Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Annals of Biological Research, 2013, 4 (1):12-21
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Egg Diapause and Metabolic Modulations during Embryonic Development in the Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

Tribhuwan Singh[1], Pramod Kumar Singh[2] and Khursheed Ahmad Sahaf[3]

[1]Research Extension Centre, Central Silk Board, Rampur Road, Una (Himachal Pradesh),
[2]Regional Sericultural Research Station, Central Silk Board, Sahaspur, Dehradun (Uttarakhand)
[3]Central Sericultural Research & Training Institute, Central Silk Board, Pampore (J&K)

ABSTRACT

Diapause, a period of arrest of growth and development enables insects to overcome and survive the unfavourable environmental conditions and represents a syndrome of physiological and biochemical characteristics. The nature of diapause in mulberry silkworm, Bombyx mori is basically determined by manifestation of genetic characters and endocrinological mechanisms under the influence of environmental stimuli, such as temperature and photoperiod. Nucleotides and carbohydrate metabolism, production and utilization of sorbitol and glycerol are also equally responsible for induction, initiation, determination, maintenance and termination of diapause. Metabolic conversion of trehalose to glycogen at the induction, glycogen to sorbitol at the initiation and sorbitol to glycogen at the termination of diapause is correlated and in each metabolic shift a key enzyme becomes active in response to hormonal and environmental stimulation.

An attempt has been made in this review article to briefly discuss the nature of egg diapause in relation to genetical and hormonal studies besides nucleotides and carbohydrate metabolism associated with induction, initiation, determination, maintenance and termination of diapause during embryonic development and its significance in mulberry silkworm, Bombyx mori.

Keywords: *Bombyx mori*, egg diapause, metabolic modulations, embryonic development

---

INTRODUCTION

Diapause is genetically programmed pre-emptive developmental response to changing seasons and environmental conditions. It is an environmentally adaptive dormancy that can occur during any stage of development (egg, larva, pupa or adult) but the diapausing stage is consistent and specific within a species. Most commonly, insects have a facultative diapauses, in which there is a developmental stage this is responsive to specific environmental 'cues' (the sensitive stage) that programme the diapause. Thus, the sensitive stage marks the physiological decision to either diapauses or delay entry into diapauses for another generation [1]. Further, diapause in insects is characterized by low metabolic rate and the induction, initiation, determination, maintenance and termination of diapause under the control of endocrinological mechanisms mediated by environmental stimuli [2]. Special attention has been paid to egg diapause of the silkworm, *Bombyx mori* (L.) and as a result, considerable information is generated on various aspects of diapause mechanism. Various physiological, biochemicals, environmental and metabolic changes are associated with the induction, initiation, maintenance, determination and termination of diapause in the silkworm, *Bombyx mori* [3-7]. The nature of diapause primarily determined by genetic characters and endocrinological mechanisms, the manifestation of which is modified by environmental factors such as temperature and photoperiod [8] and is almost maternal. Hibernating Potency Value (HV) besides nucleotide and carbohydrate metabolism is also equally responsible for induction, initiation, maintenance and termination of

diapause [9]. Since mulberry silkworm enter diapause as embryo, the duration of egg life depends on the duration of embryonic diapause and represents a syndrome of physiological and metabolic events. Metabolic conversion of trehalose to glycogen at induction, glycogen to sorbitol at initiation and sorbitol to glycogen at termination of diapause is correlated and in each metabolic shift, a key enzyme becomes active in response to hormonal and environmental stimulation.

In this article an attempt has been made to discuss briefly the advances achieved in relation to genetical and hormonal studies on diapause nature and biochemical studies associated with induction, initiation, determination, maintenance and termination of egg diapause along with nucleotide and carbohydrate metabolism and its significance in mulberry silkworm, *Bombyx mori* (L.).

I. EGG DIAPAUSE

Colouration or pigmentation of insects is mainly due to melanin, pterine, pigments, ommochrome and carotenoid. In some insects, colouration is closely correlated with diapause and colour pattern is sometimes used as an index to distinguish diapause from non-diapause. Among these pigments, ommochrome is well documented in relation to embryonic diapause [10]. Entry into diapauses is widespread among insects and can occur at any stage of the life cycle, although the specific stage at which diapauses is initiated are characteristically fixed in each species viz., egg *(Bombyx mori, Melanoplus differntialis, Aedes aegypti),* larva *(Cydia pomonella, Gilpinia polytoma, Cephus cinctus, Lucilia caesar),* pupa *(Antheraea mylitta, Hyalophora cecropia, Antheraea mylitta, Philosamia cynthia, Saturnia pavonia, Mimas tiliae)* or adult *(Leptinotarsa decemlineata* [11].

a) Ommochrome biosynthesis

The insects having only one generation in a year are known as univoltine and in such insects embryonic diapause occurs in all generations regardless of environmental conditions, is known as 'obligatory diapause', while in those having two or more generations in a year (bivoltine or multivoltine), diapause is expressed in the subsequent generation when the female receives specific stimuli from environment is termed 'facultative diapause'. The univoltine forms lay only hibernating (diapausing) eggs, which are also called 'Kurodane eggs', and multivoltine forms lay only non-hibernating eggs known as 'Nemadane eggs', while the behavior of bivoltine are intermediate [7, 12]. Bivoltine silkworm breeds lay non-hibernating eggs during first generation and hibernating in the next generation, which hatches out in the following spring and thus producing only two generations in a year. The silkworm eggs of diapause type are pigmented (dark brown) due to presence of ommochrome formed in serosa cells, while non-diapause eggs are light yellow / white due to lack of this pigment [13]. The ommochrome pigment is formed from tryptophane metabolism which in insects has been studied extensively and the pathway from tryptophane to ommochrome has been reviewed in detail [14]. In general, three major metabolites directly involved in the biosynthesis of the ommochrome pigments are formyl-kynurenine, kynurenine and 3-hydroxy kynurenine. The corresponding enzymes responsible for their production are kynurenine-formamidase, kynureninase and kynurenine-3-hydroxylase. It was presumed that kynurenine-3-hydroxylase is a precursor of serosa pigment but not transmitted directly from haemolymph to ovum and the diapause hormone accelerates its generation or synthesis in the pupal body. The egg in which, the enzymes for tryptophane metabolism is not formed remains yellow or white in colour.

