STUDIES ON THE NEUROENDOCRINE CONTROL OF EGG HATCHING AND METAMORPHOSIS IN *Philosamia ricini* (LEPIDOPTERA: SATURNIDAE)

ABSTRACT

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Based on the two families of neuroendocrine hormones, the steroid molting hormones, ecdysones and the sesquiterpene juvenile hormones, we present certain observations in the control of egg hatching and metamorphosis in the Eri silkworm, *Philosamia ricini*. The role of adenosine-3', 5'-monophosphate (cAMP) as a second messenger was also investigated.

In the present study, the obliterate effect of JH-III application on the eggs of *Philosamia ricini* was observed. Younger eggs tend to be more vulnerable to JH treatment but the percentage of hatching was also drastically reduced in older eggs as well. JH treatment to eggs also had a long term effect; most larvae that manage to hatch were not able to develop normally and may die at any stage during their postembryonic life.

Contrary to eggs treated with Juvenile hormone III (JH-III), 20-hydroxyecdysone (20-HE) treated eggs enhances hatching considerably. Although, certain stages of young eggs are susceptible to 20-HE treatment, the older treated eggs tend to response favorably in term of hatchability. Those larvae that hatched as a result of 20-HE treatment developed normally in normal time and the hormone does not seem to affect its postembryonic life as observed in JH treatment. Thus 20-HE may be regarded as a possible promoter of hatching in insects in addition to its role as a molting hormone.

In our observation, cAMP more or less mimicked the action exhibited by 20-HE, since the unhatched eggs produced as well as the pattern of effectiveness throughout the stages by both the treatments are almost similar.

The egg of *Philosamia ricini* is oval or laterally flattened ellipsoid. The freshly laid eggs are candid white in colour while the chorion is colorless and semi-transparent. Four distinct regions, the posterior and anterior poles, lateral flat sides and ventral (dorsal) edges, are easily distinguishable. The surface structure of the chorion is covered with a network pattern of fairly uniform polygons, mostly hexagonal but occasionally pentagonal or heptagonal units. The polygonal shape was common to the whole surface region. There is variation in the unit area of the polygon according to their location on the eggs.

The boundaries between polygons are made of ridges which had distinct aeropyles. The aeropyle number corresponds to the polygonal structure so that there are six aeropyles in a hexagonal structure, five in pentagonal, seven in heptagonal and so on. The variability in the size of aeropyles during egg development may be correlated with the metabolic rate of the developing embryo and the modification due to environmental conditions.

The study in term of hatching of the larvae from the eggs revealed that they gnawed their way out through the chorion membrane in accord with the process adopted by larvae of all lepidopteran insects. For the process of hatching there is no rupture of egg membrane. Line of weaknesses on egg surfaces were not observed nor the presence of egg buster or specialize spine or cuticle. The overall structure of the egg during the course of development until hatching was not observe to change except for the minute pores or aeropyles present on the ridges of the entire polygonal network.

In the present study, ligation during feeding stage (day 3) does not bring about pupal transformation other than the prodromal signs of pupation *viz.* gut purge, wandering, spinning, color change and lost of mobility in anal prologs, during their period of survival. The neck ligated larvae, showed normal developing pupae, while in the abdominal ligated larvae, the anterior part of the body bear a typical pupal character and the lower posterior region still retained the larval character of the last day 5th instar. These larvae continue their developmental processes by undergoing spinning even when the ligated thread is still intact. The pupal cuticle bearing regions are interpreted as having received enough molting hormone, ecdysone while the posterior region in the later that still bear the larval cuticle are devoid of the molting hormone due to ligation. However, complete pupal cuticle formation occurred only when larvae were ligated long after gut purge (day 3 after gut purge). Thus, first critical secretion of prothoracicotropic hormone (PTTH) & ecdysone can be assumed to occur at day 3, 5:00 hr (during feeding) onwards and this is necessary for causing prodromal signs of pupation and for transition of feeding to post-feeding stage. While the second secretions that caused the molt from larva to pupal form was probably released three days after gut purge from 4:00 hr onwards.

Further, the phenomenon underlying gut purge and pupal ecdysis seemed to be under the control of circadian clock since, larvae that undergo gut purge on day-5 and day-6 pupated exclusively on day-9 and day-10 respectively, forming a time interval of exactly 4 days in between the events.

In the present study, mosiac characters in ligated larvae were also observed. The posterior part of the abdomen had perfectly formed and tanned pupal cuticle. Failure of the anterior part to pupate (anterior inhibition) can be attributed to respiratory deficiencies by injury of the tracheal system during ligation or the involvement of the posterior darkening factor (PDF) or still another factor, the anterior retracting factor (ARF).

In the present study, emergence of adult or eclosion from pupal case under natural condition occurred during the photophase period of the last day of adult development: beginning at 15:00 hours and continuing till 18:00 hours or later. Consistently, eclosion starts quite late in the cool afternoon period when light was comparatively reduced. The timing of eclosion was observed to occur after 9-11 hours exposure to light. It was also observed that, adult eclosion in *Philosamia ricini* was restricted within a time frame or gating during precise period of the day; if the pupa completes development while the gate is open, ecdysis can occur during the same day. By contrast, if development is completed after the gate (after 15:00hr) has shut; the insect must wait until the opening of the gate on the next day so that ecdysis can occur within the time frame of 15 hr-18hr.

In the present study, considering the time of eclosion commencing at 15:00 hours on the last day of adult development, the release of eclosion hormone (EH) in *Philosamia ricini* in all probability may occur at about 1 hr prior to adult emergence *i.e.* at 14:00 hours. This was substantiated by the fact that EH extracts prepared at about this time (1 hour before normal eclosion) triggers extensibility in isolated wings of pharate adult that had not been exposed to EH activity.

The curves of oxygen consumption during both the larval cycles (4th and 5th instars) showed an inverted U-shaped, where the descending part of the curve during moulting is regarded as a curve representing protein synthesis. JH treated larvae recorded lesser amount of O_2 consumed compared to 20-HE and cAMP treated larvae. Higher O_2 consumption in larvae treated with 20-HE and cAMP may be correlated to the specific action of these compounds directly or indirectly to the epidermal cells which may induce molting and thereby higher metabolic activity. Whereas, the JH-III treated larvae may not influence molting and they may only help to maintain larval character so metabolic activity is possibly reduced and hence lesser amount of O_2 consumption. However, JH-III treated larvae almost always showed highest O_2 consumption amongst the three treatments at the ultimate days of both the instars. But by this time development of the 20-HE or cAMP treated larvae had become more advanced with low metabolic activity and are at rest and on the verge of molting, therefore O_2 consumption is low. On the other hand, the

JH-III treated larvae at the same stage are quite active and probably high levels of metabolic activity or differentiation are in progress and hence higher demand of O_2.

In the present study, the pattern of O_2 consumption in cAMP treated larvae are more or less similar to 20-HE treatment and it may be possible that cAMP directly act *via* PTTH to induce indigenous 20-HE secretion and activation which may in turn stimulate cellular activity and thereby resulted in higher O_2 consumption.

Morphology and structural changes of integuments of 4th & 5th instars are quite similar throughout development. In the present study, the late larval integument with its structural maturity can be correlated to be induced by PTTH and ecdysteriod secretions, since, such hormones are known to be at their maximal levels prior to molting. At about this time probably the molting gel is secreted and the epidermal cells undergo a period of mitosis and cell division. The epidermal cell population becomes denser, the cells are more columnar and prominent, and their apical surface is thrown into a series of fine folds. It was also observed that intercellular spaces are more prominent than in younger stages and the epicuticle and endocuticle are distinguishable probably they are ready to be shed during the preceding molt. In contrast, low level of integumentary structures up to day-3 in both the instars in term of cuticular deposition revealed that they have not been subjected to ecdysteriod action and hence cellular activity was also low. At about this time, the cuticle looks smooth and pliable while the epidermal cells and intercellular spaces are also not prominent. The epicuticle and exodocuticle are not clearly distinct from each other making them difficult to identify.

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GENERAL INTRODUCTION

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Ericulture:

Ericulture is one of the oldest cultures in North Eastern India and is almost a prerogative to the tribals of the region. The culture existed even before the Aryan migration to the region (Chowdhury, 1992). Ericulture in North Eastern India occupies 94% of the total culture of the country of which 70% are in Assam. History points out that eri silk were always identified as "Assam Silk". The earliest available reference to eri silk culture in India had been documented in 1779 (Lefroy and Ghosh, 1912). Ericulture is an ideal activity for development as subsidiary occupation in a country like India providing supplementary income to a large number of rural hilly and tribal populations and also for sericulturists because of the abundant availability of castor in the rural areas.

Large quantities of eri silk are produced each year, mainly in India among Asian countries. The production grew from 93 MT in the 1950s to over 1316 MT in 2002-2003. Most of these silks are consumed domestically in India, leaving little for export. In India at the present time, most eri silk is produced in Assam, Manipur, Meghalaya, Nagaland, Arunachal Pradesh and Mizoram, i.e. primarily in the North Eastern India. Thus ericulture from time immemorial has a close link with the culture and tradition of the people of North Eastern India involving nearly 1.20 lakh families with coverage of 7793 hectares under food plantation area. The rearing of eri silkworm is a heritage and almost a prerogative for the tribals in North Eastern India, where the chrysalis is more important as delicacy and silk cocoon is considered a bye-product. The culture is a village craft-the rearing, spinning and weaving activities are handed over from one generation to the other (Suryanarayana and Singh, 2005).

Ericulture is in peril today and is confined to the Brahmaputra valley and in some tribal inhabited districts of Assam and its neighbouring states of Meghalaya, Nagaland, Mizoram, Arunachal Pradesh and Manipur (Singh and Benchamin, 2001). Prospects of ericulture in North Eastern India will depend a lot on the ground realities of the prevailing environment and the institutions of research and development in the country as well as on the specific gains the culture will make out shifting to another mode of operation.

The insect:

Ericulture in North East India and the morphology of the insect itself have been well documented by Suryanarayanan and Singh (2005). Eri silkworm, Philosamia ricini is a polyphagous, multivoltine, non-mulberry domesticated silkworm which is reared indoor and feeds mainly on castor plant *Ricinus communis* (Castor) and other plants viz. *Manihot esculanta* (Topioca), *Heteropanax fragrans* (Kesseru), *Ailanthus excelsa* (Barkesseru), *Evodia flaxinifolia* (Payam) and *Ailanthus grandis* (Barpat). Eri silkworm is a holometabolous insect completing its life cycle by passing through four different stages *viz.* egg, larva, pupa and moth. Philosamia ricini is a domesticated form, although originally it is wild but due to taming over a long period of time by human beings and due to artificial selection to rear eri silkworms as was done in North Eastern states, it has descended to the present form. The structure of genetalia, wing pattern and chromosome number demonstate that it was derived from its closest wild ally *Samia canningi* (Hutton). The main distinguishing feature of *P. ricini* is the diffusion of the abdominal white tuffs, sometimes resulting in individuals with a solid white abdomen. The ground colour is usually gray or grayish brown, rarely reddish, but occasionally olive gray. The wing pattern is heavily marked by white in the antemedian and postmedian lines. The postmedian area is often much darkened, which is probably the easiest and quickest way to recognize this species.

Philosamia ricini is characterized by its relative availability as an experimental tool, thus providing an excellent bioassay system in the silkworm brain hormone project (cf. Ishizaki *et al.,* 1983; Ishizaki and Suzuki, 1990). This silkworm has also been studied for endocrinology (Saito and Fugo, 1990; Saito and Mukaiyama, 1990a, b; Kimura-Kawakami *et al.*, 1992; Yagi et al., 1995) and haemolymph protein biochemistry (Shimada *et al.*, 1992; Saito, 1998).

The systematic position provided to eri silkworm is as follows:

Super family - Bombycoidea, Latreill, 1802
Family - Saturniidae, Boisduval, (1837) 1834
Subfamily - Saturniidae, Boisduval, (1837) 1834
Tribe - Attacini, Blanchard, 1840
Genus - *Philosamia*, Hutton
=*Samia*, Hubner, (1819) 1816
Species - *ricini*, Donovan, (1798)
Common name - Eri silkworm

Neuroendocrine control of insect development:

Insects are characterized by an enormous diversity of morphological forms and living habits. This indirect form of development is manifested by the occurrence of immature larval stages, which in many insect groups live in a completely different environment and possess an entirely different form than those of the adults. The indirect development requires precise regulation of developmental processes and their synchronization with the changing environmental conditions. The same precise regulation occurs during metamorphosis when the transformation of the larvae into the mature adult takes place.

The regulation and synchronization of insect development is controlled by the neuroendocrine system. It record stimuli from the environment such as photoperiod, temperature, humidity, availability of food, etc., and transform these impulses into chemical messengers or hormones which in turn cause functional and developmental changes among various effector organs according to genetically determined programme. The neuroendocrine system represents a functional unit consisting of the nervous system and the endocrine glands. The most important part of the system are the neurosecretory cells located in the central nervous system. They are responsible for transmission of neural messages to the endocrine glands or other tissues. Direct effects of these secretions on 'target' tissues have the character of first order neuroendocrine functions since they stimulate secretion of hormones by other endocrine glands such as corpora allata or prothoracic glands. The hormones secreted by these latter endocrine glands represent neuroendocrine functions of the second order.

Growth and developmental processes connected with physiological changes in insect tissues are regulated by three hormones collectively referred to as developmental or metamorphosis hormones. One of theme is a neurohormone released by the neurosecretory system (Cells) while the second is released by the corpora allata and the third by the prothoracic glands. The neurosecretory cells may occur individually or in small groups.

A characteristic feature of the life cycle of insects is that all insects have eggs which hatch at the appropriate time after embryogenesis is completed, which leads into the emergence of larvae which also grow and molt in definite numbers, and then become pupae as in the case of holometabolous insects like the silkmoth, *Philosamia ricini* (Lepidoptera).

In insects, egg hatching, growth and metamorphosis (moulting, pupation and eclosion) depend on the activity of a number of endocrine organs. The neuroendocrine system of the insects control all the developments. It is known that a group of neurosecretory cells in the brain secrete a hormone, prothoracicotropic hormone (PTTH) which induces the thoracic glands to produce another hormone, ecdysone, and this second hormone stimulates growth of the body through yet other links. The PTTH is a brain neurosecretory peptide of insects that stimulates the prothoracic glands to secrete ecdysteroid, thereby inducing molting and metamorphosis (Bollenbacher and Granger, 1985; Ishizaki and Suzuki, 1994; Gilbert *et al.*, 1996; Hiruma, 2003). It is widely accepted that PTTH is released at specific times during insect development, signaling the transition of developmental stadia such as larval molting, larval-pupal transformation and adult development. Two families of hormones, the steroid molting hormones, ecdysones and the sesquiterpene juvenile hormones thus regulate development in insects. The ecdysones initiate and coordinate synthetic programmes used to make a new exocuticle during molting (Truman and Riddiford, 1999) and probably hatching of the larvae from the eggs (Hsiao and Hsiao, 1979). In contrast, the juvenile hormones, are considered "status quo" hormones that maintain the larval form and prevent metamorphosis and hatching, with regard to hatching, embryos from the basal groups response to juvenile hormone with a reduction on growth and the precocious onset of differentiation (Wyatt and Davey, 1996; Truman and Riddiford, 1999).

Juvenile hormone (JH) action:

JH is released from the corpora allata as it is produced; it is not stored. The activity of the corpora allata is regulated by humoral factors such as allatotropin and allatostatin as well as by nervous connections. Its haemolymph titer, which varies through the course of development, is consequently a product of the rate of synthesis and the rate at which it is degraded or excreted. Estimates of its half life in various insects are usually less than two hours so that sustained high titers must reflect high rates of synthesis as in cockroach (Tobe and Stay, 1985). Peptides like octopamine ehance JH synthesis in *Locusta* and *Apis,* but inhibit in adult *Diploptera* (Kaatz *et al.*, 1994). JH also has a feedback effect on its own production in the haemolymph where low titer enhances synthesis and high titer inhibits it. The inhibitory effects are produced *via* allatostatins (Stay *et al.*, 1994). Juvenile hormone (JH) plays important role in insect development and reproduction and its effect in immature insects is to modify the responses to ecdysteriod; it has no effect on its own. In adult insects, however, where JH affects sexual maturation and behavior, it acts independently of ecdysteriods. In molting and metamorphosis, it determines molt characteristics: ecdysteroid pulses in the presence of JH trigger larval-larval molts but, in its absence, cause larval-pupal, then pupal-adult metamorphosis (Riddiford, 1985). In addition to having multiple functions in blocking adult differentiation, retaining larval structures and regulating ovarian maturation, JH is a key player for phase polymorphism in armyworms, aphids and locusts and for caste differentiation in termites and ants. JH in the haemolymph usually binds to the haemolymph JH-binding protein, so that it is protected from metabolism by the general esterases (Riddiford, 1994; Hiruma, 2003). JH may function as a primer or as a regulator of the target tissue. As a primer, it acts directly on the nucleus so that the cell becomes responsive to a subsequent regulatory signal, which may be JH itself or another hormone. As a regulator, JH may act *via* membrane receptors to activate existing enzymes *via* a second messenger cascade, or it may act at the nucleus to promote protein synthesis as in *Rhodnius* (Sevala and Davey, 1989).

In many insects the application of JH or JHAs during larval development disturbs the endocrine balance and, consequently, induces abnormal development (Retnakaran *et al.*, 1985). Induction of extra molting and prolongation of the last larval instar are the most noticeable effects of JHA treatment (Cymborowski and Stolarz, 1979; Mizoguchi, 2001; Kamimura and Kiuchi, 2002). Many workers have studied the effects of JH and JHAs in larval molting and metamorphosis in insects. Kamimura and Kiuchi (2002) applied JHA, fenoxycarb topically to the starved silkworm *Bombyx mori* at beginning of the 3rd or 4th (penultimate) instar and found that fenoxycarb triggers the extra molt by inducing an additional larval molt (6th instar) of ecdysteroid surge before the last larval instar. Gu *et al.* (1997) shows the involvement of JH in regulation of PTTH transduction during the early last instar larva of *Bombyx mori*. Induction of prefect super larvae by the application of JHA to young larvae of the silkworm, *Bombyx mori* was also reported by Kamimuara (1995). Sakurai *et al.* (1989) showed that JH inhibits ecdysone secretion and responsiveness to PTTH in prothoracic glands of *Bombyx mori*.

Ecdysteriod action:

The action of ecdysteriods at cellular and molecular level has been well studied (O' Connor, 1985; Riddiford, 1985) however, much of the initial knowledge of the mechanism of action of the hormone came from studies on the effects of these hormones on transcriptional control in polytene chromosomes of *Chironomus tentans* (Clever and Karlson, 1960) and *Drosophila melanogaster* (Ashburner and Berendes, 1978). These studies prompted the theory that steroid hormones act directly on genes and ecdysteriod-regulated gene expression in insects has been reviewed by Lepesant and Richards (1989). Thus, the modern version of ecdysteriod action is that the hormone enters the cell and goes to the nucleus where it combines with the ecdysone receptor (Koelle *et al.*, 1991)and directs the transcription of the early genes, most of which encode transcription factors (King-Jones and Thummel, 2005). These factors in turn induce a secondary response by repressing some of the early genes and activating the expression of tissue-specific genes and thereby causes initiation and coordination of molting processes.

Many physiological events in insect are elicited by critical titer of ecdysteriods that include the principal molting hormone, 20-hydroxyecdysone (20-HE) and ecdysone (E), which is the precursor of 20-HE but also has a morphogenetic roles of its own. These events are usually coordinately modulated by JH. The rate of synthesis of ecdysteriods is critical for molting and metamorphosis in insect. Haemolymph level of ecdysteriods largely resulted from the balance between upstream stimulation of ecdysteriod synthesis by the brain neuropeptide PTTH and feedback inhibition of ecdysteriod synthesis by ecdysteriods themselves (Gilbert *et al.*, 2000, 2002).

Ecdysone is not stored in the prothoracic glands and its appearance in the haemolymph reflects its immediate synthesis. Synthesis is often under dual control. It is triggered by the PTTH but the effects are modulated, at least in some insects, by an inhibitory hormone and by direct neural regulation which may be either stimulatory or inhibitory (Koolman, 1995). In larval *Manduca*, ecdysteriods and JH both affect the production of ecdysone, but their effects vary (Smith, 1995). During feeding period of the larva, JH inhibits ecdysone synthesis. Subsequently, the ecdysteriods produced by the PGs have a positive feedback effect which is enhanced when JH is also present. Finally, however, when the glands are highly active, the ecdysteriods have a negative feedback and contribute to the subsequent rapid decline in haemolymph titer (Sakurai and Williams, 1989). In *Drosophila*, (Riddiford, 1993) demonstrated that the ecdysteroid (20-HE) titer increases before each larval molt and is required for triggering these development transitions. At the end of the third larval instar, a high titer peak of ecdysteroid triggers puparium formation and the onset of metamorphosis. Several pulses of ecdysteroids during metamorphosis are responsible for further differentiation of these adult structures. The ecdysteroids titer declines at the end of pupal development and is relatively low when the adult fly emerges from the pupal case.

Cyclic AMP action:

Adenosine 3', 5' monophospate (cAMP) plays a central role in mediating the action of a wide range of hormones (Robinson *et al.*, 1968). The importance of cAMP in hormone action was first recognized by Sutherland and Rall (1958) who showed that it played an essential role in the hyperglycemic actions of glucagons and epinephrine on the liver. Since then this compound has been implicated in cellular control mechanisms in a wide range of organisms from bacteria to mammals. The level of cAMP is regulated by altering the activity of adenyl cyclase, which represents the site of action of most hormones as well as certain neurotransmitters (Robinson, *et al*, 1968). Nijhout, (1994) also suggested that in experimental endocrinology it is often possible to mimic the action of a hormone on a particular target tissue by exposing that tissue to elevated concentrations of exogenous cAMP, by simple injection of cAMP or a more stable analog such as dibutyryl cAMP. This compound acts as an analogous to hormone in certain case of physiological experiments. Many hormones were shown to increase intracellular levels of cAMP in their appropriate targets. The mimicking property of cAMP is due to the presence of adenylate cyclase, the enzyme responsible for cAMP synthesis, which is located in the plasma membrane and is highly dependant on membrane structure for its activity. Hormone-sensitive adenylate cyclase is composed of at least three separable protein components, i.e., a hormone receptor (R), a guanine nucleotide regulatory protein (s) (N), and a catalytic subunit (C) responsible for production of cAMP from intercellular ATP.

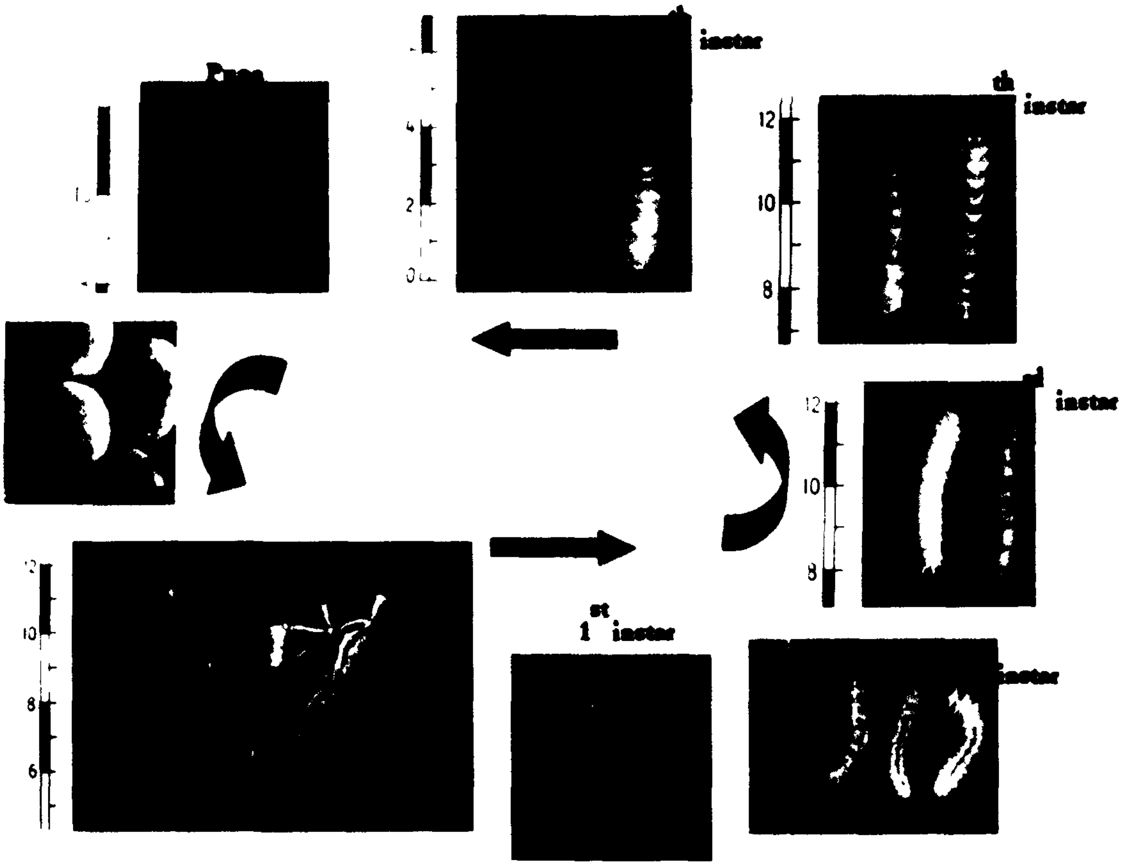
The very high concentrations of cAMP required to activate secretion when applied exogenously may be determined by several factors. Firstly, cell membranes may be relatively impermeable to these large nucleotides and the cell must be flooded with a very high concentration to enable enough cAMP to enter the cell to raise the intracellular level sufficiently to activate the secretion. Secondly, since phosphodiesterase is continually hydrolyzing cAMP, sufficient cAMP must enter to the cell to counteract the degradative process before stimulation of the effector system is apparent. Thirdly, the natural hormone may induce an increase in other intermediates such as calcium so that higher concentrations of cAMP are required to overcome the lack of these other intermediates (Treherne *et al.*, 1972; Sevala *et al.*, 1989).

Adenylate cyclase from fat body of silkworms has been described in some detail in *H. cecropia* (Filburn, and Wyatt, 1976) and *B. mori* (Morishima, 1974, 1980a, b, 1981). The role of cyclic nucleotides as a second messenger in mediating cellular response to hormones and neurotransmitters has been studied in lepidopteran insects such as *H. cecropia* and *Danaus plexipus* (Wiens, and Gilbert, 1967; Bhakthan, and Gilbert, 1968; Chang, 1974; Fain, 1982) and *B. mori* (Vanderberg, and Mills, 1974; Yanagawa, and Horie, 1978). Cyclic nucleotides also elicit eclosion behavior in *H. cecropia* (Truman, *et al.*, 1976) and *Antheraea polyphemus* (Truman and Schwartz, 1980). cAMP have also been found to play key roles in the regulation of fluid and ion secretion in many insects such as the fluid secretion by malphighian tubules in *Pieris brassicae* (Nicolson, 1976). Adenylate cyclase activation of both onset and termination of pupal diapause have been implicated in saturniid moths (*H. cecropia* and *A. pernyi*) by Rasenick, and Berry, (1981). The membrane potential of *Galleria mellonella* prothoracic glands can also be elicited by cAMP (Gersch, and Birkenbeil, 1980) and ecdysone secretion stimulation by prothoracic gland in *Manduca sexta* (Vedeckis *et al.*, 1974, 1976).

cAMP has been reported to play a role in some steroid-mediated events such as activity of pupal wing epidermis of *Hyalophora gloveri* (Applebaum and Gilbert, 1972) and premature formation of cellular autophage vacuoles in *Mamestra brassicae* (Sass *et al.*, 1983). Truman *et al.* (1976) observed that the injection of cAMP in the central nervous system of silkmoths causes hormonal release of programmed behavior. Later on Smith and Pasquarello (1989) showed that the exact roles of calcium ions and cAMP in stimulating ecdysteroid synthesis appear to differ in different developmental stages, and these control mechanisms are still under active investigation. The involvement of cyclic AMP as a potential second messenger in the neurohormonal control of ovarian steroidogenesis was also investigated in the adult female blowfly *Phormia regina* (Maniere and Vanhems *et al.*, 2000).

The modes of action of insect hormones in relation to activation of second messenger nucleotides have been reviewed by Smith (1995), Morton and Simpson (1995), Smith and Sedlmeier (1990) and Spring (1990).

Fig 1. Life Cycle of *Philosamia ricini*



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CHAPTER – 1

Effect of Juvenile hormone-III, 20-Hydroxyecdysone and Adenosine 3', 5' monophospate (cAMP) on Egg hatching.

Chapter – 1

INTRODUCTION

Juvenile hormone and 20-hydroxyecdysone has been reported to function even before the embryo's own endocrine system become functional. Rees (1995) illustrated that during oogenesis, 2-deoxyecdysone and ecdysone conjugates are put into the egg presumably to be used in development before the embryo's own endocrine system becomes functional. In the same way, Juvenile hormone is present in the newly laid egg as a result of its gonadotropic role during oogenesis, but then is metabolized by specific esterases as embryonic development begins (Roe *et a.l*, 1987; 1990; Temin *et al*, 1986). Consequently, JH is absent in midembryogenesis, when the embryo is undergoing most of its morphogenesis and growth. It then reappears prior to the beginning of the nymphal molt (Temin *et al*, 1986) and in association with the final histodifferentiation of the embryonic tissues. The delay in the appearance of JH until just prior to the nymphal molt is also seen in the cockroach *Nauphoeta* (Imboden *et. al.*, 1978.) and the bug *Oncopeltus fasciatus* (Dorn, 1983).

Effect of Juvenile Hormones (JH) and Juvenile hormone analogs (JHAs) on egg development:

Ever since the first crude extract of Juvenile hormone from the giant silk worm, *Hyalophora cecropia* was prepared (Williams, 1956), certain other substances with JH-like properties or JHAs (juvenile hormone analogs) *viz.* farnesenic acid derivatives (Williams, 1967) and Juvabione or paper factor (Bowers, *et. al.*, 1966) and others were identified. The deleterious effect of JH and JHAs on the embryonic development inside the egg has been a subject of study by different worker around the world in insects such as *Pyrrhocoris apterus* (Slama and Williams, 1966; Riddiford, 1972), *Hyalophora cecropia* and *Antheria pernyi* (Riddiford and Williams, 1967). While the majority of applied entomologists, repeating these studies in different insects with different compounds felt contented to observe their deleterious effect on hatching as such, a few felt interested in studying their effect in some detail on the developmental phenomenon. Credit doubtlessly, goes to Riddiford (1972) and her collaborators for the pioneering observation that juvenoids affect insect embryogenesis by causing developmental arrest. The used of juvenoids in treating eggs for developmental arrest of embryo has been performed successfully in *Schistocerca gregaria* (Novak, 1969), *Thermobia domestica* (Rohdendorf and Sehnal, 1973), etc.

While these interesting studies have convincingly demonstrated that juvenoid application to eggs causes a biological effect, as distinct from toxic effect, which manifests itself in the form of arrest of embryonic development. They have opened up several questions of far reaching importance. Firstly, which are the stages in insect embryogenesis when development arrest may take place-only blastokinesis and the terminal stages of embryo-larva transformation, or other stages as well? Secondly, is there some relationship between the stage of juveniod treatment and the stage when development is disrupted? Thirdly, a simple question which has largely been ignored – whether the amount of the hormonally active compound applied has any relevance in relation to the stage of developmental arrest, or generally speaking the nature of effect? Fourthly, if certain stages are more responsive to juvenoid treatment, why? Indeed there are a number of questions, which easily arise in the wake of the aforesaid studies.

Recently many workers had tested JHA, fenoxycarb as an effective insecticides in different stages of development in insects *viz.* *Chrysoperla carnea* (Celli *et al.*, 1997), tortricid species (Charmillot *et al.*, 1985), *Micromus tasmaniae* and *Chrysoperia carnea* (Rumpf *et al.*, 1997; 1998) and *Chrysoperla rufilabris* (Liu and Chen, 2001). The potential role of juvenile hormone as target of pest management has been reviewed by Chieka and Riddiford (2006).

The present study will attempt to determine the deleterious effect of JH-III of certain stages of the eggs of *P. ricini* and establish a relationship with the doses given in line with the previous study.

Role of ecdysteriods in insect development:

The development of an *in vitro* assay for vitellogenin synthesis (Pan *et al.*, 1969) led to the discovery that the ovary produced ecdysone (Fallon *et al.*, 1974; Schlaegar *et al* 1974; Hagedorn *et al.*, 1975; Hoffman *et al.*, 1980). Horn (1971) speculated that the ecdysone in adult female might play a role in ovarian development. In fact, ecdysteriods were discovered in adult females in some of the earliest work on the extraction of this hormone from insects. Adult female *B. mori* moths were found to contain large amount of ecdysteriod (Karlson, and Stamm-Menendez, 1956). Further work by Shaaya and Karlson, (1965) extended these findings by showing that two peaks of ecdysteriod activity occurred during the pupal stage and that young female moths contained four times more ecdysteriods than males. Hanaoka and Ohnishi (1974) showed the second peak was found only in female pupae and was abolished by ovariectomy. Lagueux *et al.* (1977) found that in *Locusta migratoria* ecdysones exist only towards the end of egg development, just before oviposition. Another striking fact is that most of the ecdysteroids present in some adult insects are found in the ovaries. In lepidopteran insects ecdysteriods are found in pharate adult female, ovary and eggs of *Galleria mellonella* (Hsiao and Hsiao, 1977; Bollenbacher *et al.* 1978), in *Manduca sexta* in young embryo (Kaplanis *et al.* 1973), and in *B. mori* in adults, pharate adult, embryos and eggs (Shaaya and Karlson, 1965; Hanaoka, and Ohnishi, 1974; Ohnishi, *et al.*, 1977; Mizuno and Ohnishi, 1975). There are marked quantities and qualitative differences in the free ecdysteroids found in insect oocytes. Free ecdysone and 20-hydroxyecdysone have been found in lepidopteran insects and those mostly found in ovaries and eggs are conjugated to more polar molecules (Hoffman *et al.*, 1980). Thus in eggs and embryos the conjugated ecdysteroids may play a physiological role and represent both a storage form for active hormone or its precursors, as well as its inactivation products.

Regarding the sites of ecdysteroid synthesis, there are experimental evidences that the ovary itself is the source and that its accumulation in the oocytes has been obtained for several insects in lepidopteran pharate adult of *B. mori* (Hanaoka and Ohnishi, 1974) and in adult female of *Galleria mellonella* (Bollenbacher *et al.*, 1978).

The history of the suggestion that the large amounts of ecdysteroids found in eggs and embryos might be related to the regulation of embryonic molts is interesting (Hoffman and Lagueux., 1985). Jones (1956) speculated that the hormonal control of embryonic and larval molts was homologous and concluded that the embryonic endocrine centers had an effect on molting and this led to the first evidence that that ecdysteroids are present in the eggs and they had a role in embryogenesis (Boohar, and Bucklin, 1963). The relative roles of maternal and embryonic ecdysteroids are not clear. It is possible that the maternal ecdysteroids are sufficient to regulate all of the embryonic molts, but it is also possible that the embryonic endocrine glands are functional during later development (Lagueux *et al.*, 1981).

The function of the ovarian ecdysteroids is the regulation of embryonic molting (Hoffman *et al.*, 1980) and in a preliminary experiment, injected 20-hydroxyecdysone (5µg) stimulated the appearance of the vitellogenin message and the most detailed evidence for the involvement of ecdysteroids in adults comes from the work on vitellogenin synthesis in *diptera* (Kunkel and Nordin, 1985).

Ecdysteroids has two sources, the prothoracic glands and ovaries. Presumably both hormones in the larva and adult are advantageous for controlling complex processes such as molting and reproduction which leads to increase fecundity. The importance of the two sources of ecdysteroids, perhaps, it is because the ovaries were the original source of ecdysteroids and at one time enough ecdysteroid was stored in the oocyte to fill the needs of both the embryonic and larval forms. The prothoracic glands might then be seen as a later development to fill the increasing demand for accurate control of ecdysteroid titres by the larval insect. Exogenous injection of 20-HE had also been demonstrated to mediate the release of hormone responsible for ovulation in *Rhodnius prolixus* (Ruegg *et al.*, 1982).

Ecdysteroids cannot be further viewed as a centrally produced insect hormone, they are true homeostatic factors produced by various peripheral tissues for mutual synchronization of tissue growth with some important developmental events such as ecdysis, oviposition, and hatching from the egg. Previous works done in *Galleria mellonella* revealed that, ecdysones may probably initiate hatching of eggs since the ecdysteriods found only in the ovary are reported to subsequently incorporated into the eggs (Hsiao and Hsiao, 1979) and that the follicle cells are the sites of synthesis, was demonstrated in *B. mori* (Legay *et al.*, 1976) and *L. migratoria* (Lagueux *et al.*, 1977). That high titer peak of ecdysteroids (20E) triggers hatching was demonstrated in *Schistocerca gregaria* and the ecdysteroid titer then declines when the 1st larva hatched (Riddiford, 1993). In accord with its postembryonic function, peaks of ecdysteroids are also seen during the embryonic moults (Lagueux *et al.*, 1979). Later, the involvement of ecdysteroids in embryonic development was reported in *Locusta migratoria* also (Truman and Riddiford, 1999; 2002).

The present study will thus examine the dose-dependant effect and feasibility of 20-hydroxyecdysone whether they promote hatching or not on treating at different stages of the eggs of *Philosamia ricini*.

