THE NEUROENDOCRINE REGULATION OF LARVAL DEVELOPMENT IN THE MAIZE-STEM BORER, *BUSSEOLA FUSCA* (FULLER) (LEPIDOPTERA: NOCTUIDAE)

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I have read this thesis and I approve it for examination

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ABSTRACT

Busseola fusca (Lepidoptera:Noctuidae) is one of the most important insect pests of maize and sorghum in Africa, south of the Sahara. Last instar larvae of this species survive the dry season in the stalks and stubbles of their host plants by undergoing diapause.

The present study was done with the main objective of investigating the involvement of the neuroendocrine system during the development of the last instar larvae with respect to non-diapause and diapause development. The main aspects studied were the biology of last larval instar, histology, ultrastructure and physiology of the components of the larval neuroendocrine system, particularly the type-A neurosecretory cells, corpus cardiacum, corpus allatum and prothoracic glands. Endocrine involvement in the regulation of development during the last instar larvae and in induction, maintenance and termination of diapause development were also investigated.

Studies on the biology of the last instar showed that two types of development, namely, non-diapause and diapause occur in Busseola fusca. The non-diapause form of development is characterized by the feeding phase and post-

feeding phase and the diapause form of development is characterized by only a long, non-feeding phase.

The structure of the components of the endocrine system revealed that both in non-diapause and diapause larvae, the type-A neurosecretory cells in the brain and the prothoracic glands were structurally comparable with insignificant differences. However, the corpora allata were structurally different in non-diapause and diapause larvae. The corpora allata in non-diapause development had cells which completely lacked glycogen-like deposits which were found abundantly in the corpora allata of diapause larvae. The mitochondria of the cells of the corpora allata in non-diapause development were not conspicuous and did not contain dense bodies. On the other hand, the mitochondria of the cells of the corpora allata in diapause larvae were large and pleomorphic and contained bodies within the mattrix. The corpora allata in non-diapause larvae were slightly smaller in size than those of diapause larvae. However, the prothoracic glands in non-diapause larvae were larger than those in diapause larvae.

The titers of juvenile hormone in non-diapause larvae were very low (as determined by Dysdercus bioassay, were of an average score of 0.8 out of 3.0), while those in the diapause larvae were high (average score of 2.8). The titers of the moulting hormone in non-diapause last instar larvae

were 10, 170, and 500ng/ml on days one, four and seven respectively; in diapause larvae the titer was generally low with a monthly mean titer of less than 200ng/ml.

Injection of the juvenile hormone analogue resulted in induction of diapause in non-diapause larvae. Elevation of the moulting hormone titers by experimental injection of ecdysone into the diapause larvae triggered moulting but it was usually a larval-larval (stationary) moult. Injection of the moulting hormone in isolated abdomens of diapause larvae induced progressive moult. These observations indicated that diapause development was not due to deficiency of moulting hormone. Diapause can be initiated and maintained by high titers of juvenile hormone. Thus the corpora allata of the diapause larvae were active. Precocene showed no allatocidal effect on the corpus allatum of the diapause larvae. Fluoromevalonate treatment of the late diapause larvae at a dosage of 10µg per individual prevented larval moult and delayed pupation relative to the non-treated larvae. At a dose of 5ug per individual, larval moults occurred and pupation was not delayed but the resultant adults were deformed.

In conclusion, this study has shown that non-diapause development is characterized by a last instar period of two distinctive behavioural phases whereas diapause development has only one behavioural phase. Also, diapause development

in Busseola fusca lasts up to the pre-pupal stage of the last instar. In addition, type-A neurosecretory cells stain lesser with advance of time in non-diapause and diapause development during the last instar while the presence of glycogen in the corpora allata of the diapause larvae signified storage of metabolites to be used in during the long period of diapause. Ecdysone titers in the haemolymph during non-diapause and diapause development were quite normal and so were the JH titers in non-diapause development. However, the persistent high titers of JH in the haemolymph during diapause development indicates that diapause development in Busseola fusca is primarily controlled by the juvenile hormone. Thus, both ecdysone and juvenile hormone are involved in the regulation of the type of development which occur during the last larval instar of Busseola fusca.

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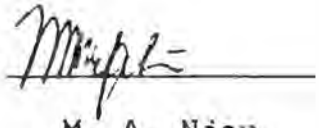
DECLARATION

It is hereby declared that this thesis is my own original work, and has not been published before or presented elsewhere for the purpose of examination



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CHAPTER 1

1 INTRODUCTION

1.1 Neuroendocrine regulation of larval growth and metamorphosis

Developmental processes in insects just like in all other living organism are modulated by hormones which are produced by endocrine glands (Wigglesworth, 1972). In insects the endocrine system consists of many glandular organs but the most important ones are the neurosecretory cells (NSC) in the brain, the retrocerebral endocrine system composed of both the corpora cardiaca (CC) and the corpora allata (CA), and the prothoracic glands (Williams, 1948). These glands have been regarded as the sources of the hormones which regulate growth, development and reproduction (Riddiford and Truman, 1978).

1.2 The classical scheme

The studies which led to the understanding of how insect hormones regulate growth and development are summarized in the classical scheme of insect endocrinology (Gilbert et al., 1980). The main aspects of this scheme have remained unchanged from the time the scheme was postulated to the present time despite the use of very modern techniques in

the study of endocrinology. The studies of Kopec (1922) established that the brain of the insect was essential during pupation of the Gypsy moth, Lymantria dispar. These findings were verified by results of other studies on Rhodnius prolixus (Wigglesworth 1934, 1936).

The occurrence of the moulting hormone in insects was implicated in the findings of the classical experiments of Wigglesworth (1934), Fukuda (1944) and Williams (1947). Later, by using isolated pupal abdomens, Williams (1952) conclusively showed that a brain hormone actually stimulated the prothoracic glands to release a moulting hormone.

Development in the absence of the corpora allata led to molting of the penultimate instar Rhodnius nymph (larva) to precocious adults (Wigglesworth, 1934). Whereas development of the final instar parabiosed to a 3rd instar larva with corpora allata produced a supernumerary larval instar. These observations led to the conclusion that a hormone present in the younger larval instar inhibited development to the adult and favoured the development of the juvenile state, thus maintaining the status quo of the insect. Thus it was called the 'inhibitory', 'juvenile' or 'status quo' hormone (Wigglesworth, 1934; 1936; Williams, 1963). There were other studies which led to the discovery of various other hormones as they were known at the time and these had been summarized in the various reviews (see Granger and Bollenbacher, 1980; Riddiford and Truman, 1978).

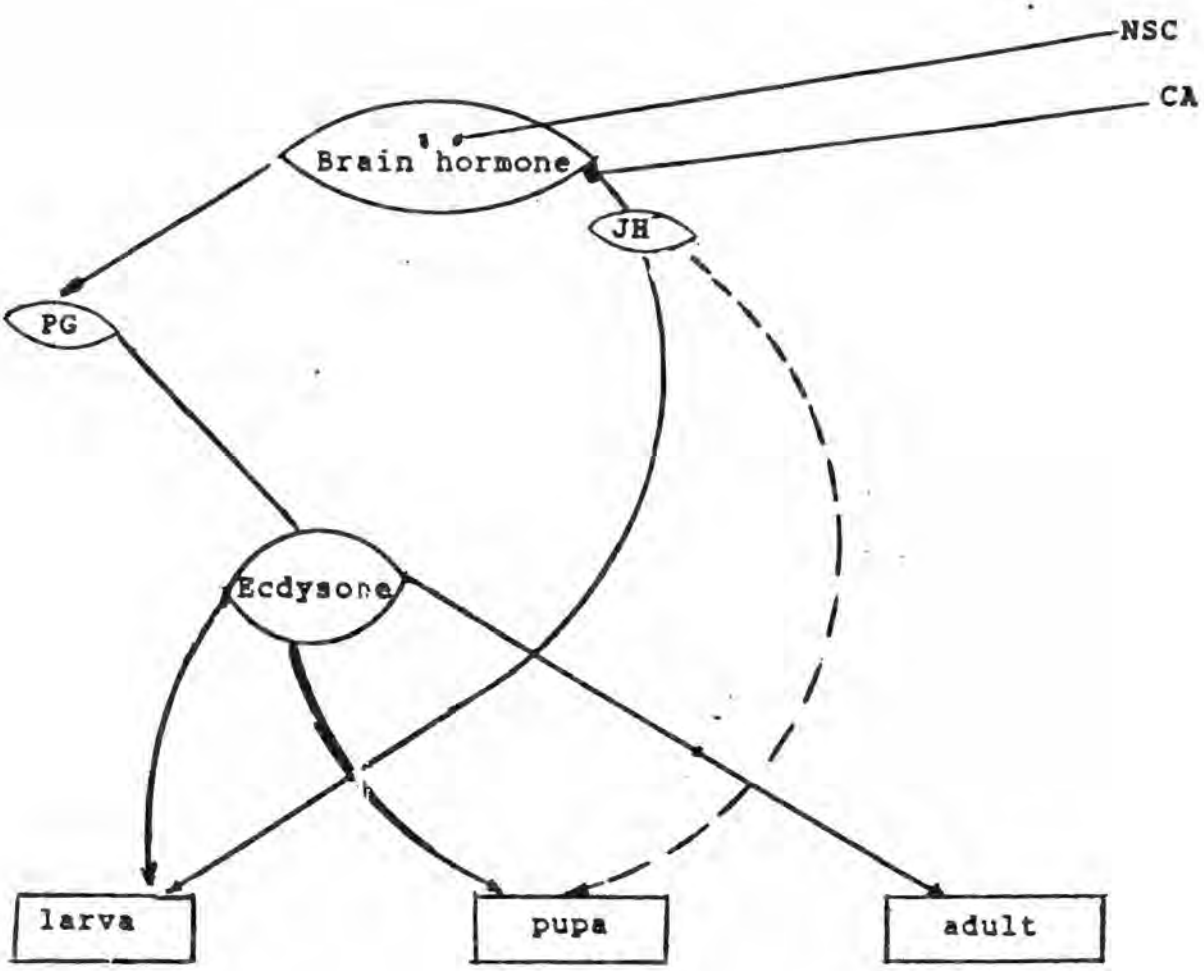

Figure 1. The Classical scheme of endocrine regulation of insect growth and metamorphosis

According to the classical scheme, neurosecretory cells (NSC) in the brain release a factor (brain hormone) into the haemolymph which stimulates the prothoracic glands (PG) to release the moulting hormone (ecdysone) which initiates moulting. The outcome of the moult, is determined by the juvenile hormone (JH) secreted by the corpus allatum (CA). Respectively, larval-larval, larval-pupal, and pupal-adult moults occur in the presence of high, intermediate and negligible titers of JH.

Since the founding of the classical scheme, more information about the endocrinology of insects has been gathered from numerous studies. Involvement of other hormones has been demonstrated and the various processes under the control of these hormones have been studied to some extent. Insect endocrine studies have also focussed on hormonal regulation of morphogenetic process as well as hormonal interaction during growth and reproduction as opposed to the endocrine regulation of metamorphosis alone as was the emphasis at the setting of the classical scheme.

With the foundations of the classical scheme on the endocrine regulation of metamorphosis well laid down by the pioneers, many studies have since been carried out not only on the hormones that were known at that time but also on new ones which were suspected to be present but had not yet been discovered. Chemical characterization of the known hormones like juvenile hormone became possible when sources rich in the hormone were identified.

Williams (1956) extracted a 'golden oil' from abdomens of male cecropia moths (Hyalophora cecropia). This substance was found to have juvenilizing effects on insects. It was chemically characterized as a mixture of three homologs, namely, JH I (Roller et al., 1967), JH II (Meyer et al., 1968) and JH III (Judy et al., 1973). The homologies were found to be present in the haemolymph in different proportions during different developmental stages of the insect. The different JHs showed morphogenetic effects in the immature stages and gonadotropic effects during the adult stage. Recently, JH III was isolated from a plant species (Tsong et al., 1988)

Fraenkel (1935) observed that the blood of the pupariating larva contained a factor which induced moulting in ligatured abdomen of fully developed fly larva. This observation led to the development of a bioassay for the moulting hormone, the Calliphora test (Fraenkel, 1935). The active principle was found to consist of two compounds, α- and β- ecdysones (see Bollenbacher et al., 1980). Other types of ecdysteroids (ecdysones) have since been isolated from insects and other arthropods as well as from plants. The plant ecdysones are generally known as Phytoecdysones. The ecdysones regulate ecdysis in immature stages and reproduction in the adult insects.

The juvenile hormone(s) from the corpora allata as discussed above are important also in the regulation of normal development. These hormones together with the other

morphogenetic hormones such as the "brain hormones" and the moulting hormone interact in the regulation of the various processes during growth and development of insects.

The "brain hormones" are now commonly referred to as Prothoracicotropic hormones (PTTH) by virtue of their stimulatory activity on the Prothoracic glands. They are found to occur in the form of two species, a large molecular species; the 22K PTTH and a small molecular species, the 4K PTTH (Nagasawa et al., 1986). Both forms have been localized in neurosecretory cells of the pars intercerebralis medialis as well as in the neurohaemal region around the corpora allata in Bombyx mori (Ishizaki et al., 1987). Although the two forms of the hormone may be found occurring together, their action seem to be rather species specific, for example, the 4K-PTTH was found to be active in brainless pupae of Samia cynthia ricini but inactive in brainless pupae of Bombyx mori (Nagasawa, et al., 1986). The chemical nature of the smaller PTTH has been determined as insulin-like (Nagasawa et al., 1984) but a lot is yet to be studied on the biosynthesis of these neurohormones.

Of the new hormones subsequently discovered are various peptide hormones such as the eclosion hormone, the tanning hormone (also known as bursicon) and the diapause hormone.

The eclosion hormone was localised in a cluster of medial neurosecretory cells in the brain (Truman, 1971), and was released to the haemolymph via the corpus cardiacum. It has been shown to be a peptide with a molecular weight of

about 9,000 daltons (Reynolds and Truman, 1980). This hormone regulates a series of events leading to adult emergence. The hormone has not been shown to regulate larval or pupal ecdyses in the lepidopterous insects.

Unlike the eclosion hormone, the tanning hormone, Bursicon, has been shown to be present in different life stages of various insect species (Riddiford and Truman, 1980). It has been shown to be a peptide with a molecular weight of about 40,000 daltons (Fraenkel and Hsiao, 1965). Its actual site of synthesis is not well established but it is prevalent in the perivisceral neurohaemal organs associated with the ganglia of the ventral nerve cord as well as in the medial part of the brain of the Diptera (Fraenkel and Hsiao, 1965). Bursicon regulates the process leading to sclerotization of the new cuticle following ecdysis.

The other well known peptide hormone is the diapause hormone which is involved in the regulation of egg diapause in the silk worm, Bombyx mori (Fukuda, 1953; Hagesawa, 1964). This hormone is produced by the subesophagial ganglion of the female adult.

The current knowledge about insect endocrinology has been obtained from relatively few insect species. Studies carried out on different insect species have reported slight but important differences in the ways species of different insects have developed the use of the various components of the endocrine system. There are some general aspects which

apply to many of the various species but the occurrence of species specific adaptations, for example, endocrine regulation of diapause, have surfaced with the examination of individual species.

1.3 The importance of maize stem-borers

Cereal stem borers are serious pests where graminaceous crops such as maize and sorghum are grown (Jepson, 1954). Quite a number of stem borer species have been documented in the tropics, but in East Africa, Busseola fusca Fuller, Chilo partellus Swinhoe, Eldana saccharina and Sesamia sp. are considered to be more important (Nye, 1960; Seshu Reddy, 1985). Studies in East Africa have reported the severity in damage and losses in cereal grain yield caused by stem borers. Duerden (1953) suggested that stem borers could cause up to 50% loss and if they are controlled by using pesticides such as DDT, about 44% increase in yield could be achieved. A larger part of the loss may be attributed to a few but major borers. For example the development of a single Busseola fusca larva inside an otherwise healthy plant brought about a reduction in yield capacity by 28% (Harris, 1962) and for every 1% reduction in Busseola fusca infestation the grain yield increased by about 35 lb/acre (Walker, 1960). In order to develop strategies and methods for the eventual control of Busseola fusca, it is important to have a thorough understanding of the biology, ecology,

to have a thorough understanding of the biology, ecology, behavior and physiology of the borer, especially, of the larvae which actually cause crop damage. The information generated would enable us to identify the weak points during the life cycle of the pest.

1.4 Justification for the studies

Despite being one of the most important stem borer species in sub-Saharan Africa, and the species which has been recorded only in Africa, there is a great paucity in the knowledge of the biology and regulation of larval development of Busseola fusca. No study of the endocrinology of Busseola fusca has ever been reported. Among the borer species in this region, Busseola fusca is one of the few which has been reported to undergo diapause as mature last instar larva, a strategy which has imparted continuity of its life cycle even during the off-season.

1.5 Objectives of the study and aspects to be investigated

The main objective of the present study is to elucidate the role played by the neuroendocrine system during non-diapause and diapause development in the larvae of Busseola fusca.

To achieve this, the following aspects were investigated:

1. Biology of Busseola fusca larva with emphasis on the last instar.

2. Morphology, histology, and fine structure of the larval neuroendocrine system.

3. Endocrine regulation of larval development.

4. Endocrine regulation of induction, maintenance and termination of diapause.

CHAPTER 2

BIOLOGY AND LARVAL DEVELOPMENT OF BUSSEOLA FUSCA

2.1.1 INTRODUCTION

The life cycle of an insect consists of embryonic and postembryonic development both of which are characterized by moulting (Riddiford, 1985). While the embryonic development occurs in the egg, the postembryonic development starts at the time of hatching and ends with the death of the adult (Wigglesworth, 1972). In the holometabolous insects, development passes through three main specialized stages, namely, the larval, pupal and the adult. The larval stage is specialized for feeding; the pupal stage is for active transformation of the larval characters to adult characters; while the adult stage is concerned mainly with reproduction (Engelmann, 1970). In lepidopterous pests, the larval stage is usually of economic importance as it is the most destructive stage due to its feeding habits. Therefore, there is a need to concentrate studies on the larval biology of species of this order with a view to generating information that may be useful in developing strategies for the control of target pests. Effective control measures of the lepidopterous stem borers involves manipulation of factors which promote growth of the larva. By knowing such factors, it becomes possible to identify the most

appropriate ones which can be manipulated in a way to bring about the control. Adequate information on such factors can be obtained from studying the biology of the larval instars so as to identify the most sensitive instar for the control methods at hand.

2.1.2 Biology of the maize stem borer. Busseola fusca.

Busseola fusca uses maize and sorghum as its main hosts. It has been recorded predominantly in areas which grow either of the two crops. In East, Central and some parts of Southern Africa, the pest is found especially on the cooler highland areas (Jepson, 1954; ICIPE, 1984 Annual Report) whereas in West Africa, it is found in the warmer coastal lowlands where most of the maize is grown (Usua, 1967). Although Busseola fusca. prefers cool areas it is so closely associated with its host plants that it can occur wherever the hosts are found.

Busseola fusca is a very successful pest. It is very much adapted to its host and the whole of its life cycle including the adult depends on the host. Although the adult do not rely on the host plants for their food, they need the plants for oviposition.

Hatching occurs on the host plants and the first instars spin silk and disperse when blown by wind to nearby plants and enter the funnels where they feed on the soft leaves leaving windows on them. As they grow the larvae bore

into the stems where they feed for the rest of their larval life. Before pupating in the stem, the mature larvae make exit holes through which the eclosing adults come out.

The feeding activities of the larvae cause great damage to the host plant. Plant mortality is only registered in the form of deadhearts in young plants with destroyed meristems. Attacked host plants which survive suffer foliar damage, stem tunneling, tassel and cob/panicle damage. Such plants are very weak and can succumb to diseases or lodge easily when blown by wind (Swaine, 1957).

Usually there are two generations of Busseola fusca during the growing season (Unnithan, 1987). The life cycle of the pest during the first generation is comprised of an embryonic period of 6-8 days (Swaine, 1957) a larval period of 24-45 days (Smithers, 1959), a pupal period of about 14 days and an adult period of about one week. Larvae of the second generation usually undergo diapause as mature last instars (Swaine, 1957; Smithers, 1959; Taylor, 1982).

2.1.3 Diapause development in last instar larvae

The mature last instar larvae of Busseola fusca enter diapause and survive on the host plant residues (stalks and stubbles) at the end of the rainy season. The occurrence of this diapause appears to be controlled by the condition of the host plants (Smithers, 1959). Larvae which were fed on

mature maize (Usua, 1973) or sorghum (Unnithan, 1987) stems entered diapause in the last larval instar.

The diapause larvae of Busseola fusca undergo stationary moulting (Usua, 1973). Diapausing larvae break diapause on the onset of the rainy season and pupate. Eclosing adults emerging from the stubble of the previous season mate and oviposit on the young plants starting at about the second week after plant emergence and reaching a peak between four to six weeks (Walters et al., 1976). Mated females select the first fully opened leaf and lay eggs on the edge of the leaf sheath in a very characteristic manner such that after oviposition the sheath falls back on the stem and conceals the eggs (Ingram, 1958; Harris, 1962).

Because of their natural habitat, that is, of feeding inside the stems of their host plants, many of the stem borers are not convenient candidates for studies on the larval behavior. This is probably one of the reasons why no studies have been undertaken on the biology of the last larval instar of Busseola fusca. In view of inadequate information on the larval biology of this species, there is a need to carry out such studies on the last instar before elucidating the endocrine system and its involvement in the regulation of development in this instar.

Thus, the objectives of this study were to undertake basic biological studies on larval development in Busseola fusca, to investigate possible behavioral phases in the last

larval instar, and to find out behavioral characteristics associated with non-diapause and diapause development.

2.2 MATERIALS AND METHODS

2.2.1 Laboratory colony

Busseola fusca has not been reared successfully on artificial diet. Therefore, the insects which were used for my (present) studies were reared on the natural host, sorghum.

A colony of Busseola fusca was started in the laboratory using pupae obtained from infested sorghum stems. The presence of a pupa in a stem was ascertained by presence of freshly made exit hole. Pupae were placed individually in 25ml plastic jam cups until adult eclosion. Adult moths were sexed, based on antennal characteristics, and male and female moths were brought together. One pair each was placed in cylindrical cage (size 30 cm X 10 cm) made of a fine wire mesh with both ends of the cylinder covered with 10.5 cm diameter plastic petri dishes. A sorghum stem with leaf sheaths and whorls, inserted in a water filled glass vial was placed inside the cage to provide oviposition site. A moist cotton wool pad was placed in the cage to provide drinking water for the moths.

The adults were maintained until egg-laying lasted. Every morning, the sorghum stem was removed and inspected

for the presence of eggs and substituted with a fresh one. Eggs still on the sheath, were incubated in specimen tubes lined with moist tissue paper and stoppered with a cotton wool.

Newly hatched larvae were introduced to cuttings of two week old sorghum seedlings inside a glass jar until they moulted into the second instar when they were transferred to three week old sorghum plants until they reached the third instar. Third instars were given four week old plants. Similarly fourth, fifth and sixth instars were reared on five, six and seven to eight-week old sorghum plants, respectively.

The duration of and growth rate of each larval instar was determined by measuring the width of the head capsule using a calibrated ocular micrometer. The mean width of the head capsule of the larva, except the first instar was divided by the mean head capsule width of the preceding instar in order to get the growth ratio from one instar to the next. The mean head capsule width of the diapause larva (head capsule, shed at the first stationary moult after collecting from the stems) was divided by the mean head capsule width of the fifth instar larva in order to find out the growth ratio for larva entering diapause and to relate it to that of the non-diapausing sixth instar larva.

2.2.2 Phase determination in the last larval instar

The larval instar preceding pupation was normally the sixth instar. Larvae of this stage were studied in details to determine phases within the instar. Non-diapause last instar larvae were placed singly in stem cuttings from six to eight week-old sorghum plants. A total of 32 larvae were used. Each larva was weighed on a Sartorius balance to the nearest 0.1 mg once daily until it pupated and weight per every 24-hour period computed. Weight increase or decrease patterns were studied to determine the growth pattern of the larvae.

Characteristics and behavior of diapausing larvae

Pigmentation, weight and size

The general appearance and body pigmentation of the last instar was taken as a criterion for distinguishing non-diapause larvae from diapausing larvae. Eight female and 8 male diapausing larvae collected from the field during the last week of July (early diapause) were kept in dry sorghum stalks which were changed after every four days. Stationary moults that occurred during the first two months under observation were recorded. Analysis of variance was done on the weights of the moulting larvae immediately after every moult for the first three stationary moults to find out the

effect of a moult on the fresh weights of the diapausing larvae.

Diapause at the end of rainy season

Studies were made to find out whether larvae diapausing at the end of long rains were similar in terms of weight compared to those diapausing at the end of the short rains. Thirty larvae were collected from sorghum stems at the end of the long rains (July) and individually weighed daily for one week. Similarly, 30 diapausing larvae were also collected at the end of the short rains (January) and weighed individually daily for one week. The data were analyzed using Statistical analysis systems (SAS).

