2. REVIEW OF LITERATURE

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

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

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

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

2.1.1 Cleavage and Blastoderm Formation

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

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

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

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

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

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

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

2.1.2. Differentiation of Blastoderm

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

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

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

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

Among Pterygotes, secondary dorsal organ present from the serosa.

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

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

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

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

2.1.3 Fate Map

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

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

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

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

2.1.4 The germ band and gastrulation

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

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

2.1.5 Extra embryonic membrane

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

2.1.6 Blastokinesis

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

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

2.1.7 Mesoderm formation

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

2.1.8 Dorsal closure

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

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

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

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

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

2.1.9 Segmentation

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

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

2.1.10 Larval Legs

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Carbohydrate

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

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

Protein

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

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

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

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

Cholesterol

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

DNA

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

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

NAD-SDH

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

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

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

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

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

NAD SDH

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

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

Trehalose

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

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

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

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

Oxidative Stress

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

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

Xanthine oxidase

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

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

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

NADH Peroxidase

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

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

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