ON THE DEVELOPMENTAL PROFILE OF HORMONES IN *SPODOPTERA MAURITIA* BOISD. (LEPIDOPTERA : NOCTUIDAE)

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CERTIFICATE

This is to certify that this is an authentic record of the research work carried out by Mrs. P.M. Mona, M.Sc. from October, 1997 to August, 2001 as a part-time student in partial fulfilment of the requirements for the Degree of DOCTOR OF PHILOSOPHY under the faculty of Science of the University of Calicut under my supervision and guidance. No part of this thesis has been presented before for any other degree. I also certify that she has passed the Ph.D. qualifying examination of the University of Calicut, held in December, 1999.

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DECLARATION

I do hereby declare that the present work is original and it has not previously formed the basis for the award of any degree or diploma.

Calicut University,
13 August, 2001.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. INSECT HORMONES: A REVIEW

Insect metamorphosis is typified by abrupt morphological transitions which are in reality smooth continua of precisely regulated events controlled by titres of several hormones. The endocrine nature of the processes and factors responsible for the regulation of moulting and metamorphosis have been understood only within the last few decades. Based on the pioneering studies by Kopec (1917, 1922); Wigglesworth (1934, 1936, 1940); Fukuda (1940, 1944); Williams (1948, 1952) and others, a basic model for the endocrine control of insect postembryonic development emerged by early 1950s. According to this basic model, the brain in response to appropriate environmental stimuli synthesises and releases *via* neurohaemal organs (corpora cardiaca or corpora allata) the brain hormone. This brain hormone now referred to as prothoracicotropic hormone (PTTH), stimulates the prothoracic glands or analogous structures (ring gland, ventral glands) to synthesise and secrete moulting hormone (MH), which initiates the events generically referred to as moult. However the character of the moult, i.e., whether it is larval-larval, larval-pupal or pupal-adult, is dictated by the relative titre of juvenile hormone (JH) which is synthesised by the corpora allata (CA) and released at or before the moult. The basic model hypothesised that presence of a high titre of JH would result in a larval- larval moult,

whereas presence of reduced titre of JH would result in larval-pupal moult. Absence of JH would result in a pupal-adult moult.

Although earlier studies demonstrated that exogenous ecdysone administered to insects could initiate moulting and metamorphosis (Karlson, 1980), one of the implicit assumptions of the basic model that MH titres fluctuated during insect postembryonic development, was not supported by direct experimental evidence until the early to mid-1960s. At this time Burdette (1962) and Shaaya and Karlson (1965a,b) using the *Calliphora* bioassay, succeeded in demonstrating that MH titres varied during larval-pupal-adult development of *Calliphora erythrocephala* and *Bombyx mori*. In accordance with the basic model, their data suggested that an increase or peak in ecdysteroid activity preceded or occurred concomitantly with the moult.

1.1. PROTHORACICOTROPIC HORMONE (PTTH)

Site of synthesis

PTTH is synthesised primarily by four large neurosecretory cells (the prothoracicotropes), two per hemisphere, whose soma reside in the dorsolateral region of the brain (Agui *et al.*, 1979; Mizoguchi *et al.*, 1990). PTTH is then transported down the axons of these neurons to the corpora

cardiaca in most of the insects whereas in lepidopterans, the axons terminate in corpora allata which serve as neurohaemal organs.

Chemistry

It has been confirmed that PTTH is a protein or peptide. Purification of PTTH from brains of *Manduca sexta* established the presence of at least one PTTH of approximately 22,000 MW and possibly a second hormone of approximately 1,000 MW (Bollenbacher and Gilbert, 1981; Gilbert *et al.*, 1981). In *Bombyx,* PTTH exists as several peptides (Ishizaki and Ichikawa, 1967). Kawakami *et al.* (1990) report that in *Bombyx* larva, the PTTH is first synthesised as a 224 amino acid polypeptide containing 109 amino acid PTTH subunits. Later studies confirmed that the mature, secreted PTTH is a homodimer containing inter and intra monomer disulphide bonds, the latter being necessary for retention of prothoracicotropic activities (Ishibashi *et al.*, 1994).

PTTH synthesis and release

At specific times during development, PTTH is released into the haemolymph (Agui *et al.*, 1980). A preliminary titre of PTTH in the haemolymph of fourth and fifth (last) instar larvae of *Manduca sexta* has been determined using the *in vitro* PTTH assay (Gilbert *et al.*, 1981). The data from this study demonstrates that the haemolymph titre of PTTH is elevated

just prior to, or concomitant with the increase in the haemolymph titre of ecdysteroids. These periods of elevated PTTH titre in the haemolymph is found to correspond temporally to the time of PTTH release (head critical periods,HCPs) as predicted by classical head ligation studies. However, direct measurement of haemolymph PTTH titres in fifth (last) instar *Bombyx* larvae shows that PTTH is released every day (Mizoguchi, 1995).

In many lepidopterans, the release of PTTH is a gated event, occurring only at certain times during the light-dark cycle. In *Manduca* and *Antheraea*, PTTH release is controlled by a light-sensitive circadian pacemaker (Truman and Riddiford, 1974; Fujishita and Ishizaki, 1982). PTTH release occurs in the larvae which have attained a critical weight before or during the time of photoperiodically determined gate that is "open" only once in every 24 hours.

One of the most important events in the timing of PTTH release during larval-pupal development is that the JH titre, levels off to an undetectable level at the halfway stage of feeding in the last instar while it remains at a detectable level in the penultimate instar prior to ecdysis. This pattern of changes in JH titre has been described in *M. sexta* (Nijhout and Williams, 1974; Fain and Riddiford, 1975; Sedlak *et al.*, 1983), *B. mori* (Sakurai, 1983) and *Trichoplusia ni* (Jones *et al.*, 1986). In *M. sexta* the precommitment drop in the JH titre is permissive for the gated release of PTTH (Rountree and Bollenbacher, 1986).

1.1.1. Control of PTTH release

The mechanism of control of PTTH release varies among the different insects. Both extrinsic and intrinsic stimuli are involved and the mediators of these stimuli can be either neural and/or endocrine.

Proprioceptive and mechanoreceptive stimuli

Studies on the mechanisms by which nervous stimuli controls PTTH release led to establishing the timing of PTTH release in insects. In *Rhodnius prolixus,* proprioceptive stimuli controlled PTTH release. Proprioception being a nervous phenomenon, the effect of abdominal stretching appears to be the cause for the release of PTTH from the brain (Wigglesworth, 1933). Stretch stimulated moulting mediated by proprioception was also demonstrated in *Locusta migratoria* (Clarke and Langley, 1963) and *Oncopeltus fasciatus* (Nijhout, 1979). In *Manduca* and other lepidopterans, proprioceptive control of PTTH release involves a form of allometry; i.e., PTTH release occurs when the brain receives information that the body has attained a certain critical weight (Nijhout, 1975). In *Manduca,* allometry may be controlling PTTH release by inactivation of the CA (Nijhout and Williams, 1974).

Photoperiodic stimuli

Photoperiodicity is a major source of external control of insect development and its apparently direct effect on the release of PTTH is expressed in either a circadian or seasonal manner. In *Manduca*, release of PTTH is photosensitive during larval development upto the second HCP of the last larval instar (Truman, 1972; Truman and Riddiford, 1974). Photoperiod does not directly control PTTH release, but acts as a primary oscillator, establishing a gate or time of day, when release can occur. The effect of seasonal photoperiodism on PTTH release is a well-established phenomenon and cues the induction and duration of diapause in insects. It is suggested that the photoreceptor and the photoperiodic mechanism in general may reside in the lateral NSC (Bollenbacher and Bowen, 1983).

Temperature stimuli

Temperature is an important external cue that regulates PTTH release, and this effect is expressed either in a circadian or seasonal context. Temperature can either act alone or in concert with a particular photoperiod, and can affect the rate of development and the induction, reversal or termination of diapause (Tauber and Tauber, 1976; Beck, 1980).

Endocrine regulators

Endocrine systems function as the chemical mediators for the environmental stimuli controlling development. In several insect species, JH appears to be a feedback inhibitor of PTTH release (Fukaya and Mitsuhashi, 1961; Fukaya and Kobayashi, 1966; Yagi and Fukaya, 1974; Hiruma *et al.*, 1978), inducing and/or maintaining larval diapause (Yin and Chippendale, 1973a,b; Takeda, 1978; Chippendale and Yin, 1979; Sieber and Benz, 1980; Chippendale and Turunen, 1981). Based on observations of the hormonal effects of 20-hydroxyecdysone on cerebral NSC, ecdysteroids may also regulate the release and/or synthesis of PTTH (Marks *et al.*, 1972; Steel, 1975; Agui and Hiruma, 1977a,b). The control of PTTH release is thus the cumulative effect of a complex and interrelated series of environmental and proprioceptive-mechanoreceptive stimuli acting *via* the nervous and endocrine systems.

1.2. THE MOULTING HORMONE

Site of synthesis

Ecdysteroids are primarily secreted by the prothoracic glands. These organs do not store significant amounts of ecdysteroids (Agui *et al.*, 1972; Chino *et al.*, 1974; King *et al.*, 1974; Bollenbacher *et al.*, 1976). Prothoracic glands from several species were cultured *in vitro* and the gland products

secreted into the culture medium were characterised and quantified by radioimmunoassay, bioassay and physico-chemical techniques. The results of these experiments established that the larval prothoracic glands of *Manduca sexta* (King *et al.*, 1974), *Bombyx mori* (Chino *et al.*, 1974), *Leucophaea maderae* (Borst and Engelmann, 1974) and *Sarcophaga bullata* (Bollenbacher *et al.*, 1976) synthesise and secrete ecdysone. Other tissues such as oenocytes, epidermis, gut, ovary and testis also seem to be the secondary sources of ecdysteroids (Delbecque *et al.*, 1990).

Chemistry

The first two moulting hormones have been isolated from *Bombyx* pupae (Butenandt and Karlson, 1954; Karlson, 1956) and are now designated as ecdysone and 20-hydroxyecdysone respectively. Both MHs proved to be polyhydroxylated ketosteroids generically referred to as ecdysteroids (Goodwin *et al.*, 1978). Ecdysone is secreted as a prohormone (Granger and Bollenbacher, 1981) which undergoes hydroxylation into the actual moulting hormone, 20-hydroxyecdysone. This process is catalysed by ecdysone 20-monoxygenase, a cytochrome P-450 mixed function oxidase present in tissues peripheral to the prothoracic glands (Smith *et al.*, 1980). Ecdysteroids possess the full C_{27} carbon skeleton of cholesterol, exhibit a cis fusion of the A and B ring, have two functional moieties in the B ring and posses hydroxyl groups at several positions. To date, 20 or so ecdysteroids have been

tentatively identified in insects or crustaceans. In addition to these zooecdysteroids, atleast 60 structurally distinct ecdysteroids (phytoecdysteroids) have been isolated from numerous phytogenetically diverse families of plants, in most cases at much higher concentrations than in arthropods (Hetru and Horn, 1980).

1.2.1 Ecdysteroids titres during postembryonic development

Ecdysteroid titres have been measured in numerous species of insects during various stages of postembryonic development. The ecdysteroid titre appears to fluctuate considerably during postembryonic development, varying from basal levels (~ 10^{-8} M) by a factor of from 20 to 30 (e.g. in *Drosophila melanogaster*) upto approximately 10^{2}-10^{3} (e.g. in *Manduca* and *Schistocerca gregaria*, Dean *et al.*, 1980; Richards, 1981).

