth and esophagus. Early development of brain starts from neuroblast distributed through large area. The proctocerbrum sometimes called the ganglion of first head segment forms the fore most and greater portion of brain and follows the

deuterocerebrum, ganglion of second head segment and send nerves to antennae and hind most tritocerebrum, the ganglion of the third or intercalary segment, from which the nerve cord to sympathetic ganglion is sent off.

Three segments of guts at first develop separately then complete alimentary canal is developed. When plugs of cells at the end of foregut, each end of mid gut and end of hind gut die and three gut segments are unite. As the anterior and posterior mid gut primordial come together they enclose remaining yolk sac within mid gut. Alimentary canal has three parts *i.e*. fore gut, mid gut and hind gut. Large amount of yolk remains in the mid gut lumen. Magnified image shows Columnar cell and goblet cell from the mid gut epithelium. In 144 hours stage mid gut reduced its width without thick yolks. Majority of yolk substances may be absorbed by the mid gut cell after digestion, leaving only part in alimentary canal.

Develop as evagination of anterior proctodeum and mark the junction between mid gut and hind gu, Malphigian tubules make first appearance as minute buds from lateral and ventro-lateral sides of the anterior region of hind gut. Gradually the length of the bud increased.

Anterior wall of tubules are continuous with the wall of midgut and posterior end with hind gut. A thin membrane stretches across the anterior end of proctodaeum, making posterior end of mid gut and is called met-enteric membrane. The region of union of posterior wall of tubules and proctodaeum differentiate from mid gut as well as from the proctodaeum, called posterior interstitial ring and later transformed to proctodaeal valve. Tubules lie anterior to the ring. Basal part of each lobule finally fused, thus forming a single vesicle from which both tubules of a pair appear to arise. Later length of each tubule has increased and become slightly twisted in the form of an inverted S. At last vesicles become coiled to form a few loops and Malphigian tubule developed.

**2.** Embryonic developments of Muga silkworm, *A. assama* during low temperature stress are delayed. After 3days refrigeration, 12 hours of eggs, embryo became larger in size. Germ band became thick. After 3days refrigeration 24 hour muga silk worm eggs became larger, covering the entire length of the egg and are showing resemblance with 48 hour embryos. After 3days refrigeration 48 hours egg is continued to grow in slower rate and are showing resemblance with 96hours embryo. As development continues

slowly 72 hours embryo after 3 days refrigeration had shown resemblance with 120 hr embryo.

To identify low temperature resistant embryonic stages from 24 hours to 144 hours embryo in eleven treatments (*viz.* 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 hours) under four temperature shocks (4, 6, 8, 10±1˚C) for ten days are taken for hatching percentage and incubation periods. 24 hours embryo at 4±1˚C show highest hatching percentage (90.85%) having non significant variation with the same embryonic stage at 6±1˚C (89.79%) and 8±1˚C (89.58%); 36 hours embryonic stages at 4±1˚C (89.85%), 6±1˚C (89.75%), 8±1˚C (89.71%); 48 hours embryonic stages at 4±1˚C (89.69%), 6±1˚C (89.58%), 60 hours at 4±1˚C (89.37%), 6±1˚C (89.53%) and 72 hours embryonic stages at 4±1˚C(89.36%), 6±1˚C (88.93%) and significantly by 24 hours embryo at 10±1˚C (83.51%) and by others. So it is observed that control treatments and the embryonic stages up to 72 hours when treated with 4±1˚ and 6±1˚, temperature stress show non significant variation in hatching percentage. As the temperature shock decreased to 8±1˚C or 10±1˚ the hatching percentage is also decreased but still close to control up to 48 hours of embryonic stage. Moreover no hatching from 84 hours onwards embryonic ages is found at 10±1˚C from 108 hour embryonic stage at 8±1˚C. It is also revealed that highest incubation period is observed at 4±1˚C low temperature stress, by 108 hours (18.72 days), non significantly followed by 4±1˚C x 120 hours (18.66 days), 4±1˚C x 132 hours (18.62 days) and 4±1˚C x 144 hours (18.60 days) and significantly by 4±1˚C x 60 hours (18.17 days), 4±1˚C x 48 hours (18.16 days), 4±1˚C x 72 hours (18.14 days), 4±1˚C x 84 hours (18.04 days), 6±1˚C x 48 hours (18.14 days) 6±1˚C x 72 hours (18.13 days) having non significant variation among them. Lowest incubation period is observed from 6±1˚C x 144 hours (11.02 days) followed non significantly by 6±1˚C x 132 hours (11.03 days)

Finally it can be said that embryonic stages up to 72 hours show better hatching percentage (90.85 to 88.93%) over control (82%) though the variations are non significant for both low temperature stresses 4±1˚C and 6±1˚C. Incubation period however, is found highest in late embryonic ages only in 4±1˚C, but for 6±1˚C up to 96 hours the incubation periods are longer. But if the embryonic age are considered to be deleted from the actual ten days shock then early ages up to 96 hours show higher incubation periods. For higher levels of temperature shock that is 8±1˚C and 10±1˚C up to 36 hours old embryo show higher hatching percentage than control though the

variations are non significant. For these two temperatures incubation period are found longer up to 72 hours. For 8±1˚C up to 96 hours can be considered as after that no hatching is observed. So it can be concluded that embryonic stages of 24, 36, 48, 60, 72, 84 and 96 hours can be identified as low temperature resistant embryonic stages, more precisely, up to 96 hours embryonic stages can be explored for improvement of cold preservation technology of seed.

Present observation shows that control treatments and the embryonic stages up to 72 hours when treated with 4±1˚C and 6±1˚C temperature stress have non significant variation in hatching percentage. As the temperature shock decreases to 8±1˚C or 10±1˚C, the hatching percentage also decreases but still close to control up to 48 hours of embryonic stage. Moreover, there is no hatching from 84 hours onwards embryonic ages at 10±1˚C and from 108 hour embryonic stage at 8±1˚C. Incubation period is longer in higher level of temperature stresses and in early ages of embryo.

**3.** Then the identified low temperature resistant stages namely 24, 36, 48, 60, 72, 84 and 96 hours old embryonic stages are given low temperature stresses from 4±1˚C to 10±1˚C with 1˚C interval that is 4±1˚C, 5±1˚C, 6±1˚C, 7±1˚C, 8±1˚C, 9±1˚C and 10±1˚C to determine the optimum low temperature for cold stress for successful low temperature preservation based on hatching percent and incubation days. Highest hatching % is observedup to9±1˚C for 24 hours, up to 7±1˚C for 36 hours and up to 6±1˚C for 48 to 72 hours of embryonic stages having non significant variation among them (90.85 to 88.23%). 36 hours embryonic stage having 8±1˚C temperature stress show hatching percentage of 87.71% while at 9±1˚C this stage show 82.56% hatching. All these hatching percentages are higher than or at per hatching in normal condition (82%).

Highest incubation period is observed from 4±1˚C temperature stress on 48 hours to 96 hours embryo (18.0 to 18.17 days); 48 and 72 hours at both 5±1˚C and 6±1˚C temperature stress (18.13 to 18.18 days) followed significantly by 60 hours (16.96 days) and 84 hours (17.86 days) embryo at 6±1˚C and also significantly with 60 hours (17.57 days) and 84 hours (17.65 days) egg at 5±1˚C and 36 hours embryo at 4±1˚C(17.58 days) having non significant variation among them. More over from 7±1˚C to higher range, performance are poor and even at 10±1˚C, hatching is not observed beyond 72 hours.

Again it is found that embryonic stages up to 72 hours performed better when the temperature stress is high **(**4±1˚C to 6±1˚C) when compared to normal condition. Though, early embryonic stages could survive well even in 10±1˚C also. More over it is observed that 60% hatching could be done up to 96 hour when treated with very low cold shock. Similarly incubation days become shorter in older embryos with the increase of temperature. However, very early embryos show longer incubation days even in 10±1˚C.

So from overall results, up to 96 hours of embryo could be identified as low temperature resistant embryonic stages excluding the in between hours (36, 60 and 84 hours) of respective day old embryo having non significant variation for better handling and easy identification during large quantity preservation. . And at the same time, low temperatures with in 4±1˚C and 6±1˚C could be applied for determination of optimum low temperature stress period.

Keeping these earlier findings under consideration the present study identifies the embryonic stages for cold shock (24 hour to 96 hours old egg). Now the optimum low temperature should be determined as cold stress from a wide range of temperature shock from 4±1˚C to 10±1˚C with an interval of 1˚C in the present study.

Highest hatching percentage is observedup to9±1˚C for 24 hours, up to 7±1˚C for 36 hours and up to 6±1˚C for 48 to 72 hours of embryonic stages having non significant variation among them (90.85% to 88.23%), 36 hours embryonic stage having 8±1˚C temperature stress shows hatching percentage of 87.71% while at 9±1˚C, this stage show 82.56% hatching. All these hatching percentages are higher than or at per hatching in normal condition (82%). So, 24 hours and 36 hours old embryo can withstand 4±1˚C to 9±1˚C and 48 hours to 72 hours old embryo can tolerate 4±1˚C to 6±1˚C regarding better hatching percentage. Now, highest incubation period is observed from 4±1˚C temperature stress on 48 hours to 96 hours embryo (18.0 to 18.17 days); 48 and 72 hours at both 5±1˚C and 6±1˚C temperature stress (18.13 to 18.18 days) followed significantly by 60 hours (17.57 days) and 84 hours (17.65 days) embryo at 6±1˚C and also significantly with 60(17.57 days) and 84 hours (17.65 days) egg at 5±1˚C and 36 hours embryo at 4±1˚C (17.58 days) having non significant variation among them. More over from 7±1˚C to higher range, performance is poor and even at 10±1˚C, hatching is not observed beyond 72 hours.

Though the first day egg (24hours and 36 hours) can tolerate up to 9 ±1˚C for quality hatching, the incubation days are not satisfactory from the preservation standpoint as incubation days become shorter in older embryos with the increase of temperature. And again, embryonic stages up to 72 hours perform better when the temperature stresses are high **(**4±1˚C to 6±1˚C) compared to normal condition. More over it is observed that 60% hatching can be done up to 96 hour when treated with very low temperature cold shock.So from overall results, up to 96 hours of embryo can be identified as low temperature resistant embryonic stages excluding the in between hours (36, 60 and 84 hours) of respective day old embryo having non- significant variation, for better handling and easy identification during large quantity preservation. And at the same time, low temperatures up to 6±1˚C are optimum for low temperature stress.

**4.** Lastly, 24, 48, 72 and 96 hours embryonic stages are given 4˚, 5˚ & 6±1˚C temperature stress at different preservation periods namely 3 days, 7 days, 10 days, 15 days and 21 days to determine the optimum periods of low temperature stress. Days delay means the period from egg laying to hatching excluding the preservation days. Highest hatching percentage is observed from 24 hour and 48 hour old embryo when preserved for 3, 7, 10 and 15 days; from 72 hours old embryo for 3,7 and 10 days and also from 96 hours old embryo for 3 days irrespective of temperature stress among 4±1˚C to 6±1˚C ( 88.93 to 90.9%) significantly followed by 72 hours when preserved for 15 days irrespective of temperatures (88.62 to 88.7%). 96 hours old embryo when preserved for 7 days show better performance also.(73.30 to 80.10%). Any embryonic age from 24 to 96 hours when preserved for 21 days irrespective of any temperature show very poor performance even no hatching from 96 hours embryo. Longest days delay is observed 9.17 days (48 hours embryo at 5±1˚C preserved for 10 days) followed by non significantly by 9.16 days (48 hours embryo at 4±1˚C for 10 days), 9.14 days (48 hours embryo at 6±1˚C for 10 days), 9.08 days (24 hours embryo at 6±1˚C for 10 days), 9.02 days (24 hours embryo at 5±1˚C for 10 days) and significantly followed by 8.96 days (24 hours embryo at 4±1˚C for 10 days), and then non significantly by 8.18 days (72 hours embryo at 5±1˚C for 10 days), 8.14 days (72 hours embryo at 4±1˚C for 10 days), 8.13 days (72 hours embryo at 6±1˚C for 10 days), 8.13 days (48 hours embryo at 4±1˚C for 7 days) , 8.1 days (24 hours embryo at 6±1˚C for 6 days) ,8.06 days (72 hours embryo at 6±1˚C for 7 days), 8.05 days (24 hours embryo at 5±1˚C for 7

days) and significantly by 8.01 days (72 hours embryo at 5±1˚C for 7 days), 8 days (24 hours embryo at 4±1˚C for 7 days), 7.99 days (72 hours embryo at 4±1˚C for 7 days).

It is observed that up to 15 days preservation early embryonic stages show good hatching percentage up to 15 days, but the days delayed regarding hatching is very short (nearly 3 days). More over 96 hours embryo show good hatching percentage when preserved for three days, but the days delayed is nearly one day short than up to 72 hours. So it can be concluded that up to ten days of preservation are found suitable for hatching percentage to ensure desired quantity of seed when required up to a delay of 9 days.

So it can be concluded that early embryos up to 72 hours can be preserved for up to 10 days at any cold shock with 4±1˚C to 6±1˚C, so that the differentiation period can be delayed to ensure seed supply at desired quantity when required. More over performances of 96 hour embryo can also meet the demand of seed supply to some extent. Keeping these problems under consideration the present study shows that highest hatching percentage is observed from 24 hour and 48 hour old embryo when preserved for 3, 7, 10 and 15 days; from 72 hours old embryo for 3,7 and 10 days and also from 96 hours old embryo for 3 days irrespective of temperature stress among 4±1˚C to 6±1˚C ( 88.93 to 90.9%) significantly followed by 72 hours when preserved for 15 days irrespective of temperatures (88.62 to 88.7%). 96 hours old embryo when preserved for 7 days shows better performance also (73.30 to 80.10%). Any embryonic age from 24 to 96 hours when preserved for 21 days irrespective of any temperature show very poor performance even no hatching from 96 hours embryo.

Longest days delay is observed 9.17 days (48 hours embryo at 5±1˚C preserved for 10 days) followed non significantly by 9.16 days (48 hours embryo at 4±1˚C for 10 days), 9.14 days (48 hours embryo at 6±1˚C for 10 days), 9.08 days (24 hours embryo at 6±1˚C for 10 days), 9.02 days (24 hours embryo at 5±1˚C for 10 days) and followed significantly by 8.96 days (24 hours embryo at 4±1˚C for 10 days), and then non significantly by 8.18 days (72 hours embryo at 5±1˚C for 10 days), 8.14 days(72 hours embryo at 4±1˚C for 10 days), 8.13 days (72 hours embryo at 6±1˚C for 10 days), 8.13 days (48 hours embryo at 4±1˚C for 7 days) , 8.1 days (24 hours embryo at 6±1˚C for 6 days) ,8.06 days (72 hours embryo at 6±1˚C for 7 days),8.05 days (24 hours embryo at 5±1˚C for 7 days) and significantly by 8.01 days (72 hours embryo at 5±1˚C

for 7 days), 8 days (24 hours embryo at 4±1˚C for 7 days), 7.99 days (72 hours embryo at 4±1˚C for 7 days.

So, it is observed that up to 15 days preservation, early embryonic stages show good hatching percentage, but the days delayed is very short (nearly 3 days) for 15 days preservation. More over 96 hours embryo shows good hatching percentage when preserved for three days, though the days delayed is nearly one day short than up to 72 hours when preserved for 3 days. So it can be said that up to ten days of preservation is suitable for hatching percentage to ensure desired quantity of seed when required up to a delay of 9 days.

**5.** Biochemcal analysis reveal the detail mechanism of During *A. assama* embryogenesis carbohydrate content is decreased from 24 hr to 72 hr old egg significantly, then has increased on 96 hr old and again decrease significantly till before hatching after 168 hr. Gradual depletion of carbohydrate content acts as utilization for embryogenic process, metabolism and chitin synthesis in *Philosamia ricini* . Present study we have also shown that in case *of A. assama* gradual decrease of carbohydrate content following anaerobic route in egg from33.98± 0.009mg/g to 25.75±0.006 mg/g. soon after aerobic path way initiated to meet extra demand of energy for histogenesis and carbohydrate content is increased to 27.64± 0.019 mg/g and gradually decreased to 13.23± 0.009 mg/g before hatching after utilizing most of stored carbohydrates

During *A. assama* embryogenesis protein content is decreased from 24 hr to 72 hr significantly and from 72 hr to 96 hr non significantly and then to 120 hr old eggs, significantly. Again, it is increased on 144 hr old eggs significantly and then decreases significantly till before hatching after 168 hr. In non diapause egg and artificially diapause terminated eggs decline in protein content much earlier than diapause egg. Initial total protein concentration in *Philosamia ricini* egg decline during early embryogenesis, rise again (on day 6) eve of emergence of first instar larva depicting intensive tissue transformation during early embryogenesis. In *A. assama* also same trend is described in present study.

During *A. assama* embryogenesis cholesterol content is increased from 24 hr (105.88±0.027) to 48 hr (111.76±0.021) old embryo non significantly then increased up 168 hr . Non-diapause eggs contain more cholesterol compared to diapause eggs. In diapause egg bulk of ecdysteroids exists as conjugated form (phosphoric esters) but in

non diapause eggs free forms coexist with conjugated forms. In non diapause eggs, Ecdysteroid (E) and 20E sharply increase from the second day (late gastrula) to the 4$^{th}$ day (organogenesis). Egg ecdysteroids are metabolized in different ways in diapause and non diapause eggs. Continuous supply of ecdysteroid 20E may be required to induce embryonic development.

