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 4th 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

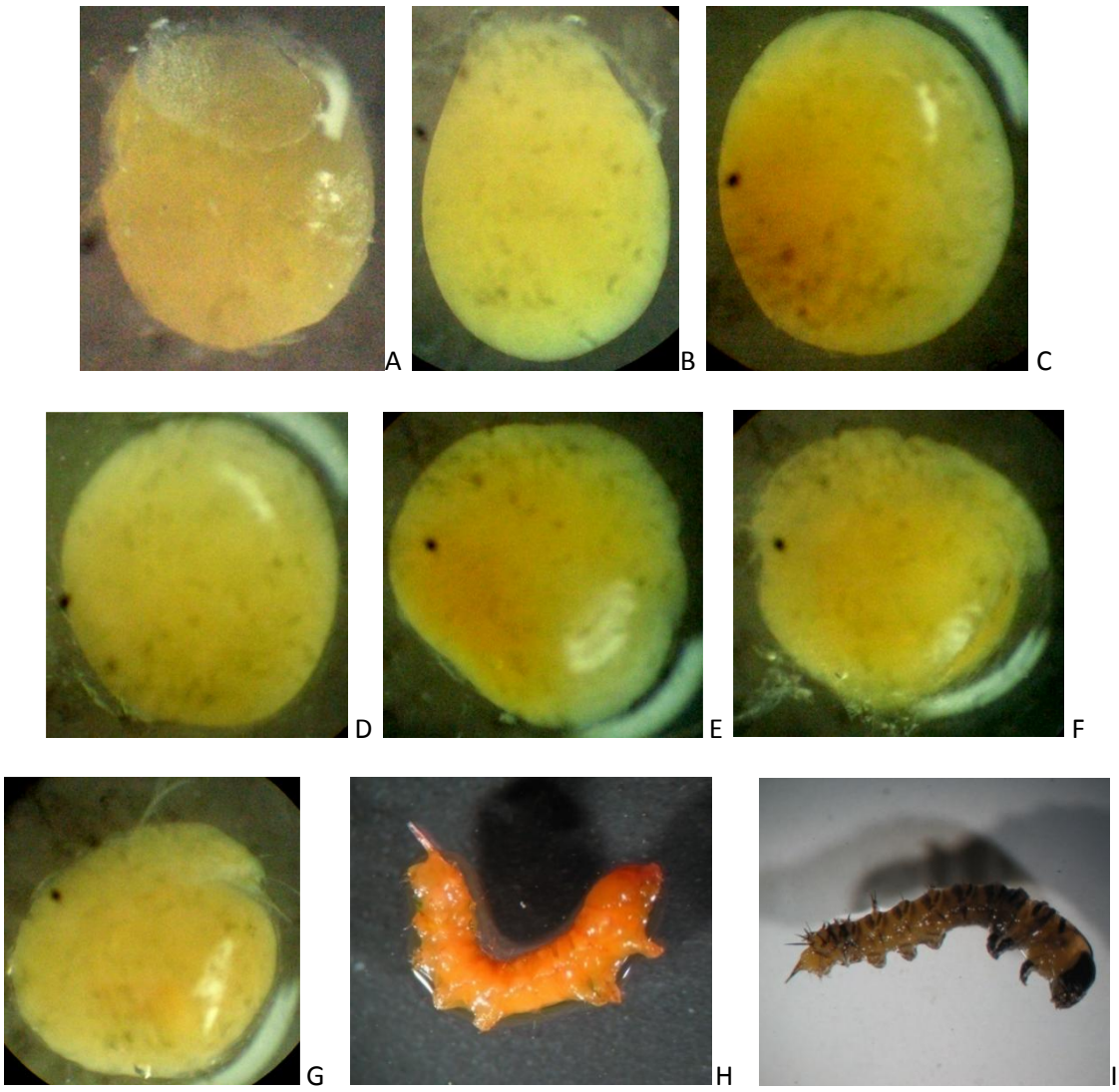


Plate 1. Showing Stereo binocular images of (A)6 Hours, (B)12 Hours, (C) 24 Hours,(D)48 Hours, (E) 72 Hours, (F)96 Hours, (G)120Hours, (H)144Hours and (I) 168 Hours of *Antheraea assama* Embryogenesis.