Silk production

Diapausing larvae produce silk during diapause. In order to find out whether silk production during diapause is related to any other aspect of development such as stationary moult and pupation, 79 larvae in late diapause were studied for a period of one month (November). Larvae were placed individually in dry conditions (in plastic jam cup containing a piece of kleenex tissue paper). Observations were made once a week (every Monday) for one month or until the larva pupated and any silk produced was recorded and removed from the cup. Stationary moults and mortality that

occurred during this period was also recorded. Larvae that escaped during this period were not included in the analysis.

Pupation.

Studies were undertaken to find out whether pupation of larvae from diapause could be compared with pupation of non-diapausing larvae. Diapausing larvae which were no longer laying down silk and were in late diapause were weighed daily until they pupated. The weights of larvae one day before the prepupal stage, during the prepupal stage and immediately after pupation were analyzed and compared with weights of similar stages of non-diapause development.

2.3 RESULTS

2.3.1 Laboratory colony

The number of instars, their duration and growth rate are given in Table 1. The increase in head capsule size shows that the mean growth ratio during different larval stages was 1.565. However, the rate of growth was higher than this during 2nd and 3rd instars and between 5th and diapausing last instar.

Instar duration of early stages (1-4) were about the same each lasting for three days. They were more than

doubled in the later instars, particularly the sixth instar which had a mean duration of 7 days (Range, 5-9 days).

2.3.2 Phases in last instar non-diapause development

Two main behavioral phases were observed in the last larval instar. These were the feeding phase and post feeding phase (Table 2). The feeding phase which was longer had several behavioral states including a resting state on the fourth day. There was voracious feeding immediately after the resting state. The post feeding phase was characterized by steady but gradual loss of weight.

Daily changes in fresh weight of the last instar non-diapausing larva is shown in figure 2. Larvae showed a decline in their fresh weights on the first two days followed by an increase in weight for two days as a result of feeding. A decline in weight at the middle of the stadium was observed for one day. This was followed by a rapid increase in weight for the next three days. Larvae then lost weight from day eight, the day they begun making exit holes. The mean rate of loss in weight of a larva per day was about 10%. A maximum loss in weight of about 12.5% was observed at pupation.

Characteristics of diapause larvae

Pigmentation

The diapause larvae assumed a different cuticular pigmentation from that of non-diapause larvae which are usually dark because of melanin granules in their epidermis. The diapause larvae appeared creamy white or immaculate due to the whitish fat body that enveloped the gut. The cuticle of the diapause larvae was waxy and relatively tougher than that of the non-diapause larvae.

Weight and size

The early diapause larvae appeared to be larger than non-diapause larvae. The fresh weights of diapause larvae over one week period at the end of both the short and the long rainy seasons are shown in figure 3. Larvae found diapausing at the end of the long rains were significantly ($P=0.05$) heavier than those found diapausing at the end of the short rains.

Loss and gain in weight was also observed during early diapause. In this case the loss or gain in weight was usually very small (less than 5%) and this pattern continued for the first 1 to 2 weeks and after this period significant loss in weight was observed only immediately after every stationary moult.

Stationary moult

Diapause larvae were found to undergo occasional stationary moults. Intermoult durations for male and female are given on Table 3. On an average, each intermoult period lasted for about three weeks under the given conditions.

The mean weights of diapause larvae at each moult were as shown on Table 4. The mean weight of the larvae immediately after the 1st, 2nd, 3rd and 4th moult were 378, 344, 304 and 255 mg respectively. It appears that larvae were losing on an average, about 12.75% of their body weight at each moult. Loss in weight after each moult was significant (P=0.05) immediately after every moult. During intermoult period there was very small change in fresh weight in the diapause larvae (Figure 3). No feeding was observed in diapause.

Silk production, moulting and pupation

The frequency of diapausing larvae that produce silk, undergoing stationary moult and pupation are presented in Table 5. The percentage of larvae in mid-diapause producing silk declined from 52% in the first week of observation to 22% in the fourth week. However, the percentage of larvae undergoing stationary moult per week remained almost the same with an average of 7.3% but the actual number of larvae

out of 79 which showed stationary moult declined from 6 (out of initial total of 79) in the first week to 4 in the third week and 5 in the fourth week. By contrast, the number of larvae which pupated increased from 1 in the first week to 5 in the fourth week. However, there was no pupation during the third week of November. These results indicate a possibility of a remote relationship between the proportion of larvae producing silk and the number of larval and pupal moults during mid-diapause. In the case of a high proportion of larvae producing silk, the number of larval moults is higher than the number of pupal moults. With increasing time lapse, and with decreasing proportion of larvae producing silk, the number of pupal moults tends to increase. Pupation was observed one week after the larvae had stopped producing silk. However, there was no apparent cyclicity in silk production.

The decline in weight during pupation of larvae under both non-diapause and diapause development is shown in figure 4. Close to pupation, larvae in non-diapause development weighed significantly more ($P = 0.05$) than those in diapause just before and during the pre-pupal stage. However, the weights of the resultant pupae were not significantly different ($P = 0.05$).

Table 1 No. of larval instars, their duration, head capsule width and growth ratio.

Instar	No. of larvae	Mean duration (days)	Head capsule width (mm mean)	Growth ratio2
1	35	3.0	0.30	
2	31	3.0	0.50	1.67*
3	37	3.0	0.87	1.74*
4	42	3.0	1.10	1.26
5	26	3.5	1.50	1.36
6	32	7.0	2.20	1.47
D[1]	51	--	2.84	1.89*

[1]diapause-larvae. [2]growth ratio = mx/px where width of head capsule of a given instar(mx) divided by the width of the preceding instar(px) except for the diapause larvae where px was of the fifth instar.

*means significantly different ($p<0.05$).

Table 2. Behavioural phases in the last larval instar under non-diapause development[1]

Phase	Behavioural activity	Duration (days, mean±s.e)
Feeding	Newly ecdysed, not feeding	1.00 ± 0.00
	Initial feeding	2.36 ± 0.33
	Resting	1.13 ± 0.21
	Second feeding	2.00 ± 0.88
Post Feeding	Exit hole making	2.10 ± 0.32
	Prepupal	1.14 ± 0.23

[1]32 larvae were used for this study.

Table 3 Frequency of stationary moults and intermoult duration

Stationary moult	No. of larvae	Intermoult duration (days mean ± S.E.)	
		females	males
1	16	18.0 ± 4.7	24.5 ± 0.7
2	16	27.0 ± 11.3	20.0 ± 2.8
3	16	20.25 ± 2.2	21.0 ± 9.0

Table 5 Silk production, stationary moult, and pupation of larvae of Busseola fusca during late diapause (November)

week	No. of larvae	% Producing silk	No. moulted[1]	No. pupated[1]	No. died[1].
1	79	51.9	6 (7.6)	1 (1.3)	2 (2.5)
2	74	31.1	6 (8.1)	3 (4.1)	1 (1.4)
3	71	23.9	4 (5.6)	0 (0)	0 (0)
4	64	21.8	5 (7.8)	5 (7.8)	2 (3.1)

[1]Figures in parenthese indicate percentages.

Table 4 Effect of stationary moult on fresh weights of diapause larvae of Busseola fusca

Moult	No. observed	Mean weight (mg)[1]
1st	12	378.33a
2nd	12	343.57b
3rd	12	303.53c
4th	12	254.80d

[1]means are significantly different from each other ($p < 0.05$). The weights were taken immediately after each moult

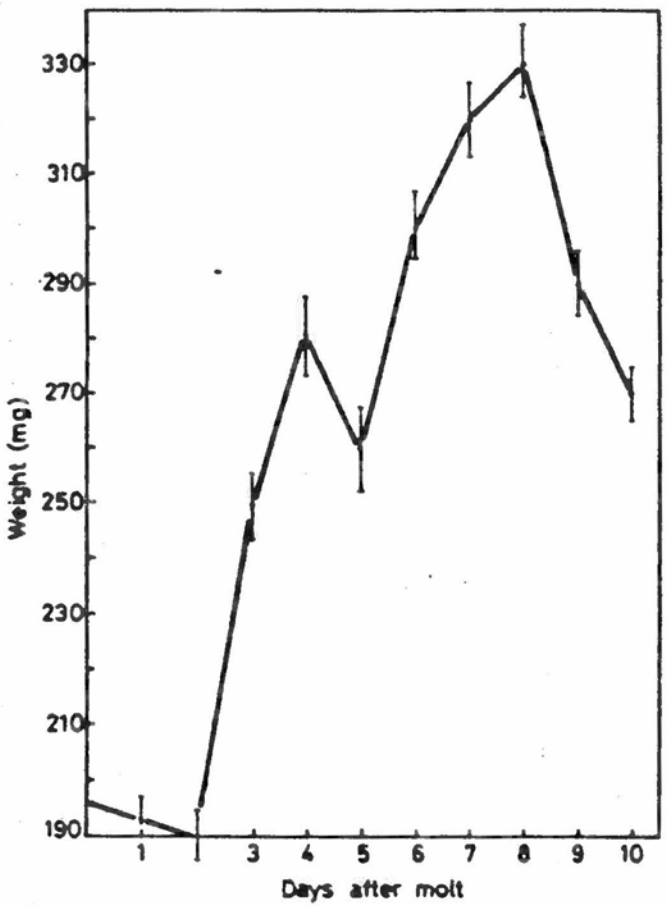

Figure 2. The growth of the last instar larvae of Busseola fusca non-diapause development.

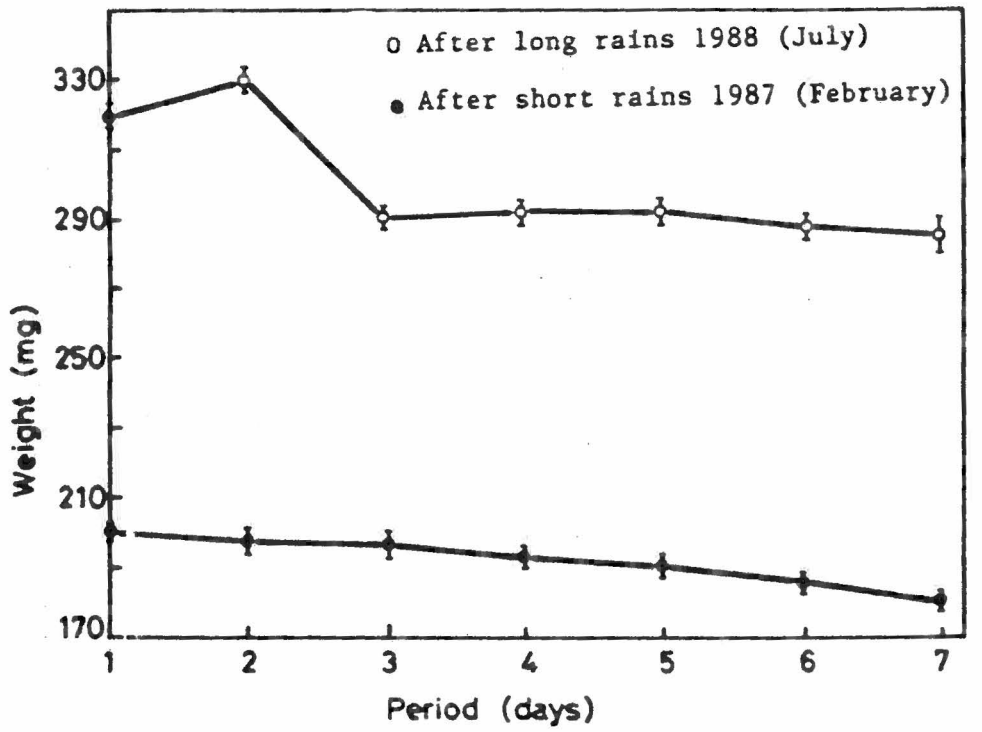

Figure 3 The fresh weights of larvae diapausing at the end of short rains compared to those diapausing at the end of the long rains.

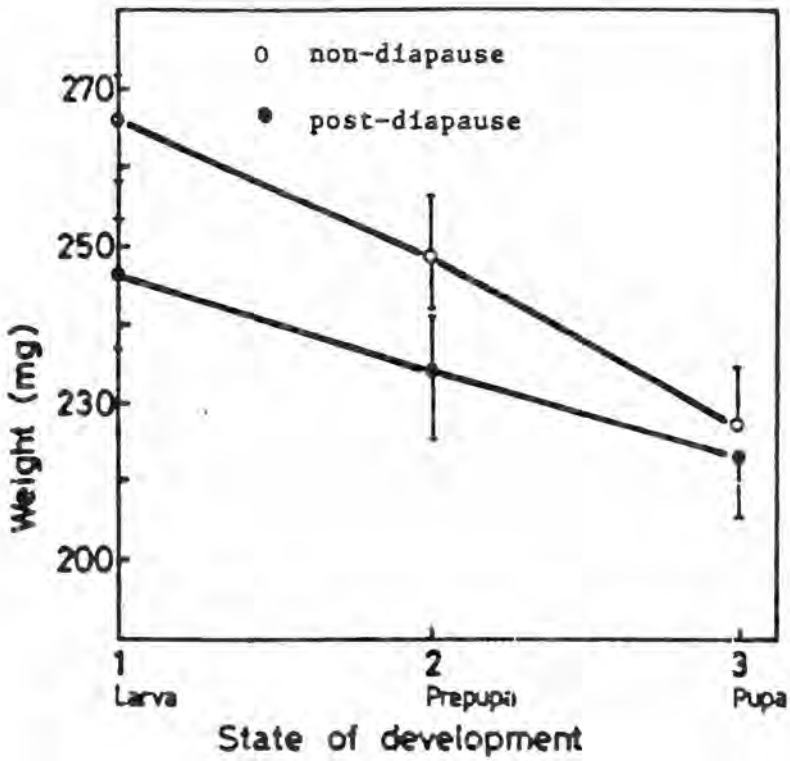

Figure 4 Mean fresh weights during pupation of non-diapause and post-diapause larvae of Busseola fusca.

2.4 DISCUSSION

The biology of the maize stem borer, Busseola fusca has been studied by several workers (Mally, 1920; Swaine, 1957; Usua, 1967; Kaufmann, 1984; Unnithan, 1987; Assefa Gebre-Amlak, 1988; among others). Only a few of these studies discuss the difficulties encountered in raising and maintaining a colony of the insect under laboratory conditions without using a standard artificial diet. However, there are efforts to develop such a diet and trials with both sorghum stem and leaf-based formulations appear promising (F. Onyango, personal communication).

The natural habitat of Busseola fusca larvae does not reveal much of their biology and behavior because the later instars are spent inside the host plant. Laboratory rearing of the insect on the cut sorghum stem does not replicate the natural environment. In the absence of any other appropriate alternatives, results from studies on insects reared in the laboratory have to be taken as they are until better rearing techniques are developed.

Despite the shortcomings of the laboratory rearing of Busseola fusca, growth and development of the larvae followed normal trends characteristic of other lepidopteran species. The overall growth ratio was slightly higher than that reported for the lepidoptera (Dyar, 1890). Growth rate in the fourth, fifth and the sixth instars were a bit lower

than those of the second, third and the diapausing sixth instar. In Busseola fusca, the number of the larval instars has been reported to be variable (Usua, 1967). Under favorable conditions, they are usually six or seven (Unnithan, 1987). It is not known why Busseola fusca has a variable number of larval instars but studies from other insects like Manduca sexta and Oncopeltus fasciatus have shown that having a variable number of larval instars may arise out of the attempt by the insect to attain a critical size and weight both of which are advantageous for reproductive fitness (Nijhout, 1975). However, in other insect species, particularly those in which diapause is expressed in the mature last larval instar and have stationary moults while in diapause, attaining a critical size and weight may not be quite distinctive and therefore there could be other parameters such as declining of juvenile hormone titer (Fain and Riddiford, 1975; Nijhout and Williams, 1974; Sakurai, 1984), which physiologically characterizes the last larval instar much more definitively. Such a mechanism can be discovered by only having a thorough knowledge of the larval biology of the target insect species.

The two main phases observed in the last instar larvae of Busseola fusca are similar to those reported for other lepidopteran larvae (Bollenbacher, 1988). Studies on other lepidopteran larvae have indicated that the last instar consists of a feeding phase during which time the larva

concentrate on feeding activities and a non- feeding phase during which the larva prepare for pupation. A critical weight is attained during the feeding phase as has been reported in Manduca sexta (Nijhout and Williams, 1974; Nijhout, 1975). Last instar larvae of Busseola fusca temporarily stopped feeding around the fourth day after moulting into the sixth instar. This was the period of the appearance of the first ecdysteroid peak and also the time when no JH activity could be detected in the haemolymph extract (Chapter, 4). The fluctuation in feeding could be related to changes in titer of JH and ecdysteroid in the haemolymph and their subsequent effect on the physiology of the gut. A fall in JH titer and initial rise in ecdysteroid titer are some of the necessary hormonal conditions required for the initiation of metamorphosis of various parts of the body including the gut of the last instar larvae of lepidopterous insects (Riddiford, 1985).

The feeding and post feeding behaviors have only been investigated in species other than the stem borers. The feeding phase in Busseola fusca lasts longer than the post feeding phase. It consisted of brief resting stages when the larvae did not take food. The most notable resting stage occurred by the middle of the feeding phase. After resting, the larvae took to voracious feeding which lasted for a day. This heavy feeding acted as a good indicator for non-diapause development. The loss in weight of the non-diapause larvae during the post feeding phase, might have been as a

result of purging and emptying of their guts. Similar findings have also been reported for the cabbage looper, Trichoplusia ni (Jones et al., 1981). By contrast, larvae destined to enter diapause did not lose much weight but showed steady and slow but consistent feeding and then out-weighed the non-diapause larvae. Larvae with melanised soft body cuticle were in non-diapause development (Schmutterer, 1969). Such larvae did not have hypertrophied fat body as was the case with diapause-bound larvae.

The difference in fresh weights of larvae found diapausing at the end of the long and short rainy seasons probably indicate the difference in the growth of the larvae during the two seasons and the the environmental conditions under-which the larvae were exposed. Larvae diapausing at the end of the long rainy season may have been exposed to a better growth environment than those diapausing at the end of the short rainy season. The behavior of the diapause larvae from the two seasons in terms of stationary moults and weight conservation appeared to be the same. This observation also show that induction of diapause in the larvae may be related more to the physiological status of the host plant than to the developmental stage of the larvae.

Although when in full diapause, Busseola fusca larvae were not feeding, they were able to maintain fairly stable body weights over a long period. They also lacked the clear-cut phases until they enter the pre-pupal stage at the end

of diapause. In addition, under laboratory conditions, the diapause larvae of Busseola fusca were observed to undergo stationary moulting. This observation raises serious questions as to the nature and extent of the type of larval diapause which occurs in Busseola fusca. The first question is whether the condition of development in the larvae of Busseola fusca which is widely regarded as diapause is actually a true larval diapause or could it be just an arrested form of larval development. The second question is to what extent do the larvae commit themselves to diapause development. Findings obtained in experiments designed to answer both questions indicate that larval diapause in Busseola fusca is true diapause and that the diapausing larvae cannot be made to break diapause during the initial 3 to 4 months (Okuda, 1988, unpublished report).

The number of stationary moults the diapause larvae undergo in field or laboratory conditions has not been investigated. Diapause larvae usually lay down silk from time to time and prepare silk-bound pupation chambers just before pupation. Silk production by diapause larvae could have hormonal significance. The diapause larvae of Busseola fusca had gone through stationary moults in the laboratory. Similar observations have been reported earlier (Usua, 1970). However, so far, there are no reports on the occurrence of stationary moult of diapause larvae under field conditions. Significant loss in weight of the larvae occurred with every stationary moult. Should stationary

moults in diapause larvae occur as frequently as has been observed in the laboratory (about once every three weeks in this study; 6 times in the entire period of diapause, Usua 1970), then it can be expected that by the end of diapause, larvae might be too small to give rise to normal pupae. It was found that even under laboratory condition, pupae obtained from diapause larvae were not significantly different in weight from those obtained from non-diapause larvae.

The results obtained from weight changes during pupation show a common trend of weight loss during the process. Larvae from non-diapause development experience a more drastic and rapid weight loss than larvae from diapause development in the process of pupation. This observation indicates that the differences in the two forms of development last only up-to the pre-pupal and do not affect the subsequent stages that follow thereafter. Therefore, the differences in weight observed prior to the pre-pupal stage reflect the occurrence of two separate forms of development during the last larval instar of Busseola fusca. In Mbita, South Nyanza, Kenya, diapause development in Busseola fusca starts in July and the pupation of the diapausing larvae starts in December (Unnithan and Seshu Reddy, 1989). Water appears to be a diapause terminating factor only at the completion of diapause development (Okuda, 1988-unpublished report; Assefa Gebre-Amlak, 1988). Recently, a diapause protein of Busseola fusca was characterized from the

diapausing larvae (Sair et al., 1989). Therefore, the occurrence of stationary moult during diapause reflects an intricate internal control by the endocrine system.

2.5 SUMMARY

In summary this study has elucidated the biology of the larval stages especially that of the last instar of Busseola fusca. Phases within the last larval instar of non-diapause development were recognized as largely consisting of the feeding phase and the post feeding phase. The feeding phase was punctuated by brief resting periods while the post feeding phase was characterized by a steady loss in fresh weight and preparation for pupation. Diapause larvae had no phases similar to those of the non-diapause larvae, but showed stationary moults. Such larvae did not feed but produced silk from time to time. Silk production ceased as the diapausing larvae approached pupation. Studies on patterns of weight changes during pupation gave a good evidence of the occurrence of two forms of development during the last larval instar. The two types of development, namely, the non-diapause and the diapause were confined to the period ending with the pre-pupal stage of the last larval instar.

CHAPTER 3

MORPHOLOGY, HISTOLOGY AND FINE STRUCTURE OF LARVAL NEUROENDOCRINE SYSTEM

3.1.1 INTRODUCTION

The neuroendocrine system which produce the hormones responsible for the coordination of biosynthetic and morphogenetic events during moulting and metamorphosis at the end of the final larval instar of several insects have been studied in detail (Wigglesworth, 1972). Following reports that the brain was essential for moulting (Kopec, 1922), numerous studies by a number of workers have attempted to find out the brain cells (neurosecretory cells) which could have been the source of the hormone (Hanstrom, 1938; Scharrer, 1952).

These neurosecretory cells were easily observed in fresh dissections largely due to their ability to refract incident light and to appear with a bluish hue (Yin and Chippendale, 1973). The cells have also been reported to be rich in cysteine-cystine (Shreiner, 1966) and stain readily with Paraldehyde fuchsin (PF) (Dogra and Tandon, 1964). However, no studies have been made to verify whether the PF-stainable cells (A-cells) are indeed the PTTH producing cells although they have been implicated to be among such cells (Lack and Happ, 1976).

The gross morphology of the neurosecretory cells and their cycles of activity has been described in the milkweed bug, Oncopeltus fasciatus (Mahon and Nair, 1975) and in various lepidopteran species (Mitsuhashi, 1963; Ilyinskaya, 1968; Takeda, 1972; Raina and Bell, 1978).

Relatively few structural studies have been made on the corpus allatum (CA) in the larval stages of the lepidoptera or other insects in other orders. Deleurance and Charpin (1978) made detailed morphological studies of the CA in the cave beetles and showed that there was evidence of cyclicity in the activities of the gland in terms of sub-cellular organelles in the three larval instars. Active glands were characterized by abundance of smooth endoplasmic reticulum, few lysosomes and a few intracellular spaces. By contrast, inactive glands contained less smooth endoplasmic reticulum and more lytic structures such as lysosomes.

Some detailed studies on the morphology of the CA have been made in adult insects of various species (Tobe and Stay, 1985). In the case of the adult Lepidoptera, two morphological cell-types of these glands have been described (Luo and Bodnaryk, 1987). These were identified as the capsulated and the isolated cell types, the former being the most common. The isolated cell type of CA occur only in members of the subfamily Hadeninae (Luo and Bodnaryk, 1987). The cell types of the larval CA have not been classified into specific groups.

However, detailed ultrastructural composition of the Busseola fusca CA in relation to specific developmental states such as diapause is not sufficiently known. Such studies have only been made in species like in the pink bollworm, Pectinophora gossypiella (Raina and Borg, 1980) and in the silk moth, Hyalophora cecropia (Waku and Gilbert. 1964), and in diapausing pre-pupae of the slug moth, Monema flavescence (Takeda, 1977). From these studies the smooth endoplasmic reticulum was reported to be prevalent in active glands.

The activities of the CA are closely linked to those of the brain and the prothoracic glands (PG). The PG were first described in the goat moth, Cossus cossus (Lyonet, 1762); they were named so largely because of the position they occupy in the larvae of the Lepidoptera. However, the position of these glands in some insect species of other orders may vary as is the case in the Diptera.