In a nondiapause egg within 24 hours of oviposition DNA content is increased in *Bombyx*. In *A. assama* DNA content is increased from 24 hr to 120 hr old egg significantly, then increase up to 144 hr old non significantly and finally increase significantly till before hatching after 168 hr . There is a sudden decrease of pyruvate content of egg from day 1 to day 3 and is continued the trend. There is a probability for utilization of pyruvate in DNA synthesis initially. From day 2 onwards increase in NAD-SDH activity confirmed the increase in fructose as well as NADPH in egg. In *Bombyx* also level of Phosphofruct kinase activities reported during early embryogenesis. Pentose phosphate pathway is hypothesized as an alternate route for carbohydrate catabolism. During early anaerobic phase of embryogenesis, fructose 5 phosphate may be converted to ribose 5 phosphate and then ribose is pulled into pathways for synthesis of deoxyribonucleotides. Thioredoxin, a cofactor is also involved in reduction of ribonucleotides utilizing available NADPH. Detail study may establish this hexose monophosphate shunt during early embryogenesis

During *A. assama* embryogenesis trehalose content is initially decreased from 24 hr to 48 hr old egg significantly, then increased up to 120 hr old significantly and again decreased significantly till before hatching after 168 hr). Trehalose is also another form to store energy in egg and also involved in organogenesis. In *Philosamia ricini* egg, reported trehalose peak on day 2 (after laying) and a larger peak on day 7 (two days before emergence) as well as increment in glucose level and decrease in trehalose during peaks.

During *A. assama* embryogenesis NAD-SDH content is initially increased from 24 hr to 72 hr old egg significantly, then decreased up to 120 hr old significantly and again increased significantly by 144 hr and finally decrease by 168 hr the shifting pattern of carbohydrate metabolism during embryonic development in non diapause egg is reported .

During *A. assama* embryogenesis NADPH-Peroxidasecontent is initially increased from 24 hr (to 48 hr old egg significantly, then decrease up to 96 hours egg significantly and again increase in 120 hr old significantly and then decrease non significantly till before hatching 168 hr.Super oxide dismutase (SOD), Catalase, Glutathione transferase and Glutathione reductase are candidate enzymes in insects. Due to lack of glutathione peroxidise effect, catalase (CAT) solely perform the job of oxidant removal in insects. NADH Peroxidase is structurally similar to glutathione reductase (GR). The charge transfer thiolate in GR is structurally equivalent to the redox active cystine in NADH Peroxidase. The activity of thioredoxin reductase (TrXR) is detected in ovaries of *Bombyx mori*, but not in eggs while neither ovaries nor eggs show glutathione peroxidise. NADH Peroxidase play active role during both crucial phases of embryogenesis.

During *A. assama* embryogenesis XO content is initially increased from 24 hr to 48 hr old egg significantly, then increased up to 120 hr old significantly and decreased non significantly but again increased before hatching after 168 hr. In *Bombyx*, super oxide anion, hydrogen peroxide and hydroxyl free radical are the witness for oxygen consumption in aerobic cell. Oxidation of hypoxanthine and xanthine to produce superoxide anion $H_2O_2$, are reflected through Xanthne oxidase. Super oxide anion is converted to $H_2O_2$ by super oxide dismutase (SOD). Like *Bombyx*,during embryogenesis XO activities increase initially on 24 hours and sharply increase from 96 hours onwards and reached highest on 168 hours reflecting ATP catabolism during embryo development .

In *A.assama*, decrease in carbohydrate level are significant during low temperature preservation. Non significant change in carbohydrate content after 3 days ‘short chill’ in 24 and 48 hr old eggs, but in 72 and 96 hr old eggs have decreased significantly. Up to 72 hr eggs there are significant decrease in carbohydrate contents are observed during different preservation period. But increase in carbohydrate content is observed only in 96 hr old eggs. However 7 and 10 days low temperature preservation cannot induce significant change in carbohydrate contents between 48 and 72 hr eggs. It may be the evidence that they have similar cold sensing capacity as well as metabolic strategy.

Soon after 3 days preservation non significant change in protein level is evident it reflects that all identified embryonic stages can with stand cold shock. But after 7 and

10 days preservation protein contents have increased significantly. It can be concluded that proteins are synthesized in response to cold shock for longer periods.

Significant decrease in cholesterol content after 3 days short chill is reported in, but during control embryogenesis, continuous increase in cholesterol content is observed, but after low temperature stress non significantly decreased. Again non significant decrease is noticed after 7 and 10 days low temperature stress. Low temperature stress may induce delay, utilizing stored energy for cellular maintenance; a 'quiescence' state can be attained by developing embryo.

Soon after 3 days 'short chill' DNA quantities are decreased in identified cold resistance embryonic stages indicating hindered cellular proliferation immediately. But after 7 and 10 days low temperature preservation DNA contents are gradually increased. After withstanding cold shock precursors for nucleotide biosynthesis are increased through converting fructose into ribose through hexose mono phosphate shunt.

Low temperature preservation induced biochemical studies reveal presence of innate mechanism to with stand stress. Metabolic shift to utilize carbohydrate as energy source during delayed development will be reflected through studies on trehalose and sorbitol dehydrogenase activity. Changes in enzyme quantity and cold with standing proteins, may contribute to protein profile during stress experiment. Cholesterol may act as source of energy as well as precursors for endocrine requirements, necessary for delayed development. Initial carbohydrate utilization may deplete precursors for DNA synthesis, but metabolic shift may lead to mono phosphate shunt after resisting low temperature stress, can induce higher DNA synthesis after prolong preservation. But detail study on the key players of two independent strategies, *i.e.* metabolic shift and oxidative stress resistance, can reflect innate mechanism and possible linkage between the pathways.

Trehalose is most dependable candidate as temperature sensor. Increase in trehalose content to withstand cold stress is observed in 24 hr, 48hr and 96 hr embryo of *A assama*. After 3 and 7 days stress significant change in trehalose content is observed, but after 10 days stress non significant change in trehalose content reflect the possibility of mechanism to withstand. 72 hr embryo is mostly engaged in developmental milieu initially, soon after it is ready to flux trehalose after 7 days preservation.

In *A. assama* initial plan for embryonic development is continued even during cold storage. So 24 hr embryo utilized energy through conversion of sorbitol to fructose until day 7 of refrigeration. In 72 hour stage NAD SDH level significantly increased after 3, 7 and 10 days preservation demands its cold resistance capacity. After that embryonic development attain a state to compensate energy as a response to cold stress. 48hr and 72 hr eggs also follow the same path, utilizing energy for minimal development and then reduction of enzyme activity recorded.

After termination of diapause , sorbitol is utilized as glycogen during embryogenesis. Chilling at 5˚C induced NAD SDH to convert sorbitol. SDH mRNA has expressed in diapause eggs after chilling at 5˚Cfor 40-50 days.

From the present study it is revealed that there is no cessation of developmental plan rather delay is possible during cold storage of nondiapause silkworm *A.assama*. Glycometabolic shift during cold storage maintain a balance between development program and stress management through fine co-ordination. Resistance of diapause eggs to the 5˚C chilling is significantly higher compared to nondiapause eggs as the hatchability increased in diapause egg but decreased in non diapause eggs after more than 30days of 5˚C chilling.

$H_2O_2$ play an importantrole in diapause initiation in *Bombyx* compared to non diapause eggs lower content of $H_2O_2$ and peak of catalase gene expression are observed in diapause eggs during diapause initiation. Significant increase in $H_2O_2$ and the marked suppression of catalase gene expression are observed when diapause initiation is prevented with hydrochloric acid. Even diapause initiation can also be prevented with exogenous $H_2O_2$

Main candidate for antioxidant enzyme system is catalase in silkworm. Diapause egg also contain higher $H_2O_2$, higher XO and lower CAT compared to non-diapause egg during 5˚C chilling, $H_2O_2$ and catalase expression in silkworms eggs are involved in diapause initiation and termination. NADH Peroxidase is an alternative antioxidant may involve in glycometabolic shift. 48hr and 96 hr embryo passed through the crucial transitions of embryogenesis where higher NADH Peroxidase activity is essential as alternate antioxidant system along with catalase. Reduction of NADPH to $NAD^+$ siphoned NADPH generated by NAD SDH and another way NAD can be utilized by NAD SDH to break sorbitol into fructose. Enhancement of enzyme activity after 7 day

in 24 hr and 72 hr embryo also establishes link between metabolism during embryogenesis and management of stress. Soon after refrigeration in 24 and 48 hours egg, NADH Peroxidase content have increased significantly, reflect its necessity to with stand oxidative stress during pre blastokinesis stages. In post blastokinetic stages significant increase in enzyme level is noticed only after 7 and 10 days preservation, where also embryo meet oxidative stress after low temperature preservation.

In *A. assama* egg due to cold shock oxidative stress is evident in early embryogenesis (i.e. 24, 48 and 72 hr) and Xanthine oxidase (XO) activity is increased to withstand the stress, and then decreased after 10 days preservation and homeostatic level is maintained. But 96 hr embryo can withstand initial chill, but due to continuous embryonic development, enhanced metabolic requirement and continued oxidative stress may lead to the enhancement of XO enzyme activity. Initial cold shock (3days) of eggs up to 72 hour , sudden significant rise in XO level confirm the idea that it is a definite candidate to monitor oxidative stress in embryo.

Present study confirms the presence of inherent homeostatic mechanism to withstand stress induced metabolic shift as well as continuation of developmental plan with inevitable delay.

# PLATES

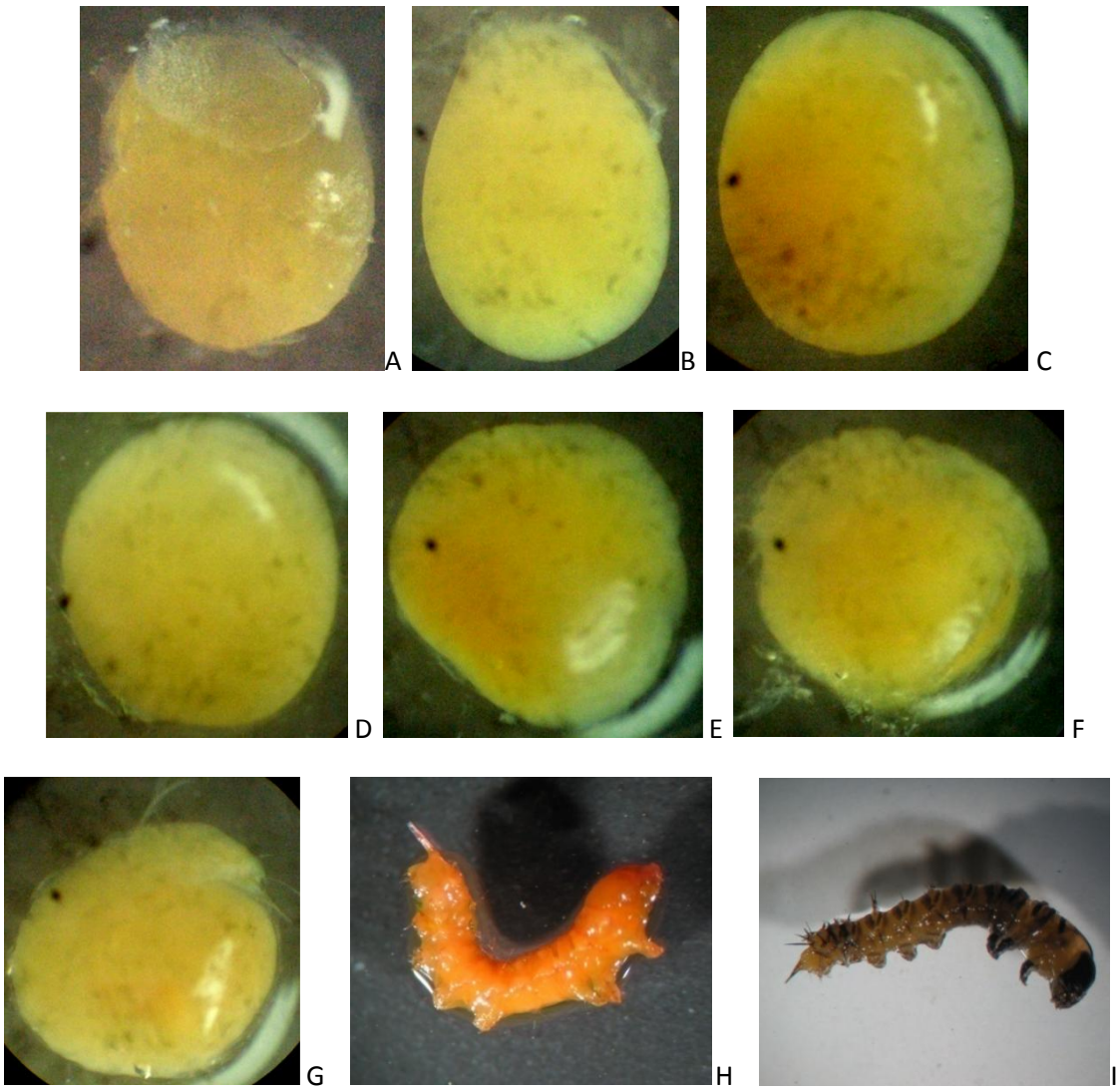



**Plate 1.** Showing Stereo binocular images of (A)6 Hours, (B)12 Hours, (C) 24 Hours,(D)48 Hours, (E) 72 Hours, (F)96 Hours, (G)120Hours, (H)144Hours and (I) 168 Hours of *Antheraea assama* Embryogenesis.

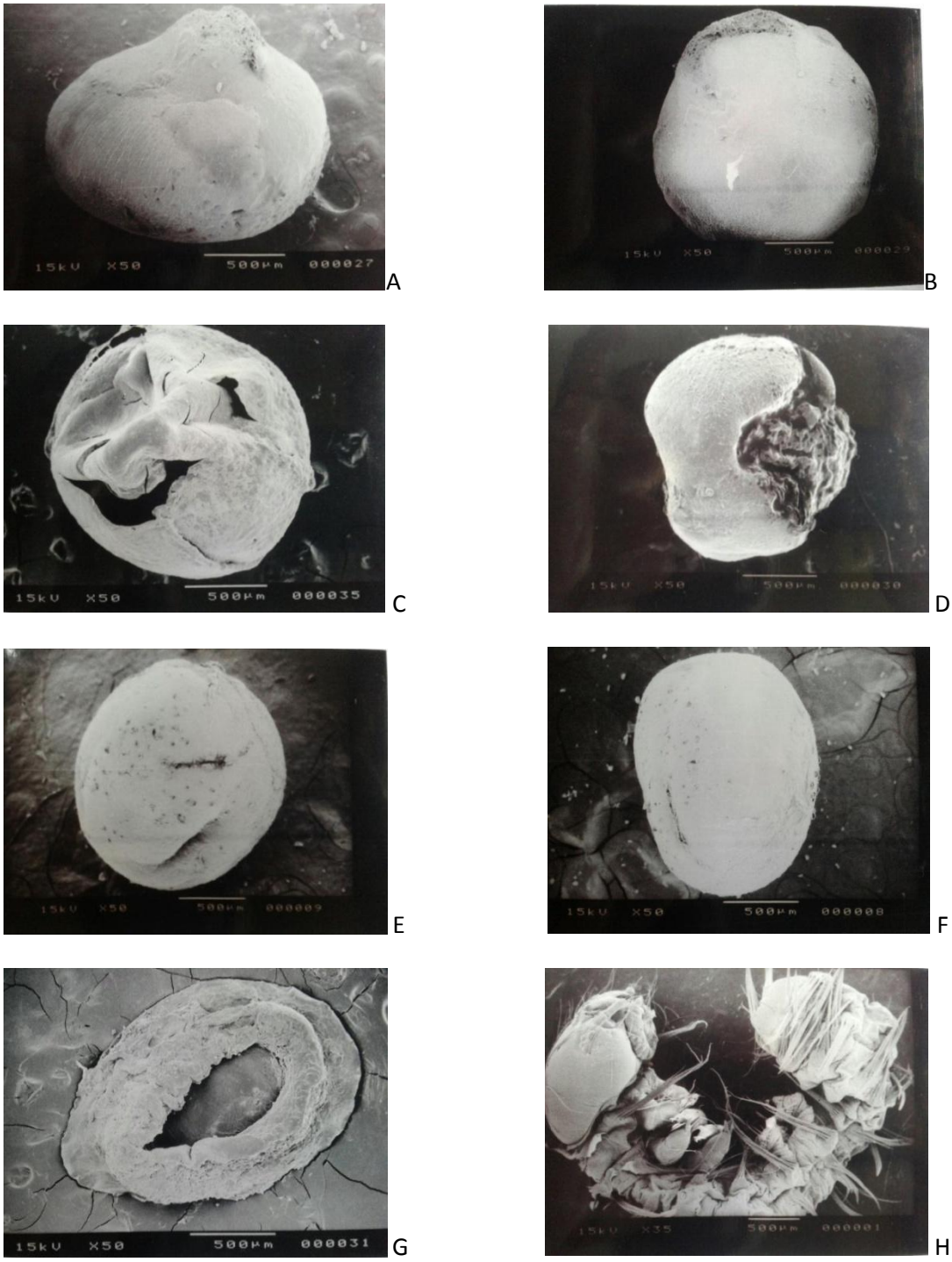



**Plate 2.** Showing Scanning Electron Microscopic images of (A)6 Hours, (B)12 Hours, (C) 24 Hours,(D)48 Hours, (E) 72 Hours, (F)96 Hours, (G)120Hr and (H)144hours of *Antheraea assama* Embryogenesis.