During larval-larval and larval-pupal development (larval-adult in hemimetabolous insects), the major peak in the ecdysteroid titre is usually present during the latter half of each stadium. Temporally and quantitatively, this titre peak is characterised by a rapid rise from basal levels before apolysis; maximal levels either slightly before, at, or slightly after apolysis; and an equally rapid drop back to low or basal levels by the time of ecdysis. The peaks preceding larval-pupal and larval-adult metamorphic moults are of slightly greater magnitude and/or duration than peaks preceding the non-metamorphic larval-larval moults.

During larval-larval development, there appears only one surge of ecdysteroid titre just preceding each ecdysis in *Galleria mellonella* (Sehnal *et al.*, 1981, 1986), *Manduca sexta* (Bollenbacher *et al.*, 1981), *Trichoplusia ni* (Jones *et al.*, 1981) and *Mamestra brassicae* (Agui and Hiruma, 1982). During larval-pupal transformation of Lepidoptera, the existence of two ecdysteroid peaks is well established (Lafont *et al.*, 1977b; Maroy and Tarnoy, 1978; Dean *et al.*, 1980; Bollenbacher *et al.*, 1981; Zimowska *et al.*, 1985; Jones *et al.*, 1986). In contrast to larval-larval and larval-pupal development, during pupal-adult development the major ecdysteroid titre peak occurs in the first half or middle of the pupal stadium (Dean *et al.*, 1980). This titre peak is characterised by a slow rise from basal levels with the onset of pupal-adult apolysis. The rise continues over an extended period during pharate-adult development, followed by a slow return to basal levels before pupal-adult ecdysis. The major ecdysteroid titre peak in pupal stadia not only differs in temporal aspects from those which occur in larval stadia but is also much greater in magnitude and duration. These major peaks in the ecdysteroid titres are responsible for initiating and/or maintaining the events associated with moulting and the pharate development which occurs during the moult, i.e., between apolysis and ecdysis. The single major ecdysteroid titre peak detected during each larval and pupal stadium is considered a "moulting peak".

In addition to the major moulting peaks, there may be one or more additional "non-moulting peaks" in the ecdysteroid titre during certain stadia, particularly the stadium leading to the first metamorphic moult (Dean *et al.*, 1980). In contrast to the major moulting peaks, these non-moulting peaks are usually present during the intermoult period and are much more subtle in magnitude and duration than the larval moulting peaks. Some of the intermoult peaks have been found to correlate temporally with activities such as DNA synthesis, RNA synthesis, lipid synthesis and organelle formations but no firm conclusions can be drawn at the cellular level (Dean *et al.*, 1980). In *Manduca sexta* a subtle intermoult ecdysteroid titre peak has been detected about day 4 of the last larval stadium. This ecdysteroid peak and comparable intermoult peaks reported for other holometabolous insects are thought to be responsible for reprogramming the epidermis from a larval to a pupal commitment (Dean *et al.*, 1980; Riddiford, 1985).

1.2.2 Factors regulating the secretory activity of prothoracic glands

The factors which regulate the production and secretion of ecdysone by the prothoracic glands are important regulators of ecdysteroid titre. Since prothoracic glands have been shown to release ecdysone immediately following its synthesis, the factors which regulate gland activity do so at the level of ecdysone biosynthesis. The principal regulator of prothoracic gland activity is the cerebral neuropeptide, the PTTH. In addition to the PTTH, other factors

also influence the rate of ecdysone biosynthesis either by indirectly affecting the synthesis and release of PTTH or by directly acting on the prothoracic glands. Such factors include environmental and neural stimuli, juvenile hormone, haemolymph proteins and ecdysteroids.

Prothoracicotropic hormone

The principal regulators of prothoracic gland activity is the PTTH from the brain (Bollenbacher and Bowen, 1983; Bollenbacher and Granger, 1985). Utilizing *in vitro* assay, it has been demonstrated that PTTH from the brain directly activates the prothoracic glands of *Manduca sexta* by eliciting an increase in the basal rate of ecdysone biosynthesis (Bollenbacher *et al.*, 1979, 1980). This activation was found to be both specific and dose-dependent. PTTH acts similarly under *in vivo* conditions and it is one of the primary physiological regulators of prothoracic gland activity.

By analogy with vertebrate steroidogenic peptides such as adrenocorticotropic hormones and leutinizing hormone, PTTH has been hypothesized to exert its effect through the action of a cyclic AMP (cAMP) as second messenger (Gilbert *et al.*, 1980). An involvement of cAMP in steroidogenesis by the insect prothoracic glands was indicated initially by the ability of phosphodiesterase inhibitors to stimulate ecdysone synthesis by the prothoracic glands of *Manduca sexta* (Vedeckis *et al.*, 1976) and to mimic

neurohormone mediated changes, in membrane potential of the prothoracic glands of *Galleria mellonella* (Gersch and Birkenbeil, 1980).

Environmental factors

Environmental factors influence PTTH synthesis and release and it is known that a light sensitive circadian clock is involved in the release of PTTH (Truman and Riddiford, 1974; Gelman and Hayes, 1982; Denlinger, 1985). Possibility exists that some environmental factors may also act directly on the prothoracic glands. A study with *Samia cynthia ricini* larvae has suggested, based on transplantation experiments that in addition to light sensitive cephalic circadian clock for the release of PTTH, these organisms also possess a light-sensitive prothoracic gland clock for the release of ecdysone (Mizoguchi and Ishizaki, 1982).

Neural factors

Neural regulation of ecdysone biosynthesis is either indirectly *via* regulation of PTTH or directly on the prothoracic glands. Studies with *R. prolixus* and *L. migratoria* have implicated that PTTH synthesis/release may be under direct neural regulation (Steel, 1978; Girardie and De Reggi, 1978; Steel and Davey, 1985). Although proof for such neural regulation has not been demonstrated, it is known that the prothoracic glands are innervated by various neural processes, including neurosecretory axons from several ventral

ganglia (Benedeczky *et al.*, 1980; Granger and Bollenbacher, 1981). In *G. mellonella* larvae, the prothoracic glands are known to be innervated by the prothoracic, mesothoracic and suboesophageal ganglia (Singh and Sehnal, 1979) and all three of these ganglia have been reported to modify prothoracic gland secretory activity either when implanted into decapitated larvae with prothoracic glands *in situ* (Mala *et al.*, 1977) or when incubated *in vitro* with prothoracic glands (Gersch, 1978).

Inhibitory and stimulatory effects of JH

Both older and more recent results suggest that juvenile hormone (JH) can regulate prothoracic gland activity. The regulatory effect of JH on prothoracic glands is either stimulatory or inhibitory depending on the stage of development.

Inhibitory effects of JH

In the last instar larvae of Lepidoptera, JH treatments within the feeding period causes a delay in metamorphosis for a few days. This has been observed in *Manduca sexta* (Nijhout and Williams, 1974; Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984a,b; Rountree and Bollenbacher, 1986). *Mamestra brassicae* (Hiruma *et al.*, 1978; Hiruma, 1980), *Spodoptera littoralis* (Cymborowski and Stolarz, 1979; Cymborowski and Zimowska, 1984),

Laspeyresia pomonella (Sieber and Benz, 1980) and *Spodoptera mauritia* (Santha and Nair, 1987).

In *Manduca sexta*, the inhibitory effects of JH or its analogues on the prothoracic glands before the commitment peak of ecdysteroids appears to be indirect. In this insect, JH appears to have an inhibitory effect on the release of PTTH by the brain, thus indirectly blocking ecdysone secretion by the prothoracic glands (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). A similar effect has been observed in the larvae of *Mamestra brassicae* (Hiruma *et al.*, 1978). In several insect species the foremost being *Chilo suppressalis* and *Diatraea grandiosella*, JH appears to be a feedback inhibitor of PTTH release inducing and/or maintaining larval diapause (Yin and Chippendale, 1973[a,b]; Takeda, 1978; Chippendale and Turunen, 1981). It has been suggested that an effect on PTTH release is a logical focus of control by JH, since release of this peptide is the initial event in the hormonal cascade leading to pupal commitment (Rountree and Bollenbacher, 1986).

However observations by other authors strongly argue against the concept that the inhibitory effect of JH on prothoracic glands is *via* PTTH. The findings on *Spodoptera littoralis* (Cymborowski and Stolarz, 1979), *Galleria mellonella* (Ciemior *et al.*, 1979), *Manduca sexta* (Safranek *et al.*, 1980; Rountree *et al.*, 1987; Watson and Bollenbacher, 1988) and *Spodoptera mauritia* (Balamani and Nair, 1992) suggest that JH directly suppresses the

function of larval prothoracic glands. Studies in *G. mellonella* revealed that JH regulates ecdysone synthesis by both these mechanisms (Sehnal *et al.*, 1981).

Stimulatory effects of JH

JH is thought to maintain prothoracic glands (Gilbert, 1962) and also to stimulate ecdysone secretion in many insects. In lepidopterans, the stimulatory effects of JH on prothoracic glands is restricted to the prepupal phase of last larval instar and to the pupal stage. The first evidence for the tropic effect of JH on pupal prothoracic glands was provided by Williams (1959). He showed that implantation of active corpora allata stimulated adult development in brainless pupae of *Hyalophora cecropia.* JH also has been shown to maintain and stimulate the pupal prothoracic glands of *M. sexta* (Dai and Gilbert, 1998).

During larval-pupal transformation in most lepidopterans, a brief burst of JH occurs in the prepupal phase of development (Varjas *et al.*, 1976; Yagi, 1976; Yagi and Kuranochi, 1976; Schooley *et al.*, 1976; Hsiao and Hsiao, 1977; Sieber and Benz, 1977; Riddiford and Truman, 1978; Bean *et al.*, 1982; Balamani and Nair, 1989[a,b], 1991). The major role attributed to this prepupal surge of JH is that this increase of JH along with the second release of PTTH is necessary for activating the prothoracic glands to their maximal rate of ecdysone secretion needed for moulting and metamorphosis (Hiruma *et al.*,

1978; Cymborowski and Stolarz, 1979; Hiruma, 1980; Sieber and Benz, 1980; Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984a,b; Cymborowski and Zimowska, 1984).

It has been suggested that JH activates prothoracic glands by eliciting the release of a prothoracic gland stimulatory factor from the fat body (Gruetzmacher *et al.*, 1984a,b). In *Manduca sexta* both the timing and magnitude of the commitment peak appear to be controlled by the interaction between three elements : PTTH, the stimulatory factor present in the haemolymph and the competence of the prothoracic glands to respond to these effectors. At the time of the first PTTH release, the titre of the factor is below the threshold for stimulation of glands (Watson *et al.*, 1987a). It appears that PTTH acts on competent prothoracic glands in the presence of low level of stimulatory factor to elicit commitment peak in the haemolymph ecdysteroid titre. At the time of the second PTTH release, the titre of this factor has risen significantly (Watson *et al.*, 1987a). Activation of competent prothoracic glands by the combination of PTTH and high level of stimulatory factor gives rise to the large peak in the ecdysteroid titre which elicits the pupal moult. In these interactions, JH regulates the release of PTTH, the haemolymph titre of the stimulatory factor and competence of prothoracic glands (Watson *et al.*, 1987b, Baker *et al.*, 1987).