The PG appears to be the most probable site of synthesis of the moulting hormone in the immature stages of insect species. The gross morphology of these glands has been well described in the larvae of some lepidopteran species (Lee, 1948). Most of them appeared to have three lobes, anterior, dorsolateral and ventrolateral. The histology of the gland has been studied in relation to the titers of the moulting hormone in the haemolymph and a good correlation between both light and electron microscopic structure of the gland cells and titers of the moulting

hormones in the haemolymph has been established (McDaniel et al., 1976; Glitho et al., 1979; Zimowoska et al., 1985). There is no information regarding the PG of Busseola fusca during diapause and non-diapause states of the last larval instar.

3.1.2 Objectives of the study

The objectives of this study were to compare the morphology, histology and ultrastructure of the neuroendocrine system, especially the median neurosecretory cells (NSC) in the pars intercerebralis, the corpora cardiaca (CC), corpora allata (CA) and Prothoracic glands (PG) in diapause and non-diapause last instar larvae of Busseola fusca. This study is expected to elucidate the main ultrastructural characteristics of the secretory activity of the CA and PG and the differences between them in view of marked differences of haemolymph JH concentrations.

3.2 MATERIALS AND METHODS

Sixth instar larvae were obtained from a colony of non-diapause insects maintained in the laboratory on sorghum stem cuttings from six week old sorghum plants. Diapausing larvae were collected from the field and kept in the laboratory at 27°C and 90% relative humidity in 25ml plastic

cups as described earlier (Chapter 2). Under these conditions the diapause lasted for 200 days.

To study the gross morphology of the larval neuroendocrine system, larvae of known age and sex (males show yellow testis underneath the abdominal cuticle) were dissected while under diethyl-ether anaesthesia and in insect ringer to reveal the brain, CA, CC and PG. The appearance of the medial neurosecretory cells through the dissecting microscope under direct illumination was noted. The positions of the CC, CA and PG were noted.

Medial neurosecretory cells (NSC) were studied in whole mount preparations of the brain stained with paraldehyde fuchsin (according to Dogra and Tandon, 1964). Freshly dissected brains along with the retrocerebral endocrine glands of larvae of known age and developmental state were fixed in freshly prepared Bouin's fluid for 24 hours at room temperature. Fixed tissues were washed in distilled water containing crystals of lithium carbonate until all the yellow colour of the picric acid had been removed. Then, they were washed in several changes of distilled water followed by oxidation in a freshly prepared oxidant, acidified potassium parmanganate solution (made by mixing 0.1ml of concentrated sulphuric acid with 0.15 gm of Potassium permanganate and 50ml of distilled water) for 2 minutes.

Tissues were then bleached in 2.5% sodium metabisulphite solution, followed by thorough washing in distilled water. After this, the tissues were dehydrated in graded series of ethyl alcohol (EtOH) ranging from 30% up to 70% ethanol (10 minutes in each change), and stained in paraldehyde fuchsin (PF) prepared according to the method of Cameron and Steele (1959). Briefly, it involved dissolving 1gm of basic fuchsin in 200ml of boiling distilled water and allowed to boil for one minute. The solution was then left to cool at room temperature, filtered and 2ml of concentrated hydrochloric acid and 2mls of paraldehyde were added to the filtrate. The mixture was left to stand for four days at room temperature followed by further filtration. The filtrate was discarded. The crystalline residue on the filter paper was dried and stored in a stoppered bottle until it was required for making the staining solution. Staining solution was made by dissolving 0.25gm of the dry stain in 50mls of 70% EtOH.

Tissues were stained for three minutes in the staining solution. Excess stain was removed using 95% EtOH and then dehydrated in 100% EtOH and cleared in Cedar wood oil followed by xylene; they were then mounted in DPX on clean slides and studied under Carl Zeiss light microscope. The location of cells which stained positive with PF was noted. Observations were also made on the number and gross morphology of such cells, their cell bodies, axons and the sites of axon terminals in the neurohaemal region.

3.2.2 Histology and fine structure of the neuroendocrine system

Larvae were anesthetized in diethyl ether and the entire brain with the attached CC, CA and the PG were dissected out in cold 2.5% glutaraldehyde buffered to pH 7.4 with 0.05M cacodylate buffer containing 5% sucrose and 0.01M $CaCl_2$. Tissues were transferred in fresh fixative and left to fix for 4 hours at room temperature. Then, they were washed in several changes of 0.05M Sodium cacodylate buffer and postfixed in 1% cacodylate-buffered Osmium tetroxide for 1 hour at 4°C. Post fixed tissues were washed in several changes of the cacodylate buffer and then dehydrated in ascending grades of ethanol starting from 30% through 50% 70%, 80%, 90% (10 minutes in each concentration) and finally dehydrated in three changes of 100% ethanol (10 minutes in each change), then cleared in propylene oxide. Cleared tissues were infiltrated in a 1:1 propylene oxide:araldite mixture overnight followed by further infiltration in araldite for 24 hour at room temperature, and finally embedded in pure araldite at 60°C (for 72 hours). Thick (1µm) and thin (500-700 Å) sections were cut on an LKB-Ultrotrome using glass knives. For histological studies 1um thick sections were collected onto glass slides and stained for general histology with 3% Toluidine blue made up in 3% borax and studied under the light microscope. Ultra-thin

sections (500-700 Å) were collected on fomvar-coated copper grids and double stained in aqueous uranyl acetate and lead citrate (Reynolds, 1963). They were examined under Philips 201 Transmission Electron Microscope operated at an accelerating voltage of 80 KV.

3.3 RESULTS

3.3.1 Morphology of the neuroendocrine system

As shown in figures 5 and 6, the neuroendocrine system of Russeola fusca consists of neurosecretory cells of the brain, CC, CA, and PG. The PG lie on top of the lateral trachea at the level of the anterior most spiracle and are innervated from the suboesophageal, prothoracic and metathoracic ganglia. The medial neurosecretory cells of the brain (NSC) send their axons through the CC and terminate in a neurohaemal area around the CA (Figure 7). The CC and CA lie on either side of the oesophagus behind the brain and below the antennal nerves (Figure 5). The CA is receive axons of neurosecretory cells from the brain through the CC with which it shares a common connective tissue.

Under direct illumination of a freshly dissected larva, one pair of neurosecretory cells (NSC) was visible on the medial part of each cerebral hemisphere. The lateral group of cells were not visible in all the preparations. The neurosecretory cells on the medial part of the brain, appeared ovoid and whitish, one cell in front of the other.

In freshly dissected larvae, the PG are a pair of discrete, white organs resembling an inverted 'Y'-shaped structure (Figures 5 and 6) attached via fine fillaments to the integument. Each gland has three branches: anterior, lateral-dorsal and lateral-ventral. The anterior branch is

innervated by the transverse nerves from the sub-oesophageal and prothoracic ganglia (Figure 6). The lateral branches receive nerves from the medial nerve of the prothoracic ganglion and transverse nerves of the anterior lateral nerve of the mesothoracic ganglion. The PG is located on the dorsal tracheal system of the prothorax and three lobes are intersected at the level of the anterior most spiracle.

3.3.2 Histology and fine structure of the neuroendocrine system

The results of whole mount staining of the brain using paraldehyde fuchsin technique are represented in figures 7, 8 and 9. Two pairs of PF-positive, A-type neurosecretory cells (NSC) occur on the medial part of the pars intercerebralis. These cells stained densely, appeared large and tear-drop shaped. In each pair, one of the cells sends its axon to the CA of the contralateral side and the other to the ipsilateral CA (Figure 10). Each cell has a large centrally located nucleus while the cytoplasm is filled with deep staining granules.

Under the light microscope and using Toluidine blue staining, brain sections revealed one pair of neurosecretory cells which differed in their staining intensity (Figure 11). The darker staining cells correspond to type-A cells. The lighter cell type (type-B cells) which was not PF-positive appeared as large as the type-A cell.

The cellular organization of the medial neurosecretory cells is given in figure 12. The cytoplasm of the type-A cell consists mainly of an accumulation of numerous electron dense neurosecretory granules of various sizes and some free ribosomes. Each cell has a large central nucleus appearing spherical. The nucleoplasm has other extranucleolar electron dense bodies with granular material. The nuclear membrane is well developed. Type-B neurosecretory cell, differed from the A-type described above by the number and nature of its electron dense granules. The cell granules occurred in groups and were rather uniform in terms of size than those of the A-cell.

Figure 13 is a photomicrograph of typical corpus cardiacum and corpus allatum cells stained with toluidine blue-borax. The two endocrine glands appear to be enveloped in the same connective tissue and adhere loosely to each other.

At the electron microscope level, the nuclei of the CC cells show convoluted or lobulate outlines, the nucleoplasm contains numerous chromatin clumps clumps (Figure 14). In the cytoplasm, mitochondria, free ribosomes, occasional short strands of rough endoplasmic reticulum, Golgi complexes and electron dense neurosecretory granules are conspicuous and relate to the secretory function. The cell surfaces attach to neighbouring cells with intercellular junctions at several points forming loose cells. The area between the loose cells is occupied by the axon profiles.

neurosecretory elements in the CC are made up of intrinsic neurosecretory cells and axons of neurosecretory cells of the brain which travel to the neurohaemal region around the CA.

The CA are glandular organs located behind the CC. They are spherical or elliptical in shape. The gland consists of two zones. The outer cortical zone stains lighter than the inner glandular zone (Figure 13). The glandular zone contains parenchymal cells as well as axons. The outer layer is continuous with that surrounding the corpora cardiaca and serve as the neurohaemal area for NSC. This peripheral area of the CA is traversed by axonal terminals containing electron dense neurosecretory granules.

Fine structure

The fine structure of the CA of the non-d apause larva is shown in figures 15 and 16. The CA secretor, cell has a nucleus with a convoluted outline and contain several nucleoli concentrated at the equatorial region. In the cytoplasm, mitochondria, lysosomal-like bodies, numerous membrane bound vesicles of different sizes and some free ribosomes are conspicuous some of the vesicles appear to coalesce and form larger vesicles. The mitochondria are numerous.

The cellular organization of the CA from diapause larva was compared to that of non-diapause larva. Figures 17-19

are electron micrographs of typical CA cells during diapause. Remarkable ultrastructural changes occur in the intracellular organelles and neurosecretory axons. The mitochondria are large and abundant in the cytoplasm. Three main types of mitochondria can be differentiated, namely round-, elongated, and oblong shape with a dense centre. Some of the mitochondria have vacuoles and contain electron dense material within the matrix. The morphological appearance of these mitochondria is unique and different from those observed in the CA of the non-diapause larvae. Apart from these large mitochondria, residual bodies are occasionally formed in close proximity to this organelle (Figure 19). The nuclei have irregular outlines and have many nucleoli scattered evenly within the nucleoplasm. In the cytoplasm, free ribosomes are detected and smooth endoplasmic reticulum show characteristic whorls (Figure 20). In addition, the cytoplasm contain glycogen-like deposits (Figure 21) unlike the situation in the CA of the non-diapause larva which did not contain any glycogen-like deposits. The nuclei show irregular outline and they contain nucleoli and chromatin bodies distributed throughout the nucleoplasm. The CA is also transversed by different types of axons containing neurosecretory granules (Figure 21). In axons located in the periphery of the gland opaque neurosecretory granules were detected (Figure 21). Spaces are formed, including empty capsules in the cytoplasm near

some of the nuclei and which indicate the transfer of the materials.

The fine structure of the prothoracic gland of non-diapause larva is shown in figures 22-28. The extensive lacunae (channel) system formed by infoldings of the cellular membranes are very prominent and appears to increase the surface area of the PG cells. This peripheral channel system contained an amorphous material and occasional microvesicles were observed within the channel spaces which appeared to be filled with granulated material. The cytoplasm of the non-diapause prothoracic gland is loaded with numerous elongated mitochondria with well developed cristae and dense matrices (Figure 24). The cytoplasm also contain numerous free ribosomes spread throughout the cytoplasm (Figure 25). Rough endoplasmic reticulum are few but no smooth endoplasmic reticulum was observed. Cytoplasmic inclusions such as dense bodies and lipid-like droplets were also observed (Figure 26). The nucleus of the PG cell appear shrunken, convoluted and contains several scattered chromatin bodies (Figure 27). Figure 28 is a transverse section of an axon of a neurone innervating the prothoracic glands of a non-diapause larva and shows granules which are contains neurosecretory material. Similar granules were observed in axons supplying the PG of diapause larva.

During diapause, the prothoracic gland has a well developed basal lamina and peripheral channels which appeared very much similar to those observed in the prothoracic glads of the non-diapause larva but were less prominent (Figure 29). The tracheole system is located in the region between the basal lamina and the cortex of the gland. The gland cells were characterized by extensive lacunar system formed by infoldings of the cellular membranes. In the cytoplasm free ribosomes and numerous mitochondria are abundant (Figure 29). Within the cytoplasm and in close proximity to the lacuna system, lytic like vacuoles are found. The cell of the PG shows also a highly ramified nucleus. The mitochondria appeared smaller in size (Figure 30) and lysosome-like structures were observed in these cells.

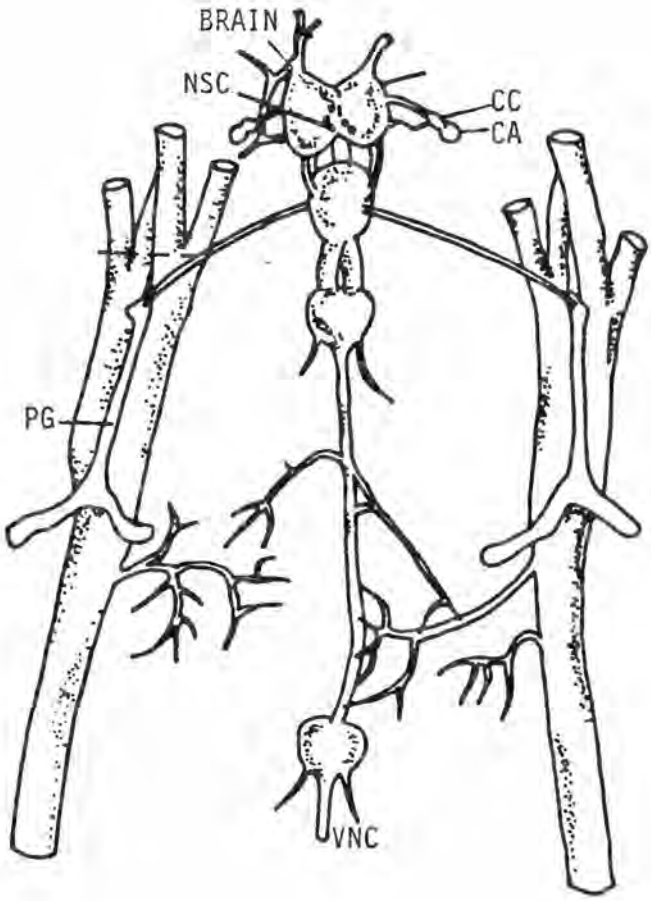

Figure 5

Diagrammatic representation of the neuroendocrine system of the sixth instar larva of Russeola fusca. CA, corpus allatum, CC, corpus cardiacum, NSC, medial neurosecretory cells, PG, prothoracic gland, VNC, ventral nerve cord.

Figure 6

A dissected preparation of the sixth instar larva showing the relative positions of the components of the neuroendocrine system: CA, corpus allatum; CC, Corpus cardiacum; NSC, medial neurosecretory cells; PG, prothoracic gland. X 250

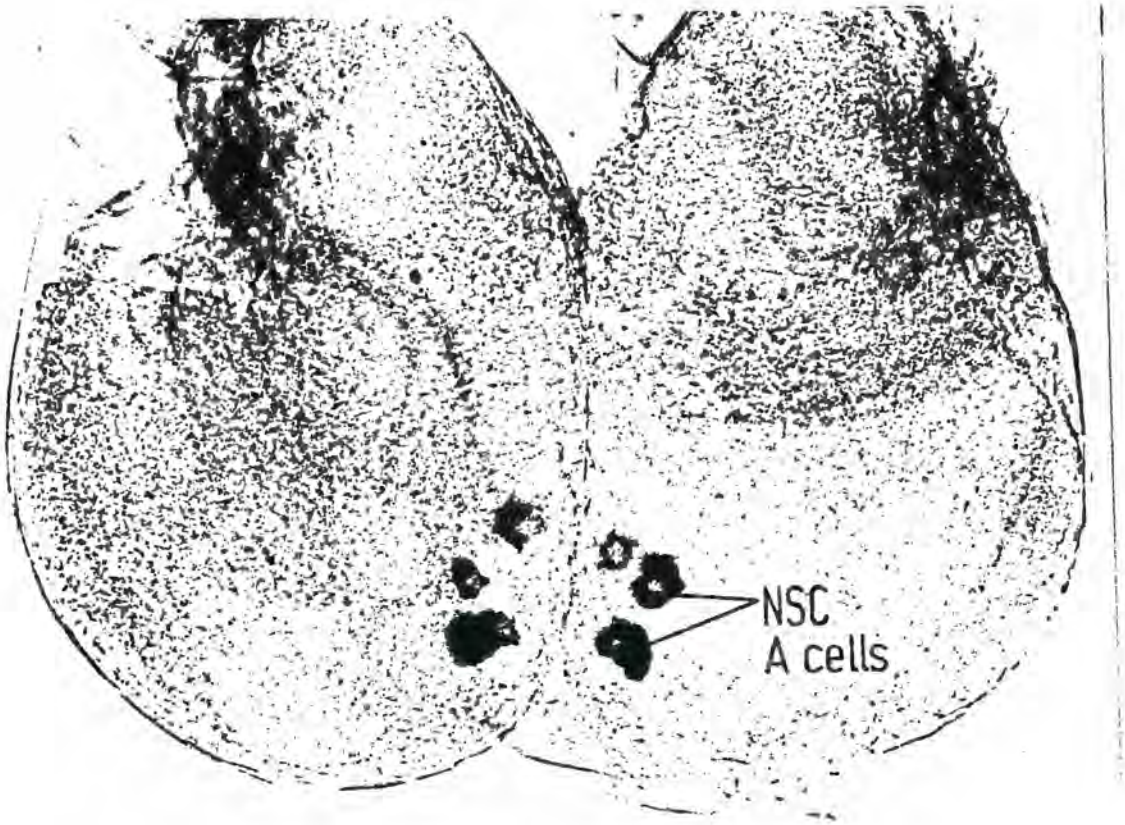

Figure 7

Photomicrograph of the whole mount preparation of the last instar larval brain stained with paraldehyde fuchsin. The A-cells (NSC) appear on the medial part of the brain. X 450

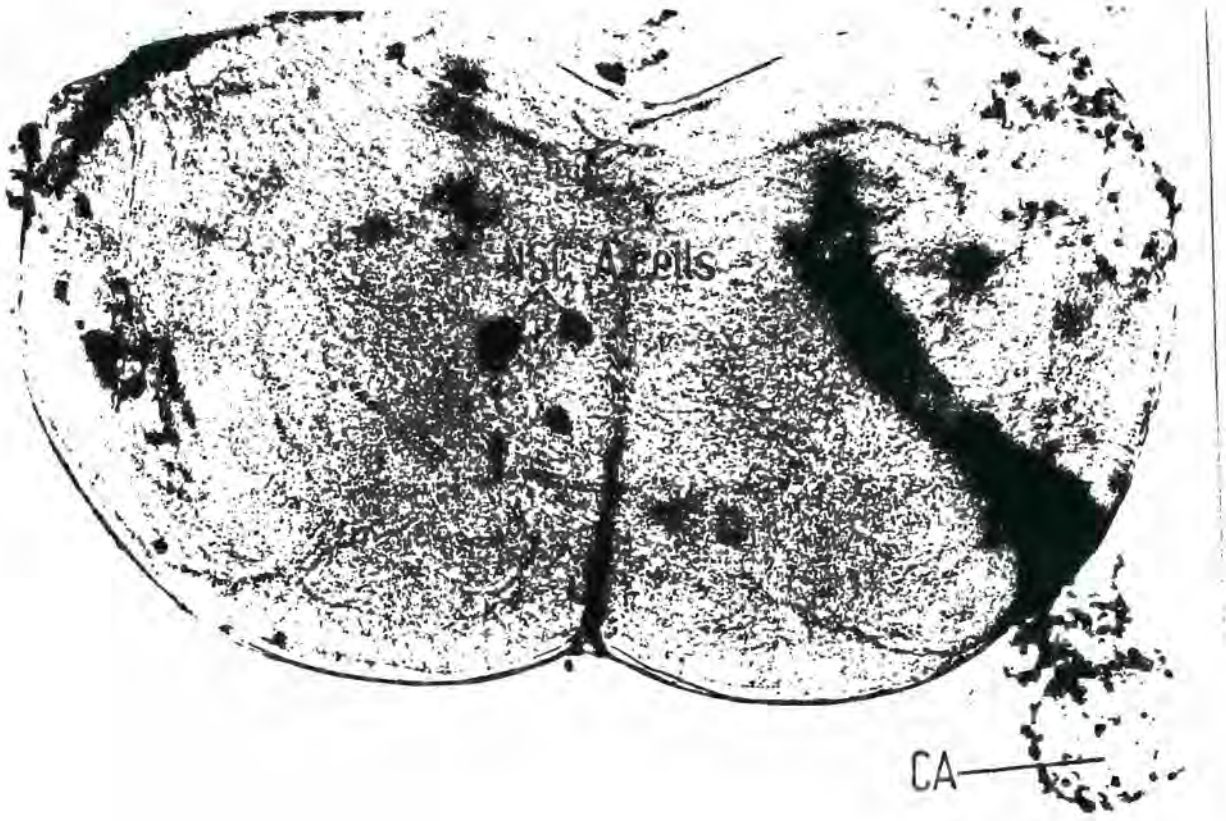

Figure 8

Photomicrograph showing whole mount preparation of prepupal brain stained with paraldehyde fuchsin. Type-A cells stained faintly. CA, corpus allatum. X 650

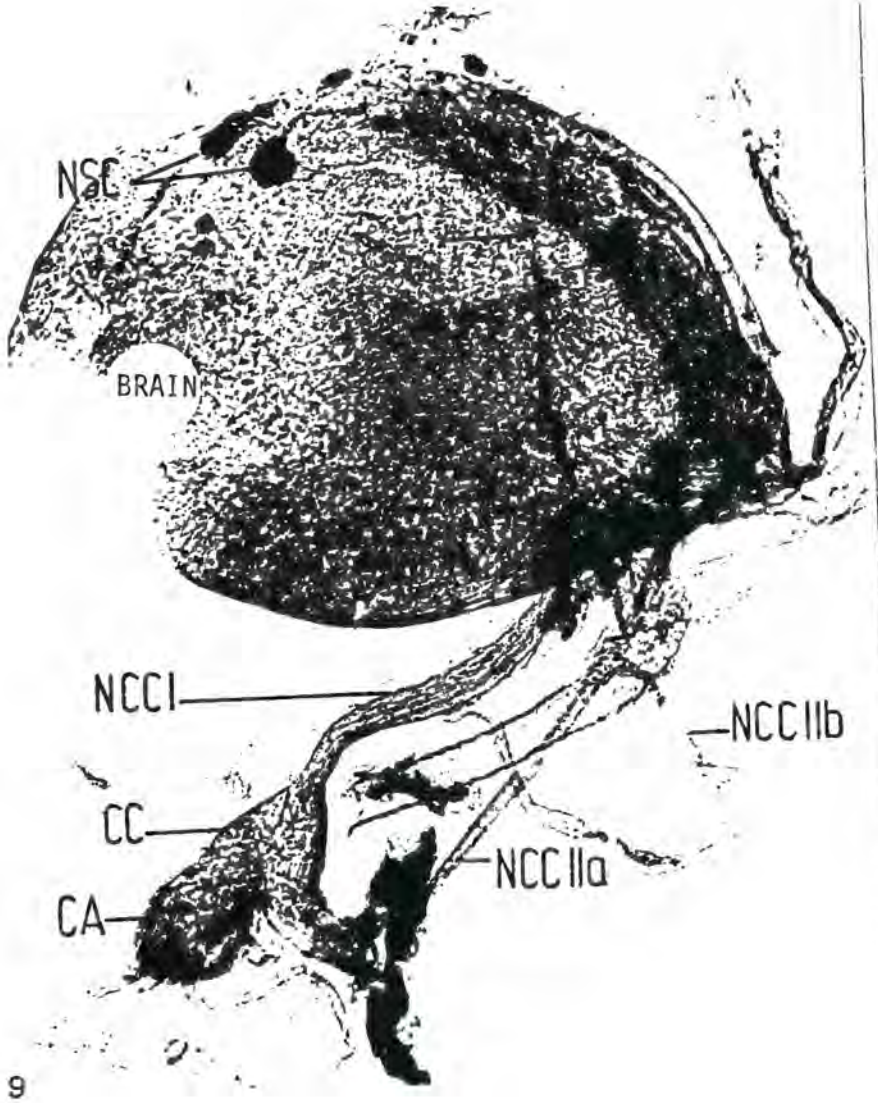

Figure 9

Photomicrograph of Busseola fusca 6th instar larval brain, CA-CC complex stained with paraldehyde fuchsin showing the relationship between the brain, corpus cardiacum (CC) and the corpus allatum (CA). The neurosecretory type-A cells (NSC), their axons (arrows) and axonal terminal around the CA stain relatively darker. X 250