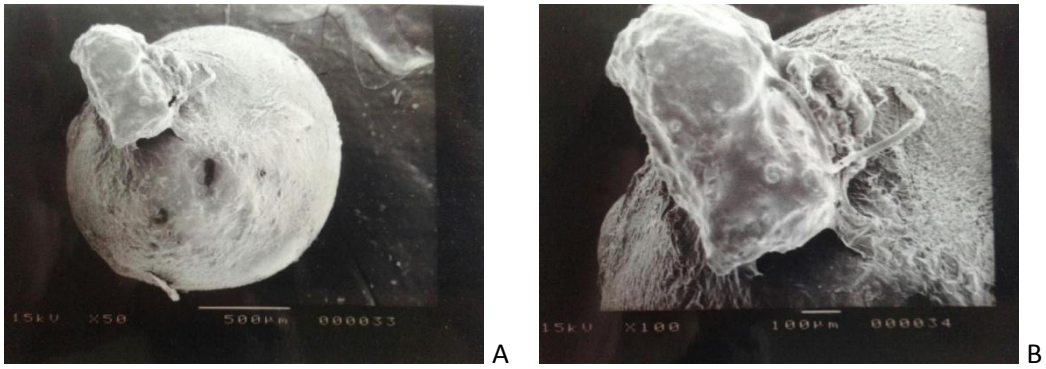



**Plate 3.** Showing Scanning Electron Microscopic image of out ward movement during ketatrepsis of *Antheraea assama* Ww embryo

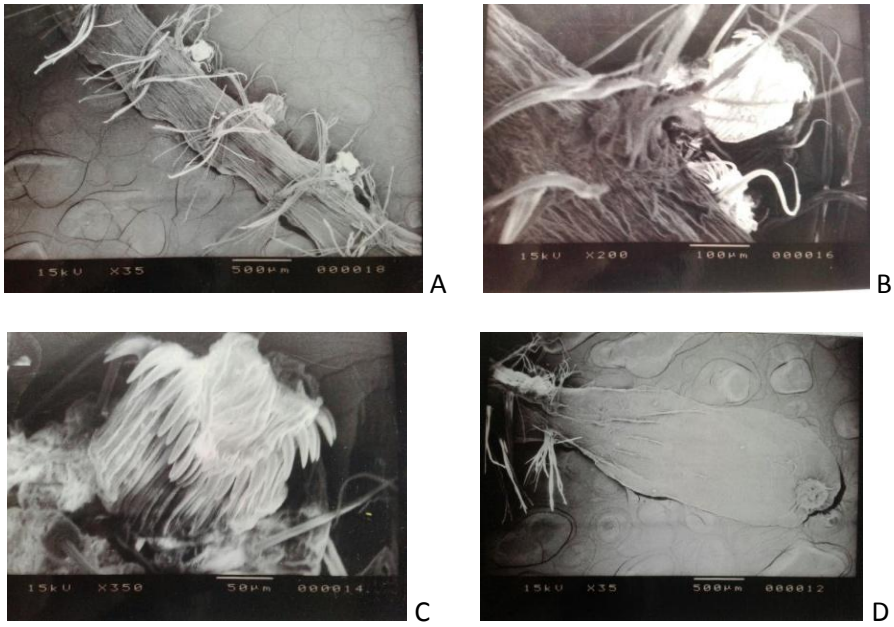



**Plate 4.** Showing appearance of setate on the embryo (A, B, C) and rectal sac (D) of *Antheraea assama*

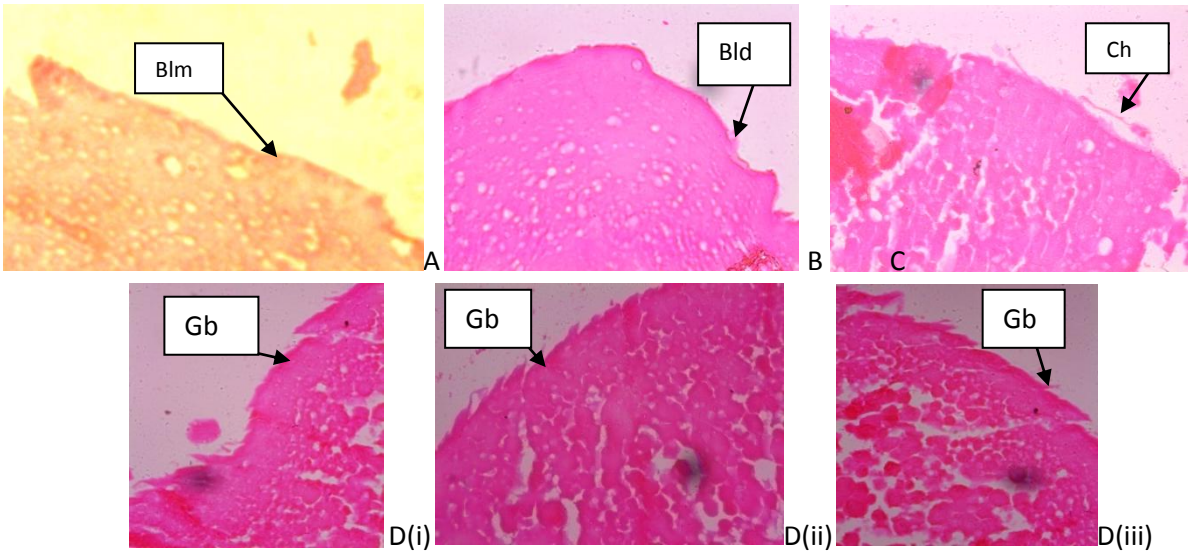



**Plate 5.** Showing section of (A)6 Hours, (B) 12 Hours, (C) & (D)24 Hours embryo of *Antheraea assama* Ww. Blm: Blastomere, Bld: Blastoderm, Ch: Chorion, Gb: Germ band

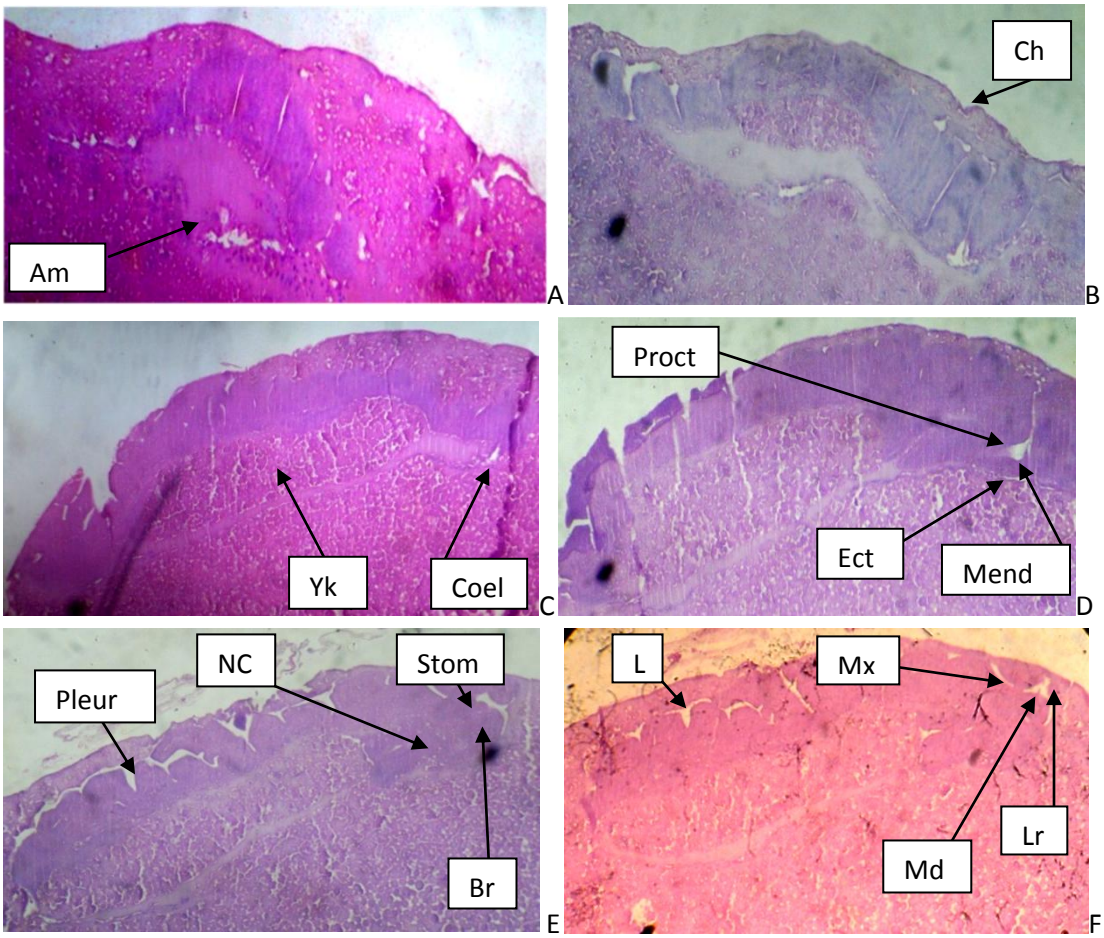



**Plate 6.** Showing sections of (A&B) 48 Hours, (C&D)72 Hours, (E&F)96 Hours of embryogenesis of *Antheraea assama* Ww. Am: Amnion, Ch:: Chorion, Proct: Proctodeum, Yk : Yolk, Coel: Celomic cavity, Ect: Ectoderm, Mend: Mesoendoderm, Br: Brain, NC: Ventral nerve cord, Stom: Stomodaeum, Pleur: Pleuropodium, L: Leg, Lr:Labrum, Mx: Maxilae, Md: Mandibles,

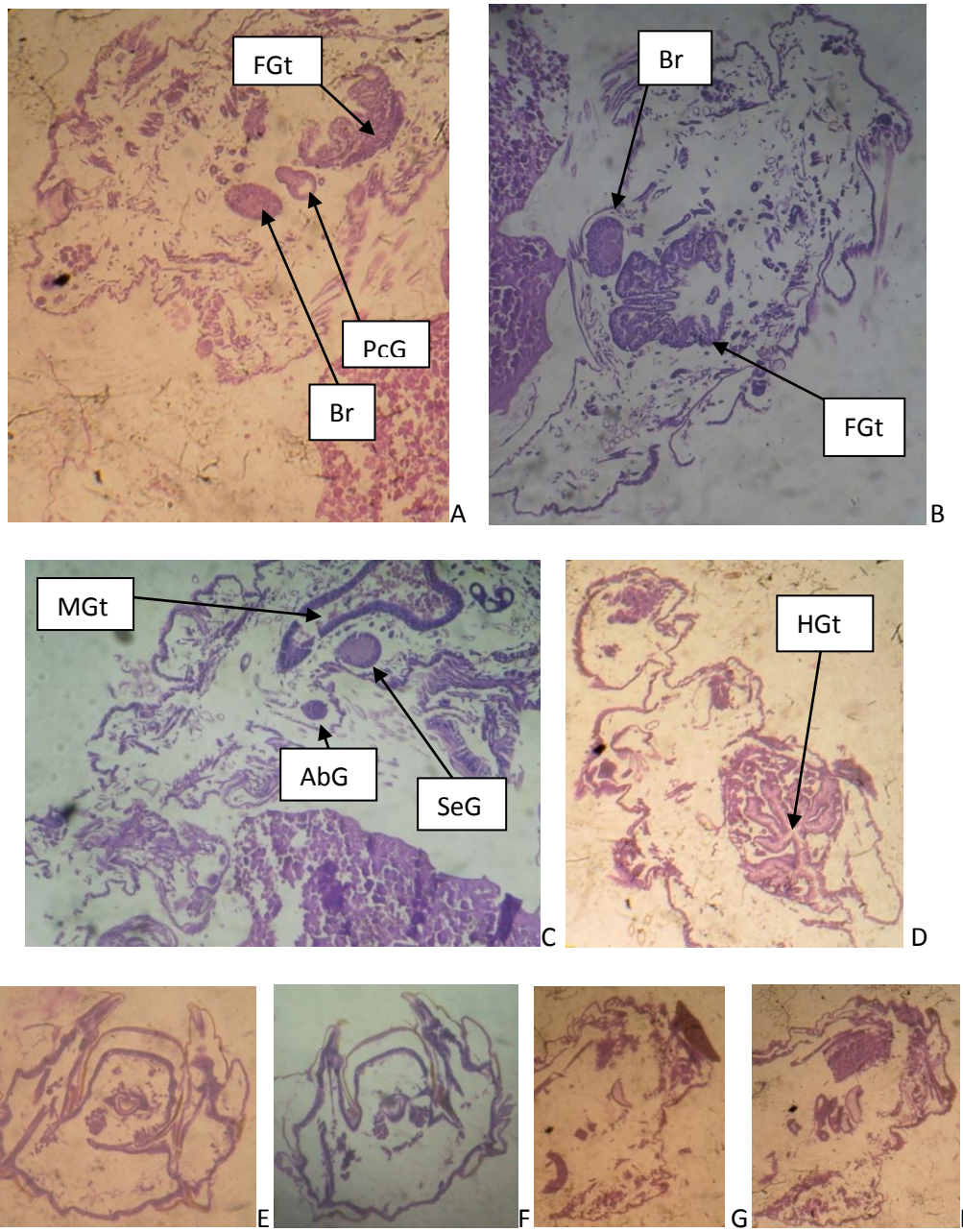
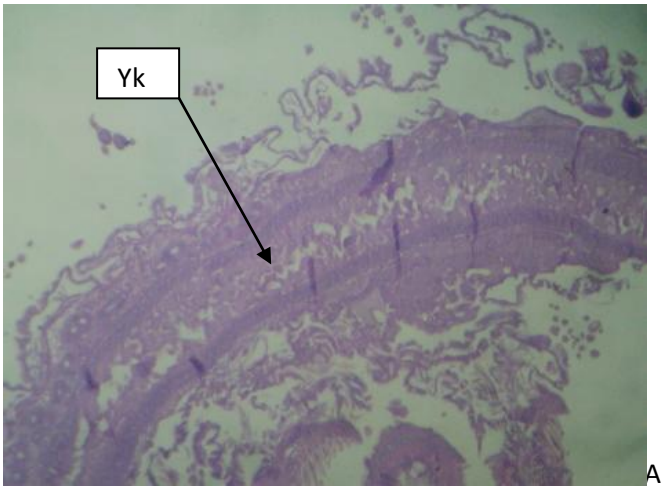
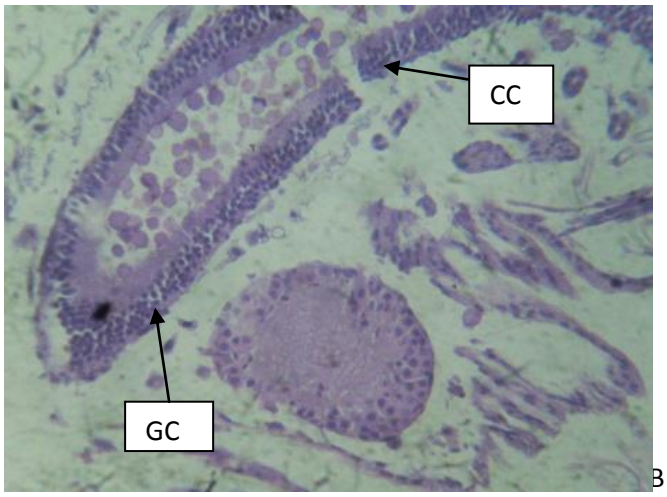


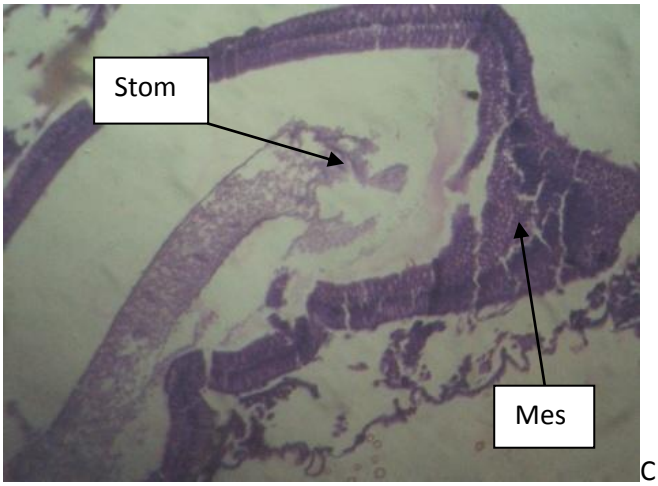

**Plate 7.** Showing section of (A)120 hours,(B,C&D)144 Hours, (E)120Hours Head, (F)144Hours Head (G) 120 Hours Caudal end and (H)144 hours Caudal end of *Antheraea assama* embryo. Br: Brain, PcG: Pro Cephalic Ganglion, SeG: Sub esophageal Ganglion, AbG: Abdominal Ganglion, FGt: Fore Gut, MGt: Mid Gut, HGt: Hind Gut




A




B




C

**Plate 8.** Showing sections of (A) 120 hours Mid Gut (B) 144 hours Mid Gut and (C) connection realized between Fore gut(Stomodeum) and Mid gut(Mesenteron) in the embryo of *Antheraea assama* Ww.Y: Yolk,CC: Columnar Cell, GC: Goblet Cell, Stom: Stomodeum, Mes: Mesenteron