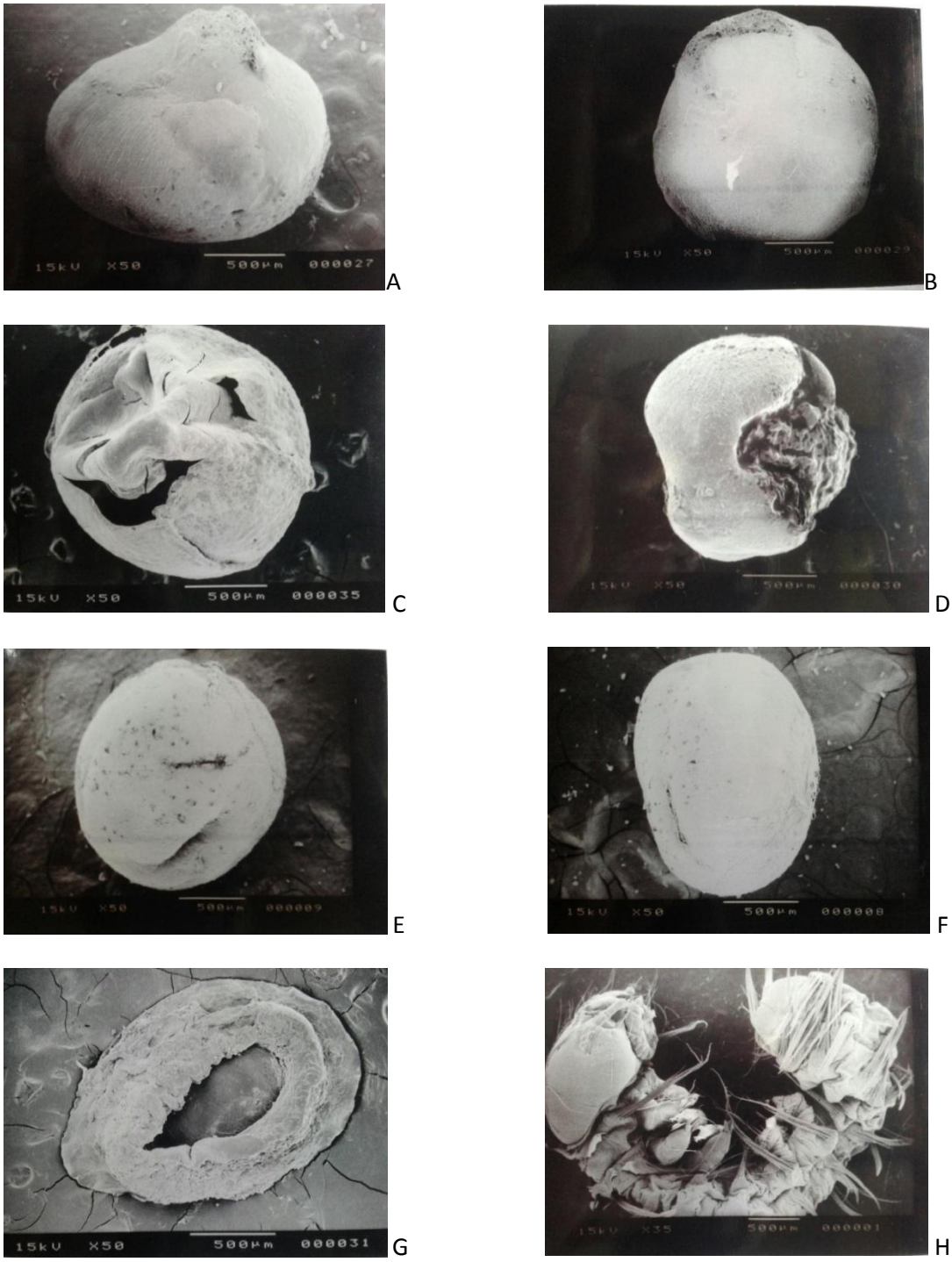


Plate 2. Showing Scanning Electron Microscopic images of (A)6 Hours, (B)12 Hours, (C) 24 Hours,(D)48 Hours, (E) 72 Hours, (F)96 Hours, (G)120Hr and (H)144hours of *Antheraea assama* Embryogenesis.

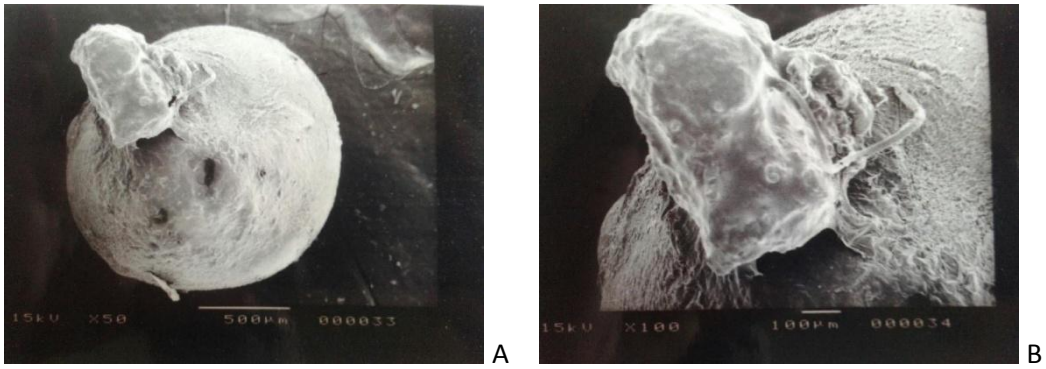


Plate 3. Showing Scanning Electron Microscopic image of out ward movement during ketatrepsis of *Antheraea assama* Ww embryo

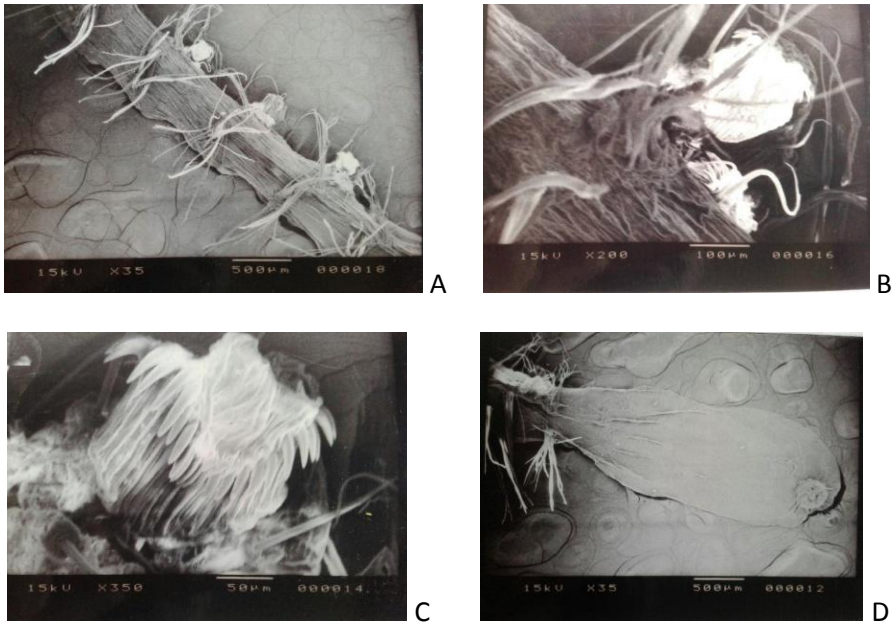


Plate 4. Showing appearance of setate on the embryo (A, B, C) and rectal sac (D) of *Antheraea assama*

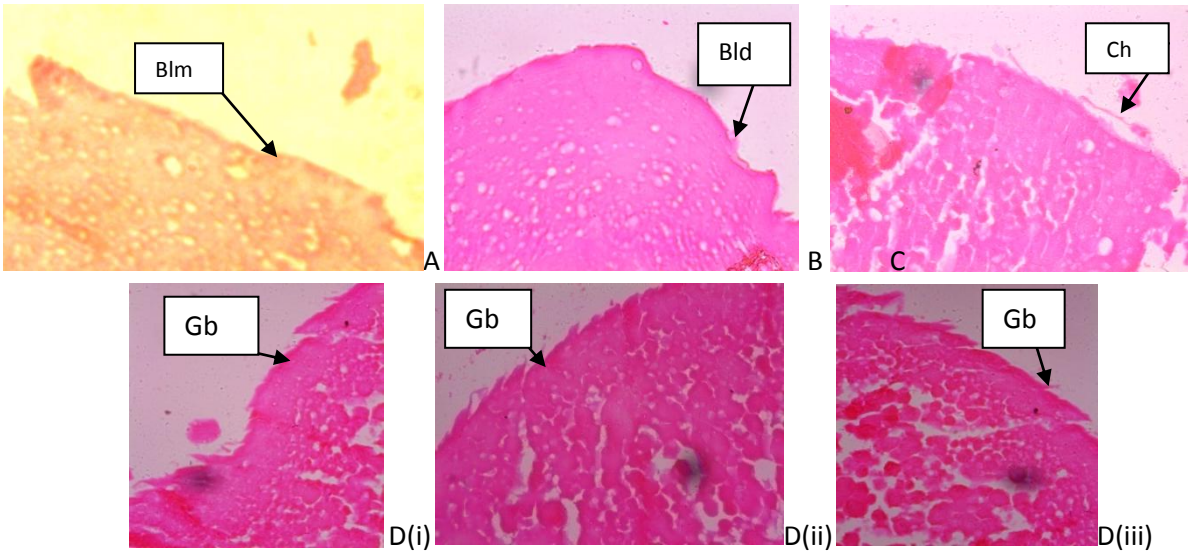


Plate 5. Showing section of (A)6 Hours, (B) 12 Hours, (C) & (D)24 Hours embryo of *Antheraea assama* Ww. Blm: Blastomere, Bld: Blastoderm, Ch: Chorion, Gb: Germ band