b) Genetical studies on diapause

The only insect embryo in which gene expression during diapauses has been studied extensively is the silkworm, *B. mori* [15]. Sericulture scientists in general assumed a series of three sex linked alleles, Hs, Hs2 and hs and three pairs of autosomal genes H1h1, H2h2 and H3h3 responsible for the hibernation and reported different kinds of voltinism in relation to 'Hibernating potency value' (H.V.). They further stated that the eggs with 0 - 1 HV value always lay non-hibernating eggs (multivoltine), while those with 11 - 12 will lay only hibernating eggs (univoltine). Bivoltine silkworm breeds have HV value in between 5 - 7. The silkworm eggs with HV value of 2 - 4 are intermediate between multivoltine and bivoltine and lays some non-hibernating eggs at high incubation temperature. Contrary to this, the silkworm eggs with HV value of 8 - 10 are intermediate between bivoltine and univoltine and lay both hibernating and non-hibernating eggs at low temperature incubation. A hypothetical genetic balance theory between major genes and constant gene of Lm to Lm^e (early maturity) as the basis of voltinism phenomenon is also proposed [16]. Genetical experiments have shown the major factor controlling diapause belongs to two loci i.e. *Lm* and *V*, and these loci as well as other loci control process other than synthesis of DH. Considering the various investigations on the genetics of voltinism, carried out by many workers, it is concluded that voltinism is maternally inherited phenomenon controlled by sex-linked genes regardless of artificial treatment of eggs [17].

c) Hormonal studies on diapause

The facultative diapause in insects is induced by environmental stimuli mediated by endocrine system [18]. In bivoltine breeds of silkworm, these stimuli (temperature and photoperiod) determine whether embryonic development in next generation is arrested or continued without interruption. Of the endocrine system, sub-esophageal ganglion (SG) has been understood to play very vital role in the induction of diapause. It is generally accepted that egg diapause in silkworm is induced by an active principle secreted from a pair of large neurosecretory cells located in SG under the control of four other neurosecretory cells. This active principle is known as 'Diapause Hormone' (DH) [10]. Two forms of DH (DH-A and DH-B) have been identified which are neuropeptides having molecular weight of 2,000 - 3,000 and consist of 12 - 14 amino acids responsible for induction of embryonic diapause in the silkworm [10, 19]. The DH-A molecules contain 14 amino acids (lysine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine) and two amino sugars (glucosamine and galactosamine), while DH-B contains the same amino acids but no amino sugars [10]. This reveals that amino sugar component of DH-A is apparently not essential for hormonal activity.

There are several differences between diapause and non-diapause eggs after oviposition. Diapause eggs showed high accumulation of 3-hydroxykynurenin, glycogen and ecdysteroids [20] with reduced accumulation of cyclic guanosine monophosphate (GMP). Among these, 3-hydroxykynurenine and glycogen exhibit dramatic changes on the commencement of diapause, where 3-hydroxykynurenine from the haemolymph in the developing eggs is oxidized to ommochrome, resulting in the dark coloration of the diapause destined eggs [21] and glycogen is mainly converted into sorbitol, acts as an anti-freeze for the diapause embryo. However, it is reported that there is no correlation between diapause and pigmentation [10] but are controlled by two different genes [13]. DH acts directly to induce expression of trehalase gene in the developing ovary, and enhances trehalase activity localized in plasma membrane of the vitellogenic follicles [22-23]. In the oocyte, glucose is immediately utilized to synthesize glycogen as a storage reserve, by which hyperglycogemia is induced in eggs, a pre-requisite for diapause initiation [10].

DH also exerts an inhibitory effect on 'esterase 'A', an enzyme essential for mobilizing the yolk needed for completion of embryogenesis [24-25]. Molecular analysis of DH action has demonstrated that DH directly induces trehalase gene expression in developing embryos, which eventually brings about hyperglycogenism in mature eggs, leading to sorbitol production at the onset of diapause [23]. DH function is conceived to be the initial and essential reaction leading to the diapause-associated metabolism in silkworm eggs [19]. The rate limiting enzymes in each pathway have been identified - trehalase in glycogen synthesis in the ovary at the induction of diapause [26]; glycogen phosphorylase in sorbitol synthesis at the initiation of diapause [3] and NAD-dependent sorbitol dehydrogenase at the termination of diapause [27]. It is also reported [28] that there is no correlation between glycogen content and diapause incidence, suggesting that diapause occurs associated with metabolism of well known glycogen-sorbitol pathway.