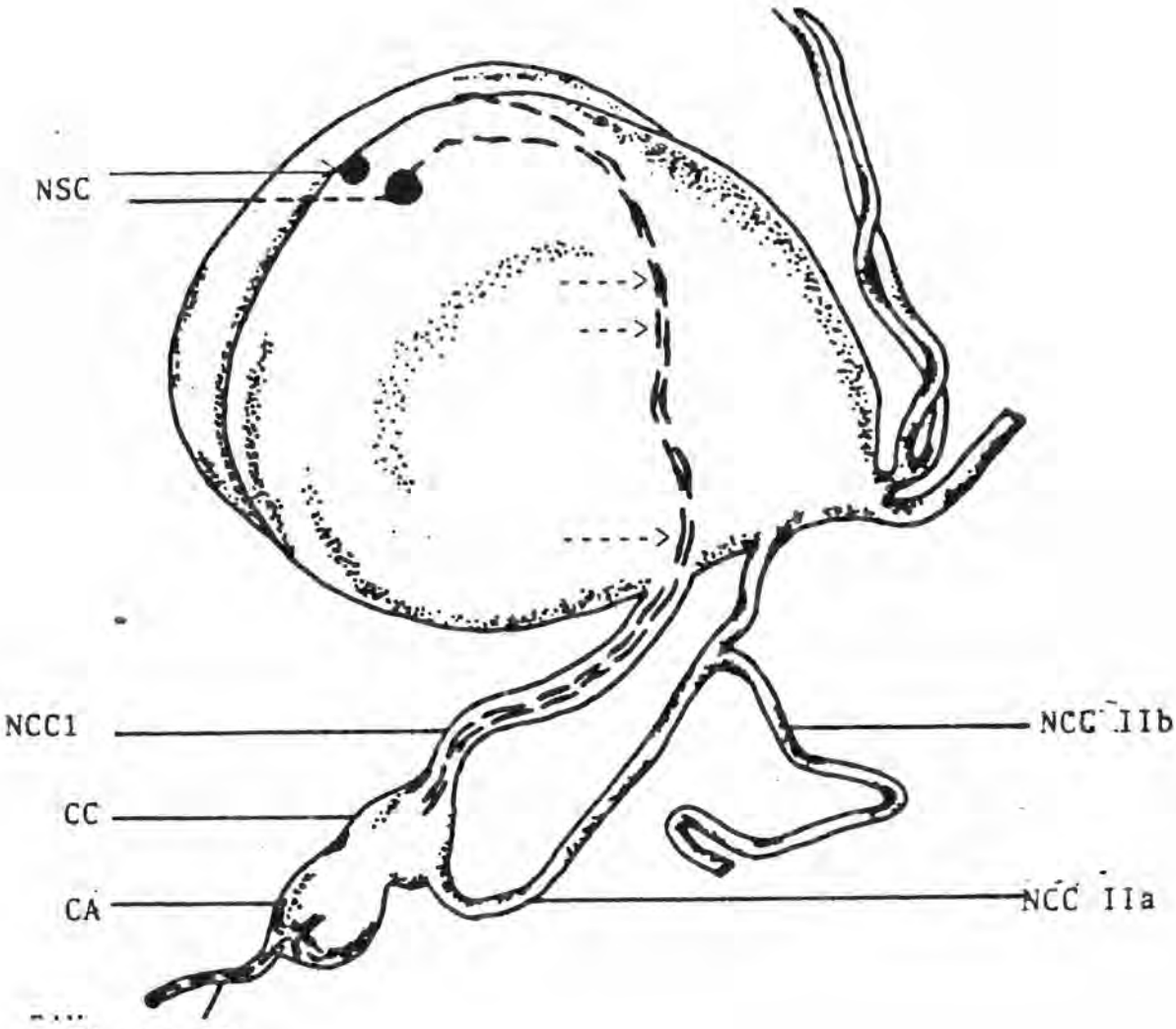

Figure 10

A schematic representation of the relationship between the medial neurosecretory type-A cells in the brain (NSC), the corpus allatum (CA) and corpus cardiacum (CC). The nervus corporis cardaci 1 (NCC1), nervus corporis cardiaci IIa (NCCIIa) and nervus corporis cardiaci IIb (NCCIIb) connect the CC to the brain

Figure 11

Photomicrograph of araldite embedded thick section through the brain from the diapause sixth instar larva stained with toluidine blue-borax. The A-type neurosecretory cells (A cell) stain intensely while B-type (B cell) stain faintly. X 450

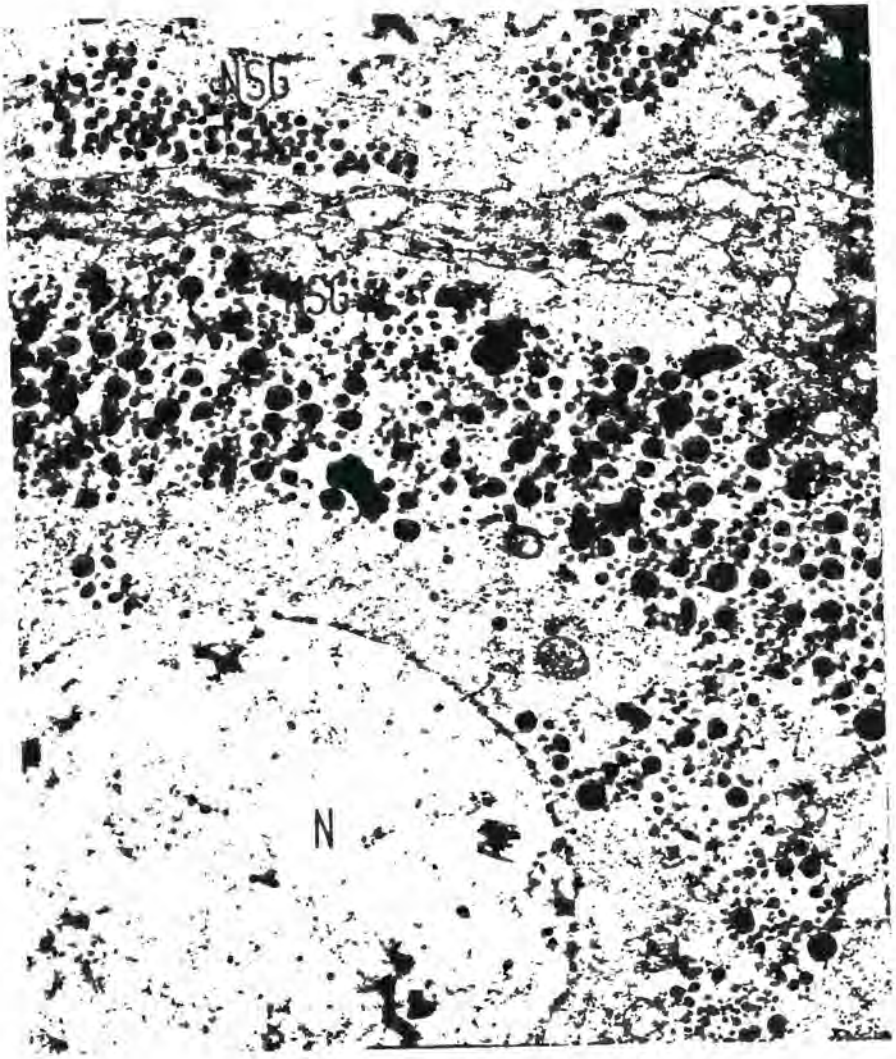

Figure 12

An electron micrograph of the last instar diapause larval brain showing neurosecretory neurone (NSG). N, nucleus.

X 10,000

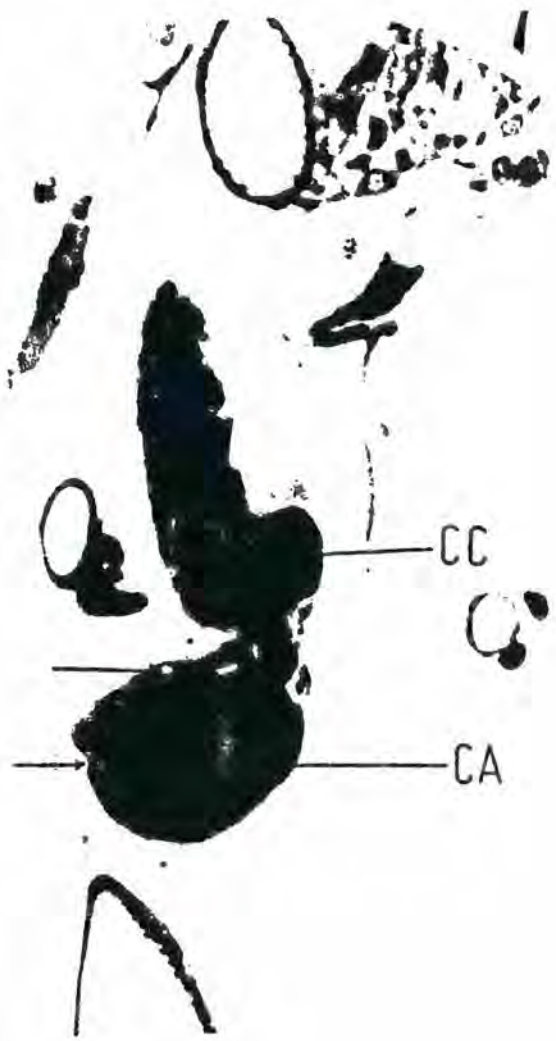

Figure 13

Photomicrograph of a thick longitudinal section through the corpus cardiacum (CC) and corpus allatum (CA) complex from the non-diapause sixth instar larva stained by toluidine-borax. The neurohaemal portion (arrow) surrounds the glandular area of the CA. X 450

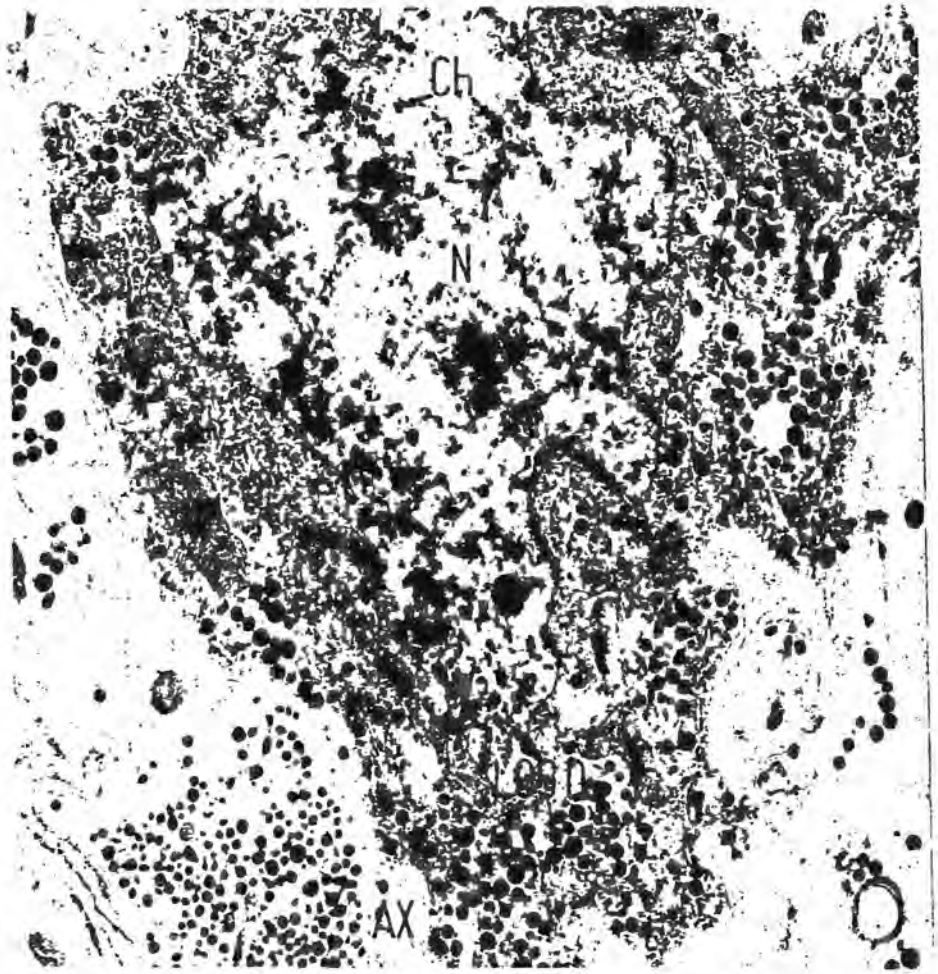

Figure 14

An electron micrograph of a portion of the CC from diapause larva showing secretory cell with large opaque neurosecretory granules (LOSD) in cytoplasm. These droplets are passed into axon-like processes (Ax) at the periphery. Mitochondria (M), and a few strands of rough endoplasmic reticulum (RER), occur in the cytoplasm. The nucleus (N) is irregular with scattered chromatin (Ch). X 30,000

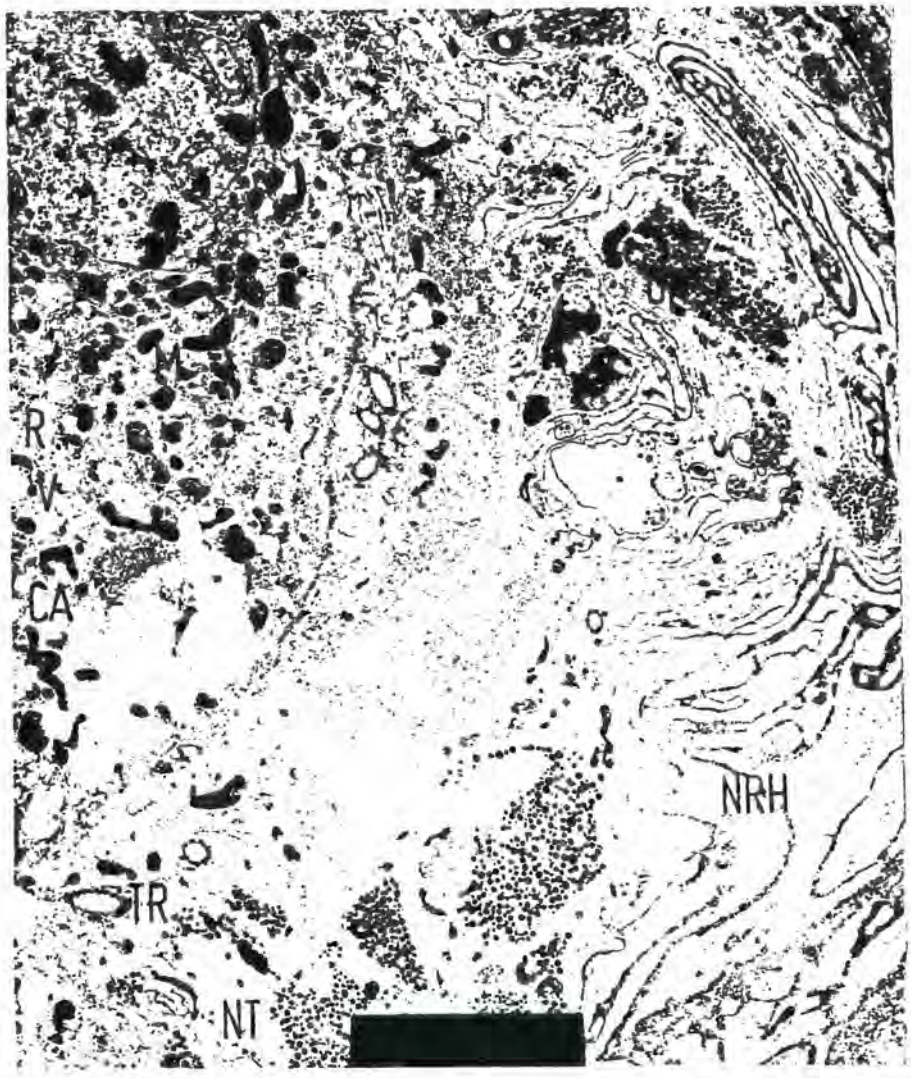

Figure 5

Electron micrograph of the periphery of the corpus allatum (CA) from non-diapause larva showing part of the neurohaemal region (NRH) with many axons filled with opaque neurosecretory granules (ONSA), and neurotubules (Nt). The nucleus (N) of the CA cell is surrounded by cytoplasm containing evenly scattered mitochondria (M), some vacuoles (V) and the ribosomes (R). X 6,750

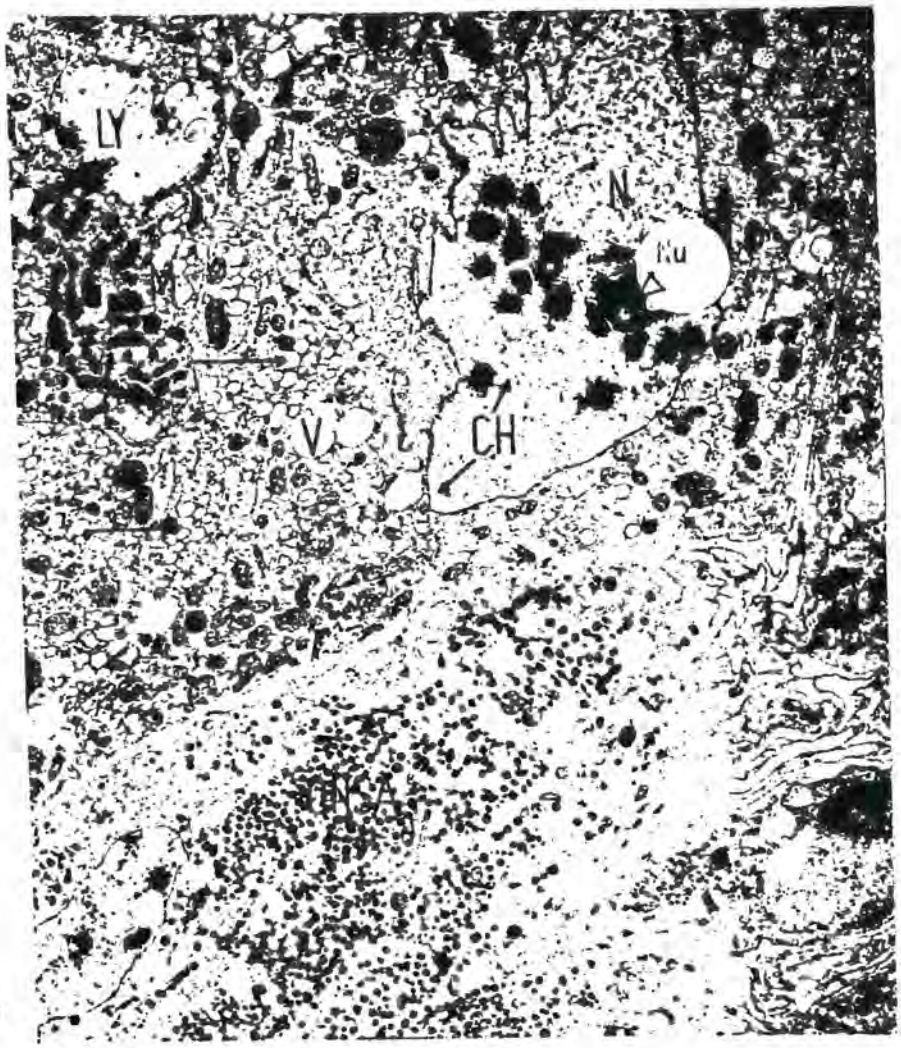

Figure 16

Electron micrograph of part of the CA cell and adjacent CC-cell from non-diapause mid-(Day 4) 6th instar larva. The CA cell cytoplasm is characterized by extensive vesiculation (arrows) which coalesce toform vacuoles (v), empty and lysosome-like bodies (LY). The nucleus (N) shows nucleoli (Nu) at the equator and diffused chromatin (Ch). CC cell is loaded with opaque granules (ONSA). X 10,000

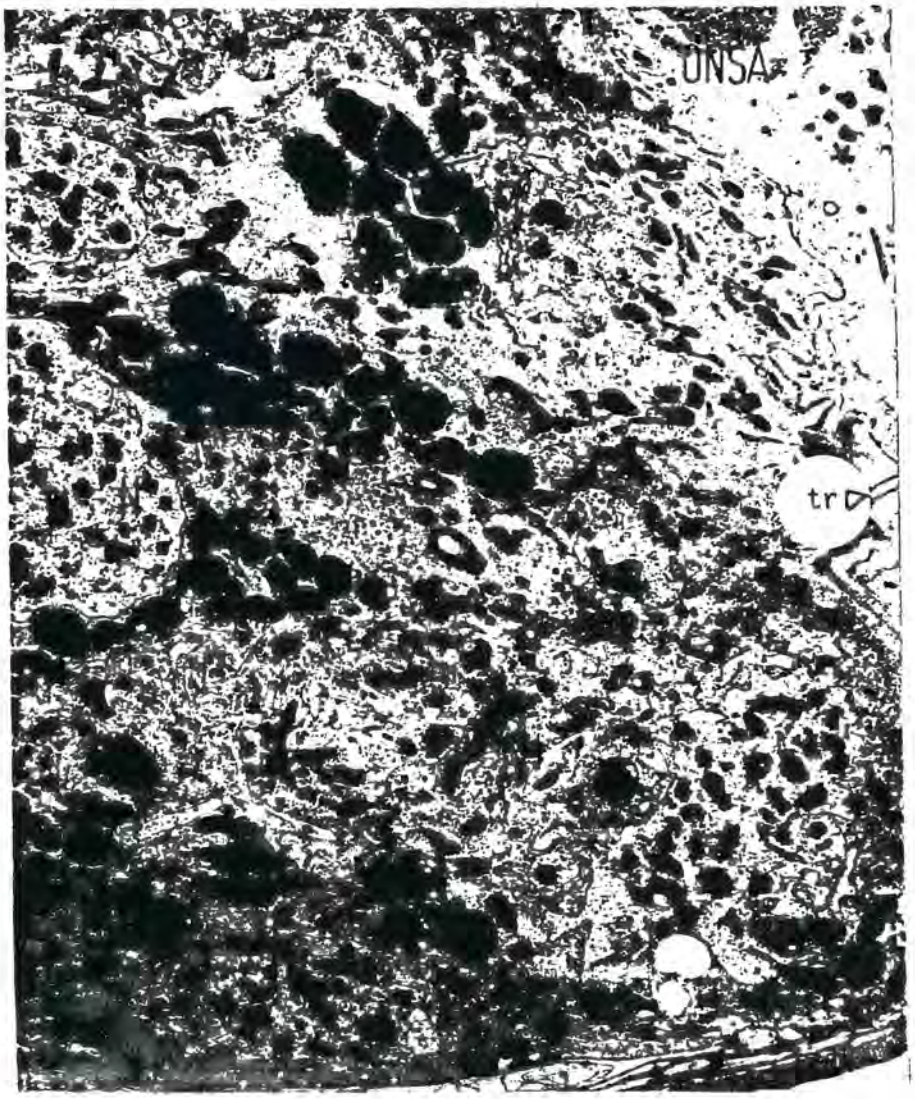

Figure 17

A low magnification electron micrograph of the corpus allatum (CA) of the diapause larva showing general architecture of the glandular cell. Bm, basement membrane, M, mitochondria, N, nucleus, ONSA, opaque neurosecretory granules, tr, trachea. X 4,500