Ecdysteroids

Ecdysteroids themselves may contribute to the regulation of ecdysone biosynthesis by feedback loops to the brain or directly to the prothoracic glands. Early evidence from parabiosis experiments with pupal *Hyalophora cecropia* indicated that the prothoracic glands could be stimulated by their own hormone (Williams, 1952). This idea was supported by Siew and Gilbert (1971) who found that the injection of 20-hydroxyecdysone into intact non-developing saturniid pupae stimulated incorporation of ^{3}H uridine into the prothoracic glands. Similar injections into pupae in which adult development had started elicited a suppression of ^{3}H uridine incorporation. These results suggest that the response of prothoracic glands to 20-hydroxyecdysone changes from positive to negative with the initiation of development. Ecdysone and 20-hydroxyecdysone have been reported to stimulate the synthesis and release of PTTH in *Leucophaea maderae* (Marks *et al.*, 1972) and *Mamestra brassicae* (Agui and Hiruma, 1977a,b).

Indications for a feedback control loop controlling the haemolymph titre of ecdysteroids has been obtained in many insect species (Mala *et al.*, 1977; Mala and Sehnal, 1978; Steel *et al.*, 1982; Beydon and Lafont, 1983). Since ecdysteroids may directly inhibit ecdysone biosynthesis in the prothoracic glands. Injections of 20-hydroxyecdysone caused a decrease in RNA synthesis in saturniid pupal prothoracic glands (Siew and Gilbert, 1971). Since

20-hydroxyecdysone was also found to elicit such inhibition in decapitated pupae, it was suggested that this steroid acts directly on the prothoracic glands (Beydon and Lafont, 1983). In *Manduca sexta*, 20-hydroxyecdysone was found to inhibit ecdysone biosynthesis in fifth instar larval prothoracic glands *in vitro* (Scott, 1982). The finding that ecdysone synthesising cells as well as the presumed prothoracicotropes and their neurohaemal organs contain ecdysteroid receptors, suggest a multiple feedback mechanism, directly on ecdysone synthesis and indirectly on PTTH synthesis/release. The inability of prothoracic glands for conversion of ecdysone to 20-hydroxyecdysone may be due to the fact that prothoracic glands are a target tissue of the moulting hormone (Bidmon and Koolman, 1989).

In summary, the hormonal influences upon prothoracic gland secretion appear to be three-fold, initial stimulation by PTTH followed by modulation by both ecdysteroids and JH. The actions of the latter two hormones appear to change during development. Both these hormones are reported to influence the prothoracic glands by two distinct routes; a direct action of the hormones on prothoracic glands and an indirect action *via* influences on the secretion of PTTH. Depending on the temporal integration of effects of primary and secondary effectors of prothoracic glands these moieties can modulate gland activity and the haemolymph ecdysteroid titre.

Competency of prothoracic glands

The competency of prothoracic glands to respond to various regulatory factors appears to be another component in the complex systems regulating prothoracic gland activity. In *M. sexta, in vitro* studies revealed that prothoracic glands exhibited an absolute refractoriness to respond to tropic stimuli during the early part of last larval instar (days 0-2) and the glands subsequently acquired the competency to respond to effectors by about days 3-4 (Ciancio *et al.*, 1986). The regulation of competency may involve humoral bioregulatory substances like JH or perhaps PTTH itself. Alternatively, it could be an intrinsic property of the glands to respond to extrinsic effectors like photoperiod (Mizoguchi and Ishizaki, 1982).

1.3 JUVENILE HORMONES (JHs)

Site of synthesis

Juvenile hormones are produced and secreted by the corpora allata (CA) (Wigglesworth, 1934, 1936), endocrine glands of ectodermal origin associated with the brain and corpora cardiaca. The corpora allata received innervations by way of cerebral and central nervous system connections from neurosecretory cell bodies located primarily in the protocerebrum and tritocerebrum, as well as by the suboesophageal ganglion. In addition, the

glands themselves contain numerous neurosecretory endings which represent the site of release of allato-regulatory peptides and amines, as well as PTTH.

Chemistry

Juvenile hormones have a terpenoid backbone and contain two hydrolytically unstable functional groups, a 10,11-epoxide and a terminal methyl ether conjugated with 2,3-double bonds (Hammock, 1985). Thus JHs are said to be methyl esters of epoxy-sesquiterpenoid acids. To date, six juvenile hormones have been identified from various insect orders. JH I and JH II the exclusively lepidopteran JHs, were the first to be identified (Röller *et al.*, 1967, Meyer *et al.*, 1968). JH III appears to occur in all orders and is the principal product of corpora allata in most insects. In the Lepidoptera, five JHs are produced, JH I, JH II, JH III, JHO and 4-methyl-JH I; the latter two are found in *Manduca* embryos and JHO in *Hyalaphora cecropia* males (Bergot *et al.*, 1980).

Synthesis and release of juvenile hormones

In vitro assays of CA activity have conclusively shown that the rate of JH release is proportional to the rate of JH synthesis over a wide range of glandular activity in *Schistocerca gregaria* (Tobe and Pratt, 1974b), *Periplaneta americana* (Pratt *et al.*, 1975a) and *Diploptera punctata* (Tobe and Stay, 1977). The species studied to date do not have any mechanism for

the storage of biosynthesised JH-III, and intraglandular hormone levels are proportional to synthetic rates.

JH titre is believed to be the primary endocrine factor influencing the "quality" of developmental events during metamorphosis. The classic experiments with hemimetabolous (Wigglesworth, 1934, 1936) and holometabolous insects (Williams, 1961) provided qualitative information on the role of JH in metamorphosis. JH inhibits somatic morphogenesis early during postembryonic development, but in the final instar, its declining titre is essential for imaginal differentiation (Sbrenna *et al.*, 1988). For the larval moult, JH must be present at the onset of the rising phase of the ecdysteroid titre (Truman, 1972; Fain and Riddiford, 1976). Appreciable quantities of JH are present at the beginning of the last larval instar as well as end of the instar. The elevated titre of JH at the beginning of the instar may control the subsequent ecdysteroid or PTTH surge responsible for initiation of ecdysis (Hiruma, 1986; Rountree and Bollenbacher, 1986). JH also is essential for the continued presence of the putative JH receptor in larval *Manduca* epidermis, particularly at times of low ecdysteroid titre. JH may also be involved in the regulation of expressions of the isoforms of the ecdysone receptor, depending on the presence of an ecdysteroid. In the absence of JH, the cells respond to ecdysteriods by switching to a metamorphic programme resulting in the production of a new type of cuticle. If JH levels remain high early in the final

instar, the insect fails to undergo successful larval-pupal or nymphal-adult transformation. Instead it becomes a giant larva or a supernumerary nymph. On the other hand, an induced depletion in the level of JH in early instars lead to irreversible precocious metamorphosis. It is generally assumed that the absence of JH is required for the metamorphosis to the adult in holometabolous insects (Riddiford, 1994). Thus the maintenance of a critical titre of JH at different developmental phases is absolutely essential for the orderly completion of insect metamorphosis, reproduction and behaviour.

1.3.1 Control of CA activity

There are a number of regulators of JH activity. The rate of JH production is an important component in the regulation of the JH titre and this in turn is regulated by neuropeptides that directly influence the biosynthetic activity of the corpora allata.

Haemolymph JH titre

Experiments utilizing either implantation of additional CA or treatment of animals with exogenous JH or analogues have clearly revealed that JH biosynthesis is suppressed in the presence of elevated JH titres (Schooneveld *et al.*, 1979; Tobe and Stay, 1980) and stimulated when JH titres are reduced (Szibbo and Tobe, 1981a). Khan *et al.* (1982) showed that suppression of JH synthesis is mediated by signals which reach the CA both

through the haemolymph and through nervous tracts from the CNS (Tobe and Stay, 1980). These results indicate that feedback loops, acting through the CNS, are responsible in part for regulation of CA activity.

Role of the brain

The brain has been implicated as a source of neurosecretory factors which control CA activity. These factors may be released in neurohaemal organs such as the CC and act *via* the haemolymph, but may also be released inside the CA themselves (Hansen *et al.,* 1982). In addition, the brain may be the origin of neural signals controlling the release of neurosecretion at the periphery, inside or outside the CA. Neurotransmitters may also have direct effects on the activity of CA cells. All these signals may be inhibitory (allatostatic) or stimulatory (allatotropic). These are peptides which are capable of stimulating or inhibiting JH production *in vitro* (Tobe and Stay, 1985). Their potency in altering JH production *in vitro* argues strongly for a role in the control of CA activity.

JH titre is determined by a complex interaction among biosynthesis, degradation, and binding to JH specific binding proteins which may differ in hemi- and holometabolous larvae. JH-binding proteins may not only afford protection for JH in the presence of catabolic enzymes, but also may be agents responsible for reducing JH levels within tissues. JH also regulates ecdysteroid titre and appears to be responsible in part for the ecdysteroid

surge that ultimately regulates the moulting process. The JH titre can be regulated by changes in the production of JH and this in turn is regulated by neuropeptides that directly influence biosynthetic activity of the corpora allata.

1.4 TESTES AS A SOURCE OF ECDYSTEROIDS

Ecdysteroids have been reported in adult males of *Drosophila melanogaster* (Hodgetts *et al.*, 1977) and *Calliphora vicina* where they are localised primarily in the testes (Koolman *et al.*, 1979). Subsequent investigations into testicular ecdysteroids in various lepidopteran species have largely been confined to immature stages, although the testes of adult *Heliothis virescens* have also been shown to contain ecdysteroids (Loeb *et al.*, 1984). In the testes of the last larval instar of the European corn borer, *Ostrinia nubilalis*, 20-hydroxyecdysone and various high polarity ecdysteroids were detected by radioimmunoassay (RIA) while in pupae and pharate adults ecdysone was additionally detected (Gelman *et al.*, 1988). The testes of the last instar larvae of *Spodoptera littoralis* was also reported to synthesize ecdysteroids (Jarvis *et al.*, 1994).

Loeb *et al.* (1982) have shown that incubation *in vitro* of the testes of *H.virescens* from mid to late last larval instar and from pupae after three days of pupal development release large amounts of ecdysteroids into the medium. Testes from the *O.nubilalis* are also capable of synthesising immunodetectable

ecdysteroids *in vitro* (Gelman *et al.*, 1989). When pupal testes of diapausing *Mamestra brassicae* were incubated for 24 h *in vitro,* 2-deoxyecdysone, ecdysone and 20-hydroxyecdysone were found in the culture medium when analysed by HPLC - RIA (Shimizu *et al.*, 1985). It has been predicted that the synthetic periods of the testes may affect, or be affected by internal changes in spermatocyst development, in addition to changes occurring outside the compartment containing spermatocysts and would also benefit from hormones released into the general circulation (Loeb *et al.*, 1984). Considerable differences among lepidopteran species is observed regarding optimum condition for synthesis, times of synthesis and the nature of ecdysteroids produced. It was also suggested that exogenous ecdysteroids is needed to initiate (Loeb *et al.*, 1988) or boost (Loeb *et al.*, 1986) endogenous ecdysteroid production by the testes *in vitro.*

Testicular metabolism of ecdysteroids has been investigated in *O. nubilalis, Lymantria dispar* and *H. virescens* by incubating the testes with [^{3}H] ecdysone *in vitro.* In pupae of *L. dispar,* the testes converted [^{3}H] ecdysone into 20-hydroxyecdysone (Loeb *et al.*, 1988) as they did in larvae, pupae and pharate adults of *O. nubilalis* (Gelman *et al.*, 1985). In *O. nubilalis*, this conversion was localised in the testes sheath (Gelman *et al.*, 1985). In *H. virescens* only the last larval instar was investigated and 20-hydroxyecdysone was again found to be the major product. However in this

case, traces of 20,26-hydroxyecdysone and 3- epi, 20-hydroxyecdysone were also identified (Loeb and Woods, 1989). In the latter study, the conversion of [^{3}H] cholesterol in the testes *in vitro* was also investigated, but no incorporation into ecdysteroids was obtained. The use of radiolabelled precursors in the last instar larvae of *Spodoptera littoralis* showed that of all the larval tissues investigated, the testes converted radiolabelled precursors into ecdysteroids most efficiently. The ability of the testes to convert [^{3}H] 2,22,25- trideoxyecdysone into ecdysteroids has been shown to vary during the last larval instar (Jarvis *et al.*, 1994).