Cyclic AMP as a second messenger:

Cyclic AMP as already stated has been used extensively in insect's endocrinological experiments for its effect and its role as a mimicking property. cAMP plays a central role in mediating the action of a wide range of hormones (Robinson *et al.*, 1968). This compound acts as an analogous to hormone in certain case of physiological experiments (Bhakthan and Gilbert, 1968; Chang, 1974; Truman *et al.*, 1976; Vedeckis *et al.*, 1976; Filburn and Wyatt, 1976; Yanagawa and Horie, 1978; Morishima, 1974, 1980a, b, 1981; Truman, and Schwartz, 1980; Gersch, and Birkenbeil, 1980; Rasenick, and Berry, 1981; Fain, 1982; Nijhout, 1994). Many hormones were shown to increase intracellular levels of cAMP in their appropriate targets. Juvenile and ecdysone are two insect hormones and it has been suggested that cAMP may be involved with the action of ecdysterone (Leenders *et al.*, 1970).

These observations have distinct evidences that cAMP has been a subject of study in different insects around the world. However, information on the effect of c-AMP on egg hatching has not been reported so far. Thus in the present study cAMP will be used to observe its mimicking property to any of the neuroendocrine hormones (JH-III and 20-hydroxyecdysone) and whether exogenous application on the eggs of *Philosamia ricini* promote hatching or not.

MATERIALS AND METHODS

1. Selection of eggs for treatment:

Philosamia ricini used in these experiments were received from, Sericulture Department, Nongpoh, Govt. of Meghalaya. Larvae were reared in a rearing cage indoor and supplied with diet of Castor leaves, *Ricinus communis*. They were reared at 25° C ±0.5 under 16L: 8D photoperiod regiment as described by Bell and Joachim (1976).

In the laboratory, eggs are laid by the moths in clusters, glued together in rows projecting from the substrate or kharika (small bundles of thatched or splited bamboo, about 30 cm long with a hook). In our condition the number of eggs laid per female ranged from 200-250 (mean 225, but sometimes 400 to 450 eggs). Six stages of the eggs after each incubation (2- hrs, 6- hrs, 9-hrs, 12-hrs, 24- hrs and 36- hrs) were chosen for treatment with the above hormones at different concentrations.

2. Topical application:

The 20-hydroxyecdysone (Sigma Chemical Ltd., USA) hormone was dissolved in 10% methanol and the hormone concentration was adjusted to 1µg, 0.5µg and 0.25µg. In case of synthetic Juvenile hormone-III (sigma Chemical Ltd., USA) and Cyclic AMP (sigma Chemical Ltd., USA) the solvent was 10% acetone and Ringer's solution (ph 7). The concentration was adjusted in the same way as that of 20-hydroxyecdysone. The different stages of the eggs chosen were topically applied with the three concentrations of each hormone by means of a glass capillary on the chorion surface of each egg. The control eggs were topically applied with 10% methanol, 10% acetone and Ringer solution (pH 7) respectively.

3. Dipping Experiments:

In case of dipping experiments freshly laid eggs were dipped in various hormone concentrations as above in a batch of 100-120 for 2-3 minutes.

After every treatment the treated eggs were immediately transferred into a culture room for incubation where the temperature is kept constant at 25°C and RH at 70-90%. The eggs were observed daily for hatching in the morning (8-10AM) and in the evening (4-6PM). The numbers of eggs hatched were then counted and scored against the unhatched eggs.

RESULTS

A. Effect of Juvenile hormone –III:

Treatment of the eggs with all the three concentrations (1 μg, 0.5 μg and 0.25 μg) was found to have deleterious effect on egg hatching irrespective of stages of the eggs. The deleterious effect of JH-III application was found to be maximum in the 2 hrs old eggs whereas least in the 36 hrs old eggs. The percentage of unhatched eggs after JH-III application was in a decreasing order as the eggs get matured. For dose 1 μg the unhatched percentages of eggs were 96.83±1.70, 93.32±0.94, 85.99±0.98, 84.13±0.84, 40.17±0.98 and 25.16±1.17 for 2hrs, 6hrs, 9hrs, 12hrs, 24hrs and 36 hrs old eggs respectively. For dose 0.5 μg the unhatched percentages of eggs were 95.99±2.22, 89.33±0.81, 80.99±0.69, 72.11±0.95, 37.75±1.17 and 17.06±0.86 for 2hrs, 6hrs, 9hrs, 12hrs, 24hrs and 36 hrs old eggs respectively. For dose 0.25 μg the unhatched percentages of eggs were 96.49±1.28, 85.99±0.98, 75.49±1.16, 67.40±0.71, 30.68±1.17 and 12.92±1.33 for 2hrs, 6hrs, 9hrs, 12hrs, 24hrs and 36 hrs old eggs respectively. Among the doses given the most deleterious was that of 1 μg followed by 0.5 μg and 0.25 μg. As the eggs get matured the effectiveness of JH-III applications irrespective of doses was found to deteriorate. In all the experiments the percentage unhatched eggs in controls were lower than that of JH-III applied eggs (**Table 1**).

It can be observed that the control bar was lowest during all the stages of treatment which indicates that all the three concentrations of JH-III was effective enough in preventing hatching especially in younger eggs of 2 hrs, 6hrs, 9 hrs and 12 hrs old. JH-III treatment in older eggs of 24 hrs and 36 hrs are however less severe (Fig. 2 and Fig. 5).

B. Effect of 20-Hydroxyecdysone:

In all the stages of the eggs it was found that the percentages of unhatched eggs were lower than that of controls irrespective of doses given (Table 1). The trend of unhatched percent was in a decreasing order from the 2hrs stage upto the 12 hrs eggs stage. In 20-hydroxyecdysone applied eggs the older the eggs the higher is the percentage of hatching. The least unhatched eggs occurred in the 36 hrs old stage (1.03± 1.03 %) with 1 μg dose whereas in the 2 hrs old eggs in the same dose the unhatched eggs was 38.33±1.23 % which was also the highest. Unhatched eggs for all the stages varies from 1.03±1.03 to 38.33±1.23 for 1 μg, 1.54±0.98 to 35.16±1.81 for 0.5 μg and 2.58±1.49 to 14.33±0.96 for 0.25 μg of 20-hydroxyecdysone. In control unhatched eggs for all stages varies from 7.40±0.86 to 41.99±2.22.

The bars representing the treatment of 20-HE against unhatched eggs followed a uniform slope with control being the highest followed by 1 μg, 0.5 μg and 0.25 μg at least upto the 12-hrs old eggs and then in the 24hrs and the 36 hrs stages of the eggs, the bar representing 0.25 μg treatment was higher than that of 0.5 μg or 1 μg treatments respectively. It can be observed that as the egg matures the slope become lower showing that hormone treatment was less effective in older developing eggs. (Fig. 3 and Fig. 5).

C. Effect of cAMP:

The percentage of unhatched eggs after application of three concentrations of cAMP was also seen to enhance hatching, with 0.25 μg being the most effective upto the 12 hrs stages which resulted in lowest percent of unhatched eggs. In the same way unhatched eggs was found to decrease as the eggs get matured after treatment of the three concentrations. In 2 hrs old stage, the higher the dose (1 μg), the higher also is the percentage of unhatched eggs (40.33±1.40 %), whereas the lower the dose (0.25

μg) of cAMP the lower the percentage of unhatched eggs (25.33±0.71). These trends continue for the younger eggs through 6 hrs, 9 hrs and upto the 12hrs stages. Interestingly, in the last two stages (24hrs and 36hrs) 1 μg was found to be more effective in bringing about lower unhatched eggs than that of 0.25 μg treated eggs. Wherein, in the former the percentage of unhatched eggs was 3.44±0.84, the later gives 13.1±0.62 % for the 24 hrs stage. In the same way, in the 36 hrs stage, the percentage of unhatched eggs treated with 1 μg was 1.20±0.81, with 0.5 μg it was 3.44±0.84 and with 0.25 μg the percentage of unhatched eggs was 11.54±0.65 %. The control experiment for all the stages always gives higher percentages of unhatched eggs than any of the three concentrations of cAMP treated eggs.

The bars representing cAMP treatment are more or less similar to that of 20-HE, except that they are a bit higher especially in older stages of the eggs. This indicates that cAMP treatment bring about much higher percentage of hatching and may also promote late embryonic development (Fig. 4 and Fig. 5)

D. Cumulative effect of JH-III, 20-HE and cAMP on egg hatching.

From the data (Table 2), it can be seen that the effect of all the treatment are significantly different from each other at all stages of the treatments. However the significant level between 20-HE and cAMP treatments are much closer and almost similar than that of JH-III treated. The cumulative effect of JH-III, 20-HE and cAMP in all stages of treatments follows a similar trend in which the younger eggs (2-12 hrs) are more prone to deleterious effect of the treatments than the older eggs (24-36 hrs). Analysis of data shows that JH-III treatment resulted in high percentage of unhatched eggs (81.183 in 2 hrs, 74.993 in 6 hrs, 67.12 in 9 hrs, 62.030 in 12 hrs, 32.453 in 24 hrs and 17.237in 36 hrs). In 20-HE treated eggs although the unhatched was more in 2 hrs (32.454 %) and 6 hrs stages (24.445 %), it falls abruptly to 12.413 % in 9 hrs to 9.001

% in 12 hrs then to 3.768 % in 24 hrs and finally to 3.143 % in 36 hrs. While cAMP treated eggs also showed high percentage of unhatched eggs in 2 hrs (35.288 %), 6 hrs (29.411 %) and 9 hrs (18.950 %), the unhatched eggs were much lower in 12 hrs (13.609 %), 24 hrs (9.367 %) and 36 hrs (6.245 %) old eggs.

Table. 3 represent the effect of concentrations of all the treatments in all stages of eggs. JH-III at 1 μg showed the maximum percent of unhatched eggs at 70. 93±5.79 followed far behind by cAMP (18.60±2.97) and 20-HE (14.74±2.84). Similarly, at 0.5 μg and 0.25 μg JH-III (65.54±5.97 and 61.50±6.24) showed highest percent of unhatched eggs followed again by cAMP (15.80±2 and 16.34±1.27) and 20-HE (11.61±2.66 and 8.47±1.36).

DISCUSSION

Slama and Williams (1966) and Enslee and Riddiford (1977) observed the deleterious effect of juvenoid applications on the eggs of *Pyrrhocoris apterus*, Riddiford (1972) in *Hyalophora cecropia*, Riddiford and Williams (1967) in *Antheria pernyi*, Novak (1969) in the locust *Schistocerca gregaria*, Rohdendorf and Sehnal (1973) in *Thermobia domestica*. In our study we also found the deleterious effect of Juvenile hormone-III application on the eggs of *Philosamia ricini*. The effectiveness was more on the younger eggs. As the eggs get matured the effectiveness of Juvenile hormone-III application deteriorated but the deleterious effect did not stop. Comparatively, among the three treated doses taken for JH-III treatments, 1 μg was found to be the most effective followed by 0.5 μg and 0.25 μg in which the unhatched eggs after treatments were 70.93±5.79 %, 65.54±5.97 % and 61.50±6.24 % respectively for all the stages of eggs (Table 3).

Our results indicated that application of JH-III to eggs resulted in decreased survival rates and prolonged development times when treated at different stages and at different concentrations. JH-III showed significant ovicidal effect on *P. ricini* eggs, with very low survival rates, depending on the stages treated and the concentrations used. Generally, the higher concentrations exhibited greater effects on the egg stages treated. Similar effect has been reported on *C. carnea* by Celli *et al.* (1997) and *Chrysoperla rufilabris* eggs by Liu and Chen (2001). JHA (fenoxycarb) also shows ovicidal effects on some insects (including *Chrysoperla*). Charmillot *et al.* (1985) observed that fenoxycarb showed more severe effects on the eggs laid singly than those laid in egg masses.

Novak (1969) and other workers working in different insects reported that as a result of such treatment, development may cease at any stage of embryogenesis from cleavage of the zygote to the fully formed embryos or developmental arrest may first take place at the blastoderm stage or it may be blocked at any other "functionally demanding phase" like invagination of the germ band or blastokinesis. In contrary, our studies finds that younger treated eggs are more susceptible to hormones applied, whether it is deleterious or it stimulate hatching. In *Philosamia ricini* we believed that the "functionally demanding phase" which is the most sensitive stage during embryogenesis must probably be the stages from 2-12 hours. These stages showed maximum unhatched eggs in term of percentages for all the three treatments (Table1).

Also of interest are the findings that larvae, which hatch out, were not able to feed and mature normally and they soon die prematurely. Ridifford and Williams (1967) observed in the eggs of the moths *Hyalophora cecropia* and *Antheria pernyi* that as a result JH treatment, embryonic development had stopped. They also observed that in those cases in which hatching did occur, the larvae often suffered with some abnormalities and further, apparently normal larvae may die during their postembryonic life, or their metamorphosis may be disrupted. In a further detailed study in *Pyrrhocoris apterus*, Riddiford (1972) concluded that once an egg has been fertilized, treatment with a juvenoid will not arrest its development until blastokinesis and occasionally the development of the nervous system is adversely affected in as much as the consolidation of the thoracic and abdominal ganglia does not take place. We observed that these abnormalities of the larvae occurred especially to those that hatched from younger eggs and even if the larvae seems to develop normally they may die at some point of time during the larval instars, pupal development or during emergence (Fig. 6). We observed that some larvae that survived had difficulty spinning cocoons. Some did not have silk to make the cocoon, whereas others produced isolated silk threads, but could not make a complete cocoon. Some larvae managed to pupate even in an incomplete cocoon. All those that did not make cocoons or did not have a complete cocoon died at the pupal stage or as pharate adults. Enslee and Riddiford (1977) ascribed the disruption of development to the abnormal breakage of the embryonic membranes and also observed that JHA treatment may affect dorsal closure, consolidation of the ganglia and the extension of the appendages during the embryo-larva transition.

In *Earias fabia*, a lepidopteran insect, Srivastava (1983) reported that, 'larger doses bring about development arrest of embryos much earlier than smaller doses', and indeed a relationship can be established between the quantity of the juvenoid applied and the rate of its inhibitory effect on development. As was in the case of JH-III treated eggs, 1 μg of both 20-hydroxecdysone and cAMP produced the same results in which the higher the dose, the higher are the percentage of unhatched eggs but this effect is pronounced only in younger stages of eggs (Table 3).

Although data obtained from the present study have been sufficient to decide upon the use of JH as insecticide in IPM semi-field and field studies are still needed. More research on the effects of JH on other insects under different agroecosystems is also needed to elucidate how to use IGRs in IPM programs. Further, base on the present study it is apparent that effectiveness of JH largely depend on the timing and stages of applications.

Contrary to the observations made for JH-III treated eggs, 20-hydroxyecdysone and cAMP treated ones produced much more hatching percentages. It can be suggested that 20-HE promote hatching at least if treated to older eggs since the percentage of hatching is quite high compared to the younger eggs. In this respect it will be important to note that Hsiao and Hsiao (1979) reported that ecdysones might probably initiate hatching of the larvae from the eggs and the fact that ecdysteriods are found in many different forms in lepidopteran insects. Riddiford (1993) demonstrated that high titer peak of ecdysteroids (20E) triggers hatching. In our observation, cAMP more or less mimicked the property exhibited by 20-hydroxyecdysone, since the unhatched eggs produced as well as the pattern of effectiveness throughout the stages by both the treatments are almost identical (Table 2 and 3). In experimental endocrinology it is often possible to mimic the action of a hormone on a particular target tissue by exposing that tissue to elevated concentrations of exogenous cAMP, by simple injection of cAMP or a more stable analog such as dibutyryl cAMP (Nijhout, 1994).

Table 1. Effect of different concentrations of JH-III, 20-HE and cAMP on egg hatching at different stages of egg development. The results are expressed as mean ± SEM, *n*=4

Treatment	Conc-tration	% of unhatched eggs					
		2 (hrs)	6 (hrs)	9 (hrs)	12 (hrs)	24 (hrs)	36 (hrs)
JH-III	**Contrl**	35.41±1.40	31.33±1.56	25.99±1.12	24.47±1.17	21.2±0.70	13.79±0.62
	0.25 µg	96.49±1.28	85.99±0.98	75.49±1.16	67.40±0.71	30.68±1.17	12.92±1.33
	0.50 µg	95.99±2.22	89.33±0.81	80.99±0.69	72.11±0.95	37.75±1.17	17.06±0.86
	1.00 µg	96.83±1.70	93.32±0.94	85.99±0.98	84.13±0.84	40.17±0.98	25.16±1.17
20-HE	**Contrl**	41.99±2.22	30.32±0.88	23.99±0.71	22.06±1.28	9.13±0.51	7.40±0.86
	0.25 µg	14.33±0.96	19.83±0.56	5.16±1.03	3.26±0.95	2.92±1.33	2.58±1.49
	0.50 µg	35.16±1.81	22.16±0.41	5.16±0.83	3.78±0.71	1.89±0.70	1.54±0.98
	1.00 µg	38.33±1.23	25.49±0.99	15.32±1.24	6.89±0.48	1.37±0.84	1.03±1.03
c-AMP	**Contrl**	44.83±1.25	35.16±0.83	24.99±1.23	21.02±0.65	12.92±0.33	8.78±0.65
	0.25 µg	25.33±0.71	23.33±0.54	16.33±0.63	8.96±0.39	13.1±0.62	11.54±0.63
	0.50 µg	28.16±1.25	27.16±1.25	18.16±1.03	10.14±0.96	7.75±0.65	3.44±0.84
	1.00 µg	40.33±1.40	31.99±0.97	20.33±0.88	14.30±0.51	3.44±0.84	1.20±0.81
LSD$_{0.05}$		**1.618**	**0.797**	**0.706**	**0.979**	**0.834**	**0.533**

Table 2. Cumulative effect of JH-III, 20-HE & cAMP on egg hatching.

Treatment	% of unhatched eggs					
	2 (hrs)	6 (hrs)	9 (hrs)	12 (hrs)	24 (hrs)	36 (hrs)
JH-III	81.183 a	74.993 a	67.12 a	62.030 a	32.453 a	17.237 a
20-HE	32.454 b	24.445 b	12.413 b	9.001 b	3.768 b	3.143 b
c-AMP	35.288 c	29.411 c	18.950 c	13.609 c	9.367 c	6.245 c
$LSD_{0.05}$	1.401	0.690	0.611	0.848	0.723	0.462

<u>Note:</u> Results are expressed as mean±SEM, Duncan's Multiple Range Test, n=16

At each stage of larval development, fig. with common alphabet does not differ significantly.

Table 3. Overall percentage of unhatched eggs irrespective of stages of the eggs after treatment of three concentrations each of JH-III, 20-hydroxyecdysone and cAMP.

Results are expressed as mean±SEM,

	Percentage of unhatched eggs		
	JH-III	20-hydroxyecdysone	cAMP
Control	25.36±1.50	22.48±2.52	24.62±2.60
1 µg	70.93±5.79	14.74±2.84	18.60±2.97
0.5 µg	65.54±5.97	11.61±2.66	15.80±2
0.25 µg	61.50±6.24	8.47±1.36	16.43±1.27

Fig 2. Percentage of unhatched eggs after treatment with three concentrations of JH-III on different stages of egg development of *Philosamia ricini*. Each bar represents the mean ± SEM, *n*=4

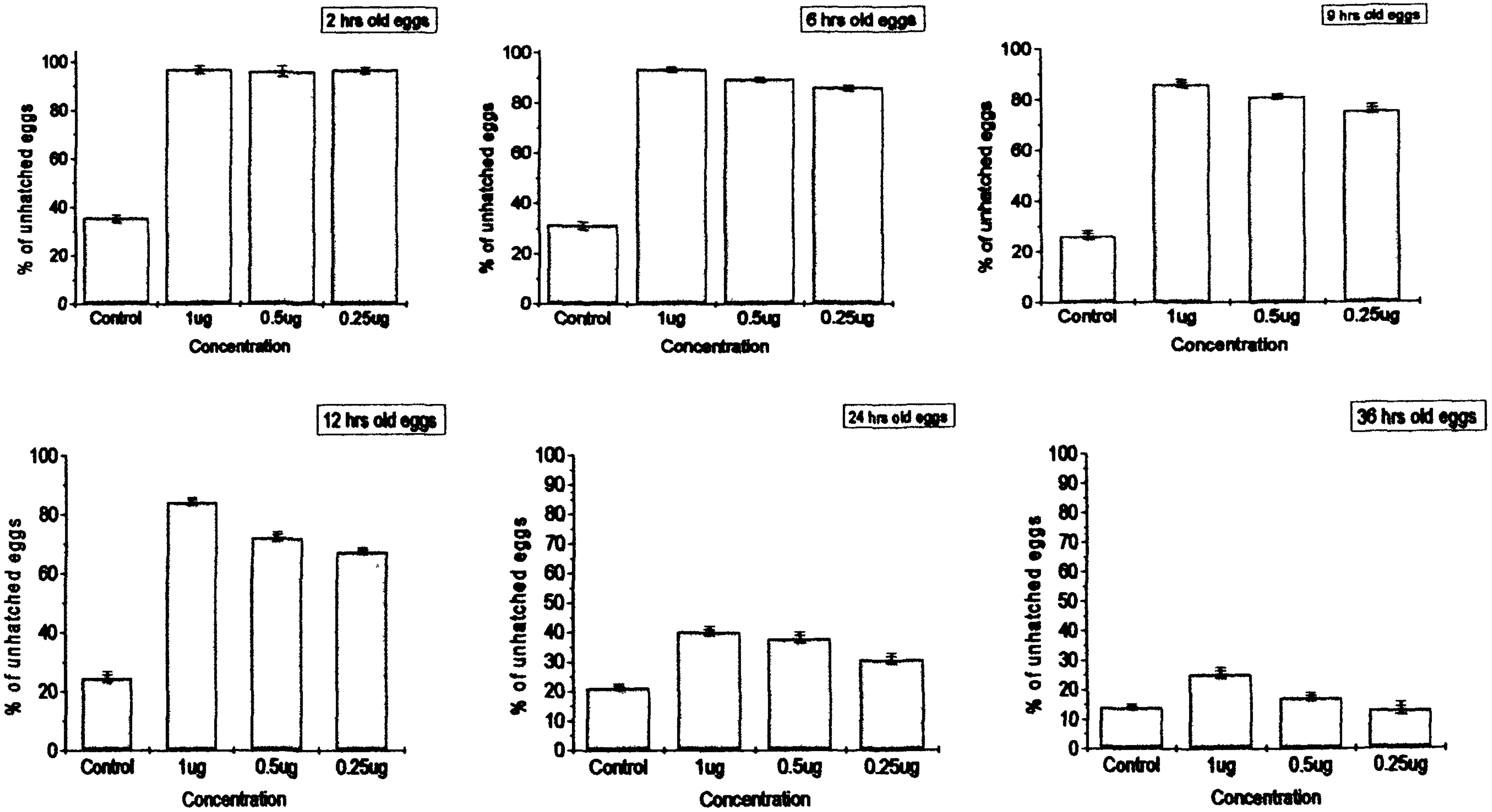


Fig 3. Percentage of unhatched eggs after treatment with three concentrations of 20-HE on different stages of egg development of *Philosamia ricini*. Each bar represents the mean ± SEM, n=4

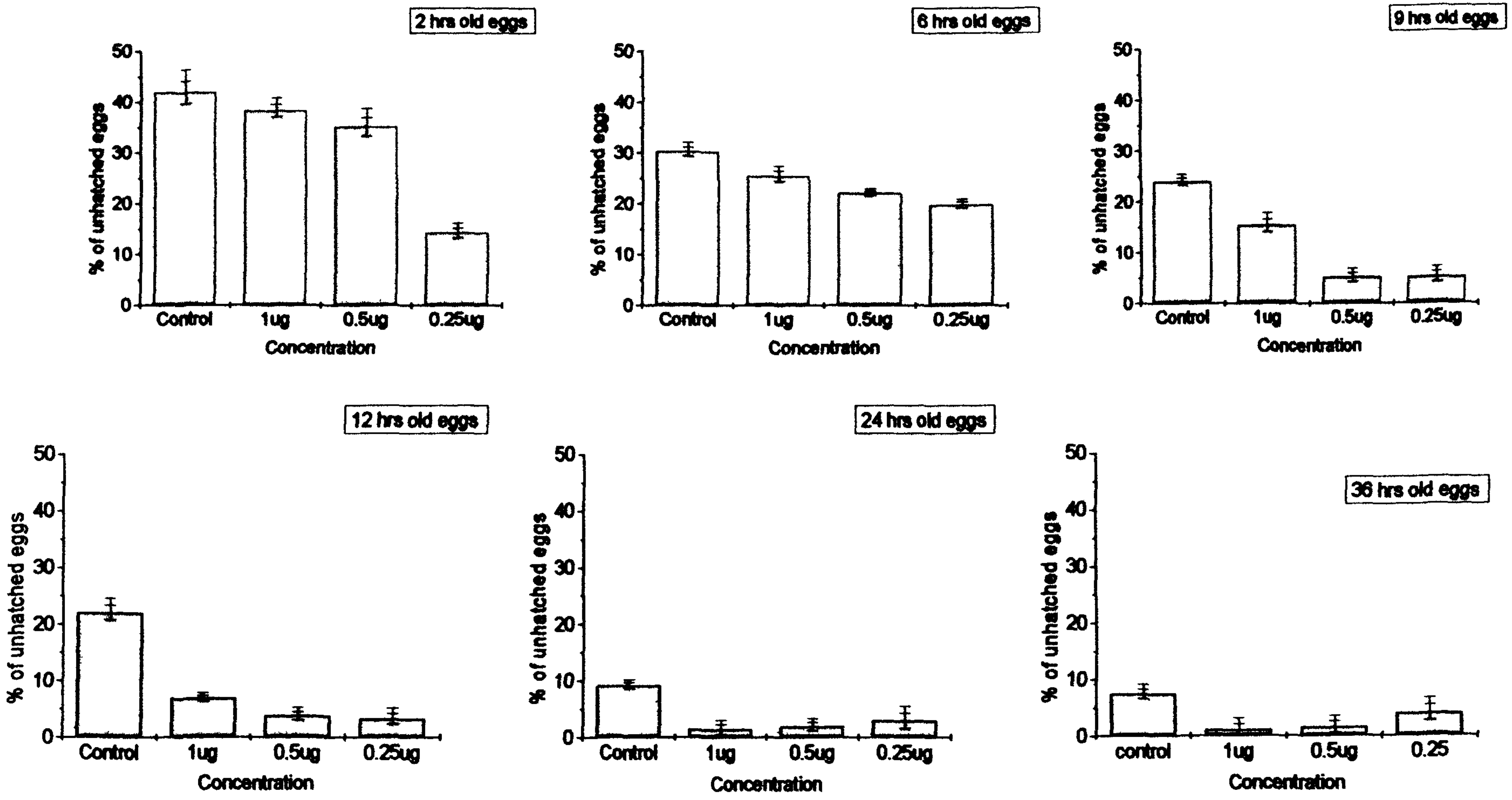


Fig 4. Percentage of unhatched eggs after treatment with three concentrations of cyclic AMP on different stages of egg development of *Philosamia ricini*. Each bar represents the mean ±SEM, n=4

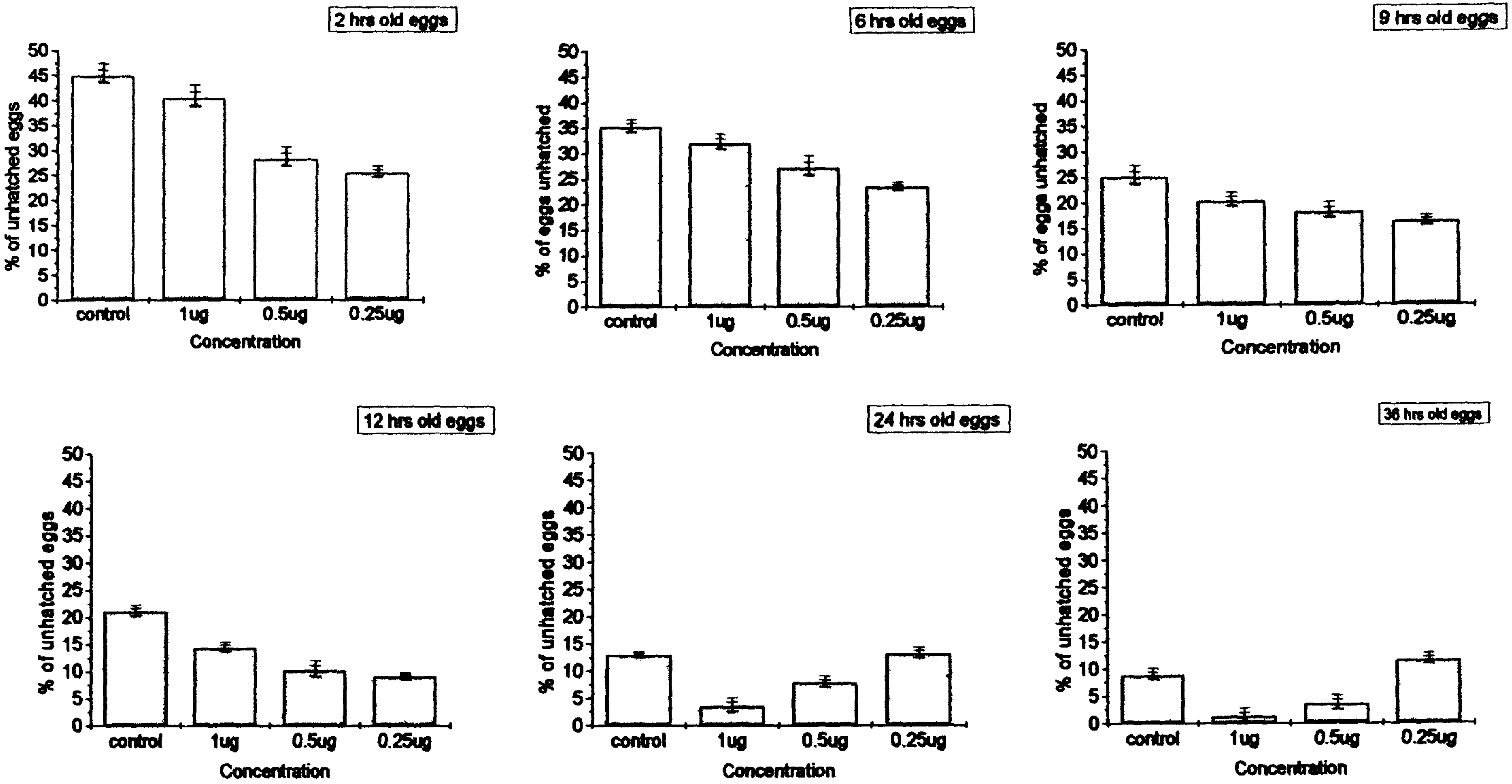


Fig 5. Comparative effect of three concentrations each of JH-III, 20-HE and cAMP. Each bar represents the mean ± SEM, *n*=4.

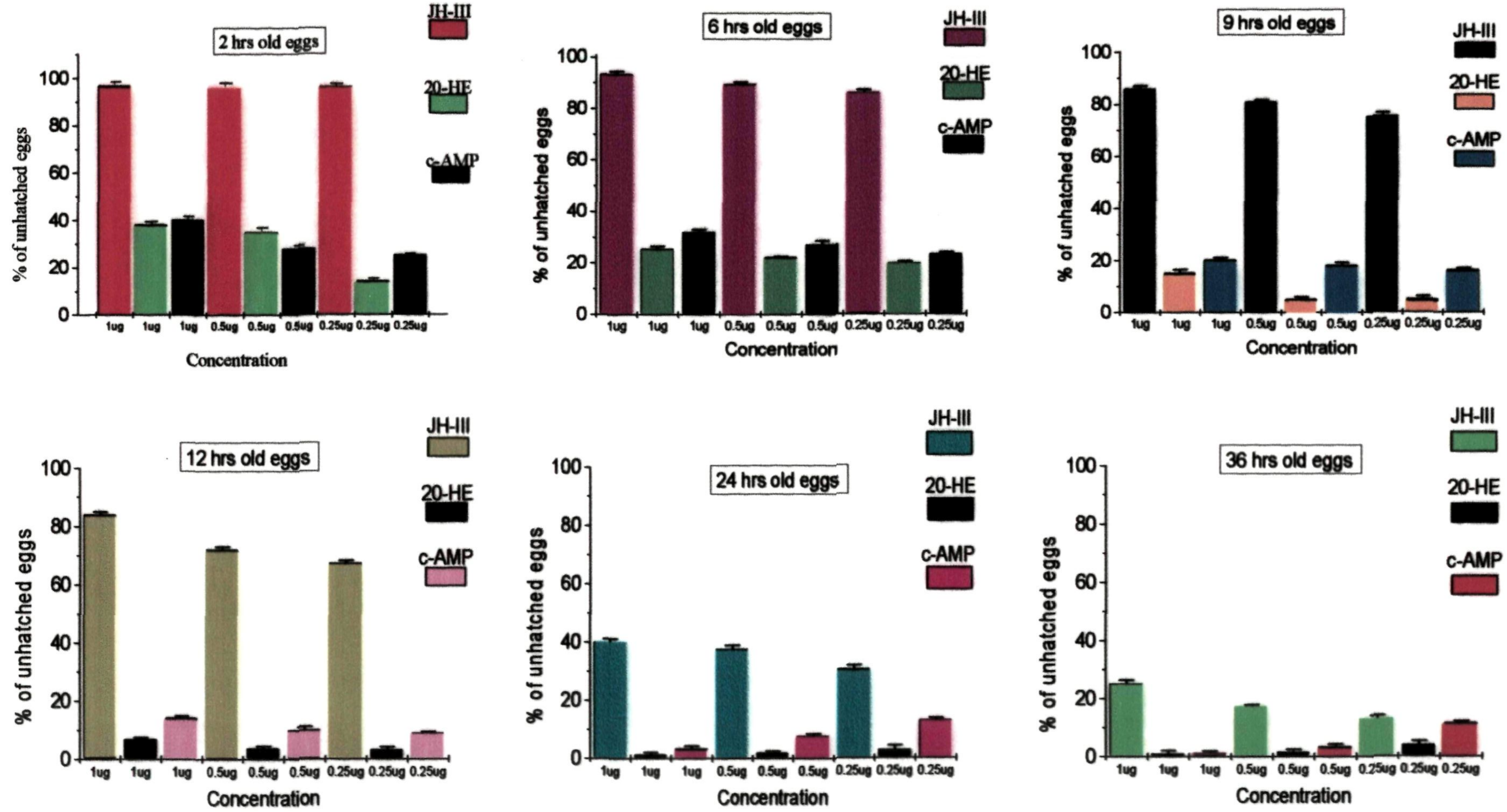
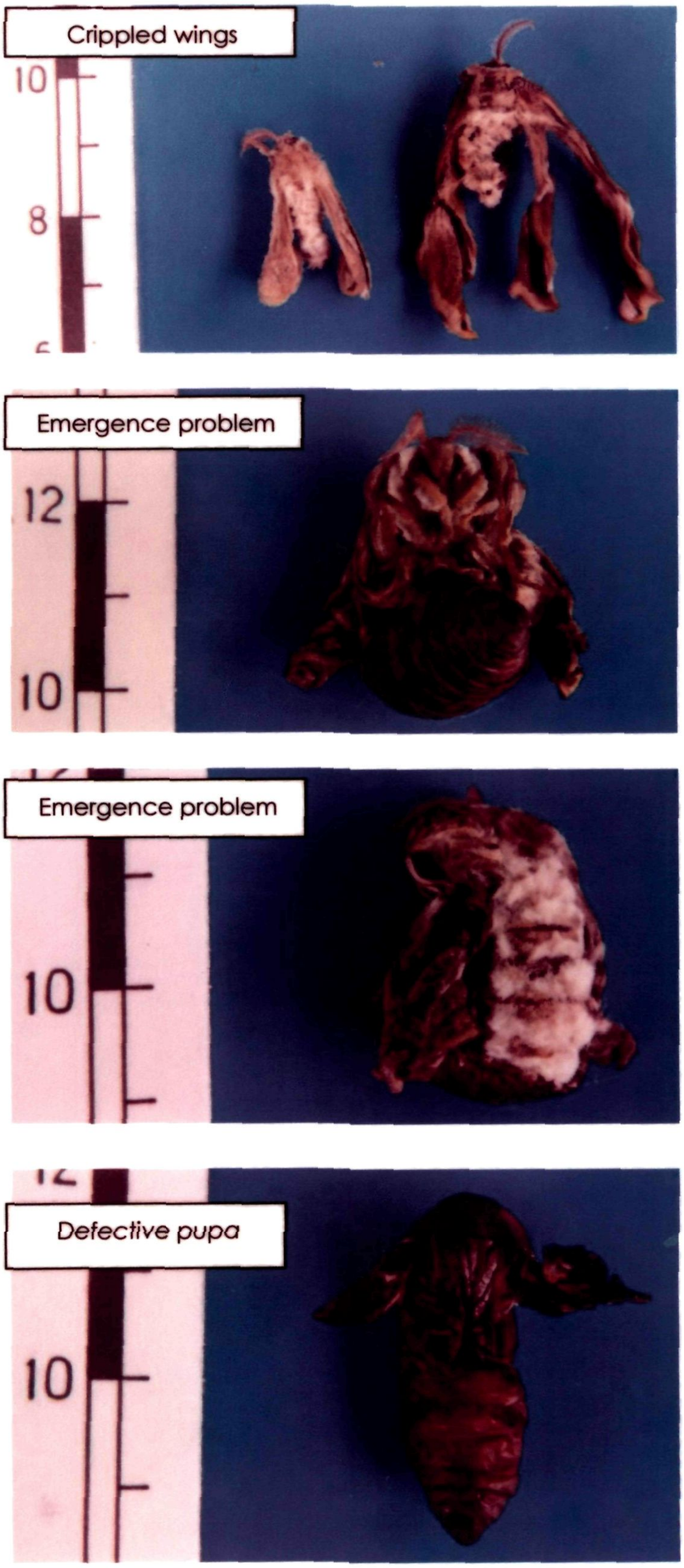


Fig 6. JH induced defective adult development.