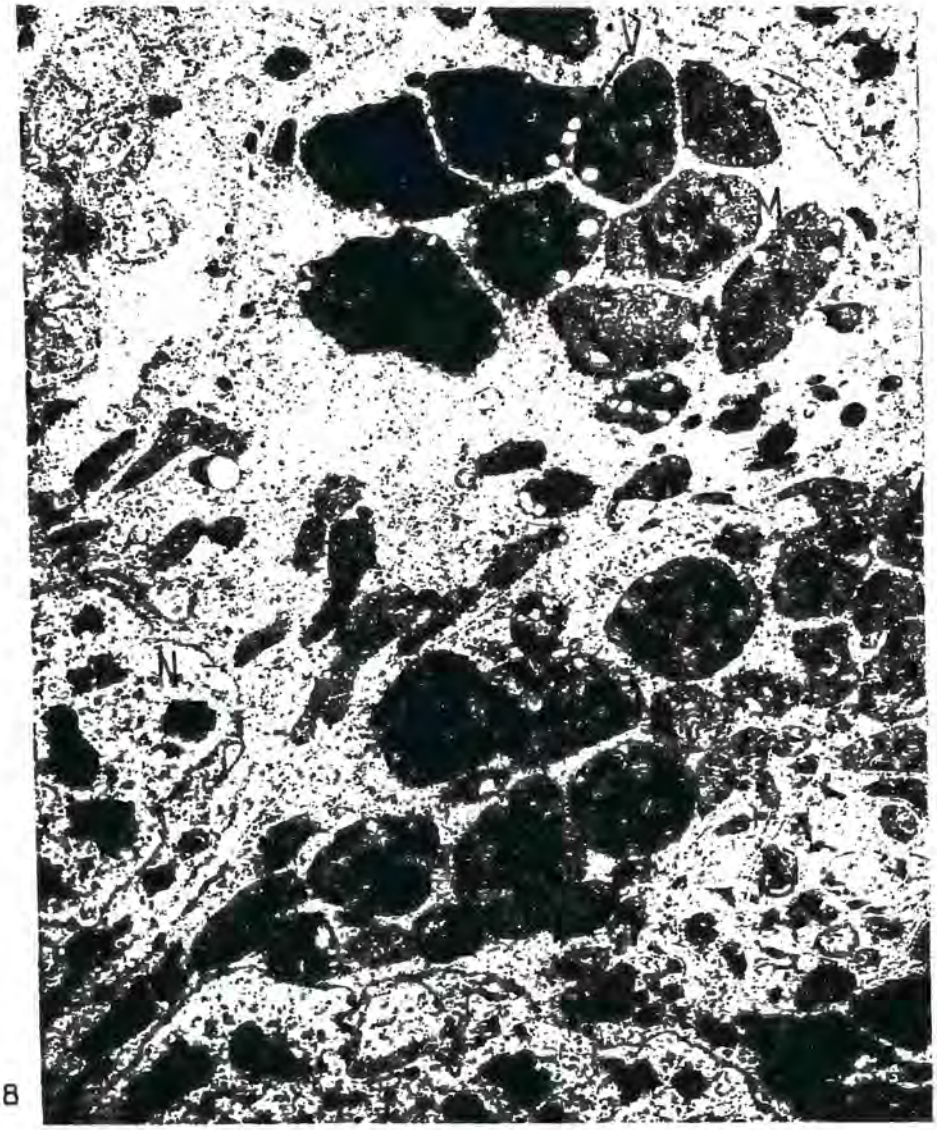

Figure 18

Part of the corpus allatum cell from diapause larva showing large pleomorphic mitochondria (M). Small vacuoles (v) (arrows) are formed inside those mitochondria containing electron-dense material. N, nucleus with fragmented nucleoli. X 30,000

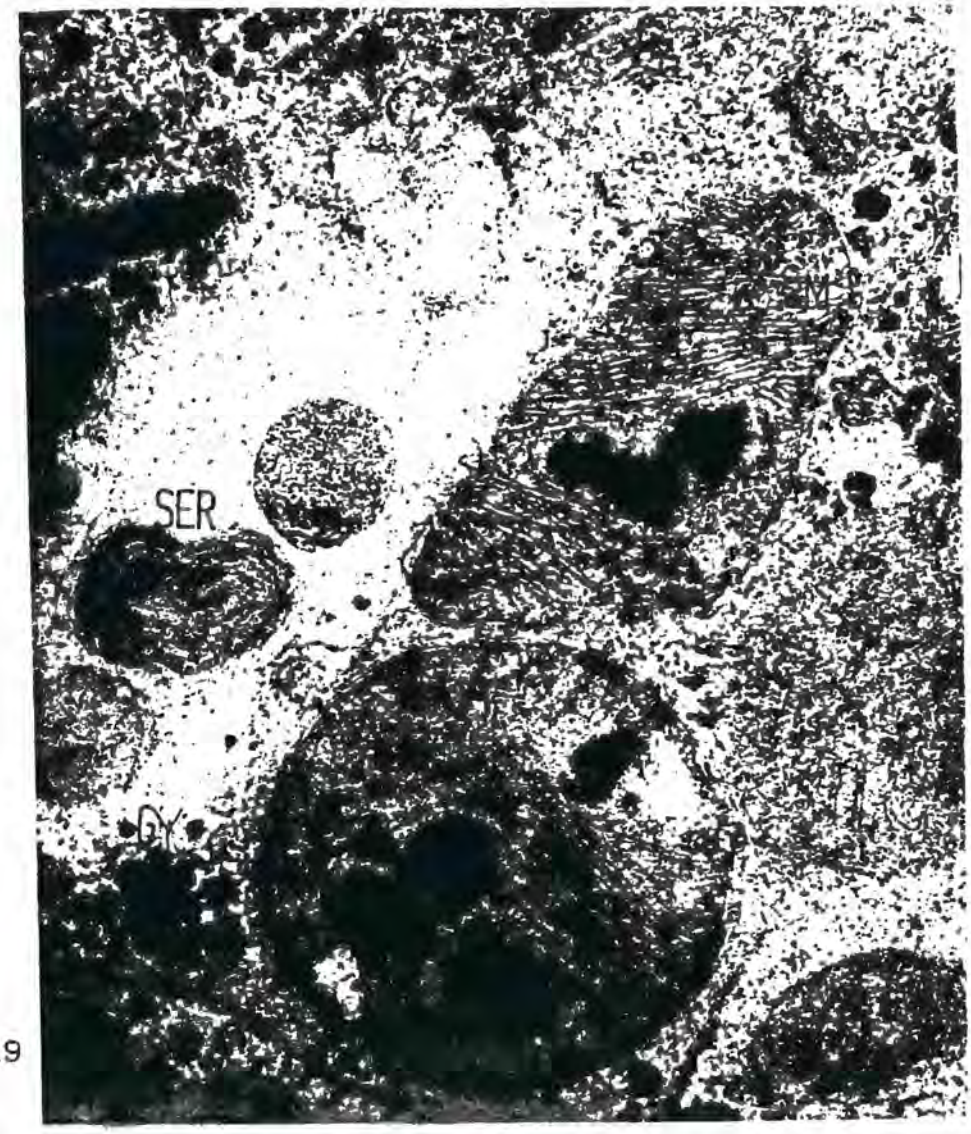

Figure 19

A high magnification of CA cell, showing detailed structure of mitochondria (M), from diapause larva. Electron-dense material (DB) are located in the mitochondrial matrix. Glycogen-like deposits(GY) are localized in the cytoplasm directly adjacent to the mitochondria. X 45,000

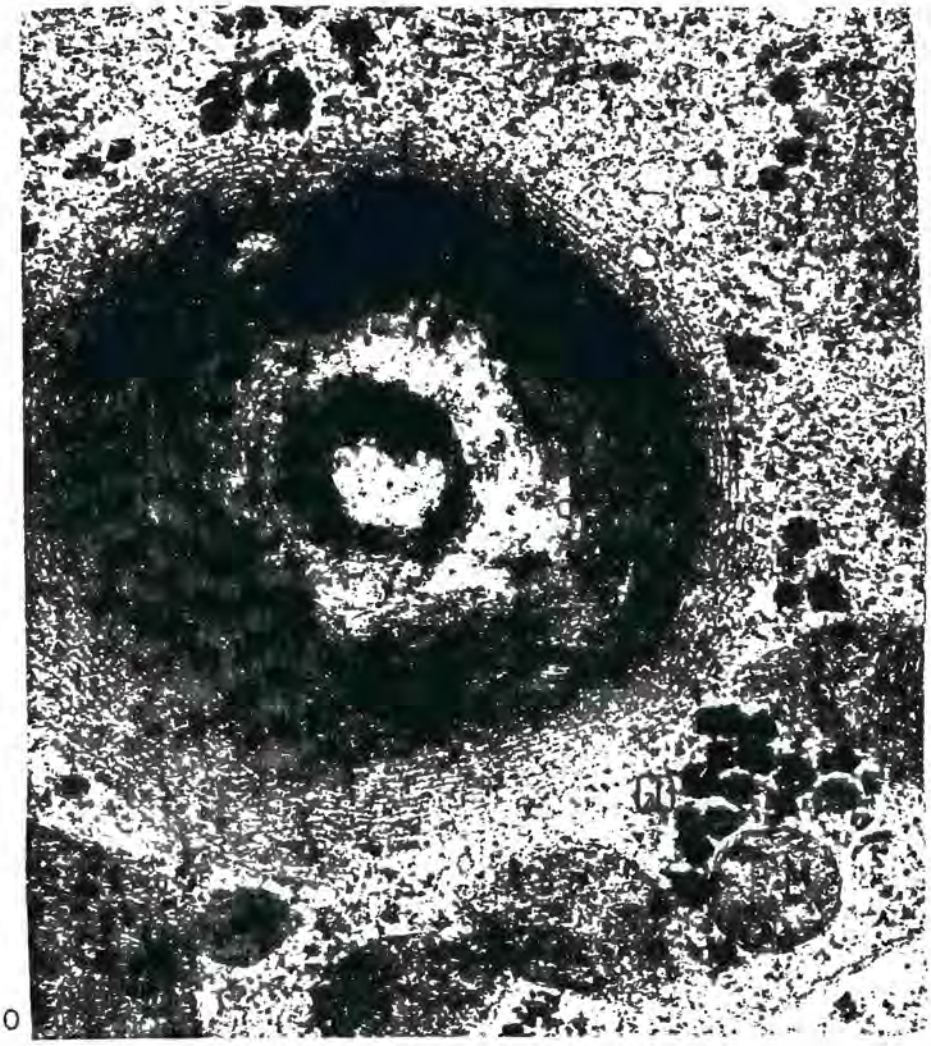

Figure 20

Smooth endoplasmic reticulum (SER) in the corpus allatum cell of the diapause larva forms concentric whorls around dense body. Glycogen deposits (GD) and mitochondria (M) are located near the lamellated SER. X 45,000

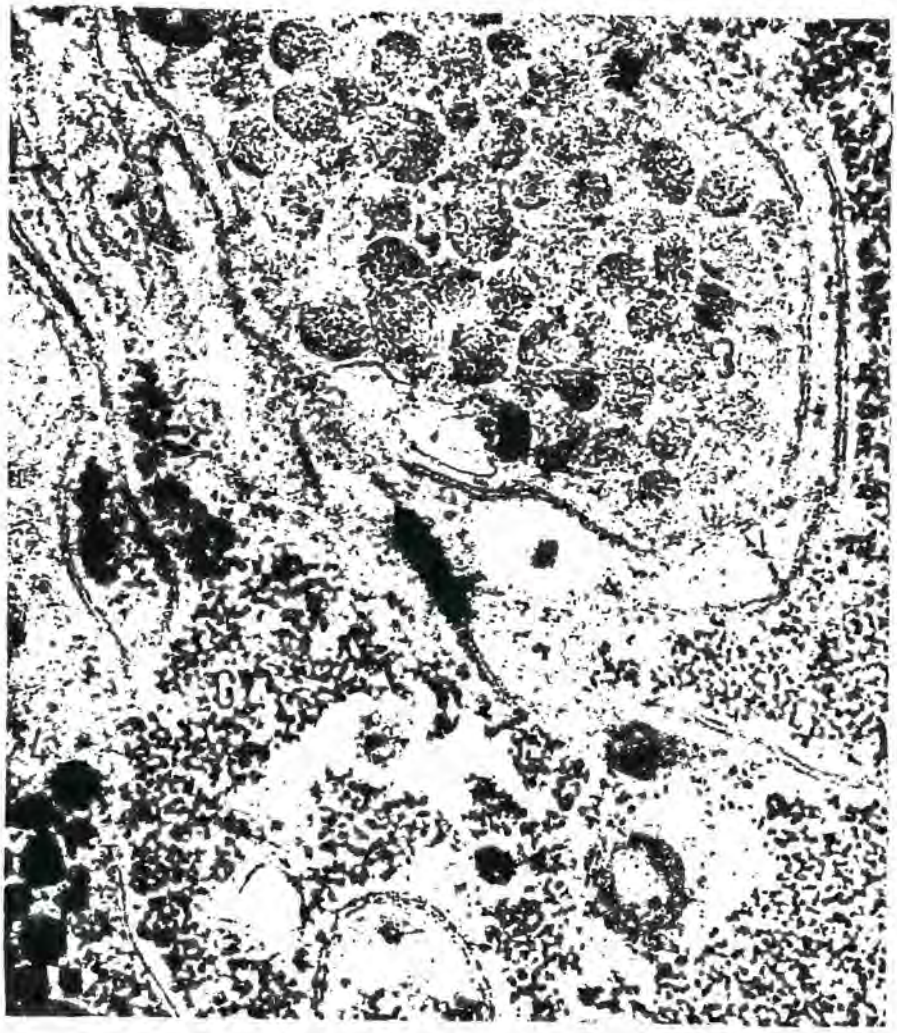

Figure 21

An electron-micrograph of the glandular part of the corpus allatum of the diapause larva showing two types of neurosecretory axons (3 and 4). X 45,000

Figure 22.

Periphery of the prothoracic gland from the non-diapause larva showing the basement membrane (Bm), and the peripheral channel system. X 6,750

Figure 23

Peripheral part of the prothoracic gland of non-diapause larva showing large vacuoles (V) which are part of the lacunar system. Vacuoles containing fibrous material creating islands of cytoplasm with mitochondria (M) and free ribosomes (R). X 45,000

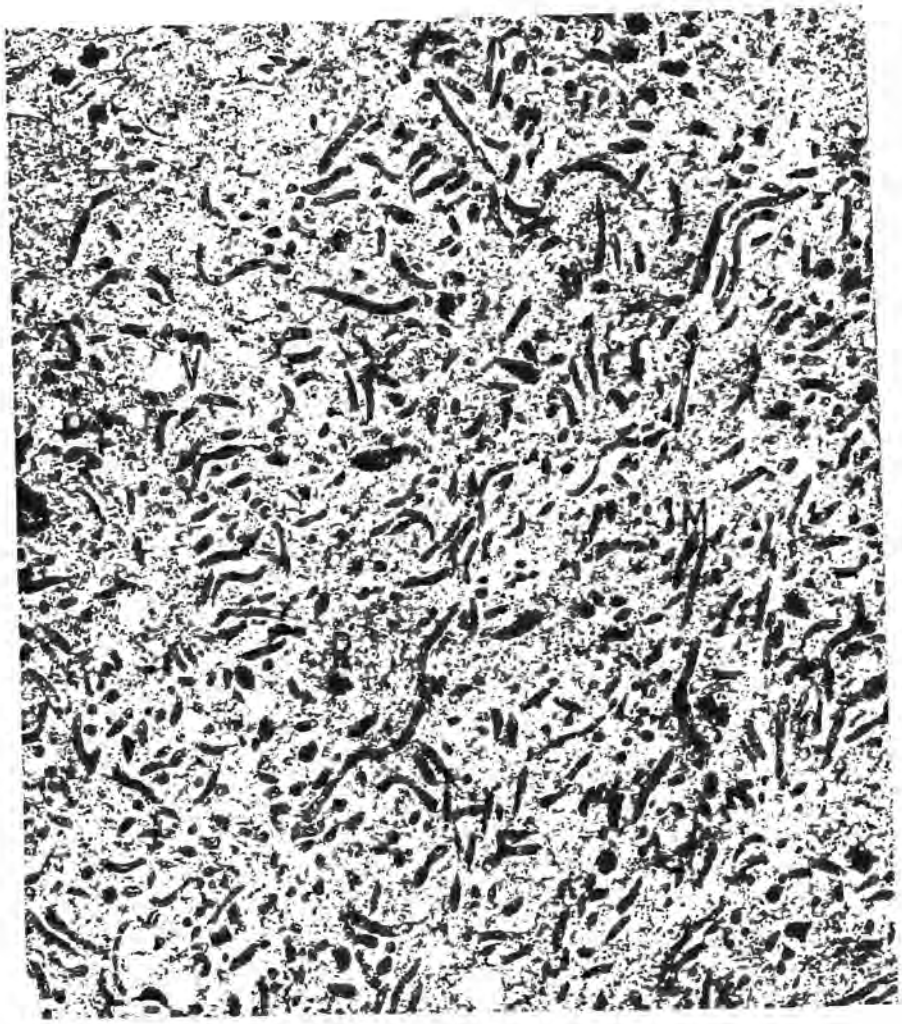

Figure 24

A portion of the cell of the prothoracic gland from non-diapause last instar larva showing numerous elongated mitochondria (M), free ribosomes (R) and vacuoles (V). X 10,000

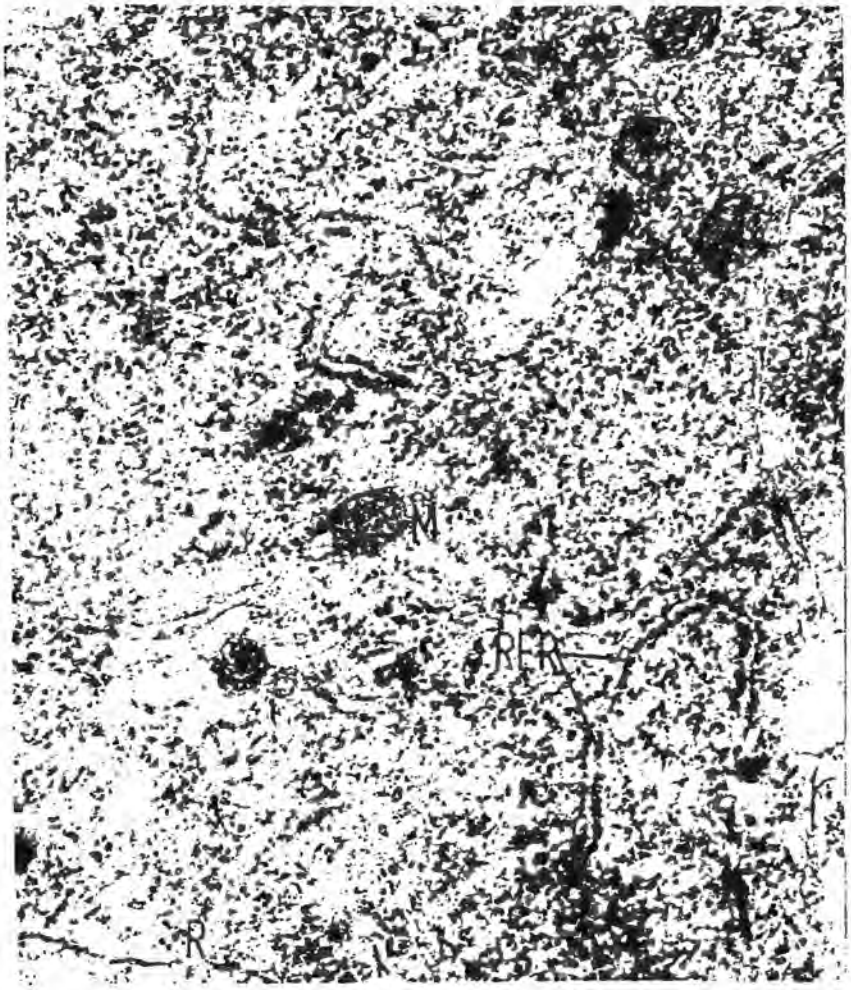

Figure 25

An electron micrograph of the cell of the prothoracic gland from non-diapause larva showing rough endoplasmic Reticulum (RER), free ribosomes (R), and mitochondria (M). X 45,000

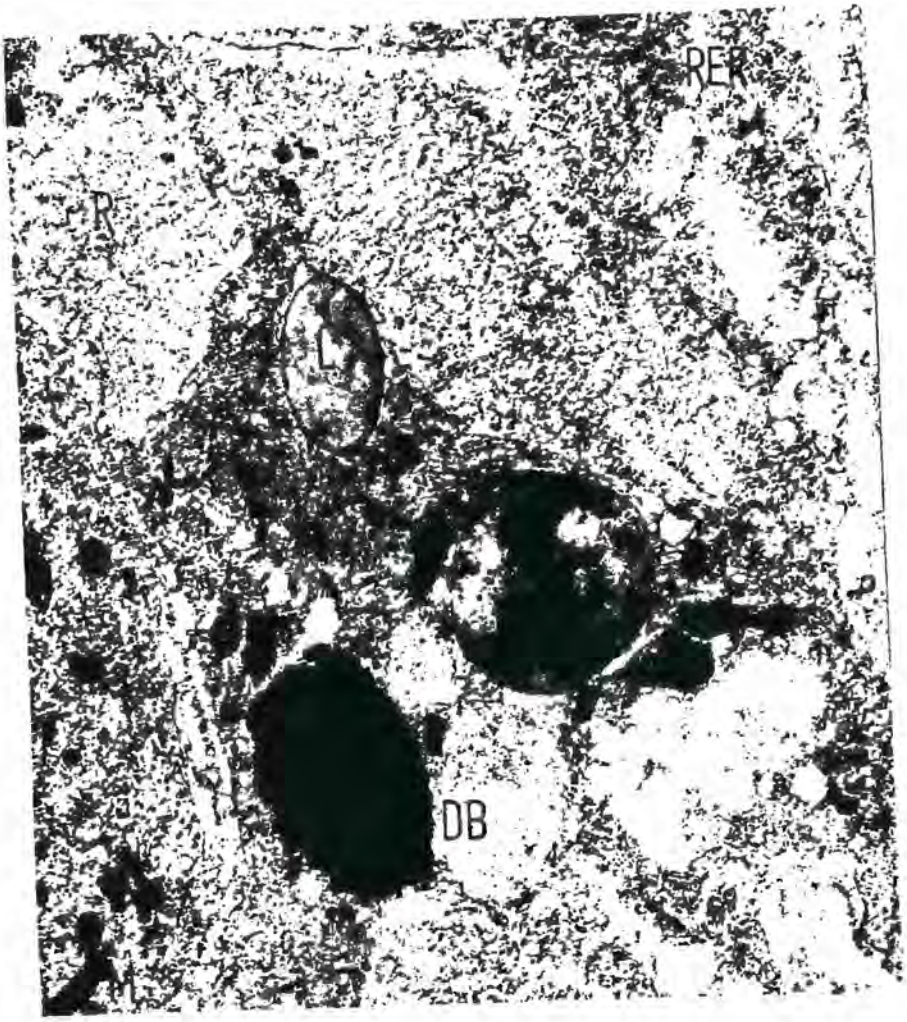

Figure 26

Part of prothoracic gland cell from non-diapause larva containing electron dense body (DB), lipid body (L) and elongated mitochondria (M). RER, rough endoplasmic reticulum, and vacuoles (v) filled with amorphous material. X 10,000

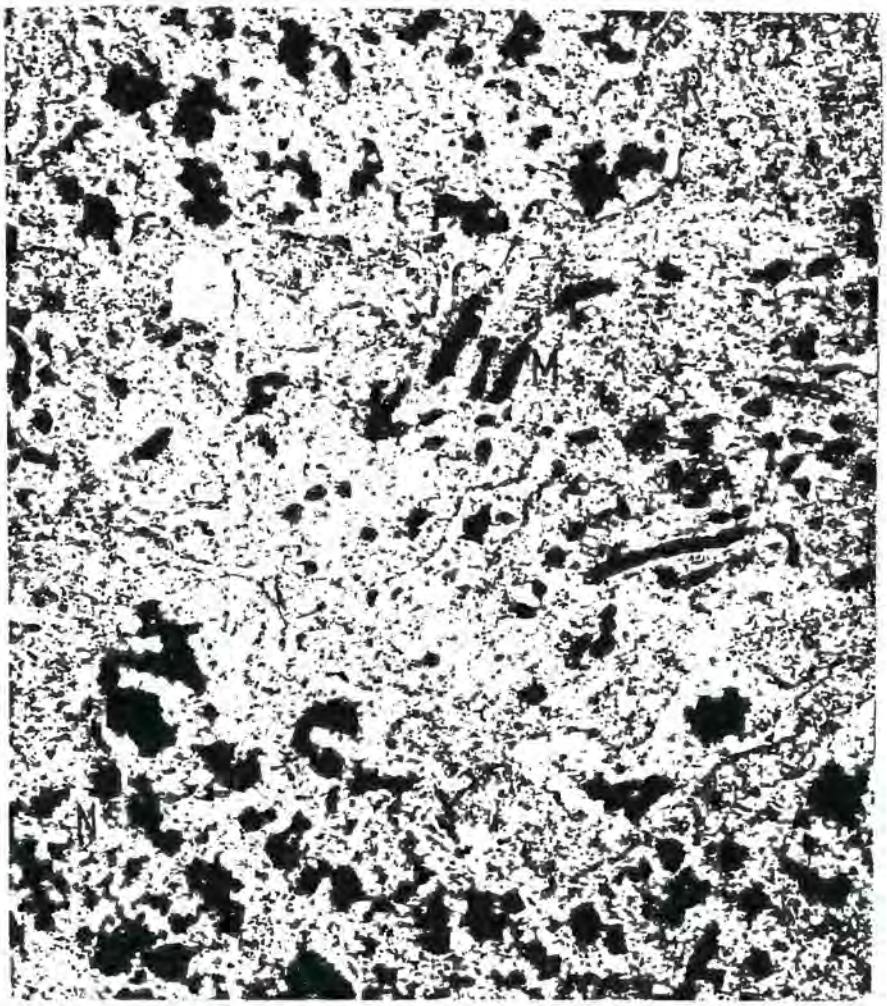

Figure 27

Part of the prothoracic gland from non-diapause larva showing lobulated nucleus (N), mitochondria (M) and free ribosomes (R). X 15,000