Table 1. Effects of temperature and photoperiod on egg diapause in bivoltine breeds of *Bombyx mori*

Temperature and photoperiod during incubation		Temperature ^{0}C Developmental stage		Nature of eggs laid by resulting silk moths
Temperature	Photoperiod	I – II	IV – pupal	
25^{0}C	Light	25 or 20	25	Diapause
	Dark	25 or 20	25	Diapause
20^{0}C	Light	25 or 20	25	Diapause
	Dark	20	25	Diapause << Non-diapause
		25	25	Diapause < Non-diapause
		25	20	Diapause >> Non-diapause
15^{0}C	Light	20	25	Diapause < Non-diapause
		25	25	Diapause > Non-diapause
		25	20	Diapause >> Non-diapause
	Dark	25 or 20	25 or 20	Non-diapause

Note: Light -16 hrs light and 8 hrs dark; Dark -16 hrs dark and 8 hrs light

d) Diapause induction

The diapause determination is almost maternal in mulberry silkworm *(Bombyx mori)*, i.e. temperature and photoperiod are most efficient at the embryonic stage of previous generation (Table 1) and only supplemental in the post-embryonic stages. The sensitive embryonic stages begin just after blastokinesis. Incubation of bivoltine eggs at high temperature results in induction of diapause in the next generation and low temperature incubation results in the production of non-diapause eggs [29]. Incubation as low as 15^{0}C causes production of non-diapause eggs in the next generation, whereas, diapause eggs are induced by incubation at 25^{0}C. When eggs are incubated at intermediate temperature of 20^{0}C, the developmental fate remains undetermined in the embryos. High

temperature at younger larval stages and low temperature at late larval stages acts to induce diapause eggs. Egg diapause is regulated by photoperiod as well as temperature during embryonic stage of the female and is completely independent of photoperiod during post-embryonic development. Thus, photoperiod becomes effective in regulation to development only when eggs are incubated at an intermediate temperature. In these eggs, long photoperiod causes induction of diapause and short photoperiod non-arrested state of development.

In silkworm, injection of Uranyl nitrate, quabain [30] and 5'-AMP [31] into females destined to lay diapause eggs caused them to lay non-diapausing eggs. Converse was demonstrated by injection of KCI and quabain into pupae of non-diapause type [32]. However, despite extensive studies, the mechanism of action of these chemicals remains unknown. Diapause is classified into three groups based on the requirement of water for embryonic development viz., sufficient water is stored in the egg at the time of oviposition, so that embryogenesis is completed till hatching as in *Bombyx mori*; water is absorbed into egg before diapause establishment and utilized for post-diapause development as in crickets; and water is absorbed mainly after diapause termination for the completion of embryogenesis as in grasshoppers [33]. In silkworm, *Bombyx mori*, hydrocarbons comprise the major lipid of the egg shell and are believed to take part in water evaporation. Insect diapause results due to the exposure of sensitive stages to distinct stimuli. This stage is usually fixed in the life history of insect and is characteristics of each species.

e) Diapause initiation

In diapause eggs, the oxygen uptake is reported to be 30 µl / g of eggs / hr. Within 4 hrs of oviposition reached to 100 µl / g of eggs / hr to 24 hrs and thereafter declined rapidly reaching to 10 µl / g of eggs / hr on 10^{th} day and 8 µl / g of eggs / hr on 70^{th} day. The decrease in oxygen uptake by day 10 at 25^0C indicates the establishment of a stable physiological status of diapause. However, after chilling of eggs for 70 days and releasing at 25^0C, increases in oxygen uptake. But when eggs are chilled at 48 hrs after oviposition and released to 25^0C, 18 µl / g of eggs / hr is reported until chilling for 50 days. This clearly indicates that decrease in oxygen uptake proceeds even at 5^0C and physiological status of eggs exposed to 5^0C after incubation at 25^0C for 2 days after oviposition is different than in eggs exposed to 5^0C for 15 days of oviposition. The ability to break diapause is correlated with the increased rate of oxygen uptake and it is stated that when eggs showed oxygen uptake of 70 µl / g of eggs / hr are incubated at 25^0C, more than 80% of larvae hatched. Oxygen uptake will be about 70 µl / g of eggs / hr in acid treated eggs (after one hour) chilled for 30 days at 48 hrs of age of eggs. This value is almost three folds higher found in the eggs before acid treatment (20 µl / g of eggs / hr). At 48 hr of age, diapause is initiated but an intense status of diapause is not established. Initiation of diapause occurs around one to two days after oviposition and a complete physiological status of diapause is established after incubation at 25^0C for 10 days after oviposition when the oxygen consumption is at decreasing trend. Also, sorbitol accumulates two days after oviposition and mitotic figures become undetectable in embryonic cells after three to four days of oviposition and this stage is termed as 'pre-diapause'. The oxygen uptake increases to a high level on prolonged chilling which coincides with complete recovery of glycogen from sorbitol and the stage of decreasing glycerol [5, 34].

f) Diapause termination

Exposure of silkworm diapause eggs to oxygen is one of the artificial methods of termination of diapause. The exposure of diapause-destined eggs to oxygen gas can prevent the expression of diapause [35] and eggs resumed embryonic development while under an oxygen-deficient environment non-diapause eggs ceased their development. However, mechanism involved in terminating the egg diapause by exposure to oxygen is still not known. Diapause in silkworm eggs can be terminated to obtain effective hatchability by means of –

I. Cold storage - chilling (hibernation schedule)
II. Hydrochlorization (hot or cold acid treatment)
III. Cold storage and hydrochlorization (chilling and acid treatment)

The application of these methods depends on the programme of hatching desired. Chilling is one of the effective methods of terminating, diapause. Exposure of diapausing silkworm eggs to a temperature as low as 5^0C over 60 days completely terminates diapause and embryogenesis resumes when these eggs are transferred at 25^0C. Optimum temperature to break the embryonic diapause is in between 5^0C - 7.5^0C. The required chilling duration to break the diapause depends on the time gap the eggs have been kept for aestivation at 25^0C after oviposition. Various comprehensive hibernation schedules for preservation of bivoltine eggs for different durations to get the desired hatching at appropriate time have already been recommended and some of them are in-vogue [36-39] to meet the demand of seed supply throughout the year.