1.4.1 Ecdysteroid titres of the testes in relation to haemolymph titres

The total ecdysteroid levels in the haemolymph and testis exhibited a similar pattern of fluctuation during the fifth (final) instar and pupal stage of *H. virescens* (Loeb *et al.*, 1982), *O. nubilalis* (Gelman *et al.*, 1988) and the pupal stage of *M. sexta* (Friedlander and Reynolds, 1992). However, the testicular ecdysteroid levels differed from those in the surrounding haemolymph at certain times depending on the stage of development. The levels of all ecdysteroids were found to be lower in the testes than in the haemolymph (Gelman *et al.*, 1988[a,b]). In *C. vicina* no ecdysteroids were detected in the haemolymph of the adult flies by Koolman *et al.*, (1979) even though large amounts of ecdysteroids were found in the testes. In *Heliothis virescens*, the quantity of ecdysteroids in the testes of the adult were found to be lower

than in the gonads of late mid-last instar larvae and pupae, nevertheless, adult testes were found to be capable of statistically significant ecdysteroid synthesis *in vitro* (Loeb *et al.*, 1984). The existence of a blood-testis barrier has also been suggested (Kambysellis and Williams, 1972; Szollosi *et al.*, 1980). Further, Jarvis *et al.* (1994) reported that the ecdysteroids of both the testis and haemolymph of last instar larvae of *S. littoralis* were different both in the timing of their fluctuations and in their content.

1.4.2 Factors regulating synthesis of testicular ecdysteroids

Previous estimates suggest that neurosecretory stimuli which induce ecdysone synthesis by the prothoracic glands may be closely related to stimuli which induce ecdysteroid synthesis by the testes.

Hence, testicular ecdysteroid synthesis was suggested to be under the control of a brain peptide, testis ecdysiotropin (Loeb *et al.*, 1987). Regulation of the maturation of male genital tissues by inducing the synthesis of growth factors by the testis and fat body has also been suggested (Loeb and Hakim, 1991; Loeb, 1991b, 1994). However, no neurosecretory nerves has been found entering the testis of Lepidoptera (Szollosi, 1982). But a unique heart-testis complex has been reported in the last instar larvae of *H. virescens* (Meola and Loeb, 1995). This cardiotesticular muscle complex, attaches the heart to the testis, which in turn is innervated by neuroendocrine nerves. This has been proposed to be the route by which the brain peptide, testis ecdysiotropin

reaches the testis. Meola and Loeb (1995) also suggested that the cardiotesticular muscle and the products sequestered in the cardiotesticular sinus may be involved in the fusion of the paired testis and maturation of the male reproductive system, since they were present only during this stage of development.

The testis ecdysiotropic peptide from the brain of *L. dispar* has been purified and its structure determined to be NH_2-Ile-Ser-Asp-Phe-Asp-Glu-Tyr-Glu-Pro-Leu-Asn-Asp-Ala-Asp-Asn-Asn-Glu-Val-Leu-Asp-Phe-OH. The peptide is biphasic in activity with maximal activity in the pupal testis (Wagner *et al.*, 1997). It was suggested that the strongly bioactive portion of the molecule probably resides in residues 1-15. This molecule requires cations like Ca^{2+} for its biological activity (Loeb, 1991b). Atleast five natural testis ecdysiotropic analogues or LTE have been identified in *L. dispar* brains (Loeb *et al.*, 1997).

Role of testes sheath in the synthesis of ecdysteroids

Immunocytochemical techniques have implicated the testes sheath as the site of ecdysteroid production. Studies revealed sensitivity in and around cells of the inner layer of the testes sheath and the extensions of the inner layer that form the walls of each of the four testes follicles (Loeb, 1986). Transmission electron micrographs of sheaths of lepidopteran testes (Szollozi *et al.*, 1980, Szollozi, 1982) indicate that the tissue of the inner layer is

structurally different from that of the outer layer, the inner layer contain lipid and glycogen deposits, abundant mitochondria, and a peculiar network of swollen channels containing flocculent material. The channels if proved to be swollen smooth endoplasmic reticulum, the sheath of insect testes resemble other tissues capable of steroid synthesis.

Analysis of the contents of testes of *H. virescens* indicated that approximately half the ecdysteroids were in the sheath and half were in the luminal fluid with minor amounts in the spermatocysts (Loeb *et al.*,1984). Giebultowicz *et al.* (1987) showed that premeiotic spermatogonia can initiate meiosis and elongation *in vitro* if they are provided with calf serum and testes sheath factors. Their data suggest that testes sheath tissue releases a factor (s) stimulating meiosis. On the other hand, it is also suggested that the sheath may provide nourishment or other conditions for survival of cysts, thus allowing meiosis to occur as a step in germ cell autodifferentiation. The spermatogenesis promoting effect of testes sheath was found to be dose dependent and varied with the donor's age.

1.4.3. Insect Growth Regulators

Insect growth regulators (IGRs) are a group of compounds which interfere with normal growth, development and reproduction in insects by disrupting hormonally regulated physiological processes of insects. There are mainly three categories of IGRs: (a) compounds which directly influence

hormones regulating development and reproduction (e.g., juvenile hormone analogues, anti-juvenile hormone agents, ecdysone mimics, anti-ecdysteroids, neurohormone analogues and their antagonists); (b) compounds which inhibit cuticle formation through an effect on chitin synthesis (e.g., Benzoyl phenyl ureas) and (c) compounds with miscellaneous mode of action (e.g., Azadirachtin). Several such compounds are known and their effects on metamorphosis and reproduction in a number of insect species have been extensively studied and reviewed (Retnakaran *et al.*, 1985; Staal, 1986; Darvas and Varjas, 1990; Nair, 1993). These compounds are considered to be useful in pest control programmes because they are target specific, non-persistent, biodegradable and environmentally benign substances to which insects may not develop resistance.

IGRs based on insect hormones have great significance as pesticides of the future and also as excellent chemical probes to elucidate the role of hormones in the basic physiological processes of insects. Manipulation of the hormone levels in the haemolymph will cause derangement of hormone dependent processes of morphogenesis and reproduction. Based on this concept many hormone analogues and antihormones have already been developed.

(i) IGRs based on neurohormones

Since many of the neurohormones of insects have not been fully characterised, neuropeptide based IGRs for insect pest control is not well advanced. The diversity and complexity of neurohormones offer lot of possibilities for design and development of neurohormone analogues. The neurohormone biosynthesis, release, transport or receptor binding are all vulnerable steps for manipulation in insect pest management.

(ii) IGRs based on juvenile hormones

Williams (1967) suggested that juvenile hormone or its analogues could be used to control insect pests. Since then, numerous analogues with JH activity has been discovered and their biological and insecticidal properties have been well studied. Some of them are Epofenonane, Methoprene, Hydroprene, Kinoprene and Phenoxy carbamate. Changing the haemolymph JH titer by treatments with JHAs at critical periods during the life cycle of an insect will interfere with normal metamorphosis and reproduction.

The limited scope of JHAs as control agents led to the discovery of compounds with anti-JH activity. Examples of anti-JH agents are Precocenes, Fluoromevalonolactone (FMev), ETB, EMD, Compactin, Piperonyl butoxide, Allylic alcohols, Bisthiol carbamate etc. Anti-JH agents disrupt normal development of early larval instars and inhibit JH dependent reproductive

activities (Sam Mathai and Nair, 1984a,b,c; Santha and Nair, 1986, 1988, 1991; Santha *et al.*, 1987; Nair, 1993).

(iii) IGRs based on ecdysteroids

Many compounds with ecdysone activity have been isolated from plants. Ponasterones, with moulting hormone activity was discovered by Nakanishi *et al.* (1966). Since then, many synthetic and natural steroids with moulting hormone activity have also been discovered. They are divided into four groups: Zooecdysoids, Phytoecdysoids, Synthetic ecdysoids and non-steroidal ecdysone agonists. An example of a zooecdysoid is the crustacean moulting hormone. The important phytoecdysoids are ponasterone, inokosterone, isoinokosterone etc. Synthetic ecdysteroids are steroids with moulting hormone activity. e.g., 22-isoecdysone.

Wing (1988) reported that a non-steroidal benzoyl hydrazine analogue, code named as RH 5849 possesses ecdysone mimetic properties. Treatment of insects with minute doses of RH 5849 interferes with feeding activity and induce a premature lethal moult (Wing, 1988; Sakunthala and Nair, 1995). RH 5849 was found to be effective against insects belonging to various orders. Yet another non-steroidal ecdysone mimic (RH 5992) discovered by Rohm and Haas Company, U.S.A. induces an incomplete moult in *Choristoneura fumiferana* (Palli *et al.*, 1995). This compound also induces spermatogenesis

reinitiation in isolated abdomens of diapausing *Cydia pomonella* larvae (Friedlander and Brown, 1995) RH 5992 acts similar to 20-hydroxyecdysone by binding to the ecdysone receptor (Wing, 1988; Wing *et al.*, 1988). RH 5992 inhibited oviposition in *Spodoptera littoralis* (Smagghe and Degheele, 1997).