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CHAPTER – 2

1. Ultra Structure of chorion membrane.
2. Mechanism of egg hatching process

Chapter - 2

INTRODUCTION

1. Structure of chorion membrane

For studying the involvement of endocrine factors (hormones) in insect's egg development, the knowledge of the chorion morphology is of prime importance. Insect eggs are typically large relative to the size of the females that produce them because they contain a great deal of yolk. It is generally believed that the eggs of endopterygota contain less yolk and are smaller than those of exopterygota (Anderson, 1972). To some extent the size may depend on the types of ovariole they possess. However, egg size is also affected by factors other than the type of ovariole. Amongst Lepidoptera from temperate regions, species overwintering in the egg stage have larger eggs than species that overwinter in some other stage, and species feeding on woody plants have bigger eggs than those feeding on herbaceous plants (Reavey, 1992). Individual females of at least some butterflies lay smaller eggs as they grow older, and females of the comborer moth, *Ostrinia nubilalis*, lay smaller eggs if they do not receive adequate nutrition (Leahy and Andow, 1994).

Insect eggs have a wide variety of forms. Commonly, as in Orthoptera and many Hymenoptera, they are sausage shaped but they can be conical as in *Pieris*, or rounded, as in many moths and Heteroptera. The eggs of Nephidae and some Diptera have respiratory horns (Hinton, 1961), while the eggs of many parasitic Hymenoptera have a projection called a pedicel at one end. The eggs of *Encyrtus* (Hymenoptera) are unusual in consisting of two bladders connected by a tube (Hinton, 1981).

The chorion of insect egg is produced by the follicular cells while the egg is in the ovary and is unique in its structure. The outer surface of the chorion is often sculptured, frequently with a basically hexagonal pattern reflecting the shapes of the follicle cells which secrete it. Outside this, grasshoppers have an additional layer, the extrachorion which differ from the chorion in being produced by gland cells in the oviducts rather than by the follicle cells.

The outer covering of the egg of the moth, *Micropteryx*, is unusual in consisting of a forest of knobbed projections. These are produced in the hour following oviposition by exudation from the oocyte itself. The exudate forces the viscous outer layer of the shell, produced by the female accessory glands, outward to form the projections (Chauvin and Chauvin, 1980).

Because the chorion is laid down in the ovary, some provision is necessary to allow the subsequent entry of sperm. This takes place in the form of micropyles, which are funnel shaped pores passing right through the chorion, usually near the anterior pole of the egg as in *Samia cynthia ricini* (Kawaguchi *et al.*, 2000).The pores are usually 1-2 µm in diameter but variable in different insects, often with a wider funnel at the surface of the chorion. The number of micropyles varies in different insects, in dipteran eggs only one micropyle is present, in Acrididae it is 30-40 (Roonwal, 1954), while in different species of Corcidae the number varies from 4-60 and pentatomids it is 10-70. The petal number surrounding the micropyle are also variable in number *viz.* 12-20 in *Samia cynthia ricini* (Kawaguchi *et al.*, 2000), 9-11 in *Bombyx mandarina* and 8-13 in *Bombyx mori* (Kawaguchi *et al.*, 1996a).

Gaseous exchange occurs via the extensive airspaces called aeropyles that are usually present in the chorion of most terrestrial insects. These aeropyles are located in the chorion adjacent to the oocyte and, in species that lay their eggs in moist environments, these airspaces may extend through the entire thickness of the chorion. The layer of air in the inner chorion has direct access to the ovum through pores in the innermost layer of the chorion and connects with the outside air via aeropyles (Hartley, 1962). In many other species, the connections are even more restricted. In *Calliphora* (Diptera), the outer meshwork and aeropyles connecting with the inner meshwork are absent over the greater part of the egg, and are present only between the hatching lines (Hinton, 1960; Anderson, 1960). Eggs of *Ocypus* (Coleoptera) have an equatorial band of aeropyles connecting with the inner airspaces, *Rhodnius* eggs have a ring in aeropyles just below the cap (Beament, 1946), and those of *Carausius*, have a single small pore at which the reticular inner chorion is exposed at the egg surface. The respiratory horns of some Diptera and the Nepidae (Hemiptera) serve the same function of connecting the inner layer of air with the atmosphere outside, while at the same time restricting the area through which rapid loss of water can occur (Hinton, 1961). Where only a single aeropyle is present, as in the beetle, *Callosobruchus*, its size is critically important for gaseous exchange (Daniel and Smith, 1994). Eggs of some terrestrial insects are also modified to counter periodic flooding by maintaining a layer of air around the egg so that the chorion become hydrophobic in nature and the gas from the surrounding water may diffuse. Thus the chorion also acts as a plastron.

The chorion thus serves as the reproductive machinery until hatching, allowing sperm entry, protecting the embryo from environmental hazards such as temperature fluctuation, excess humidity and desiccation and microbial invasion, while ensuring an adequate oxygen supply and carbondioxide discharge. These rather paradoxical events are achieved by the highly ordered architecture of the chorion, which has openings of elaborate size and shape, penetrating the chorion layers (Hinton, 1970; Margaritis, 1985). The morphological features of the chorion have been analysed on lepidopteran insects such as the domesticated silkworm (Akai, 1957, 1976; Kanda *et al.*, 1974; Katsuno, 1984a, b; Kawaguchi *et al.*, 1993), the wild silkmoth, *Bombyx mandarina* (Omura and Kataoka, 1943; Sakaguchi *et al.*, 1990, 1998; Nho, 1990; Kawaguchi *et* *al.,* 1996a, b), the wild silkmoths, *Antheraea polyphemus* (Mazur *et al.*, 1980, 1982, 1989; Regies *et al.*, 1982), *Hyalophora cecropia* (Smith *et al.*, 1971) and the muga silkworm, *Antheraea assama* (Dey *et al.*, 2003). These achievements have indicated that the chorion structures in terms of either the surface pattern made of the micropyle, the variable number, structure and size of aeropyles and polygonal network or the internal structures including the sperm ducts and lamellar layers are more or less variable from species to species, reflecting the taxonomic diversity of the lepidoptera. This situation provides a rationale for additional comparative analyses of the chorion structures in different species.

2. Mechanism of egg hatching process

Most insects force their way out of the egg by exerting pressure against the inside of the shell. The insect increases its volume by swallowing the extra-embryonic fluid and in some cases by swallowing air which diffuses through the shell. Then, waves of muscular contraction pump haemolymph forwards so that the head and thoracic regions are pressed tightly against the inside of the shell. In grasshoppers, and perhaps in other insects, these muscular waves are interrupted periodically by a simultaneous contraction of the abdominal segments which causes a sudden increase in pressure in the anterior region. These sudden contractions ultimately ruptures the shell as in *Schistocerca gragaria* (Bernays, 1972a, b).

The position of the rupture generally depends on where the insect puts pressure on the chorion. In grasshoppers, the chorion is split transversely above the ampullae; in the water beetle, *Agabus*, the split is longitudinal, while in some species it is variable in position. The chorion of some species has a line of weakness along which it splits. The egg of *Calliphora*, has a pair of longitudinal hatching lines running along its length (Anderson, 1960; Hinton, 1960) and in Heteroptera a hatching line runs round the egg where the cap joins the body of the chorion (Beament, 1946). In eggs of *Aedes*, there is a line of weakness in the serosal cuticle and a split in the chorion follows this passively, perhaps because the serosal cuticle and chorion are closely bound. In species with a thick serosal cuticle, such as Arididae and Hetroptera, hatching is aided by an enzyme that digests the serosal endocuticle before hatching begins.

Cuticular structures known as egg bursters also aid hatching in a number of insects. These are usually on the head of the embryonic cuticle of Odonata, some ortoptera, Heteroptera, Neuroptera and Trichoptera, but are on the cuticle of the first stage larva in Nematocera, Carabidae and Siphonaptera. These cuticular structures may be in the form of different shaped central tooth *viz.* row of spines in *Cimicomorpha* (Southwood, 1946), pair of spines (sucking lice), cuticular tooth (fleas and mosquitoes). In *Agabus*, the egg-buster is a spine on either side of the head, but many Polyphaga have egg-busters on the thoracic or abdominal segments of the first stage larva. The larva of *Dacus* (Diptera) uses its mouth hooks repeatedly protruding them until they tear the chorion. The blades in lice and the spines in *Cimex* are used to tear the vitelline envelope, the chorion then being broken by pressure (Sikes and Wigglesworth, 1931).

Special muscles which assist the hatching process are known to occur in some insects. They usually break down soon after hatching.

In lepidoptera, the larva gnaw their way through the chorion and after hatching, continue to eat the shell until only the base is left. In *Pieris brassicae*, where the eggs are laid in a cluster, newly hatched larvae may also eat the tops off adjacent unhatched eggs (Chapman, 1998).

In the present study, we have focused our attention on the ultra surface structure of the chorion membrane and the mechanism of egg hatching of *Philosamia* *ricini*, by using binocular microscope and scanning electron microscope (SEM). It will also be of interest to examine whether there are surface changes in chorion morphology during development.

MATERIALS AND METHODS

Insects.

Philosamia ricini silkworm maintained at the Sericulture farm, Nongpoh, was used for the experiment. Larvae were brought to the laboratory and reared on fresh castor leaves (*Ricinus communis*) under natural photoperiod. Shortly after emergence, female moths were allowed to copulate with males for 12 h and then to oviposit in the dark. Eggs to be examined were collected at the same time (10 AM) every day until hatching. After every collection the eggs are washed thoroughly with distilled water, blotted and air dried. Then they are processed further for Scanning Electron Microscopy. All procedures were performed at 25°C.

Observation of chorion by Binocular and Scanning Electron Microscope.

Whole eggs were fixed in Neutral buffer formalin solution for 4 to 5 days at 4°C and observed for overall appearance under a binocular microscope. Alternatively, whole eggs were dehydrated in a graded ethanol series, freeze dried with *tert*-butyl alcohol, mounted on a stub with double-stick carbon tape, sputter coated with gold and observed for surface morphology with Scanning Electron Microscope. The egg dimensions (lengths of major and minor axes and surface area) and the unit areas of the polygonal network patterns were measured using photographs as described by Kawamura and Nakada, (1981) and Kawaguchi *et al.* (1996a). Each value was the average of 40 eggs from egg batches deposited by five moths (15 eggs per batch). For calculation of the average polygonal unit area and aeropyle diameter, 150 and 30 units were arbitrarily chosen from the eggs respectively.

RESULTS

The egg of *Philosamia ricini* is oval or laterally flattened ellipsoid; freshly laid eggs are candid white while the chorion is colorless and semi-transparent. The lengths of the major and minor axes were 1.67±0.25 mm and 1.41±0.12 mm (mean ±SD, N=40), respectively while the diameter is 1.37± 0.22mm and the weight of the egg 6. 018 ± 0.34 g (Table 4).

The surface of the chorion is covered with a network pattern made of fairly uniform, mostly hexagonal (occasionally penta- or heptagonal) units. These network pattern are polygons, which are imprints of the follicular epithelial cells that secrete the chorion proteins. The polygonal shape was common to the whole surface region. The boundaries between polygons made ridges, which, corresponding to the junctions of follicular epithelial cells, had distinct acropyles at three-cell junctions. Each polygon can be said to have aeropyles at each ridges so that there are six aeropyles in a hexagonal structure, five in pentagonal, seven in heptagonal and so on. There is variation in the unit area of the polygon according to their location in different region of the eggs. The unit area for the anterior and posterior pole regions are 407.2±45.3 and 455.7±82.3μm^2 (mean ±SD, N=150), respectively, whereas 561.4±33.1 and 448.5±72.25μm^2 (mean ±SD, N=150) for the lateral flat and marginal regions, respectively (Table 4).

During the course of development from day-3 until hatching, no significant structural changes were observed in either the polygonal structures and the overall morphology of the egg (Fig 7-17). Line of weaknesses which are usually present in some insects' eggs are not observed even at time of hatching or close to it. However, the size of the pores or aeropyles kept on changing as the egg matures. At day-3 of egg development the aeropyle size was 0.84 ±0.33 µm then it increases to 1.3±0.10 µm and 1.56 ±0.22 µm in day-5 and day-7 respectively. At day-8 (1.58 ±0.20 µm) the aeropyle size is almost the same as that of day-7 but then it increases again to 1.875±17µm in day-9 which is the maximum size attained during all the days of egg development. After day-9, the aeropyle decreases to 0.85±0.15 µm (day-12) and to 0.97±0.33 µm (mean ±SD, N=30) at the time of hatching (Table 4).

From our observation we found that the hatching process of the newly emerge larvae are through gnawing (Fig. 17). The larva eats their way out through the chorion membrane mostly from the anterior region but they may also emerge from lateral or any other sites of the egg. Egg buster or spine which aid in hatching are not present in the newly emerge larvae.

DISCUSSION

The chorion of lepidopteran insects has spatially and morphologically differentiated surface regions. In the present study the chorion of *Philosamia ricini* was observed to lack the aeropyle crown region. In this respect the chorion of this species was fundamentally similar to those of *B. mandarina* and *B. mori* (Kawaguchi *et al.*, 1996a) and *Samia cynthia ricini* (Kawaguchi *et al.*, 2000). In the chorion surface of the mature *A. polyphemus* reveals four clear-cut regions distinguished by differences in cell imprints, i.e. flattened sides, aeropyle crown region, meridional stripe region and miropyle region (Mazur *et al*, 1982). Also in *B. mori* (Ohtsuki *et al.*, 1977a, b; Kawaguchi *et al.*, 1993) and *B. mandarina* (Kawaguchi *et al.* 1996a, b) four distinct regions, the posterior and anterior poles, lateral flat sides and ventral (dorsal) edges, are apparent, although there is no aeropyle crown region.

The egg size was larger in *Philosamia ricini* in term of length of major and minor axis and the area of lateral flat region than those of other lepidopteran insects *viz. B. mori* and *B. mandarina* (Kawaguchi *et al.*, 1996a, b). The number of petals surrounding the external micropyle in *Samia cynthia ricini* (Kawaguchi *et al.*, 2000) was larger than in *Bombyx*, while the rosette pattern made by petals was found to be simpler in the former than in the latter, where in two-to four-petaled patterns predominate (Kawaguchi *et al.*, 1993, 1999). In the present study although the petals surrounding the micropyle are not detected, it is assumed that they may be similar to that found in *Samia cynthia ricini* as above. The polygonal network patterns especially at the lateral flat and marginal regions are regular, there is no significant variation in the unit area of the polygon exhibited. At the posterior pole, knobs residing inside the polygonal units are visible prominently.

The variability in the size of aeropyles during egg development is difficult to distinguish functionally. However, since they mainly serve for gaseous exchange between the egg and the outside environment, it may be that gradual increase in size at least up to the day-9 demands more oxygen uptake by the developing larva. Too small a pore will not permit the entry of an adequate supply of oxygen and the eggs of two strains of *Callosobruchus maculatus* with different metabolic rates have differently shaped aeropyles (Daniel and Smith, 1994). Again it is presumed that the decrease of the aeropyle size after day-9 up to the time of hatching may be brought about by the dryness of the egg as the fluid inside continue to dehydrate due to development or may be due to its prolonged exposure to dry environment. A very interesting case in the eggshell of Hawaiian species *D. grimshawi* which is unusually thick and possibly for that reason has developed numerous but very narrow aeropyles which might function during embryogenesis in dry environment (Margaritis, 1985). Thus the variability in the size of the aeropyles during egg development requires the insect to modify its egg structure according to the demand of the environmental condition.

The study in term of hatching of the larvae from the eggs revealed that they gnawed their way out through the chorion membrane in accord with the process adopted by all larval lepidopteran insects (Chapman, 1998). For the process of hatching there are no rupture of egg neither from line of weaknesses nor presence of egg buster or specialize spine or cuticle.

In the present study, the overall structure of the egg during the course of development until hatching was not observe to change except for the minute pores or aeropyles present on the ridges of the entire polygonal network.

Table 4. Description of overall measurement of egg of *Philosamia ricini*.

Site	Measurement	
*Length of egg (Chorion surface)	Major axes	1.67±0.25 mm
	Minor axes	1.41±0.12 mm
	Diameter of egg surface	1.37±0.22 mm
*Whole egg	Weight	6.108±0.33 mg
** Unit area of polygonal structures	Anterior	407.2±45.3 μm^2
	Posterior	455.7±82.5 μm^2
	Lateral flat region	561.4±33.1 μm^2
	Marginal region	448.5±72.25 μm^2
***Aeropyle diameter	Day-3	0.84 ±0.33 µm
	Day-5	1.3±0.10 µm
	Day-7	1.56 ±0.22 µm
	Day-8	1.58 ±0.20 µm
	Day-9	1.875±17µm
	Day-12	0.85±0.15 µm
	Day-13	0.97±0.33 µm

*mean±SD, N=40; ** mean±SD, N=150; *** mean±SD, N=30

Fig 7. Chorion morphology at day 3.

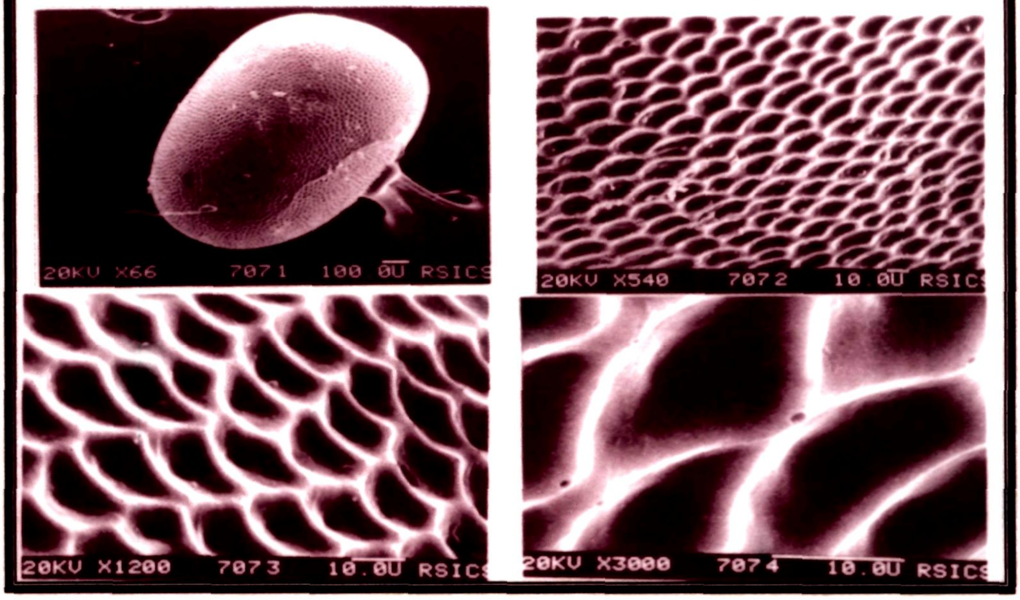


Fig 8. Chorion morphology at day 4.

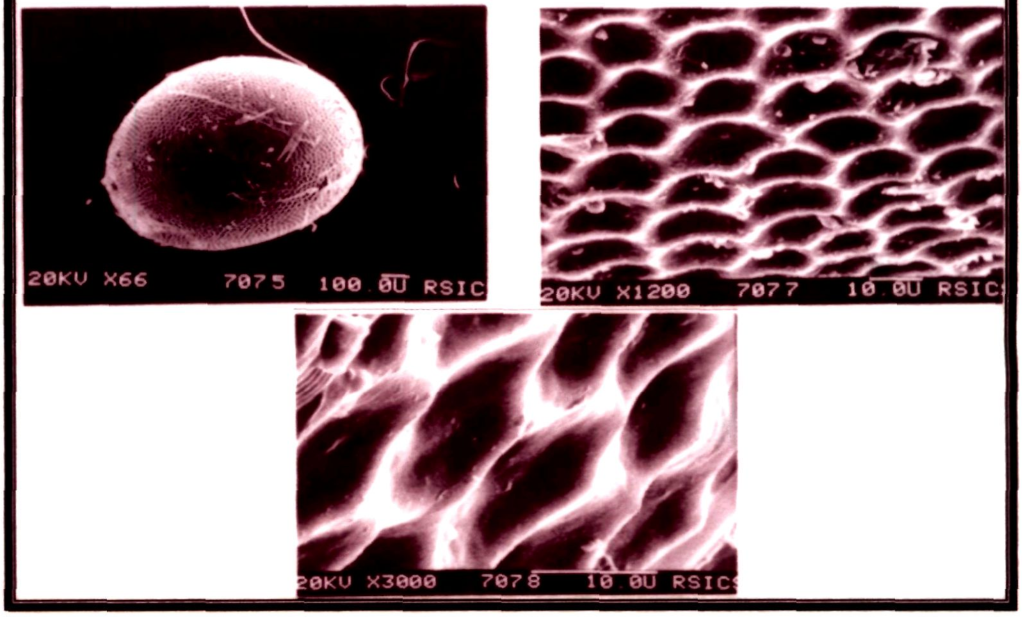


Fig 9. Chorion morphology at day 5.

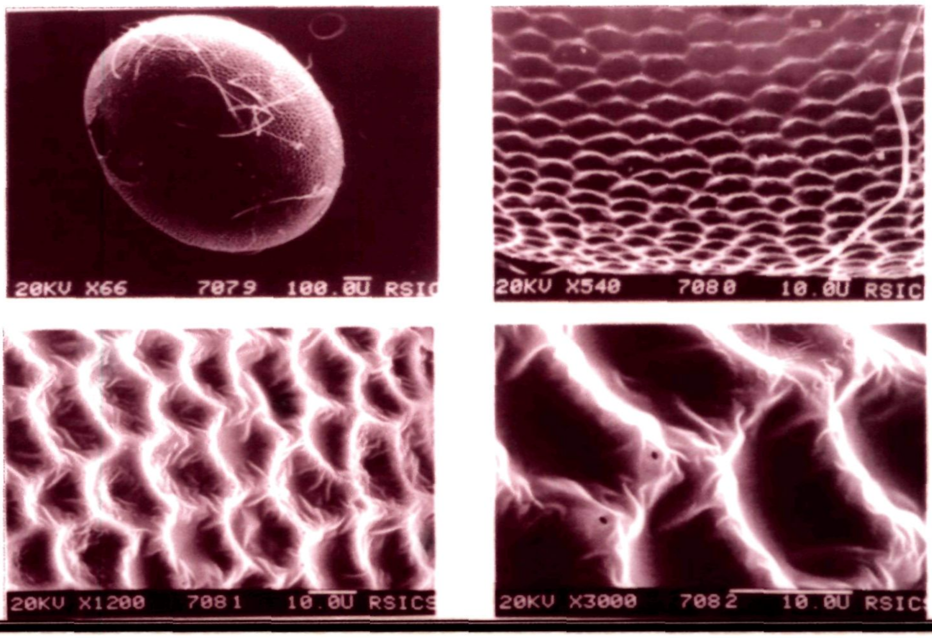


Fig 10. Chorion morphology at day 6.

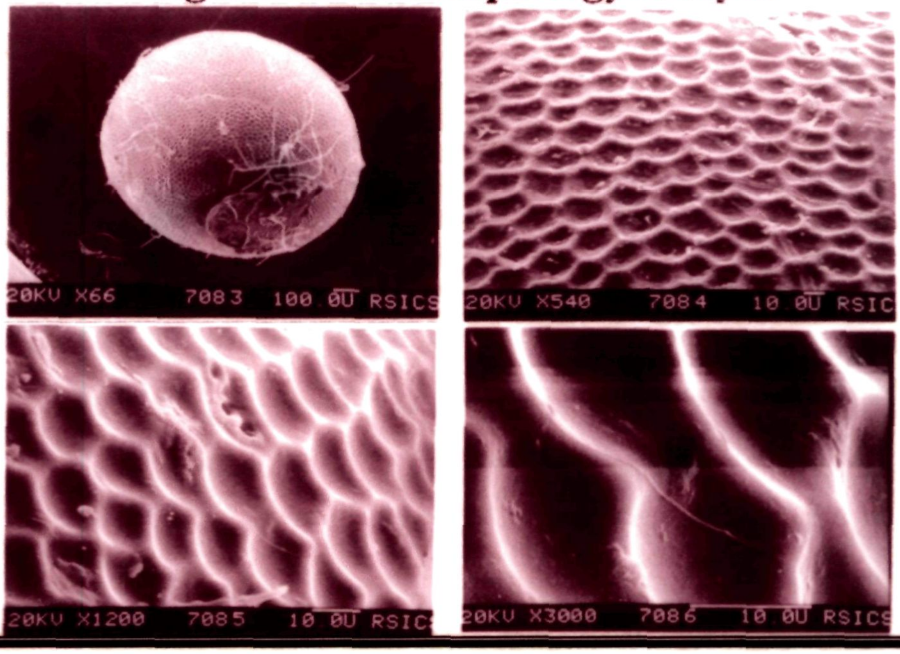


Fig 11. Chorion morphology at day 7.

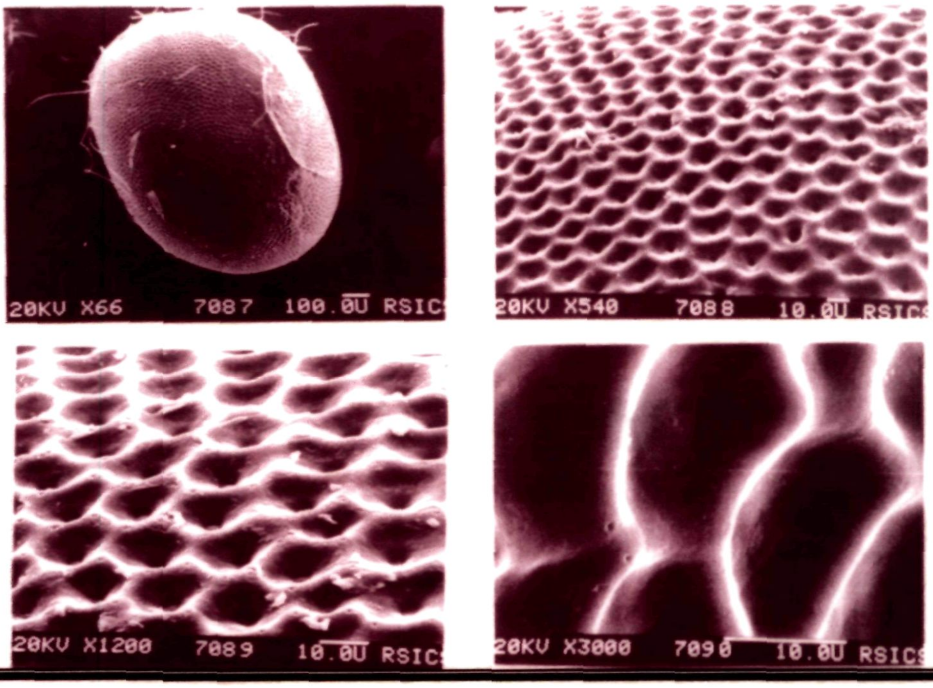


Fig 12. Chorion morphology at day 8.

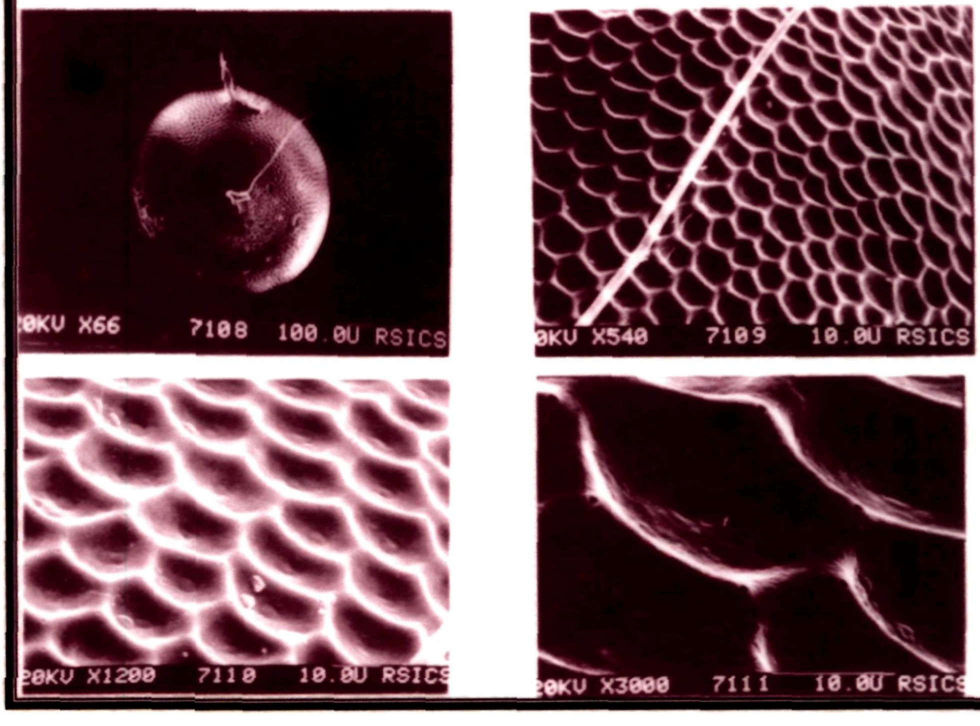


Fig 13. Chorion morphology at day 9.

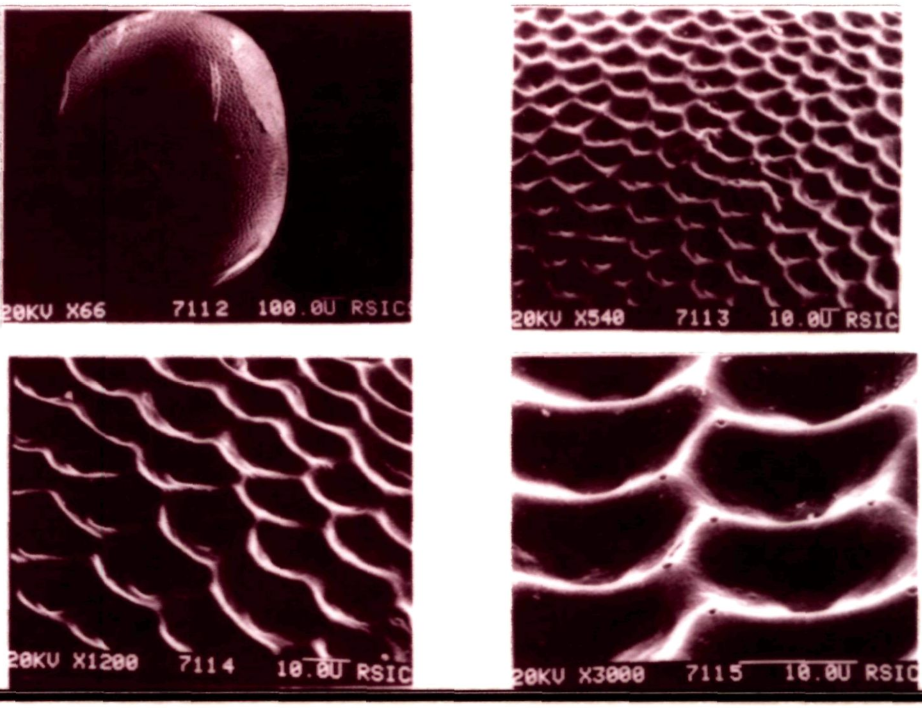


Fig 14. Chorion morphology at day 11.

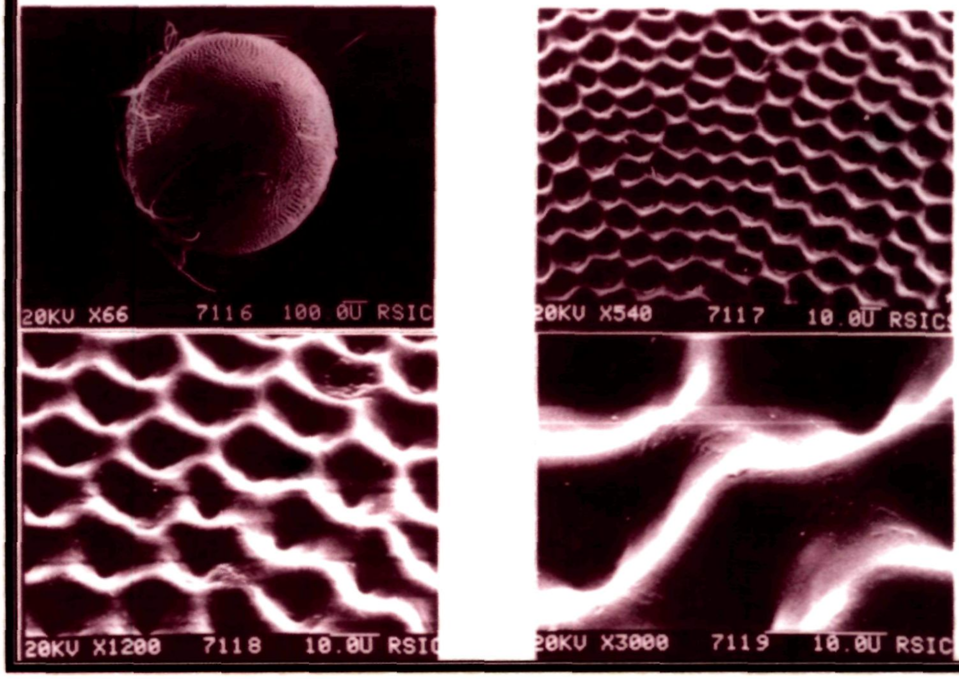
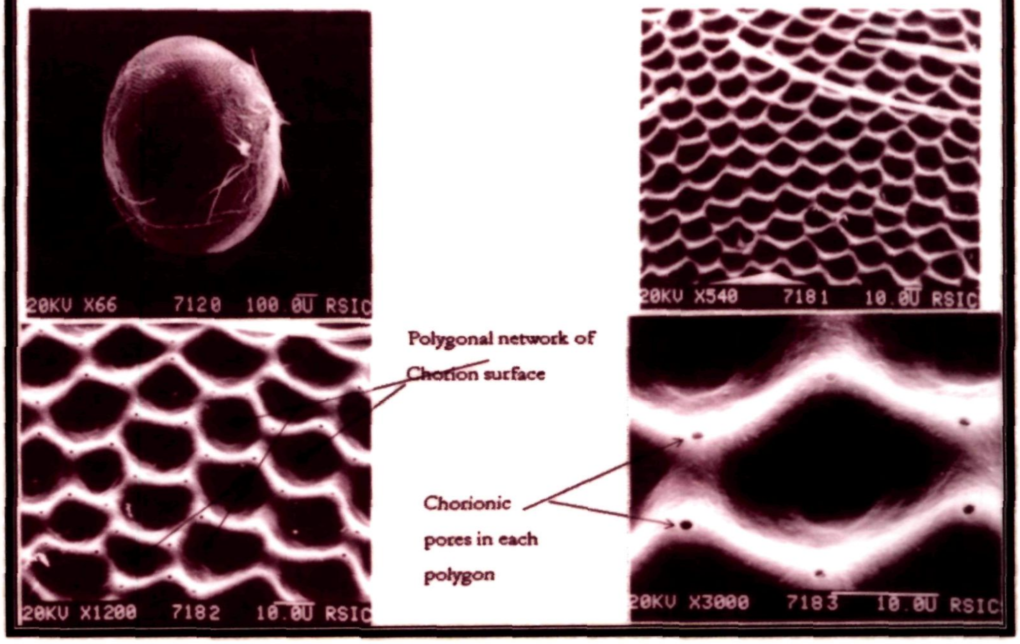


Fig 15. Chorion morphology at day 12.



Polygonal network of Chorion surface

Chorionic pores in each polygon

Fig 16. Chorion morphology at day 13.