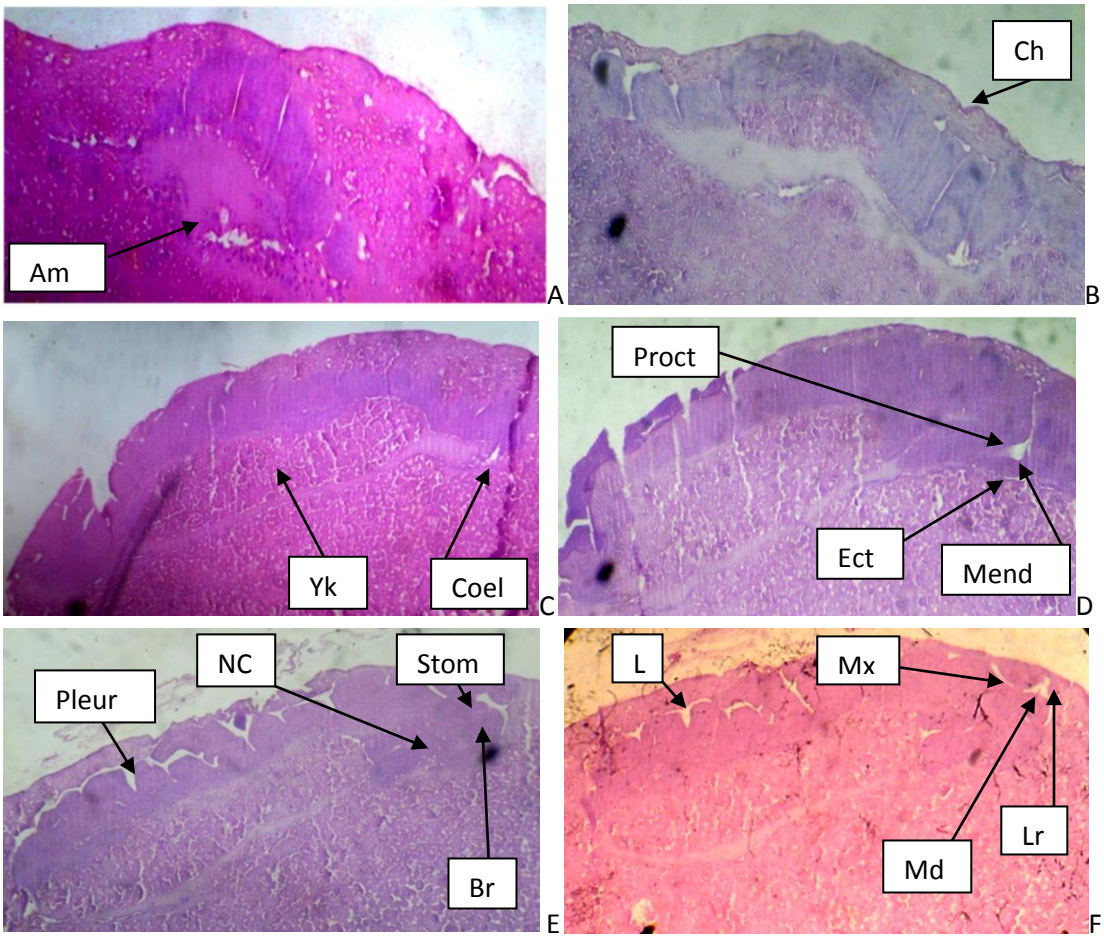


Plate 6. Showing sections of (A&B) 48 Hours, (C&D)72 Hours, (E&F)96 Hours of embryogenesis of *Antheraea assama* Ww. Am: Amnion, Ch:: Chorion, Proct: Proctodeum, Yk : Yolk, Coel: Celomic cavity, Ect: Ectoderm, Mend: Mesoendoderm, Br: Brain, NC: Ventral nerve cord, Stom: Stomodaeum, Pleur: Pleuropodium,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

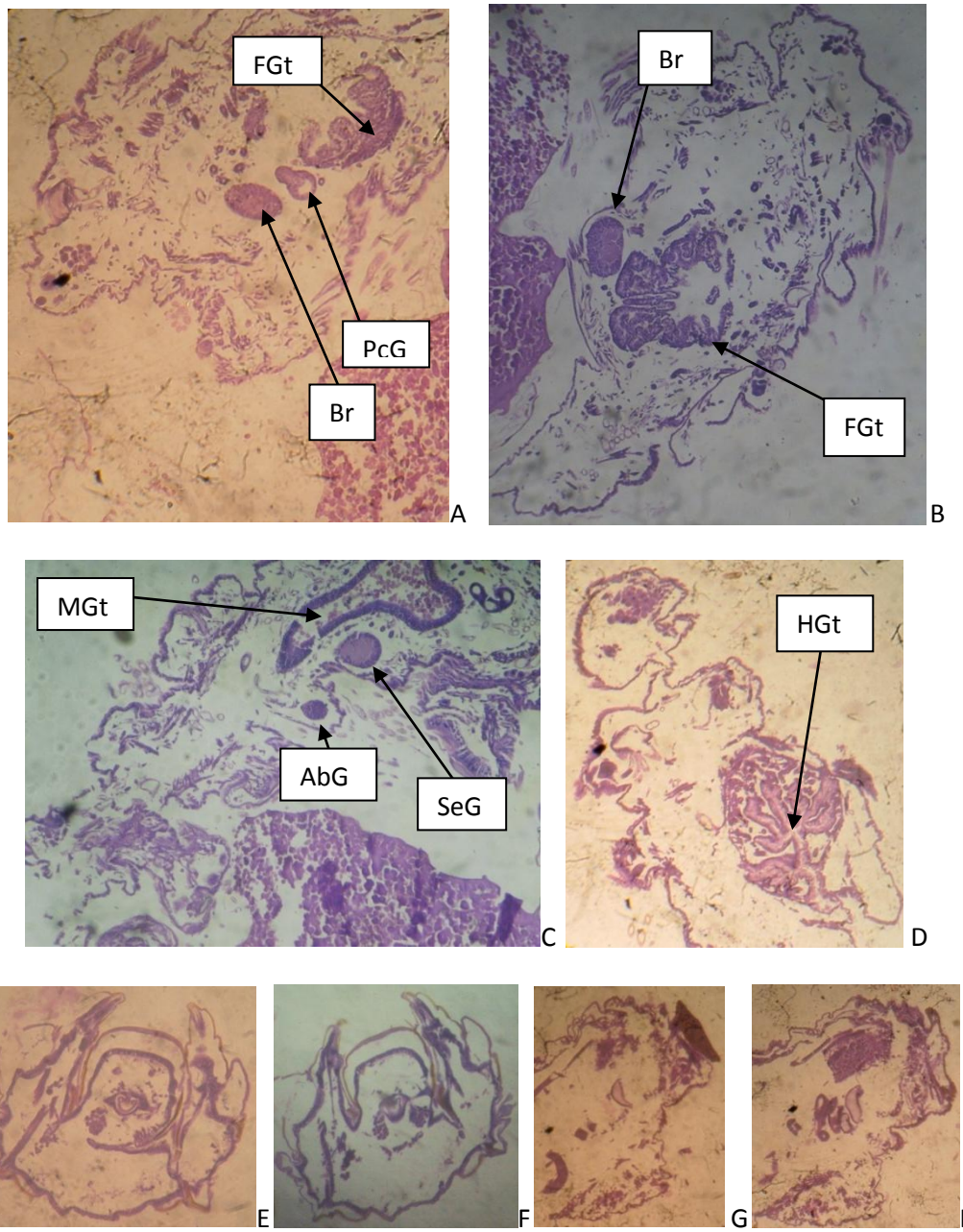
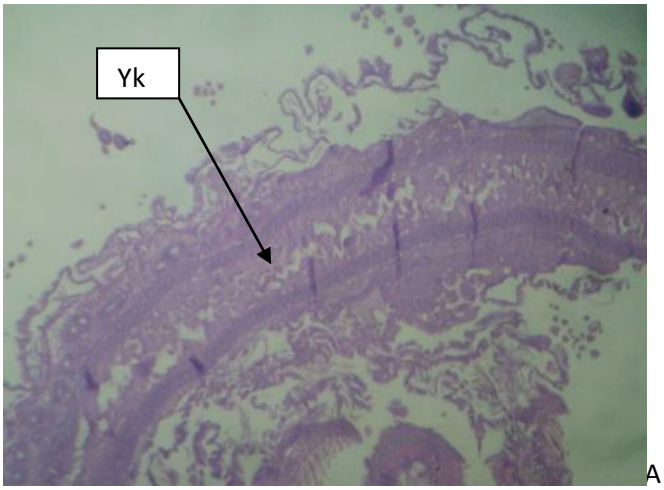
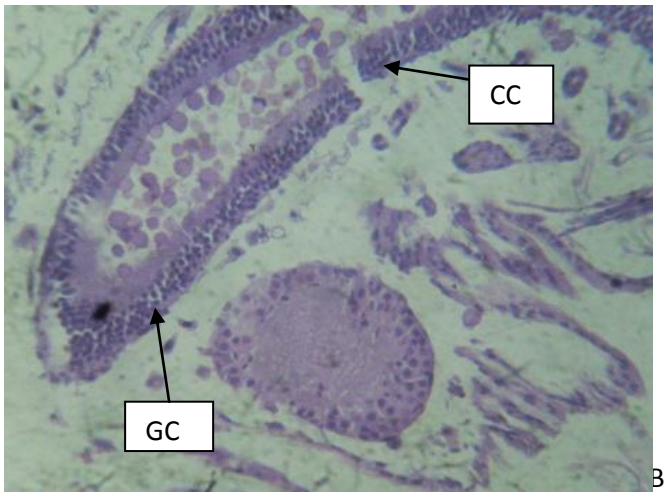