1.5. OBJECTIVES OF THE INVESTIGATION

Growth and development in insects, which are punctuated by periods of moulting are regulated by endogenous titres of ecdysteroids, JH and PTTH. These hormones are also involved in the regulation of reproduction of insects. Therefore, information on the occurrence, titre changes and factors regulating the hormone titres is essential to elucidate the role of hormones during insect development and reproduction. Although ecdysteroid titres during postembryonic development have been determined for a number of lepidopteran species, vast differences occur in the time sequence of developmental events in insects. Hence hormone titres obtained from one species at any given point of time may not accurately coincide with the same in another species. Hence the present investigation is undertaken to study the ecdysteroid titre changes in the haemolymph and testes during larval development and metamorphosis of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). This lepidopteran insect is a sporadic pest of paddy in the state of Kerala, in India. This insect has been subjected to a number of experimental studies in our laboratory concerning the effect of insect growth regulators with

hormonal and anti-hormonal activity on larval development, metamorphosis and reproduction (Nair, 1980, 1981, 1993; Sam Mathai and Nair, 1983, 1984a,b,c,d; Santha and Nair, 1986, 1987, 1988; Santha *et al.*, 1987; Nair and Rajalekshmi, 1989; Pradeep and Nair, 1989; Sam Mathai *et al.*, 1989; Balamani and Nair, 1989a,b; Santha and Nair, 1991; Balamani and Nair, 1991, 1992, 1994; Jagannadh and Nair, 1992; Sakunthala and Nair, 1995; Vengopalan *et al.*, 1994; Benny and Nair, 1999). The interpretation of results obtained from these studies was often hampered by the lack of data concerning the timing of release of hormones during larval development and metamorphosis. Hence one of the major objectives of the present investigation is to analyse the timing of release of the principal morphogenetic hormones namely PTTH and JH and determination of haemolymph and testicular ecdysteroid titres. For this, the classical approach of ligation (neck or thorax) extensively utilised in endocrinological laboratories is employed. Further, the effects of exogenous treatment of a JH analogue (JHA), hydroprene and a non-steroidal ecdysteroid agonist, RH 5992 on haemolymph and testicular ecdysteroid titres are also determined. The fluctuations in hormone titres are correlated with morphogenetic changes occurring during metamorphosis of normal insects and those of treated ones. Further, fluctuations in the testicular ecdysteroid content during testicular development and spermatogenesis are studied. The effects of treatments of hormone analogues/agonists on testicular ecdysteroid titres are also analysed. It is

hoped that the results of this investigation will lead to a better understanding of the endocrine regulation of larval development, metamorphosis and spermatogenesis and will form the basis for future investigations on various aspects of metamorphosis and reproductive maturation of *S. mauritia*.

Chapter 1 deals with a detailed review of insect hormones, testicular ecdysteroids and factors regulating their synthesis and release.

Chapter 2 provides basic information on the pest status and a detailed account of the rearing and maintenance of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) under laboratory conditions. The general experimental techniques employed in this investigation are also included.

Chapter 3 examines the haemolymph ecdysteroid profile during larval-pupal and pupal-adult development of *S. mauritia*. It also incorporates the timing of PTTH release during larval-pupal development. Effects of hormones/hormone analogues on the ecdysteroid titres of the haemolymph are also analysed.

Chapter 4 examines the testicular ecdysteroid profile during larval-pupal and pupal-adult development in *S. mauritia*. Effects of hormones/hormone analogues on testicular ecdysteroid titres are also analysed.

CHAPTER 2

MATERIALS AND METHODS

2.1 PEST STATUS OF *S. MAURITIA*

Spodoptera mauritia Boisd. (Lepidoptera : Noctuidae) commonly known as rice swarming caterpillar, or army worm is a sporadic pest of *Oryza sativa*. The pest attack assumes serious proportions and cause considerable damage especially when there is a good start of monsoon followed by a prolonged dry spell. This insect is distributed all over India and usually occurs on paddy from July to September. Large swarms of larvae appear suddenly and destroy whole fields of paddy and then march on to the next. This tendency of the pest to migrate from field to field in large numbers gives it the name army worm. The larvae attack the nursery and early growing stages of paddy. The seedlings in the nursery are cut and completely eaten up as though grazed by cattle. Larval feeding progresses from the leaf margins leaving behind the leaf midribs thus completely destroying the young paddy seedlings. In a severe outbreak, serious damage results in heavy loss of crop. The loss in yield due to larval infestation ranges from ten to twenty percent. The pest status of *Spodoptera mauritia* is further complicated by their ability to migrate to alternate host plants (for eg. *Ischaemum aristatum*) during off-season periods at the end of which they make a full scale comeback on the nursery stages of paddy. *Spodoptera* species have been widely utilised in physiological, biochemical and endocrinological, research, holding the tenth position in the group of insect species often used for research.

2.2. REARING AND MAINTENANCE OF THE LARVAE OF *S. MAURITIA*

The female adult moths which were attracted to the light during night were collected using a sweeping insect net. In the laboratory, these moths were then transferred to glass chimneys closed at both ends with muslin cloth. Adult moths were fed on 10% solution of honey. Cotton swabs soaked in 10% solution of honey were kept in the glass chimneys for this purpose. The female laid eggs on the cloth or on the sides of the chimneys, from which the first instar larvae hatched after 3 days.

The larvae were reared in glass chimneys at the initial stages. The larvae were supplied fresh, tender leaves of the grass *Ischaemum aristatum* collected from paddy fields. The food material was changed every day. Uniform rearing conditions were provided to larvae and they were kept away from wind and intense light. The larvae were maintained at room temperature, RH 90 $\pm$ 3% and 12:12 light : dark, photoperiod regime. Larvae were transferred to large plastic troughs as they grew in size. During summer days, the cloth covering the trough was wetted frequently. The pupae were kept seperately in beakers for adult emergence.

2.3 BIOLOGY OF *S. MAURITIA*

The *S. mauritia* larvae developed at a uniform rate and underwent six larval instars before pupating under the conditions provided in the laboratory.

(i) First instar larva

The newly hatched larvae congregated on the cloth covering of the chimney. The first instar larvae were characterised by the presence of a large, black head shield and light green coloured body. On each segment of the body setigerous, small, wart-like, dark, pigmented tubercles were present which were arranged in a cross wise row. The larvae moved in a characteristic leaping manner. They descended by means of silken threads to the tender grass leaves supplied for feeding. Newly hatched larvae did not feed immediately. The first instar larvae measured about 1 mm in length and 0.5 mm in width. These larvae moulted to the second instar after 2-3 days.

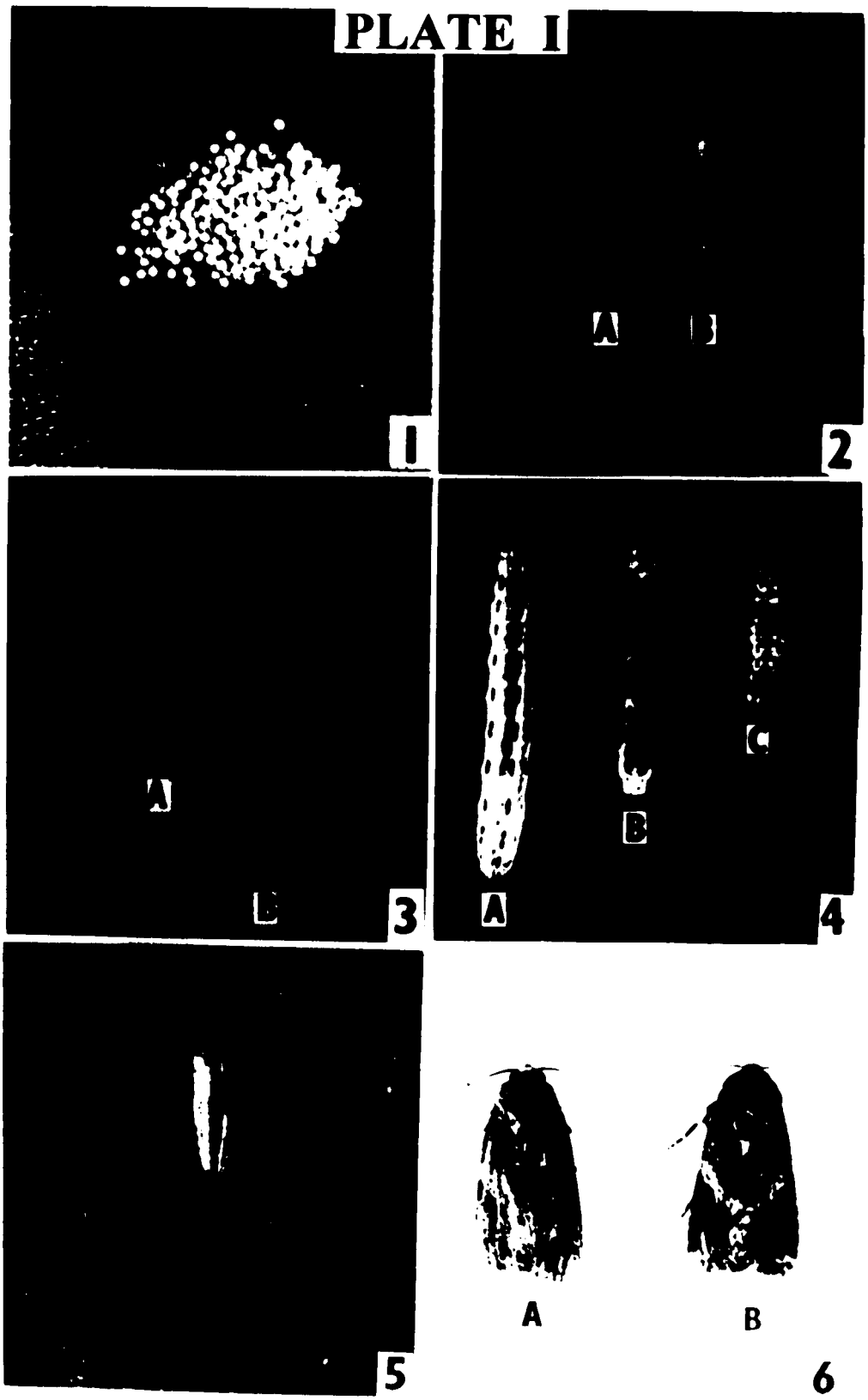
(ii) Second instar larva

In second instar larvae, three white longitudinal stripes appeared on the dorsal surface of the body extending from the prothorax to the last abdominal segment. The larval body was green in colour with two pairs of white, longitudinal, lateral stripes, one pair being more prominent. On each segment, setigerous, small, wart-like, dark tubercles were present. The second instar larvae also descended using silken threads. Newly moulted

PLATE I

Fig. 1. Egg mass
Fig. 2. Fourth instar larvae
A. Day 1 larva
B. Day 3 larva
Fig. 3. Fifth instar larvae
A. Day 1 larva
B. Day 3 larva
Fig. 4. Sixth instar larvae
A. Day 3 larva
B. Day 4 larva (wandering stage)
C. Prepupa
Fig. 5. Pupa
Fig. 6. Adults
A. Female
B. Male

PLATE I



second instar larvae measured about 2.5 mm in length and 0.5 mm in width. The duration of this instar was 2-3 days.

(iii) Third instar larva

The larvae no longer used silken threads to descend to the grass leaves. The third instar larvae possessed three white longitudinal stripes on the dorsal side and two pairs on the lateral side of the body extending from anterior to the posterior end. These larvae also had reddish black supraspiracular stripes extending from anterior to posterior end. The newly moulted third instar larvae measured 6 mm in length and 1 mm in width. After 2-3 days, larvae moulted to fourth instar.

(iv) Fourth instar larva

The three, dorsal longitudinal stripes became dull white in colour. Two lateral reddish black stripes were present, one on each side of the body. Body became greyish in colour. Black intermittent dots appeared on each segment dorsolaterally which broadened towards the later stages of the instar. The dorsum of the larva was paler than the supraspiracular area and was over laid with strands and flecks of brown. The newly moulted larva measured about 11.5 mm in length and 2 mm in width. The fourth instar larval period extended upto 2-3 days (Pl : I , Fig. 2).

of gut purge, larvae start the wandering behaviour which lasts for 24 h. During this period the larvae measured 24 mm in length and 5 mm in width. These larvae wandered about at the bottom of the rearing trough. At the later stages of wandering, the body of larvae shrunk gradually and the movements reduced as the abdominal prolegs got retracted. The larvae thus got transformed into the prepupal stage. The prepupal stage was characterised by a highly wrinkled larva which underwent larval-pupal apolysis after 24 h. The prepupal stage measured 20 mm in length and 5 mm in width.