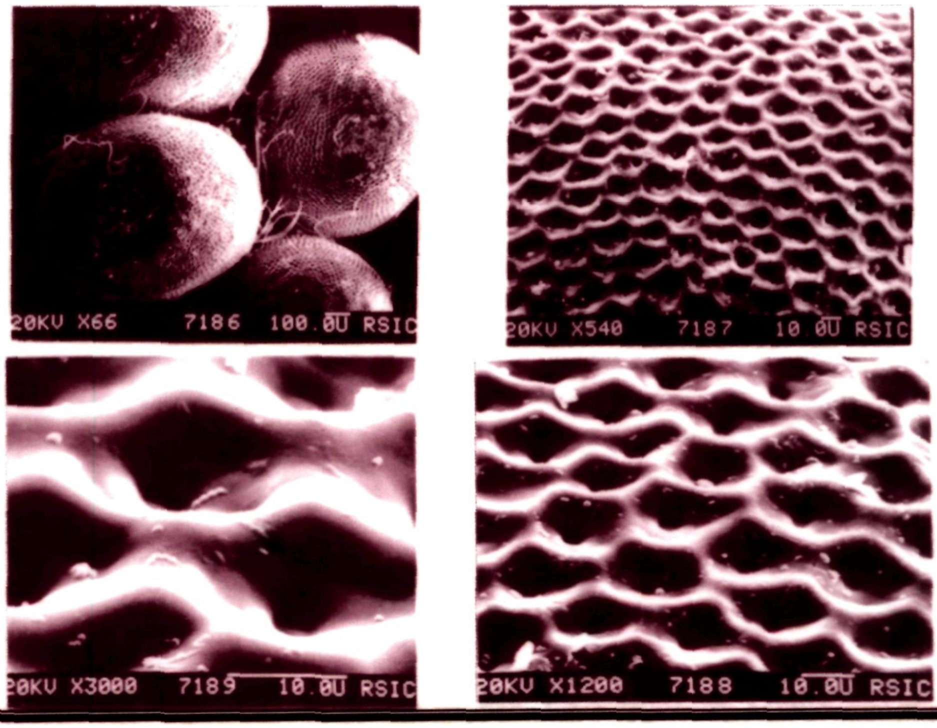
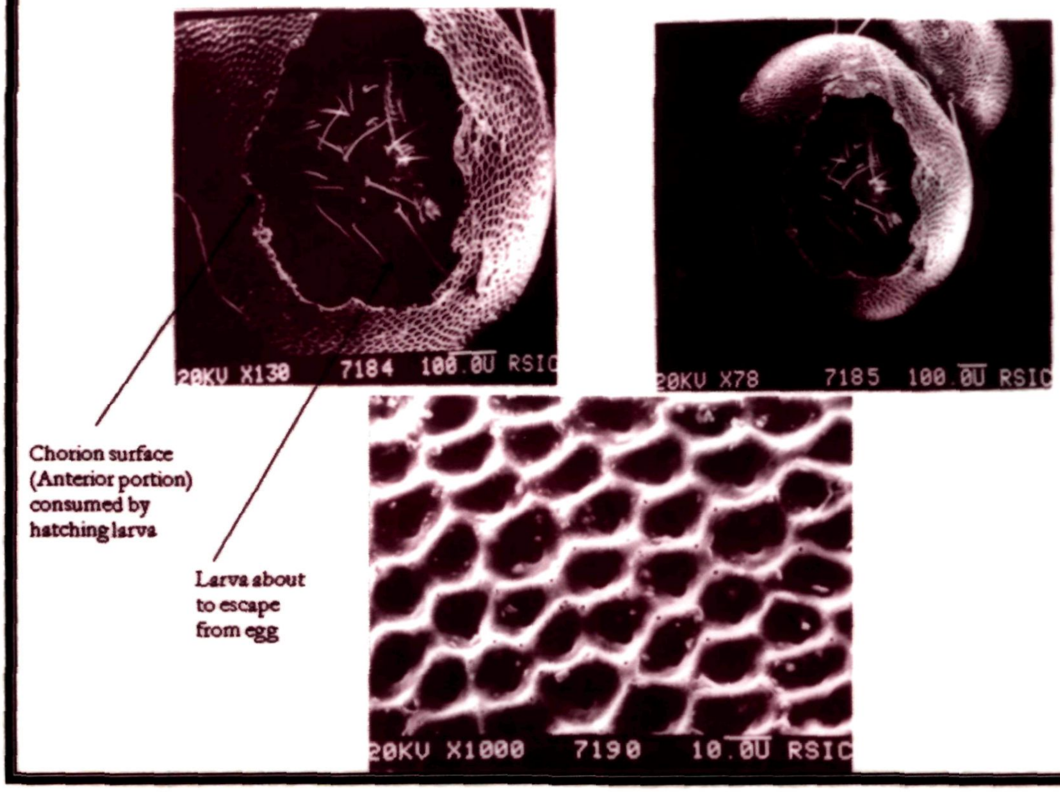


Fig 17. Chorion morphology at hatching (day 13)



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CHAPTER – 3

Hormonal influence underlying Pupation by ligation

Chapter – 3

INTRODUCTION

The onset of a molting cycle and the initiation of metamorphosis are intermediate events in the life of most and perhaps all insects. Both processes are tightly linked to growth and nutrition; insect do not molt by clock, nor do they count their instars to determine when they will metamorphose, even though under optimal growing conditions the molting cycle occurs with predictable regularity, and the time of metamorphosis can often be predicted to the nearest day. Every molting cycle, whether it is a larval, pupal, or an adult molt, begins with the secretion of (prothoracicotropic hormone) PTTH by the brain. PTTH stimulates the secretion of the molting hormone, ecdysone, and it is the action of ecdysteroids on the epidermal cells that is responsible for stimulating the biochemical and morphological events of the molting cycle (Nijhout, 1994).

The endocrine role of the insect brain was first recognized by Kopec (1917, 1922). The simple technique of dividing an insect into various parts by ligatures has serves as a powerful tool in the development of the field of insect endocrinology. The early studies of Kopec (1922), which involved placing ligatures around the necks of lepidopteran larvae, first indicated the importance of the brain to the molting process. Kopec (1917) studied the control of molting in the gypsy moth, *Lymantria dispar* and noted that when a ligature was placed around the middle of the body of early final instar larva, the anterior portion pupated normally but the posterior portion never did and concluded that something in the anterior half of the body was needed for normal metamorphosis. Removal of the brain from a mid-final instar larva likewise inhibited pupation, but removal of the subesophageal ganglion did not. From this experiment

Kopec concluded that the brain was the source of the metamorphosis-stimulating factor, and that the brain did not exert this effect via nerves, since removal of the subesophageal ganglion effectively severed the nervous connections between the brain and the rest of the body.

The endocrine role of the brain was confirmed by Wigglesworth (1934) in *Rhodnius* and *Williams* (1947) in silkmoth, *Hyalophora cecropia*. The role of the prothoracic glands in supplying the insect hormone (ecdysone) was later shown by experiments, which consisted in ligaturing caterpillars between the thorax and abdomen (Fakuda, 1944; Williams, 1952). When ligatures were applied before the time of prothoracic gland activity, the abdominal compartment subsequently showed no signs of molting. When ligated after this critical period, however, the abdomens underwent a complete molt. A mixed response was often seen when ligatures were applied during the time of prothoracic gland activity (Fakuda, 1944). In such abdomens part of the epidermis retracted and secreted a new cuticle. This type of mosaic response was sometimes also seen when isolated abdomens were induced to molt by injection of ecdysone (Sehnal, 1972). In *Manduca sexta*, abdomens isolated during the period of ecdysone secretion show a similar mosiac molt (Truman, 1972; Truman and Riddiford, 1974). Apparently at some point during their response to ecdysone, the epidermal cells become "competent" to secrete a new cuticle, at which time they become independent of any further influences of the prothoracic glands. The results obtained using *Manduca* showed that the epidermal cells become autonomous according to a reproducible, stereotyped pattern within an abdominal segment. Furthermore, this pattern of independence appeared to be related to the pattern of sensitivity to juvenile hormone (JH) in the same tissues (Truman, 1972).

One of the earliest and clearest demonstrations of the head critical period was proved by Wigglesworth (1934) who showed that in *Rhodnius* the molt became independent of the head about three days after the blood meal in fourth instar larvae and about seven days after the blood meal in fifth instar larvae. It is assumed that PTTH secertion in *Rhodnius* begins very soon after the blood meal because ecdysteroid titers in the hemolymph begin to rise shortly after a meal is taken (Steel, *et al.*, 1982).

The prothoracicotropic hormone (PTTH) is a brain neurosecretory peptide of insects that stimulates the prothoracic glands to secrete ecdysteriod, thereby inducing molting and metamorphosis (Bollenbacher and Granger, 1985; Ishizaki and Suzuki, 1994; Gilbert, *et al.*, 1996). It is widely accepted that PTTH is released at specific times during insect development, signaling the transition of developmental stadia such as larval molting, larval-pupal transformation and adult development. However, little is known about the factor(s) and mechanism(s) that regulate the timing of PTTH secretion. The factor most extensively studied as a candidate for the regulator of PTTH secretion is the juvenile hormone in some lepidopterans including *Manduca sexta* (Nijhout and Williams, 1974 a, b; Safranek, *et al.*, 1980; Roundtree and Bollenbacher, 1986), *Spodoptera littoralis* (Cymborowski and Stolarz, 1979) and *Bombyx mori* (Akai and Kobayashi, 1971; Gu, *et. al.*, 1997; Mizoguchi, 2001).

In view of the above fact the present study will demonstrate the involvement of pupation factors/hormones responsible in bringing about events leading to pupation by ligation experiments. It will also be of interest to determine the time of ecdysis in relation ligations. The varied morphological changes attained during the transition from larval to prepupal development will also be studied and correlated to the anticipated hormonal secretions.

MATERIALS AND METHODS

Experimenting animals:

Philosamia ricini used in these experiments were received from, Sericulture Department, Nongpoh, Govt. of Meghalaya. Larvae were reared in a rearing cage indoor and supplied with diet of Castor leaves, *Ricinus communis*. The larvae were maintained at 25±0.5° C and RH 70-80% under 12L: 12D photoperiod regimen with 24:00 designating lights-on which is taken as the beginning of a new day (Truman, 1972). The age of 5th instar larvae was abbreviated to L5 day *n* where day 0 indicates the day of ecdysis to this instar. Post-feeding larvae were restaged from the day of gut purge (day 0) and their age was given in days after the purge (Fujishita and Ishizaki, 1982). To facilitate the start of spinning, larvae, shortly after wandering phase, were placed in a bamboo made mountage or 'Chandraki' of desirable diameter, a device used commonly in Eri culture.

In these experiments the proper staging of larvae was of paramount importance. The 5th instar larvae, the beginning of metamorphosis are marked by the cessation of feeding and the entry into the 'wandering stage'. An animal in this condition has purged its gut and yellow colour of the body has developed. These larvae were due to the secretion of PTTH and ecdysone during the preceding day (Truman and Riddiford, 1974). Therefore in order to identify the anticipated time of hormonal secretions, morphological and developmental changes following pupation, ligatures were done extensively at different times. For neck ligation, blood-tight ligatures were placed around the neck, just behind the head and for abdominal ligation, ligatures were done around the 2nd & 3rd abdominal segments. Transition from larval to post feeding prepupal stage was studied using gut purge as a marker. Mobility lost of prolegs of the anal and the 3rd to 6th abdominal segments were used to underline the endocrine events for the first and the second release of PTTH and ecdysone respectively (Fujishita & Ishizaki, 1981, 1982). All morphological and behavioral changes in the last larval instar including timing of gut purge and pupal ecdysis were recorded by observation.

RESULT

1. Changes in the morphology and behavior of the last 5th instar larva:

After continuous feeding for 6-8 days, the larva emptied the gut content with the excretion of massive fluid. This phenomenon of emptying fluid from their gut is known as gut purge (Nijhout and Williams, 1974a; Truman and Ridifford, 1974). During gut purging the head part will be tilted slightly upward accompanied by an abrupt and clear-cut release of fluid faeces. Later additional intermittent surges of 3-4 times in minute quantity follow and the whole process is completed within half an hour. After gut purge the larva will slightly change its color to light yellow (which is its natural color) the color prior to gut purge was due to the presence of fat (wax) particles embedded to its integument. Gut purging usually occur during photophase period before 12 noon. After few minutes, the larva move around in search of suitable place for spinning and this stage is known as 'wandering phase'. When the larva had settled down at the appropriate site, spinning start forthwith lasting 1-2 days. As spinning advances, there is remarkable weight loss and the morphology of the larva changes gradually. The first morphological change was observed in the anal proleg in which it losses its mobility initially and then later on became buried in the basal region (Fig. 20). Later, the ocellar contraction takes place and the prolegs of the 3rd – 6th abdominal segments lost their ability to contract and expand as did the anal proleg one day earlier. Insects which have lost the mobility in all the prolegs lie inside the cocoon assuming a typically prepupal form.

The lost of the mobility in the anal prolegs was used to underline the endocrine events that occur prior to pupation. Ligated larvae generally exhibited varying degree of gut purges, colour changes, body shrinkages and complete loss of mobility of the anal prolegs. Those animals which did not lose the anal proleg mobility never showed any signs of the prodromes leading to pupation. A direct correlation with the timing of ligation was observed in all the events that bring about pupation *viz.* the lost in the mobility of the anal prolegs. When ligation was done long after gut purge, the 3rd – 6th abdominal prolegs always showed lost of mobility while it was not so when ligation was done during the feeding stages. Thus, it can be assumed that, firstly, the functional degeneration of the anal prolegs are induced by hormones released during the feeding stage, though its completion is attained as late as day-2 after gut purge. Whereas, in the 3rd – 6th abdominal prolegs, the mobility loss can be assumed to be preprogrammed at much later time after gut purge. In order to determine the endocrine events underlying the secretions of PTTH and ecdysones responsible for pupal development the following experiments were done.

2. Timing of gut purge in relation to pupal ecdysis.

Transition from larval to post feeding prepal stage was studied using gut purge as a marker, since this process occur abruptly with clear-cut start which enable to record accurately the time of its action.

Larvae which had ecdysed to the 5th instar in the same day shows different timing of gut purge and pupal ecdysis. The time of gut purging for those larvae which underwent on day-5 and day-6 are confined only to the photophase regimens of their respective days i.e. within 12:00 noon. Pupal ecdysis occurred within the 17 hr period of day 9 and day-10 respectively i.e. larvae that had purged their gut content on day-5 pupated exclusively on day-9, while those which had purged their gut on day-6 pupated on day-10, thus forming a time interval of 4 days in between gut purge and pupal ecdysis (Fig. 18). This experiment apparently explained that pupal ecdysis is under the control of an endogenous circadian clock.

3. Anticipated first release of PTTH & Ecdysone. (Ligation during feeding stage at day 3, 5:00 hr onwards)

The loss of the anal proleg mobility was first observed when larvae were ligated at 5: 00 hr of day 3. The other signs of pupations *viz.* gut purge, change of body colour, initiation of wandering phase and body shrinkage also occurred (Fig. 19). The larvae could even spin a cocoon, however the intensity of spinning is greatly reduced (Fig. 23 A & B). Ligations at different intervals prior to 5:00hr of day 3 did not show any sign of pupation. Again, ligations applied at different times prior to 11:00 hr of day 4 show same result as that when ligation was done at 5:00hr of day 3 (Table. 5). The larvae lost the ability to contract and expand of the anal prolegs and thereafter they became buried in the soft distal part of the abdomen. However, these larvae never show any sign of formation of pupal cuticle other than the induction signs of pupation during their survival (Table. 5). Thus, first critical secretion of PTTH & ecdysone can be assumed to occur at day 3, 5:00 hr (during feeding) onwards and this is necessary for transition of feeding to post-feeding stage.

4. Anticipated second release of PTTH & Ecdysone. (Ligation during post-feeding stage at day 3, 4:00 hr onwards).

As mentioned previously, ligated larvae during feeding stage were unable to produce pupal cuticle. Accordingly, it was anticipated that the second step of PTTH and ecdysone secretions must be released in the post feeding stage following gut purge. Neck ligations were then performed at various times after gut purge to examine the 2nd critical release of PTTH and ecdysone that are responsible for formation of pupal cuticle.

When larvae were randomly ligated long after gut purge they pupated in nearly normal time, *i.e.* within 5 days after the gut purge. Ligations prior to 4:00 hr of day 3 show no sign of ocellar retraction, the first sign of pupal cuticle formation. However, when ligation was done at 4:00 hr day-3, pupal cuticle starts to form accompanied by ocellar retraction and the 3rd to 6th abdominal prolegs lost their mobility (Fig. 20 b) during their survival of 15-25 days. Observations similar to the above condition continue to occur when ligations were done at different intervals upto the 10:00 hr of day 3. Therefore it is assumed that second PTTH secretion begin at least 4:00hr thereon followed by the second ecdysone secretion (Fig.21).

5. Endocrine induced larval-pupal morphology by ligations.

a. Neck and abdominal ligations prior to gut purge during feeding stage: Neck ligated larvae results in the development of complete pupa identical to normal one except the head, where it resemble the old larval character. This head could not tuck in for further development due to blockage by ligating thread. Cuticle is sclerotized and tanned. (Fig. 24. A & B). However, abdominal ligation, showed interesting larval-pupal structures. The anterior part of the body bear a typical pupal character whereas the lower posterior region still retained the larval character of the last day 5th instar (Fig. 22. a, b & c). The larva however, continue its developmental processes by undergoing spinning while the ligated thread is still intact (Fig. 23. A & B). The most observable larval characters are the abdominal segments with their appendages.

b. Ligation long after anticipated hormonal secretion -Possible role of ARF & PDF. A posterior to anterior gradient was seen in the medley abdomens in that each segment showed slightly more new pupal cuticle than did its anterior neighbour. The posterior part of the abdomens had perfectly formed and tanned pupal cuticle while the anterior part still bear partial larval characters. The larval characters revealed the head, thoracic legs, segments of thorax and 1st -2nd abdominal segments (Fig. 25 A, B &C).

Typically, the patches of pupal cuticle were completely formed on the posterior region only, of normal sculpturing and thickness, and tanned. While on the entire anterior region bear margin of thin, untanned larval cuticle, which eventually darkened but always a few days later than the normal cuticle.

DISCUSSION

In addition to reprogramming of the epidermis, the first peak of ecdysteriods in last instar larvae of holometabolous insects also causes dramatic changes in the insect's physiology and behavior. The first and most obvious behavioral response is that the animal stops feeding and voids its gut contents. Most larvae then move away from their food source and go in search of an appropriate site for pupation. In the larvae of Lepidoptera and Diptera this activity period is referred to as the "wandering phase". The larvae of many Lepidoptera that pupate above ground wander in search of an appropriate substrate, and there they undertake an elaborate and stereotyped cocoon-spinning behavior. In larvae of Saturniidae the entire complex sequence of behaviors requires to build a peduncle, then an outer and then and inner cocoon (often with escape hatches built in), followed by the impregnation of the inner cocoon with a predator repellant fluid from the Malpighian tubules, is entirely preprogrammed in the central nervous system and triggered by the secretion of ecdysteriods (Lounibos, 1975, 1976; Giebultowicz *et al.*, 1980). Among the interesting biochemical changes that take place early during the wandering phase of certain Lepidoptera is the lost of the blue hemolymph protein, insecticyanin, and the synthesis of red ommochromes in the dorsal epidermis (Buckmann, 1959; Riddiford, 1985). This result in the distinctive change in the color of the larva by which the wandering phase can be readily identified even when the animal is temporarily quiescent or experimentally restrained. Wandering, burrowing, and spinning behavior all occur during the period between the two peaks of ecdysteriods. The second peaks of ecdysteriods terminates the wandering phase and, with the onset of apolysis, initiates the stages commonly called the *prepupa*, during which the imaginal disks grow and evert, and the cuticle of the pupal stage begins to be deposited. The mechanism by which ecdysteriods cause this elaborate array of molecular and physiological changes is not known.

It is of interest to note that during pupation, the transition of the feeding larva to prepupa take place presumably at the onset of the first PTTH and ecdysone secretions. The induction of ocellar contraction and formation of pupal cuticle was later supposed to be brought about by the second release of PTTH and ecdysone (Table. 5). After attaining both the events of secretions, the larva undergo completion of pupal development in normal time. These two steps of hormonal secretions in larval-pupal development are the same as has been reported in *Manduca* (Truman and Riddiford, 1974; Bollenbacher, *et al.*, 1975; Truman, *et al.*, 1974; Riddiford, 1976). The switchover to pupal commitment during the larval-pupal molt in *Manduca* requires ecdysteroids, but only occurs in the absence of juvenile hormone during the last larval instar. Reprogramming of the epidermal cells and some of the imaginal disks is induced by the first peak of ecdysteroid that occur in the absence of JH. Although the endocrine control of pupal commitment has been best studied in the Lepidoptera, it is now generally believed that in all insects whose metamorphic molt is preceded by two periods of ecdysteroid secretion, the first peak causes reprogramming of the epidermis from a larval to pupal commitment, while the second peak induces the molt (Smith, 1985; Sehnal, 1989).

The last larval instar thus differs from all the other instars in the pattern of ecdysteroid secretion and in the physiological response to ecdysteriods. There are two peaks of ecdysteriod secretion instead of one. The first peak of ecdysteroids reprograms the response pattern of the epidermis, which now becomes committed to secrete a pupal instead of a larval cuticle and triggers the sequence of physiological and behavioral events that prepare the animal for pupation. The second peaks provoke a normal molting cycle during which the altered commitment of the epidermal cells is manifested. Both the altered pattern of ecdysteroid secretion and the novel physiological responses to ecdysteriods have a common cause, namely the disappearance of JH from the hemolymph. The corpora allata cease to secrete JH during the last larval instar, and the lost of JH is intimately involved in reprogramming the molecular and physiological responses to ecdysteriods. Thus we can conclude that both the peaks of PTTH and ecdysone secretions as observed occurred during the period underlying the induction of prodomal signs leading to pupation.

The time interval between PTTH and ecdysone secretions that leads to pupation is not known in the present study. A wide separation in a magnitude of days of the critical period for ecdysone secretion is not uncommon, however. This interval has been reported to be 5 days in *Hyalophora cecropia* (Williams, 1952), 5 days in *Bombyx mori* (Fakuda, 1944), and 2 days in *Pieris brassicae* (Lafont, *et. al.*, 1977) and 6 days for the pupal-adult development in *Hyalophora cecropia* (Williams, 1952). From our observations, PTTH and ecdysone secretions are gated at a particular time so that further stages of development are directed accordingly in response to the endogenous signal.

The secretions of PTTH and ecdysone during the whole events leading to pupation seemed to be under the control of circadian clock, since both the secretions are capable of entering the photophase and scotophase regions of the light regimen. This condition has been reported for the larval-larval development in *Samia ricini* (Fujishita and Ishizaki, 1981). Further, the phenomenon underlying gut purge and pupal ecdysis also seemed to be under the control of circadian clock since, larvae that undergo gut purge on day-5 and day-6 pupated exclusively on day-9 and day-10 respectively forming a time interval of exactly 4 days in between the events

(Fig.19).Various changes in the light-dark condition did not affect in the timing that brought about signs for pupal development (Fujishita and Ishizaki, 1982). Thus, neither pupal ecdysis nor hormonal secretions are controlled by a photo sensitive circadian clock. Several factors including nervous elements have been implicated for initiation of hormonal release for ecdysis (Wigglesworth, 1934, 1936; Edwards, 1966; Sehnal and Edwards, 1969; Alexander, 1970; Pipa, 1971) and humoral agents (Nijhout and Williams, 1974a, b; Safranek, *et. al.*, 1980) and which mechanism operates seems to vary according to species and stages.

Concerning the first step of PTTH and ecdysone secretion, the gate for release of the two hormones was observed to reside in the photophase region. By contrast, this gate resides in the scotophase in the larval-larval development in *Samia cynthia ricini* (Fujishita and Ishizaki, 1981) and *Manduca sexta* (Riddiford, 1980a, b). However, our observation do not take into account the precise timing of hormonal secretions and that ligation experiments explained only the time when such hormonal release become necessary for further development. The phase of PTTH gate was reported to vary depending upon the developmental stages in *Manduca* (Truman, 1972; Truman and Riddiford, 1970; 1974).

Since ligation was done after the critical period long after gut purge i.e. after the 2nd released of PTTH had occurred, the whole body of the larva had received enough ecdysone to render them capable of pupal formation. Although the head had not purge in and still show the larval character, but this probably may be due to ligation at the neck region making the head impossible to tuck in due to blockage by ligation (Fig.24. b). Besides, becoming autonomous from the prothoracic glands, the epidermal cells also become committed to the type of cuticle that they will secrete (Truman *et al.*, 1974). From this relationship it was concluded that the epidermis became committed to pupal differentiation by the time that it acquired its independence (Truman, 1972).

In *Manduca sexta*, the 5th instar larva initiates the pupation process by the release of PTTH before wandering and after the wandering stage. The first release of PTTH and ecdysone provoked the onset of wandering stage, but did not cause the formation of the pupa. The molt to the pupal stage began 2 days later with a second released of PTTH late on day 1. When ligation was done prior to the 2nd peak of PTTH secretion they routinely showed mosiac molting response (Truman and Riddiford, 1974). Our observation also showed intermediate character of larval-pupal epidermal cuticle (25. a-c.). This tissue presumably had received sufficient exposure to ecdysone at the time of ligation to render it capable of forming the new pupal cuticle. The rest of the epidermis, which had not molted, apparently required a longer exposure to ecdysone before independence was attained. However the most readily retained larval structures were the anterior thoracic legs.

A posterior to anterior gradient was seen in the mosiac abdomens in that each segment showed slightly more new pupal cuticle than did its anterior neighbour (Fig. 25. a-c.). The posterior part of the abdomens had perfectly formed and tanned pupal cuticle. This shows that the posterior epidermis has gained independent of PTTH secretion according to reproducible pattern (Truman, *et al.*, 1974). The posterior epidermis has been exposed enough to molting hormone, ecdysone to render them capable enough to form pupal characters. The hormonal conditions for pupation behavior and for tanning of the puparial cuticle are present many hours before the actual processes begin. Larvae ligated behind the fused central nervous system and ring gland after the critical period, when the whole body is already committed to pupation, ultimately tan in both parts of the body (Kerkut and Gilbert, 1985). Failure of the anterior part to pupate (anterior inhibition) under certain circumstances is rather common (Ratnasiri and Fraenkel, 1974a) and it can also be attributed to respiratory deficiencies by injury of the tracheal system during ligation (Ratnasiri and Fraenkel, 1974b).

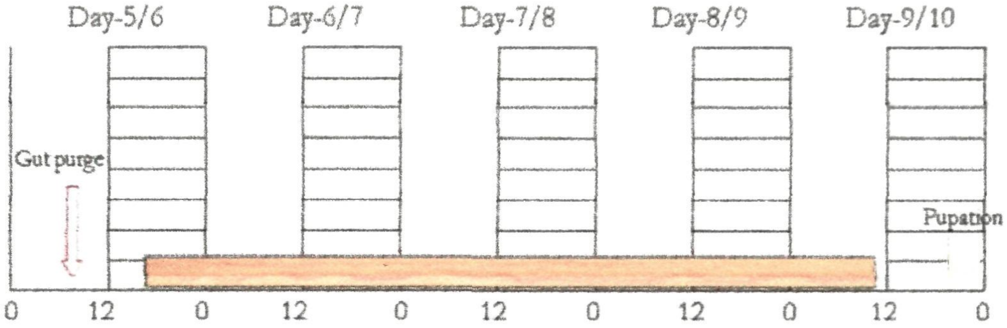
A factor, so far hypothetical, has been postulated to be involved in the control of pupariation. It is supposed to be produced in the posterior region of the larval body and its assumed function is to stimulate tanning (darkening) of the cuticle. Hence it has been named the posterior darkening factor by Whitehead (1974). Thus, in the present study, the darkening of the posterior part of the larval-pupal cuticle may be caused by the posterior darkening factor (PDF).

Still another factor, the anterior retracting factor (ARF) (Sivasubramanian *et al.*, 1974) may be responsible to render the anterior part incapable to pupate due its absence. The activity is low or absence at all stages prior to initiation of pupation, rises to a maximum during the first 2 hours of tanning, is maintained for several hours, and then wanes (Seligman *et al.*, 1977). Further, the Posterior retracting factor (PTF) was injected to the posterior of ligated part, tanning of the cuticle was faster than that of the anterior region (Zdarek and Fraenkel 1969). So in the case of the present study, the occurrence of pupal character in the posterior region of the ligated part indicates the maximal activity of PTF.

Fraenkel (1935) showed that a mature blowfly larva tightly tied below the ring gland with cotton thread, pupated in the anterior portion only, and that the posterior portion could be induced to pupate by injecting haemolymph from larvae which had just undergone pupation. The hormone activity present was later identified as ecdysone (Butenandt and Karlson, 1954). Later, the 'puparium test for ecdysone' was also utilized for discovery and further search for ecdysoids of plant origin, and it is currently used until now. Base on this, test works has been done in *Cecropia* silkworms (Fraenkel and Zdarek, 1970) and other lepidopteran larvae (Sato, *et al.*, 1968). Thus, in the present experiment (Fig. 22. a – c) the anterior portion of the body had received enough ecdysone for formation of pupal cuticle whereas the posterior half was deprived of the molting hormone by ligation.

In conclusion, it is of interest to note that neck ligated larvae shows mobility loss of the anal prolegs when ligation was done at appropriate time which thus explained that sufficient quantity of hormones was released. The critical period of hormonal release denotes the first observable effect of morphological change without further supply of the hormones. This timing may vary depending on the events of choice (Truman and Riddiford, 1974; Riddiford, 1980a, b).

Fig 18. Timing of gut purge in relation to pupal ecdysis.



Note. Yellow bar represents wandering, spinning, weight lost, mobility lost (anal &abdominal prolegs), body shrinkage & color change

Fig 19. Neck ligations on day-3 (5:00 hrs)(Red arrow) during feeding stage brought about responses leading to pre-pupal characters.

Red line indicate appearance of prodromal signs of pupation (gut purge, wandering phase, color change, etc. & yellow line indicate disappearance of anal prolegs).

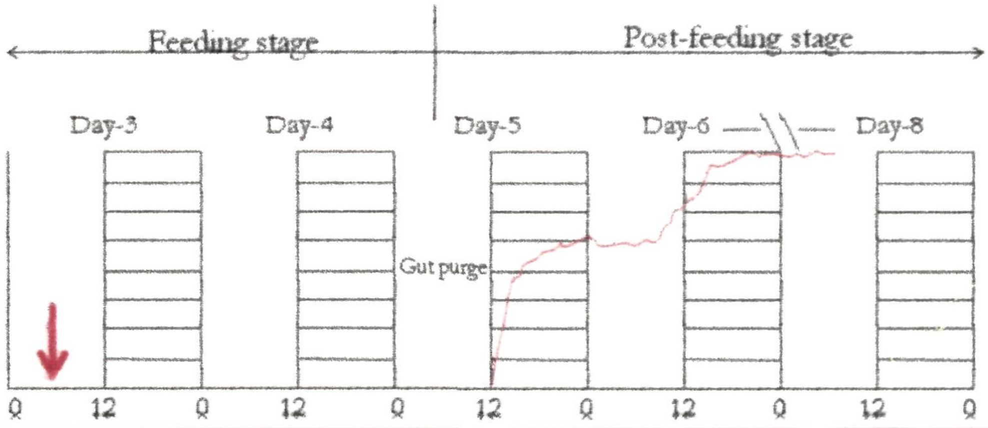


Fig 21. Ligations on day-3, 4:00 hrs (yellow arrow) after gut purge showing ocellar retraction (red line) and pupal cuticle formation (yellow line).

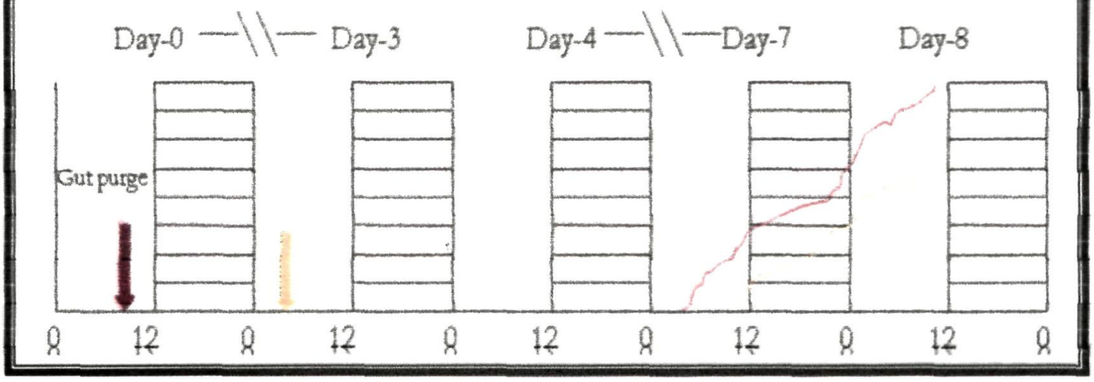


Table 5. Morphological changes due to ligation at different times.

Stage of larva	Time of Ligation		Prodromal signs of pupation				Mob. lost		Patch of P. cuticl (A-D)	Spinning	Ocell retraction	Pupation		
			Gut purge	Body shrink	Color chnge	Wander	AP	ABP				Ant	Post	Mosaic
5th instar feeding stage	Day- 2 (0-24 hr)	N	-	-	-	-	-	-	-	-	-	-	-	-
		A	-	-	-	-	-	-	-	-	-	-	-	-
	Day-3 (0-4:30 hr)	N	-	-	-	-	-	-	-	-	-	-	-	-
		A	-	-	-	-	-	-	-	-	-	-	-	-
	Day-3 (5-24 hrs)	N	+	+	+	+	+	-	NA	+-	NA	-	-	-
		A	+	+	+	+	+	-	NA	+-	NA	-	-	-
	Day-4 (0-11 hrs)	N	+	+	+	+	+	-	NA	+-	NA	-	-	-
		A	+	+	+	+	+	-	NA	+-	NA	-	-	-
5th instar post feeding	Day- 2 (0-24 hr)	N	+	+	+	+	+	-	NA	+-	NA	-	-	-
		A	+	+	+	+	+	-	NA	+-	NA	-	-	-
	Day-3 (0-3:30 hr)	N	+	+	+	+	+	-	NA	+-	NA	-	-	-
		A	+	+	+	+	+	-	NA	+-	NA	-	-	-
	Day-3 (4-10 hrs)	N	+	+	+	+	+	+	+-	+-	+-	+	+-	-
		A	+	+	+	+	+	+	+	+	+	+-	+-	+-
	Day-4 (2-24 hrs)	N	+	+	+	+	+	+	+	+	+	+	+-	+-
		A	+	+	+	+	+	+	+	+	+	+	+	+-

Note: N=Neck ligation, A= Abdominal ligation, NA= Not applicable, Ant= Anterior,

Post= Posterior, AP= Anal proleg, ABP= Abdominal prologs, A-D= Antero-dorsal, P= Pupal

Fig 20. Ligation during feeding & post feeding stages induced prodromal signs of pupation.

(A.) Ligation at day-3; 5:00 hr-Lost of mobility in the anal proleg (day-5). (B.) Sclerotized anal proleg (single arrow) buried partially in basal part & lost of mobility in the 3rd-6th abdominal prolegs (double arrow) but still intact.

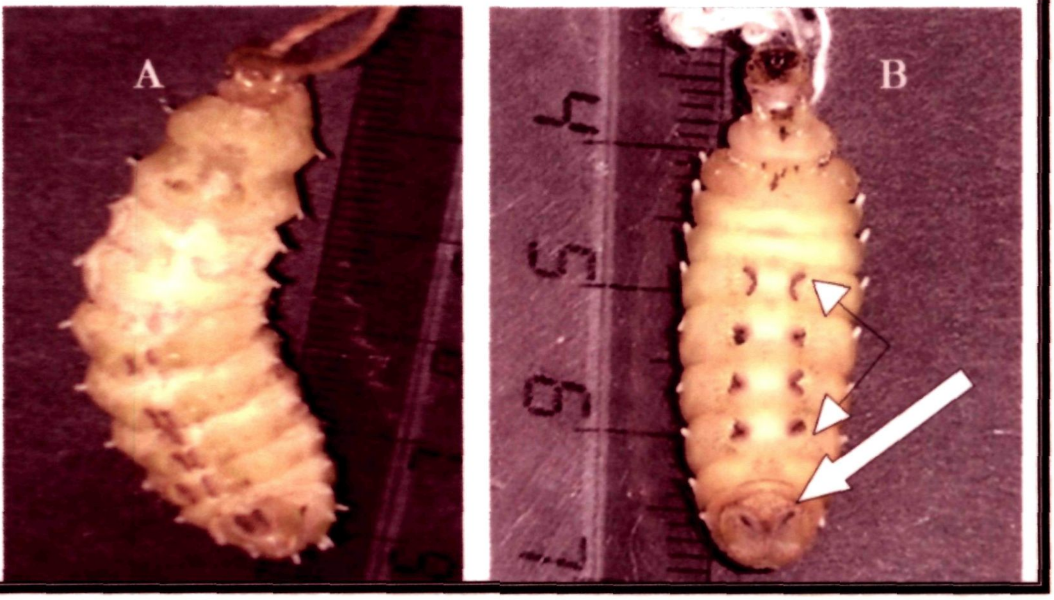


Fig 22. Abdominal ligations before anticipated hormone secretions blocked posterior pupal cuticle formation.
(a. Lateral view; b. Dorsal view; c. Ventral view.)

(a.) (b.) (c.)

Fig 23. Abdominal ligation before anticipated hormone secretion.
(A & B). Cocoon spun by larva while the ligated thread is still intact.

Fig 24. Normal developing pupal characters

(A). Normal developing pupa.
(B). Neck ligation long after anticipated hormonal secretions (after gut purge) developed normal pupal character but head part was blocked from invagination due to ligation.

