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).

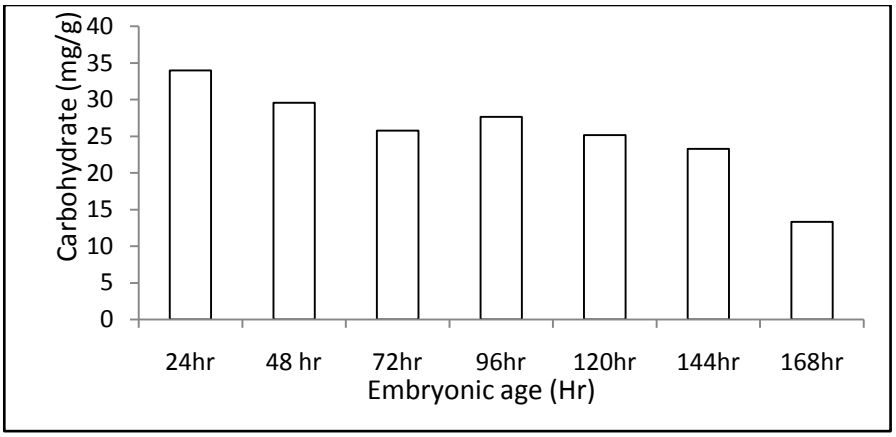

Fig1. Showing changes in carbohydrate contents during normal differentiation of *A. assama*

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).

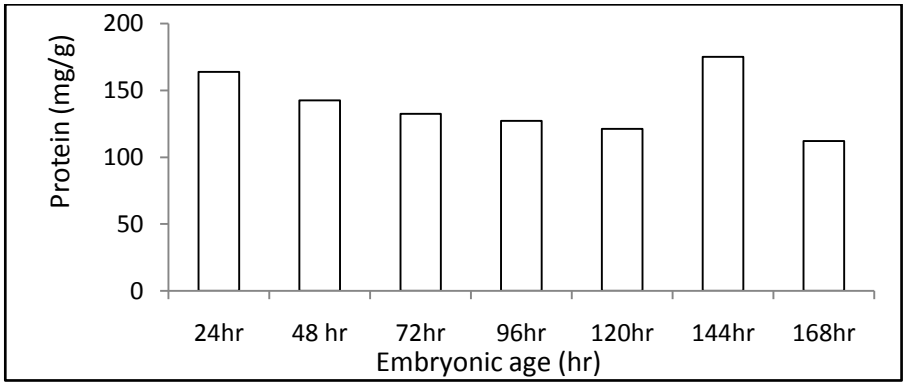

Fig 2. Showing changes in protein contents during normal differentiation of *A. assama*

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.

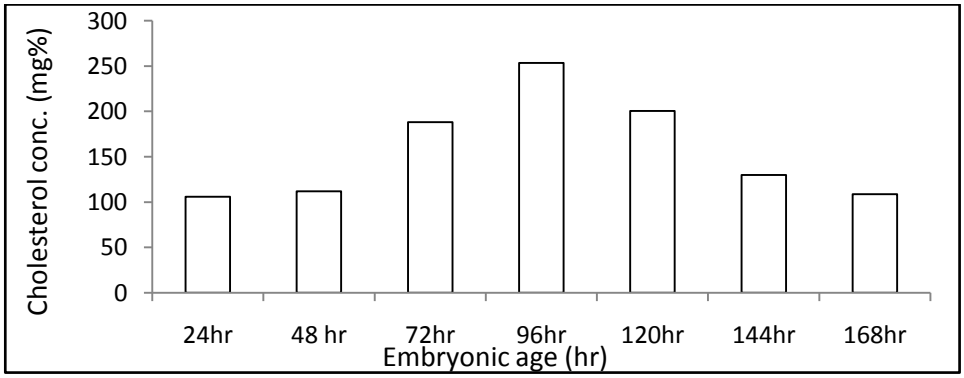

Fig 3showing changes in cholesterol contents during normal differentiation of *A. assama*