HCI treatment (hydrochlorization) of silkworm diapause eggs has been the method of choice for blocking the diapause both at commercial and basic research laboratory level. There are two methods of HCI treatment. In the first method 20 - 24 hrs old oviposited eggs are soaked in HCI solution (specific gravity: 1.075 at 15^0C) at 46.1^0C for 5 minutes. This avoids the eggs to enter into diapause and hence when incubated at 25^0C, larvae hatches in 10 -11

days after treatment. In the second method 20 - 24 hrs old oviposited eggs are soaked in HCI solution (specific gravity 1.10 at 10^0C) at room temperature for 60 - 90 minutes. Sometimes, to block the diapause for longer period in order to get hatching at desired time, chilling followed by acid treatment is also used. In this method, 48 hrs old eggs are first chilled at 5^0C for more than 30 days and then soaked in HCI solution (specific gravity 1.10 at 15^0C) at 48^0C for 5 minutes. This treatment causes diapause eggs to hatch within two months after oviposition. However, it is still an open question how hydrochlorization blocks diapause. HCI treatment is reported to stimulate the activity of specific esterase isozyme [40-41] and RNA synthetic activity [42] in treated diapause eggs. Activity of 'esterase A' increases dramatically within 30 minutes of HCI treatment. The esterase attains maximum activity before the eggs are competent to develop and it is suggested [43] suggested that activation of 'esterase A' is pre-requisite for resumption of development.

Different low temperatures specifically affect carbohydrate metabolism and process of diapause termination. Effect of low temperature on glycogen, sorbitol and glycerol contents at the initiation of diapause can be observed when eggs are preserved at 5^0C - 10^0C after 3 days of oviposition. The conversion of glycogen to sorbitol and glycerol during initiation phase of diapause is not dependent upon ambient temperature [44-45]. After the initiation of diapause stage and exposure of eggs to 5^0C for 200 days for diapause termination, it is reported that sorbitol decreased from 100^{th} day, however, glycerol increased till 120^{th} day and then decreased. Trehalase and glycogen phosphorylase functions as regulators of carbohydrate metabolism, the first being specific to induction and later to initiation of diapause. NAD-dependent sorbitol dehydrogenase is responsible for termination of diapause) [27] in silkworm eggs.

II. METABOLIC CHANGES DURING EMBRYONIC DEVELOPMENT

Since the first detailed study on the development of silkworm *(Bombyx mori)* eggs, embryological studies were confined especially in solving the practical problems such as identification of suitable stages for refrigeration of early embryos and the induction, initiation, continuation and termination of diapause in order to develop an effective system for long-term cold storage of silkworm eggs. In these studies, morphological changes of embryos were used to assess their long-term survival. Moreover, the tolerance of eggs to cold storage varies with the stage of embryonic development besides genotypes because of adaptation phenomenon, metabolic changes and also genetic variations etc [46] and hence relatively recent studies on silkworm eggs becomes more concern with physiology, biochemistry and metabolic activity associated with termination of embryonic diapause [5-6, 47] for effective handling.

a) Biochemical composition of silkworm egg

The biochemical composition of silkworm egg varies depending upon variety, season and environmental conditions [47]. Freshly laid eggs of silkworm are composed of protein (~ 10%), lipids (~ 8.5%), glycogen (~ 2.5%) and chorion (~ 18%) and water (~ 60%) [48]. Of the total protein, more than 95% consists of yolk protein: vitellin (~ 40%), 30 kD protein (~ 35%) and egg-specific protein (ESP) (~ 20%). These proteins are quite different from each other in their physiochemical and biological properties [49]. Each protein exhibits a unique profile of degradation during embryonic development, viz, -

- Egg-specific protein is utilized during early embryonic development and completely disappeared by the time of hatching.
- Vitellin begins to decrease at later stage of embryogenesis development and about 40% of the initial amount remains unutilized even in hatched larvae [4]. Thus, vitellin metabolism appears to be independent of the diapause phenomenon.
- 30 kD proteins are less utilized during embryonic development [49].

ESP is degraded during embryonic development by hydrolysis process and catalyzed by trypsin like seryl protease and alters the carboxyl site of Lys^{114} and $Argenine^{210}$ of ESP. The developmental increase in activity is due to increase at transcription of mRNA for this enzyme protein. Therefore, the utilization of ESP is a programmed event correlated with embryogenesis.

Lipid (~ 8.5%), the second major component of egg is composed of triglycerols (~ 80%) and phospholipids (~ 20%). Other constituents such as free fatty acids, mono and triglycerides are usually present in small quantities. Most of the metabolic energy (approximately 70% of the total energy) utilized during embryonic development is derived from the oxidation of triglycerol. The oxidation of lipids is advantageous for embryogenesis of terrestrial cleidoic eggs because large amount of metabolic water (1.07 g/g lipids) is released. Phospholipids are distributed as major component of yolk protein and used for formation of embryonic cells. Lipid is the major component consumed during diapause and the lipid concentration is higher in diapausing silkworm eggs than in non-diapausing eggs [50]. Approximately, $2/3^{rd}$ of the total CO_2 exhaled by the eggs is by degradation of

tricyglecerides [51]. Diapausing ovaries accumulate high level of glycerol and sorbitol indicates that diapause is achieved by unique metabolic route that is not needed for non-diapausing eggs [10].