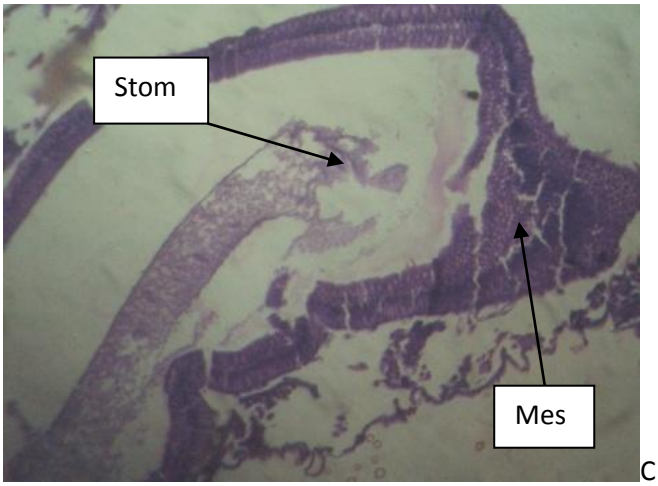
Plate 7. Showing section of (A)120 hours,(B,C&D)144 Hours, (E)120Hours Head, (F)144Hours Head (G) 120 Hours Caudal end and (H)144 hours Caudal end of *Antheraea assama* embryo. Br: Brain, PcG: Pro Cephalic Ganglion, SeG: Sub esophageal Ganglion, AbG: Abdominal Ganglion, FGt: Fore Gut, MGt: Mid Gut, HGt: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

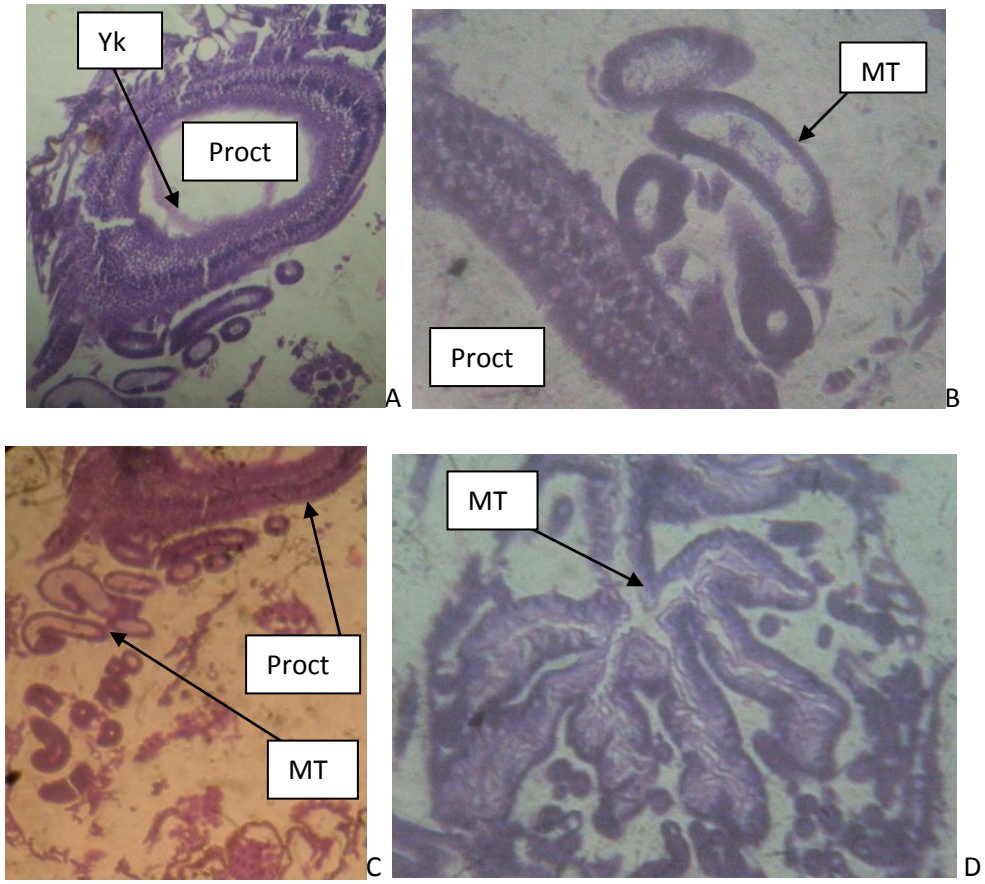


Plate 9. Showing section of 120hours embryo having bud from proctodeum (A,B,C) and 144hours embryo with Malphigian tubules(D) in *Antheraea assama* Ww. Proct: Proctodeum, Yk: Yolk, MT: Malphigian tubule