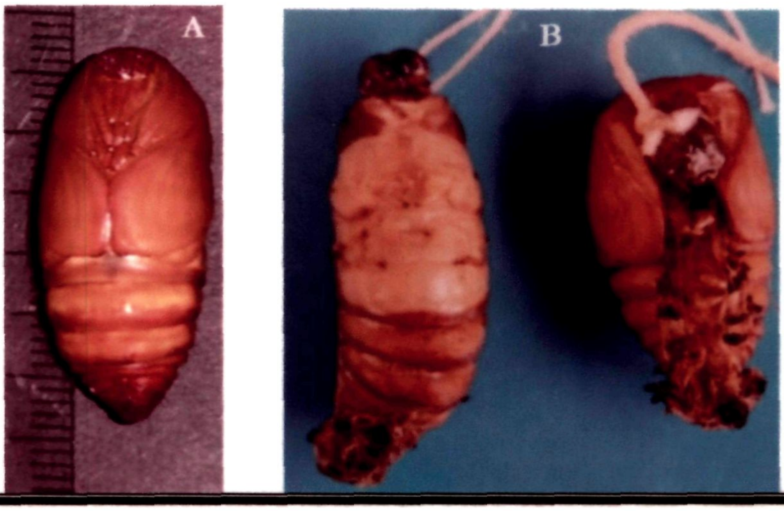
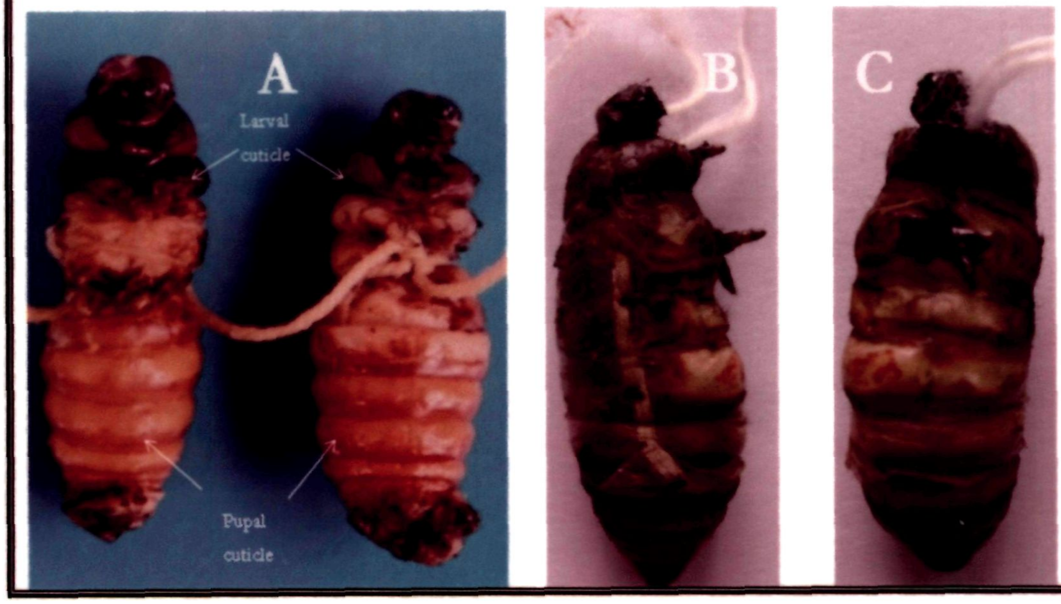


Fig 25. Ligation long after anticipated hormonal secretion -Possible role of ARF & PDF

(A). Abdominal ligation - larval-pupal intermediate (anterior-larval & posterior-pupal characters).
(B & C). Mosaic response in neck ligation – thoracic leg still intact. (Lateral & Ventral views)



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CHAPTER – 4

Study of Eclosion behavior and effect of EH extract on wing extensibility

Chapter – 4

INTRODUCTION

Growth and development in insects occurs in stages that are punctuated by molting, a set of biochemical and behavioral events. The behavioral events, termed pre-ecdysis and ecdysis, served to loosen the old cuticle (called the exuvium) from the underlying new cuticle. During this period the insect is often referred to as being a pharate stage or in case of lepidopteran insect, a pharate moth (Hinton, 1946). After the insect escape from the old skin, its new cuticle is then expanded into its proper form and hardened. The process of insect ecdysis involves more than the insect simply crawling out of the old skin. It demands the coordinated interplay of a number of behavioral, physiological and developmental processes. Ecdysis itself is composed of rhythmic abdominal peristaltic waves that terminate in the thorax with vigorous shrugging of the wing bases. The movement of the exuvium back along the abdomen is generally sufficient to free the legs and antennae. The ecdysis of silkmoths is interrupted by processes involved in escape from the surrounding cocoon.

The first indication of a hormonal control over ecdysis came from experiments on adult ecdysis (eclosion) in the giant silkmoths, where it was suggested that ecdysis or eclosion of the moth from its pupal cuticle could be evoked by a brain- centered neuroendocrine mechanism (Truman and Riddiford, 1970). Eclosion in these moths is confined to a specific time of day determined by the light-dark cycle. When the brain was removed early in adult development, the resulting brainless moths eventually ecdysed from the old pupal cuticle but their behavior was abnormal. These ecdysis attempts were spread randomly throughout the day and night. When a brain was implanted into the abdomen of similar debrained insects, these 'loose-brain' moths subsequently showed a normal ecdysis that occurred at its characteristic time of day.

Moreover, implantation of the brain from a different species into a debrained host resulted in the recipient eclosing at the time characteristic of the donor species. This clearly indicated that the brain regulated the timing of adult eclosion and furthermore, that it could exert this control through a blood-borne factor. The release of this neurohormone into the blood is a vital link in a chain of events that leads from a circadian clock, located in the brain (Truman 1971). Thus the brain clock (which in turn is entrained by environmental variables) is able to determine the time at which eclosion occurs by controlling the release of the eclosion hormone. Once the hormone appears in the blood, eclosion inevitably follows. In the Saturniid silkmoths, the interval between eclosion hormone release and the performance of eclosion behavior is relatively invariant (Truman, 1973c); in *Manduca sexta* (a Sphingid), on the other hand, the actual initiation of eclosion behavior once eclosion hormone has been released is somewhat subject to the influence of environmental variables (Reynolds, *et al*, 1979).

The hormonal basis for eclosion demonstrated in silkmoths showed that the behavioral could be evoked by extracts of brains and corpora cardiaca from pre-eclosion insects (Truman and Riddiford, 1970). Injection of EH- containing extracts into pharate adult *H. cecropia* invariably resulted in the prompt display of the pre-eclosion, eclosion and wing expansion behaviors (Truman, 1971). The ecdysial behaviors appeared to be a programmed response of the insect's nervous system to the hormonal signal. A direct verification of the above conclusion was made possible by the observation that abdomens isolated from pharate *H. cecreopia* responded to EH injection by performing the abdominal components of the pre-eclosion and eclosion behaviors (Truman and Endo, 1974). These results indicated that the chain of abdominal ganglia contained the information necessary for the generation of the two behaviors as was seen in *Manduca sexta* (Taylor and Truman, 1974). EH in some other insect like *Schistocerca* is involved in the pre-ecdysial cuticular softening in which the cuticle in stage 3 of ecdysis increases in softness, which facilitates its extraction from the old cuticle and its rapid expansion to adult size. As suggest by Elliott (1981) the timing of this change is consistent with the action of an EH-like factor.

The eclosion hormone originates in the mid-protocerebrum of the brain, presumably in the median neurosecretory cells, and is transported from there to the corpora cardiaca, where it is released into the insect's hemolynph. The existence of an eclosion-stimulating factor was confirmed by showing that extracts prepared from the brain of corpora cardiaca from pharate adult moths caused precocious eclosion of *Antheraea pernyi* moths (Truman, 1973a). The *Antheraea pernyi* eclosion assay as described is a whole insect bioassay method that is very simple to perform. Given large numbers of insects, a large number of tests can be done simultaneously. The assay utilizes the ability of the eclosion hormone to cause precocious eclosion of pharate adults of *Antheraea pernyi* when injected on the last day of pharate adult life, when adult development is essentially complete, and when the insects are 'waiting' for their normal emergence gate. The *Antheraea pernyi* bioassay is semi-quantitative in that the latent period between injection and subsequent eclosion is reduced when larger amounts of hormone are injected.

Truman (1973c) described the eclosion hormone as acting to 'switch off' pupal behavior patterns, and 'switch on' adult behaviors. Perhaps the eclosion hormone also serves to integrate a wide variety of physiological changes with the behavioral events of eclosion. The amount of activity that could be recovered from the corpora cardiaca precipitously declined at the time of eclosion, suggesting release of material into the haemolymph. Moreover, the haemolymph of eclosing moths triggered eclosion when injected into other pharate adults. This can be substantiated by the observation that such neurohormone was able to elicit sequence of behavior patterns in the differentiated abdominal CNS still within the abdomen (Truman, 1978b). Subsequently, isolated abdominal CNS preparations were shown to respond to EH by generating a complex program of motor activity. Thus, the brained normally triggered adult eclosion through the release of eclosion hormone (EH). Few years later, the active 'eclosion hormone' from pharate adults was also shown to evoke molting behavior in pharate larvae (Copenhaver and Truman, 1982) and pharate pupae (Truman *et al.*, 1980) of *Manduca sexta*, leading to the hypothesis that eclosion hormone evokes all post- embryonic ecdyses by a direct action on the central nervous system (Truman, *et al.*, 1981a, b).

The eclosion hormone also has a number of other effects on the long and short-term physiology and behavior of the insects in which it is known to occur. A good candidate for the release of preparatory behaviors (a series of vigorous abdominal rotations and these movements loosen the attachments between the two cuticles) prior to eclosion, is the ecdysteriod titre. In all insects studied thus far, a marked fall in circulating ecdysteriods occurs towards the end of the molt. Thus, the regulation of ecdysis was found to be under the influence of ecdysteriods. Once, the developmental events such as endocuticle digestion and molting fluid resorption are accomplished, the neuroendocrine system are activated for initiating ecdysis by the decline in ecdysteriod titres (Slama, 1980). This decline also renders the insect competent to release EH although other factors such as time of day or the attainment of a suitable ecdysis perch may also be important. The release of EH then triggers a series of rather stereotyped behaviors including the last stages of the preparatory phase and ecdysis itself. The eclosion hormone appears to initiate the 'programmed cell death' of the abdominal intersegmental muscles, which are used in eclosion behavior itself, but which are useless thereafter (Truman, 1973a). In addition, the eclosion hormone is required for the appearance of many characteristically adult behavior patterns (Truman, 1976).

Other factors that play a role in influencing cuticle extensibility as in *Rhodnius*, is a material released from peripheral nerve endings that has local effect on nearby cuticle to cause plasticization during blood meal (Maddrell, 1966). Around the time of ecdysis, bursicon has a marked effect on cuticle extensibility. This action is well-documented as a preparation for the post ecdysial expansion of the cuticle in newly emerged blowflies (Cottrell, 1962a, b, c; Reynolds, 1976). In *Manduca*, bursicon also causes a marked plasticization of the wings of freshly ecdysed moths (Reynolds, 1977). The changes in wing extensibility induced by bursicon differ from those caused by EH in that the effects of the former are transient and are followed by stiffening of the wing as tanning takes place, whereas those produced by the later are stable and persist for a number of hours. The respective roles of bursicon and EH in the control of cuticle extensibility during ecdysis begin with softening of the wing in response to EH and this aids in removal of the wing from its sheath of pupal cuticle. Although more extensible than it was, the wing is still stiff enough to withstand the stresses and abrasions that result from the movements. After the insect has attained a wing expansion site, bursicon is then released, which causes a second stage of wing softening. This second increase in extensibility is presumably a necessary aid for the expansion of the wings by hemolymph pressure. The effect of bursicon is transient, and is followed by the hardening of the wing in its adult shape (Reynolds *et al.*, 1979).

Based on the ability of the extracts obtained from the precise time of developmental stage of the insect to cause eclosion behavior in the test insect, a number of bioassays have been employed to quantify eclosion hormone activity in tissue or hemolymph samples especially in lepidopteran insects, *viz. Antheraea pernyi* (Truman, 1973a; Truman *et al.*, 1980) and *Hyalophora cecropia* (Truman and Riddiford, 1970; Truman, *et al.*, 1981b) and in a sphingid moth, *Manduca Sexta* (Reynolds and Truman, 1980; Reynolds, 1980a, 1980b; Copenhaver and Truman, 1982). Subsequently, the assays developed measured the ability of extracts to cause precocious eclosion in pharate adult insects. The activity brought about as a result of application of the extracts was quantified and expressed in terms of units, with one EH unit defined as the amount of biological activity present in the corpora cardiaca of a pharate adult *Manduca sexta* (Reynolds and Truman, 1980). These insects were very convenient to use because their normal time of eclosion was quite predictable.

The non- behavioral bioassay for the involvement of EH in altering cuticle extensibility was based on the ability of the eclosion hormone to cause an increase in the extensibility of the wing cuticle of pharate adult *Manduca Sexta* while the moth is still enclosed within the pupal case (Reynolds, 1977). Generally in insects, before emergence all of the adult cuticle has assumed their final dimensions and has hardened, with the exception of the wings. Reynolds isolated wings that had not been exposed to ecdysones and injected with EH extract (obtained from pre eclosed pharate adults) into the wings through wing venation. He observed that such injections would cause extensibility in wings after subjecting to load. The assay is generally used in pupal ecdysis because of its high sensitivity, ease of accomplishment and absence of false-positives. Further, the isolated wings require small amount of injected materials (5-10 μl). However, this assay cannot be used when samples contain bursicon since the latter peptide causes the wings to tan, thereby rendering them inextensible. The new cuticle formed during a molt is typically larger than that of the preceding stage. In many cases it expands to its appropriate size as the insect is extricating itself from the old cuticle. The expansion of some of the cuticular structures may aid in the rupturing of the overlying exuvium. This process of cuticular expansion is facilitated by changes, in the mechanical properties of the cuticle which make it more extensible (Reynolds, 1980b). The isolated wing is very sensitive to EH and serves as a rapid, sensitive bioassay for EH activity. In this bioassay the effect of the eclosion hormone is independent of the CNS, and although much evidence suggests that the active factor causing plasticization is indeed identical with the eclosion hormone, the possibility that the wing plasticizing factor might ultimately prove to be distinct from the eclosion hormone.

Thus, based on the works emanating from earlier studies, it is proposed to demonstrate the effect of pre-eclose extracts of pharate adults on extensibility of wings in *Philosamia ricini* in line with the method described by Reynolds (1977). The study will also brought about into light the presume time of eclosion hormone release based on the behavioral events underlying eclosion processes.

MATERIALS AND METHODS

1. Assessment of behavioral patterns of preparatory phase and eclosion process:

Philosamia ricini silkworms maintained at the Sericulture farm, Nongpoh were used for the experiment. Larvae were brought to the laboratory and reared on fresh castor leaves (*Ricinus communis*). Developing 5th instar larvae were kept under natural photoperiod of 13L: 12D at 25±2°C and 75±5% RH until adult emergence. Post-feeding larvae were restaged and their ages were given in days after spinning.

The time following spinning until adult emergence was recorded by observation for all the behavioral changes that took place. The recognizable pre-eclosion behavioral patterns or preparatory phase particularly pertaining to a series of vigorous abdominal rotations (as these movements loosen the attachments between the two cuticles), developmental markers such as the darkening of the metathoracic bars (the latter serve to split the exuvium during ecdysis), digestion of endocuticle and resorption of molting fluid which renders the eclosing pupa comparatively dry in appearance were given due importance during observation (Jungreis, 1979).

To facilitate spinning, larvae, shortly after wandering phase, were placed in a bamboo made mountage or 'Chandraki' of desirable diameter, a device used commonly in Eri culture.

2. Selection of test insects and preparation of eclosion hormone extracts for injection:

Based on the behavioral patterns during preparatory phase as described, last day i.e. 25 days old after spinning pharate adults were selected for extraction of EH. It is worth noting that in all Saturniid silk moths, *viz. Antheraea pernyi* and *Hyalophora* *cecropia* the presumed time of EH release occurs at about 1 hr or more before eclosion (Truman, 1971; 1973c). Hence, considering the closeness of *Philosamia ricini* to *Antheraea pernyi* and *Hyalophora cecropia* (Peigler & Naumann, 2003) and the fact that eclosion occurring at 15:00 hr (by observation) of the last day of adult development, the test insects for extraction were chosen at 14:00 hr or 1 hr less before normal eclosion, so that in all probability there is presence of EH in the extracts. Release of eclosion hormone presumably does not occur under our condition until 14:00 hr or later of the last day developing pharate adult stage. This precise timing is essential for the success of the experiments. Reynolds *et al* (1979) reported that in *Manduca sexta*, isolated wings before 1hr of normal eclosion are capable of responding to eclosion hormone exogenously. With slight modification so as to make sure that the wings are not yet expose to EH, the forewings are isolated from the pharate adult at about 13:00hr *i.e.* 2 hr before normal eclosion and subsequently kept in damp filter paper in a petri dish (Fig. 27).

Haemolymph samples (50 µl) of the last day pharate adult were collected from the incisions made in the dorso-lateral side of abdomen. The samples were stored at -20°C until the time of its usage. The extracted materials were homogenized and diluted with Ringer's solution to make concentrations in such a way that each 10 µl solution contain 5µl, 10 µl, 15 µl, 20µl, 25 µl and 30µl of designated EH extracts.

Wings are then injected with the 10 µl solution of different concentrations of extracts into the sub-costa and gripping the vein with forceps proximal to the injection site to prevent leakage from the cut ends of the veins, at the wing articulation. The contralateral wings that are injected with Ringer's solution alone served as control. After injection, the wings are incubated in room temperature in a moist chamber for 60-90 minutes. After incubation, the extensibility of the test wings are assessed by stretching them under load using a 3 gm load (in the form of a small 'bulldog clamp'. Measurements are taken after 3 min. and the effects of the test solutions on cuticle extensibility are expressed as the difference between the test wing to that of a paired, contralateral, control wing, which has been injected with Ringer's solution alone, and which was tested at the same time as the experimental wing. Average scores of above 0.5 mm extensibility were considered indicative of hormone activity when based on 4-5 determinations as described by Reynolds and Truman (1980) (Fig. 26).

RESULT

1. The course of behavioral processes underlying eclosion:

The time taken for complete development since spinning was 25 days. Prior to eclosion there are certain development markers showing sign of maturation for eclosion. Eclosion was observed to be confined to a specific time of day occurring at about 15:00 hr and continuing upto 18:00 hr on day 25 after spinning. Ecdysis behavior starts at around 13:00 hr and the developmental events such as endocuticle digestion and molting fluid resorption are well accomplished before the 14:00 hr of the last day. Ecdysis processes in *Philosamia ricini* was preceded by various preparatory behaviors, which anchor the old cuticle to the substrate and loosen the attachments between the old and new cuticles.

In the present study, three major phases on the basis of movements during ecdysis were classified as reported by Truman (1985). These movements begin much earlier than the eclosion event itself starting with the preparatory phase, ecdysial phase and post-ecdysial phase. In all cases the major movements involved in shedding of the old cuticle are rhythmic peristaltic waves, which push the exuvium posteriorly along the insect. The peristaltic waves are often co-ordinated with various specialized movements, which withdraw the appendages from their sheath of old cuticle as described below:

a. Preparatory phase:

The preparatory phase can be easily distinguished, which last about 1 hour and consists of 20 min period of frequent bouts of rotary movements of the abdomen followed by a 25-30 min of quite phase. Air swallowing in order to aid expansion of new cuticle may probably taken place later. The preparatory phase is typically quite flexible and can be easily disrupted by disturbance to the insect.

b. Ecdysial phase:

The behaviors shown during the ecdysial phase are quite rigid and stereotyped. They begin with behaviors, which eventually result in the rupture of the exuvium. During this period rhythmic abdominal peristaltic waves resulted in its termination in the thorax and the movement of the exuvium back along the abdomen freed the legs and antennae. The ecdysial phase ends when the insect pulls free from the old cuticle. Some expansion of the new cuticle may occur during this time as an aid to ecdysis. Once ecdysial phase is initiated, the insect is committed to completing the behavior.

c. Post-ecdysial phase:

The insect during post-ecdysial phase generally seeked an appropriate site for cuticular expansion and then exhibit the various behaviors necessary to inflate any unexpanded new cuticle to its proper dimensions through vigorous shrugging of the wing bases. Hardening and darkening of the cuticle begins shortly after wing expansion.

2. Effect of EH-extracts on wing extensibility:

After incubation of 60-90min, the extensibility of the test wings are assessed by stretching them under load using a 3 gm load after 3 min. The first indication of wing extensibility was observed for those injected with 5 μl, but only those wings injected with 10 μl which showed expansion of 0.85±0.020mm were taken into account for measurement. Wings injected with lower concentration of 5 μl showed extension of 0.375±0.032mm and were not taken as indicative of EH activity since it cannot attain the minimum expansion of 0.5mm. When the wings are injected by raising the concentrations by 5 μl from 10 μl to 15 μl, 20 μl, 25 μl and 30 μl there was increase of wing expansion by 1.725±0.314mm, 2.95±0.064mm, 3.862±0.062mm and

4.412±0.071mm respectively at each concentration. The increase is exponential with 30 µl showing maximum increase of 4.412±0.071mm. (Table. 6 and Fig. 28)

Expressing the increase in extensibility in term of percentage, we observed that when concentration was increased from 5µl to 10 µl, the isolated wings showed an increase of 129.72%. However, on raising the concentrations from 10 µl to 15 µl reducing value of 102.30% was obtained. Further, increased of 15 µl to 20 µl, 20 µl to 25 µl and 25 µl to 30 µl in concentration of EH extracts lowered wings extension to 71.51%, 30.84%, 14.24% respectively (Table. 7).

DISCUSSION

1. Time of eclosion (adult emergence):

An impressive feature of ecdysis is that it often occurs during a precise period of the day. In all the insects that have been thoroughly investigated, the timing of adult emergence/eclosion seemed to be under the influence of light. Most of the eclosion processes in insects occurred during the photophase period even under variable photoperiod regimens. Further, the timing of adult emergence although occurring during the photophase, can take place at variable times during the day and this can vary from species to species and even in the same species as well. In *Antheraea pernyi*, the normal emergence gate occurs from 11-15 hours after lights-on in a long day condition of 17L: 7D photoperiod (Truman, 1973c). Under the same condition, normal eclosion in *Hyalophora cecropia* begins about 1 hour after lights-on, which suggest that the release of hormone probably was triggered by exposure to light (Truman, 1971). Whereas, in *Manduca sexta* (a sphingid) reared under 12L: 12D photoperiod, the release of eclosion hormone begins after 20 hours i.e. 8 hours after lights-on (Reynolds, 1977).

In the present study, taking into consideration the importance of correct assessment of the development stages, emergence of adult from pupal case under natural photoperiod condition of 13L: 11D occurred during the photophase period beginning at 15:00 hours and continuing till 18:00 hours or later at the last day of adult development. Consistently, eclosion starts quite late in the cool afternoon period when the light was comparatively reduced preferably under shade. This timing of eclosion was observed to occur at about 10:00 hours after lights-on. It was also observed that, adult eclosion in *Philosamia ricini* followed the same pattern as that of other Saturniid insects in that last day pupal development was restricted within a time frame or gating during precise period of the day, if the pupa completes development while the gate is open, ecdysis can occur during the same day. By contrast, if development is completed after the gate (after 15:00hr) has shut, the insect must wait until the opening of the gate on the next day so that ecdysis can occur within the time frame of 15 hr-18hr (Fig. 27). The eclosion of the adult of many insects, including *Manduca* and the giant silkmoths, shows gating to that of *Drosophila* (Truman, 1971, 1978a; Truman and Riddiford, 1970; Hughes, 1980). In the saturniid moths of *Antheraea pernyi* and *Hyalophora cecropia*, adult eclosion is restricted to gates that occur at specific times of day (Truman, 1971). This is especially dramatic in the synchronous 'hatches' of mayflies and other aquatic insects (Truman *et al.*, 1981b). In many of these ecdyses there is clear evidence that the restriction of the behavior to a precise temporal gate is controlled by circadian clock. The occurrence of pupal ecdysis is non-gated in *Manduca* and can be accurately predicted by the appearance of various developmental markers such as the darkening of the metathoracic dorsal bars that later serve to split the exuvium during ecdysis (Truman *et al.*, 1980). Once dorsal bars appear, pupal ecdysis begins irrespective of time of day or night. The brain does not only regulate the time of adult eclosion; it also contains the endocrine trigger for this behavior. Thus ecdysis may be controlled in at least two different ways. In the non-gated pattern, ecdysis occurs at constant time after the initiation of the molt by PTTH and ecdysteriods (Truman *et al.*, 1983). By contrast, in gated ecdyses the insects may be forced to wait until a certain time of day to ecdyse, even though their development is apparently completed.

2. **Release of eclosion hormone:**

The eclosion hormone as described originates in the mid-protocerebrum of the brain, presumably in the median neurosecretory cells, and is transported from there to the corpora cardiaca, where it is released into the insect's hemolymph (Truman, 1973c).

The timing of release of eclosion hormone from corpora cardiaca to haemolymph in bringing about final eclosion of adult is variable in different insects. In *Manduca sexta*, where the time of release has been specifically investigated, the hormone is massively released into the haemolymph during a very short period (< 20 min) at about 2.5-3 hours prior to eclosion event (Reynolds *et. al.*, 1979). In *Hyalophora cecropia* release into the haemolymph would appear from indirect evidence to take place about 1 hour before the beginning recognizable eclosion behavior (Truman, 1971). Whereas in *Antheraea pernyi*, the release of EH into the haemolymph was a little more than an hour (Truman, 1973a).

In the present study, considering the timing of eclosion commencing at 15:00 hours of the last day of adult development and taking into consideration the fact that the release of eclosion hormone in its close relative insects (at least for *Hyalophora cecropia* and *Antheraea pernyi*) occurring in less than 2 hours before eclosion, the release of EH in *Philosamia ricini* in all probability may occur at around the same time i.e. 14:00 hours period before adult emergence assuming adult eclosion in *Philosamia ricini* following the same pattern of hormonal release as did the other saturniid moths. Thus, extracts from brain-corpora cardiaca complex for obtaining EH were prepared around 1 hour before normal eclosion so as to avoid false positive interference of other factors *viz.* material release from peripheral nerve endings (Maddrell, 1966) and bursicon (Cottrell, 1962b; Reynolds, 1976).

The present study employed extracts obtained only from the brain-corpora cardiaca as described, although EH can be released either from centers in the head or from sites in the chain of ventral ganglia. However, our study is confined only to adult eclosion and it would be worth noting that EH release occurs from the brain-corpora cardiaca axis in adult ecdysis and from the ventral ganglia for pupal ecdysis and for all of the larval ecdysis (Truman, 1985). Interestingly, in pharate larvae and pupae no activity was detectable in the corpora cardiaca (Taghert *et al.*, 1980; Copenhaver and Truman, 1982). The amount of activity lost from the corpora cardiaca was about 0.7 units, an amount consistent with the dosage of EH that must be injected into a pharate adult Manduca in order to induce precocious eclosion. The ventral ganglia, the other site of EH storage in the pharate adult – show no loss of stored activity at the time of adult eclosion and therefore appear to make no contribution to the circulating EH (Truman *et al.*, 1981a). On the other hand, marked depletion of 40-70% of the stored activity at the time of ecdysis suggests a release of the material rather than degeneration of hormone in the axon (Truman *et al.*, 1981b). The release hypothesis was supported by the fact that measurements during adult, pupal and fifth instar larval ecdyses showed the appearance of EH activity in the haemolymph at the time of the depletions (Reynolds, *et al.*, 1979; Truman, *et al.*, 1981b; Copenhaver and Truman, 1982).

3. **Effect of eclosion extracts on wing extensibility:**

In the present study, although injection with lowest dose of 5 μl showed an increase of 0.37±0.0323mm of wing extensibility, they are not considered as indicative of having EH activity since expansion of wings cannot attain the 0.5 mm minimum response as suggested by Reynolds (1977). Hence the results are discarded off. In *Hyalophora cecropia*, the lowest consistency dose of purified EH that can trigger a stereotyped eclosion behavioral response in isolated abdomen was 0.72 μg (Reynolds and Truman, 1980). The same purified hormone with lowest concentration of 120ng/ml can trigger the performance of a normal sequence of eclosion behavior when present in the saline, bathing the completely isolated *H. cecropia* abdominal CNS (Truman, 1978a). Further, in *Manduca sexta* it was reported that 5ng of purified EH material elicited an average wing score of 1.00 mm (Reynolds and Truman, 1980). It can thus be observed that there are differences in the concentration of EH materials for bringing about elicitation of certain eclosion behavioral response even within the same species and in other species as well. The present study confirms the suggestion of Reynolds and Truman (1980) that the various biological activities attributed to eclosion hormone on the basis of experiments with unpurified extracts of nervous tissue are in fact, due to the action of a single neurohormone rather than a complex of hormones.

In the present experiment, it was observed that increase in concentration of extracts at each injection, reduces the wing extensibility if counted from the last attained expansion (Table.7). These patterns of reduced wing extensibility expressed on the basis of last wing expansion attained at a particular concentration, may be due to the interference of bursicon, the tanning hormone, which harden and sclerotyized the newly formed cuticle. The release of bursicon was shown to be associated with ecdysis in all insects that have been studied (Cottrell, 1962a; Fraenkel and Hsiao, 1962; Reynolds, 1985). In many cases such as in *Leucophaea manderae* (Srivastava and Hopkins, 1975) and *Schistocerca gregaria* (Padgham, 1976a, b), release may occur at or soon after the splitting of the old cuticle. The other instances are typified by *Calliphora erythrocephala* (Fraenkel, 1935; Fraenkel and Hsiao, 1962) and *Manduca sexta* (Truman, 1973b) it may be delayed for a varying period after ecdysis until the insect reaches a site suitable for cuticle expansion and tanning. This association of bursicon release with ecdysis suggest an involvement of EH in the control of the former.

It may be mentioned that hardening or stiffening of cuticle is a process which mean that the cuticle becomes less extensible. This irreversibility of sclerotization implies that its timing (eclosion) must be very carefully controlled (Seligman, 1980). In accordance with the suggestion made by Reynolds (1985), in the present experiment, the process thought to be governed by bursicon in reducing wing extensibility from 129.72% to 14.24% irrespective of doses, does not begin too soon after ecdysis as this would prevent the attainment of the new cuticle's proper shape, and might event prevent ecdysis altogether.

In the present study, the doses at which the extracts elicited wing expansion were quite high in comparison to earlier observations performed by different workers. It is worth mentioning that purified EH are obtained after complex isolation and purification processes using highly sensitive sophisticated instruments (Reynolds and Truman, 1980). They are free of impurities and very stable. Administration of such purified substance usually resulted in prompt response by target tissues even in minute quantity. In contrast, the present study used EH materials obtained directly from unpurified extracts in accordance with the suggestion of Truman (1973c) and the experiments may be considered more or less qualitative. Further, it is highly attributed that the higher requirement of doses for elicitation of eclosion behavior may be due to the presence of other biological substances embedded with EH so that the potency of the extracts is reduced considerably. The differences in dosages for inducing wing expansion may also be derived probably due to the hormone being degraded rapidly after injection. Once in the hemolymph, the eclosion hormone has a rather brief existence, being rapidly eliminated from the blood, presumably by enzymatic activity. In *Manduca*, the half-life of the hormone is about 45 min (Reynolds *et al.* 1979). This is entirely in keeping with the postulated "trigger" role of the eclosion hormone in initiating behavioral and other changes (Truman, 1978a). Interestingly, its tissues appear to be insensitive to the eclosion hormone except at times close to the normal time of release. In *A. pernyi*, behavioral responses to the eclosion hormone first occur only during the penultimate day of adult development. In *Manduca*, the situation is even more extreme; responsiveness to the eclosion hormone is not seen in either the CNS or in the wing epidermis until about 4 hour before the hormone's normal time of release (Reynolds *et al.* 1979). In this latter case, the development of hormonal responsiveness is rather precisely timed.

Fig. 26. Wing assay method (Reynolds, 1977)

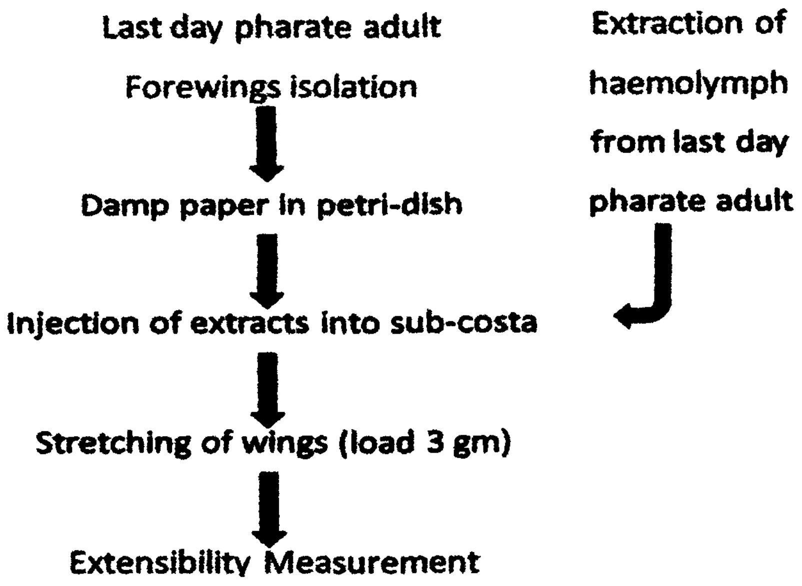

Fig 27. Last two days adult development in *Philosamia ricini* showing timing of eclosion (yellow bar) under natural photoperiod of 13L: 11D. The time of wing isolation and selection of insects for EH extraction are also shown in arrows.

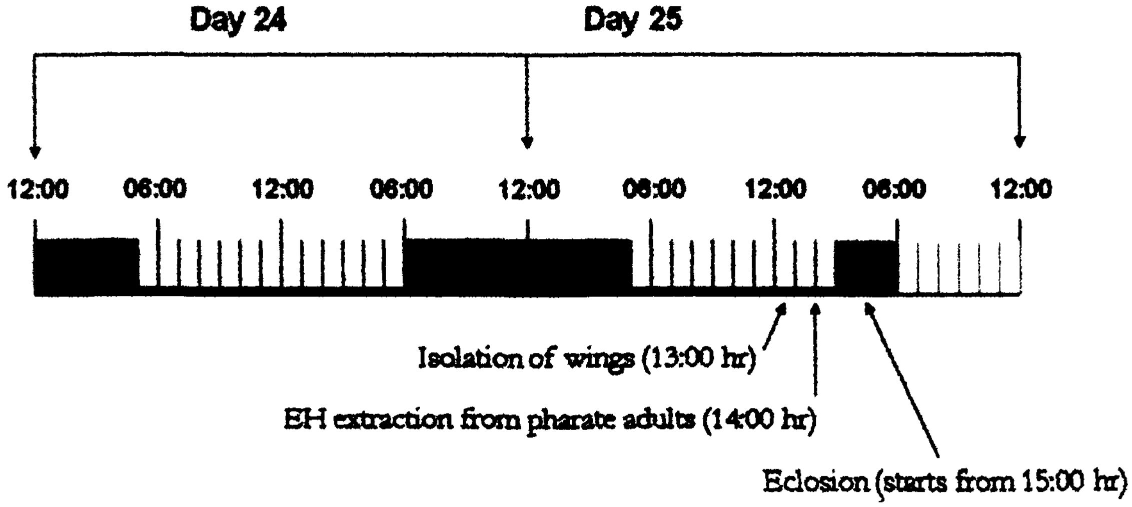

Fig.28. Extensibility of wings after treatment with different concentrations of EH extracts of pharate adult *Philosamia ricini*.

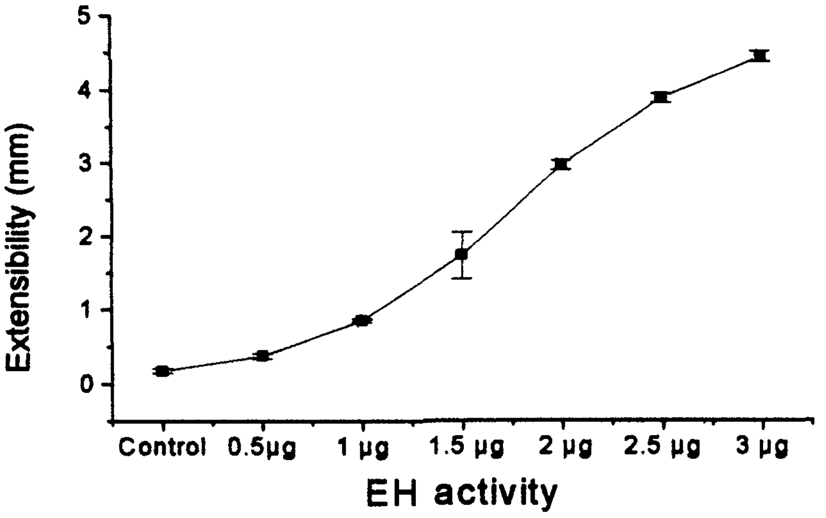

Table. 6. Effect of different concentrations of EH extracts on extensibility of isolated wings of pharate adult of *Philosamia ricini*.

Sl. No.	Concentrations	Extensibility (mm) Mean ±SE
1.	Control	0.175±0.032 a
2.	5 µl	0.375±0.032 b
3.	10 µl	0.85±0.020 c
4.	15 µl	1.725±0.314 d
5.	20 µl	2.95±0.064 e
6.	25 µl	3.862±0.062 f
7.	30 µl	4.412±0.071 g

Note: At 0.05 level the means are significantly different.
Fig. with common alphabet does not differ significantly.

Table.7. Gained in extensibility of isolated wing of *Philosamia ricini* on raising concentration by 5 µl.

Results are expressed as mean ±SEM. n=4

Sl. No.	Raising conc. by 5µl		Gain in extensibility (mm)	Gain in extensibility (%)
	From	To		
1.	5 µl	10 µl	0.48±0.026	129.72
2.	10 µl	15 µl	0.875±0.167	102.3
3.	15 µl	20 µl	1.225±0.189	71.50
4.	20 µl	25 µl	0.876±0.063	30.84
5.	25 µl	30 µl	0.55±0.067	14.24

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CHAPTER – 5

Effect of Juvenile hormone-III, 20-Hydroxyecdysone and Adenosine 3', 5' monophospate (cAMP) on oxygen consumption during larval development

CHAPTER - 5

INTRODUCTION

The preparation for molting in insect is accompanied by a 50 to 1900% increase in oxygen consumption by the whole animal (Poulsen, 1935; Nyst, 1941; Scudamore, 1947; Edwards, 1950, 1953; Schneiderman, 1952; Bliss, 1953; Schneiderman and Williams, 1953), which means that the metabolism of some or all of the tissues is vastly increased. In *Gecarcinus lateralis* oxygen consumption of the integumentary tissues increases during exocuticle formation before ecdysis (Skinner 1962).

All the studies concerning the effects of the activation hormone (PTTH) on respiratory metabolism confirm that it has a stimulatory effect on oxygen consumption of whole body or isolated body parts (Thomsen, 1949; Slama, 1965; Minks, 1967). Removal of the source of AH, i.e. neurosecretory cells or corpora cardiaca usually result in the decreased metabolic rate. In particular the most effected metabolic functions are directly or indirectly associated with food digestion and utilization (Minks, 1967; Slama, 1964). Slama (1960) has therefore recognized three components in the respiratory metabolism of adult *Pyrrhocoris* females, each component having a characteristic response to hormones. (i.) basal cellular metabolism which occurs after removal of all the known neuroendocrine centers or in diapause; (ii.) digestive metabolism which is dependent on AH (PTTH); (iii.) reproductive metabolism which is dependant on the corpora allatum hormone and growth of the ovaries in adult females. The last two examples of hormone stimulated respiratory activity can occur independently. Thus, the overall metabolism of an active female is controlled by the simultaneous cooperation of hormones; the CAH regulates metabolism in tissues associated with reproductive functions, whereas the AH regulates metabolism in tissues associated with trophic activity. Removal of the corpora cardiaca from larvae cause a decrease in respiratory metabolism to a low level, while removal of the corpora allata had a relatively minor effect on respiration (Slama, 1965). Removal of the prothoracic glands from larvae of *Pyrrhocoris* does not inhibit digestive metabolism but after prolonged periods during which ecdysis does not occur there is a continuous decrease in respiratory rate due to hypertrophy of the body and limits. The PTTH is also known to stimulate corpora cardiaca for ecdysteriod secretion and this enhances respiratory metabolism of the whole body (Nijhout, 1994).