Glycogen in silkworm eggs undergoes specific changes during diapause. In the diapause eggs, the following reversible reaction occurs with the initiation and termination of diapause –

Glycogen ⟷ Sorbitol + Glycerol

With the initiation of diapause, the above reaction is right oriented while during termination; it is left oriented [52]. The experiment with 14^C glycine showed that sorbitol is totally derived from glycogen, while glycerin is produced only when glycogen content reached the lowest level. About the mechanism of reversible reaction, following steps exists –

Glucose + NADPH ⟷ Sorbitol + $NADP^+$
Glucose-6-p / fructose-6-p + NADPH ⟷ Sorbitol-6-p + $NADP^+$
Glyceraldehydes + NADPH ⟷ Glycerin + $NADP^+$
2 - OH acetone-p + NADH ⟷ 2-p glycerin + NAD^+

b) Nucleotide metabolism

In silkworm eggs, diapause is decided during the maturation process of the eggs in the ovary of pupal body. Therefore, there is a close relationship between diapause occurrence and metabolism of egg cells. In insects, nucleic acid is not only related to the expression of genes but also influence protein synthesis, cell division, growth and development. In univoltine genotypes, sub-oesophageal ganglion if removed at early pupal stage, the female will lay non-diapausing eggs, while normal female laid diapause eggs. If the mature eggs inside the ovariole of above two groups taken out, it is found that DNA content of diapause eggs is 25.29% lower than that of non-diapause eggs and RNA content of diapause mature eggs is 25.48% less but the DNA / RNA ratio of these two groups were the same (Table 2,3). Hence, it is inferred that DNA content of mitochondria of diapause eggs is probably lower than non-diapause eggs.

Table 2. Relationship between diapause and metabolism in mature eggs of the silkworm, *Bombyx mori* [53]

Characters	Nature of eggs	
	Non-diapause	Diapause
DNA (µg / mg)	0.87 (100)	0.65 (74.71)
RNA (µg / mg)	18.84 (100)	14.04 (74.52)
Glycogen (%)	1.60 (100)	1.87 (116.88)
Lipids (%)	26.5 (100)	27.5 (103.77)
Oxygen consumption (mm^3 / g / h)	26.82 (100)	13.64 (50.19)

Table 3. Nucleic acid metabolism during early embryonic development of diapauses and non-diapause eggs in *Bombyx mori* [53]

Embryo development (hrs)	*24	*48	**72	**120
DNA (µg / mg)				
Non-diapause	1.37	1.67	1.86	12.25
Diapause	1.00	1.49	1.54	1.58
RNA (µg / mg)				
Non-diapause	15.84	15.98	15.54	17.76
Diapause	14.27	14.33	14.00	13.58
DNA / RNA				
Non-diapause	0.086	0.104	0.120	0.127
Diapause	0.071	0.103	0.110	0.117

**Pre-diapaused period; ** Diapaused period*

RNA plays major role in protein metabolism and embryo morphogenesis. It is reported that mRNA carrying out the early morphogenesis information is synthesized during the egg formation process and is deposited in the cytoplasm of egg before fertilization. The entrance of sperm activates the egg and the mRNA in latent condition is activated first. During the pre-diapause stage, the DNA content in both non-diapause and diapause eggs are used very rapidly. But 48 hrs after oviposition, the DNA metabolism of these two types of eggs are quite different (Table 3). The DNA content of diapause eggs keeps constant after 48 hrs of oviposition is perhaps the biochemical pre-condition for stoppage of embryo morphogenesis and initiation of embryonic diapause. On contrary, the DNA content of non-diapause eggs continues to increase rapidly. In non-diapause eggs within 24 hrs of oviposition, with the increase of DNA content, RNA content decreases. After 72 hrs of oviposition large amount of RNA is synthesized and accumulated in the non-diapausing eggs. This is closely related to the synthesis of protein during embryonic development. On contrary, in diapause eggs within

three days of oviposition, the RNA content keeps constant though large quantity of DNA is synthesized and accumulated. Within 24 hrs of oviposition the RNA / DNA ratio in both the types of eggs (diapause and non-diapause) increases rapidly but after 48 hrs they reach the same level. Later the DNA / RNA ratio of non-diapause eggs increased rapidly.

Di and tri phosphates of adenine, guanine, inosine and uredine in the brain under specific environmental condition are related to the acceptance of environmental stimuli during embryogenesis and serves as diapause induction [54]. This nucleotide content is high in the brain of pupae when the eggs are exposed to high temperature (27^0C) and continuous light.