(vii) Pupal instar

Pupae were of the obtect type, dark brown in colour and measured 16 mm in length and 5 mm in width (Pl : I, Fig. 5) Adults emerged out of the female pupae after 7 days. Male pupae took 8 days for adult emergence.

(viii) Adults

Adults were medium sized moths and had a conspicuous spot on the forewings, which had wavy pattern on the fringe. Insects measured about 15 mm in length and had a wing span of 30-35 mm. Adult moths exhibited sexual dimorphism in their morphological characters. Males were dark greyish with white markings on forewings and were provided with large tufts of hairs on the forelegs. Females lacked both these white markings and tufts of hairs (Pl : I, Fig.6). Mating took place in the night within 24 h after

emergence. Egg laying commenced 24 h after mating. Eggs were laid in masses of 100-500 each and were covered with buff coloured silken hairs (Pl : 1,Fig. 1). On the whole, embryonic period lasted 2-3 days, larval period 19-23 days and pupal period 7-8 days.

2.4 EXPERIMENTAL ANIMALS

The larvae/pupae/adults used for various experiments were collected from the laboratory colony reared and maintained as described above. Newly moulted larvae were isolated from the stock culture and reared in separate containers to facilitate the synchronous development of larvae and also for the precise determination of the age of the larvae used for experimental work. Larvae which showed head capsule slippage were kept isolated on the day prior to the moulting. The newly moulted larvae which had pale colour were separated on the day of the ecdysis itself. In the case of sixth instar larvae, to observe the determination and timing of gut purge, the experimental larvae were placed in separate containers, the bottom of which was lined with filter paper in order to absorb the fluid faeces. Gut purged larvae were separated using the criteria of loss of weight, colour change and the observation of fluid faeces in the filter paper. Sexing was possible in the late fifth instar stage when the undifferentiated testes became clearly visible through the transparent cuticle of male larvae. Insects were sexed in the fifth instar larval stage and reared separately. The experimental insects were reared and

maintained as in the case of stock culture. The percentage of mortality during each instar was also observed.

Prepupae were separated from the stock culture to obtain newly moulted pupae. Newly ecdysed (day 0) pupae were easily recognised as they appeared pale cream coloured. Soon tanning of the pupal cuticle commenced changing the colour to light brown and finally dark brown. Adults (male) emerged after 8 days.

The age of larvae and pupae and adults were abbreviated to day *n* where day 0 indicates the day of ecdysis to this stage. Newly ecdysed larvae/pupae/adults were treated as day 0, larvae/pupae/adults 36 h old as day 1 and so on. Slight variations in the intermoult duration was frequently observed in the laboratory stock culture. So larvae showing synchronous development from the same egg mass were utilised for conducting experiments.

2.3 GENERAL EXPERIMENTAL TECHNIQUES

In order to analyse the role of hormones in insect postembryonic development especially during metamorphosis and spermatogenesis, the classical approach of neck/thorax ligation to eliminate the endogenous source of hormones and the hormone replacement therapy were employed. Sixth instar larvae of day 1 and day 4 were used for the experiments. Larvae of

these age groups were selected because the head critical periods occur supposedly on day 1 and day 4 of sixth instar larval stage. Formation of spermatids and initial differentiation of sperm bundles also take place at this stage (Venugopalan *et al.*, 1994). In one set of experiments, sixth instar larvae were 'neck-ligated' to remove the endogenous source of cerebral neurosecretory factors and JH. In another set, the sixth instar larvae were 'thorax-ligated' to remove secretions of the cerebral neurosecretory cells and prothoracic glands.

In yet another series of experiments, neck-/thorax-ligated day 1/day 4 larvae and pupae were topically treated with a JHA, hydroprene or with a non-steroidal ecdysteroid agonist, RH 5992. The effects of these treatments on metamorphosis and spermatogenesis were carefully analysed.

Ligations

Sixth instar day 1 and day 4 larvae were mildly anaesthetised using diethyl ether and tightly ligated using a cotton thread. Ligations were carried out either around the neck or between the pro- and mesothorax. The former is designated as 'neck-ligated' larvae which lacked brain and corpora allata and the latter as 'thorax-ligated', which lacked not only brain with retrocerebral endocrine glands but also prothoracic glands.

Chemicals

The juvenile hormone analogue (JHA), hydroprene (ethyl 3,7,11-trimethyl dodeca-2,4-dienoate) was a gift from Dr. G.B. Staal, Zoecon Corporation, California, USA. The non-steroidal ecdysteroid agonist RH 5992 (Tebufenozide, 1,2-dibenzoyl-1-tert-butyl hydrazide) was obtained as a gift from Rohm and Haas Company, Spring House, Pennsylvania, USA.

JHA or RH 5992 was dissolved in acetone to prepare a solution of 1 μg JHA/RH 5992 per μl acetone or 5 μg JHA/RH 5992 per μl acetone. For various treatment procedures, measured quantities of these compounds were used utilizing a Hamilton microsyringe.

Treatments

(a) Treatment of hydroprene on unligated larvae

Sixth instar day 0 larvae were treated topically with repetitive daily dose of 20 μg JHA (day 0 – day 3) on the abdominal tergites using the microsyringe. Subsequent treatments were done at approximately the same time of the day. Haemolymph was extracted and the testes dissected from day 1 onwards. Control larvae were treated with the same volume of acetone.

(b) Treatment of RH 5992 on unligated larvae

Sixth instar day 0 and day 4 larvae were treated topically with a single dose of 2 μg or 5 μg RH 5992 on the abdominal tergites using the microsyringe. Control larvae were treated with the same volume of acetone.

(c) Treatment of hydroprene on ligated larvae

Neck-/thorax-ligated sixth instar day 1 larvae were treated topically with a daily dose of 5 μg JHA (day 1 – day 3) on the abdominal tergites, using the microsyringe. The first dose was given thirty minutes after ligation and the second and third doses were given at the same time on subsequent days. Haemolymph was extracted and the testes dissected from the treated larvae on the next day of completion of hormone treatment. In a similar manner, neck-/thorax-ligated day 4 larvae were treated with a single dose of 5 μg JHA and the haemolymph was extracted and the testes dissected on the subsequent days. Control larvae were treated with a similar volume of acetone.

(d) Treatment of RH 5992 on ligated larvae

Neck-/thorax-ligated sixth instar day 1 and day 4 larvae were treated topically with a single dose of 2 μg or 5 μg or 10 μg RH 5992 on the abdominal tergites using the microsyringe. Haemolymph was extracted and the testes dissected from the treated larvae on the day of treatment and on all

subsequent days of survival. Control larvae were treated with the same volume of acetone.

(e) Treatment of hydroprene on newly ecdysed pupae

Newly ecdysed pupae (day 0) of *S. mauritia* were treated topically with 0.5 µg and 1 µg JHA using the microsyringe. Control pupae were treated with the same volume of acetone. The haemolymph was extracted and the testes dissected from the treated pupae on all days of survival.

(f) Treatment of RH 5992 on newly ecdysed pupae

Newly ecdysed pupae (day 0) of *S. mauritia* were treated topically with 5 µg or 10 µg RH 5992 using the microsyringe. Control pupae were treated with the same volume of acetone. The haemolymph was extracted and the testes dissected from the treated pupae on day 2 and day 5 of the pupal stadium.

Preparation of haemolymph samples for RIA

Haemolymph was extracted in 300 µl of 75% ice cold methanol in 1.5 ml Eppendorf tubes. The methanolic extracts were vortexed, refrigerated for at least 30 minutes and centrifuged at 14,000 rpm at 4°C for 5 min. Aliquots of the supernatant were evaporated in vacuo in 6 x 50 mm borosilicate tubes using Savant speedvac lyophiliser and stored in the freezer. Ecdysteroid titres were determined by radioimmunoassay following the methods of Borst and

O'Connor (1974), Bollenbacher *et al.* (1975) and Gelman *et al.* (1997). The antiecdysone, a gift from W.E. Bollenbacher was prepared from a hemisuccinate derivative of ecdysone (at the C-22 hydroxyl group) that had been coupled to thyroglobulin. Competitive studies showed that the antibody had a high affinity for ecdysone, 20-hydroxyecdysone and makisterone A (Gilbert *et al.*, 1977; Kelly *et al.*, 1981). Standards (50-4000 pg) were prepared from 20-hydroxyecdysone (Calbiochem. Corp., La Jolla, CA). The radioactively labelled antigen was tritiated ecdysone (63.5 Ci/mmol: New England Nuclear, Boston, M.A., USA). The antigen-antibody complex was precipitated with saturated ammonium sulphate yielding a final concentration of 50% ammonium sulphate. The precipitate was washed with 50% ammonium sulphate and then dissolved in 25 µl of distilled water. After the addition of 300 µl of Ecoscint A (National Diagnostics, Atlanta, GA, USA), each tube was vortexed and counted for [^{3}H] in a Beckman (Columbia, MD, USA) LS 3801 Scintillation Counter. The concentration of ecdysteroids was determined from curves generated by the 20-hydroxyecdysone standards (50-4000 µg) that had been assayed with the samples, and was expressed as pg 20-hydroxyecdysone equivalents/µl haemolymph.

Preparation of testes samples for RIA

The testes were homogenised in 1.5 ml Eppendorf tubes containing 100 µl ice cold 75% methanol using a plastic homogenizer. The homogenate and a

200 µl wash of the homogenizer were vortexed, refrigerated for at least 30 min and centrifuged at 14,000 rpm at 4°C for 5 min. Aliquots of the supernatant were evaporated in vacuo in 6 x 50 mm borosilicate tubes using Savant speedvac lyophiliser and stored in the freezer. Ecdysteroid titres were determined by radioimmunoassay by the methods described earlier.

Statistical analysis of data

Ecdysteroid titres of the haemolymph and testes have been represented as line graph or as histogram. Each point or column in the graph/histogram represents the mean of at least three separate determinations. Student t-tests were employed to determine whether haemolymph/testicular ecdysteroid peaks were significant. The mean peak value was compared with the two mean baseline values on either side, not necessarily two adjacent points, but rather points that delineated the peak from the rest of the curve.

CHAPTER 3

DEVELOPMENTAL PROFILE OF HORMONES DURING METAMORPHOSIS AND THE EFFECTS OF HORMONE MIMICS

3.1 INTRODUCTION

During metamorphosis in lepidopteran insects, developmental changes occur in a sequential order as has been demonstrated in *Bombyx mori* by Kiguchi *et al.* (1985). These sequential events have been assumed to be under the control of ecdysteroids, JH and brain hormones (For review see Gilbert *et al.*, 1996). Changes in haemolymph titre of ecdysteroids have been determined in the last larval stadium of various lepidopteran insects and a small ecdysteroid peak occurs immediately before gut purge (Dean *et al.*, 1980; Smith, 1985). The small peak coincides with the occurrence of pupal commitment of epidermal cells in *Manduca sexta* (Bollenbacher *et al.*, 1975; Riddiford, 1978) and is thus designated as the commitment peak. The low ecdysteroid titre peaks before the gut purge have been correlated with initiation of nucleolar activity, RNA synthesis and organelle formation in the fat body and epidermis (Dean *et al.*, 1980) and development of wing discs in *Bombyx* (Kawasaki, 1995). A major peak precedes pupal development which triggers apolysis and subsequent events that lead to pupation (Riddiford, 1976). During pupal-adult development, a single large peak occurs in the first half of pupal stage which probably promotes apolysis and pharate adult development in all the lepidopterans examined. However, the prothoracic glands have been reported to undergo apoptosis (programmed cell death)

during the pupal stage (Dai and Gilbert, 1997). Hence the glandular source of ecdysteroids during pupal stage is still unclear.