Each of the developmental cycles in insects (larval or pupal molt cycles, reproduction cycles in females) are characterized by distinctive changes in respiratory metabolism. Cyclic changes in oxygen consumption during larval instars and the U-shaped course of oxygen consumption in pupae has long been known (Slama, 1960). During the period of intensive differentiation there occurs a U-shaped decrease of metabolic rate, which is common to metamorphosis in the Endopterygotes. In the initial period of intensive growth at the beginning of an instar the presence or absence of JH has little effect on the respiratory rate (Slama, 1965; Slama and Williams, 1965).

In an earlier paper Slama (1964) showed that the respiratory rate in females of *Pyrhocoris* is cyclical, increasing amid decreasing periodically in close connection with the cycles of reproduction and oviposition. The cycles disappear after the corpus allatum or the ovaries are removed; the females then maintain a constant intermediate rate of respiration. When the centers engaged in the release of the activation hormone (i.e., the neurosecretory cells of the brain or the corpora cardiaca) are also removed, the postemergence growth of the females is suppressed and the respiration rate decreases to the level characteristic of diapause.

From these results it is evident that the removal of the corpus alatum has little effect on the respiratory metabolism of *Pyrrhocoris* males. This contrasts with the situation in females, where allatectomy considerably depresses the rate of respiration (Slama and Hrubesova, 1963).

An increase in JH titer in the haemolymph of adult insects, especially females is followed by an increase in respiratory metabolism. Conversely, for example allectomized females have a lower rate of oxygen consumption. This has been demonstrated in such different groups as diptera (Slama and Hrubersova, 1963), hemiptera (Slama, 1964) and dictyoptera (Sagesser, 1960). However, it has become apparent that JH has no general metabolic effect but influences metabolism indirectly by affecting growth and physiological functions of certain organs or tissues (Slama, 1964, 1965).

In vivo measurements of oxygen consumption in Lepidopteran pupae injected with *Cecropia* extracts showed certain deviation from a common U-Shaped course of the respiratory curves, which normally occur in metamorphosis of all Endopterygotes. For example, there occurred a slight acceleration in the rise of respiration corresponding to the right hand part of the U-shaped curve, but no basic differences were found. Quite similar results are observed in *Tenebrio* pupae with farnesylmethylether treatment. Here the U-shaped decline of the respiratory curves did not reach the bottom values registered in the control group. It was suggested that there is possible similarities between these effects and those found by Slama (1965) after CA implantations in metamorphosis of Hemiptera exist.

The rate of oxygen consumption in different insects and even in the same species are variable during various development cycle which often led to conflict interpretation among different workers. There are many factors which are directly unrelated to endocrinology that influence oxygen consumption in insects. It is clear that oxygen consumption and total metabolism in general in insects are governed by factors such as environment, physiology, food, body size, muscle work, water and other external influences. In these lines, it will be important to note the works of various authors on the oxygen metabolism in different insects.

Bartholomew and Lighton (1985) reported that in cockroach, *Blaberus gigantus* (mean mass 4.33± 0.81g) the rate of oxygen consumption was 0.160 ml O_2/g/h (±0.02 s.d., N=5) when motionless. Similarly, Phillipson and Watson (1965) reported the respiratory metabolism of the terrestrial isopod, *Oniscus asellus* and oxygen consumption of single mosquitoes was measured using a differential pressure transducer (DPT) (Gaoussou, *et al.*, 1999). Kemp, *et al.* (2004) also reported the effect of temperature on oxygen consumption during the life cycle of the prepupa-wintering bee, *Megachile rotundata* (F) and the adult-wintering bee, *Osmia lignaria* Say (Hymenoptera: Megachilidae). Stusek, *et al.*, (2000) reported the possibility of detecting the presence of wood-boring insects in wood by measuring the oxygen consumption between different species as well as between adults and larvae of one species in different environmental conditions.

Danks (1987) used individual respiratory rate measurements to specify the physiological status of the larvae, because it is known that oxygen consumption is lower during diapause than during development. The design of the respirometer was similar to that used by Slama (1984). In the chestnut weevil, *Curculio elephas* Gyllenhal (Coleoptera: Curculionidae), with larval diapause, as in the Colorado potato beetle with imaginal diapause (Ushatinskaya, 1978), oxygen consumption is lower during a prolonged diapause than during the winter diapause. However, in contrast to

Ushatinskaya's statement, the cumulative energy consumption during prolonged diapause is high, which is compensated-for by extra lipid reserves in the larvae.

In the alien pest beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), oxygen consumption ranges from 0.5 μmol/g fresh mass (FM)/h at 12 °C to 3.4 μmol/g FM/h at 24 °C. At 12 °C, oxygen consumption decreased continuously during an entire period of starvation. However, at 16, 20 and 24 °C, beetles display a marked hyperactivity that leads to an increase in the oxygen consumption level during the first week of starvation, followed by a steep decrease until the end of the starvation period. Oxygen consumption either does not decline in fed beetles (observed at higher temperatures) or declines at a much shallower rate than in starved beetles (observed at cooler temperatures) (Renault *et al.*, 2003).

The oxygen consumption rates of inactive male house crickets, *Acheta domesticus* L. (Orthoptera: Gryllidae), increased with male body mass at a rate similar to that found in other studies of insect aerobic metabolism (Hack, 1997).

Low metabolism is a primary index of diapause in insects. In the boll weevil, *Anrhonomus grandis grandis* Boheman, the rates of oxygen (O_2) consumption and carbon dioxide (CO_2) production are lower in diapausing than reproductive insects. Respiration was more variable in reproductive than diapausing adults probably because of differences in reproductive rates among individuals and a greater capacity for these adults to respond to changing environments. Adult feeding on bolls did not influence respiration (Wagner and Villavaso, 1999).

In *Drosophila melanogaster* oxygen consumption remains relatively constant over the majority of the life span of each line of flies, aerobic efficiency declines with advancing age. This loss of aerobic efficiency manifests itself as a decline in total body metabolism as measured by heat production, and appears to be associated with an age-specific increase in damage inflicted upon mitochondria by oxygen free radicals (Ross, 2005).

Working on the larvae of *Rhodnius,* Zwicky & Wigglesworth (1956) correlated the cytological changes that take place in the epidermis at various times after feeding during the moulting cycle with the rate of oxygen consumption and concluded that the latter largely represents the rate of protein synthesis during growth and development. Other studies on *Rhodnius prolixus* larvae in which moulting has either been inhibited or temporarily arrested by heat-treatment lend support to this theory (Okasha, 1968 a, b*)*.

The metabolic rate of an organism can be estimated from the food consumption, the energy released as heat, or the amount of oxygen used in oxidation processes to obtain the energy. All three methods can be employed, but they are not equally satisfactory. However, the determination of oxygen by estimation from the amount of oxygen used in oxidation processes to obtain the energy is technically easy, gives good results, and, in fact has been used so much that when metabolic rate is mentioned, it usually means rate of oxygen consumption (Schmit-Neilsen, 1961). The oxygen consumption using the above method is also the most satisfied method for determining the metabolic rate of pupae (La Due, 1964). During this phase the metabolic rate falls during the early stages and rises during the latter part of the phase.

MATERIALS AND METHODS

Larvae were collected from Sericulture Farm, Govt. of Meghalaya, Nongpoh and were raised in the Laboratory with *Ricinus communis* (Castor), their usual food plant. The effect of JH-III, 20-hydroxyecdysone and cAMP on the oxygen consumption of the test insects in course of their development, were determined by injection of the test materials with three concentrations each prepared as follows. Injections were given only to those larvae which had just ecdysed to 4th and 5th instars.

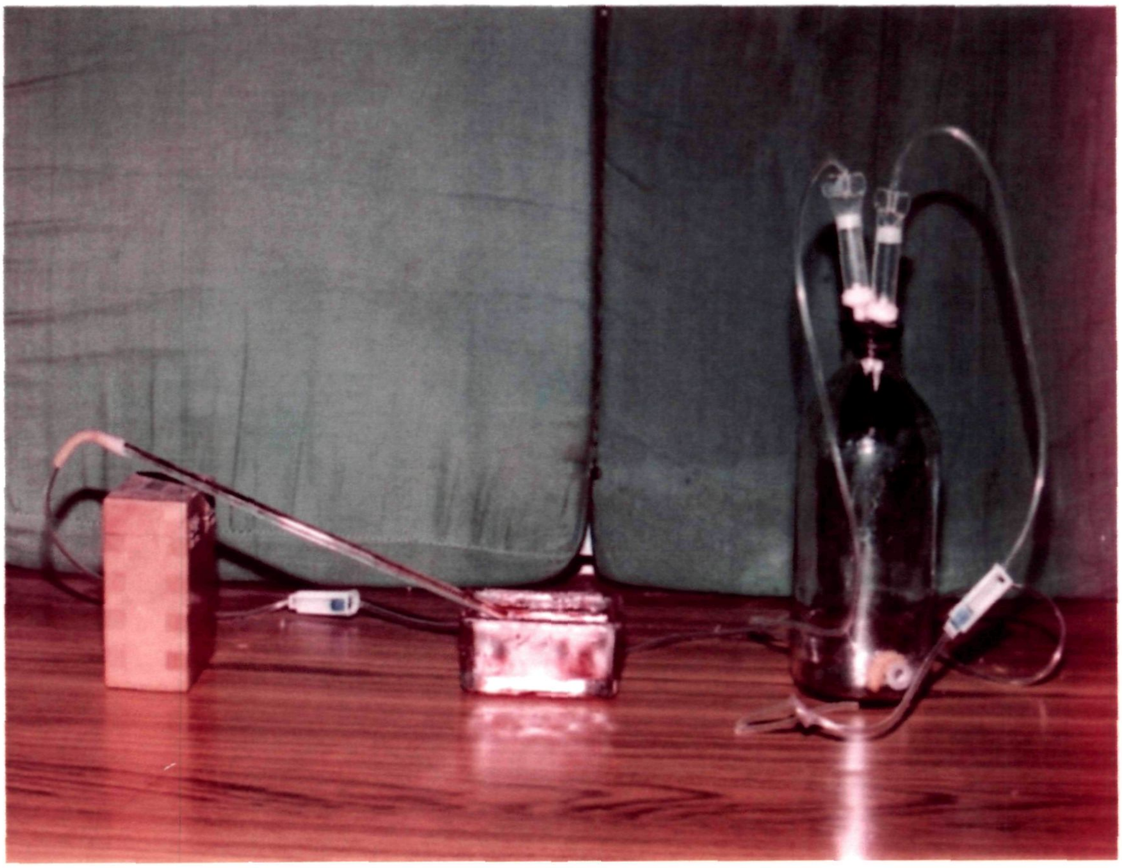
The 20-hydroxyecdysone (Sigma Chemical Ltd., USA) hormone was dissolved in 10% methanol and the hormone concentration was adjusted to 1µg, 0.5µg and 0.25µg. In case of synthetic Juvenile hormone-III (sigma Chemical Ltd., USA) and Cyclic AMP (sigma Chemical Ltd., USA) the solvent used was 10% acetone and Ringer's solution (ph 7). The concentrations were adjusted in the same way as that of 20-hydroxyecdysone. Injections were given with a dose of 10 µl/insect of the three concentrations of each solution on the very first day of the 4th and 5th instars respectively. The control insects received 10 µl each of 10% methanol, 10% acetone and Ringer solution (pH 7) respectively. Prior to injection, the larvae are kept in refrigerator for 3-5 min in low temperature to make them slightly immobile for easy operation. After injections the wound that occurred during injection were sealed with molten wax to prevent leakage of haemolymph. Oxygen consumption of the larvae were recorded daily at appropriate time in course of their development.

The method described by Schmidt-Neilsen (1961) for the determination of oxygen consumption in which he employed for the oxygen consumption in rats was followed with some modifications. Information on the methods and selection of experimental subjects was also obtained orally from Prof. B. Kharbuli and Prof. B. B. Gupta, *Dept. of Zoology, NEHU, Shillong, Meghalaya.*

A simple apparatus was assembled and the equipment included glass bottles, one-hole rubber stoppers, copper wire mesh, NaOH crystal pellets and graduated pipettes (Fig. 29). The NaOH pellets, which were used to absorb the CO_2 expired by the larvae, were kept inside the bottles and covered with copper wire mesh in such a way that it did not come in contact with the experimenting larvae during experiments. The bottles were then placed in an insulated campers ice box to keep the temperature constant. A sheet of glass was then placed over the top of the box to ensure these conditions, and the readings were done through the glass. The temperature inside the box was 25°C ± 2 at all times.

During measurement, the bottles were placed in a slanting position so that the NaOH was held into one corner of the bottom of the bottle and the copper wire mesh act as barrier between the larva and the NaOH. The insects were placed into the bottles and a period of time allowed to pass before the insertion of the drop of water into the end of the pipette. This was done to allow the temperature of the bottles to return to normal after being handled. A control was also set up to determine if all conditions were correct. For movement of the column of water, red dye (eosin solution) was added to the water which was inserted into the pipette. Weighing of the insects were done before and after the tests on an analytical balance. The time during all operations and the movement of the fluid (red dye) in the pipette were recorded and the oxygen consumption of the test insects were expressed in ml O_2 consumed/ gram body weight/ hour.

Fig 29. A simple assembled respirometer used for measuring Oxygen consumption



RESULT

1. Effect of JH-III on oxygen consumption.

4th instar larva: From the data analysed it can be observed that as concentration of JH-III increases the amount of oxygen consumed also increases (Table 8). Highest oxygen consumption occurred to larvae that are treated with 1 µg increasing from 1.129±0.143 ml at day-1, 2.541±0.080 ml at day-2, 2.918±0.107 ml at day-3, 3.069±0.045 ml at day-4 and decreasing to 2.935±0.033 ml at day-5. At 0.5 µg concentration the amount of oxygen consumed increases from day-1 to day-4 as 1.06±0.051, 2.24±0.048, 2.53±0.042 and 2.74±0.085 respectively but decreases to 2.23±0.015 at day-5. Similarly, larvae treated with 0.25 µg of JH-III increases as above but the amount of oxygen consumption are lowest recording from day-1 to day-1 as 1.05±0.083, 1.52±0.125, 2.15±0.077, 1.13±0.024 and 0.04±0.010 respectively. Control larvae shows the least amount of oxygen consumed at least upto the 3rd day (0.95±0.094 at day-1, 1.38±0.023 at day-2 and 1.92±0.050 at day-3 respectively) but higher than 0.25 µg treated larvae at day-4 (1.84±0.037) and day-5 (1.22±0.071). In all cases irrespective of concentrations, it can be observed that the course of oxygen consumption followed an inverted U-shaped curved increasing from day-1 to day-4 and finally decreases at day-5 (Fig. 31).

5th instar larva: At day-1, oxygen consumption was found to be highest in 0.5 µg treated larvae (1.949±0.068) followed by 0.25 µg (1.834±0.067), control (1.744±0.055) and 1 µg (1.065±0.004). In the rest of the developing larvae oxygen consumption was highest to those treated with 1 µg from day-2 to day-5 (2.634±0.131, 2.942±0.061, 3.712±0.073 and 3.606±0.138). From day-2 to day-5, 0.5 µg treated larvae resulted in oxygen consumption of 2.440±0.046, 2.845±0.042, 3.026±0.100 and 1.938±0.10, whereas in 0.25 µg treated larvae oxygen consumption was 2.228±0.012, 2.316±0.096, 2.802±0.107 and 2.802±0.107 respectively. Control group recorded 2.117±0.009, 2.396±0.091, 2.869±0.049 and 2.367±0.023 in oxygen consumption from day-2 to day-5 respectively (Table 10 & Fig. 34).

2. Effect of 20-HE on oxygen consumption.

4th instar larva: The amount of oxygen in ml/gm. body weight/hr were observed to be highest in those larvae treated with 20-HE. Here also as concentration increases amount of oxygen consumed also increases but only upto the 3rd day and then it decreases in the 4th and 5th day (Table 8). Larvae treated with 1 μg recorded the highest oxygen consumption which increases from 1.629±0.046, 3.221±0.071 and 3.840±0.049 upto 3rd day but decreased to 1.837±0.059 in 4th day on the day of molting. Comparatively, 0.5 μg treated larvae consumed lower amount of oxygen compared to 1 μg treated larvae which are recorded as 1.433±0.090, 3.040±0.0, 3.453±0.010, 1.629±0.082 and 0.961±0.022 from 1-5 days respectively. Similarly, 0.5 μg treated larvae recorded lowest oxygen consumption of 1.242±0.037, 2.933±0.037, 3.229±0.011, 1.356±0.089 and 0.153±0.012 from 1-5 days respectively. Control group recorded lowest amount of oxygen consumed upto 3rd day (1.039±0.033, 1.466±0.042 and 2.082±0.044 respectively) but highest in 4th day (1.960±0.044) and the 5th day (1.447±0.084).

5th instar larva: The increase in oxygen consumption for control group and 0.25 μg treated larvae were upto the day-4 which then decreased in day-5 while for 0.5 μg and 1 μg treated larvae it increases upto day-3 and then falls in the 4th and 5th day of larval development (Table 10). Oxygen consumption was highest in 0.5 μg treated at day-1 (2.259±0.124), 1 μg treated larvae at day-2 (3.833±0.043) and day-3 (3.958±0.098), 0.25 μg (3.942±0.111) at day-5 and control group at day-5 (3.767±0.061).

The course of oxygen consumption also follows an inverted U-shaped curve but the slope at one end is much straighter (4th instar, Fig. 32 & 5th instar, Fig. 35)

3. Effect of c-AMP on oxygen consumption.

4th instar larva: c-AMP treated larvae also recorded high oxygen consumption which are almost similar to 20-HE treated larvae. From the data it was observed that as concentration increases amount of oxygen consumption also increases (Table 8). From day-1 to day-5, 1 ug treated larvae recorded highest oxygen consumption (1.744±0.081, 2.845±0.050, 3.436±0.081, 2.062±0.005 and 1.750±0.067) followed by 0.5 μg (1.426±0.044, 2.650±0.082, 3.042±0.036, 1.739±0.075and 0.56±0.104) and 0.25 μg (1.252±0.01, 2.454±0.040, 2.843±0.043, 1.250±0.059 and 0.131±0.018) treated larvae. In control oxygen consumption was recorded to be lowest (1.13±0.039, 2.360±0.020, 2.767±0.072, 1.614±0.075 and 1.347±0.047 from day 1-5 respectively). The oxygen curve was similar with that of 20-HE treated (Fig. 33).

5th instar larva: Oxygen consumption was highest in 1 μg treated larvae at day-2 (3.035±0.092) and day-3 (3.729±0.037) while in day-4(3.620±0.039) and day-5(3.342±0.050) control group recorded highest O_2 consumption. At day-1, 0.5 μg treated larvae showed highest O_2 consumption of 2.051±0.047 (Table 10). c-AMP treated larvae at different concentrations also showed same pattern of O_2 consumption curve (Fig. 36).

4. Comparative effect of all treatments (JH-III, 20-HE and c-AMP).

4th instar larva: 20-HE and cAMP treated larvae did not show significant different in the amount of oxygen consumption at day-1 (1.336 and 1.388) and day-4 (1.695 and 1.677) whereas in all other days these two treatments along with JH-III show significant different. Oxygen consumption was highest with cAMP (1.388) treatment followed closely by 20-HE (1.336) and JH-III (1.046) at day-1. In day-2 and day-3, 20-HE (2.665 and 3.151) treated larvae shows the highest amount of oxygen consumed followed by cAMP (2.577 and 3.022) and JH-III (1.918 and 2.378). JH-III treated larvae showed highest oxygen consumption in day-4 (2.196) and day-5(1.608) while 20-HE treated lowest in day-4 (1.695) and c-AMP lowest in day-5(0.949) (Table 9).

5th instar larva: From the data (Table 11), it can be observed that 20-HE application resulted in highest O_2 consumption from 1st day of development up to the 4th day (1.939, 3.294, 3.552 and 3.262 respectively) irrespective of concentrations. JH-III treated larvae recorded highest O_2 consumption only on day-5 (2.437). Oxygen consumption was not significantly different for JH-III (1.648) treated and cAMP (1.694) treated at day-1 while it was for 20-HE (2.049) and cAMP (2.130) treated at day-5.

DISCUSSION

The curves of oxygen consumption during both the larval cycles (4th and 5th instars) showed an inverted U-shaped (Figs. 31-36), thus being similar to most of endopterygotes as that of *Rhodnius* larvae when the descending part of the curve during moulting cycle which may be regarded as a curve representing protein synthesis (Zwicky & Wigglesworth, 1956; Slama, 1960). Indeed, histochemical studies concerning changes that occur in the follicular epithelium of the ovary in *Rhodnius* during the reproductive cycle as described by Patchin & Davey (1968) support this view. It has been found, moreover, that the rate of oxygen consumption mirrors the proteolytic activity in the mid-gut of *Rhodnius* adults at various times after feeding (Thomsen & Moller, 1963).

Chamberlin and King, (1998) observed variable developmental changes in midgut aerobic metabolism when assessed by comparing tissue respiration, oxygen consumption of isolated mitochondria, and the activity of citrate synthase (CS) of larval instars of *M sexta*. During the last larval instar of *Manduca sexta* (tobacco hornworm), the insect enters the wandering phase, in which the animal ceases feeding and the structural reorganization of the midgut begins. In the present study, molting of 4th instar to 5th instar and prior to wandering in 5th instar may be correlated to *M. sexta* in which one day prior to wandering there was a significant decline in tissue respiration and mitochondrial succinate oxidation and these conditions may contribute to reduced O_2 consumptions in *Philosamia ricini* as well.

On the other hand the oxygen consumption from day 2 significantly increases mostly upto the 4th day of both larval developments which are higher than that of day-5. The oxygen consumption is comparatively very less at the beginning of the instars or synthesis of cuticle. The stages, immediately preceding molt (ecdysis), are marked by no further synthesis of exoskeleton, thereby decreases continuously in oxygen consumption gradually. This experiment shows that oxygen consumption increases exponentially during the preparatory molting cycle until it reach its maximum value before ecdysis and after that, decreases gradually. Chamberlin (2004) stated that the respiration rate of day-4 midguts was significantly higher than that of day-5 midguts in *M. sexta*. These metabolic changes may be early indicators of the more dramatic changes in midgut function that are to occur upon reaching the wandering phase. It is argued that midgut function is modulated by the commitment peak of ecdysteroids, which induces the external and behavioral characteristics associated with the wandering phase (Chamberlin and King, 1998). Moreover, high titres of ecdysteriod were recorded in *Samia cynthia ricini* toward the end of preparatory phase of molting (Jiang *et al.*, 2005). In the present study the peak of oxygen consumption which normally increased in day-3 or day-4 of both developing instars can also be correlated with the high titres of ecdysteriod occurring at these times as described.

It is well established that JH maintained larval characters and prevent molting in insects whereas ecdysteriods in haemolymph are responsible for inducing molting and metamorphosis in insects (Riddiford, 1994, 1996). Whereas, cAMP has been implicated to act as a micmicking substance to hormone activities (Nijhout, 1994; Maniere and Vanhems, 2000). In the present study, we have found that JH treated larvae recorded lesser amount of O_2 consumed compared to 20-HE and cAMP treated larvae during the middle part (2-4 days old) of larval development. Such variable O_2 consumption rates are expected considering the different properties the hormones have. Higher O_2 consumption in larvae applied with 20-HE and cAMP may be correlated to the specific action of these substances directly or indirectly to the epidermal cells which may induce molting and thereby higher metabolic activity (Table 9). Whereas, the JH-III treated larvae may not influence molting and they may only help to maintain larval character so metabolic activity is possibly reduced and hence lesser amount of O_2 consumption. However, JH-III treated larvae almost always showed highest O_2 consumption at the ultimate day of both the instars (Table 10 & 11). But by this time larval development of the 20-HE or cAMP treated larvae has become more advanced with low metabolic activity and animal at this stage are resting and on the verge of molting therefore they may consumed low level of O_2. In JH-III treated larvae at the same stage are quite active and probably high levels of metabolic activity or differentiation are still going on and hence higher amount of O_2 may be needed.

cAMP has been reported to play a role in some steroid-mediated events in *Hyalophora gloveri* (Applebaum and Gilbert, 1972), *Mamestra brassicae* (Sass *et al.*, 1983) and hormonal release of programmed behavior in silkmoths (Truman *et al.*, 1976). In the present study, the pattern of O_2 consumption in cAMP treated larvae are more or less similar to 20-HE treatment and it may be possible that cAMP directly act via PTTH to induce indigenous 20-HE secretion and activation which may in turn stimulate cellular activity and thereby resulted in higher O_2 consumption. In *Galleria mellonella* the membrane potential of prothoracic glands was also reported to be elicited by cAMP (Gersch, and Birkenbeil, 1980) and ecdysone secretion stimulation by prothoracic gland in *Manduca sexta* (Vedeckis *et al.*, 1974, 1976).

Overall O_2 consumption of the larvae in both the 4th and 5th instars are quite similar and here it was convenient to described the result of hormones and cAMP treatments in common approach. On the other hand, JH-III treated larvae which comparatively showed lower O_2 consumption during most of the developing instars are in accord with the suggestion at the beginning of an instar the presence or absence of JH has little effect on the respiratory rate (Slama, 1964; 1965; Sehnal and Slama, 1966).

Fig. 31. Effect of three concentrations of JH-III on the Oxygen consumption of the 4th Instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

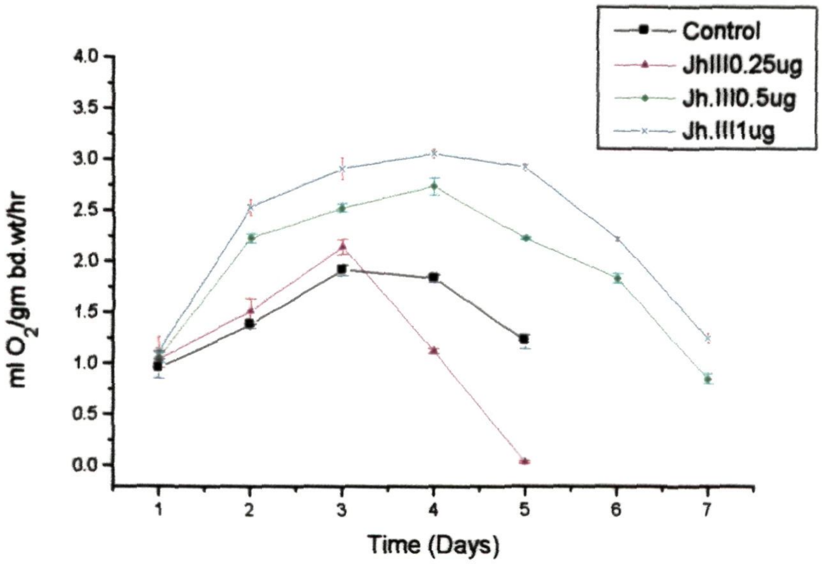


Fig.32. Effect of three concentrations of 20-Hydroxyecdysone on the Oxygen consumption of 4th Instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

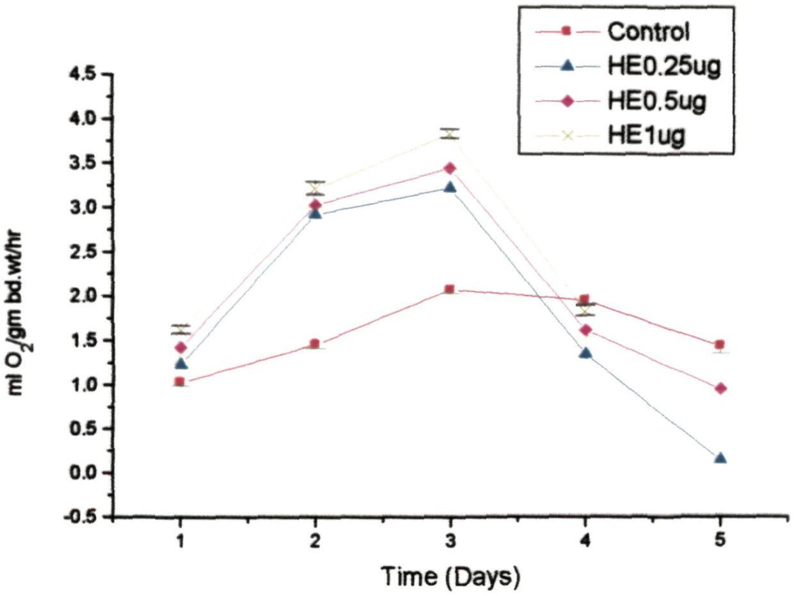


Fig.33. Effect of three concentrations of c-AMP on the Oxygen consumption of 4th Instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

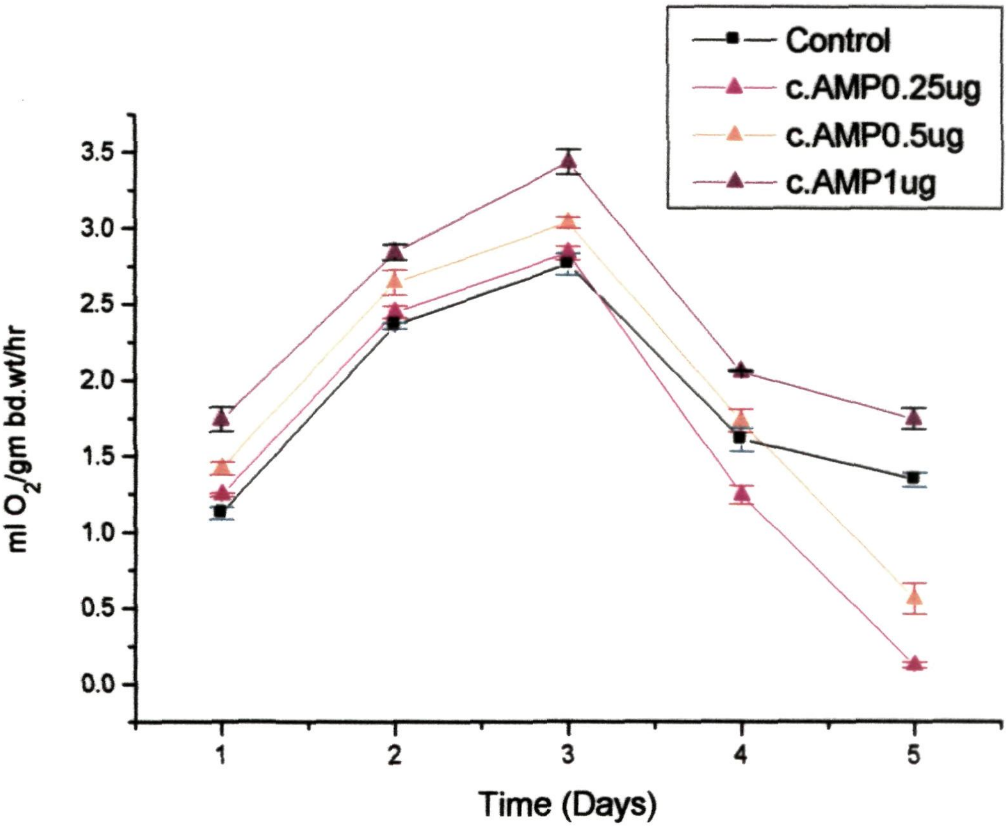


Fig.34. Effect of three concentrations of JH-III on the Oxygen consumption of the 5th Instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

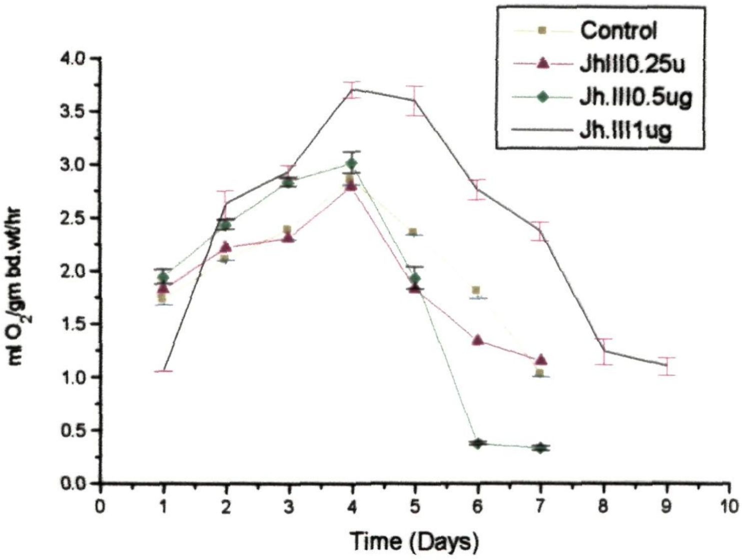


Fig.35.Effect of three concentrations of 20-Hydroxyecdysone on the Oxygen consumption of the 5th instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

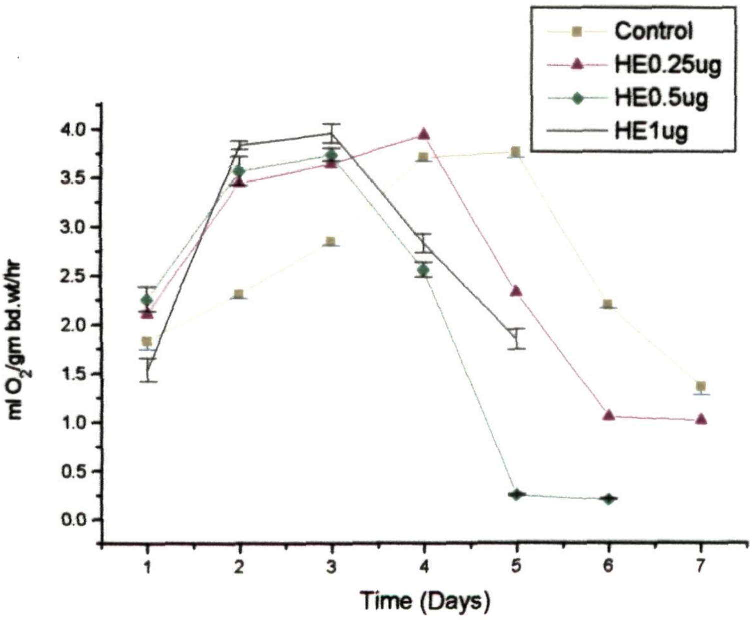


Fig.36. Effect of three concentrations of c-AMP on Oxygen consumption of the 5th Instar larva of *Philosamia ricini*. Each point represents the mean ± SEM, *n*=4

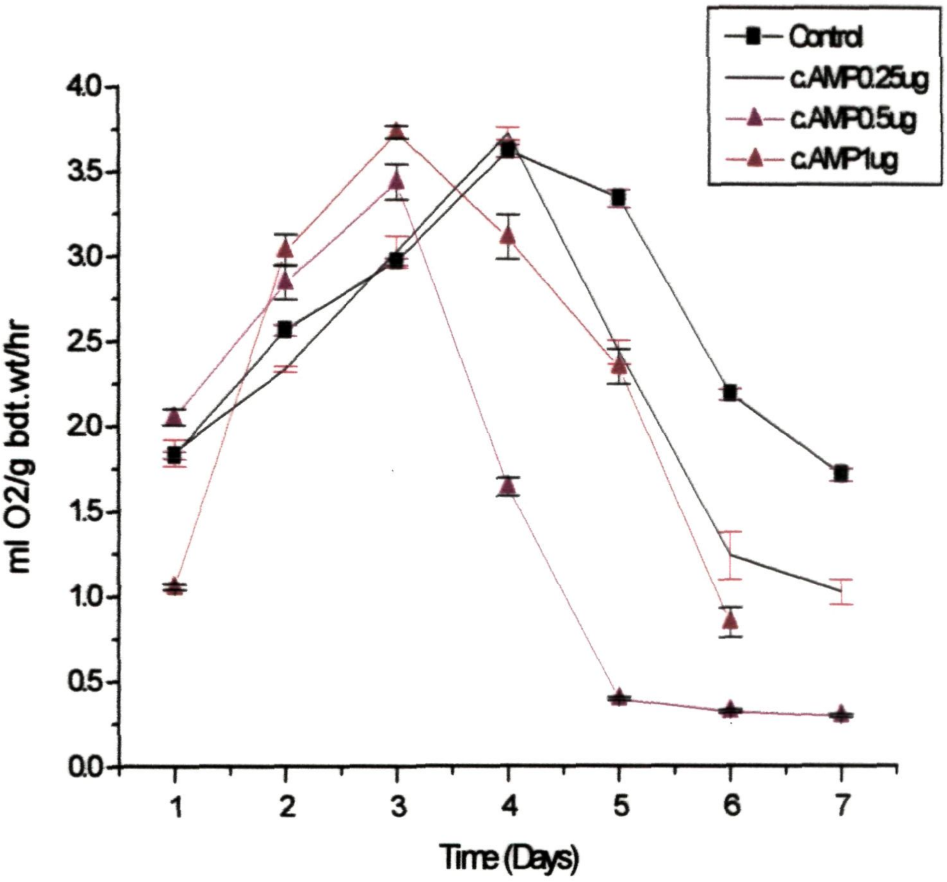


Table.8. Effect of different concentrations of JH-III, 20-HE and c-AMP on oxygen consumption of 4th instar larvae. The results are expressed as mean ± SEM, *n*=4

Hormone	Concen-trations	O_2 consumption in days (ml O_2/gm bd. wt/hr)				
		1	2	3	4	5
JH-III	Control	0.95±0.094	1.38±0.023	1.92±0.050	1.84±0.037	1.22±0.071
	0.25 µg	1.05±0.083	1.52±0.125	2.15±0.077	1.13±0.024	0.04±0.010
	0.50 µg	1.06±0.051	2.24±0.048	2.53±0.042	2.74±0.085	2.23±0.015
	1.00 µg	1.12±0.143	2.54±0.080	2.91±0.107	3.06±0.045	2.93±0.033
20-HE	Control	1.039±0.033	1.466±0.042	2.082±0.044	1.960±0.044	1.447±0.084
	0.25 µg	1.242±0.037	2.933±0.037	3.229±0.011	1.356±0.089	0.153±0.012
	0.50 µg	1.433±0.090	3.040±0.030	3.453±0.010	1.629±0.082	0.961±0.022
	1.00 µg	1.629±0.046	3.221±0.071	3.840±0.049	1.837±0.059	-
c-AMP	Control	1.13±0.039	2.360±0.020	2.767±0.072	1.614±0.075	1.347±0.047
	0.25 µg	1.252±0.01	2.454±0.040	2.843±0.043	1.250±0.059	0.131±0.018
	0.50 µg	1.426±0.044	2.650±0.082	3.042±0.036	1.739±0.075	0.56±0.104
	1.00 µg	1.744±0.081	2.845±0.050	3.436±0.081	2.062±0.005	1.750±0.067
	$CD_{0.05}$	**0.119**	**0.097**	**0.095**	**0.101**	**0.085**

Table. 9. Effect of hormones on oxygen consumption of 4th instar larvae (irrespective of concentrations).