c) Carbohydrate metabolism

Carbohydrate metabolism is the main pathway of biochemical regulation of diapause in the silkworm. It is proved that induction; initiation, maintenance and termination of diapause are related to carbohydrate metabolism. Amount of glycogen accumulated in diapause eggs is 1.7 times higher than in non-diapause eggs [45, 55]. More than 90% of carbohydrate accumulated in the silkworm diapause eggs is glycogen. The glycogen initially present in the diapaused eggs rapidly broken down into sorbitol and glycerol at the onset of diapause. Glycogen accumulated in the silkworm egg is from the glycogen stored in the fat body during pupal stage, which is converted into trehalose and is released into haemolymph and then absorbed by developing oocyte. The trehalase localizes in plasma membrane of vitellogenic follicles where haemolymph trehalose hydrolyses into glucose to be taken up by oocytes. The glucose is immediately used to synthesize glycogen as a storage reserve, by which hyperglycogenia is induced in diapause eggs. Consequently, DH provides the metabolic state leading to diapause-associated carbohydrate metabolism in silkworm eggs [19]. It has been demonstrated that enzyme which is involved in trehalose synthesis include trehalase, hexokinase, phosphoglucomutase, UDP-glucose-pyrophosphatase and glycogen synthetase. Of this trehalase is a membrane bound restriction enzyme existing on the surface of oocytes and follicle cells. The amount of trehalose in the pupal body fluid entering the ovary and the amount of glycogen synthesized in the egg is related to the trehalase activity. Thus, the increase of glycogen content in diapauses eggs depends on the increase of trehalase activity. At the initiation of diapause, the stored glycogen is converted into sorbitol by the activation of phosphorylase 'b' to 'a' under anaerobic condition (Yamashita *et al*., 1975) [3]and thus during the whole diapause stage, the glycogen content in the silkworm eggs keeps at a very low level but the sorbitol content keeps at a very high level. The pathway of glycogen to sorbitol is coupled to pentose phosphate pathway [56] through the supply of reducing power (NADPH) [57]. In these eggs, activity of glucose-6-phoaphate (G-6-P) dehydrogenase is about twice higher than that of phosphofructokinase and G-6-P amounts equivalent to about 10% of the accumulated sorbitol are oxidized through pentose-phosphate pathway. The other fraction of G-6-P is eventually metabolized to glycerol [5]. After the termination of diapause, sorbitol is again converted into glucose and the key enzyme involved in the activity is NAD-sorbitol dehydrogenase, which plays significant regulatory role in the metabolic cycle. Termination of diapause is expressed in terms of hatchability and when eggs are fully activated; their embryogenesis is completed in 14 days [50].

Accumulation of huge quantity of sorbitol in the diapausing eggs is generally regarded as a protecting agent of eggs from cold injury in winter. One of the remarkable difference between diapause and non-diapause eggs within few days of oviposition is that glycogen rapidly disappears in the diapausing eggs but remains at its initial level in the non-diapause eggs. In the metabolic pathway from sorbitol to glycogen, 'Nicotinamide Adenine Dinucleotide Sorbitol Dehydrogenase' (NAD-SDH) catalyses the reaction from sorbitol to fructose [27, 58]. Incubation of diapause eggs at 25^0C does not induce the activity of NAD-SDH until 90 days [6]. Moreover, the activity appears at very low level between 90 -160 days. Similarly, chilling at 5^0C for less than 30 days does not induce activity but 40 days period of chilling effectively stimulates activity. In contrast, 0.5^0C never induces activity even with an exposure exceeding 250 days. Utilization of sorbitol in diapause eggs thus appears to be controlled by NAD-SDH activity through mediation of temperature [47]. In non-diapause eggs, the sorbitol content increased on 2^{nd} day of oviposition and then decreased rapidly [59].

d) Sorbitol production and utilization

There is a significant biochemical difference at the time of oviposition between eggs destined to become diapause and non-diapause. The eggs developed in the presence of diapause hormone accumulate large quantity of glycogen [45]. When oogenesis proceeded in the absence of diapause hormone, the laid eggs becomes non-diapause with less accumulation of glycogen and during embryogenesis, conversion of glycogen to sorbitol does not occur. Sorbitol is an ideal metabolite to assess the diapausing state of silkworm eggs [60]. In diapause eggs, the initiation of sorbitol synthesis is correlated with the time when embryogenesis progressively slowed down. Conversion of glycogen to sorbitol at this stage is monitored by the enzyme 'glycogen phosphorylase-a' activity. Conversion of glycogen phosphorylase-'b' to 'a' is being reported absent in non-diapause eggs under normal conditions [3].

Glycogen phosphorylase-b kinase responsible for conversion of 'b' to 'a' form is identified in silkworm eggs and is reported to have some similarities in kinetic properties to that in other insect tissues [61]. There is no difference in their activity between diapause and non-diapause eggs. When newly laid non-diapause eggs are exposed to low temperatures, sorbitol is accumulated and the extent of accumulation is closely dependent on the temperatures of exposure. In this case, glycogen is also used as an initial substrate for the formation of sorbitol. Thus, in non-diapause eggs, sorbitol production seems to be a biochemical adaptation against low temperature stress [62].

In diapause eggs, sorbitol began to decrease after continuous chilling for at least two months or by treatment with HCI on one month chilled eggs. The more advanced fall in sorbitol took place in eggs chilled for longer period. In all cases, sorbitol is stoichimometrically reconverted to glycogen at the time of termination of diapause [60]. Thus, the conversion of sorbitol to glycogen is specific sign signally the breakdown of diapause in silkworm eggs. In the metabolic pathway from sorbitol to glycogen, NAD-sorbitol dehydrogenase, which catalyses the reaction from sorbitol to fructose is noticed to be the key enzyme. This activity is not being detected in eggs un-chilled or chilled for less than two months and abruptly appears in eggs chilled for more than two months. The duration of diapause may be determined by analyzing the amount of oxygen required to prevent the decrease of sorbitol content in the eggs [63], which indicates that conversion of sorbitol to glycogen upon termination of diapause is closely related to the respiratory system via - GP cycle. Diapause termination is also closely related to the oxygen consumption during preservation of eggs [63] and that the activity of NAD-SDH enzyme is associated with the oxygen required for the termination of diapause. It is established that supply of oxygen to diapause eggs affect the maintenance of diapause period and when eggs are kept under anaerobic condition, the conversion of sorbitol to glycogen is delayed leading to delayed diapause termination.