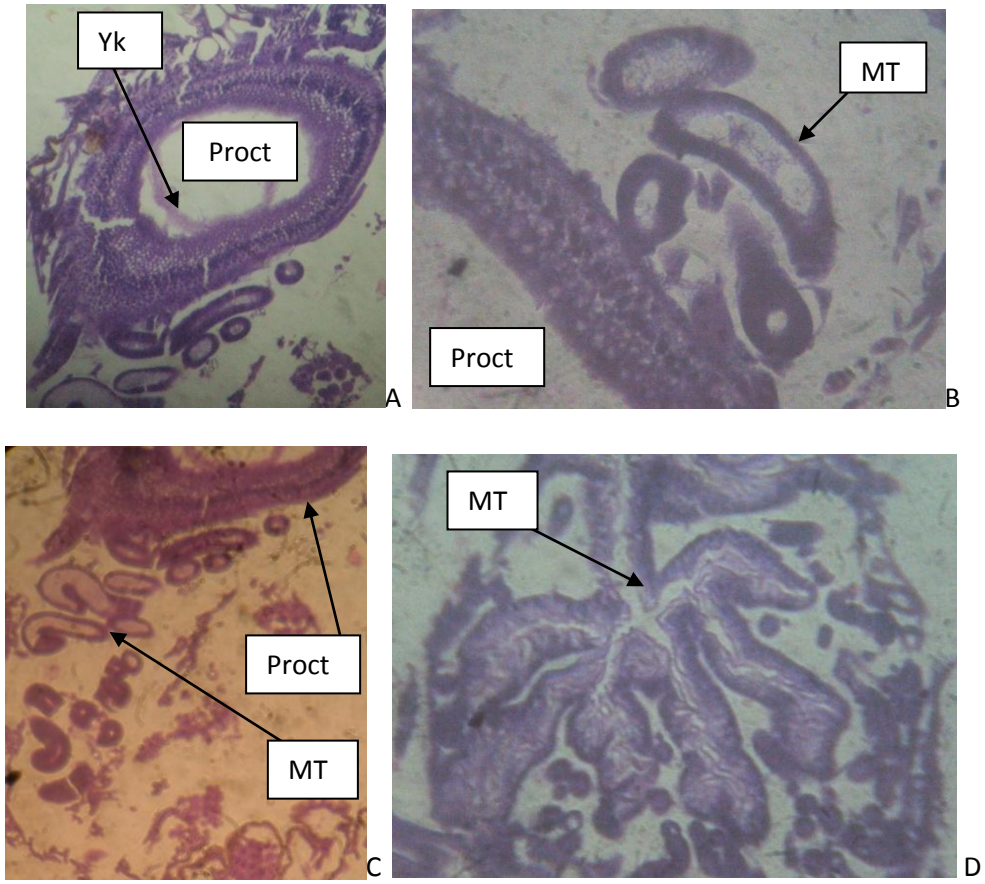



**Plate 9.** Showing section of 120hours embryo having bud from proctodeum (A,B,C) and 144hours embryo with Malphigian tubules(D) in *Antheraea assama* Ww. Proct: Proctodeum, Yk: Yolk, MT: Malphigian tubule

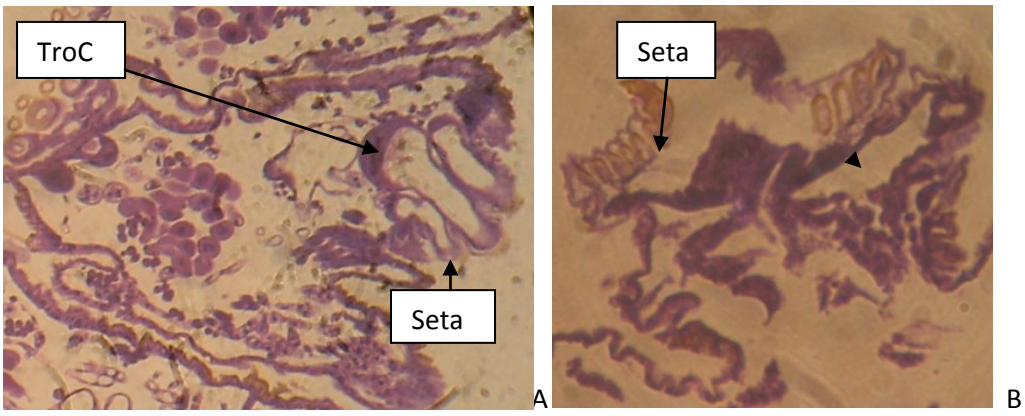



**Plate 10.** Showing section of (A) 120hours embryo with Trichogen cell and (B)144 hours embryo with seta during embryogenesis of *Antheraea assama* Ww. TroC: Trichogen cell

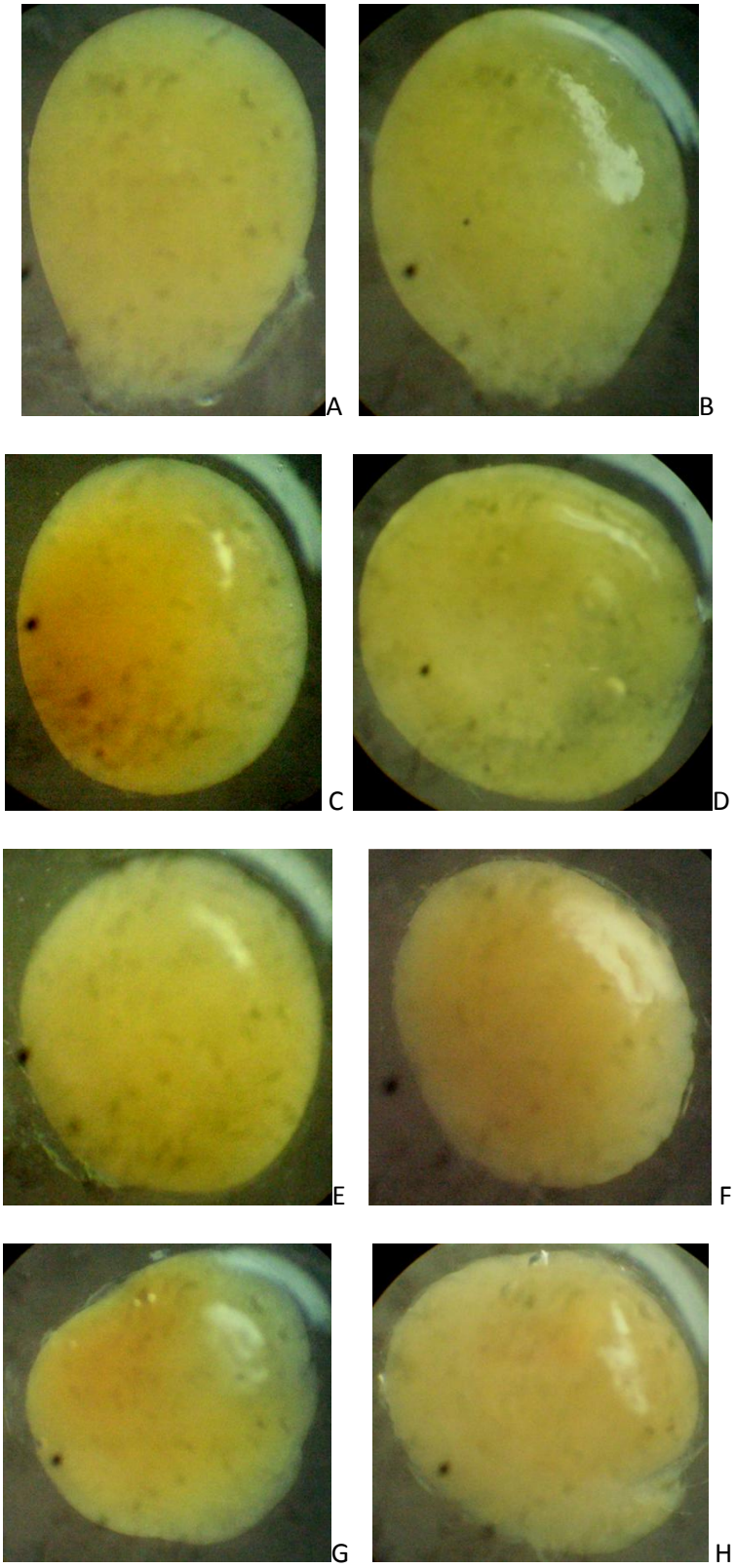
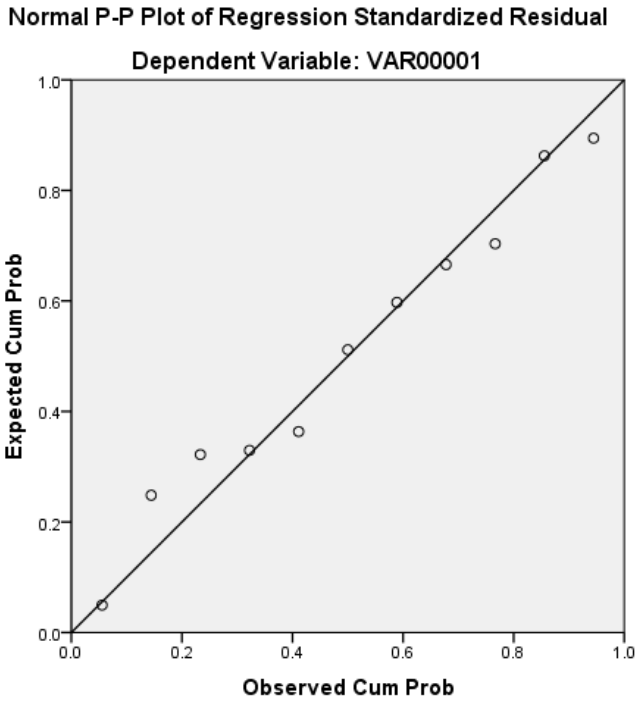
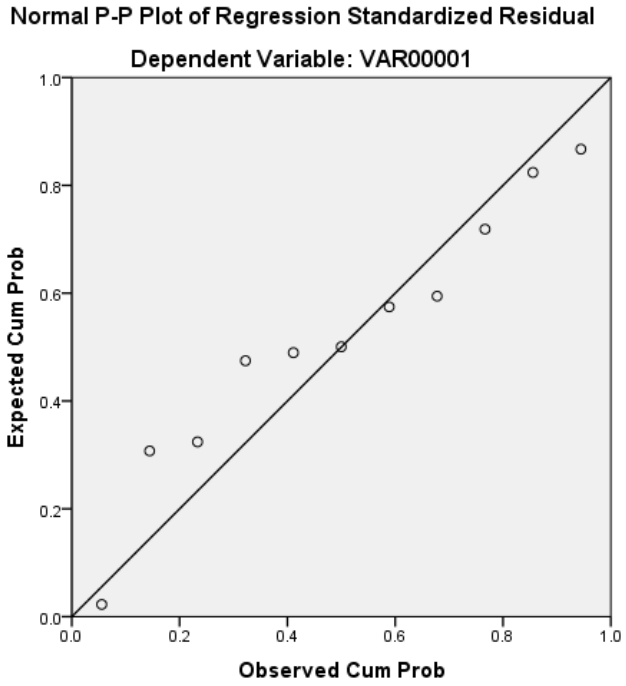



**Plate 11.** Showing Stereo binocular images of (A)12 Hours, (B)12 Hours after 3days refrigeration, (C) 24 Hours,(D)24Hours after 3days refrigeration, (E) 48 Hours, (F)48 Hours after 3days refrigeration, (G)72Hours and (H)72Hours after 3days refrigeration, at 5˚C during *Antheraea assama* Embryogenesis.

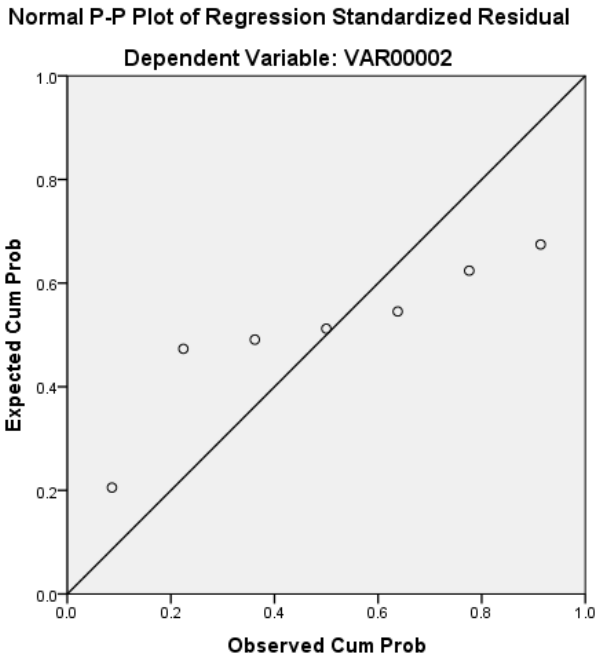



A

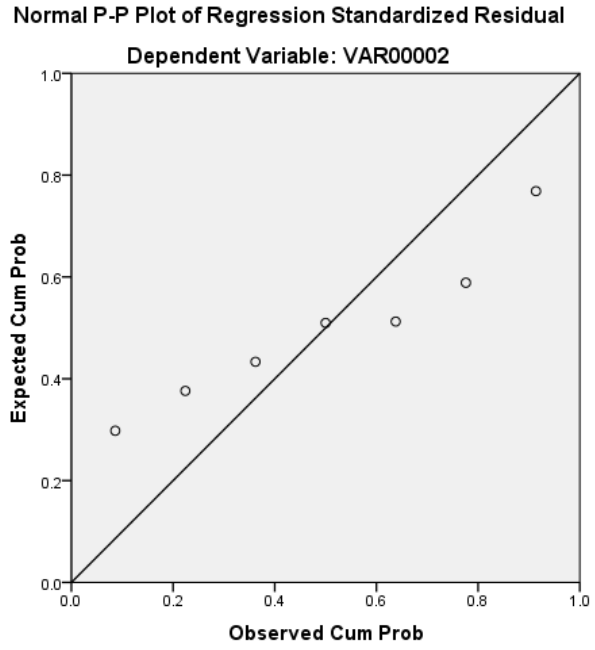



B

**Plate 12.** Regression Plot showing effect of temperature stress on different embryonic stages of *A.assama* on A) hatching percentage and B) incubation period

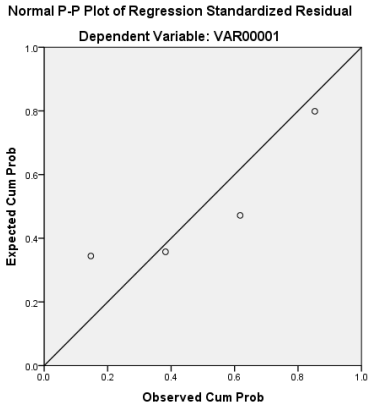



A

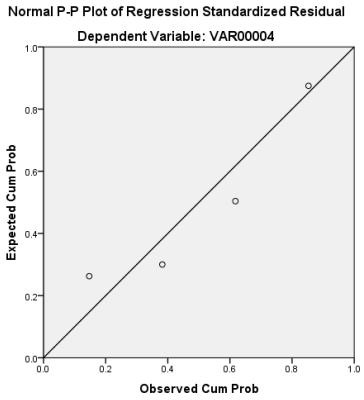



B

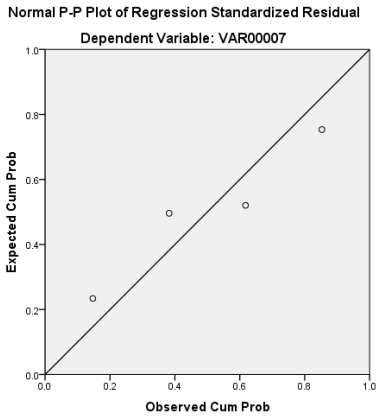
**Plate 13.** Regression Plot showing Effect of different low temperature stress in identified low temperature resistant embryonic stages of *A.assama* on A) hatching percentage and B) incubation period.



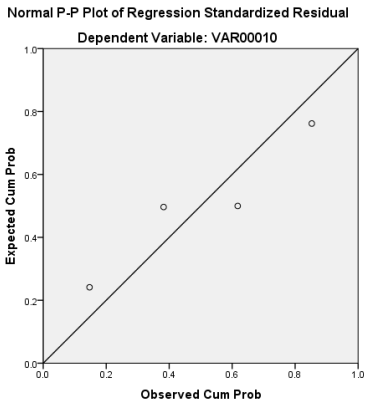

A



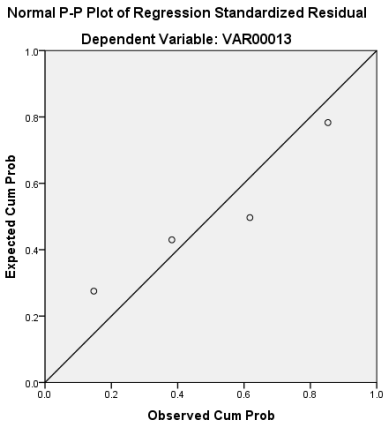

B




C

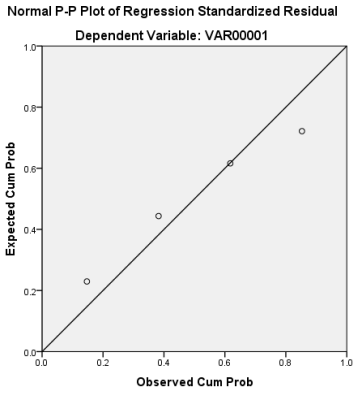



D

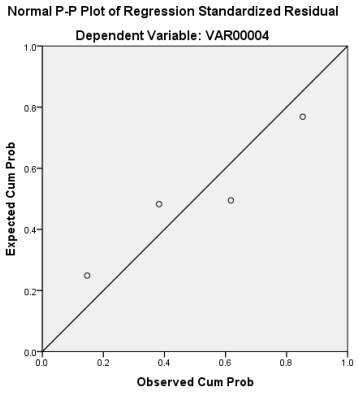



E

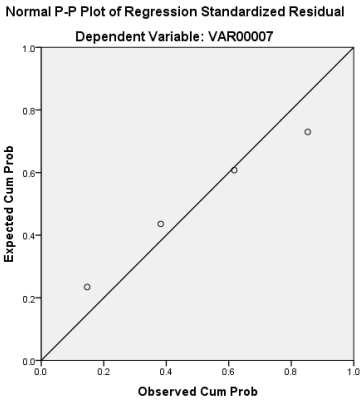
**Plate 14.** Regression Plot showing Effect of preservation period [A) 3days; B) 7days; C) 10 days; D) 15 days and E) 21 days] after low temperature stress to the identified embryonic stages of *A.assama* on hatching percentage.