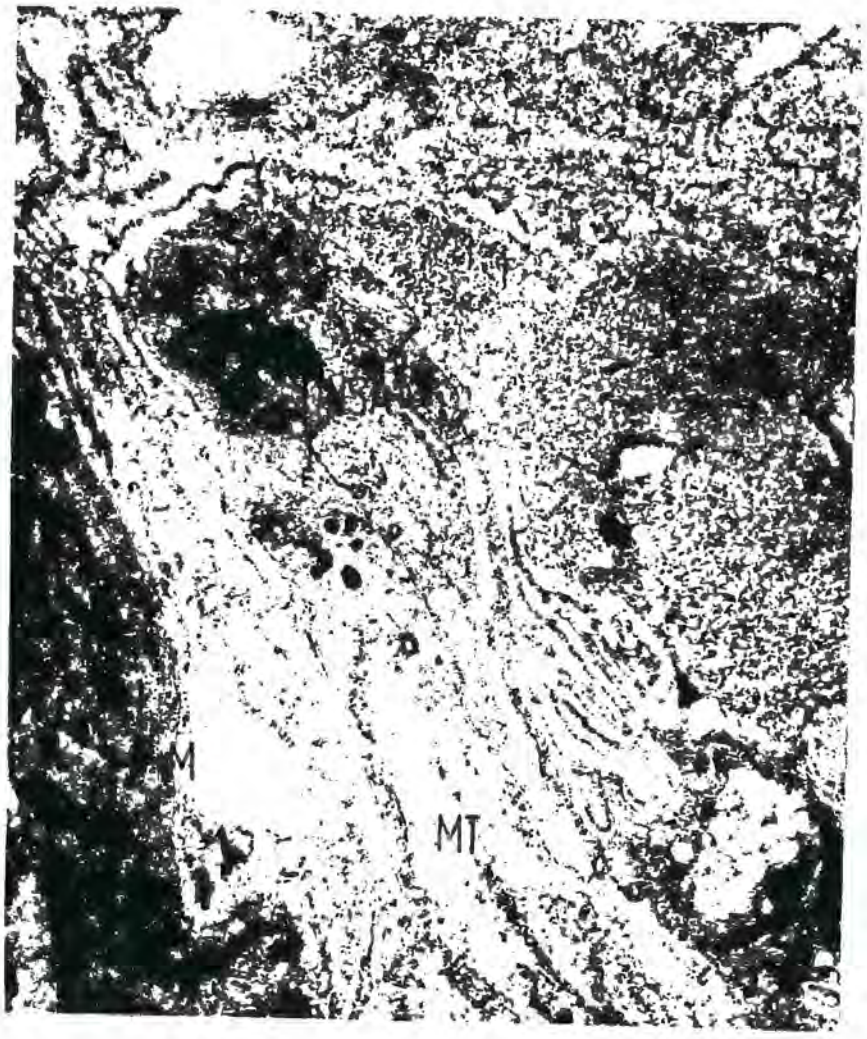

Figure 28

Periphery of the prothoracic gland of non-diapause diapause last instar larva showing an axon with opaque neurosecretory granules (ONSA) and microtubules (Mt). M, mitochondria. X 15,000

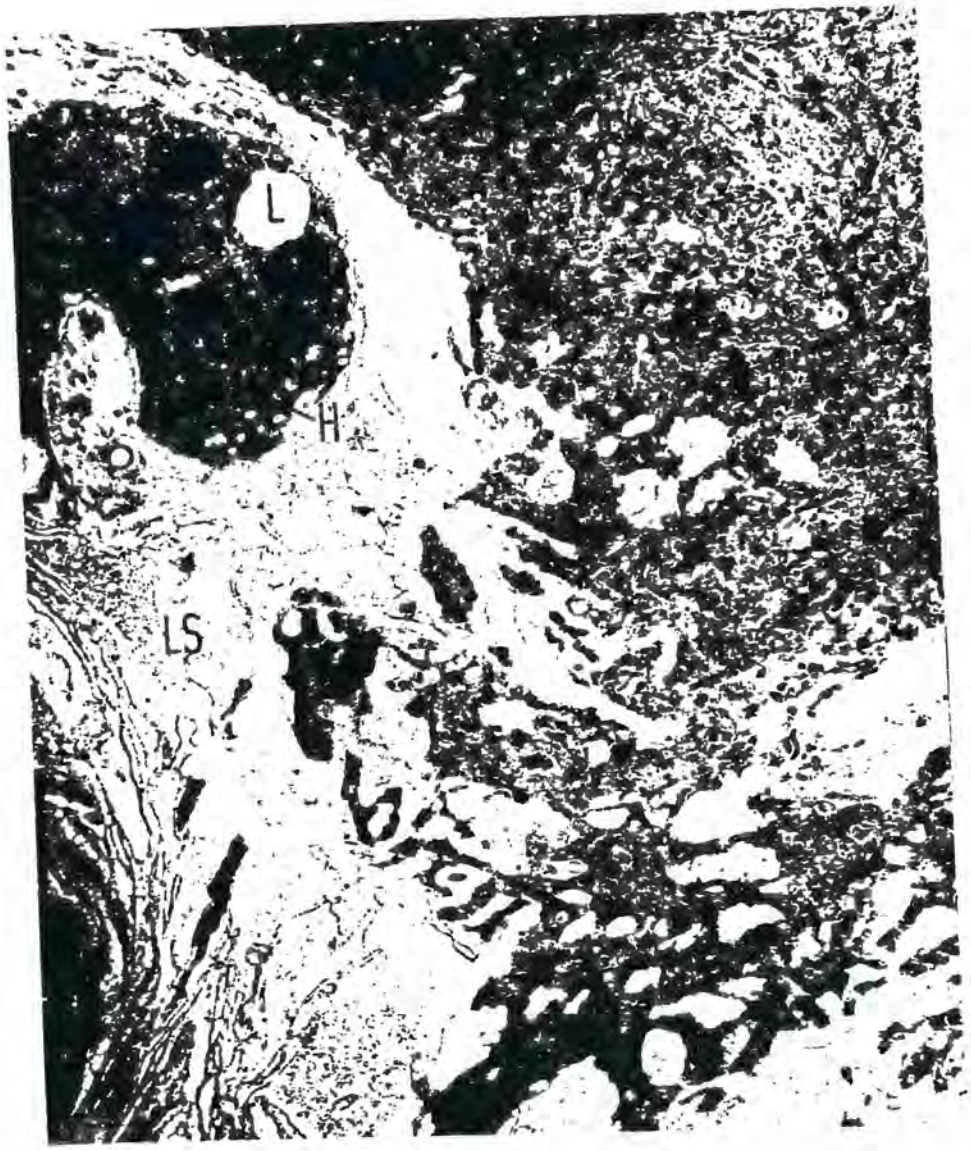

Figure 29

Prothoracic gland of diapause larva showing the peripheral channel system, numerous mitochondria and haemocyte (H) in the infolding. X 4,500

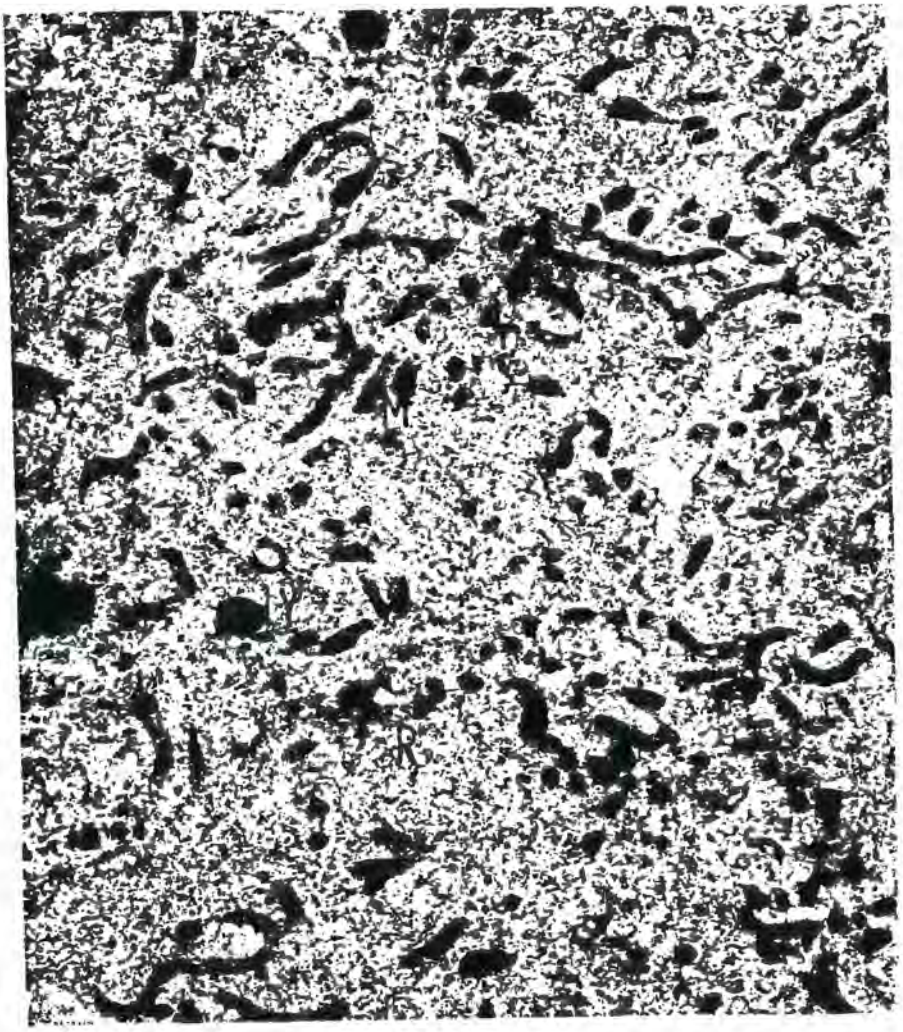

Figure 30

Electron micrograph of part of the prothoracic gland cell from the diapause larva showing cytoplasm with small and elongated mitochondria (M), lysosomes(LY) and free ribosomes (R) X 15,000

3.4 DISCUSSION

The gross morphology of the components of neuroendocrine system of Busseola fusca was similar to that of other lepidopterous larvae. The innervation pattern of the retrocerebral endocrine glands, the corpora cardiaca and CA conforms to that described in Diatraea grandiosella by Yin and Chippendale (1973). Relative to what has been reported in other insect species, two pairs of A-type neurosecretory cells is rather on the low side. In the case of the Pink Bollworm, Pectinophora gossypiella, four groups of such cells were reported (Raina and Borg, 1978). The location of these cells may probably be species specific. In Rhodnius prolixus, (Wigglesworth, 1940) and Megoura viciae (Steel, 1978), such cells were found in the medial part of the pars intercerebralis, whereas in Manduca sexta (Agui et al., 1979) they were observed on the lateral part, and in Hyalophora cercropia (Williams, 1947) they occurred on both medial and lateral parts of the pars intercerebralis. In common to other insect species, the significance of PF positive cells in the larvae of Busseola fusca in terms of PTTH production remain to be established but in Bombyx mori, these cells have been implicated as the possible sources of the brain hormone (Nagasawa et al., 1986). Most of the initial studies employing histological (Hanstrom, 1938) as well as brain tissue transplantation (Wigglesworth, 1940)

had indicated that the Medial neurosecretory cells (M-NSC) were among the possible sources of the brain hormone. Since then, further studies have shown that prothoracicotropic hormone (PTTH) activity was localized in M-NSC of some insect species like Rhodnius prolixus (Wigglesworth, 1940), Hyalophora cecropia (Williams, 1948) and Megoura viciae (Steel, 1978). In the case of Manduca sexta, one of these cells was eventually shown to be the source of PTTH (Agui et al., 1979).

Immunohistochemical studies of the cells producing immunoreactive materials to PTTH were reported in the medial neurosecretory cells of Bombyx mori and Samia cynthia ricini (Nagasawa et al., 1986). These studies also localized the area around the CA as immunoreactive. These and other studies indirectly tend to support that the PF-positive cells in the brain were among the group of cells which produced PTTH. The hormones from the neurosecretory cells are released to the haemolymph via the CC in some insects, or CA in others. These acted as the neurohaemal organs. The present study show that the neurohaemal area of the medial neurosecretory cells in Busseola fusca is around the CA. Similar arrangement has also been reported in other lepidopterous as well as non-lepidopterous species. In Bombyx mori, for instance, the neurosecretory cells sending their axons to the CA have cell bodies in the pars intercerebralis medialis (Nagasawa et al., 1984). Also a similar arrangement has been described in Leptinotarsa

decemlineata brain where their entire morphology was mapped out using retrograde diffusion of horse-radish peroxidase (Khan, 1983).

Apart from acting as the neurohemal organ for the neurosecretory material from the brain, the CA is an important endocrine gland in insects because it is the sole producer of juvenile hormones (Cassier, 1979; Tobe and Stay, 1985). In terms of structure, the CA of Bussecola fusca have both glandular and interstitial non glandular cell types. The glandular cell type show changes in subcellular organelles depicting states of activity. In non-diapause last instar larvae where the gland appear to show low activity, there is little smooth endoplasmic reticulum. By contrast in the diapausing larvae the smooth endoplasmic reticulum is abundant which indicates a state of high activity. In Schistocerca gregaria, development of smooth ER parallels hormonal activity, implicating it in the elaboration of JH (Odhiambo, 1966). The extent of the occurrence of the pleomorphic mitochondria especially in the CA of diapause larvae indicates the possibility that these organelles may also be involved in certain aspects of JH production in diapausing larvae of Bussecola fusca.

The structure of PG in Bussecola fusca larvae during non-diapause development showed interesting features such as the peripheral channel system which at the light microscope level failed to pick up stain. Features characteristic of active glands reported in studies on the glands of other

lepidopterous larvae, like prominent rough endoplasmic reticulum, pallisade microvilli and plasma membrane, large vesicles and flocculant material, ring nucleoli, myelin figures and lipid droplets (McDaniel et al., 1976; Glitho et al., 1979) were also observed in the PG in non-diapause Busseola fusca. However, cytoplasmic organelles depicting inactive cells of the PG such as glycogen rosettes, microtubules and apposed basement membrane were not observed in the diapause larvae. A possible explanation for this may be the retention of the capacity to respond and resume activity during diapause so as to facilitate production of the moulting hormone to induce the occasional stationary moults.

3.5 SUMMARY

The histology and fine structure of the larval neuroendocrine system in the last instar larvae of Busseola fusca were studied. The neurosecretory cells on the medial part of the brain were found to stain with Toluidine blue in giving two main cell types. The type-A cell stained positive with Paraldehyde fuchsin (PF) and stained darker with Toluidine blue another type of cell which stained lighter with toluidine blue was also observed. Both cell types contained neurosecretory granules but the type-A cells appeared to contain larger amount of granules of greater size and electron density. The other type of cells showed

sparse and fewer clusters of the neurosecretory granules. The medial neurosecretory cells in the brain and the intrinsic neurosecretory cells of the corpora cardiaca sent their axons to the neurohaemal area around the CA. The corpora cardiaca was comprised of two neurosecretory elements and non-neurosecretory cells. Cells of the CA of diapause larvae contained large mitochondria which had dense bodies and vacuoles which was absent from mitochondria of non-diapausing larvae. The cells also contained glycogen-like granules which were not found in cells of the non-diapause CA. There was however no clear-cut structural appearance of the PG which could be useful in distinguishing glands of non-diapause larvae from those of diapausing larvae.

CHAPTER 4

ENDOCRINE REGULATION OF LARVAL DEVELOPMENT

4.1.1 INTRODUCTION

Holometabolous insects pass through a number of larval instars and a pupal stage before maturing to adults (Wigglesworth, 1972). In each larval stage, there is feeding accompanied by an increase in weight and size. Such larval development is regulated by a number of factors some of which are endocrine in nature. These include the hormones which control the processes of moulting and metamorphosis.

In the case of the Lepidoptera, development is characterized by larval and metamorphic moults. Larval moult occurs in the presence of a moulting hormone (ecdysone) and the juvenile hormone (JH). The ecdysone is required for all types of moulting, but the juvenile hormone secreted by the corpora allata is present only when the genetic programming of the insect requires growth without differentiation. Thus the juvenile hormone inhibit metamorphosis during the immature stage. At metamorphosis, the larva moults into a pupal form and finally to adult. The metamorphic moults usually take place in negligible titers of JH.

Moulting and metamorphosis in insects are coordinated and controlled by the neuroendocrine system. There is cytological evidence that the medial neurosecretory cells of

the brain are involved during development. Cyclic changes in the activities of these cells have been observed during non-diapause and diapause development of many insect species.

4.1.3 Objectives

The objectives of the present study were to investigate the involvement of the neuroendocrine system in the development of the last instar larvae of Busseola fusca. Studies were undertaken on different aspects of the endocrine system in non-diapause and diapause larvae, such as morphometric characteristics of the corpora allata and the prothoracic glands, histology of the Paraldehyde fuchsin (PF) positive cells, estimation of JH and ecdysone titers, investigation of the critical period for the endocrine glands to exert their influence.

4.2 MATERIALS AND METHODS

4.2.1. Morphometric studies on the Corpora allata and prothoracic glands and estimation of the activities of median neurosecretory cells

Morphometric studies of the corpora allata and prothoracic glands were undertaken to find out the role played by these glands during non-diapause and diapause development of the larvae. Histological studies of the medial neurosecretory

cells (MNC) were also carried out in order to investigate their activity patterns during larval development.

For studies on the morphology of the corpora allata and the prothoracic glands, non-diapause and diapause larvae of different states were taken. These were early- (immediately after moulting), mid- (on the fourth day) and late (on the seventh day) of the last instar non-diapause larva and diapausing larvae in early- (July), mid- (September) and late-(November) diapause. The larvae were anesthetized in diethyl-ether and dissected under insect ringer (Ephrussi and Beadle, 1934). The maximum width of the left and right corpora allata, and the width of the base of the anterior lobe of both the left and right prothoracic glands were measured using an ocular micrometer. The brain and the corpora cardiaca and corpora allata complex of these larvae were fixed in Bouin's fluid and processed for bulk staining with Paraldehyde fuchsin as described previously (Chapter 3). The intensity of staining of the cytoplasm was visually scored on a scale of 1-3 where 1 was for faint and 3 was for maximum intensity. The width of the nucleus of the cell was assessed in the same manner.

4.2.2 Determination of the haemolymph JH titer

The role of JH during development of the last larval instar was investigated by determining the relative titer of JH in the haemolymph of the penultimate instar, and in the last

instar non-diapausing and diapausing larvae. The relative titers were determined indirectly by assaying hexane extracts of the haemolymph for JH activity on the last instar nymphs of Dysdercus cingulatus.

Extraction and bioassay of JH.

Haemolymph samples were collected from 20 each of 5th instar, early and late (prepupal) 6th instar non-diapausing and diapausing larvae. The abdominal proleg was cut-off and 50µl of haemolymph was collected in a capillary tube from each larva. Pooled haemolymph (1ml) from 20 larvae of each group was transferred to a cryotube containing a few crystals of phenylthiourea to prevent tyrosinase activity and stored frozen until extraction. For extraction the haemolymph was pipetted into 5 ml glass test tube and 1 ml of methanol and 2 ml of hexane were added. The mixture was vortexed vigorously and centrifuged at 4,000rpm for 15 minutes at 4°C. The hexane epiphase was pipetted out and the solvent removed by evaporation over a water bath. Extraction procedure of each sample was repeated twice. The resultant residue, a golden yellow oil; was stored at 4°C until the time of the bioassay.

The hexane extract of haemolymph was diluted 10, 100 and 1000 times (volume/volume) in acetone. These were assayed for morphogenetic effect on larva (nymph) Dysdercus cingulatus. Freshly moulted fifth instar nymphs from a colony of the insects maintained on cotton seeds in the laboratory were anesthetized in diethyl-ether, and 1 µl of the test solution was applied topically on 5 insects each, for each dose; they were replicated 3 times. For control, nymphs were similarly treated with 1µl acetone. The treated and control insects were fed on wet (soaked) cotton seeds and were kept until the next moult or until they died and the morphogenetic effect of the extract and mortality, if any were determined. The morphogenetic effect were measured quantitatively by a 0-3 score based on the degree of development of the fore wings and the number of tarsal segments. Those with three tarsal segments (as in adults) and fully differentiated fore wings with horizontal bars were regarded as adults (score 0) and those with two tarsal segments (as in nymphs) and short, and less developed fore wings without the horizontal bars were considered larval (supernumerary) (score 3) and those showing varying degrees of larval and adult characters were considered intermediates (score 1-2).

Critical period for the release of ecdysone.

The critical period for the release of ecdysone before pupation in the last instar larvae was determined by isolating the abdomens from the anterior part of the body containing the prothoracic gland by a ligation between the thorax and the abdomen applied at daily intervals after the last larval moult. Further development of the isolated abdomen was studied. The number of larvae ligated on each day after the last moult was 21. The ligated larvae were maintained individually in plastic jam cups with perforated lids and containing a piece of moist kleenex tissue paper. Observations were made on the abdomens until they pupated or for a period of three weeks. The number and percentages of abdomens pupating in each group was recorded.

4.2.3 Determination of the ecdysone titer

Ecdysone titers in the haemolymph of non-diapausing and diapausing last instar larvae of different ages were determined by radioimmunoassay (RIA) (Borst and O'Connor, 1977).

Haemolymph samples.

Haemolymph samples were collected daily for 7 days after moulting from last instar non-diapause larvae and monthly,

(July-October) from diapause larvae. Five larvae were used for each stage and 50µl haemolymph was taken from each larva (total 250µl). Haemolymph samples were labelled and stored frozen until the time of assay.

Extraction and assay.

Extraction of ecdysone was done as follows. For each assay 10ul of haemolymph, in replicate of three for each group was extracted with 40µl of ethyl alcohol. Three 10ul units of the supernatant were each transferred into individual borosilicate glass tube and the solvent evaporated using a gentle stream of nitrogen gas. The quantity of ecdysone in the residue was determined by RIA.

To each tube with the extract, 100ul of ^{3}H-ecdysone (New England nuclear, specific activity 63.5Ci/mmole) and 100ul of the antibody solution were added and thoroughly mixed by vortexing followed by incubation in the cold room for overnight. The tritiated ecdysone and the unlabelled extract competed for binding with the antibody (I am most grateful to Prof. Riddiford for the labelled ecdysone and the antibody). The bound antibody-antigen complex was then precipitated from the unbound mixture using protein A (purchased as Pansorbin from CalBiochem £ 507861) followed by centrifuging. The precipitate in the form of a pellet was recovered after the supernatant had been aspirated out. It was then mixed with 50ul of distilled water and 450µl of

liquiscint (insta-gel, United technologies, Packard, Illinois). Radioactivity of the precipitate was determined by scintillation counting. For uniformity, the data was normalized to nanogram ecdysone equivalent per ml of haemolymph.

4.3 RESULTS

4.3.1 Morphometric studies on the Corpora allata and prothoracic glands and estimation of the activities of median neurosecretory cells

There was no significant difference in size of the CA between non-diapause and diapause larvae or in CA between the two sexes in both states of development (Table 6).

In non-diapause larvae, the width of the prothoracic glands was significantly greater ($p<0.05$) than in diapause larvae. There were no significant differences in the width of PG between the sexes.

Results of Paraldehyde fuchsin (PF) staining of neurosecretory cells in the brain showed evidence of variable activity in non-diapausing larvae. In early (newly) moulted and late (7th day) sixth instar non-diapausing larvae only the perikaryon picked up the stain but in 4 day-old larvae the perikaryon and axons of all the A-cells were stained (Figure, 8; Chapter 3). In the case of diapausing larvae especially in early- (July), mid- (September) and

late- (December) diapause the intensity of staining with PF steadily decreased. Nuclear width and cytoplasmic staining of such cells at the stages considered are shown in table 7. The widths of the cell nuclei were not significantly different ($P<0.05$). By contrast, the perkaryon staining of the cells in early diapause were significantly more intense than those at late diapause.

4.3.2 Critical period:pupation of isolated abdomens

The effect of post ecdysial age at abdominal ligation of the last instar larvae on subsequent development of the isolated abdomens is summarized in table 8. None of the abdomens isolated from head and thorax, within two days after moulting pupated. Less than a quarter of abdomens isolated 3 days after moult pupated. However, as the larvae aged, more and more of them could proceed with development in the absence of the head and thorax which contain the neuroendocrine system regulating moulting and pupation. Thus 23-98% of the abdomens ligated off 3-7 days after the previous moult pupated. Therefore, the critical period before which the brain can activate the prothoracic glands and the later to start secreting/releasing ecdysone appeared to be at least 3 days. More than 50% of the abdomens pupated without PG after a post moult period of 5 days.