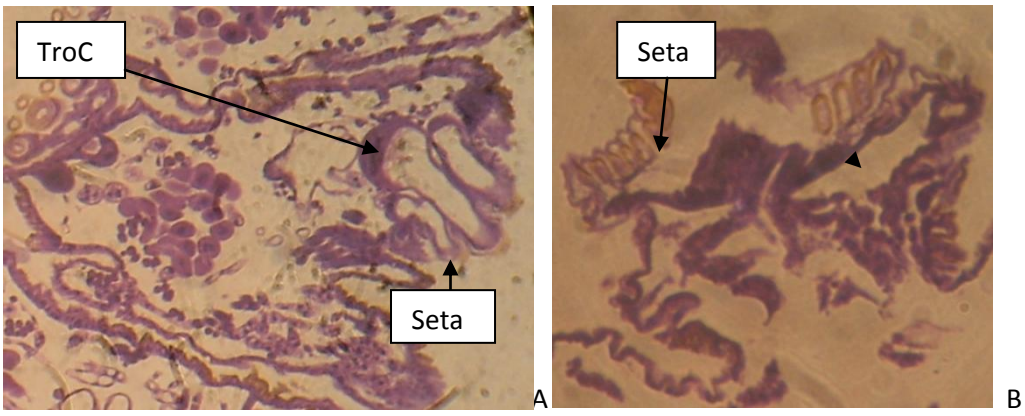


Plate 10. Showing section of (A) 120hours embryo with Trichogen cell and (B)144 hours embryo with seta during embryogenesis of *Antheraea assama* Ww. TroC: Trichogen cell

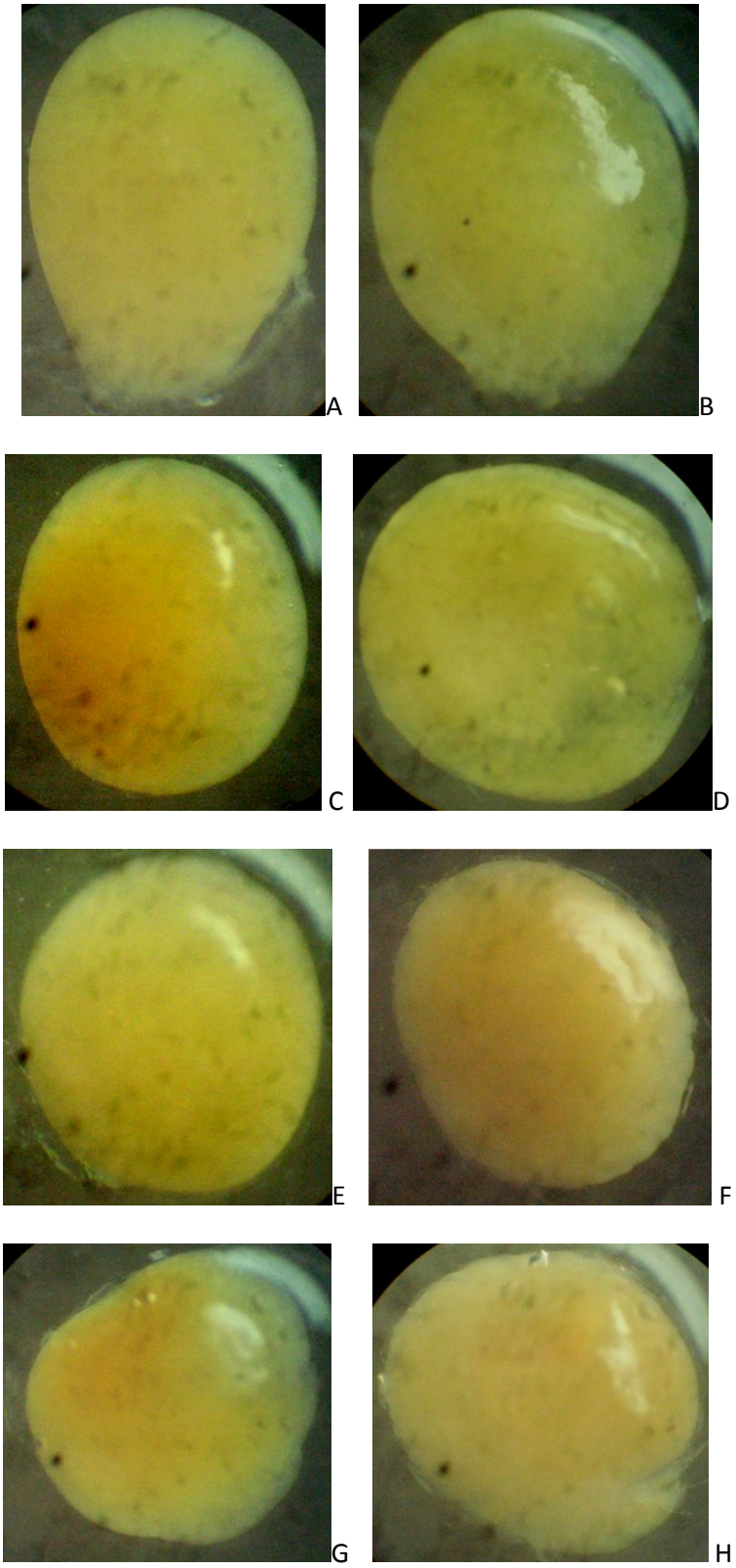
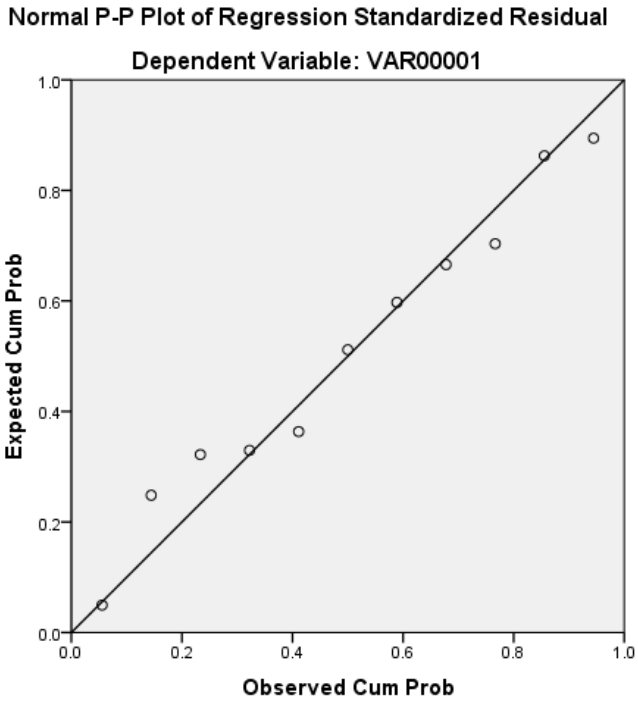
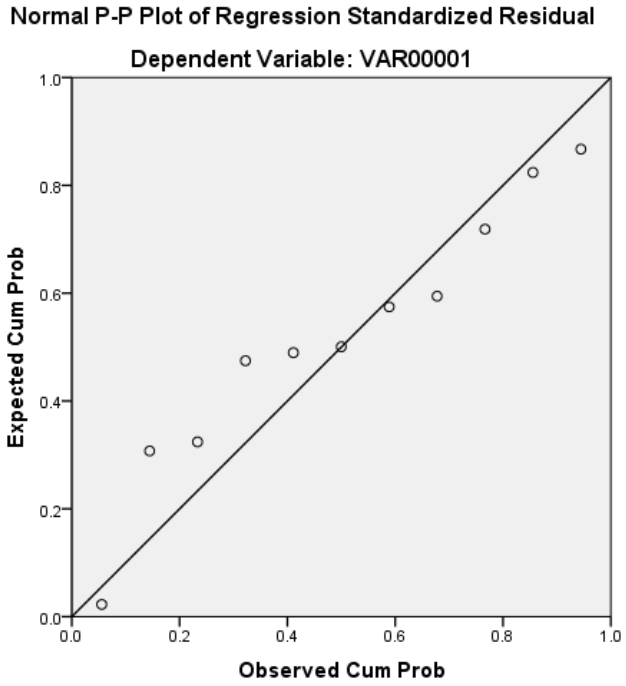