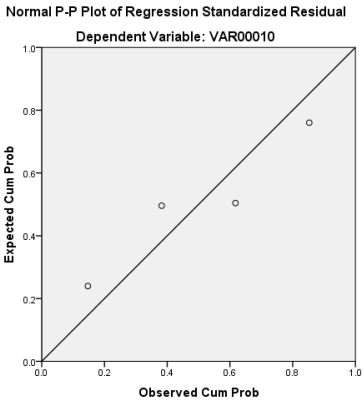

A



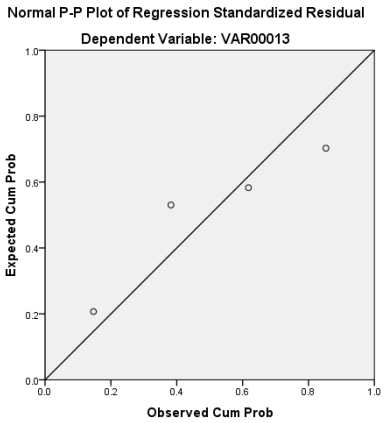

B




C



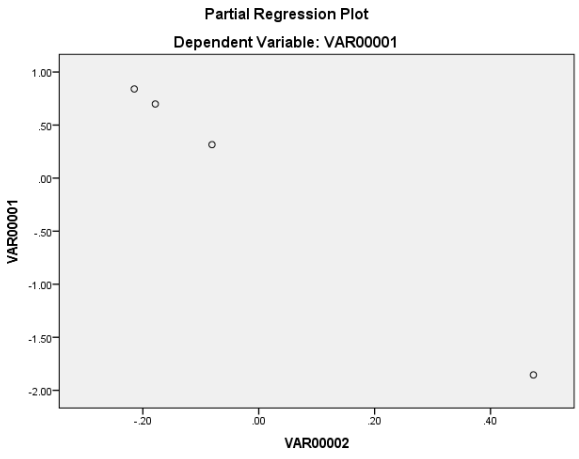

D



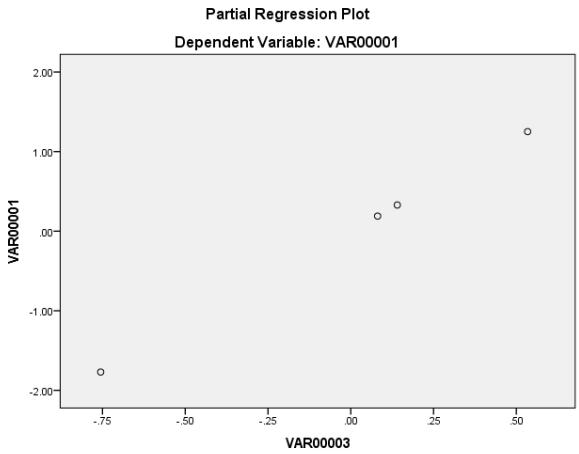

E

**Plate 15.** Regression Plot showing effect of preservation period [A) 3days; B) 7days; C) 10 days; D) 15 days and E) 21 days] after low temperature stress to the identified embryonic stages of *A.assama* on days delay of hatching.

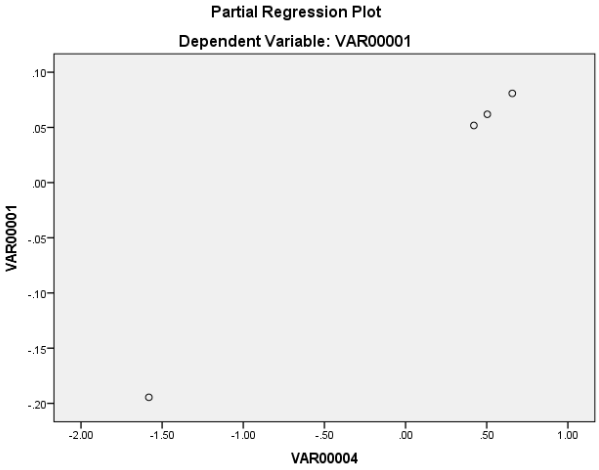
.




(A)

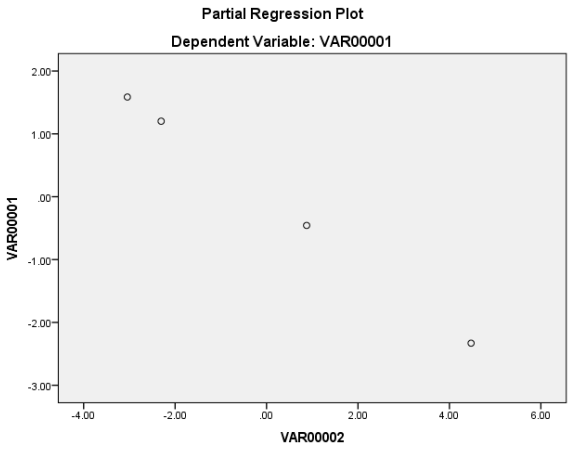



(B)

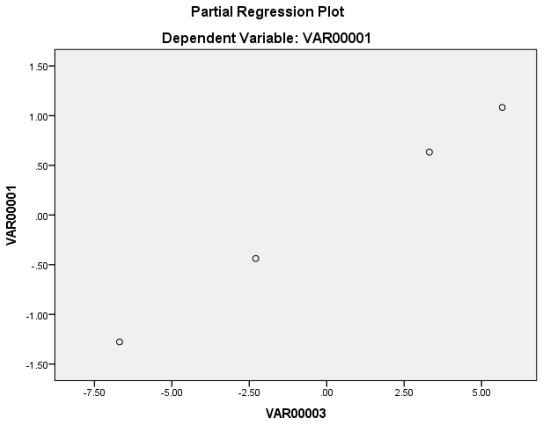



(C)

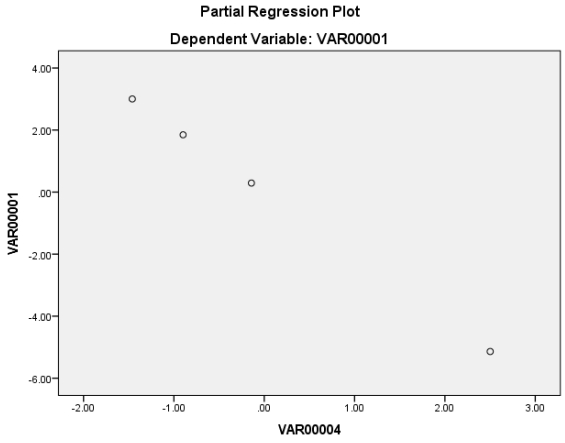
**Plate 16.** showing regression plot for NAD SDH activity for 24,48,72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4




(A)

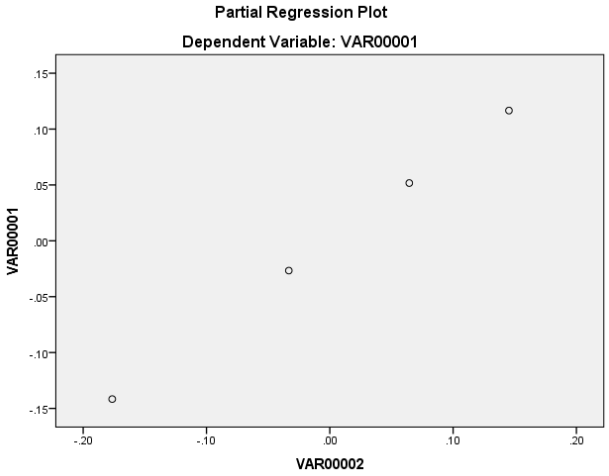



(B)

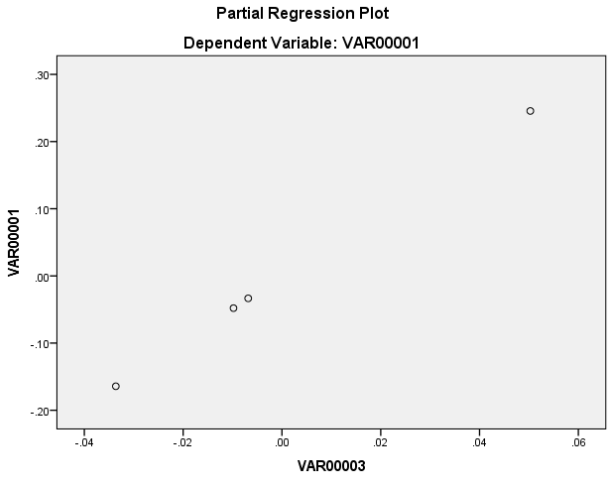



(C)

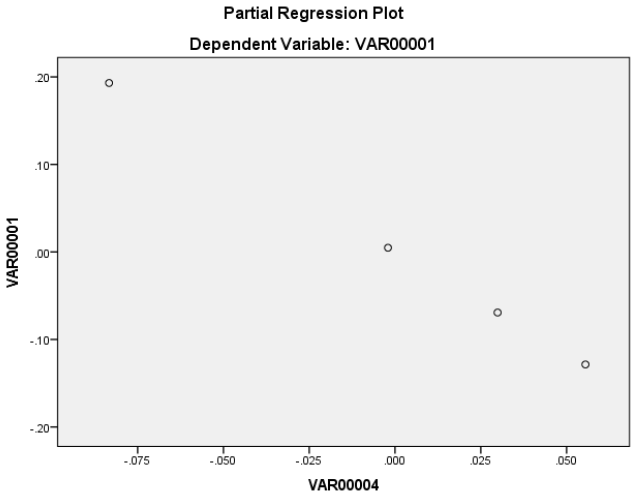
**Plate 17.** showing regression plot for Trehalose quantity for 24, 48, 72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4




(A)

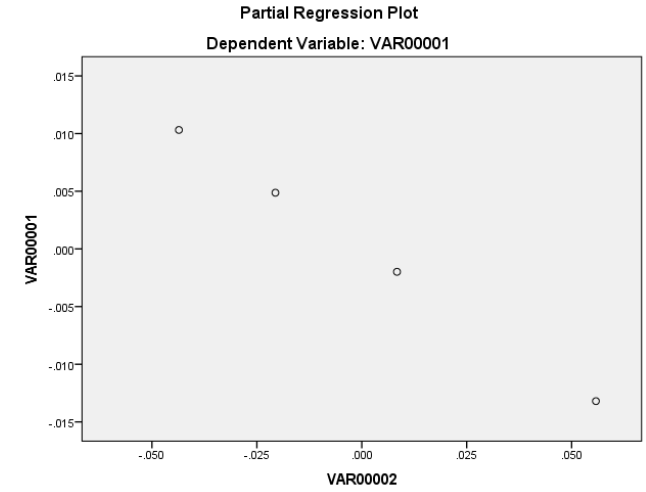



(B)

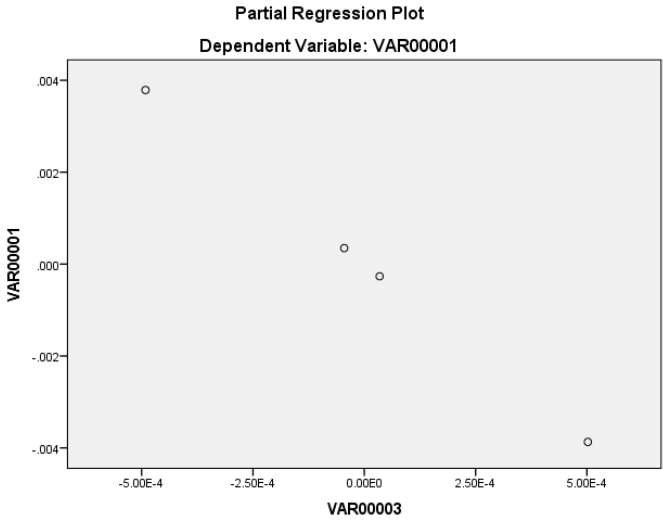



(C)

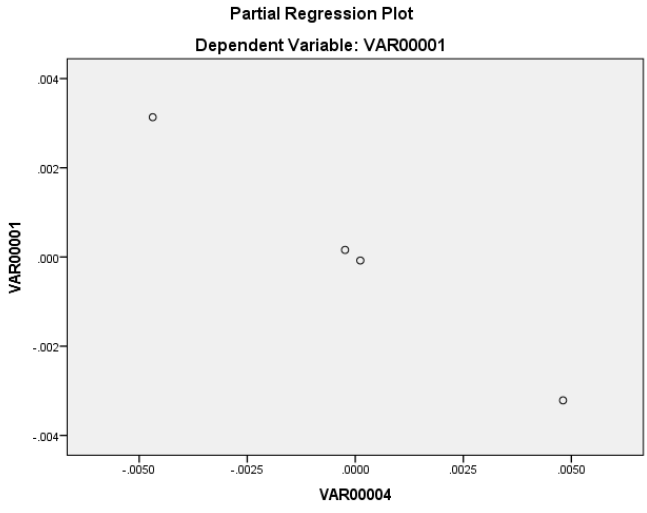
**Plate 18.** Showing regression plot for Xanthine oxidase activity for 24, 48, 72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4




(A)




(B)




(C)

**Plate 19.** showing regression plot for NADPH Peroxidase activity for 24, 48, 72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4

# 7. REFERENCES


Agrell, L.P.S., & Lindquist, A.M. (1973). Physiological and Biochemical changes during insect development. In M. Rockstein (Eds.), *The Physiol. Insecta* (pp. 159 – 247).

Akitamo, S., Egi, Y., Nakamura,Y., Suetsigu, Y., Oishi, K. & Sakamoto, K.,(2017). Genome wide micro array screening for Bombyx mori genes related to transmitting the determination outcome of whether to produce diapause or non diapause eggs. *Insect. Sci*, 24 (2), 187 – 193.

Amy, R.L. (1961). The embryology of *Habrobracon jugl&is* (Ashmead). *J. Morph*., 109, 199-217.

Anderson, D.T. (1962). The embryology of *Dacus tryoni* (Frogg.) [Diptera]. *J. Embryol. Exp. Morph.*, 10, 248-292.

Anderson, D.T. (1972). The development of hemimetabolous insects. In S.J. Counce, & C.H. Waddington, *Developmental Systems- Insects* (pp 95-163, 165-242).

Anderson, D.T. (1973). *Embryologyand Phylogeny in Annelids & Arthropods*. London: Pergamon Press.

Anderson, D.T., & Wood, E.C. (1968). The morphological basis of embryogenic movements in the light brown apple moth, *Epiphyas postvittana* (Walk.) (Lepidoptera: Tortricidae). *Aust. J. Zool.*, 16, 763-793.

Angelini, D.R., & Kaufman, T.C. (2005). Functional analysis in the milkweed bug *Oncopettus fasciatus* (Hemiptera) support the role for Wnt signalling in body segmentation but not appendage development. *Dev. Biol*., 283, 409 – 423.

Audrewarthe, H.G., & Birchi, L.C. (1954). *The distribution of animals*. USA: The University of Chicago Press.

Baba, H., Kuwabara, N., & Iwashita, Y. (1997). Scanning electron microscopic observation on the embryogenesis in the wild silk moth *Antheraea yamani. J. Seri. Sci. Japan*, 66 (2), 99-106.

Bale,J.S., & Hayward, S.A. (2010). Insect over wintering in a changing climate. *J. Expt. Biol*., 213, 980 – 994.

Balnisky, B.I. (1981). *An Introduction to Embryology*. Philadelphia: Saunders College.

Bardoli, S., & Hazarika, L.K.(1994). Body temperature and thermoregulation of *Antheraea assama* larvae. *Entomologia Experimentalis et Applicata*,72, 207 – 217.

Becker,A., Schlöder, P., Steele, J.E., & Wegener, G.(1996). The regulation of trehalase metabolism in inscets. *Experientia*, 52, 433-439.

Beenakkers, A.M. Th., Van-der -Horst, D.J., & Marrewijk, W.J.A. (1981). Role of lipids in energy metabolism. *Energy Meta. Insects*, 53 – 100.

Berdu Jr, J.G., & Pereira, M.F. (2013). Silk Production in Latin American Regions – Constrains & the way forward. *Sericologia*, 53(1), 15-23.

Bergmeyer, H.U., Gawehn, K., & Grassl, M. (1974). In H.U. Bergmeyer (Eds.), *Methods of Enzymatic Analysis* ( Volume I, pp. 521-522). New York, NY : Academic Press Inc.

Biswas, I., and Ray, N. (2007). Studies on short term cold preservation for supply of seed during main commercial crop rearing of muga silkworm. *J.Exp., India.,* 10(2), 271 -276.