4.3.3 Juvenile hormone titer

Morphogenetic effects of haemolymph JH extracts of penultimate and last instar non-diapausing and diapausing larvae of Bussecla fusca are presented in Figures 31 & 32. Supernumerary larval moult (average score 3) was induced in the 5th instar Dysdercus treated with 1 µl of 10 times diluted haemolymph extract of the penultimate (5th) instar of Bussecla fusca. The nymphs which were treated with a thousand dilution of the extract from the 5th instar showed an average score of 2.8. From these results, it can be deduced that the titer of juvenile hormone in the haemolymph of the fifth instar larvae of Bussecla fusca was high. By contrast, the average juvenilizing effect of the corresponding dose of the extract from the sixth instar non-diapause larvae was only 0.8. Most of the treated nymphs moulted into slightly malformed adults. These results showed that the extract from the non-diapausing sixth instar had a very low titer of JH. Extracts from early diapausing larvae diluted 10, 100 and 1000 times showed average juvenilising scores of 2.8, 2.4 and 1.8, respectively. Similarly, for corresponding doses of extracts from late diapausing larvae resulted in average scores of 2.8, 1.2 and 0.2. These results indicated that diapause larvae contained a higher titer of JH than the non-diapausing last instar larvae. Also, titer of juvenile hormone in late diapause was

comparatively lower than that found in the larvae during early diapause.

Ecdysone titer

Ecdysone titers showed two peaks in the final larval instar during non-diapause development (Figure 33). The first occurred on day 4 and was relatively small compared with the second peak on day 7, the beginning of the pre-pupal stage. The first peak coincides with the critical period determined by the abdominal ligation studies above, indicating that after the first surge of ecdysones on the sixth instar, abdominal development (pupation) can proceed without the involvement of the prothoracic glands. Ecdysone titers in the haemolymph of the diapausing larvae is given in Figure 34. Although the titers were generally low it was present in the haemolymph samples taken in July-October (mean monthly titer less than 200ng/ml).

Table 6. Mean diameters of Corpora allata (CA) and Prothoracic glands (PG) during non-diapause and diapause development in Busseola fusca

Developmental state	sex[1]	Mean diameter (arbitrary units) CA	PG.
Non-diapause	male[1]	0.356	0.347a[2]
	female[1]	0.339	0.376a
Diapause	male[1]	0.456	0.330b
	female[1]	0.469	0.284b

[1]no. larvae used = 8.

[2]means in same column followed by same alphabet are not significantly different from each other ($p<0.05$)

Table 7 Activity of neurosecretory cells (ns) during different stages of diapause and non-diapause: Nuclear size and intensity of cytoplasmic (expressed in mean arbitrary units).

Developmental status	anterior ns cell		posterior ns cell	
	nucleus (size)	cytoplasm (staining intensity)	nucleus	cytoplasm (staining intensity
non-diapause	1.25a	1.0a	1.37a	1.0b
early-diapause	1.25a	1.4b	1.18a	1.5a
late-dapause	1.3a	1.1a	1.3a	1.4ab

Means in the same column followed by same alphabet are not significantly different from each other ($p < 0.05$).

Table 8 The effect of age at ligation on pupation of isolated (ligated) abdomens of the last instar larvae of Busseola fusca

(n = 21)

Larval age at ligation (days)	% Abdomens pupated (mean ± s.e.)
1	0.00
2	0.00
3	23.3 ± 1.17
4	27.0 ± 2.24
5	60.0 ± 2.58
6	90.0 ± 2.24
7	98.0 ± 0.75

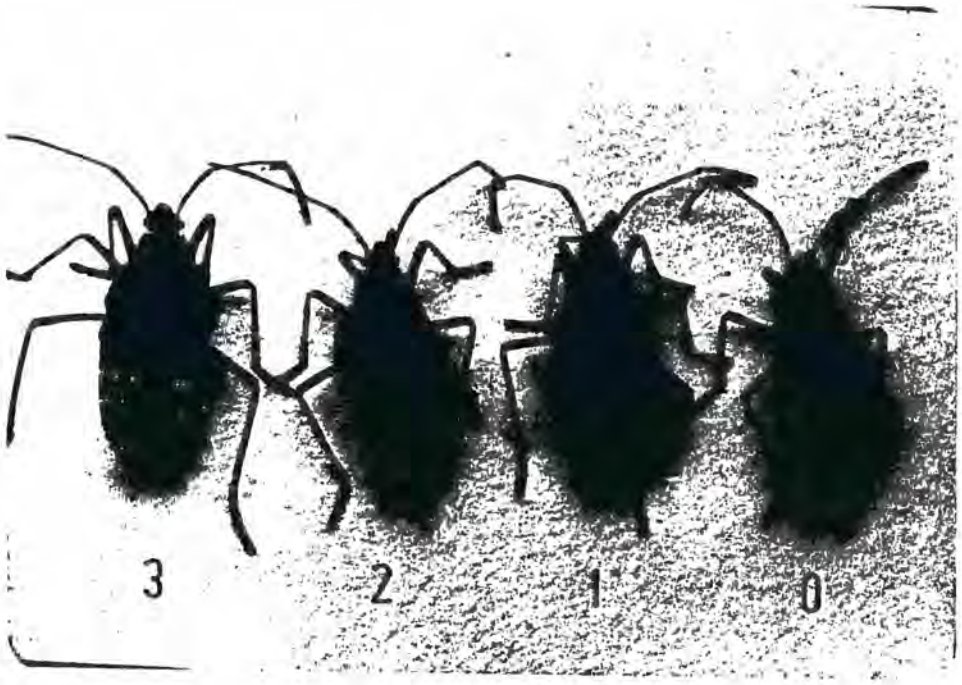

Figure 31 Juvenilizing effect of different doses of haemolymph extracts of Busseola fusca on Dysdercus cingulatus. (The scores 3, 2, 1, 0 were assigned according to the extent of juvenile appearance of the resultant insects moulting after treatment).

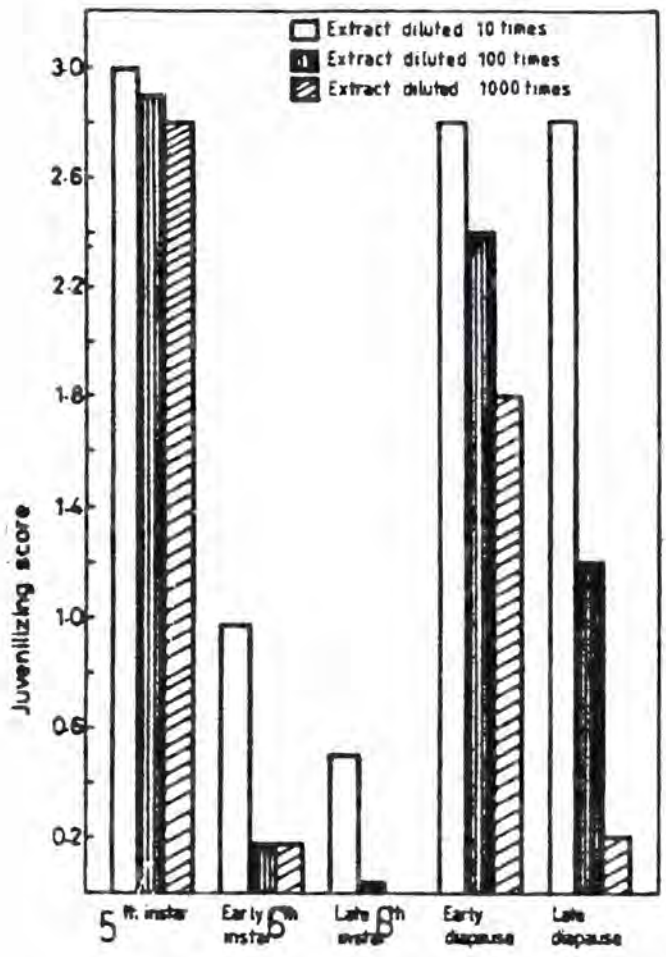

Figure 32 Juvenilizing effects of different concentrations of haemol extracts from 5th and 6th instars and diapausing larvae of Busseola fusca on the last instar nymphs of Dysdercus.

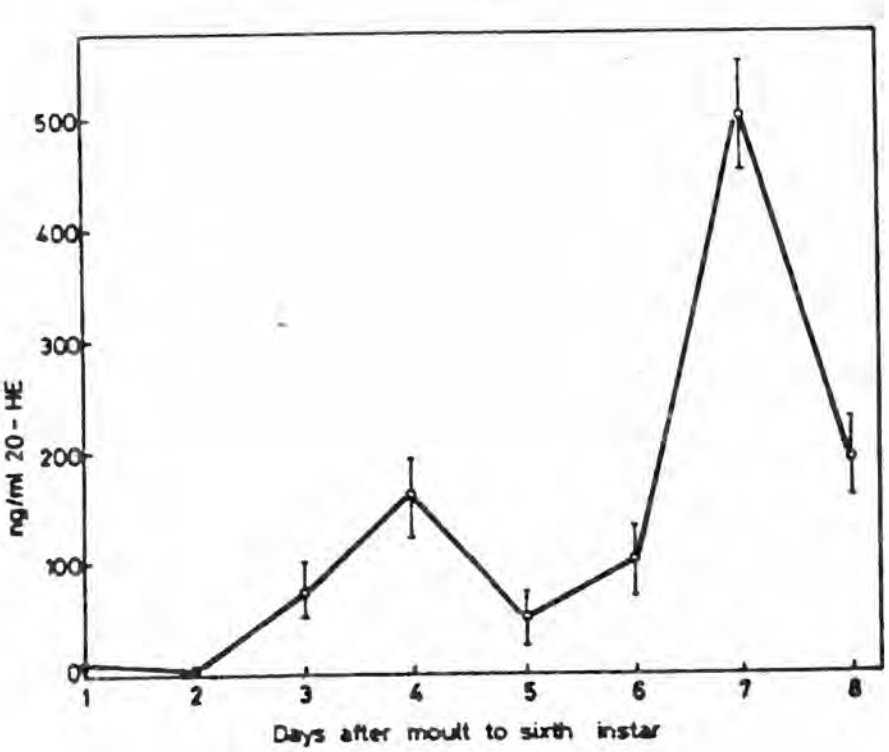

Figure 33 Daily titers of ecdysone in the haemolymph of the non-diapause larvae

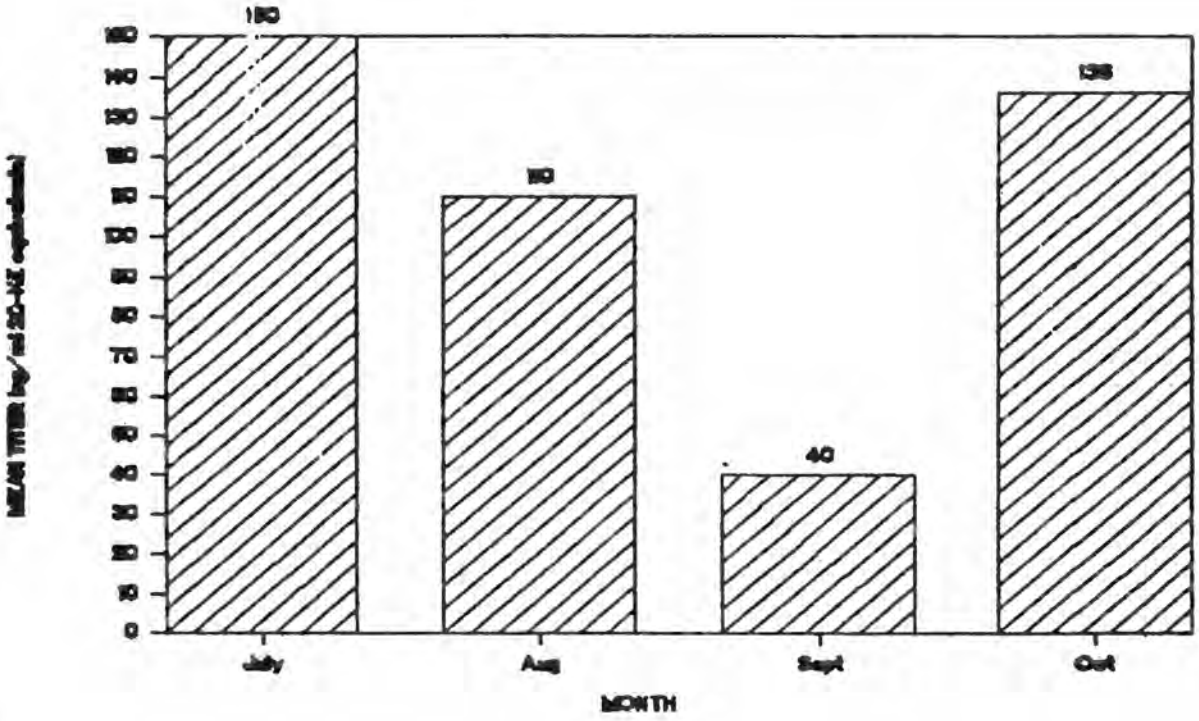

Figure 34 Mean monthly titers of ecdysone in the haemolymph of the diapause larvae

4.4 DISCUSSION

The size of the corpora allata have been used widely to depict activity patterns of the glands (Odhiambo, 1966; Lanzrein et al., 1979;). The lack of clear-cut significant differences in size of the corpora allata in the non-diapause and diapause larvae indicate that morphological studies alone may not be adequate to measure the secretory activity of these glands in the last instar larvae. The corpora allata of diapause larvae continue to produce JH during diapause. The slightly bigger CA in the diapausing larvae than in non-diapausing larvae may be a sign of increased activity.

Correlation between the size of the cells of the Prothoracic glands and their activity pattern has been reported in other insects. For example, bigger cells were reported during maximum ecdysone synthesis in the army worm Spodoptera litoralis (Zimowoska et al., 1985). In Busseola fusca, the prothoracic glands are probably less active during the diapause state but this does not mean that they are refractory to stimulatory agents in the haemolymph. The size of the prothoracic glands in Busseola fusca corresponded well with the haemolymph titers of moulting hormone.

Ligation procedure is useful for investigating the role and critical period activity of the neuroendocrine system.

By ligation the brain, corpora allata and the prothoracic glands were separated from the rest of the body of the larva thus leaving the abdomen devoid of further source of hormones from these glands. Depending on the time of ligation, the abdomen of the last instar larvae can either remain as it is, or continue with progressive development. Further development of the abdomen will depend on the presence the moulting hormone in the haemolymph. Under normal conditions ecdysone production by the glands depends on their stimulation by the brain hormone. Thus, the brain is required at least for some critical period during the last instar. The present experiment did not reveal the critical period for the stimulation by the brain through PTTH, but it appeared to be within the first three days of the moult because sufficient quantity of ecdysone appeared to be present in the haemolymph to induce pupation in more than 20% of the isolated abdomens.

The low levels in the titer of the juvenile hormones in the haemolymph are characteristic of the last instar larvae of most lepidopteran species (Bollenbacher, 1988). Probably this minimal titers of juvenile hormone is related to the type of behavior observed in the last instars, such as feeding. Low levels of juvenile hormone in the haemolymph may also affect the activities of the other endocrine glands. For example, the declining titers of JH in Manduca sexta permitted small release of PTTH which triggered synthesis and release of ecdysone by the prothoracic glands

(Riddiford and Truman, 1978). Ecdysone titers were elevated and showed a commitment peak by the middle of the instar (Bollenbacher, 1988). Rising titers of ecdysone influences the activities of the cells in the gut lining (Riddiford, 1985) and this may be reflected in the feeding behavior of the larvae. In the last larval instar of Busseola fusca, there was a subtle rise of titer of moulting hormone about the middle of the instar. This peak coincided with a resting behavior in the larvae (see chapter 2).

Unlike in last instar larvae of other lepidopterous species (Riddiford, 1985), the JH titer in the final instar larvae of Busseola fusca remained low throughout the stadium. The decline in JH titers of other species which have similar life patterns of development in the last instar as Busseola fusca has also been reported, for example in the Southwestern corn borer, Diatraea grandiosella (Yin and Chippendale, 1976) and also in the European corn borer Ostrinia nubilalis (Beck and Shane, 1973). However, in other insects JH titer rises during the post commitment phase which prevent precocious development of adult characters during pupation as has been observed in Manduca sexta (Kiguchi and Riddiford 1978, Whisenton et al., 1987). In Manduca sexta, the CA switch from synthesis of JH during the feeding stage to synthesis of JH acid in the pre-pupal stage (Bhaskaran et al., 1986).

Unlike the JH levels in the last instar in non-diapause development of Busseola fusca, the moulting hormone

levels show fluctuations characteristic of many lepidopteran species. In Busseola fusca, moulting hormone titers were low at ecdysis to the last instar, then rose slightly reaching a small peak on day 4, declined before steadily rising again to the final peak which initiated apolysis and synthesis of the new pupal cuticle.

Titers of the moulting hormone in the haemolymph as a result of synthesis and release by the prothoracic glands follow closely the appearance of the brain hormone and other stimulatory factors in the haemolymph (Watson et al., 1986; 1988). Before the onset of the pre-pupal stage in Busseola fusca, at the middle of the instar, there is intense staining of both the perikaryon and axons of medial neurosecretory cells of the pars intercerebralis which could presumably be related to PTTH production/release. In such a case, there will be least titer of PTTH in the haemolymph. However, there could also be a high titer of other stimulatory factors acting on the prothoracic glands triggering the small ecdysone peak observed about this time. A combined stimulation exerted by PTTH and the haemolymph factors would trigger maximal synthesis of the moulting hormone. This will inevitably lead to a rise in the titers of the moulting hormone to the post commitment peak level that is required for apolysis and synthesis of the new cuticle. Finally metamorphosis will occur at the end of non-diapause form of development in Busseola fusca.

4.5 SUMMARY

Morphometric measurements appeared useful criterion to distinguish differences in endocrine activity between non-diapause from diapause development only with regards to the prothoracic glands. In terms of size, the corpora allata of non-diapause larvae were not significantly different from those of diapause larvae.

PF-positive cells in the pars intercerebralis stained variably during the different states of development of larvae in non-diapause and diapause development. The cytoplasm of these cells stained more intensely during non-diapause and early diapause than in late diapause. The critical period for the prothoracic glands to produce the required titer of ecdysone for further development of the abdomens was by the third day of the last instar during non-diapause development in Bussenla fusca. In non-diapause larva the titers of the moulting hormone in the haemolymph peaked twice, the first peak occurred on day four and was smaller than the second peak which occurred on day 7. Juvenile hormone extracts from the haemolymph of the last instars, both in non-diapause and diapause development showed morphogenetic effects on the last instar nymphs of Dysdercus. The extent of juvenilization of the nymphs by such extracts was taken as a measure of relative titer of JH present. Extracts from the fifth instar larvae of Bussenla fusca and larvae in early diapause gave higher juvenilizing

scores than those from the non-diapausing sixth instar larvae whose score was even lower than that of the larvae in late diapause. This means that high titers of JH are found in the penultimate instar and in early diapausing larvae. There was a decline in titer of JH in late diapause larvae. The JH titer in the last instar non-diapause larva was low.

CHAPTER 5

ENDOCRINE REGULATION OF INDUCTION, MAINTENANCE AND TERMINATION OF DIAPAUSE IN BUSSEOLA FUSCA

5.1 INTRODUCTION

Stem borers, especially those of crops like maize and sorghum usually encounter a period when there are no suitable host plants. Under such conditions diapause form of development is taken up by larvae as a means to survive the unfavorable period until the onset of the next season when food becomes available again. Studies investigating the incidence and physiology of diapause in insects have shown it to be a very complex state of development regulated by the endocrine system.

5.1.1 Diapause development in insects

Diapause is a state of developmental arrest that is utilized as a means of surviving an adverse environmental condition in such a way that the active stages of its life cycle are synchronized with availability of food (Denlinger, 1985). Diapause has been found to occur at different developmental stages in different insects and therefore different

regulatory mechanisms are involved in these insects depending on the stage at which it is expressed.

Juvenile hormone from the corpora allata is involved in regulating larval diapause in a number of lepidopterous species studied. Most of these studies have found that exogenous treatment of the last instar non-diapause larvae by juvenile hormone or its analogs such as Methoprene can induce diapause development (Yagi and Fukaya, 1974; Scheltes, 1978; Yin and Chippendale, 1979).

Environmental factors such as day-length (photoperiod), temperature etc, are known to be important as source of cues in the induction, maintenance and termination of diapause. For example, in stem borer Chilo partellus, the seasonal change of food quality is believed to be the primary cue for induction of larval diapause (Scheltes, 1978). Diapause is maintained as long as the inductive environmental conditions last and is terminated at the onset of the favorable conditions for the growth of the host plants. Once larval diapause has been induced it has to be maintained for the required period. It has been shown in some insect species, that larval diapause is maintained by intermediate titers of JH (see Chippendale, 1977 for review). Presumably, termination of larval diapause followed by pupation may be related to declining activities of the corpora allata.

The role of ecdysone in development of larval diapause is not clear. In some insect species, larval diapause has

been implicated to be due to deficiency of ecdysone. In such insects, diapause was terminated by ecdysone injections (Bhatnagar-Thomas, 1976; Sieber and Benz 1980). By contrast, ecdysone injection in diapausing larvae of Chilo suppressalis (Yagi and Fukaya, 1974), Diatraea grandiosella (Yin and Chippendale, 1976) and Chilo partellus (Scheltes, 1978) did not terminate diapause but triggered stationary (larval-larval) molts.

Diapause development has been reported in the mature last instar larvae, usually of the second generation of the maize stem borer, Busseola fusca (Swaine, 1957; Usua, 1970; Unnithan 1987; Assefa Gebre-Amlak, 1988). The host plants (sorghum or maize) on which such larvae feed were in a well advanced state of maturity and therefore could have provided the necessary environmental signals essential for the induction of diapause development in the larvae. The role of the environment in diapause maintenance n Busseola fusca has not been studied. However, diapause termination in this species has been suggested to be influenced by the environment (Assefa, Gebre-Amlak, 1989). Transducing environmental cues for diapause development with respect to its initiation, maintenance and termination is normally mediated by the endocrine system (Denlinger, 1985).

5.1.2 Objectives

The objectives of the study reported here were to investigate the endocrine regulation, particularly, the role of juvenile hormone and ecdysone, in induction, maintenance and termination of larval diapause in Busseola fusca. Experiments were carried out to study the effects of JH-analog on non-diapausing larvae, JH and Ecdysone and anti-juvenile hormone compounds (Precocene and Fluoromevalonate) on diapause larvae.

5.2 MATERIALS AND METHODS

5.2.1 Effect of the juvenile hormone analog, Methoprene on non-diapause last instar larvae.

In order to determine whether JH can induce larval diapause, larvae reared under non-diapause inducing conditions (fed on young sorghum stems, Unnithan, 1987) were injected with the JH analog, Methoprene (ZR 515) (received from Zoecon Corporation, Palo Alto. California). Methoprene was dissolved in paraffin oil to give 1µg methoprene/1ul paraffin oil. Forty non-diapausing larvae at the outset of the final larval instar were anesthetized and were injected with 1µl each of the solution using a Hamilton micro-syringe. For control, 40 larvae each were injected with 1µl paraffin oil alone. Experimental and control larvae were

maintained under non-diapause inducing conditions (on young sorghum stem cuttings) until they pupated or for the duration of the observation period of 7 weeks. Mortality, frequency and quality of moulting and morphogenetic effects were noted.

5.2.2 Effects of 20-hydroxyecdysone (20-HE) on diapause larvae

Various doses of 20-HE (Calbiochem, California, USA) were injected into diapausing Busseola fusca larvae with an objective of finding out whether diapause in these larvae was due to the inactivity of the prothoracic glands and lack of moulting hormone. The 20-HE was dissolved in distilled water. Serial dilutions to give 1, 5 and 10µg of the compound per 1µl of water were made. Diapausing larvae were anesthetized in diethyl-ether and injected with different doses (1, 5, and 10µg) of the ecdysone solution on the dorsal part of the first abdominal segment. The controls received 1 µl of distilled water. Both the experimental and the control larvae were maintained individually in dry environment that does not favour termination of diapause. Observations were made on the type of moult that occurred. The results were recorded after one week.

In order to investigate whether reducing the JH titer in the circulating haemolymph can induce diapause termination, the abdomens of diapausing larvae were isolated

from the rest of the body by a ligature between last thoracic segment and first abdominal segment. This was done to ensure complete isolation of the haemolymph of the abdomens from the anterior parts of the larvae. Both portions, anterior and posterior to the ligature were induced to moult at different intervals after ligation namely 1, 3, 5 and 7 days, by injection with 10µg of ecdysone dissolved in distilled water and the quality of the moult (whether stationary larval/larval, larval/pupal intermediate or pupal) recorded. In a separate experiment, abdomens which were 7 days old were each injected with 1µg Methoprene and followed by injection with 10µg ecdysone six hour later. The results in terms of the quality of the moult were recorded for a period of one month.