Hormone	O_2 consumption in days (ml O_2/gm bd. wt/hr)				
	1	2	3	4	5
JH-III	1.046 a	1.918 a	2.378 a	2.196 a	1.608 a
20-HE	1.336 b	2.665 b	3.151 b	1.695 b	0.640 b
c-AMP	1.388 b	2.577 c	3.022 c	1.677 b	0.949 c
$CD_{0.05}$	**0.103**	**0.084**	**0.083**	**0.087**	**0.073**

Note: Results are expressed as mean±SEM., Duncan's Multiple Range Test, n=16.
At each stage of larval development, fig. with common alphabet does not differ significantly.

Table. 10. Effect of different concentrations of JH-III, 20-HE and c-AMP on oxygen consumption of 5th instar larvae. The results are expressed as mean ± SEM, n=4

Hormone	Concen-trations	O_2 consumption in days (ml O_2/gm bd. wt/hr)				
		1	2	3	4	5
JH-III	Control	1.744±0.055	2.117±0.009	2.396±0.091	2.869±0.049	2.367±0.023
	0.25 µg	1.834±0.067	2.228±0.012	2.316±0.096	2.802±0.107	2.802±0.107
	0.50 µg	1.949±0.068	2.440±0.046	2.845±0.042	3.026±0.100	1.938±0.104
	1.00 µg	1.065±0.004	2.634±0.131	2.942±0.061	3.712±0.073	3.606±0.138
20-HE	Control	1.844±0.090	2.317±0.043	2.859±0.045	3.716±0.042	3.767±0.061
	0.25 µg	2.115±0.116	3.451±0.079	3.65±0.120	3.942±0.111	2.329±0.077
	0.50 µg	2.259±0.124	3.573±0.148	3.740±0.066	2.560±0.078	0.253±0.009
	1.00 µg	1.541±0.118	3.833±0.043	3.958±0.098	2.831±0.095	1.846±0.101
c-AMP	Control	1.829±0.021	2.566±0.030	2.970±0.021	3.620±0.039	3.342±0.050
	0.25 µg	1.847±0.079	2.338±.017	3.029±0.095	3.723±0.039	2.439±0.070
	0.50 µg	2.051±0.047	2.844±0.099	3.436±0.104	1.640±0.052	0.394±0.009
	1.00 µg	1.051±0.017	3.035±0.092	3.729±0.037	3.109±0.131	2.347±0.102
	CD$_{0.05}$	**0.132**	**0.127**	**0.134**	**0.139**	**0.134**

Table.11. Effect of hormones on oxygen consumption of 5th instar larvae (irrespective of concentrations).

Hormone	O_2 consumption in days (ml O_2/gm bd. wt/hr)				
	1	2	3	4	5
JH-III	1.648 a	2.355 a	2.625 a	3.102 a	2.437 a
20-HE	1.939 b	3.294 b	3.552 b	3.262 b	2.049 b
c-AMP	1.694 a	2.695 c	3.291 c	3.023 a	2.130 b
$CD_{0.05}$	**0.114**	**0.110**	**0.116**	**0.120**	**0.116**

Note: Results are expressed as mean±SEM., Duncan's Multiple Range Test, n=16.
At each stage of larval development, fig. with common alphabet does not differ significantly.

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CHAPTER – 6

Structure of larval integument during development: Correlation with hormonal titres

CHAPTER-6

INTRODUCTION

The bodies of arthropods are encased in essentially nonliving external skeletons. In order for the tissues within to grow, the volume and surface area of the external skeleton must increase. Insects have solved the problem of growth within a nonliving exoskeleton in two different ways. The primitive method, by which their integument grows, shared with all other arthropods, is by molting. This involves the periodic casting away of the old confining cuticle and the manufacture of a new larger one. During this process, much of the old cuticle is digested for reuse. Various steps in the molting cycle are designed to protect the new cuticle from digestion by the enzymes that attack the old cuticle, while keeping the former soft and pliable so it can expand rapidly when the old cuticle is finally shed.

The cuticle is the outer covering of the insect and is its exoskeleton to which the muscles are attached. The outermost layer is called the epicuticle; under this is the exocuticle followed by the endocuticle. In some systems the exo- and endocuticle are classed together as the procuticle. In some insects only the epi- and exocuticle are deposited before ecdysis with the endocuticle following after ecdysis whereas in others, some endocuticle may be deposited before ecdysis (Condoulis *et al.*, 1966). The epicuticle is composed of protein whereas the exo- and endocuticle contain both chitin and protein in varying proportions depending on the type of cuticle, i. e. whether rigid or flexible. Chitin is a polymer of N-acetylglucosamine and can be cross-linked to the protein components of the cuticle in a process called sclerotization or hardening which usually occurs in the exocuticle just after the shedding of the old cuticle and expansion of the new cuticle. After sclerotization the insect is able to move, feed, fly, etc. The rigid parts of the cuticle are then set and cannot be expanded whereas flexible cuticle may expand either due to a simple unfolding of the new epicuticle or in response to hormonal signals. When the epicuticle has completely unfolded, further expansion is impossible and the larva must molt in order to grow further. Molting is also necessary at the end of larval life for metamorphosis (Mitchell, *et al.*, 1971; Marks and Sowa, 1976).

The epidermis is a single cell layer that makes the cuticle that lies above it. During the intermolt period the epidermis actively deposits lamellate endocuticle, especially in those regions where the cuticle is extensible. The chitin and protein are secreted as plaques at the tips of the microvilli at the apical surface of the epidermal cells (Ferkovich *et al.*, 1981). Above the plaques in the extracellular space, the cuticle arises by self-assembly of the chitin microfibrils and the secreted proteins. As the larva grows, the epidermal cells underneath the extensible cuticle also grow. During this cell growth, there is DNA synthesis and the epidermal cells may become polyploid (Dyer *et al.*, 1981). In the case of a soft bodied insect such as the larva of the tobacco hornworm, *Manduca sexta,* the epicuticle is deposited in folds to allow for growth during the succeeding intermolt period during which time the epicuticle then unfolds. In this instance, the underlying endocuticle expands via a series of apical expansion points created by the deposition by the epidermal cell of vertical chitin microfilaments during the first day after ecdysis. To accommodate these two processes that must occur at the same time, an unstructured layer is deposited between the epicuticle and the lamellate procuticle (Riddiford, 1976; 1978).

At the onset of the molt, the epidermal cells detach from the overlying cuticle and the cells including those that are polyploidy, may divide. Endocuticle synthesis ceases and is followed by secretion of 'molting gel' that fills the space between the old cuticle and the apical border of the epidermis. Later activation of these enzymes at the end of the molt leads to digestion of the old cuticle. Other cellular events preparatory to the deposition of the new cuticle such as cellular shape changes that prefigure the surface conformation also occur (Wielgus and Gilbert, 1978; Wolfgang and Riddiford, 1987).

Both embryonic and postembryonic growth and development are punctuated by periodic molts known to be initiated by the ecdysteriods. Regulation of cuticle production is under hormonal control, both by ecdysteriods and juvenile hormone (Riddiford, 1994). The role of ecdysteriods in molting is important in early cellular changes for proliferation and differentiative changes. Pertaining to the control of 20-HE during ecdysis and molting in insects, innumerable works have been done. The cellular events in the life of epidermal cells have been correlated with ecdysteriod titres in several insects' orders *viz.* odonata (*Aeshna cyanera*, Schaller and Charlet, 1980), orthoptera (*Gryllus domesticus*, Gnatzy and Romer, 1980; *Locusta migratoria*, Hirn *et al.*, 1979; Brehelin and Aubry, 1982), Hemiptera (*Oncopeltus fasciatus*, Smith and Nijhout, 1981; *Rhodnius prolixus*, Steel *et. al.*,1982), Lepidoptera, (*Calpodes athlius*: Dean *et al.*, 1980; Locke and Huie, 1980; *Galleria mellonella*: Hwang-Hsu, *et al.*, 1979; Sehnal *et al.*, 1981; *Bombyx mori*, Kiguchi and Agui, 1981; *Manduca sexta*, Fain and Riddiford, 1973; Riddiford, 1976, 1978, 1981; Bollenbacher *et. al.*, 1981; Wolfgang and Riddiford, 1981; *Peiris brassicae*: Mauchamp *et al.*, 1979; Cassier *et al.*, 1980).

It has been associated that during the rise of 20-HE, the epidermis responds by going through a preparatory phase that includes RNA and protein synthesis, often DNA synthesis and cellular shape changes. During this initial phase all of the ecdysteriod-dependent cellular events for later cuticle deposition occurred and the relatively short exposures to 20-HE *in vivo* are sufficient, and in fact the removal of hormone is required for later normal deposition (Marks *et al,* 1970, 1976; Oberlander, *et al.*, 1974; Ferkovich *et al.*, 1981; Fristrom *et al.*, 1982).

The principal function of the insect epidermis is cuticle secretion, and although cuticle deposition prior to esdysis is elicited by the molting hormone, ecdysone, there is ample evidence that cuticle secretion continues after ecdysis into the next instar (Weis-Fogh, 1952; Neville, 1963; Whitten, 1969; Filshie, 1970; Mitchell *et al.*, 1971; Zelazny and Neville, 1972). The exact regulatory mechanism for cuticle secretion between ecdyses (intermoult) is not known although there is evidence in *Sarcophaga falculata* that post emergence cuticle deposition is induced by a substance from the ocellar nerve (Schlein, 1972) while a humoral factor from the head of *Calpodes ethlius* appears necessary (Condoulis and Locke, 1966). For cuticle synthesis to occur during intermolt period the brain secrete a releasing or stimulating factor that causes the fat body to synthesize or release substrates or other substrates necessary for cuticle synthesis (Wielgus and Gilbert, 1976). It has also been postulated that minute quantities of β-ecdysone induce intermoult cuticle secretion in both *Calpodes* (Wang and Locke, 1975) and *Bombyx mori* (Kimura, 1973).

However, the requirement of ecdysteriods for cuticular deposition varies even in the same animal. Insects appear to be sensitive to ecdysones at all stages of their development. If a sufficient amount of an ecdysone is applied to an insect, the epidermal cells of the insect promptly secrete a cuticle (Schneiderman, 1972). In *Manduca sexta*, the larval dorsal abdominal epidermis requires 12h exposure to the same concentration of 20-HE in the presence of JH, but again no larval cuticle is produced until about 12-24h later (Mitsui and Riddiford, 1978). It has been reported that the duration of the period of epidermal mitosis during the rising phase of ecdysteriod titer is shorter than in the normal adult molt (Smith and Nijhout, 1981). In both the larval and adult molts of *Rhodnius prolixus*, where feeding initiates both growth and molting, the appearance of mitosis which was triggered by the increasing cell size occurs just before the molting rise in ecdysteriod (Steel *et al.*, 1982). Hence, epidermal cells have different strategies for increasing the surface area of the new epicuticle produced at a larval molt and for changing the types of cuticular structures produced at all molts. In all cases these events are initiated by the rising phase of the ecdysteriod titer and DNA synthesis is usually involved *viz. Bombyx mori* (Kato, 1977; Kato and Oba, 1977) and *Manduca sexta* (Dyer *et al.*, 1981).

The titers of ecdysones are known precisely for the last larval instar of *Manduca sexta* (Bollenbacher *et al.*, 1975) and *Samia cynthia ricini* (Jiang *et al.*, 2005) and also bioassays for juvenile hormone activity have long been investigated in numerous species of insects (Gilbert and Schneiderman, 1960; Williams, 1961). Therefore it would be of interest to study the cuticle morphology of the penultimate 4th instar and the last larval 5th instar and correlate the same with ecdysteriod and also JH titers during development of the silkmoth, *Philosamia ricini* (Lepidoptera: Saturniidae).

MATERIALS AND METHODS

1. The Insect:

Philosamia ricini used in these experiments were received from, Sericulture Department, Nongpoh, Govt. of Meghalaya. Larvae were reared in a rearing cage indoor and supplied with diet of Castor leaves, *Ricinus communis*. They were reared at 25° C ±0.5 under 16L: 8D photoperiod regiment as described by Bell and Joachim (1976).

In the laboratory, eggs are laid in clusters, glued together in rows projecting from the substrate or kharika (small bundles of thatched or splited bamboo, about 30 cm long with a hook). In our condition the number of eggs laid per female ranged from 200-250 (mean 225, but sometimes 400 to 450 eggs). The oviposition period lasted 7 to 8 days and after incubation and hatching the larvae were given soft and succulent young leaves of castor of which they fed immediately. Later during the course of their growing process the leaves were given as per their requirement in term of quality and quantity. Initially, the larvae live gregariously in young instars, but later they tend to segregate and feed freely. Thus, the larval activity and feeding occurred from the date of hatching until when they shortened and spun cocoon. Between feeding periods, 1st to 3rd instars orient themselves on the underside of leaves and later feed voraciously. After the larvae stop feeding, they emptied their gut content and undergo wandering for few hours. Then the larvae prepared cocoons individually between the leaves and pupated inside the cocoon within 5-7 days.

In the present study it would be convenient to describe only the time following the molt of the 3rd instar to the 4th instar and the following developmental stages upto the period prior to pupation.

For studying the integumental development changes, some physiologically synchronous 4th instar larvae were fist selected at day-1, just few hours after molting of the last day 3rd instar to 4th instar. These larvae were then kept for histological study, while the rest of the larvae were allowed to continue feeding and growth. As soon as the larvae had reached day-3 of development, some larvae were again separated as has been done in the first day and the collection and separation was preferably done at the same time. In our condition, the duration of development of the 4th instar lasted exactly for five days and accordingly the larvae were selected at this time. Later, those larvae that molted to the fifth instar were again selected on the same day of molting and they were designated as day-1 fifth instar. In the same way, the developing 5th instar larvae were allowed to continue feeding and growth as in the 4th instar. The larvae were chosen for study on the 2nd, 3rd, 5th and 8th days of the last larval instar development and their age were designated as day-2, day-3, day-5 and day-8 respectively. The last larval instar feeds upto the 5th day and then emptied its gut content, undergo wandering and spin a cocoon within the same day although with low intensity. As spinning advances, there is remarkable weight loss and the morphology of the larva changes gradually. After spinning for 1-2 days the larvae lie inside the cocoon and lost the mobility of their prolegs assuming a typically prepupal form and then finally pupated on the 9th day. However, for convenient and ease of differentiation, larvae were selected on the 8th day for histological study of the integument.

2. Histological procedure:

Larvae were anesthetized with chloroform and then dissected for integumentary structures. The dorsal and dorso-lateral sides of the integument of appropriate sizes were cut and were always used for the study. To prevent shrinking of the cuticle, the larvae were initially decapitated; the gut content emptied and the haemolymph were drained and washed thoroughly in clean water. They were then fixed in freshly prepared Bouin's fixative so that the tissue's morphology were are preserved, cells killed, autolysis and bacterial infection prevented. This will minimize swelling and shrinkage and convert the chemical constituents into stable compounds and also prevent or minimize their displacement from their original locations, harden the material without making brittle so that it can withstand subsequent physical and chemical treatments and render the material stainable. The tissues were kept submerged in fixative for 24-48 hours or more. After fixation pieces of integument were dissected out neatly, cleaned of adhering tissues, washed and dehydrated in ascending series of ethyl alcohols (30 %, 50 %, 70 %, 90 % and 100 %) in appropriate time at each stage. After dehydration, the tissues are transferred in a container containing absolute alcohol and xylene in the ratio of 1:1 for five minutes, then immersed in xylene (5 min) followed by xylene-paraffin solution (1:1) and incubated with Paraffin wax (58-60°C, Merck, India Ltd.) overnight. After incubation, the tissues are embedded in paper boats of appropriate sizes and processed further according to standard histological procedures. The embedded tissues were sectioned at 6-8 μ, affixed to coated slides and stained in Mallory's Triple Stain (Humason, 1966). Observations and all photomicrography were performed using Microscope.

The Mallory's triple stain was used in the study because this stain can differentiate integumentary structures based on their chemical constituents and they can be observed accordingly in different shade of colours. For instance, Aniline blue which is acidic can stains connective tissues and cartilages, Orange-G stains blood cells, myelin and muscle while the Acid fuchsin stains the rest of the tissue including the muscle in grades of pink and red. Thus, the lipid epicuticle will reveal blue colour, cuticulum epicuticle-red and endocuticle will stain bluish, while the rest of the tissue will give colour in grades of pink and reddish or brownish.

3. **Preparation of Malory's Triple Stain:**

a. **Mallory's I:**

i. Preliminary mordant: Saturated $HgCl_2$ in 100ml of distilled water mixed with 5 % H_3COOH (acetic acid).

ii. Acid fuchsin (Sigma Chemical Ltd., USA), 1 % in distilled water.

iii. Phosphomolybdic acid, 1 % in distilled water.

b. **Mallory's II:**

i. Aniline blue (Gurr) - 0.5 g

ii. Orange-G - 2 g

i.) and ii.) were dissolved in 100 ml distilled water.

4. **Proceedure of staining:**

a. Removal of paraffin in xylene - 5-10 min

b. Removal of xylene in absolute alcohol - 5 min

c. Hydration in descending series of alcohols - 5 min each

d. Mordant in $HgCl_2$-H_3COOH solution - 10 min.

e. Rinse in d. w. and stain in Acid fuchsin soln. - 15 sec.

f. Differentiate by washing in d. w. - 10 sec or as required (to differentiate red colour)

g. Keep in Phosphomolybdic acid soln. - 1-5 min

h. Wash in d. w. and stain in Mallory's II - 2 min

i. Drain and wipe the back of the slide in d.w.

j. Dehydration in ascending series of alcohols. - 5 min each

k. Differentiate Aniline blue w. s. in 90 % alcohol - 10 sec or more as required.

l. Dip twice in Absolute ethyl alcohol - 10 sec each.

m. Keep in xylene for 10 min and mount in DPX.

METHODOLOGY

(Routine Paraffin Histological Methods):

Dissection (Saline solution)
↓
Fixing of tissues (Bouin's fluid)
↓
Dehydration (up grade) 20 minutes each
↓
Clearing or de-alcoholisation

1. Abs alcohol: xylene (1:1) (20 – 30 minutes)
2. Xylene (20 – 30 minutes)

↓
Infiltration

1. Xylene: Paraffin (1:1) at 58° C – 60° C (20 – 25 minutes)
2. Pure Paraffin (20 – 30 minutes) or incubate overnight

↓

Embedding

1. Paper boards applied with glycerine
2. Cooling 15 – 40 minutes

↓
Trimming of the block

1. Face – 1mm
2. Base – 3-4mm

↓
Sectioning (5 – 8 μm in thickness)
↓
Affixation of slide Mayer's solution
↓
Staining Mallory Triple Stain
↓
Mounting in DPX
↓

Observations and photographs

RESULT

1. Whole animal growth:

To provide a basis for the interpretation of cellular growth, whole animal weight was determined (Weilgus and Gilbert, 1978). The resulting weight curve (Fig. 30) suggest the presence of two distinct physiological phases in each case (4th and 5th instars): first, a period of growth from days 1-4, characterized by a logarithmic weight increase, and second, a period of preparation for larval molting (4th instar to 5th instar) and pupation from 5-6 or 7 days, characterized by weight lost and then weight stabilization (Table. 12). In the fifth instar larva the transition between the two phases is marked by cessation of feeding and elimination of the gut contents, accounting for the precipitous weight loss (Fig. 30 B). In the 4th instar larva the weight curve exhibit a logarithmic increase up to the last day of the instar (Fig. 30 A). Comparatively both the instars shows contrasting weight curves towards the end of each developing stages respectively (Fig. 30 C).

2. Histology of overall integumentary structures:

The larval integument is composed of sclerotized and unsclerotized cuticle at different stages of development, epidermal cells and a thick basement membrane. On day 1 of both the 4th and 5th instars, the epidermal cells exhibits cuboidal or low columnar morphology (Fig. 37 and Fig. 40), but become distinctly columnar as growth ensues on day 3. Adjacent cells also maintain close contact until day-3 in 4th instar (Fig. 38) and day-2 in 5th instar (Fig. 41) when intercellular spaces become visible and but persist afterwards (Fig. 39, 43 & 44). The epidermal cells continue to deposit cuticle after ecdysis to the fifth instar and secrete the thickest lamellae at day-5 in 4th instar and day-8 in 5th instar respectively (Fig.39 and Fig. 44). The retraction of cells during apolysis from the cuticle follows an interior to posterior, dorsal to ventral gradient, but cells at the intersegmental membranes adhere until the approximate time of apolysis in the prolegs. Following apolysis little morphological change can be observed at the light microscopic level other than secretion of pupal cuticle in 5th instar and no significant cell death can be detected at any time during the instar.

3. Cuticle morphology at day-1 & day-3 of 4th instar (Fig. 37 & 38) and day-1, day-2 & day-3 of 5th instar (Fig.40, 41 & 42).

As the epi and exocuticles are being deposited, the epidermal cells begin to secrete the endocuticle above it as can be seen from cuticle of early instars (1-3 days old) which exhibits most prominent and smooth endocuticle, while epi and exo-cuticles are thin and not distinct from each other (Fig. 37 & Fig 40). This pattern of cuticle growth are seen up to the 3rd day in both instars while the integumentary structures are more prominent in the 5th instar (Fig. 40 & 41).

The fourth instar at day-1, revealed a very prominent and probably soft endocuticle which occupy most of the integumentary structures (Fig. 37). The basement membrane are not prominent while the epidermal cells are visible lightly with very low columnar morphology. As the larva grow, the basement membrane and the epidermal cells become more distinct (Fig. 38). However, morphology of the epicuticle and the endocuticle does not change much compared to the previous one at day-1. Overall the sclerotization of cuticle layers are low. Basement membrane which contained epidermal cells along with intercellular spaces are faintly observed.

The cuticular structures of the young 5th instar are similar to the growth pattern of the 4th instar at the same stages. At day-1 and day-2, the endocuticle are most prominent and while the basement membrane and the overlying epidermal cells exhibit low columnar morphology suggesting low activity of the cells. The outer cuticular structures of epicuticle and exocuticle are much less defined showing that they are not yet sclerotize and in the process of growing (Fig. 40 and Fig. 41). Bristle along with its base can also be observed as outgrowth originating from the integument and forming a canal through the entire structure (Fig. 41). As the larva became older, the integument continues to grow and increases in size and the cellular structures became more defined with appearance of intercellular spaces at day-3 (Fig. 42). The epicuticle and the exocuticle are also more deeply stained and can be distinguished clearly while the basement membrane show adhering columnar epidermal cells suggesting growth has taken place in all integumentary structures.

4. Cuticle at day-5 in 4th instar (Fig. 39) and day-5 & day- 8 in 5th instar (Fig.43 & 44).

Integuments of late larval instars (4th instar-Day 5; 5th instar-Day 5-8) exhibits rough endocuticle with thickest lamellae produced at these times. The epi and exo-cuticles are distinct with prominent sclerotization. Basement membrane along with epidermal cells with prominent intercellular spaces are also observed.

In the present study it was observed that the last stages of cuticle deposition in 4th and 5th instars separates the cuticle from the epidermis and the formation of plagues in between them (Fig 39 and Fig. 43 & 44). This apolysis which is distinguishable can be correlated with, and presumably due to, the disappearance of the attachment plaques. Sclerotization and roughness of the overlying cuticle above the basement membrane are the most observable characters which emphasized that sclerotization of outer cuticle had completed and the cuticle are ready for molting (Fig. 39 and 44). At about these times the molting gel is probably secreted and mitosis and cell division had occurred as the epidermal cell becomes denser, more columnar, and their apical surface is thrown into a series of fine folds especially for the integument at day-5 in 4th instar and day-8 in 5th instar respectively (Fig. 39 and Fig. 44).

In the 4th instar at day-5 *i.e.* last day of developing instar, thickest lamellae secreted between 2-4 days can be observed prominently (Fig. 39). It can be observed that epicuticle and exocuticle are highly sclerotized and appear to be hardened which suggest that they are ready for the molt to the fifth instar in a short time. The epidermal cells are highly columnar while the plagues becoming wider showing that they are also ready to be filled up with the molting fluid which are due to be secreted at the time of molting.

In the fifth instar at day-5, the thick lamellae with the appearance of intercellular spaces in between the epidermal cells are almost the same as observed in day-3 of 4th instar, however in the fifth instar the epicuticle and the exocuticle appear to be unsclerotized which shows that the integumentary structures are still growing and are not yet ready for molting process (Fig. 43). The overall appearance of the integument seemed to be soft and pliable so that it can create spaces for more expansion as it continue to grow even for a short while although larva at this stage (day-5) had almost cease feeding. As larval development continue by undergoing wandering, gut purging, spinning and attaining pre-pupal character, the integument at day-8 showed matured structures (Fig. 44). The epicuticle and exocuticle appeared hardened and heavily sclerotized and looked to be almost detached from the underlying endocuticle implying prodromal sign of pupation or ecdysis. The integument secreted the thickest lamellae at this time as can be seen in the structure of the endocuticle. The basement membrane along with the epidermal cells are also prominently displayed showing high columnar morphology. Cellular activity at this time have probably undergone division and are ready to be shed so that the larval character are dispose to make room for differentiation into the pupal cuticle.

DISCUSSION

Correlation of cuticular growth and structures with hormonal secretions

Morphology and structural changes of integuments of 4th & 5th instars are quite similar throughout development. The high columnar morphology of the late larval integument with its structural maturity (prominent epidermal cells & intercellular spaces) can be correlated to be induced by prothoracicotropic hormone (PTTH) and ecdysteriod secretions, since, such hormones are known to be at their maximal levels prior to molting. It is established that during the last instar development of *Samia cynthia ricini,* ecdysteriod level changes dramatically. Haemolymph ecdysteriod level was low (<2 ng/ml) during the first half of the instar which was similar to ecdysteriod synthesis by (prothoracic gland) PG *in vitro* and also the actual synthesis from both the pair of prothoracic glands. High titer of PTTH just before this high peak of ecdysteriod were observed which show that ecdysteriod secretion was regulated by PTTH since during the period of low titer of ecdysteriod PTTH activity was not seen (Jiang *et al.*, 2005). Haemolymph level of ecdysteriods is dominantly regulated by PTTH (Mizoguchi *et al.*, 2001), although prothoracicostatic peptide plays a role in this regulation in some insect species (Hua *et al.*, 1999). In many insects endocuticle deposition continues throughout the entire intermolt period, so that the cuticle grows progressively thicker. Endocuticle deposition stops when apolysis for the next molting cycle begins.

This low ecdysteriod level can presumably relate to low cuticular growth as can be seen from the smooth and pliable nature of the cuticle while the epidermal cells and intercellular spaces are also not prominent. At this time the epicuticle and exodocuticle are not clearly distinct from each other making them difficult to identify.

This condition of cuticular structures revealed that they have not been or yet to be exposed to required amount of ecdysteriod or may be ecdysteriod have not been secreted at this time since the cuticle seemed to be unprepared for undergoing the molting process.

Again in *Samia cynthia ricini* haemolymph ecdysteriod level at the start of wandering phase increased from 15±3 ng/ml on day-7 and reached 86±11 ng/ml on day-9 and decreased steadily to a low level (~5 ng/ml) before pupation. This rise of ecdysteriod level is similar from that secreted by protharacic gland *in vitro* and from the individual glands itself (Jiang *et al.*, 2005). In the present study cuticle morphology were not observed beyond day-8 because the secretion of PTTH and ecdysteriod are highly expected by this time. However, comparing the rate of ecdysteriod synthesis and cuticular structure, we can assume that peaks of high ecdysteriod synthesis corresponds to hectic activity of epidermal cells. It was observed that in both the late larval instars (day-5 in 4th instar and day-5 and day-8 in 5th instar) intense activity of integumentary structures. At about this time probably the molting gel is secreted, the epidermal cells undergo a period of mitosis and cell division. The epidermal cell population becomes denser, the cells more columnar and prominent, and their apical surface is thrown into a series of fine folds. When cell divisions are completed, the epidermal cells secrete the cuticulin layer of the new epicuticle. Cuticulin is made up of lipoproteins that quickly become cross-linked and sclerotized with other proteins. This tanning process renders the cuticulin layer refractory to dissolution by acids, solvents and enzymes, although it remains quite permeable to water and many small molecules. We found that intercellular spaces more prominent than in younger stages and the epicuticle and endocuticle are distinguishable probably they are ready to be shed during the preceeding molt.

Usually, haemolymph ecdysteriod level and ecdysteriod synthesis by PGs in vitro change in parallel (Sehnal *et al.*, 1988). Most lepidopteran insects exhibit two ecdysteriod peaks in the last larval instar. The first, a subtle peak or plateau, is presumably associated with changes in larval behavior and reprogramming of the epidermis from the larval –type to pupal –type synthesis. The second peak is much larger. It is the hormonal stimulus for the molt from larva to pupa (Smith, 1985; Sehnal, 1989). Similar to *B. mori* (Mizoguchi *et al.*, 2001), the first peak of ecdysteriods was not significant in *Samia cynthia ricini* although PTTH activity was observed on days 1-2 (Jiang *et al.*, 2005) however it was reported that ecdysteriod are secreted only after the third day of larval development (Fujishita and Ishizaki, 1982). Therefore, in the present study, low level of integumentary structures upto day-3 in both the instars in term of cuticular deposition revealed that they had not been subjected to ecdysteriod action and hence cellular activity was also low. Further, the advanced integumentary structure observed in the late stages of larval development are highly expected to have been exposed to ecdysteriod since on days 9-10 in *Samia cynthia ricini*, right before the molt from larva to pupa, a big peak of PTTH and ecdysteriods was observed in the haemolymph and the medium incubated with PGs at this stage (Jiang *et al.*, 2005). The PTTH activity should contribute to the big peak of ecdysteriod which thus bring about cuticular changes for preparation of the molting process.

In any species of insects extensibility of the new cuticle is further enhanced by the action of two hormones, eclosion hormone and bursicon, that are secreted just prior to ecdysis. These hormones cause the release of plasticizing factors into the cuticle, probably via the pore canals. The plasticizing factors are believed to weaken some of the noncovalent bonds that hold cuticular proteins and chitin molecules together

(Reynolds, 1985). The extent to which the new cuticle can expand during ecdysis is determined entirely by the fine folds laid down in the outer cuticulin layer at the beginning of the molt cycle. During the pre- and postecdysial expansion the epicuticle stretches to a nearly flat surface. Cuticles that must remain extensible during the intermolt period, such as those of the larval abdomen of the blood sucking bug *Rhodnius prolixus*, retain a finely puckered cuticulin layer, which allows for some additional expansion when the animal takes a blood meal. In the present study although the cuticulin layer of soft-bodied holometabolous larvae were not taken into acccount, such caterpillars, are also finely corrugated at the beginning of an instar and gradually smooths out as the animal grows and the cuticle expands. If such provision for surface amplification of the epicuticle is not made, any further expansion in volume during the intermolt would be restricted to the amount that the abdomen can extend by telescoping. The space between the epidermal cells and the cuticle also known as plagues, which are formed in the late developing larvae (day-3 onwards) presumably are filled with a gelatenous solution, the molting gel, which contains, enzymes that will eventually digest part of the old cuticle. It was reported that in insects when cell divisions are completed, the epidermal cells secrete the cuticulin layer of the new epicuticle. Cuticulin is made up of lipoproteins that quickly become cross-linked and sclerotized with other proteins. This tanning process renders the cuticulin layer refractory to dissolution by acids, solvents and enzymes, although it remains quite permeable to water and many small molecules (Locke and Huie, 1979).

Again cuticular structures both in early and late instars can be correlated to activity JH titers. The action of JH on development is always associated with ecdysteriod action. JH does not prevent prevent ecdysteriod action in inducing the molt, but modulates its action. In the lepidopteran tobacco hornworm, *Manduca sexta,* rising ecdysteriod titers in the presence of JH cause a larval molt, and the critical period for the presence of JH is at initiation of the ecdysteriod rise (Hiruma, 2003). This condition of hormone interplay might have also occurred during the larval development of 4th instar (Fig. 37 & 38) where JH activity is high and during its molt to the 5th instar in day-5(Fig. 39) where JH activity is comparatively low. Again, the JH titer declines precipitously because of the cessation of secretion of JH by the corpora allata and the increase of JH esterase activity for degradation of JH so that JH become undetectable at the time of pupal commitment. This condition allow the brain to secrete PTTH so that it induce the PGs to secrete ecdysteriod (20-HE) and absence of JH this time causes larval-pupal commitement of the epidermis. Therefore in day-8 of 5th instar (Fig. 44), the integument can be considered that they have been committed to larval-pupal morphology.

Table.12. Larval weight of 4th and 5th instars of *Philosamia ricini* after ecdysis.