Prothoracic glands of both larvae (Gibbs and Riddiford, 1977; Mala *et al.*, 1977) and pupae are stimulated by PTTH from the brain (Agui *et al.*, 1980). The head critical periods (HCP) in the last larval stadium of lepidopteran insects have been determined by neck ligation. HCP has been defined as the period during which a linearly increasing percentage of neck-ligated larvae exhibit dorsal vessel exposure during the same gate as intact larvae (Truman and Riddiford, 1974). However, later studies suggested that HCP signifies a change in the responsiveness of the prothoracic glands to PTTH (Sakurai *et al.*, 1998).

In addition to environmental factors, the release of PTTH is regulated by endogenous factors such as JH. At the beginning of the last larval instar, JH may have a direct inhibitory effect on prothoracic gland function (Watson and Bollenbacher, 1988; Sakurai *et al.*, 1989), in addition to suppressing PTTH release (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). In contrast to the inhibitory effect of JH early in the final instar, JH exerts a stimulatory effect following the commitment peak of ecdysteroids. At this time, JH increases haemolymph ecdysteroids and accelerates pupation (Cymborowski and Stolarz, 1979; Safranek *et al.*, 1980; Gruetzmacher *et al.*, 1984b). Juvenoids have also been reported to prevent apoptosis of pupal

prothoracic glands and promote synthesis of ecdysteroids in the treated pupae (Dai and Gilbert, 1998).

The prothoracic glands have been shown to possess ecdysteroid receptors (Bidmon and Sliter, 1990). Hence ecdysteroids or ecdysteroid agonists (RH 5849, RH 5992) themselves may have a feedback effect on the prothoracic glands (Sakurai and Williams, 1989; Warren and Hetru, 1990). Further the nature of the ecdysteroid feedback effect (positive or negative) depends upon the secretory history of the prothoracic glands (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989). The present study attempts to identify the timing of release of the principal morphogenetic hormones PTTH and JH and determine the haemolymph ecdysteroid profile during larval-pupal and pupal-adult development of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). Further, this study examines the effects of exogenous treatments of a JH analogue (JHA), hydroprene and a non-steroidal ecdysteroid agonist, RH 5992 on haemolymph ecdysteroid titres. The present study also demonstrates that the fluctuations in the titre of ecdysteroids can be correlated with specific developmental events occurring during larval-pupal and pupal-adult metamorphosis.

3.2 MATERIALS AND METHODS

Animals

Larvae and pupae of *S. mauritia* were taken from the laboratory stock culture reared and maintained as described previously. The present studies were conducted on sixth instar larvae (final instar) and pupae of various ages.

Ligations

Ligations were done as described earlier (see page 45).

Chemicals

Chemicals were obtained from various sources as mentioned earlier (see page 46).

Treatments

Larvae and pupae were treated with different dosages of chemicals as described previously (see page 46).

Collection of haemolymph

Sixth instar larvae of different age groups were mildly anaesthetised with diethyl ether and 5-20 µl of haemolymph was collected in a calibrated micropipette through an incision made on a proleg. Pupal haemolymph (2 µl) was collected in a similar manner by means of punctures made near the head

region. Haemolymph samples could not be taken from adults owing to the fat body dispersed in the haemolymph.

Preparation of haemolymph samples for RIA

Haemolymph samples were prepared (see page 48) and RIA done as described earlier.

3.3 RESULTS

3.3.1 Haemolymph ecdysteroid titres of larvae, pupae and pharate adults

Sixth (final) instar larvae of *S. mauritia* fed voraciously during the first three days. On day 4, the larvae stopped feeding, emptied their guts (gut purge) and transformed into wandering stage. Wandering larvae showed a reduction in size and transformed into prepupae by day 5. The prepupal stage is characterised by a highly wrinkled body. Prepupae underwent pupation within 20-24 h. On the whole, the sixth instar larval period extended up to 6 days. The pupal period lasted 7 days in females and 8 days in males. Dissection of pupae revealed that pharate adult development commenced on day 4.

The present study was undertaken to determine haemolymph ecdysteroid titres of male sixth instar larvae, pupae and pharate adults. Ecdysteroid titres in the haemolymph samples were determined by means of

RIA and the results correlated with known developmental events occurring during larval-pupal-adult development and metamorphosis of *S. mauritia*. For a few stages sampled, haemolymph ecdysteroid titres of males were not significantly different from those of females. Results are expressed as pg 20-hydroxyecdysone equivalents/µl haemolymph.

(i) (a) Haemolymph ecdysteroid titres during sixth instar larval stadium

Haemolymph ecdysteroid titres for sixth instar larvae of *S. mauritia* are shown in Fig. 7. In the newly moulted (white head capsule) sixth instar larvae (day 0), the titres were 37 ± 21 pg/µl which decreased to 15 ± 2 pg/µl on day 1. Hormone titres gradually increased on day 2 (26 ± 5 pg/µl) to form a peak on day 3 (39 ± 20 pg/µl). Titres increased to more than 100 pg/µl haemolymph for the first time on day 4 (372 ± 13 pg/µl). It may be recalled that on day 4, larvae exhibit gut purge and show wandering behaviour. The increasing trend of hormone titres continued on day 5, the prepupal stage when it rose steeply to reach its maximum (2524 ± 453 pg/µl). Only the peak on day 5 was significant ($P < 0.05$).

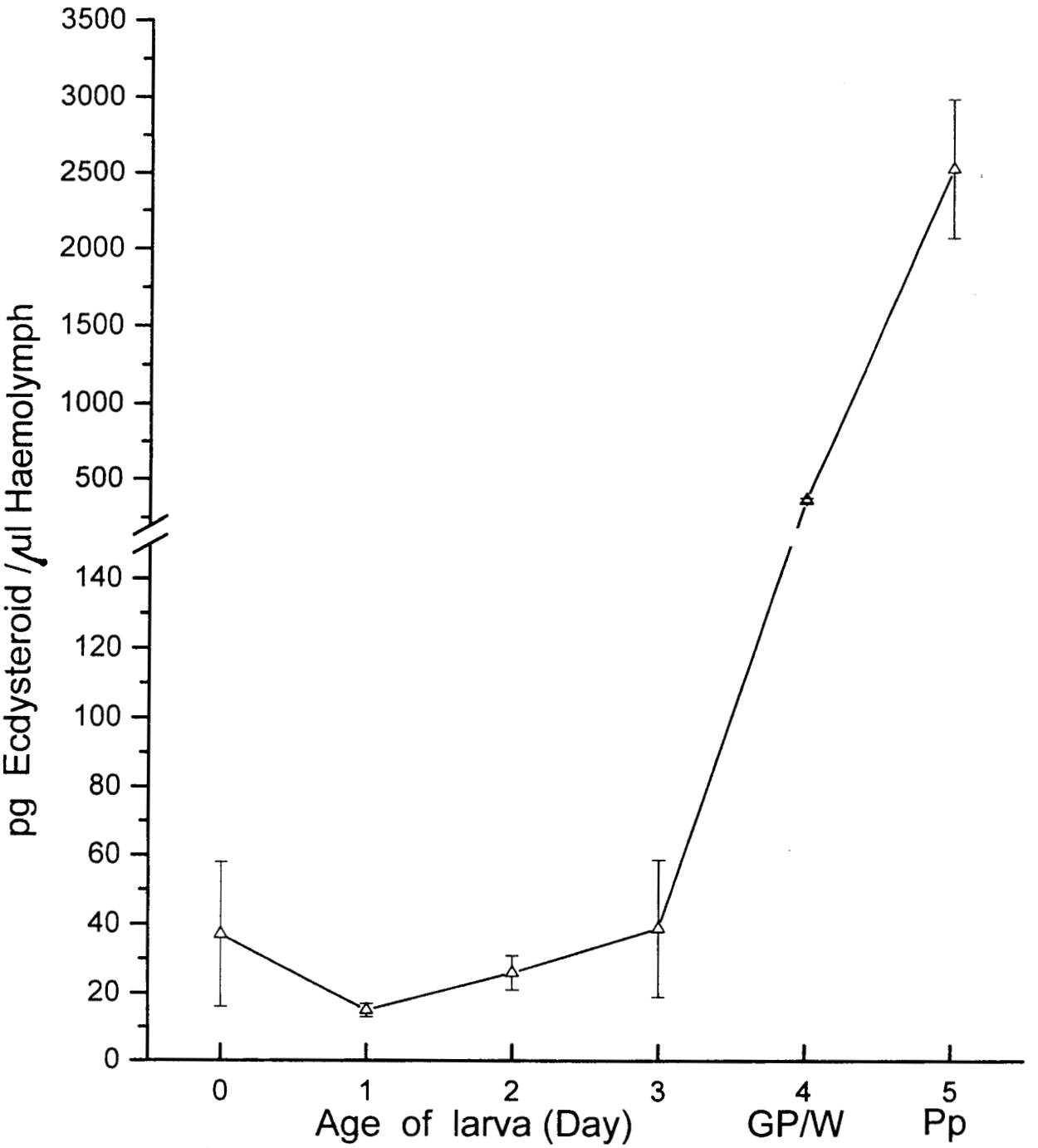


Fig. 7 Changes in haemolymph ecdysteroid titres during the sixth instar larval development.

GP – Gut purge ; W - Wandering ; Pp – Prepupa

(b) Changes in haemolymph ecdysteroid titres of sixth instar larvae at 6 h intervals

Ecdysteroid titres of sixth instar larvae from 0 h to 132 h post ecdysis are shown in Fig. 8. In the newly moulted (white head capsule) sixth instar larvae (day 0), titres were just under 15 pg/µl namely, 11 ± 4 pg/µl, 9 ± 3 pg/µl, and 4 + 0 pg/µl at 0 h, 6h and 12 h respectively. The small peak (Peak I) (21 ± 3 pg/µl) observed at 18 h (day 1) was significant at $P < 0.05$ (relative to ecdysteroid titres at 12 and 24 h). The titres then dropped to 6 ± 3 pg/µl at 24h, increased slightly at 30 h (7 ± 3 pg/µl) and then showed a small peak (Peak II) at 36 h (16 ± 5 pg/µl). There was a reduction in titres on day 2, at 42 h and 48 h when the titres were 10 ± 3 pg/µl and 4 ± 1 pg/µl respectively. A gradual rise occurred at 54 h (10 ± 2 pg/µl) leading to a significant peak (Peak III) at 60 h (47 ± 17 pg/µl) ($P < 0.1$, relative to titres at 54 and 78 h). After the 60 h stage, the titres gradually decreased on day 3 at 66 h (19 ± 9 pg/µl), increased slightly at 72 h (25 ± 0 pg/µl) and then declined at 78 h and 84 h to 10 ± 2 pg/µl and 15 ± 2 pg/µl. The hormone profile increased considerably on day 4 at 90 h and 96 h to 58 ± 15 pg/µl and 278 ± 56 pg/µl respectively and developed a shoulder at 102 h (156 ± 25 pg/µl). The larvae purged their guts at 96 h. An increase in hormone titres was observed towards the end of day 4 at 108 h (492 ± 78 pg/µl). This was the first instance in the sixth instar larval stage when hormone titres increased beyond 100 pg/µl haemolymph.