Plate 11. Showing Stereo binocular images of (A)12 Hours, (B)12 Hours after 3days refrigeration, (C) 24 Hours,(D)24Hours after 3days refrigeration, (E) 48 Hours, (F)48 Hours after 3days refrigeration, (G)72Hours and (H)72Hours after 3days refrigeration, at 5˚C during *Antheraea assama* Embryogenesis.

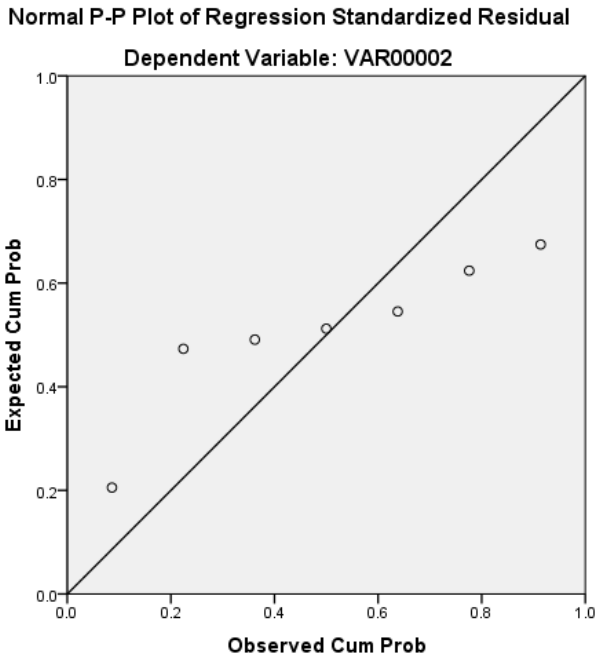


A

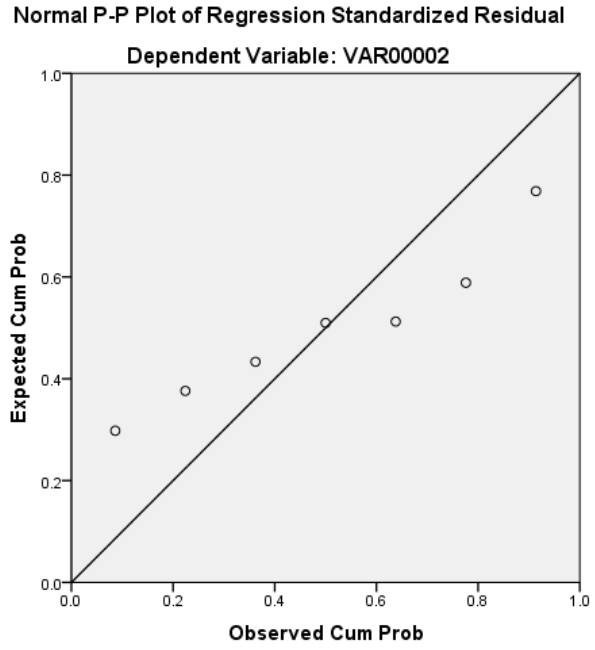


B

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

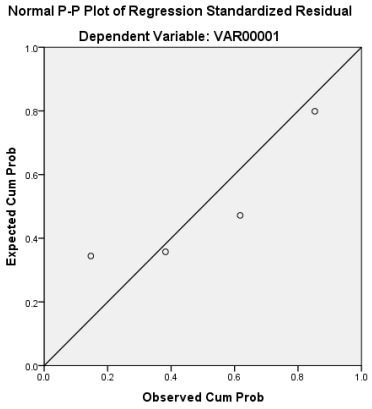


A

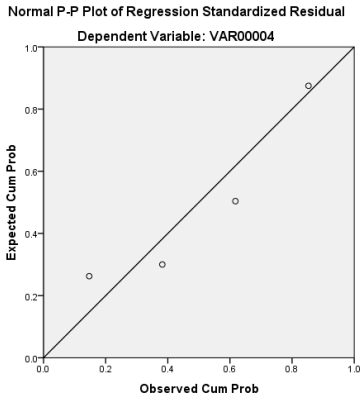


B

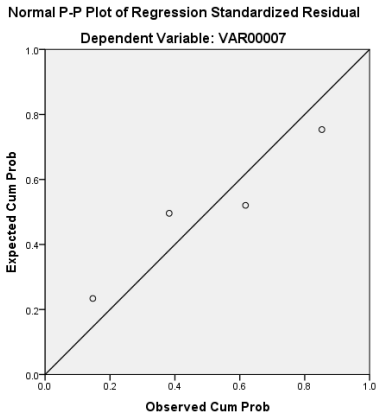
Plate 13. Regression Plot showing Effect of different low temperature stress in identified low temperature resistant embryonic stages of *A.assama* on A) hatching percentage and B) incubation period.