Diapausing silkworm eggs chilled at 5^0C for at least three months when transferred to 25^0C, the eggs resumed embryogenesis and hatches within two weeks [60]. Soaking the pre-chilled diapausing eggs into hot solution can also bring about hatching [64]. In these eggs, sorbitol content began to decrease after continuous chilling for at least two months or by treatment with HCI on one month chilled eggs. Greater fall in sorbitol content took place in eggs chilled for longer periods. Conversion of sorbitol to glycogen does not take place if eggs are kept continuously at 25^0C [60] even for more than six months. After diapause, all sorbitol in the eggs is converted into glycogen. During embryogenesis, the main carbohydrate consumed is glycogen. The glycogen-sorbitol-glycogen metabolic process is roughly the same as the process of diapause onset, maintenance and termination. In other words, the change of carbohydrate metabolism of the silkworm eggs is a close relative to the phenomenon of diapause.

e) Changes in amino acid

Significant changes in some free amino acids occurred during the initiation and termination of diapause. In particular, a sudden large increase in alanine content (about 50µ mol / g eggs) occurred at the initiation of diapause [65-66]. Afterwards alanine declines gradually with the increase of glutamate and especially proline. Proline content is low during the initiation and maintenance of diapause but increased suddenly during the termination period indicating the conversion of alanine to glutamate and proline in diapausing eggs. Proline accumulated may be utilized during embryogenesis. In diapausing insects, high concentration of free amino acids as well as of polyols and sugars serves to decrease the super cooling point [67]. The super cooling point of *Bombyx mori eggs* is lower during diapause and hibernation [68] may be due to increase of total amino acids and the accumulation of alanine or proline. Proline serves as an energy source for later stages of embryonic life [65]. On shifting the diapause eggs on 10^{th} day after oviposition from 25^0C to 4^0C, the high alanine level in the diapausing eggs decreased, whereas, the levels of praline, glutamate and glutamine increased until day 70^{th}, suggesting metabolic conversion of alanine to praline. In diapausing eggs of silkworm, pyruvate produced from glycogen by the glycolytic pathway appears to be converted first to alanine and then to praline via glutamate to maintain energy sources during diapause for resumption of embryogenesis. Therefore, the diapause eggs can survive for one year or more if necessary, despite their small reserves [65].

REFERENCES

[1] DL Denlinger, *Annu Rev Entomol*, **2002**, 31, 239-264.
[2] V Kostal, J *Insect Physiol*, **2006**, 52, 113-127.
[3] O Yamashita, K Suzuki and K Hasegawa, *Insect Biochem*, **1975**, 5, 707-718.
[4] K Irie and O Yamashita, *J Insect Physiol*, **1980**, 26, 811-817.
[5] T Yaginuma, M Kobayashi and O Yamashita, *J Comp Physiol*, **1990a,** 8, 160-177.
[6] T Yaginuma, M Kobayashi and O Yamashita, *Comp Biochem Physiol*, **1990b**, 97 B, 495-506.