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

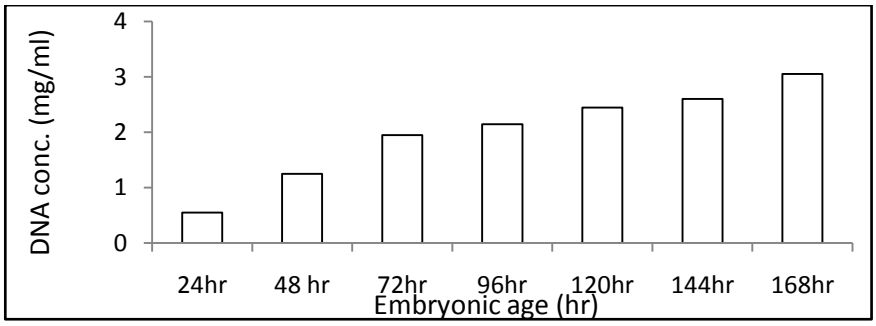

Fig 4 showing changes in DNA contents during normal differentiation of *A. assama*

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.

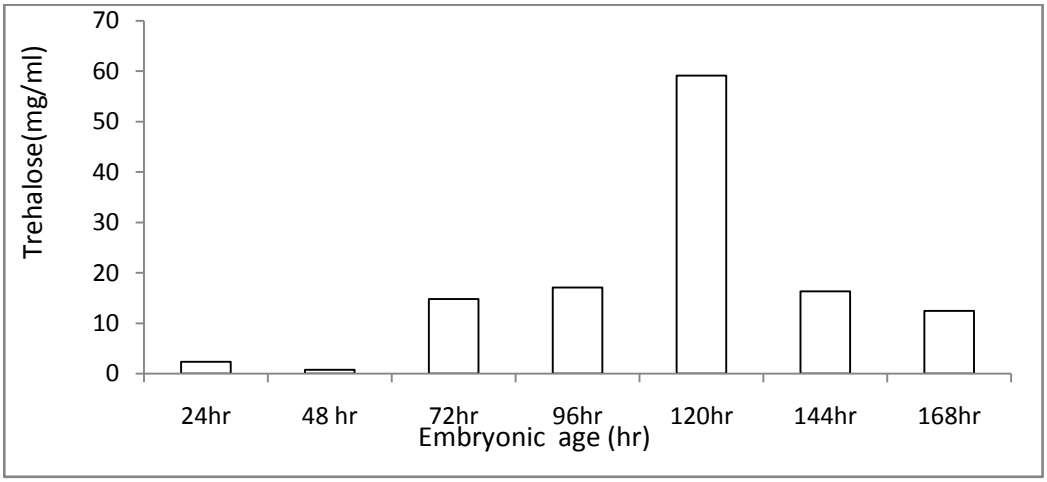

Fig 5 showing changes in trehalose contents during normal differentiation of *A. assama*

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).

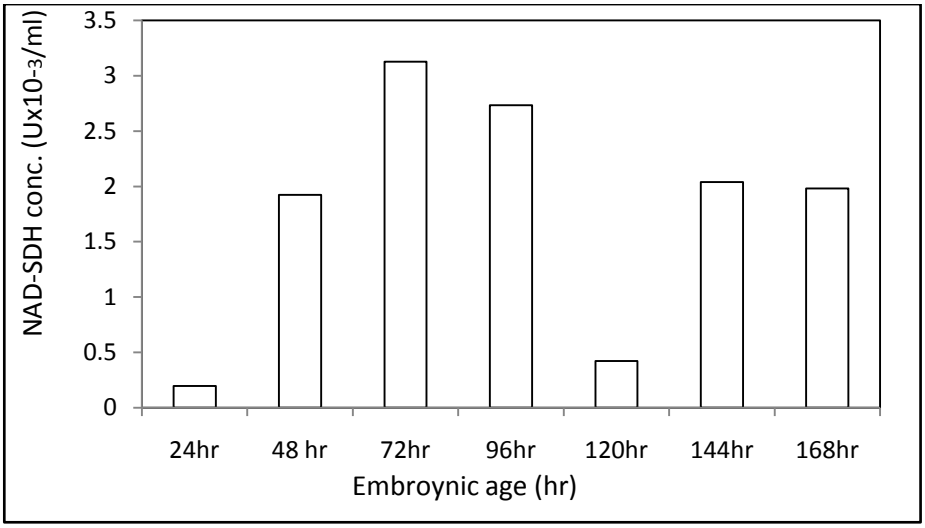

Fig 6showing changes in trehalose contents during normal differentiation of *A. assama*

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.