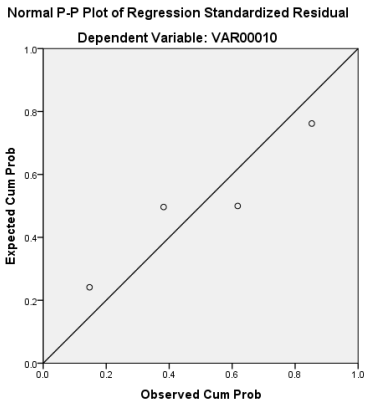
A



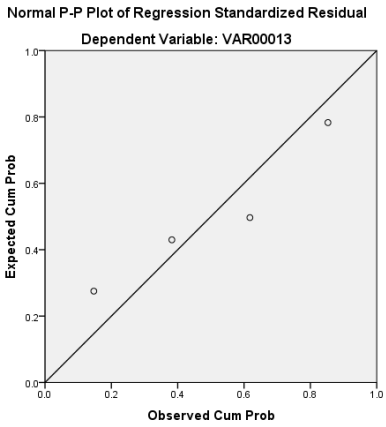
B



C

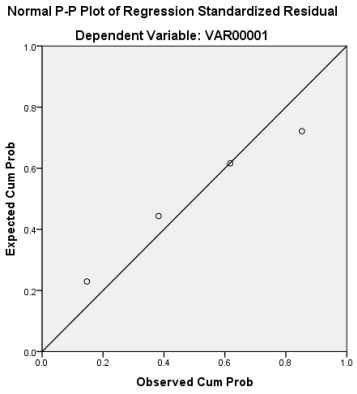


D

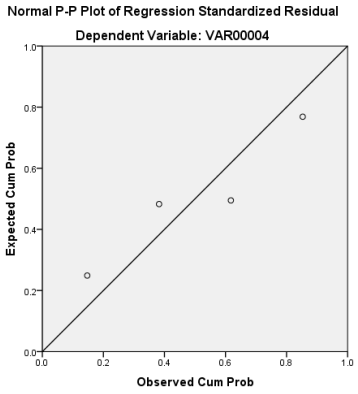


E

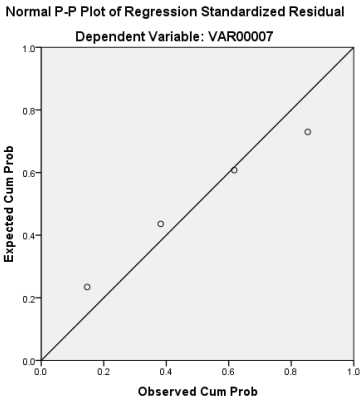
Plate 14. Regression Plot showing Effect of preservation period [A) 3days; B) 7days; C) 10 days; D) 15 days and E) 21 days] after low temperature stress to the identified embryonic stages of *A.assama* on hatching percentage.



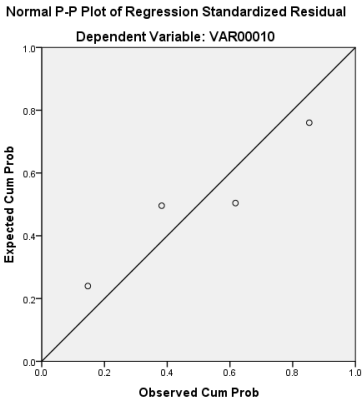
A



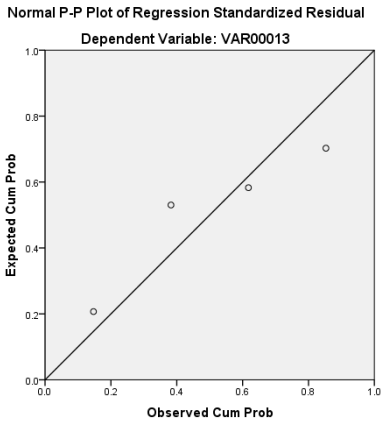
B



C



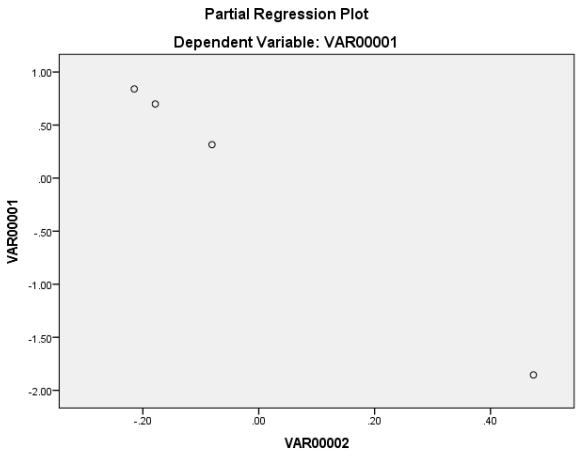
D



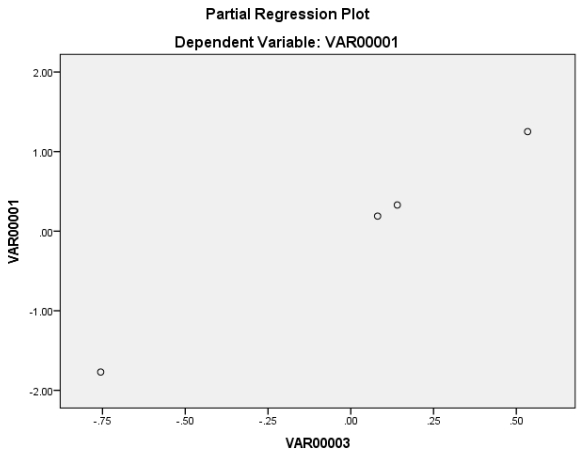
E

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.

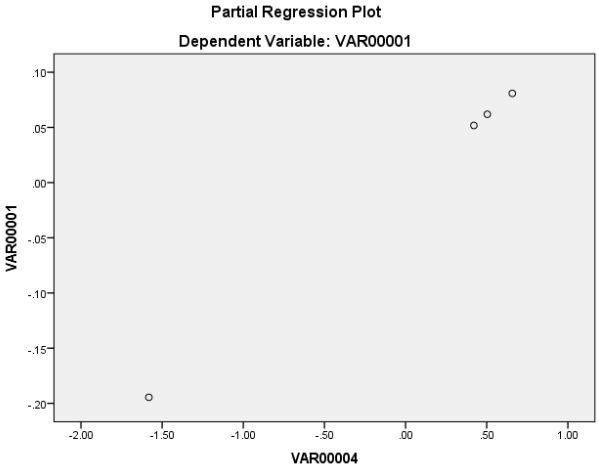
.



(A)

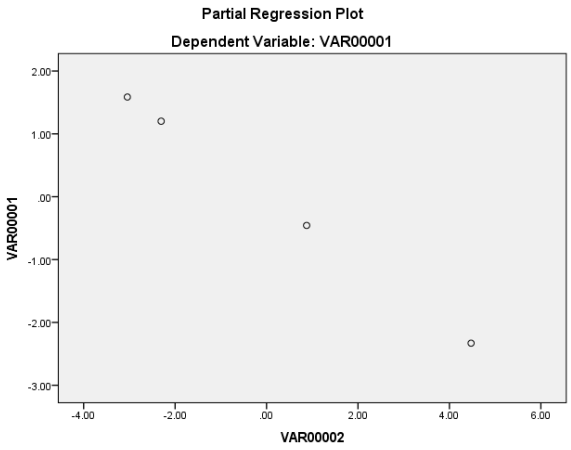


(B)

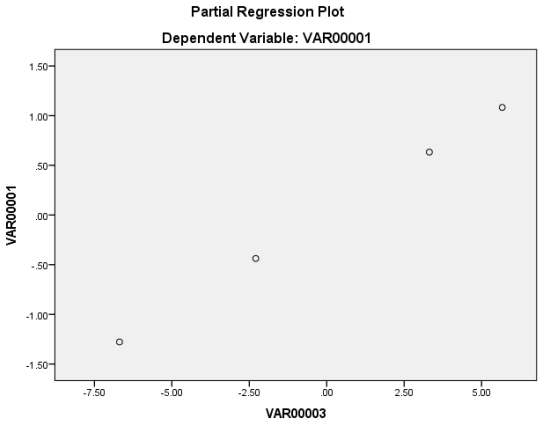


(C)

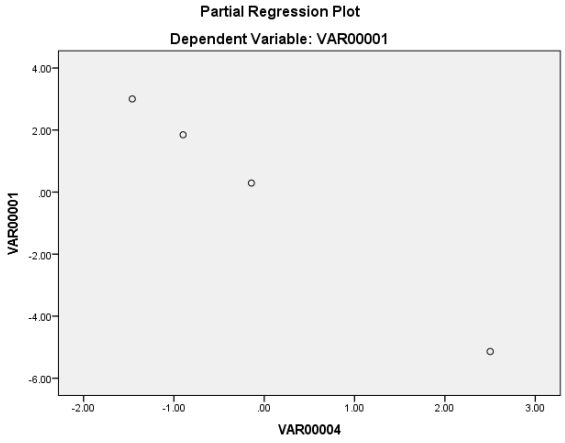
Plate 16. showing regression plot for NAD SDH activity for 24,48,72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4