[7] T Singh and B Saratchandra, *Principles and Techniques of Silkworm Seed Production*, Discovery Publishing House, New Delhi, **2004,** 1-361.
[8] KP Jayaswal, *Bull Ind Acad Sci*, **2002**, 6, 1-20.
[9] T Singh, MM Bhat and MA Khan, S*ilkworm Egg Science – Principles and Protocols*, Daya Publishing House, New Delhi, **2010**, 1-278.
[10] O Yamashita and K Hasegawa, In: GA Kerkut and LI Gilbert LI (Ed.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Pergamon Press, Oxford, **1985**, 1, 407-434.
[11] T Singh, *Indian Silk*, **1989**, 28, 2-3.
[12] Y Tazima, *Silkworm Egg*, Central Silk Board, Bangalore, **1978**, 1-40
[13] B Jaike, *Canye Kexue*, **1989**, 15, 184-188.
[14] B Linzen, *Adv Insect Physiol*, **1974**, 10, 117-246.
[15] SM Hong, SK Nho, NS Kim, JS Lee and SW Kong, *Zool Sci*, **2006**, 23, 517-528.
[16] S Morohoshi, *Growth Physiology in Silkworm*, Tokyo University Press, Tokyo, **1976.**
[17] G Subramanya and A Murakami, *Indian J Seric*, **1994**, 33, 103-109.
[18] RF Chapman, *The Insects: Structure and Function* (4th Ed.), Cambridge University Press, **1998**, 1- 403.
[19] O Yamashita, *J Insect Physiol*, **1996**, 42, 669-679.
[20] M Coulon, *Comp Biochem Physiol*, **1988**, 89, 503-509.
[21] DL Denlinger, In: GA Kerkut and LI Gilbert (Ed.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Pergamon Press, Oxford, **1985**, 8, 353-421.
[22] M Ikeda, Z Su, H Saito, K Imai, Y Sato, M Isobe and O Yamashita, *J Insect Physiol*, **1993**, 39, 889-895.
[23] ZH Su, M Ikeda, Y Sato, H Saito, K Imai, M Isobe and O Yamashita, *Biochem Biophys Acta*, **1974**, 1218, 366-374.
[24] H Kai and Y Haga, *J Seric Sci Japan*, **1978**, 47, 125-133.
[25] X Shiqing, T Guoqin, K Hidennori, X Junliang, Z Biping, C Xilin and S Yanghu, *Canye Kexue*, **2000**, 26, 140-145.
[26] O Yamashita, K Hasegawa and M Seki, *Comp Endocr*, **1972**, 18, 515-523.
[27] T Yaginuma and O Yamashita, *Insect Biochem*, **1979**, 9, 547-553.
[28] N Katagiri, O Ando and O Yamashita, *J Insect Physiol*, **1998**, 44, 1205-1212.
[29] T Yokoyama, In: RE Smith, IE Miller and CN Smith (Ed.), *History of Entomology*, **1973**, 267-284.
[30] S Takeda and K Hasegawa, *J Insect Physiol*, **1975**, 21, 1995-2003.
[31] S Takeda and K Hasegawa, *J Seric Sci Japan*, **1976**, 45, 337-344.
[32] K Suzuki, S Tsuda and K Miya, *Appl Ent Zool*, **1978**, 13, 48-50.
[33] Y Ando, *Appl Ent Zool*, **1974**, 9, 261-270.
[34] T Yaginuma and O Yamashita, *J Seric Sci Japan*, **1977**. 46, 5-10.
[35] H Sonobe, A Matsumoto, Y Fukuzaki and S Fujiwara, *J Insect Physiol*, **1979**, 25, 381-388.
[36] SB Dandin, J Jayaswal and K Giridhar, *Handbook of Sericulture Techniques*, Central Silk Board, Bangalore, **2000**, 1-198.
[37] A Manjula and HK Hurkadli, *Indian Text J*, **1995**, 106, 70-74.
[38] A Manjula and HK Hurkadli, *Sericologia*, **1996,** 36, 665-675.
[39] GV Reddy and MV Samson, *Current Tech Semi on Silkworm Seed Tech*, Bangalore, **1995**, 71-80.
[40] H Kai and K Hasegawa, *J Insect Physiol*, **1973**, 19, 799-810.
[41] H Kai and K Nishi, *J Insect Physiol*, **1976**, 22, 1315-1320.
[42] S Kurata, K Koga and B Sakaguchi, *Insect Biochem*, **1979**, 9, 107-110.
[43] H Kai, T Kawai and Y Kawai, *Insect Biochem*, **1987**, 17, 367-372.
[44] T Furasawa, M Shikata and O Yamashita, *J Comp Physiol*, **1982,** 157, 21-26.
[45] O Yamashita, T Yaginuma and K Hasegawa, *Ent Gen*, **1981**, 7, 195-211.
[46] H Sonobe, K Maotaini and H Nakajima, *J Insect Physiol*, **1986**, 32, 215-220.
[47] O Yamashita and T Yaginuma, In: RE Lee and DL Denlinger (Ed.), *Insects at Low Temperature*, Chapman and Hall, New York, **1991**, 424-445.
[48] O Yamashita and K Irie, *Nature*, **1980**, 283, 385-386.
[49] J Zhu, LS Indrasith and O Yamashita, *Biochem Biophy Acta*, **1986**, 882, 427-436.
[50] SE Kim, M Shikata and H Akai, *J Seric Sci Japan*, **1981**, 50, 94-100.
[51] S Nakasone, J *Seric Sci Japan*, **1979**, 48, 526-532.
[52] Y Horie, T Kanada and Y Mochida, *J Insect Physiol*, **2000**, 46, 1009-1016.
[53] S Kurata, T Yaginuma, Y Kobayashi, K Koga and B Sakaguchi, *J Seric Sci Japan*, **1980,** 49, 107-110.
[54] C Wu, T Yamashita, H Taira and K Suzuki, *J Insect Physiol*, **1996**, 42, 441-447.
[55] T Furasawa, T Yaginuma and O Yamashita, *Zool Jb Physiol*, **1992**, 96, 169-178.
[56] T Kageyama, *Insect Biochem*, **1976**, 6, 507-511.
[57] N Horoki, H Takemori, Y Nonaka, H Sonobe and M Okamoto, *Eur J Biochem*, **2000**, 267, 6914-6920.
[58] T Yaginuma and O Yamashita, *Insect Biochem*, **1986**, 16, 677-685.

[59] T Furasawa and W Yang, *J Seric Sci Japan*, **1987**, 56, 143-149.
[60] O Yamashita, T Yaginuma, M Kobayashi and T Furasawa, In: F Sehnal, A Zabza and DL Denlinger (Ed.), *Endocrinological Frontiers of Physiological Insect Ecology*, Wroclaw Tech. Univ. Press, Wroclaw, **1988**, 263-275.
[61] R Ziegler, M Ashida, AM Fallen, LT Wimer, SS Wyatt and GR Wyatt, *J Comp Physiol*, **1979**, 149, 321-332.
[62] T Furasawa and M Shikata, *J Seric Sci Japan*, **1982**, 51, 77-83.
[63] T Furasawa, K Shimizu and T Yano, *J Seric Sci Japan*, **1987**, 56, 150-156.
[64] O Yamashita, *Adv Invert Rep*, **1984**, 3, 251-258.
[65] M Osanai and Y Yonezawa, *Insect Biochem*, **1986**, 16, 373-379.
[66] K Suzuki, M Hosaka and K Miya, *Insect Biochem*, **1984**, 14, 557-561.
[67] L Somme, *Comp Biochem Physiol*, **1982**, 73(A), 519-543.
[68] K Suzuki, M Fujita and K Miya, *J Seric Sci Japan*, **1983,** 52, 185-190.