5.2.3 Effect of precocene II and Fluoromevalonate on diapause larvae

In order to interfere with the activity of the corpora allata of diapausing larvae of Busseola fusca, allatocidal compounds like precocene and fluoromevalonate were used.

Early diapause larvae of Busseola fusca were anesthetized in diethyl ether and treated topically on the dorsal side with different doses of precocene II (Calbiochem, California, USA) dissolved in acetone. The doses used were: 10, 20, 40 and 500µg per larva. Nine-ten larvae were used per dose. For control 18 larvae were

treated with equivalent volume of acetone alone. Treated and control larvae were observed for a period of one month, and any mortality or moults that occurred during this period were recorded.

Fluoromevalonate (FMev) (received from Zoecon Corporation Palo alto. California), shown to have anti-juvenile effect on lepidopteran larvae (Staal et al., 1981) was also used to inhibit CA activity in diapausing Bussenla fusca larvae which had been in diapause for a period of three months (mid-diapause). Larvae were first anesthetized in diethyl-ether and then treated topically with 1, 5 or 10µg of Fmev dissolved in 1ul of acetone on the dorsal part of the fourth abdominal segment. For each dose 30 insects were used. Another 10 larvae each received 2µg FMev. For control, the larvae were topically treated with 1µl each of acetone. Larvae were then placed individually in numbered 25ml plastic cup with a cover and lined with a piece of Kleenex tissue paper and observed for a period of six weeks. Any mortality, moulting or pupation which occurred during this period was recorded.

5.3 RESULTS

5.3.1 Effect of methoprene on non-diapause larvae

Table 9 shows the effect of methoprene injection on non-diapausing larvae. None of the 40 treated larvae pupated

within the observation period of 7 weeks. Treated larvae assumed the characteristics of diapausing larvae. These larvae grew bigger, as a result of prolonged feeding, lost their melanin pigment and acquired the immaculate coloration typical of the diapause larvae. Methoprene treated larvae also underwent stationary (larval-larval) moults which was another characteristic of diapause larvae of Busseola fusca. One stationary moult was observed and it occurred in the third week after treatment. The methoprene treated larvae failed to break diapause and eventually died without pupating. On the other hand all the controls pupated within a period of 2 weeks after the treatment.

5.3.2 Effect of 20-HE on intact and isolated abdomens of diapause larvae

The effect of injection of 20-HE on intact early diapausing larvae is summarized in table 10. Doses of 5 and 10µg induced moulting. Nearly all (70-80%) of the larvae moulted into another larval instar and only 10% of those which received 5µg dose pupated at their first moult.

Abdominal ligation of diapausing larvae prevented further development. Neither the posterior nor the anterior portion moulted within 1 month period. If however, 10µg 20-HE was injected into the ligatured abdomens, moulting occurred (Table 10). When ecdysone was injected immediately after ligation, 75% underwent a larval moult. By contrast,

when the abdomens were injected 3 or more days after ligation, 70% underwent a pupal moult, and the remaining moulted into larval-pupal intermediates. These results suggest that the level of JH in the abdomen was high at the time of ligation and it gradually decreased with advancing time after ligation, then, resulting in progressive development. This was further supported by the finding that when 10µg methoprene was applied 3 days or more after ligation prior to injection of ecdysone, 6 hours later resulted in only larval moults (Table 11).

5.3.3. Effect of precocene and fluoromevalonate on diapause larvae

The effect of topical application of precocene II on the diapause larvae is shown in table 12. With the exception of a lethal effect, precocene II had no apparent effect on diapausing larvae of Busseola fusca. Treated and the control larvae remained in diapause and pupated at the end of diapause.

The effects of anti-JH fluoromevalonate on mid-diapause larvae are summarized in figure 35. There was increasing mortality rate with increased dosage of the compound. First larval moults were observed in the third week in the controls. The maximum number of stationary moults observed in any one larva were 2. The first moult occurred at the 3rd day of the experiment and the second one followed after one

month. The first larval moult for larvae that received 5ug of fluoromevalonate occurred during the second week after treatment and in the fourth week for those on 1µg of fluoromevalonate. There was no larval moult during the third week or later after the fluoromevalonate treatment.

By week 6, 7 of the untreated controls had pupated whereas only 2 of those treated with 5µg FMev pupated. For those treated with 10µg FMev, there was no pupation for over a period of 3 months although they eventually pupated. The resultant adults were all malformed.

Table 9 Effect of juvenile hormone analog (methoprene) on the development of the last instar non-diapause larvae of Busseola fusca

Larval age (days)	Treatment	No. used	Condition after 7 weeks			
			No. died	Stationary moults	Larvae	Pupae[1]
1	control	10	1	0	0	9
	Methoprene	10	2	1	8	0
2	control	10	0	0	0	10
	Methoprene	10	3	0	7	0
3	control	20	4	0	0	16
	Methoprene	10	3	0	7	0
Total	control	40	5	0	0	35
	Methoprene	30	8	1	22	0

[1]Pupae eclosed to normal adults.

Table 10 Effect of various doses of ecdysone on moulting in early diapause larvae of Busseola fusca

Dose (µg/larva)	No. used	% Larvae moulting into : Larvae	Larval-pupal intermediate	Pupae
0 (control)	20	0	0	0
1	20	0	0	0
5	20	70	20	10
10	20	80	20	0

Table 11 Effect of ecdysone (20-HE) injection and methoprene application prior to ecdysone injection on ligatured (isolated) abdomens of diapausing larvae at different intervals after ligation.

Time of injection (days after ligation)	no. larvae used	% Abdomens moulting into: larva	larval-pupal intermediate	pupa
1[a]	20	25.0	50.0	25.0
3[a]	20	40.0	40.0	20.0
5[a]	20	0.0	40.0	60.0
7[a]	20	0.0	20.0	80.0
7 Methoprene[a,b]	20	100.0	0.0	0.0
controls[c]	20	0.0	0.0	0.0

[a]Injected with 10µg ecdysone

[b]Injected with 10µg methoprene before ecdysone injection

[c]Freshly ligatured abdomens injected with 10µl of dstilled water

Table 12 The effect of precocene II treatment on diapause larvae of Busseola fusca one month after treatment

Treatment (µg/larva)	No. used	No. died	No. moulting (larvae)[1]	No. moulting (pupae)[1]
10	9	1	7	1
20	10	2	6	2
40	9	3	5	1
500	10	8	1	1
acetone (control)	18	8	9	1

[1]One month after treatment.

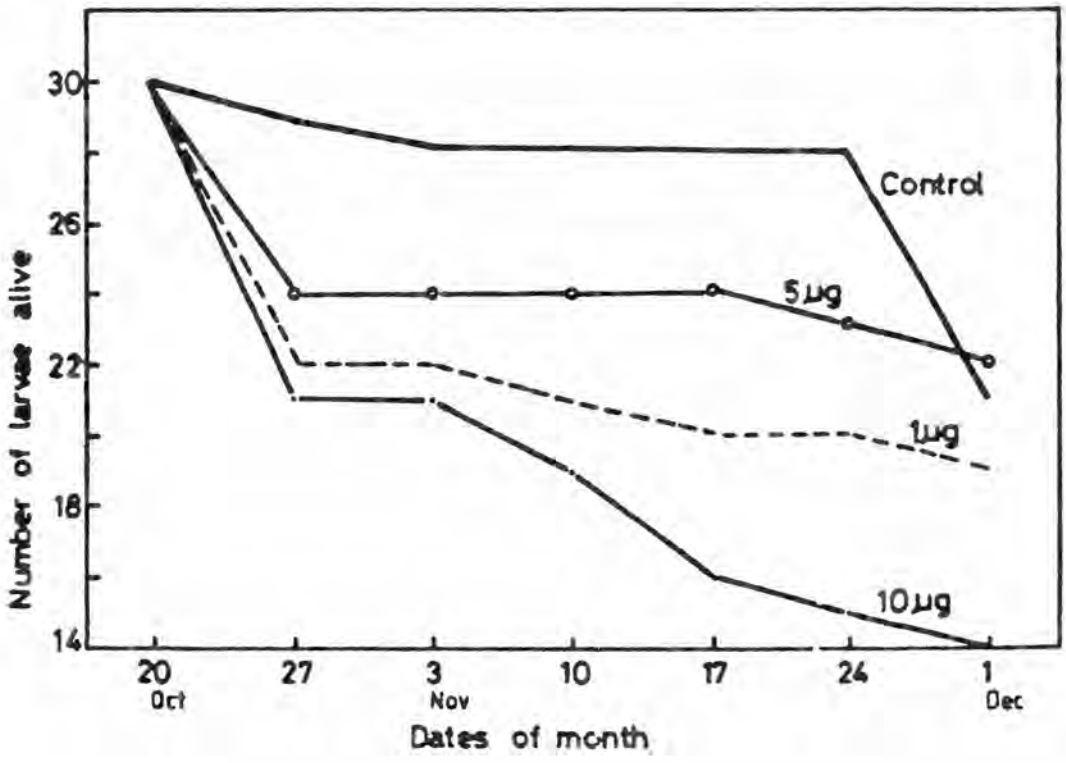

Figure 35 The effect of Fluoromevalonate on survival of Busseola fusca larvae in mid-diapause

5.4 DISCUSSION

Diapause in the maize stem borer Busseola fusca is a survival strategy and therefore a very important form of development that requires intricate control especially by the endocrine system.

In Busseola fusca, diapause development is probably induced by JH because the juvenile hormone analog, methoprene induced full diapause syndrome in last instar non-diapause larvae of Busseola fusca. These larvae were observed to undergo stationary moulting. For those insects which undergo larval diapause, it is the juvenile hormone which is largely involved in regulating diapause (Chippendale, 1977). This hormone plays a major role in the induction and maintenance of larval diapause in the pyralid Chilo suppressalis (Fukaya and Mitsuhashi, 1961; Yagi and Fukaya, 1974), Diatraea grandiosella (Yin and Chippendale, 1973, 1976, 1979), and in pre-pupal diapause of Monema flavescence (Takeda, 1978). Juvenile hormone has also been found to play a definitive role in the control of certain phases of diapause induction in Laspeyresia pomonella (Sieber and Benz, 1977) and Ostrinia nubilalis (Chippendale and Yin, 1979). Since larval diapause is a programmed delay in pupation its regulation also must require the participation of other developmental hormones especially the

brain hormone and the molting hormone (J. Truman, personal communication).

Although larval diapause is generally considered to be controlled by the juvenile hormone the ways with which the hormone brings about this regulation in the diapausing larvae of various species is quite different. Four main types of regulatory schemes have been documented. The first scheme involves maternal determination as evidenced in the parasitic Hymenoptera (Ryan, 1965) and the Calliphoridae (Vinogradova and Zinovjeva, 1972). The second scheme is where juvenile hormone is involved in the initiation and maintenance as seen in Chilo supressalis (Yagi and Fukaya, 1974), Chilo partellus (Scheltes, 1978), Diatraea grandiosella (Yin and Chippendale, 1979) and the pre-pupal diapause of Monema flavescence (Takeda, 1978). The third scheme is where juvenile hormone is involved only in the initiation of the diapause as observed in Ostrinia nubilalis (Yagi and Akaike, 1976) and Laspeyresia pomonella (Sieber and Benz, 1977; 1980) The fourth scheme is as in the case of Nasonia vitripennis (De Loof, et al., 1979) where juvenile hormone is not involved at all. With such a variability in regulatory roles by just a single hormone it is difficult to state with any certainty that a given unstudied species showing larval diapause will fall into a definite regulatory pattern of a known species. On the basis of the current results, Busseola fusca appears to belong to the group of

insects in the second scheme where JH is involved in the initiation and maintenance of larval diapause.

The corpora allata of diapause larvae of Busseola fusca maintained high levels of activity especially during early diapause. Previously it has been suggested that fluctuations in haemolymph JH titers also affected changes in the levels of production of the other hormones particularly that of the ecdysones (Nijhout and Williams, 1974; Watson et al., 1986). Although juvenile hormone titer was found to be high during the early stages of diapause (see Chapter 4), it is quite possible that with time it may rise and fall in such a way that there is an average level, which declines subtly.

The diapause larvae of Busseola fusca undergo stationary moulting (Usua, 1973). The frequency of stationary moult depends on the environment of the diapause larvae and can be as high as once every three weeks. These moults essentially mean that the prothoracic glands were releasing sufficient quantity of moulting hormone to promote moulting. In the event that the stimulation of the prothoracic glands was not from the brain hormone, then other sources such as the haemolymph stimulating factor may be involved (Watson et al., 1986; 1988).

Ordinarily, diapause larvae do not moult by the time non-diapause larvae would be expected to moult. The inability to moult observed in diapause larvae was accounted for as due to lack of moulting hormone and consequently diapause in the last instar larvae was explained as due to

deficiency of ecdysone. For example removal of the prothoracic glands of non-diapause larvae of the South-western corn borer, Diatraea grandiosella, resulted into failure of the treated larvae to pupate (Yin and Chippendale, 1975). This anomaly could only be corrected by re-implantation of the prothoracic glands or ecdysone injection into the treated larvae. The prothoracic glands of diapausing larvae of Diatraea grandiosella (Yin and Chippendale, 1975) and the prepupae of the slug moth, Monema flavescens (Takeda, 1976) were shown to be structurally inactive. Larvae of the codling moth, Laspeyresia pomonella destined for diapause were found to have extremely low titers of ecdysone (Sieber and Benz, 1980). However, exogenous treatment of ecdysone to diapausing larvae in several insect species has shown variable results. For example, in Laspeyresia pomonella (Sieber and Benz, 1980), and in the larvae of the khapra beetle, Trogoderma granarium (Bhatnagar-Thomas, 1976), diapause development was terminated by application of ecdysone. Diapausing larvae of Ostrinia nubilalis (Beck and Shane, 1969) and prepupae of Monema flavescens (Takeda, 1976) responded to ecdysone treatment by producing larval-pupal intermediates.

Injection of 20-HE to intact diapausing larvae of Bussenla fusca resulted in larval-larval moults; this suggests that the titers of JH are sufficiently high to prevent progressive moulting in diapause larvae. The results obtained from injection of ecdysone into isolated abdomens

showed further that progressive moulting could occur in the absence of JH. These findings were further supported by the occurrence of larval-larval moults in ligated abdomens when juvenile hormone analogs were applied before the injection of ecdysone (see table 11). The elevated titers of JH were made possible by active corpora allata and hence if the CA function can be interfered with anti-JH compounds like precocene and fluoromevalonate it could lead to premature termination of diapause. However, precocene was not allatocidal in diapause larvae of Busseola fusca. This observation is not surprising because the precocenes have not been shown to be effective even in non-diapause larvae of lepidopterous species. For example they were shown to have no effect in the larvae of the African army worm, Spodoptera exempta (McCaffery and McDowell, 1987). However, repeated doses of precocene was reported to affect development in Spodoptera mauritia (Mathai and Nair, 1984 Santha and Nair, 1986).

But studies in susceptible species have revealed that the precocenes induce cellular degeneration of the corpora allata (Unnithan et al., 1977; Bowers and Martinez-Padro, 1977; Pener et al., 1978) leading to destruction of their ability to synthesize juvenile hormone (Brooks et al., 1979; Pratt et al., 1978). The stationary moult which occurred after Fmev treatment cannot be attributed to the substance because it could have taken place as any other ordinary moult like that observed in the controls. It is possible

that fluoromevalonate was allatocidal to the corpora allata at the dosage of 10ug per larva but there was no ecdysone to cause a moult. At the lower doses fluoromevalonate did not affect the status of the corpora allata since larval-larval moult still took place. Thus neither precocene nor fluoromevalonate treatment led to termination of diapause. However, detailed and more analytical studies are required to find out the effects of fluoromevalonate on the corpora allata of diapausing Busseola fusca larvae.

5.5 SUMMARY

Diapause development in the mature last instar larvae of Busseola fusca can be induced by the juvenile hormone analog, methoprene. Diapause cannot be terminated by ecdysone injection. Progressive moulting (Pupal) occurred in isolated abdomens with reduced or no JH content, of diapausing larvae when injected with ecdysone. Simultaneous treatment of isolated abdomens of diapausing larvae with methoprene and ecdysone resulted in only stationary moulting. These results show that diapause development in this insect is induced and maintained by juvenile hormone. They also show that diapause in Busseola fusca is not due to ecdysone deficiency. Precocene had no antiallatotropic effect on the diapausing larvae. Fluoromevalonate also did not terminate diapause but at effective doses, it delayed pupation. It appears that termination of diapause occurs at

reduced level of JH or in the absence of it in the haemolymph. How this is brought about is yet to be known.

CHAPTER 6

GENERAL DISCUSSION

The evidence presented in the previous chapters shows how non-diapause and diapause development in the last larval instar of Busseola fusca are controlled by the neuroendocrine system. Since the onset of studies which culminated into the current branch of Insect endocrinology, many workers have attempted to unravel the ways by which hormones regulate post embryonic development in insects (see Granger and Bollenbacher, 1981; Hagedorn, 1985; Steel and Davey, 1985).

In the case of Busseola fusca, just as in other insect species, three kinds of hormones produced from the various components of the neuroendocrine system, in particular, the type-A neurosecretory cells of the brain, the corpora allata and the prothoracic glands appear to play a major role in the regulation of the type of development occurring during the final larval instar.

Very little work, particularly in the interendocrine regulation of insect development (see for review Steel and Davey, 1985) has been carried out on insect species despite the obvious fact that the type of regulation between the various endocrine axes in insects is a process central to

the control of development among other physiological processes.

The lack of information on endocrine interactions in insect species is largely due to the lack of convenient experimental procedures for investigating the integrated functions of the hormonal systems. This problem is compounded by the slow rate with which the relevant scientific equipments are reaching laboratories.

The components of the neuroendocrine system which are involved in the regulation of development during the last larval instar of Bussenla fusca have been investigated and over the chapters, knowledge of the physiology of these systems has tentatively shown the basic inter-regulatory mechanisms which could be involved.

The cytological and ultrastructural evidence for the activities of the medial neurosecretory cells presented in chapters 3 and 4, indicates that in Bussenla fusca, synthesis of the neurosecretory material is not limited to any particular period although the rates may be variable. The question which remains unanswered is the exact identity of the PTTH-producing cells, the neuropeptide and its components which is generally referred to as the brain hormone, PTTH. In several insect species PTTH has been shown to consist of different homologies synthesized by various groups of neurosecretory cells in the brain (Bollenbacher and Granger, 1985; Bollenbacher et al., 1984; Nagasawa et al., 1986; Kataoka et al., 1987; Flanagan et al., 1988).

The occurrence of more than one neurohormone from a single neurosecretory cell has also been reported in some invertebrate species (Scheller and Axel, 1984; Joosse, 1986). Presumably, they may arise out of a larger precursor molecule, a prohormone. The sub-units usually subserve different endocrinological functions. In Busseola fusca, the medial neurosecretory cells terminating on the corpora allata (chapter, 3) may have regulatory influence on the CA as was reported in Diploptera punctata (Rankin et al., 1986). Yet, the most recognized role of PTTH is its tropic effect on the prothoracic glands. It has been reported in Manduca sexta, that the brain and its neurohaemal organ contain sufficient PTTH to trigger synthesis of ecdysone by the prothoracic glands at any given time in Mamestra (Bollenbacher, 1988). Therefore the most important regulation of PTTH titer in the haemolymph is exerted at the site of its release.

Ordinarily, in the last larval instar, the release of PTTH has been suggested to occur twice, preceding each of the two ecdysone peak titers. However, in Busseola fusca it was not possible to establish the exact periods of release of the brain hormone despite the presence of PAF-positive material in the cell bodies and in the neurohaemal area around the corpora allata. Therefore, it may be of future interest to characterize the products of the medial neurosecretory cells of Busseola fusca with the objective of identifying the molecular species of PTTH prevalent in this

species and at the same time determining the exact periods of their release.

Haemolymph extracts show very little juvenile hormone activity during the last larval instar under non-diapause development. Relative to the penultimate larval instar, the haemolymph extract of early last instar in Busseola fusca show a marked drop in juvenilizing activity. In some other lepidopteran larvae such as Spodoptera litoralis (Cymborowski and Stolarz, 1979), Manduca sexta (Fain and Riddiford, 1975), Trichoplusia ni (Jones and Hammock, 1985; Newitt and Hammock, 1986), the titers of juvenile hormone have also been reported to fall to very low levels within a few days after ecdysis to the last instar.

The decline in the titer of juvenile hormone in the haemolymph could probably be a result of inhibition of the corpora allata by the brain (Feyersein, 1985; Bhaskaran et al., 1980; Granger et al., 1987; Granger and Jansen, 1987). Apparently, the decline in the haemolymph JH titer is one of the initial requirements for initiating the series of events leading to pupal metamorphosis. Thus in Busseola fusca a low haemolymph juvenile hormone titer is characteristics of not only the last larval instar, but also the occurrence of non-diapause form of development since it makes the larvae attain the required physiological condition for pupal development. This non-diapause form of development in Busseola fusca is limited to only a brief period of about a week. Therefore all endocrinological events associated with

the regulation of development in this instar proceed sequentially in order to bring about the desired control.

Physiologically, the drop in juvenile hormone titer is essential for the prothoracic glands to acquire competence to respond to priming by the brain hormone (Bollenbacher, 1988). In the case of last larval instars of Bussenla fusca under non-diapause development, this occurs during the precommitment period.

Prior to the decline in the titer of juvenile hormone early in the last instar larvae of Bussenla fusca, the prothoracic glands appear to be in a state of basal activity (chapter 3) and the haemolymph ecdysone titers are at a very low level (chapter 4). Similar findings have also been reported in Manduca sexta (Wolfgang and Riddiford, 1986) but in Calpodes ethlius a small increase in ecdysone titer was found to occur just a few hours after ecdysis to the last larval instar (Dean et al., 1980).

Two peaks of ecdysone titers were found in Bussenla fusca during non-diapause development (chapter 4). The first peak which occurred on day four after ecdysis to the last instar presumably results from the induction by the first PTTH peak release. The second ecdysone peak is significantly larger than the first one, and occurred on day seven of the last instar. The factors which cause the larger peak may be more than the PTTH alone. In the diapausing larvae, the ecdysone titers were generally low (chapter 4) but gradually increased to critical levels over a period of time.

However the effect of juvenile hormone on the prothoracic glands of the last larval instar varies with respect to the larval phenology whereby a switch from an inhibitory role during the early phases to a stimulatory role in the late phases of the instar occurs (Cymborowski and Stolarz, 1979; Hiruma and Agui, 1982). Such a stimulatory role of JH on the prothoracic glands has been implicated in the occurrence of stationary moults in ligated bodies of diapausing larvae of Chilo suppressalis following JH treatment (Yagi, 1975). Similarly, for the case of diapausing larvae of Busseola fusca, the presence of persistently high juvenile hormone titers in the haemolymph may be responsible for allowing the stimulation of the prothoracic glands by PTTH to produce sufficient titers of ecdysone for the observed periodic stationary moults. Thus a decline in the haemolymph juvenile hormone early in the last larval instar may prevent a surge of ecdysone from the prothoracic glands in response to PTTH.

Juvenile hormone has been reported to regulate the titer of a haemolymph factor which promotes ecdysone synthesis by prothoracic glands in vitro (Watson et al., 1988). It remains to be established whether the extent of gland stimulation by such a factor is enough for the production of the critical titer of ecdysone required to induce a moult in diapausing larvae of Busseola fusca.

Resumption of juvenile hormone production at later stages of the last larval instar is essential for normal

metamorphosis (Watson et al., 1986). In some insect species, research findings have implicated that the regulation of the activities of the corpora allata during this period is primarily influenced by the precommitment ecdysone peak titer via the brain-corpora cardiaca axis (Bollenbacher, 1988). In the case of Busseola fusca larvae during non-diapause development, ecdysone titers peaked for the first time on day four. At this stage, presence of juvenile hormone activity in the haemolymph extracts was quite low and did not become detectable during the pre-pupal stage. Similar mechanisms may also operate at the end of diapause development in this species.

Therefore, the occurrence of non-diapause or diapause development in the last instar larvae of Busseola fusca depends on the way the larval neuroendocrine system, in response to the stimuli from the environment is directed to act. It has already been shown in Chilo partellus, a species which show similar physiological responses to stimuli from similar host plants like Busseola fusca, that feeding on the young hosts promote non-diapause development while feeding on mature host lead to diapause development and that the hormone responsible for the regulation of induction and maintenance of diapause development was JH (Scheltes, 1978). In the light of the knowledge obtained from the present studies, it can be concluded that, in Busseola fusca, the interendocrine interactions that control development of the last larval instar were also centered on JH. Since diapause

development, as an escape means of surviving adverse environmental conditions, is regulated by JH, it can be concluded that hormonal procedures of controlling Busseola fusca should focus on the control of JH production during the larval stages of the insect.

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