(A)

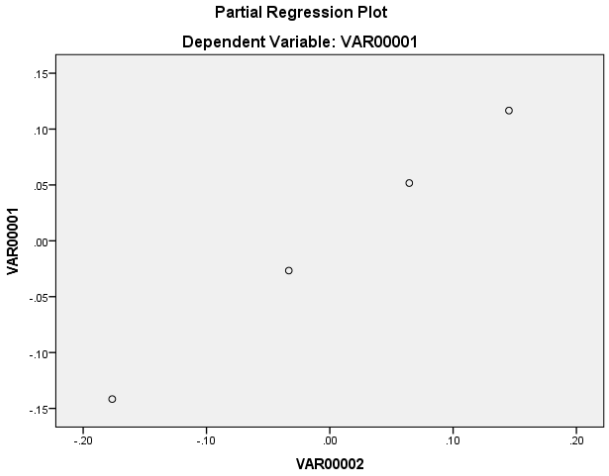


(B)

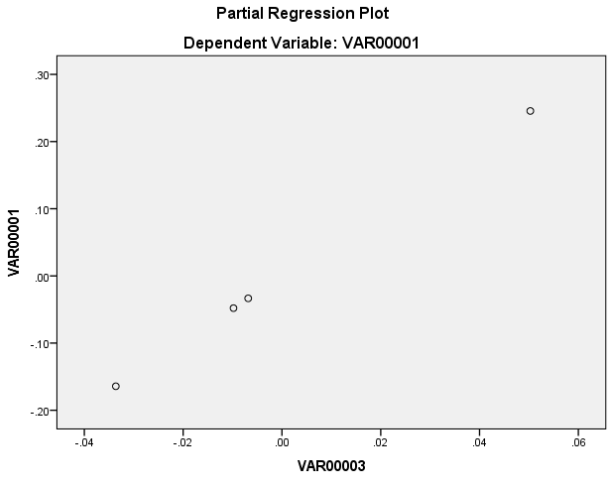


(C)

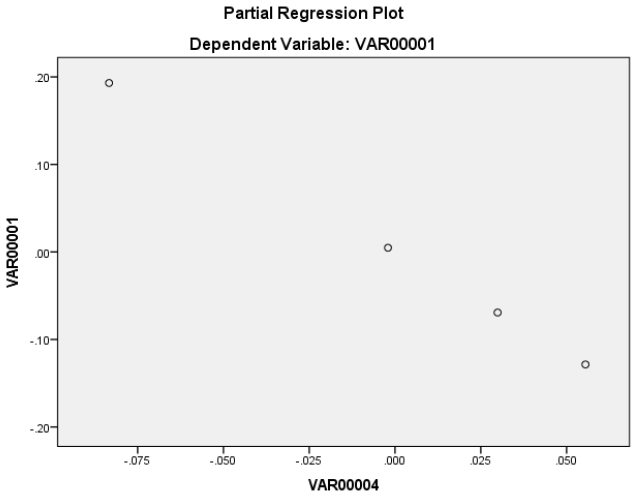
Plate 17. showing regression plot for Trehalose quantity for 24, 48, 72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4



(A)

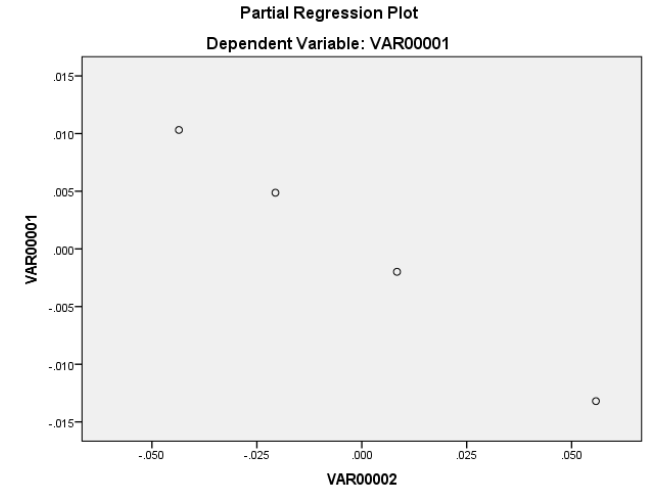


(B)

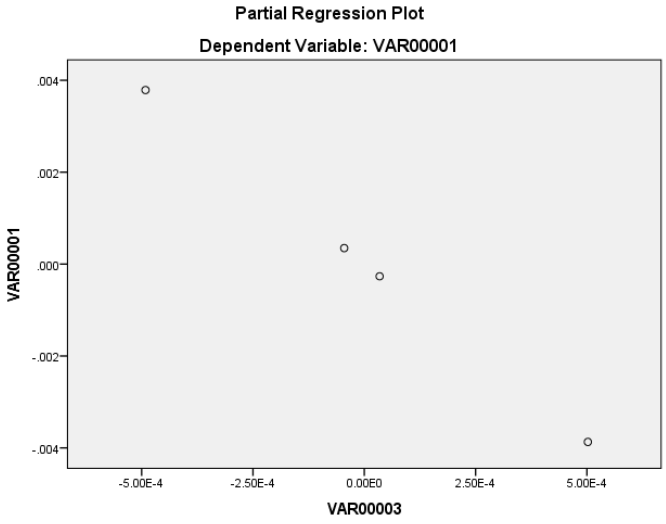


(C)

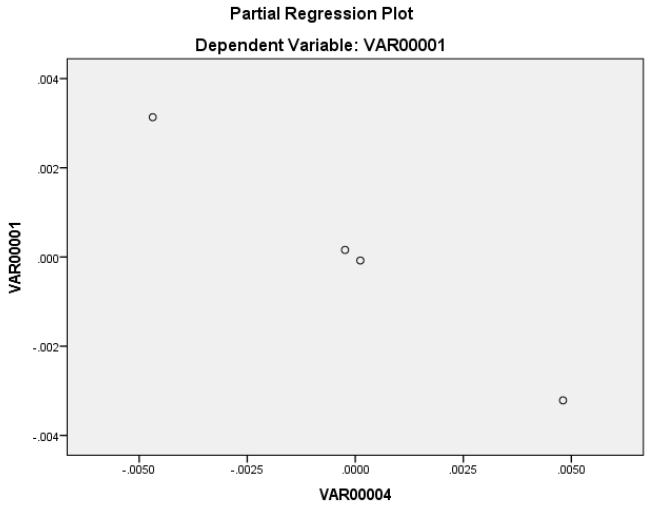
Plate 18. Showing regression plot for Xanthine oxidase activity for 24, 48, 72 and 96 hour embryo, where VAR 1: Control; VAR2: 3days; VAR3:7 days and VAR4: 10days of refrigeration. A: VAR1x Varriable2; B: VAR1x VAR 3; C: VAR1 x VAR 4



(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