Bloter, C.J., & Chefurka, W., (1990). The effect of the phosphate treatment on superoxide dismutase, catalase and peroxidise in the granary weevil, *Sitophilus granaries*. *Pestic. Biochem. Physiol.*, 36, 52-60.

Bora, A., Borah, B., & Sengupta, A.K. (1990) Studies on presentation of muga cocoons at high altitude and low temperature. *Ann Report (1988-90), R.M.R.S. Boko,* 30 – 32.

Bora, D.S. (2006). Sensitivity of *Antheraea assama* Westwood to interaction of photo period and thermoperiod. In Muga silkworm : Bio chemistry, Bio technology, and Molecular Biology (pp. 67–74).

Bosewell, R.E., and Mahowald, A.P. (1985). Cytoplasmic determinants in embryogenesis. In G.A Kerkut & L.I. Gilbert (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Vol. I., pp. 387-405). Oxford: Pergamon Press.

Boyer,R.F. (2006). *Mordern experimental Biochemistry* (pp.389-405). New Delhi: Pearson.

Brachet, J. (1957). *Biochemical Cytology*. NewYork: Academic Press.

Brachet, J. (1962). Nucleic acid in development. *J. Cell. Comp. Physiol*., 60, Supple.I,1-18.

Casperson, T. (1950).*Cell growth and cell function*. New York: W.W. Norton Company Inc.

Chandrasekhar, P.M., & Bali, G. (1987). Glycogen level and glycogen phosphorylase activity in eggs of silk worm *Bonbyx mori* L. *Proc. Indian Aacad. Sci. (Anim. Sci.)*, 96(1), 49-54.

Chaudhury, A. (1998). Biochemical studies of certain enzymes of carbohydrate metabolism at some stages of development of silk worm *Antheraea assama*, Westwood and *Philosamia ricini*, Boisduval. (Ph.D. Thesis), Guwahati University.

Chen, P.S., & Brigel, H. (1965). Studies on the protein metabolism of *Culex pipiens*, L.V. changes in free amino acids and peptides during embryonic development . *Comp. Biochem. Physiol.*, 14, 463 – 473.

Chen, Y.R., Jiang, T., Zhu, J., Xie, Y.C., Tang, J.C., Chen, Y. H., Tang, S,M., Hao, B.F., Wang, S.R. Huang, J. S., Shen, X.J.(2017). Transcript sequencing reveals potential mechanism of diapause preparation in bivotine silk worm, Bombyx. ( Lepideptora: Bombycidae) *Comp. Biochem. Physiol. Part D. Genomics proteomics*, 24, 68 – 78.

Chino, H. (1957a). Carbohydrate metabolism in the diapause egg of the silkworm *Bombyx mori*. I Diapause and change in glycogen content. *Embryologia*, 3(4), 295-316.

Chino, H. (1958). Carbohydrate metabolism in the diapause egg of the silkworm *Bombyx mori*. II Conversion of glycogen into sorbitol and glycerol during diapauses. *J. Insect Physiol*, 2, 1-12.

Chippendale, G.M. (1970). Metamorphic changes in the fat body proteins of the south western corn borer, *Diateraea grandiosella*. *J. Insect Physiol*., 16, 1057-1068.

Chippendale, G.M. (1978).The function of carbohydrate in insect life process. In M. Rockstein (Eds.),Biochemistry of Insects (pp1-55). Academic Press.

Choudhury, B., & Sahu, A.K. (2000, June 13-14). Muga Seed Production by private grainers. Paper presented at the workshop on *Development of Muga & Eri Silk industries through improved technologies*, N. Lakhimpur, Assam.

Choudhury, B., Bhattacharya, A., Singh, B. K., Siddiqui, A.A., Das, P., & Jayprakash,P.(2012). Disease incidence dynamics of muga silk worm (*Antheraea assamensis*, Helfer) and their correlation with meterological factors. *J.Appl.Biosci*., 38(1),101-104.

Choudhury, P., Baruah, S., & Devi, D. (2013). Changes in carbohydrate and protein in the developing healthy and pebrine infected embryos of *Antheraea assamensis*, Helfer. *IJBPAS*, 2(8), 1627-1632.

Choudhury, S.N. (1981). *Muga Silk Industry*. Assam: Directorate of Sericulture.

Choudhury, S.N. (2006, Feb. 17-18). Problems and prospects of muga silkworm seed production. Paper presented in the workshop on *Problem and prospect of muga silkworm seed production* (pp. 1-10). Muga silkworm seed organization , Guwahati.

Choudhury. B., Bhattacharya, A., Singh, B.K., & Provakar, C.J. (2010) Effect of global warming in the rearing performance of muga silkworm *Antheraea assamensis*, Helfer. *J. Assam Sc. Soc*., 51(1), 134-140.

Christensen, P. J. H. (1943), Embryologische und zytologische studien űber die erste und frűhe Eientwicklung bei Orgyia antique Linne (Fam. Lymantriidae, Lepidoptera). *Vidensk. Meddr dansk naturh. Foren.*, 106, 1-223.

Colombo, G., Benassi, C.A., Allegri, G. & Longo, E. (1962). Free amino acids in eggs of *Schistocerca gregaria* F. during development. *Comp. Biochem. Physiol*., 5, 88 – 93.

Copf, T., Schroder, R., & Averof. M. (2004). Ancestral role of candal genes in axes elongation and segmentation. *Proc. Natl. Acad. Sci. USA*., 101, 17711 – 17715.

Counce, S.J. (1961). The analysis of insect embryogenesis. *A. Rev. Ent*., 6, 295-312.

Counce, S.J. (1973). The causal analysis of insect embryogenesis. In S.J. Counce, & C.H. Waddington, *Developmental Systems- Insects-2* (pp 1-156).

Damen, W.G. (2007). Evolutionary conservation and divergence of the segmentation process in arthopods. *Dev. Dyn*., 236, 1379 – 1391.

Das, P. (2000, June 13-14). Base Paper of Muga Silk Industry in Assam. Paper presented at the workshop on *Development of Muga & Eri Silk industries through improved technologies,* N. Lakhimpur, Assam.

Dash, A.K., & Nayak, B.K. (1988). Effect of refrigeration on hatching of egg of the tasar silk moth *Antheraea mylitta,* Drury,(Saturindae) *J. Res. Lepid*., 27 (3-4), 259 -271.

Datta, R.K., Sengupta, A.K., & Biswas,S.N.(1972). Studies on refrigeration of multi voltine silkworm eggs at low temperature. *Indian J. Seric.*, 11, 20 – 27.

Davis, G.K., Patel, N.H. (2002). Short, long and beyond molecular and embryological approaches to insect segmentation. *Annu. Rev. Entomol*. 47, 669–699.

Dearden, P., & Akam, M. (2001). A role for wings in segment morphogenesis but not segment formation in the grasshopper *Schistocerca gregaria*. *Dev. Genes Evol*., 210, 329-336.

Denlinger, D.L.,( 2002). Regulation of diapause. *Annu. Rev. Entomol.*, 31, 239 –264.

Denlinger, D.L. (1986). Dormancy in tropical insect. *Anu. Rev. Entomol*., 47, 93 – 122.

Denlinger, D.L., & Lee, Jr. R.E. (2010).*Low temperature Biology of Insects*. NewYork: Cambridge University Press.

Dolin, M.I. (1957). *Journal of Biological Chemistry,* 225, 557-573.

Dukes, H.H. (1955). The physiology of domestic animals. New York: Camstock Publishing Associates.

Dutta, R.K., Sengupta, K., & Biswas, S.N.(1972). Studies on the refrigeration of multiroutine silkworm eggs at low temperatures. *Indian Journal of Science*, 11, 20-27.

Dutta, R.K.,(1988). Embryological studies of non-diapause silkworm *Bombyx mori* L. *Ind. J. Seric*., 27, 1-6.

Engelmann, F. (1970). *The Physiology of Insect Reproduction*. Oxford & New York: Pergamon Press.

Felton, G.W., & Sumners, C.B. (1995). Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.*, 29, 187-197.

Fujiwara, Y., Tanaka, Y., Iwata, K., Rubio, R.O., Yaginuma, T., Yamashita, O., & Shiomi, K. (2006). ERK/MAPK regulates ecdysteroids and sorbitol metabolism for embryonic diapauses termination in the silk worm *Bombyx mori. J. Insect Physiol*., 52(6), 569-575.

Fullilove, S.L., & Jacobson, A.G. (1971). Nuclear elongation and cytokinesis in *Drosophila montona* . *Dev. Biol.*, 26, 560-577.

Furusawa, T., Indrasith, L.S., & Shikata, M. (1985). Changes in DNA, RNA content and RNA/DNA ratios during the embryonic diapause of *Bombyx mori*. *J. Seric. Sci. Jpn*., 54(1),53-62

Furuswa, T. & Yang, W.J. (1987). Fluctuation of free sugars with embryonic development of non diapause eggs of silk worm *Bombyx mori*. *J. Seric. Sci. Jpn.*, 56, 143-149.

Gerlach, U.,& Hiby, W. (1974). In H.U. Bergmeyer (Eds.), *Methods of Enzymatic Analysis* ( Volume II, pp. 569-573). New York, NY : Academic Press Inc.

Ghosh, S., & Ray, N. (2005). Effect of low temperature preservation delayed hatching of eggs of of eggs of muga silk worm, *Antheraea assama*, Westwood. In U. Chakraborty & B. Chakraborty (Eds.), *Stress Biology* (pp117-121). New Delhi: Naraosa.

Ghosh, S., & Ray, N. (2006). Embryological studies of silk worm *Antheraea assama* Ww., Paper presented at the Workshop on problems & prospects of Muga silk worm seed production, MSO, CSB, Guwahati, 17-18th Feb. (pp. 29).

Goswami, D., Singh, N.I., Ahamed, M., & Giridhar, K. (2013). Embryonic development in Muga silk worm, *Antheraea assamensis* Helfer (Lepidoptera: Saturniidae). *Mu. Entomology & Zoology*, 8 (2): 852-857.

Govindan, R., Devaiah, C., Rangaswami, H.R., & Thippeswami, C. (1980). Effects of refrigeration of the eggs of Eri Silkworm, *Samia Cynthia Ricini*, Biosduval on hatching. *Indian J Seric*., 19, 13 -15.

Gross, R.J. (1952). The early embryology of the book louse, *Liposcelis divergens* Badonnel (Poscoptera: Liposcelidae). *J. Morph*., 91, 135-167.

Gross, R.J. (1953). The advanced embryology of the book louse, *Liposcelis divergens* Badonnel (Poscoptera: Liposcelidae). *J. Morph*., 92, 157-191.

Gutzeit, H.O., Zissler, D., Grau, V., Liphardt, M., & Heinrich U.R. (1994). Glycogen stores in mature ovarian follicles and young embryos of Drosophila. Ultra structural changes in some biochemical correlates. *Eur. J. Cell. Biol*., 63, 52- 60.

Görg, R.J. (1952). Untersuchungen am Kein von Hierodula (Rhombodera) crassa Giglio- Tos, ein Beitrag zur Embryologie der Mauotiden (Mantodea). *Dt. Ent. Z.*, 6, 389-450.

Hagan, H.R.(1951). *Embryology of the viviparous insects*. New York: Ronald Press Co.

Hakim, R.S., Boldwin, K., & Snagghe, G. (2010). Regulation of Midgut Growth, Development and Metamorphosis. *Annu. Rev. Entomol*., 55, 593-608.

Han, Q., Li, G., & Li, J. (2000a). Chorion peroxidase mediated NADH/$O_2$ oxidoreduction cooperated by chorion malate dehydrogenase- catalyzed NADH production: a feasible pathway leading to H $_2O_2$ formation during chorion hardening in *Aedes aegypti* mosquitoes. *Biochim. Biophys. Acta*, 1523 (2-3), 246-253.

Han, Q., Li, G., & Li, J. (2000b).Purification and characterization of chorion peroxidise from *Aedes aegypti* egg. *Arch. Biochem. Biophys*., 378 (1), 107-115.

Hansen, H. (1946). The theoretical aspect of insect metamorphosis. *Biol. Rev*., I. 21.

Hasegawa, K., & Yamashita, O. (1965). Studies on the mode of action of the diapauses hormone in the silkworm *Bombyx mori*, VI- the target organ of diapauses hormone. *J. Exp. Biol*., 43, 271-277.

Hayakawa, Y., & Chino, H. (1981). Temperature dependent inter conversion between glycogen and terhalose in diapausing pupae of *Philosamia Cynthia ricini* and *pryeri.Insect Biochem.*,11 (1), 43-47.

Hayakawa, Y., & Chino, H. (1982). Phosphofructokinase as a possible key enzyme regulating glycerol and trehalose accumulation in diapausing inscets. *Insect Biochem*.,12 (6), 639-642.

Hiratsuka, K. (1975). *Text Book of Tropical Sericulture*. Tokyo: Japanese Overseas Co-operation Volunteers.

Hochachka, P. W., Buck, L. T., & Land, S.C. (1996). Unifying theory of hypoxia tolerance: molecular / metabolic defense and rescue mechanisms for surviving oxygen lack. Proc. Natl. Acad. Sci. USA, 93, 9493-9498.

Horie,Y., Kanda, T., & Moehida, Y. (2000). Sorbitol as an arrester of the embryonic development in diapausing eggs of the silkworm *Bombyx mori. J.Insect Physiol*.,46,1009-1016.

Howe, R. H. (1967). The temperature effects on embryonic development in insets. *Annu. Rev. Ent*., 12, 15- 42.

Huettuer, A.F. (1923). The Origin of the germ cells in *Drosophila melanogaster*. *J. Morph.*, 37, 385-423.

Hutcher, P., & Battery, N. (2011). Sericulture: Silkworms & mulberries. In *Biological Diversity Exploiters & Exploited* (Chapter 5, pp73 – 96). London: Wiley & Blackwell.

Ibohal Singh, N., Ibotombi Singh, L, Somen Singh, L., & Suryanarayana, N., (2005). An overview of the salient features of *Antheraea frithii*, Moore- a promising wild silk moth of India. In *Proceedings of 20th International Congress of wild silk moth* (Vol II, pp. 74-77), Bangalore: Central Silk Board.

Ikada,E. (1910). Embryonic development and morphological changes of *Bombyx mori*. *Sangyoh Shinpoh*, 18, 26-42.

Ikada,E.(1912).Morphological change of silk worm eggs untill 2 days from oviposition. *Sangyoh Shinpoh*, 20,9-11; 43-47.

Ikekawa, N., Saito, S.M., Kobayashi, M., & Tsuda, K. (1966). Studies on the sterol of *Bombyx mori* L. IV. Conversion of Sterol in the silk worm. *Chem. Pharm. Bull*., 14,834-836.

Imanishi, S., Sinbo, H.,& Kiguchi, K.(1996). Cryopreservation of silk worm eggs. *J. Seric. Sci. Jpn.*, 65(4), 235 – 240.

Indira, T. (1963). Biochemical and cyto chemical studies during development on ovarian growth in *Spheroderma molestum* (Dub) Ph. D. Thesis, Annamalai University, India.

Jaworski, T., & Hilszezanski (2003). The effect of temperature and humidity change of insect development and their impact on forest ecosystem in the context of expected climate change. *Forest Research Papers.* ,74(4), 345 – 355.

Jayaprakash, P., Bhattacharya, A., Choudhury, B., Singh, B.K., & Hajarika, H.K.(2012). Muga silk production- its tribulations, prospects and inroads made in the face of unprecedented impugnations. In M. Chutia, S. A. Ahmed, K. Neog, R. Kumar & K. Das (Eds.), *Compendium of Seminar Papers*. Paper presented at the National Seminar on Recent Trends in Research & Development in Muga Culture-Ideas to action. MSO, Guwahati, 3-4May 2012 (pp. 141-150).Guwahati: Central Silk Board.

Johanusen, D., & Butt, A.H. (1941). *Embryology of insectsand Myripods*. New York: Mc Graw- Hill.

Jolly M.S. (1979). *FAO Agricultural Services bulletin (291).* Rome: Food and Agricultural Organisation of the United Nations.

Jolly, M.S. (1983). Organization of bivoltine grainage for tropics. In *Sericulture, Project-3*. Mysore :Central Sericultural Research and Training Institute.

Jolly,M.S., Sen, S.K., & Ahsan, M, (1974). Tasar Culture. Bombay: Ambika Publishers.

Jura, C. (1972). Development of a pterygote insects. In S.J. Counce & C. H. Waddington (Eds.), *Development Systems –Insects* ( Vol.-I). London &New York: Academic Press.

Jura, C. (1972). Development of Apterygote insects. In S.J. Counce, & C.H. Waddington, *Developmental Systems- Insects-1* (pp 49-94).

Karlson, P. & Hofmeister (1963). Zur bigenese des ecdysons. I. Unwandlung von cholesterin in ecdyson hoppe. *Seyler's Z. Physiol. Chem*., 331, 298.

Karlson, P. (1963). Chemie and biochemie der insekt euhormone. *Angew. Chem.*,75, 257-265.

Karnavar,, G.K., & Nair, (1969) Changes in body weight, fat, glycogen and protein during diapauses of *Trogoderma granarium. J. Insect Physiol*., 15, 95-102.

Katsuki, M., Murakami, A., & Watanabe, I. (1980). Fate mapping of some tussues in the genetic mosaics of the silk worm *Bombyx mori*. *Zool. Mas*. 89, 269-276.

Kawaguchi,Y., Kawakami, K., Kusakabe, T., & Koga, K. (2004). Histological Observation of eggs of the embryonic lethal mutation set in Bombyx mori. *J. Insect Biotech.and Sericology*, 73,101 –106.