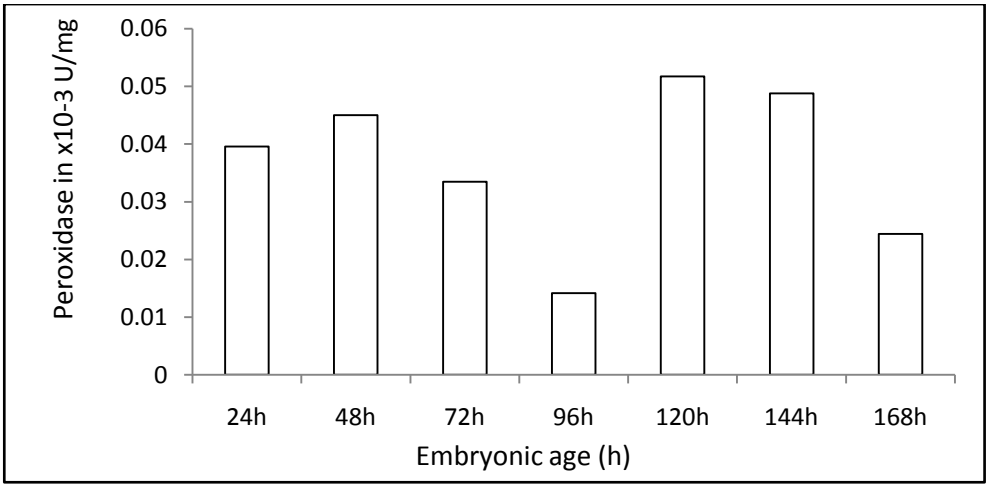

Fig7showing changes in NADH Peroxidase contents during normal differentiation of *A. assama*

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).

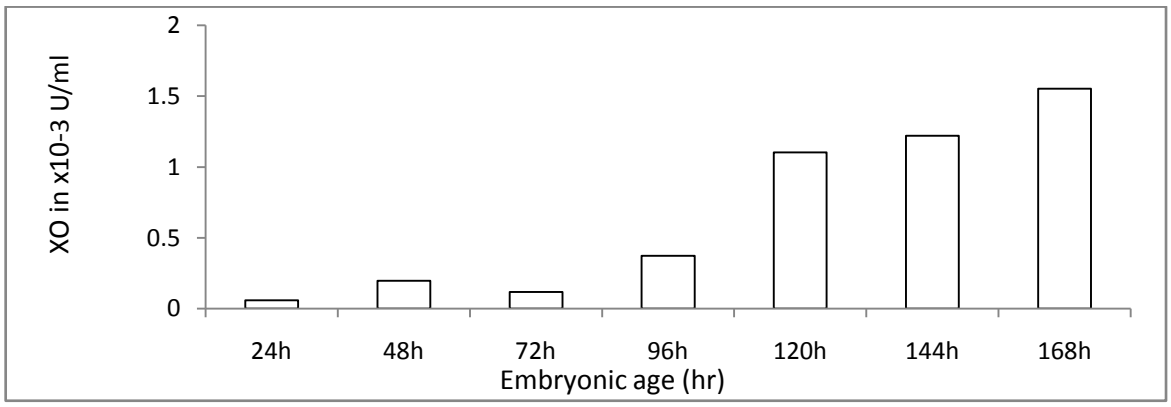

Fig 8showing changes in XO contents during normal differentiation of *A. assama*

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.