Days after Ecdysis	Weight of 4th Instar larvae (gm). Mean ± SD	Weight of 5th Instar larvae (gm). Mean ± SD
1	1.05±0.035	2.65±0.041
2	1.31±0.015	3.14±0.024
3	1.86±0.031	3.71±0.015
4	2.55±0.023	5.24±0.025
5	2.73±0.028	7.14±0.034
6	-	5.42±0.032
7	-	5.03±0.027
8	-	4.92±0.023
9	-	4.51±0.027

Fig.30. Weight of 4th instar (A), 5th instar (B) larvae and comparative weight (C) of *Philosamia ricini*

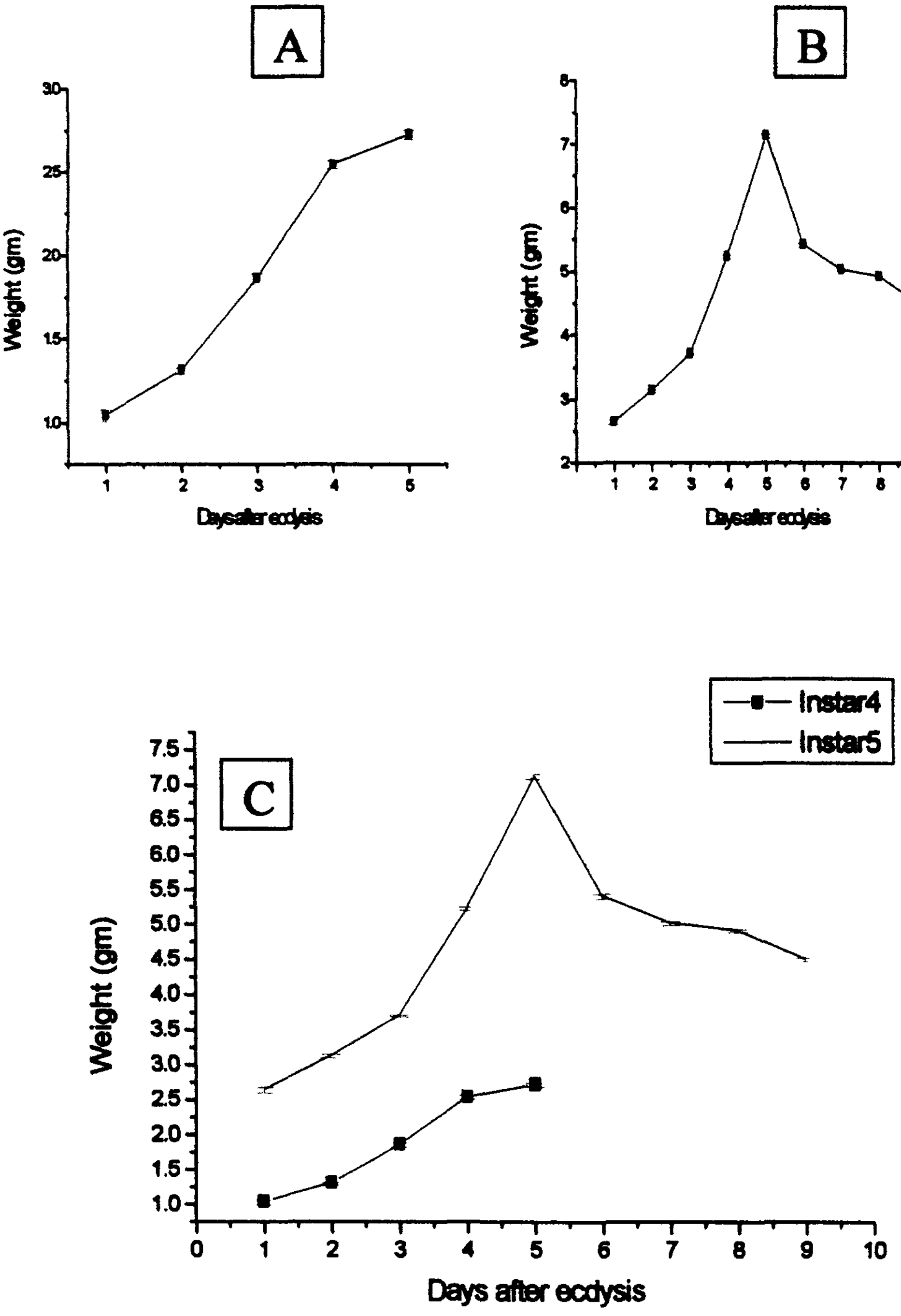


Fig.37. Integument of 4th instar at day 1

Scale bar A-C=50 µm; D=25 µm

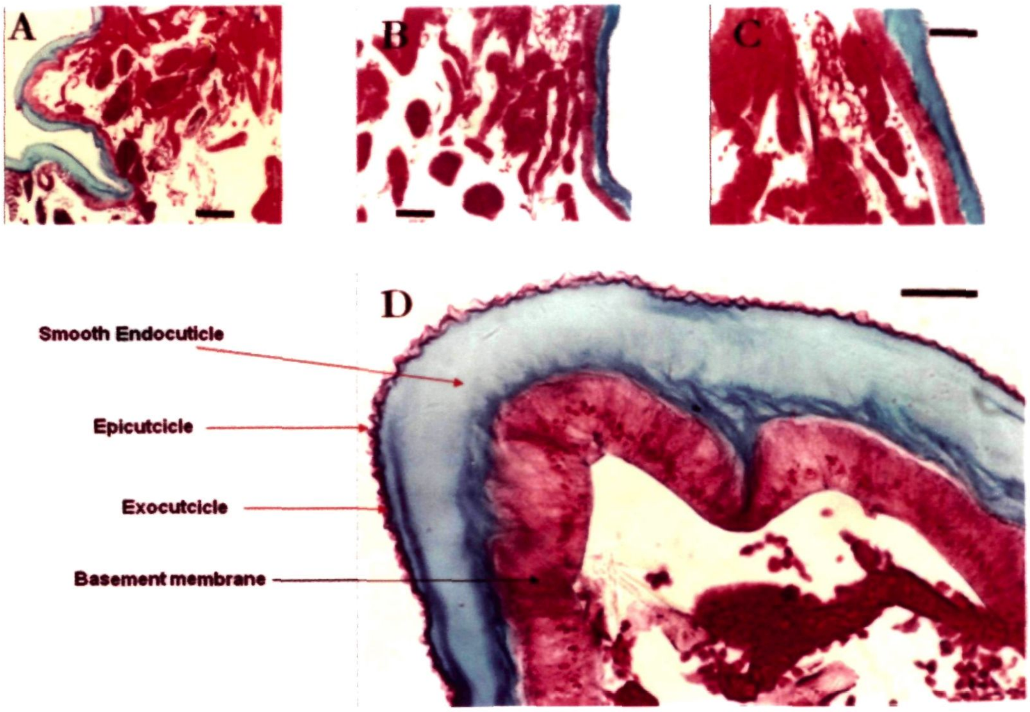
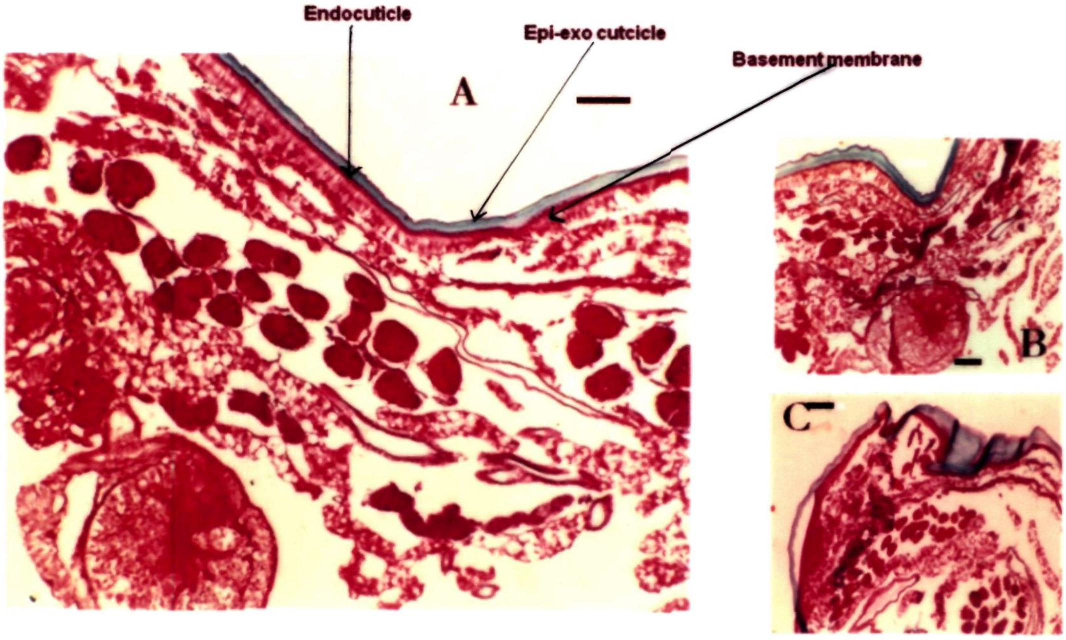


Fig. 38. Integument of 4th instar at day 3
Scale bar A-C=50 µm



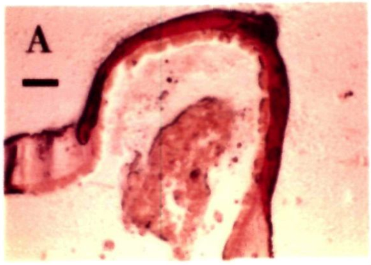


Fig. 39. Integument of 4th instar at day 5 illustrating the thick cuticular lamellae secreted between 2-4 days. Intercellular spaces are also present.
Scale bar A & B=25 µm

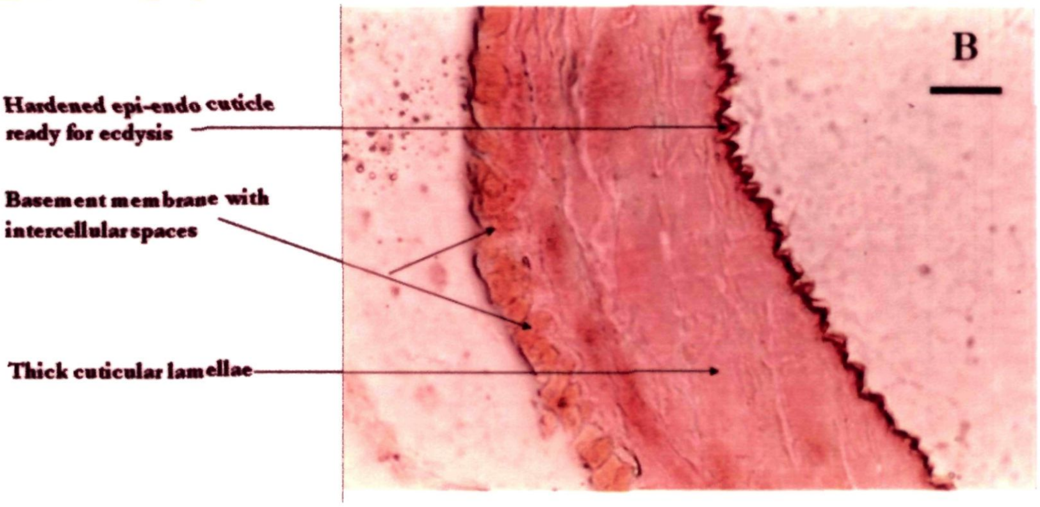


Fig. 40. Integuments of 5th instar larvae at day 1. Scale bar A-D=25 μm

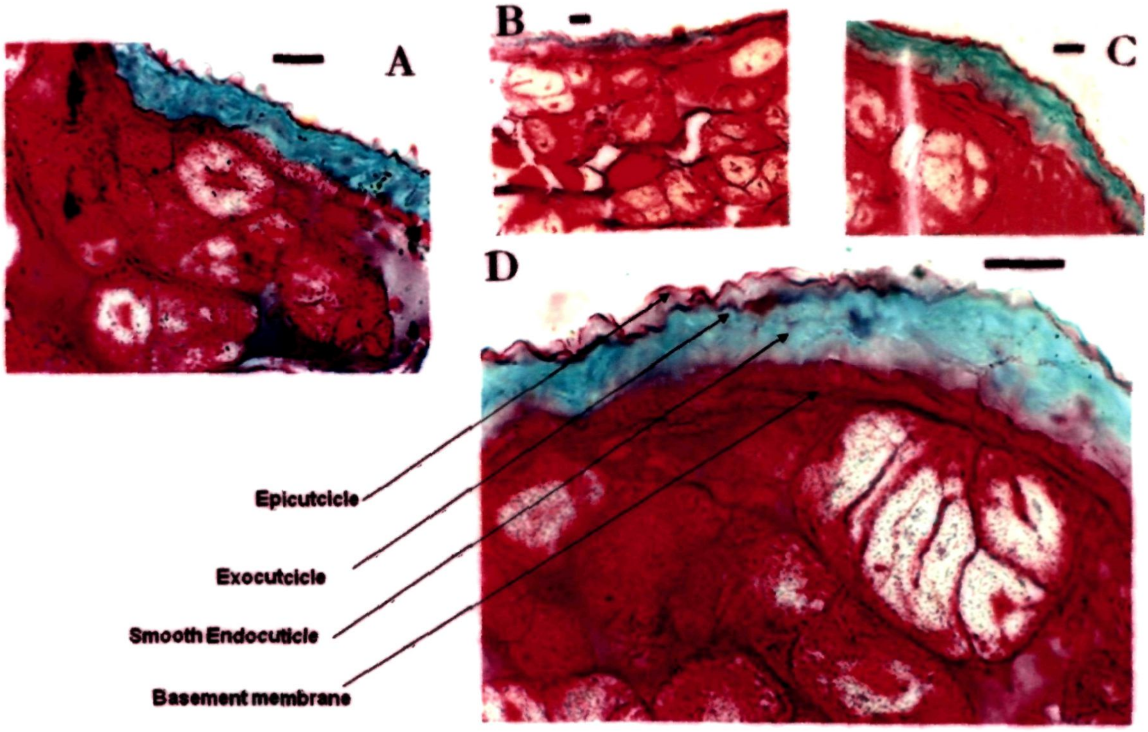


Fig. 41. Integument of 5th instar at day 2. Scale bar 25 μm

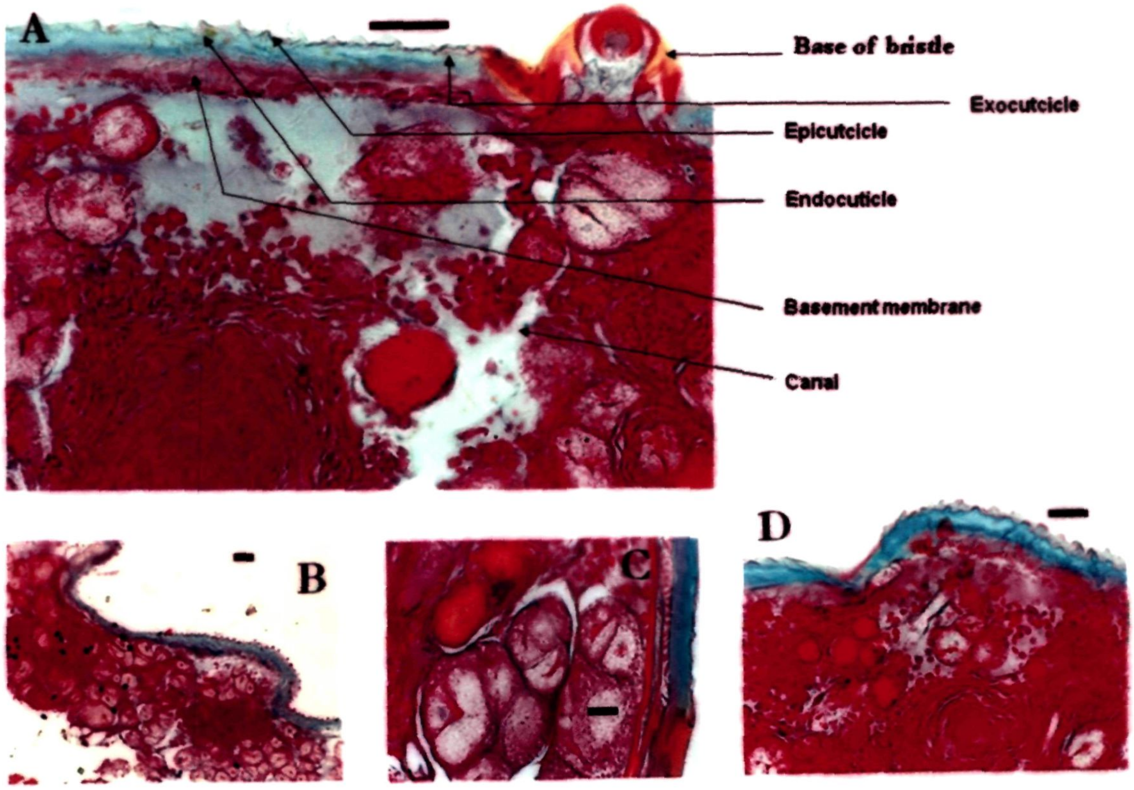


Fig. 42. Integument of 5th instar at day 3. Scale bar A-D=25 μm

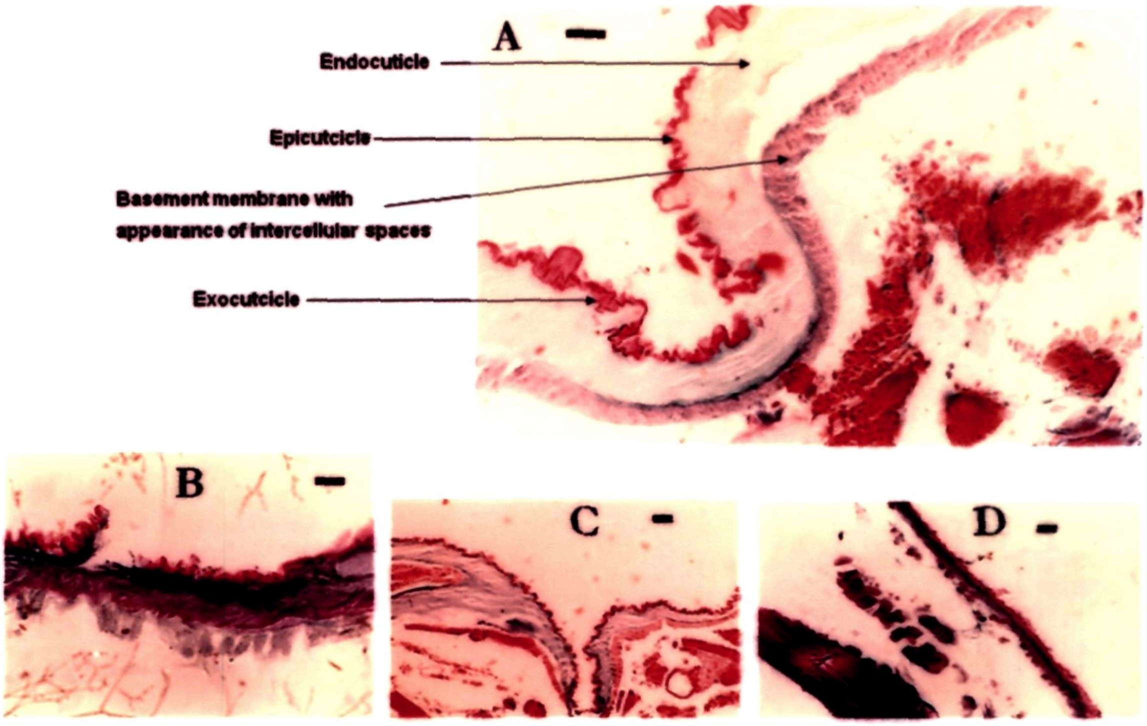


Fig. 43. Integument of 5th instar at day 5 illustrating thick cuticular lamellae secreted between day 2 and 4. Intercellular spaces are also present.
Scale bar A-D=25μm

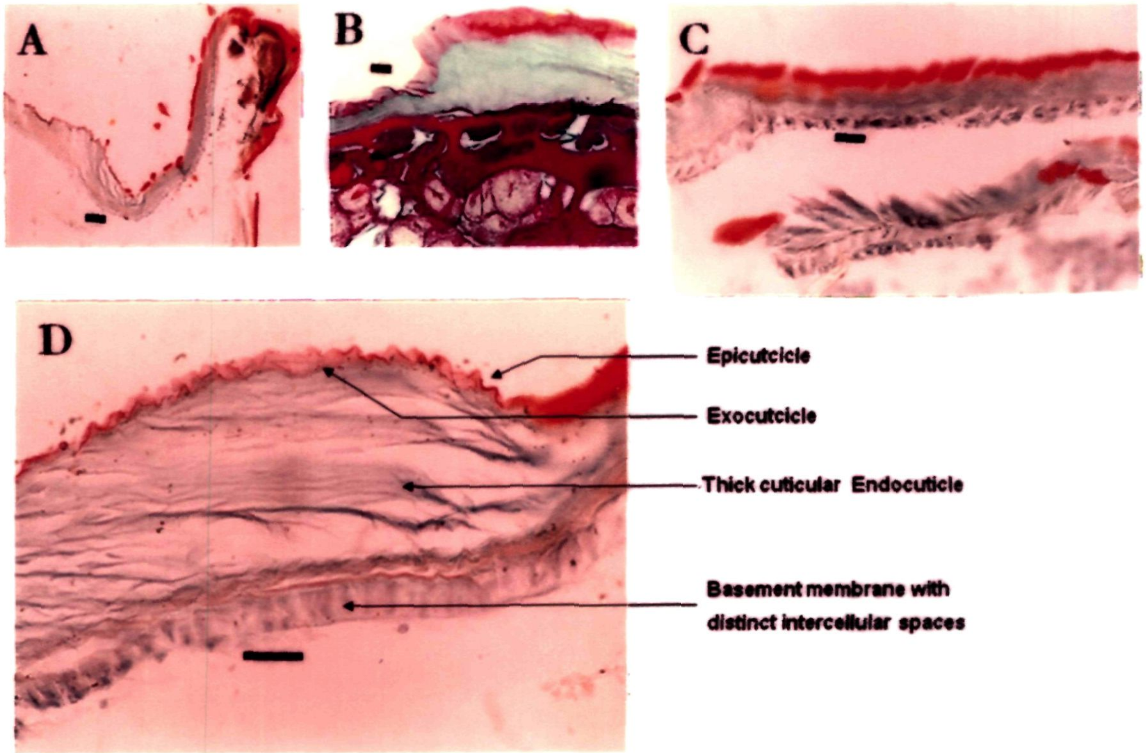
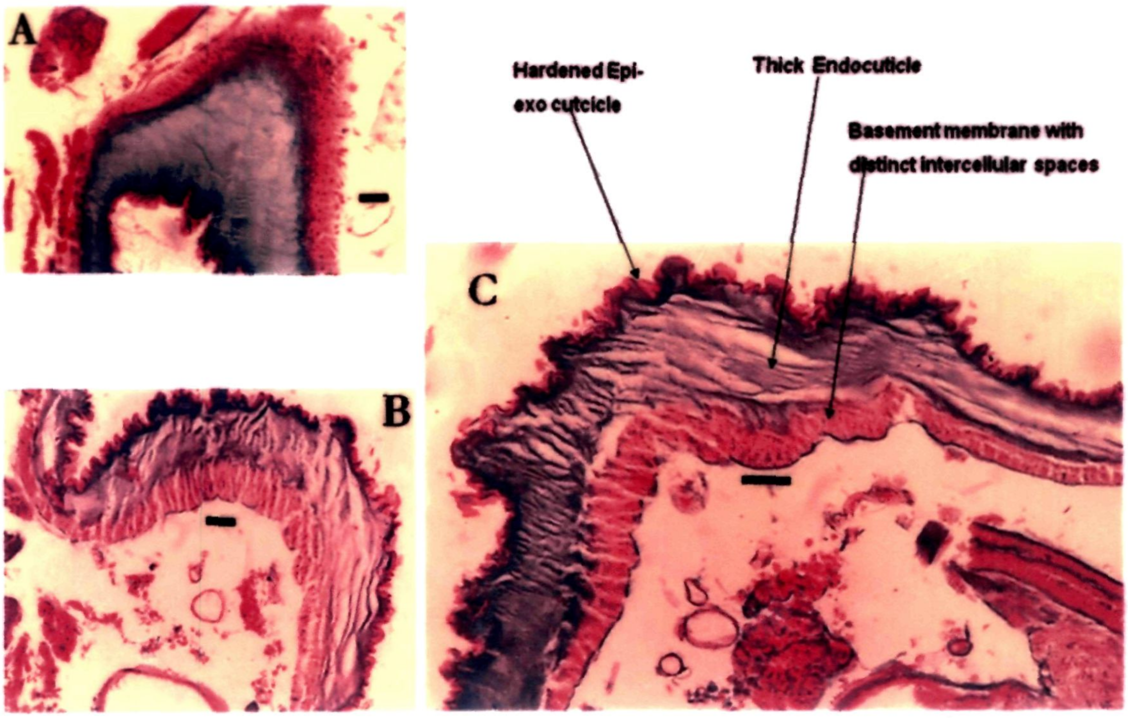


Fig. 44. Integument of 5th instar at day 8. Scale bar A-C=25 μm



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GENERAL CONCLUSION

GENERAL CONCLUSION

1. Effect of JH-III, 20-HE and cAMP on Egg hatching:

In our study we observed the deleterious effect of JH-III application on the eggs of *Philosamia* ricini. The effectiveness was more on the younger eggs. As the eggs get matured the effectiveness of JH-III application deteriorated but the deleterious effect did not stop.

Juvenoids are all fat-soluble compounds and it is quite possible that when they are administered, they get into the yolk mass and become available to the developing embryo through slow diffusion and as and when yolk is consumed by it. When larger doses are administered, however, they become immediately available and the inhibitory action starts forthwith.

As a result of JH treatment, developing embryo may cease at any stage of embryogenesis or at any other "functionally demanding phase". In *Philosamia ricini* we believed that the "functionally demanding phase" which is the most sensitive stage during embryogenesis must probably be the stages from 2-12 hours. These stages showed maximum unhatched eggs in term of percentages for all the three treatments.

Also of interest are the findings that larvae, which hatch out, were not able to feed and mature normally and they soon die prematurely. In those cases in which hatching did occur, the larvae often suffered with some abnormalities and further, apparently normal larvae may die during their postembryonic life, or their metamorphosis may be disrupted. We also observed that these abnormalities of the larvae occurred especially to those that hatched from younger eggs and even if the larvae seems to develop normally they may die at some point of time during the larval instars, pupal development or during emergence.

However, contrary to the observations made for JH-III treated eggs, 20-HE and cAMP treated eggs produced much more hatching percentages. Although, 20-HE and cAMP seemed to be have deleterious effect only to younger eggs but they enhance or promote hatching in older eggs. In our observation, cAMP more or less seemed to mimic the property exhibited by 20-HE, since the unhatched eggs produced as well as the pattern of effectiveness throughout the stages by both the treatments are almost identical. It may be possible that 20-HE enhance embryonic development as a result of the treatment but further investigation at the molecular level will emphasize the feasibility of the hormone.

2. Ultra structure of chorion and mechanism of hatching:

The chorion of lepidopteran insects has spatially and morphologically differentiated surface regions. In the present study the chorion of *Philosamia ricini* was observed to lack the aeropyle crown region. But four distinct regions, the posterior and anterior poles, lateral flat sides and ventral (dorsal) edges, are apparent.

The egg size was larger in *Philosamia ricini* in term of length of major and minor axis and the area of lateral flat region than those of other lepidopteran insects viz. *B. mori and B. mandarina.* The polygonal network patterns especially at the lateral flat and marginal regions are regular, there is no significant variation in the unit area of the polygon exhibited. At the posterior pole, knobs residing inside the polygonal units are visible prominently.

The variability in the size of aeropyles during egg development is difficult to distinguish functionally. However, since they mainly serve for gaseous exchange between the egg and the outside environment, it is assumed that insects modify the aeropyle structure during egg development according to the demand of the environmental condition.

The study in term of hatching of the larvae from the eggs revealed that they gnawed their way out through the chorion membrane in accord with the process adopted by most lepidopteran insects. For the process of hatching there are no rupture of egg neither from line of weaknesses nor presence of egg buster or specialize spine or cuticle.

In the present study, the overall structure of the egg during the course of development until hatching was not observe to change except for the minute pores or aeropyles present on the ridges of the entire polygonal network.

3. Study of hormonal influence underlying Pupation by ligation:

The first and most obvious behavioral response during preparatory period for pupation in *Philosamia ricini* is that the animal stops feeding and voids its gut contents (gut purging). Most larvae then move away from their food source and go in search of an appropriate site for pupation; the activity period is referred to as the "wandering phase". The larvae wander in search of an appropriate substrate, and there they undertake an elaborate and stereotyped cocoon-spinning behavior. The entire complex sequence of behaviors requires the insect to build a peduncle, then an outer and then and inner cocoon (often with escape hatches built in), followed by the impregnation of the inner cocoon with a predator repellant fluid from the Malpighian tubules. This result in the distinctive change in the color of the larva by which the wandering phase can be readily identified even when the animal is temporarily quiescent or experimentally restrained.

In the present study, during pupation, the transition of the feeding larva to prepupa take place presumably at the onset of the first PTTH and ecdysone secretions. The induction of ocellar contraction and formation of pupal cuticle was later supposed to be brought about by the second release of PTTH and ecdysone. After attaining both the events of secretions, the larva undergoes completion of pupal development in normal time.

The time interval between the first step of PTTH and ecdysone secretions is 31 hrs which induced prodomal signs of pupation, while the interval between the secretions in the second step is comparatively narrow (7 hrs) and this bring about ocellar contraction with formation of pupal cuticle at least locally towards the antero-dorsal margin.

Further, the phenomenon underlying gut purge and pupal ecdysis seemed to be under the control of circadian clock since, larvae that undergo gut purge on day-5 and day-6 pupated exclusively on day-9 and day-10 respectively forming a time interval of exactly 4 days in between the events.

Our observation also showed intermediate character of larval-pupal epidermal cuticle. The pupal tissue (anterior to ligation) presumably had received sufficient exposure to ecdysone at the time of ligation to render it capable of forming the new pupal cuticle. The rest of the epidermis (posterior larval form), which had not molted, apparently had not been exposed to the molting hormone or ecdysteriods.

In contrast, mosiac characters of posterior to anterior gradient was seen in that each posterior segment showed slightly more new pupal cuticle than did its anterior neighbour. The posterior part of the abdomens had perfectly formed and tanned pupal cuticle. Failure of the anterior part to pupate (anterior inhibition) can also be attributed to respiratory deficiencies by injury of the tracheal system during ligation. Another factor, so far hypothetical, may be postulated to be involved in the control of the darkening and pupal formation in the posterior part of the ligated insect; this condition may be caused by the posterior darkening factor (PDF). Still another factor, the anterior retracting factor (ARF) may be responsible to render the anterior part incapable to pupate due its absence.

4. Study of Eclosion behavior and effect EH extract on wing extensibility:

In the present study, emergence of adult from pupal case under natural photoperiod condition of 13L: 11D occurred during the photophase period beginning at 15:00 hours and continuing till 18:00 hours or later at the last day of adult development. Consistently, eclosion starts quite late in the cool afternoon period when the light was comparatively reduced preferably under shade. This timing of eclosion was observed to occur at about 10:00 hours after lights-on. It was also observed that, adult eclosion in *Philosamia ricini* followed the same pattern as that of other Saturniid insects in that last day pupal development was restricted within a time frame or gating during precise period of the day, if the pupa completes development while the gate is open, ecdysis can occur during the same day. By contrast, if development is completed after the gate (after 15:00hr) has shut, the insect must wait until the opening of the gate on the next day so that ecdysis can occur within the time frame of 15 hr-18hr.

In the present study, considering the timing of eclosion commencing at 15:00 hours of the last day of adult development, the release of EH in *Philosamia ricini* in all probability may have occurred at around the 14:00 hours period *i. e.* 1 hr before adult emergence. EH extracts prepared around this time induced wing extensibility in the pharate adult wings devoid of any EH activity.

In the present study, the doses at which the extracts elicited wing expansion was quite high in comparison to earlier observations performed by different workers. But the present study used EH materials obtained directly from unpurified extracts and the experiments may be considered more or less qualitative. Further, it is highly attributed that the higher requirement of doses for elicitation of eclosion behavior may be due to the presence of other biological substances embedded with EH so that the potency of the extracts is reduced considerably or probably due to the hormone being degraded rapidly after injection.

5. Effect of JH-III, 20-HE and cAMP on oxygen consumption during larval development:

The curves of oxygen consumption during both the larval cycles (4th and 5th instars) showed an inverted U-shaped, thus being similar to most of endopterygotes where the descending part of the curve during moulting cycle which may be regarded as a curve representing protein synthesis. If the above-mentioned interpretation is correct, then the course of oxygen consumption may be considered as reflections of the rates of syntheses of the specific developmental proteins.

In the present study, the reduced O_2 consumptions during molting (4th instar to 5th instar) and prior to wandering (5th instar) may be correlated with significant decline *in tissue respiration and mitochondrial succinate oxidation*. The oxygen consumption is comparatively very less at the beginning of the instars. The stages, immediately preceding molt (ecdysis), are marked by no further synthesis of exoskeleton, thereby decreases continuously in oxygen consumption gradually. However, oxygen consumption increases during the preparatory molting cycle until it reach its maximum value before ecdysis and after that, decreases gradually. These metabolic changes may be early indicators of the more dramatic changes in midgut function that are to occur upon reaching the wandering phase. In the present study the peak of oxygen consumption which normally increased in day-3 or day-4 of both developing instars can also be correlated with the high titres of ecdysteriod occurring at these times.

In the present study, we found that JH treated larvae recorded lesser amount of O_2 consumed compared to 20-HE and cAMP treated larvae. Higher O_2 consumption in larvae treated with 20-HE and cAMP may be correlated to the specific action of these compounds directly or indirectly to the epidermal cells which may induce molting and thereby higher metabolic activity. Whereas, the JH-III treated larvae may not influence molting and they may only help to maintain larval character so metabolic activity is possibly reduced and hence lesser amount of O_2 consumption. However, JH-III treated larvae almost always showed highest O_2 consumption at the ultimate day of both the instars. But by this time larval development of the 20-HE or c-AMP treated larvae has become more advanced with low metabolic activity and larvae at this stage are resting and on the verge of molting therefore they consumed low level of O_2. The JH-III treated larvae at the same stage are quite active and probably high level of metabolic activity or differentiation are in progress and hence higher demand of O_2.

In the present study, the pattern of O_2 consumption in cAMP treated larvae are more or less similar to 20-HE treatment and it may be possible that cAMP directly act via PTTH to induce indigenous 20-HE secretion and activation which may in turn stimulate cellular activity and thereby resulted in higher O_2 consumption.

Overall O_2 consumption of the larvae in both the 4th and 5th instars are quite similar and here it was convenient to described the result of hormones and cAMP treatments in common approach. 20-HE treated larvae showed highest O_2 consumption and this concurred with the findings that extracts prepared from corpora cardiaca stimulate respiratory metabolism of the whole body. On the other hand, JH-III treated larvae which comparatively showed lower O_2 consumption during most of the developing instars are in accord with the suggestion that at the beginning of an instar, the presence or absence of JH has little effect on the respiratory rate.

6. Structure of larval integument during development: Correlation with hormonal titres.

It is established that during the last instar development of most insects, ecdysteriod level changes dramatically. Thus, correlating ecdysteriod titre to integumentary structure may be convenient to explain the changes and growth during development.

Morphology and structural changes of integuments of 4th & 5th instars are quite similar throughout development. The high columnar morphology of the late larval integument with its structural maturity (prominent epidermal cells & intercellular spaces) can be correlated to be induced by PTTH & ecdysteriod secretions, since, such hormones are known to be at their maximal levels prior to molting.

In contrast, the low ecdysteriod level can presumably relate to low cuticular growth as can be seen from the smooth and pliable nature of the cuticle while the epidermal cells and intercellular spaces are also not prominent. At this time the epicuticle and exodocuticle are not clearly distinct from each other making them difficult to identify. This condition of cuticular structures revealed that they have not been or yet to be exposed to required amount of ecdysteriod or may be ecdysteriod have not been secreted at this time since the cuticle seemed to be unprepared for undergoing the molting process.

In the present study, comparing the rate of ecdysteriod synthesis and cuticular structure, we can assume that peaks of high ecdysteriod synthesis corresponds to hectic activity of epidermal cells. At about this time probably the molting gel is secreted, the epidermal cells undergo a period of mitosis and cell division. The epidermal cell population becomes denser, the cells more columnar and prominent, and their apical surface is thrown into a series of fine folds. We found that intercellular spaces more prominent than in younger stages and the epicuticle and endocuticle are distinguishable probably they are ready to be shed during the preceding molt.

In the present study, low level of integumentary structures upto day-3 in both the instars in term of cuticular deposition revealed that they had not been subjected to ecdysteriod action and hence cellular activity was also low. Further, the advanced integumentary structure observed in the late stages of larval development are highly expected to have been subjected to hormone action: the time before the molt from larva to pupa, prothoracicotropic hormone (PTTH) and ecdysteriods are known to increase in volume both in the haemolymph and the prothoracic glands (PGs). The PTTH activity may contribute to the high titer of ecdysteriod which thus bring about cuticular changes for preparation of the molting process.

BIO-DATA

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Paper and Abstracts:

1. Raghu Varman, A., Hajong, SR., Kharbuli, B., Marngar, D. and Renthlei, **CZ. 2002.** Role of Vitamin T (Torutilin) in the differentiation of soldier caste in the termite, *Odontotermes distans* (Holmgren). Proc XX Symp Reprod Biol Comp Endocrinol, 2002. pp. 5-6.
2. Raghu Varman, A., Dey, S., Gupta, A., Syntem, R., Hajong, SR., Mukhim, DBK., Kharbuli, B., Chatterjee, K. and **Renthlei, CZ**. 2002. Role of haeme pigments in the colour vision of workers of the honeybee, *Apis cerana* var.

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3. Raghu Varman, A., Hajong, SR., Kharbuli, B., Ao, B., **Renthlei, CZ.** and Kroeger, H. 2003. Induction of puffs in the polytene chromosomes of the salivary gland cells of the middle IV instar larvae of *Chironomus khesiya* by spermine, RNA-polymerase and cyclic-AMP. Proc XXI Symp Reprod Biol Comp Endocrinol. pp. 63.
4. **Renthlei, C.Z.,** Chakravorty, R., Kharbuli, B. and Raghuvarman, R. (2007). Effect of Juvenile hormone III (JH III), 20-hydroxyecdysone and adenosine-3', 5'-monophosphate (cAMP) on egg hatching of eri silkworm, *Philosamia ricini* (Lepidotera: Saturnidae). *Regional Symposium on Current Research Thrust in Animal Sciences: Interface with End Use Researchers and Stake Holders.* NEHU, Shillong. March 15-16, 2007. Page 43.
5. **Renthlei, C.Z.,** Chakravorty, R., Kharbuli, B. and Raghuvarman, R. (2007). Logarithmic weight increase and morphology of last instar integument of the eri silkworm, *Philosamia ricini* (Lepidotera: Saturnidae). *Regional Symposium on Current Research Thrust in Animal Sciences: Interface with End Use Researchers and Stake Holders.* NEHU, Shillong. March 15-16, 2007. Page 41.

Seminars/Symposiums/Workshops attended:

1. The National Academy of Science, India, 72nd Annual Session, (October 25th-27th, 2002) held at North Eastern Hill University, Shillong-22.
2. National Roving Seminars on Patenting in Biotechnology (October 27th, 2002) organized by the Dept. Of Biotechnology, Ministry of Science and Technology, New Delhi, at the Bioinformatics Centre, North Eastern Hill University, Shillong-22.
3. Thirteenth National Symposium on Environment (June 5th-7th, 2004). Focal Theme: Mining of Energy Resources-Environmental Management, Sponsored by Board of Research in Nuclear Sciences. Dept. of Atomic Energy, Govt. of India.
4. Regional Consultation on Farmers' Issues (Eastern India) (3rd-4th Nov. 2004), organized by The National Commission on Farmers (NCF), MS Swaminathan Research Foundation (MSSRF) and United Nation's World Food Programme (UNWFP) at the ICAR Research Complex, Umiam, Meghalaya.

5. International Year of Rice 2004 (5th –6th Nov. 2004). Workshop on Rice Heritage of the Northeast – Challenges, Opportunities and Strategies for the future, organized by MS Swaminathan Research Foundation (MSSRF) and ICAR Research Complex for NEH Region, Umiam, Meghalaya.
6. National Symposium on Citriculture: A road map (22nd –24th Feb. 2006). Organised by Indian Society of Citriculture, Nagpur in collaboration with National Research Centre for Citrus, Nagpur and ICAR Research Complex for NEH Region, Umiam, Meghalaya.
7. Attended seminar on HPLC: Principle and Applications (25th April, 2006), organized by Waters India Ltd. Kolkatta and SAIF, NEHU, Shillong at Bijini Complex, Lautumkhrah, Shillong.

Fellowship Awarded:

1. Mizoram State Fellowship, Directorate of Higher and Technical Education, Government of Mizoram
2. North Eastern Council Fellowship, Govt. of India, Shillong.