Keino, H., & Takesue, S. (1982). Scanning electron microscopic study on the early Development of silkworm Eggs (*Bombyx mori* L.). *Develop. Growth & Differ.* , 24 (3), 287-294.

Khanikar, D.P., & Choudhury, S.N.(1997, Nov 7-8). Effects of preservation of muga seed cocoons at low temperature. Paper presented at the Seminar on *Environmental potential and conservation of nature and natural resources* (pp.22) Sibsagar College, Sibsagar, Assam.

Khanikar, D.P. ,& Dutta, S.K. (1998b). Effect of induced pupal diapause on rearing performances and cocoon parameters of muga silkworms, *Antheraea assama* Westwood. *J. Agric. Sci. Soc., North East India*, 11 (20), 140 – 143.

Khanikar, D.P., & Dutta, S.K. (1998a). Low temperature preservation of seed cocoons for delaying moth emergency in muga silkworm, *Antheraea assama* ,Westwood (Lepideptera: Saturmidae ). *Int. J. Wildsilkmoth and silk*, 5, 216 -220.

Khanikor ,D.P. and Dutta, S.K. (1998, Nov 11 – 14). Low temperature to seed cocoons for delaying moth emergence in muga silk worms (*Antheraea assama* Westwood) Lepideptora : Saturnidae. Paper presented at the *3rd International Conference on Wild Silk moths.* Bhubaneswar, Orissa.

Kihara,F., Itoh, K., Iwasaka, M., Niimi, T., Yamashita, O., & Yaginuma, T. (2009). Glycerol kinase activity and glycerol kinase-3 gene are up regulated by acclimation to 5˚C in diapauses eggs of *Bombyx mori*. *Insect. Biochem. Mol. Biol*, 39 (11),763-769.

King, R.C. (1970). *Ovarian Development inDrosophila melanogaster*. New York: Academic Press.

Kodri, K. D., Bednaraova, A., Zemavova, M. & Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects- an update. *Int. J. Mol. Sci*., 16, 25788-25816.

Krause, G. (1957). Neue Beitrague zur Entwicklungsphysiologie des Insekten Keimes. *Verh. dt. zool. Ges.(Graz)*, 396-424.

Krishnappa, B. L., Reddy, D.N.R., and Narayanaswamy, K.C. (2001) Changes in carbohydrate, lipid and moisture contents during the embryonic development of *Samia cynthia ricini*, Biosdual. *Indian J. Seric*., 2,180-181.

Kűhn, A. (1971). *Lectures on Developmental physiology*. Berlin & New York: Springer.

Kumaresan, P., & Quadri, S.M.H.(2012). Growth & instability of mulberry silk production in India. *Indian J. Seric.*, 51(1),64-71.

Kumereshan, P., Thangavelu, K., & Sinha, R.K.(2004). Studies on long term preservation of eggs of Indian tropical multi-voltine silk worm (*Bombyx mori*, L.) genetic resources. *Int. J. Indust. Entomol*. 9(1). 79-87.

Kurata, S., Yaginuma, T., Kobayashi, M., Koga, K., & Sakaguchi, B. (1980). DNA content and cell number during embryogenesis of *Bombyx mori*. *J. Sericult. Sci. Jpn*., 49 (2), 107-110.

Kuwana, J., & Takami, T. (1957). *Insecta. An Embryology of Invertebrates*. In M.Kume & K. Dan, (pp287-343). Tokyo: Baifukan .

Lalouette, L., Williams, C.M., Hervant, F., Sinclair, B.J., & Renault, D. (2011). Metabolic and oxidative stresses in insects exposed to low temperature thermal fluctuation. *Comp. Biochem. Physiol.*, 158, 229-234.

Larinink, O. (1997). Apomorphic & plesiomorphic characteristics in Archeognatha, Monura and Zygentoma. *Pedobiologia*, 41, 3-8.

Lee, Jr. R.E. (2010). A primer on insect cold tolerance. In D. L. Denlinger & Jr. R.E. Lee(Eds.), *Low temperature Biology of Insects* (Chapter 1). NewYork: Cambridge University Press.

Legay, J.M. (1974). Sur la croissance sequentielle de l'ovocyte dans le tube ovarique an *Bombyx mori* pendant le stade nymphal. *Annls. Embryol. Morph*. 7, 277-282.

Leopold, R.A. (2000). Short term cold storage of housefly (Diptera: Muscidae) Embryos : survival and quality of subsequent stages. *Annals of Entomological Society of America* Vol 93 (4), 884 – 889.

Levinson, Z.H. (1960). The evolution of sterol requirements.the function of dietary syterol in phytophagus insects. Paper presented at *the 11$^{th}$ Int.Congr. Ent.*, 3, (pp.145-156).

Lim, S.H., Kim, Y.T., Lee, S.P., Rhee. I.J., Lin, J.S., & Lin, B.H. (1990). Sericulture training Manual. *FAO Agricultural Services Bulletin 80*. Rome: Food & agricultural training organization of the United Nations.

Liu, P. Z., & Kaufman, L. C. (2005). Short and long germ segmentation: unanswered questions in the evolution of a developmental mode. *Evol. Dev*., 7(6). 629 – 646.

Liu, W. (2012). Functional analyses in the silk worm, *Bombyx mori*, support a role for Notch signalling in appendage development but not the groucho-dependent pair rule process. *J. Exp. Zool. (Mol. Dev. Evol.)* 9999B, 1 – 12.

Lonvet, J.P. (1964). La Segregation du mesoderme chez lembryoudu Phasme *Carausius morosus* Br. *Bull. Soc. Zool. Fr*., 89, 688-701.

Lowry,O.H., & Passonneau, T.V. (1972). *A flexible system of enzymatic Analysis* (pp. 71-77,179-182). New York: Academic Press.

Lynech, J. A., Brent, A. E., Leaf, D.S., & Plutz, M.A . Desplan.C.(2006). Localized maternal orthodenticle patterns anterior and posterior in the long germ wasp *Nasonia. Nature* ,439, 728 – 732.

Mahowald, A.P. (1963). Electron microscopy of the formation of the cellular blastoderm in *Drosophila melanogaster*. *Exp. Cell Res.*, 32, 457-468.

Makka, T., Seino, A., Tomita, S., Fujiwara, H., Sonobe, H. (2002) A possible role of 20-hydroxyecdysone in embryonic development of the silk worm *Bombyx mori*. *Arch. Insect Biochem. Physiol.* 51, 111-120.

Makka,T., & Sonobe, H. (2000). Ecdysone metabolism in diapauses and non diapause eggs of silk worm *Bombyx mori*. *Zool. Sci*., 17, 89-95.

Martini, A., Baldassari, N., & Baronis, p. (2006). Embryonic development in *Neodiprion sertifer*. *Bulletin of Insectology* ,59 (1), 45-52.

Mc Gregor, D.A.,& Loughton, B.G. (1979) Yolk protein degradation during embryogenesis of the African migratory Locust. *Can. J. Zool*., 7, 907 – 917.

Mc Gregord, A. and Loughtoub, G. (1974). Yolk- protein degradation during embryogenesis of the African migratory locust, *Can. J. Zool.*, 52, 907 – 917.

Miya, K. (1950). *Trans. Sapporo Nat. Hist. Soc.*, 19, 36-39.

Miya, K. (1953). The presumptive genital region at the blastoderm stage of the silk worm egg. *J. Fac. Arg. Iwate. Univ.*, I, 223-227.

Miya, K. (1958). Studies on the embryonic development of gonad in the silk worm *Bombyx mori* L. Part I: Differentiation of germ cells. *J. Fac. Arg. Iwate. Univ.*, 3, 436-467.

Miya, K. (1960). Electron microscopic studies on the embryonic development in the silkworm *Bombyx mori*, II, Fine structure of the serosa. *J. Seric. Sci. Jpn*., 29,273.

Miya, K. (2003). *The early embryonic development of Bombyx mori: An ultra structure point of view*. Tokyo: GENDAITOSHO.

Miyawaki, K., Mito, T. Sarashina, I. Zhang, H., Kinmyo, Y., Ohuchi, H.,& Noji, S. (2004). Involvement of Wingles / Armadilo signalling in the posterior sequential segmentation in the cricket. *Gryllus bimanculatus* (orthoptera) as revealed by RNAi analysis. *Mech. Dev*.,121, 119 – 130.

Mizuno, T., Watanbe, K., and Ohushi, E.(1981). Developmental changes of Ecdysteroids in the silk worm *Bombyx mori*. *Develop. Growth and Differ*., 23(5),543-552.

Moorthy, S.M., Krishnan, N., Bhattacharya, T., & Choudhuri, A. (2007). Changes in the titre of protein and cholesterol content in non diapause, artificially diapause terminated and diapauses eggs of silk worm, *Bombyx mori*. *Int. J. Indust. Entomol.*, 15(2), 165-169.

Myer, B.L.B. and Burggrem, W. W. (2010) Metabolic Allometry during development and metamorphosis of the silk worm *Bombyx mori*. Analysis Patterns and Mechanisms. Physiological and Biochemical Zoology, 83(2), 215 – 231.

Nace, G.W. (1970). Specific macromolecular changes during development. In O.A. Schjeide & J. Devellis (Eds.), *Cell Differentiation* (pp. 322-350). New York : Norstrand Reinhold Company.

Nagina, N., & Nagesh Chandra, B.K. (1988). On the effect of low temperature on egg hatch and subsequent development of *Samia Cynthia Ricini* Biosduval (Lep:Sat). *Sericologia*, 28 (3), 337 – 341.

Nagy, L., Riddiford, L., & Kiguchi, K. (1994), Morphogenesis in the early embryo of the Lepidopteran *Bombyx mori*. *Dev. Biol.*,165(1) , 137-151.

Nagy, L.M. & Carrol, S. (1994). Conservation of wingless pattering functions in the short germ embryo of *Tribolium castaneum*. *Nature*, 367, 460–463.

Nakada, T. (1932). The resistance of Silkworm embryo to abnormal high temperature and dryness on the external morphology of the embryo. *Bull. Fukuoka Sericult. Expt. Stn.*, 1, 1 -26

Nakao, H. (2010). Characterization of Bombyx embryo segmentation process : expression profile of engrailed , even-skipped, Caudal and wnt 1/ Wingless homologue. *J. Exp. Zool. (Mol. Dev. Evol.)*, 314B, 224 – 231.

Nakao,H. (2012). Anterior and posterior Centres jointly regulate *Bombyx* embryo body segmentation. *Dev. Biol*. http: // dx.doi.org/10.1016/j.ybdio. 2012.08.029.

Narayanaswamy, T.K.,& Govindan, R. (1987). Effect of Refrigeration of eggs of pure Mysore silkworm, *Bombyx mori*, at blue stage. *Entomon*, 12, 105-107.

Nation, J.L. (2008). *Insect Physiology and Biochemistry*. Boca Raton, USA: CRC Press.

Nayak, B.K. & Dash, A.K.(1989). Effect of refrigeration egg incubation period of the Tasar silk *Antheraea mylitta* Drury (Saturnidae) *J. Lepidopteran Society*., 43(2), 152 -153.

Newman, S. A. (2011). Animal egg as Evolutionary Innovation : A solution to the "Embryonic Hourglass" Puzzle. *J. Exp. Zool. (Mol. Dev. Evol. )* 316, 467 – 483.

Niimi, T., & Yaginuma, T. (1992). Biosynthesis of NAD- Sorbitol dehydrogenase induced by acclimation at 5˚C in diapauses eggs of the silk worm *Bombyx mori. Comp. Biochem. Physiol*., 102,169-173.

Niimi,T., Yamashita,O., & Yaginuma, T. (1993). Developmental profile of the gene expression of the Bombyx homolog of mammalian sorbitol dehydrogenase during embryogenesis of non diapauses eggs. *Comp. Biochem. Physiol*. B, 106 (2), 437-442.

Ohnishi, E., Mizuno, T., Chatani, F., & Sakurai, S. (1977). 2- Deoxy-d-ecdysone from ovaries and eggs of the silk worm, *Bombyx mori*. *Dev. Growth Differ*., 19, 67-70.

Ohtsuki, Y., Mori, S., Kanda, T., & Kitazawa, T. (1976). Morphological observation on embryonic moult in the silk worm, Bombyx mori. *J. Sericult. Sci. Japan* ,45(3), 225-231.

Ohtsuki,Y. (1979).Silk worm eggs. In The Japanese Society of Sericultural Science (Eds.), *A general text book of Sericulture* (pp.156-173). Tokyo: Nihon Sanshi Sinbun Sha.

Okazaki, T. & Yamashita, O. (1981). Changes in glucose and fructose contents during embryonic development of silk worm, *Bombyx mori*. *J. Seric. Sci. Jpn*.50(3),190-196.

Othsuki, R., Sato, S., Nagai, O., Horigone, I., Yoshitake, S., Goto, S., Sato, S., Totani, C., Nagashima, E., & Yoshitake, S. (1997). Silk worm egg production. New Delhi: Oxford and IBH Publishing Co. Pvt. Ltd.

Overqaard, J., & Mac Millan, H.A. (2017). The integrative physiology of insect chill tolerance. *Annu. Rev. Physiol*., 79,187-208.

Pandey, R.K., Luoklam. R., Das, P.K., & Noamani, M.K.R.(1992). Effect of cold storage on the hatchability of oak tasar silkworm. *Indian Silk*, 31(2), 155 -157.

Panfilio, K.A. (2008). Extraembryonic development in insects and the acrobatics of blastokinesis. *Dev. Biol.* 313, 471 – 491.

Pant, R. & Nautiyal, G.C. (1974). Change in protein, glycogen, free sugar content and active phosphorylase activity during embryogenesis of *Philosamia ricini*. *Proc. Indian Aacad. Sci. (Sec. B)*, 80, 121-126.

Pant, R., & Sharma, K.K. (1976). Variation in glycogen, total carbohydrate, free sugars, proteins, total free amino acid and some enzymes (active phosphorylase , acid and alkaline phosphatases) during embryonic development of *Antheraea mylitta*. *Curr. Sci*., 45, 125-128.

Park, K.E., & Yoshitake, N. (1969). Effect of incubation temperature on nucleic acid and protein synthesis during embryonic development of the silk worm *Bombyx mori* L.(Lepidetera: Bombycidae). *Appl. Ent. Zool*., 4(4), 171-176.

Peel, A.D., Chapman, A.D., & Akam, M. (2005). Arthopod segmentation : beyond the *Drosophila* paradigm. *Nature Rev. Genet*., 6, 905-916.

Pflugfelder, O. (1958). *Entwicklungsphysiologie der Insekten*. Leipzig: Akademische Verlagsgesellschaft.

Plummer, D.T. (1998). *An Introduction to Practical Biochemistry* (pp. 216-219).New Delhi: Tata Mc Graw-Hill.

Polten, K.A, Davidowitz, G., & Woods, H.A. (2011) Gross stage consequences of egg temperature in the insect *Manduca sexta*. *Functional Ecology*, 25, 548 – 556.

Ponnuvel, et. al. 2010. Ponnuvel, K.M., Murthy, G.N., Awasthi, A.K., Rao, G., & Vijayprakash, N.B. (2010). Differential gene expression during early embryonic development in diapause and non diapause eggs of multivoltine silk worm *Bombyx mori*. *Indian J. Exp. Biol.*, 48(11), 1143 – 1151.

Sarkar, B.N., Sarmah, M.C., Dutta, P., & Dutta, K.(2012). Embryo Isolation and egg preservation technology of Eri silkworm *Samia ricini* (Donovan) (Lepidoptera: Saturniidae). *Munis Entomology & Zoology*, 7 (2), 792-797.

Rabecea, L.F., & Palfey, B.A. (2010).Flavin independent enzymes. In *Reference module in Chemistry, Molecular Science and Chemical engineering: Comprehensive natural products II*, Vol. 7, Cofactors, (pp.37-43).

Raikhel, A.S., & Dhadialla, T.S.(1992). Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol*., 37, 217 – 251.

Ranjan, R.K., & Hazarika, U. (2012). Constrains in muga culture: strategies & research programmes undertaken at CMERTI Lahdoigarh. In M. Chutia, S. A. Ahmed, K. Neog, R. Kumar, & K. Das (Eds.), *Compendium of Seminar Papers*. Paper presented at the National Seminar on Recent Trends in Research & Development in Muga Culture-Ideas to action. MSO, Guwahati, 3-4May 2012 (pp. 3-11).Guwahati: Central Silk Board.

Ranna, B., Luikham,R., Chalapatty, M.V., Debaraj,Y., Singh, N .I., Sinha, R.K., & Thangavelu, K. (2002). Effect of refrigeration on the hatchability of different ages of Oak tasar silkworm eggs. *Sericologica,* 42(4), 581 -584.

Read, E. M., & Day, M.R. (1966). Embryonic movements during development light Brown Apple Mouth. *Aust. J. Zool*., 14, 253-263.

Richards, O.W., & Davies, R.G. (1977). *Imms general text book of entomology*. London: Chapman & Hall.

Rockstein ,M. (1974). The insect and external environment. In *The physiology insects* (Vol –II). NewYork: Academic Press.

Roonwal, M.L. (1936). Studies on the embryology of the African Migratory Locust, *Locusta migratoria migratorioides*, I. *Phil. Trans. R. Soc. B.* 226, 391-421.

Roonwal, M.L. (1937). Studies on the embryology of the African Migratory Locust, *Locusta migratoria migratorioides*, II. *Phil. Trans. R. Soc. B.* 227, 175-244.

Rubio, R.O., Suzuki,A., Mitsumasu,K., Homma,T., Niimi,T., Yamashita, O. & Yaginuma, T. (2011).Cloning of cDNAs encoding sorbitol dehydrogenase-2a and b enzyme characterization and up regulated expression of the gene in Bombyx mori diapauses eggs exposed to 5˚C. *Insect. Biochem.Mol. Biol.,* 12 (4),361-366.

Ruuge, E.K., Ledenev, A.N.; Lakomkin, V.L.; Konstantinov, A.A., & Ksenzenko, M.Y.(1991). Free radical metabolites in myocardium during ischemia and reperfusion. Am. J. Physiol., Suppl., 261, 81-86.

Sahu, M., Mukherjee, P.K., & Mondal, T. (1998, Nov. 11-15) Constrains during pre seed crop rearing of *Antheraea assama*, Ww. at seed research level in lower Assam. Paper presented at the *3rd International Conference on wild silk moths*, Bhubaneswar, Orissa.

Saito, M., Yamazaki, M., & Kobayashi, M. (1963). Steroid biosynthesis in the silk worm. *Nature,* 198, 1324.

Sakano, D., Furusawa, T., Sugimura, Y., Storey, J.M., & Storey, K.B. (2004). Metabolic shifts in carbohydrate metabolism during embryonic development of non diapauses eggs of silk worm *Bombyx mori*, *J. Insect Biotech.& Sericol*., 73, 15-22.

Salt, R.W. (1961). Principles of cold hardiness. *Annu. Rev. Ent*., 6, 55- 74.

Sander, K. (1976), Specification of basic body pattern in Insect embryogenesis. Adv. Insect Physiol. 12, 125-238.

Sanders,E.J. (1975). Aspects of furrow membrane formation in the cleaving Drosophila embryo. *Cell. Tiss. Res.* 156, 463-474.

Satio, S. (1937). *J. Fac. Agric. Hokkaido Imp Univ. ,* 40, 35-109.

Saunders , D. S. (1982). *Insect clocks*. Oxford: Pergamon Press.

Seidiel, F. (1961). Entwicklungsphysiologisch Zentren im Eisystem der Iusekten. *Verh. dt. zool. Ges.*, 121-142.

Sengupta, K. & Singh, K.(1974). Some studies on the effect of refrigeration of muga *Antheraea assama* Ww. cocoons on moth emergence, egg laying and hatching of eggs. Paper presented at the *First International Seminar on Non- mulberry silks* (pp.174). CTR & TI, Ranchi.

Shingleton, A.W., Geoffroy, C., Sisk & David L Stern . Diapause in the pea aphid phia (*Acysthosiphonpisum*) is a slowing but not a cessation of development. *BMC Developmental Biology,*

Shinmyo, Y., Mito, T., Matsushita, T., Sarashina, I.,Miyanaki, K., Ohuchi, H.,& Noji, S. (2005). Caudal is required for gnathal and thoracic patterning and for posterior elongation in the intermediate germband in cricket *Gryllus bimaemlatus*. *Mech. Dev*., 122, 236 – 239.

Singh, S.P., & Singh, J. (1980). Carbohydrate turn over in relation to trehalase activity in eri silk moth *Philosamia ricini*, during embryogenesis. *Acta Physiol. Acad. Sci. Hung*., 55,149-152.

Singha, B.B., Sahu, A.K., & Das, P.K. (1998, Nov. 11-14). *Identification of low temperature resistant embryonic stages in muga silkworm, Antheraea assama*. Ww. Paper presented at the 3rd International conference on wild silk moth, Bhubaneswar, Orissa.

Sinha, A.K., Sinha, U.S.P., & Sengupta, K. (1989). Changes in the free amino acids during embryonic development of *Antheraea proylei*. *Indian J. Sericulture*, 28(1), 118-120.

Sinha, A.K., Sinha, U.S.P., Sengupta, A.K., & Sengupta, K. (1987).Changes in free amino acid in the larval and pupal haemolymph of *Antheraea proylei*, J. Paper presented at the *XV Inernational Sericultural Congress*, Thailand

Sinha, U.S.P., Sinha, A.K. & Sinha, S.S.(1991). Changes in concentration of proteins and carbohydtares in the developing healthy and pebrine infected embryos of tropical tasar silk worm , *Antheraea mylitta*, Drury. *Indian J. Seric*., 30(2), 155-156.

Snobbe, H., Masumoto, T., Tokushige, H., & Makka, T.(1997). Developmental changes in accumulation and metabolism of ecdysteroids in diapause eggs and non diapause eggs of silk worm Bombyx mori. In S. Kawashima, & S. Kikuyama (Eds.), Advances in Comparative Endocrinology (Vol. I, pp 185-189). Bologna: Monduzzi Editorem.

Sohal,R.S., Aggarwal, S., Dubey, A., & Orr, W.C. (1993). Protein oxidative damage associated with life expectancy in house flies. *Proc. Natl. Acad. Sci. USA*, 90, 7255-7529.

Sonobe, H., Maotaini, K., & Nakajima, H. (1986). Studies on embryonic diapause in the PND mutant of the silkworm, *Bombyx mori*, Genetic control of embryogenesis. *J Insect Physiol.*, 32, 215-220.

Sonobe, H., & Yamada, R. (2004). Ecdysteroids during early embryonic development in silk worm *Bombyx mori*: metabolism and functions. *Zool. Sci.*, 21, 503-516.

Sonobe, H., Tokushige, Makka, T., Hara, N.,& Fujimoto, Y. (1999).Comparative studies of ecdysteroid metabolism between diapause eggs and non diapauses eggs of silk worm *Bombyx mori*. *Zool.Sci.*,16, 935-943.

Srivastava, U.S. & Bahadur, I. (1961). The development of the Malpighian in *Dysdercuskoemigi* (Hemiptera). *Quarterly Journal of Microscopical Science*, 102 (3), 347 – 60.

Steele, J. E. (1982). Glycogen Phosphorylase in insects. *Insect Biochem*., 12, 131 – 147.

Storey, K. B., & Storey, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev*., 68, 27-84.

Storey, K., & Storey, J.M. (2012). Insect cold hardiness: metabolic, gene, and protein adaptation. *Can. J. Zool.*, 90, 456-475.

Storey, K.B. (1982). Phosphofructokinese from the over wintering gall fly larva, *Eurosta solidaginis* : control of cryoprotectant polyol sysnthesis. *Insect Biochem*., 12, 501-505.

Storey, K.B., & Storey, J.M. (1991).Carbon balance and energetic of cryoprotectant synthesis in a freeze tolerant insect: responses to perturbation by anoxia. *J. Comp. Physiol.B*, 160, 77-84.

Storey, K.B., & Storey, J.M.(1990).Biochemistry of cryoprotectants. In D. Denlinger and R.E. Lee (Eds.), *Insects at low temperature* (pp.64-93). New York: Chapman Hall.

Stribel, H. (1960). Zur Embryoualentwicklung der Teumiten. *Acta trop*., 17, 193-260.

Stuart, J.A., & Brown, M.F. (2006). Energy quiescence and the cellular basis of animal life spans. *Comp. Biochem. Physiol*., A, 143, 12-23.

Sujuki, K., Fujita, M., & Miya, K. (1983). Changes in super cooling point of the silk worm eggs . *J. Seric. Sci. Jpn*., 52, 185-190.

Takami, (1969). *A general text book of the silk worm eggs*. Tokyo, Japan : Zenkoku Sanshu Kyokai.

Takami, T. and Kitazawa, T. (1960). Eternal observation of embryonic development in the silkworm. *Sanshi Shikenjo, Kokoku, Sericult. Expt. Stn. Tech. Bull*., 75, 1-31.

Takei,R., & Nagashima,E. (1975). Electron microscopic investigation on the early developmental stages of diapauses and non diapauses eggs in the silk worm *Bombyx mori* L. *J. Seric. Sci. Jpn*., 44, 118-124.

Takesue, S., Keino, H., & Onitake, K. (1980). Blastoderm formation in the silkworm egg (Bombyx mori L.). *J. Embryol. Exp. Morph*, 60, 117-124.

Takesue, S., Onitake, K., & Keino, H. (1977). Studies on the blastoderm & germband formation during early embryogenesis in the silk worm, *Bombyx mori* L. *Zool. Magazine* , 86, 345.

Takimi, T. (1946). *Seibutsu*, 1, 208-211.

Tang, S. & Li, Y.(1994). primary studies on changes in quantity of phospholipids and one day hatchability of Bluish silk worm egg by different Cold -storage Treatments. *B.B.A.*, 1218(3), 366 – 374.

Tao, H., Liu, H.J., Cheng, Y.Q., Sima, Y., Yin, W.M., & Xu, S.Q. (2015). Parental environmental exposure leads to glycometabolic disturbances that affect fertilization of eggs in Silkworm, *Bombyx mori*: parental transcript legacy. *J. Comp. Physiol.*, 185(1), 47-55.

Tayadae , D.S. (1987). Heterosis effect on etonomic traits of new hybrids of silkworm, *Bombyx mori* L. *Sericologia*, 27, 301-307.

Tayede, D.S., Jawale, M.D., & Uncheganixar, P.K.(1987). Effect of refrigeration on hatchability of eggs of multivoltine race *Bombyx mori* L. *Sericologia*, 27(2), 297.

Tazima, Y. (1964). *The Genetics of the silkworm*. London: Academic Press.

Tazima, Y. (1978). *The silkworm: an Important Laboratory Tool*. Tokyo: Kodausha.

Tazima, Y. (1984). Silkworm Moths. In I.L. Mason (Eds.), *Evolution of Domesticated Animals* (pp.416 -424). London: Longman.

Thangavelu, K., Bhagowati, A.K. & Chakraborty, A.K. (1985a). Preservation of muga cocoon for seed purposes. *Indian Silk*, 23(12), 25- 26.

Thangavelu, K., Bhagowati, A.K., & Chakraborty, A.K. (1985). Introduction of quiescence in muga silkworm *Antheraea assama*Westwood. *Current Science*, 54(19), 1011 -1013.

Thangavelu, K., Bhagowati,A.K. and Chakraborty, A.K (1987). Diapause potential in muga silkworm *Antheraea assama*. *Insect Sci. Let.* 8(1), 61 -63.

Thangavelu, K., Bhagowati,A.K. and Chakraborty, A.K(1985 b). Induction of quiescence in muga silkworm *Antheraea assama* Westwood. *Curr.Sci*., 54(19) 1011 -1013.

Thangavelu, K.; Chakraborty, A.K.; Bhagowati, A.K.,& Isa, Md. (1988). *Handbook of muga culture*. Bangalore: Central Silk Board

Thangavelu,K and Sahu, A.K.(1986). Further studies on the indoor rearing of muga silkworm (*Antheraea assama* Westwod) (Lepidoptera :Saturnidae). *Sericologia*, 26 (2), 215 – 224.

Tomita, S., & Kikuchi, A. (2009). Abd – B suppresses lepidopteran proleg development in posterior abdomen. *Developmental Biology*. 328, 403 – 409.

Toyama, K. (1896). Structure of the eggs and development of the embryos in the silkworm, *Bombyx mori*, L. *Ngakukai-hoh*, 28, 28-80.

Toyama, K. (1902). Contribution to the Study of Silk Worm, I. On the embryology of the silk worm. *Bull. Coll. Agric. Tokyo Imp. Univ.*,5, 73 -118.

Trevlyan, W.E., & Harrison, J.S. (1952). Yeast metabolism: fractionation and microdetermination of cell carbohydrate. *Biochem. J*., 50, 298-303.

Tricohniroff , A. (1879). Uber die entwicklungsgeshichte des Seidenwurm. *S. Zool. Anz*., 2, 64-67.

Turner, F.R, & Mahowald, A.P. (1976). Scanning electron microscopy of Drosophila embryogenesis. I. The Structure of the egg envelop & formation of the cellular blastoderm. *Dev. Biol*. 50, 95-108.

Upadhyay, V.B., & Pandey, A.K. (2000). Effect on refrigeration on eggs and pre refrigeration period on the survival of larvae of *Bombyx mori*. *Ind. J. Ent*., 62(1), 28-33.

Vemenanda Reddy, G., Veeraiah, T.M., & Samson, M.V.(2004). Silk worm seed production schedules for bivoltines-New dimensions. *Indian J. Seric*. 43(1), 25-34.

Viswakarma, S.R. (1982 – 83) Effects of Refrigeration of Erik Silkworm, *Philosamia ricini* Hutt. eggs on hatching (Lep. Sat.). *Indian J. Seric.*, 36 – 39.

Waddington, C.H. (1967). *Principles of Developmentand Differentiation*. New York: Macmillan.

Waddington,C.H.(1962). *New patterns in genetics and development*. New York: Columbia University Press.

Walters, F.L. (1967). Effects of Temperature on Hatching & Development of *Tribolium confusum*. *J. Stored Products*, Referral to in Howe, R.H. *Annu. Rev. Ent*., 12, 15- 42.

Wiggelsworth, V.B. (1972). *The principles of insect physiology*. London: Methnen & Co.

Wyatt, G.R. (1967). Biochemistry of sugars and polysaccharides in insects. In J.W. Beament, J.E. Treherne and V.B. Wigglesworth (Eds.), *Advances in Insect Physiology* (pp. 287-360).London & NewYork : Academic Press.

Wyatt, G.R., & Kalf, G.F. (1957). The chemistry of insect haemolymph. *J. Gen. Physiol*., 40, 833.

Yaginuma, O. & Yamashita, T.(1979). NAD-dependent sorbitol dehydrogenase activity in relation to the termination of diapause in eggs of Bombyx mori. *Insect Biochem*., 9(5), 547- 553.

Yaginuma, T., & Yamashita, O. (1978). Polyol metabolism related to diapauses in Bombyx eggs : different behaviour of sorbitol from glycerol during diapause and post diapause. *J. Insect. Physiol.*, 24, 347-354.

Yaginuma, T., & Yamashita, O. (1979). NAD- dependent sorbitol dehydrogenase in relation to the determination of diapauses in eggs of *Bombyx mori. Insect. Biochem*., 9(5), 533-547.

Yaginuma, T., & Yamashita, O. (1999). Oxygen consumption in relation to sorbitol utilization at the termination of diapauses in eggs of the silk worm *Bombyx mori*. *J. Insect Physiol*., 44,621-627.

Yaginuma, T., Kobayashi, M., & Yamashita, O. (1990a). Effect of low temperatures on NAD sorbitol dehydrogenase avtivity and morphogenesis in non diapauses eggs of the silk worm *Bombyx mori. Comp. Biochem. Physiol*., B. 97 (3), 495-506.

Yaginuma, T., Kobayashi, M., & Yamashita, O. (1990b).Distinct effects of different low temperatures on the induction of NAD sorbitol dehydrogenase activity in diapauses egg of the silk worm of *Bombyx mori. J. Comp. Physiol*., B, 160, 277-285.

Yamada, R., & Sonobe, H. (2003). Purification, kinetic characterization and molecular cloning of novel enzyme ecdysteroid- phostatase. *J. Biol.Chem*., 278, 26365-26373.

Yamahama, Y., Seno, K., & Hariyama, T. (2008). Changes in lipid droplet localization during embryogenesis of the silk worm *Bombyx mori*. *Zool.Sci*., 25, 580-586.

Yamashita, O. (1965). Carbohydrate metabolism during embryonic development of the silk worm, *Bombyx mori*, L. *J. Seric. Sci. Jpn*., 34, 1-7.

Yamashita, O. and Hasegawa, K. (1985). Embryonic diapauses. In G. A. Kerkut, & L.I. Gilbert (Eds.), *Comprehensive Insect Physiology Biochemistry and Pharmachology* (pp. 407-434). Oxford: Pregamon Press.

Yamashita, O., & Hasegawa, K. (1976). Diapause hormone action in silkwormovaries incubated *in vitro* $^{14}$C- trehalose incorporation into glycogen. *J. Insect Physiol*., 22(3),409-414.

Yamashita,O., & Yaginuma, T. (1991). Silk worm eggs at low temperatures: implications for sericulture. In R.E. Lee & Denlinger, D.L. (Eds.), *Insects at low temperature* (pp. 424-445). NewYork : Chapman Hall.

Yamashita,O., & Yaginuma, T. (1991). Silk worm eggs at low temperatures: implications for sericulture. In R.E. Lee & Denlinger, D.L. (Eds.), *Insects at low temperature* (pp. 424-445). NewYork : Chapman Hall.

Yamato, K., Banno, Y., Fujji, H., Maike, F., Kashige, N., & Aso, Y. (2006). Catalase from the silk worm *Bombyx mori*: gene sequence, distribution and over expression. *Insect Biochem. Mol. Biol*., 35, 277-283.

Yuyin, C. (2000). Technical standards for commercial egg production of bivoltine silkworm varieties used in China. *Sericologia*, 40,185-201.

Zhao, L.C., Hou, Y.S., Sima, Y.H.(2014). Changes in glutathione redox cycle during diapause determination and termination in the bivoltine silk worm *Bombyx mori*. *Insect Science*, 21 (1), 39-46.

Zhao,L.C., & Shi, L.G. (2010). Metabolism of hydrogen peroxide between diapauses and non diapauses eggs of the silk worm *Bombyx mori* during chilling at 5˚C. *Arch. of Insect Biochem. Physiol*., 74 (2), 127-134.

Zhu, I., Indrasiith, L.S., & Yamashita, O. (1986). Characterization of vitellin, egg specific protein and 30- kDa proteins of *Bombyx* eggs and their fates during oogenesis and embryogenesis. *Biochem. Biophys. Acta*, 882, 427-436.