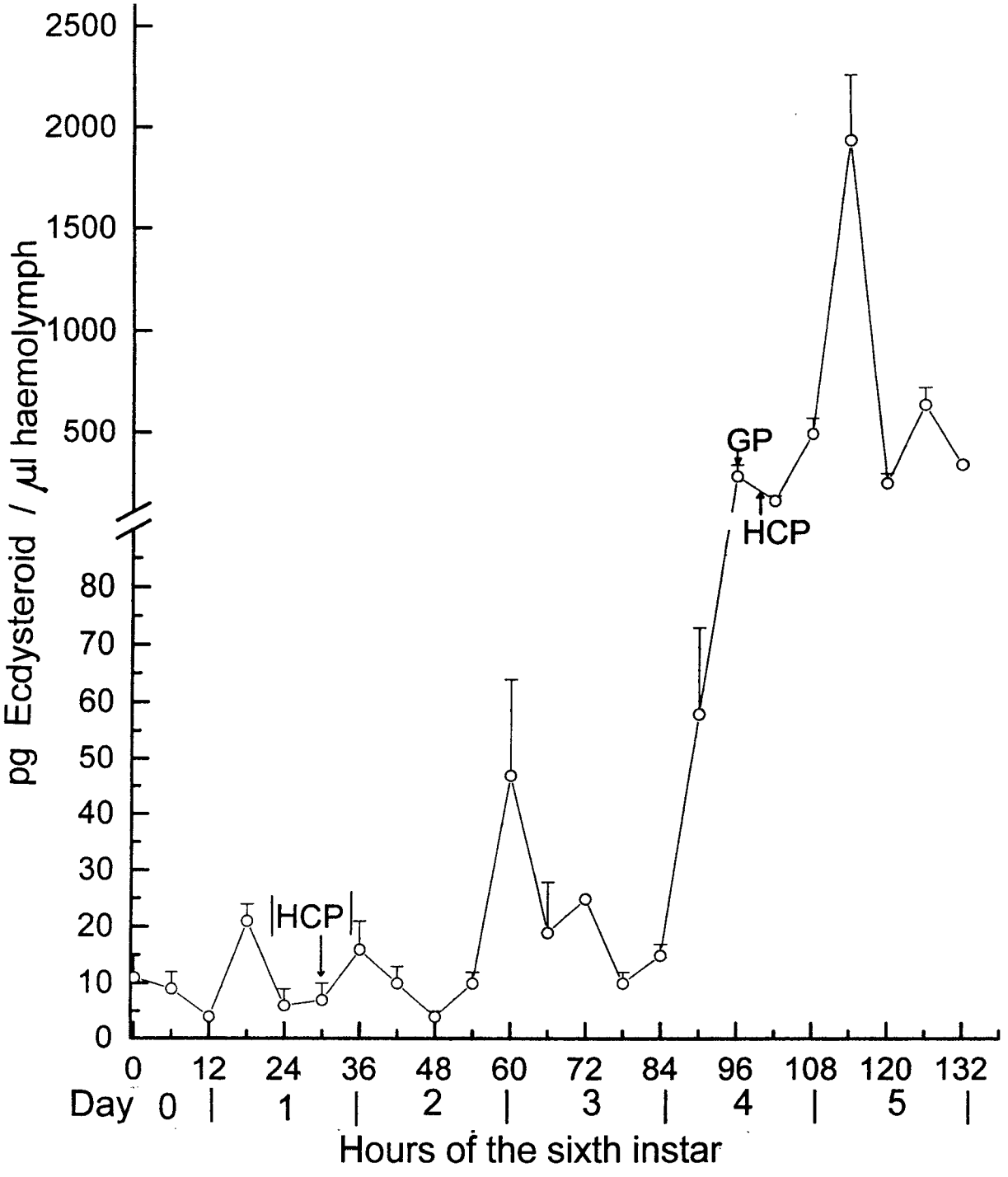


Fig. 8 Changes in haemolymph ecdysteroid titres at six hour intervals during the sixth instar larval development.
GP – Gut purge ; HCP – Head critical period

Hormone titres rose steeply thereafter and reached a significant peak on day 5 at 114 h (1924 ± 324 pg/µl) ($P < 0.05$, relative to titres at 84 and 120 h). This formed the premoult peak (Peak IV) in *S. mauritia* after which a decrease was seen at 120 h (243 ± 48 pg/µl) and then a small but significant peak (Peak V) at 126 h (638 ± 85 pg/µl) ($P < 0.05$, relative to titres at 120 and 132 h) which decreased at 132 h (336 ± 34 pg/µl).

(ii) Haemolymph ecdysteroid titres during pupal stadium

Ecdysteroid titres of haemolymph from insects 0 - 7 days post-pupation are shown in Fig. 9. The haemolymph titres were low in the newly moulted pupae (day 0, 686 ± 63 pg/µl). Titres increased on day 1 (1973 ± 521 pg/µl) after which it rose rapidly and formed a significant peak on day 2 (14221 ± 1458 pg/µl, $P < 0.05$ relative to ecdysteroid titres on day 1 and $P < 0.1$ relative to ecdysteroid titres on day 5). Thereafter, hormone titres decreased gradually on day 3 (7831 ± 478 pg/µl), day 4 (5915 ± 1159 pg/µl), day 5 (894 ± 74 pg/µl), day 6 (574 ± 186 pg/µl) and day 7 (467 ± 185 pg/µl). Titres reached a minimum on day 7 which was almost the same as that on day 0.

3.3.2 Determination of head critical period (HCP) during larval-pupal transformation

This study was undertaken to assess the temporal patterns of PTTH secretion during larval-pupal transformation of *S. mauritia*. Sixth instar

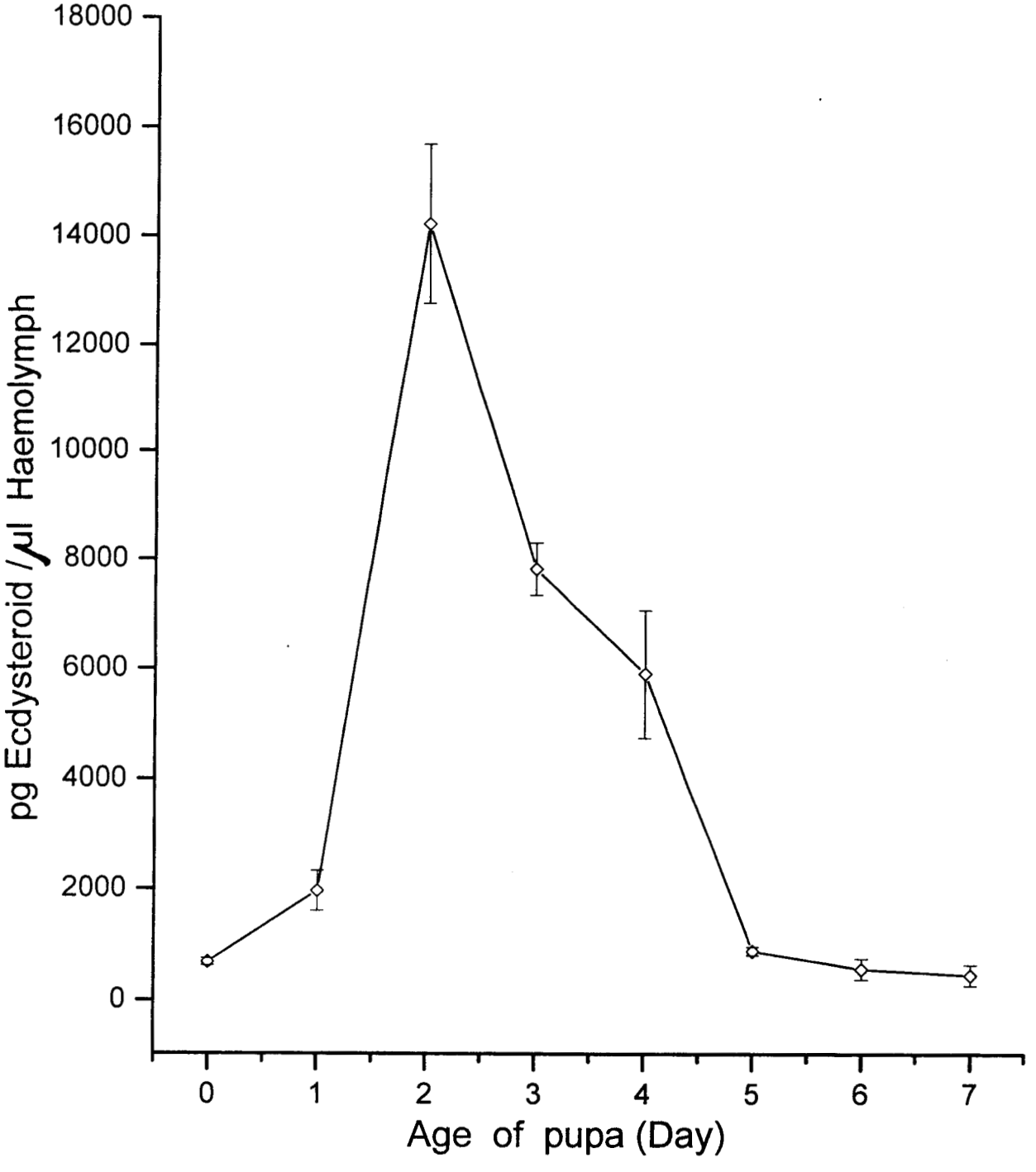


Fig. 9 Changes in haemolymph ecdysteroid titres during pupal development.

larvae were 'neck-ligated' at six hour intervals from the time of ecdysis to the sixth instar to remove the endogenous sources of cerebral neurosecretory factors and JH. The ligated larvae were then closely observed for signs of gut purge and pupal cuticle secretion.

The duration of development of normal, unligated sixth instar larvae of *S. mauritia* was 6 ± 0 days. Sixth instar larvae at the termination of the phagoperiod exhibited gut purge which occurred 95 ± 1 hours after ecdysis. These larvae underwent pupation 48 h after gut purge. These developmental phenomena, i.e. gut purge and pupation have been utilised to assess the temporal patterns of PTTH secretion during larval-pupal transformation. Fig. 10 shows the effect of neck ligation upon the timing of gut purge and pupation. Larvae ligated within 24 h showed no developmental response during their survival of seven days. Of the larvae ligated at 30 h and 36 h, 70% showed gut purge after 4.5 ± 0.5 days. The incidence of gut purged larvae was 100% when ligations were performed at 42 h, 48 h, 54 h, 60 h, 66h, 72 h, 78 h, 84 h, and 90 h. The time taken for gut purge was broadly distributed over a period of 5-7 days. The neck-ligated larvae survived for 11 ± 1 days but never underwent pupation. Larvae neck-ligated at 96 h survived for 12 ± 1 days but never pupated. Post-feeding larvae neck-ligated at 102 h and 108 h after ecdysis underwent pupation in 7 ± 1 days. Occurrence of pupation was 100% when prepupae were neck-ligated. Prepupae neck-ligated at 120 h,

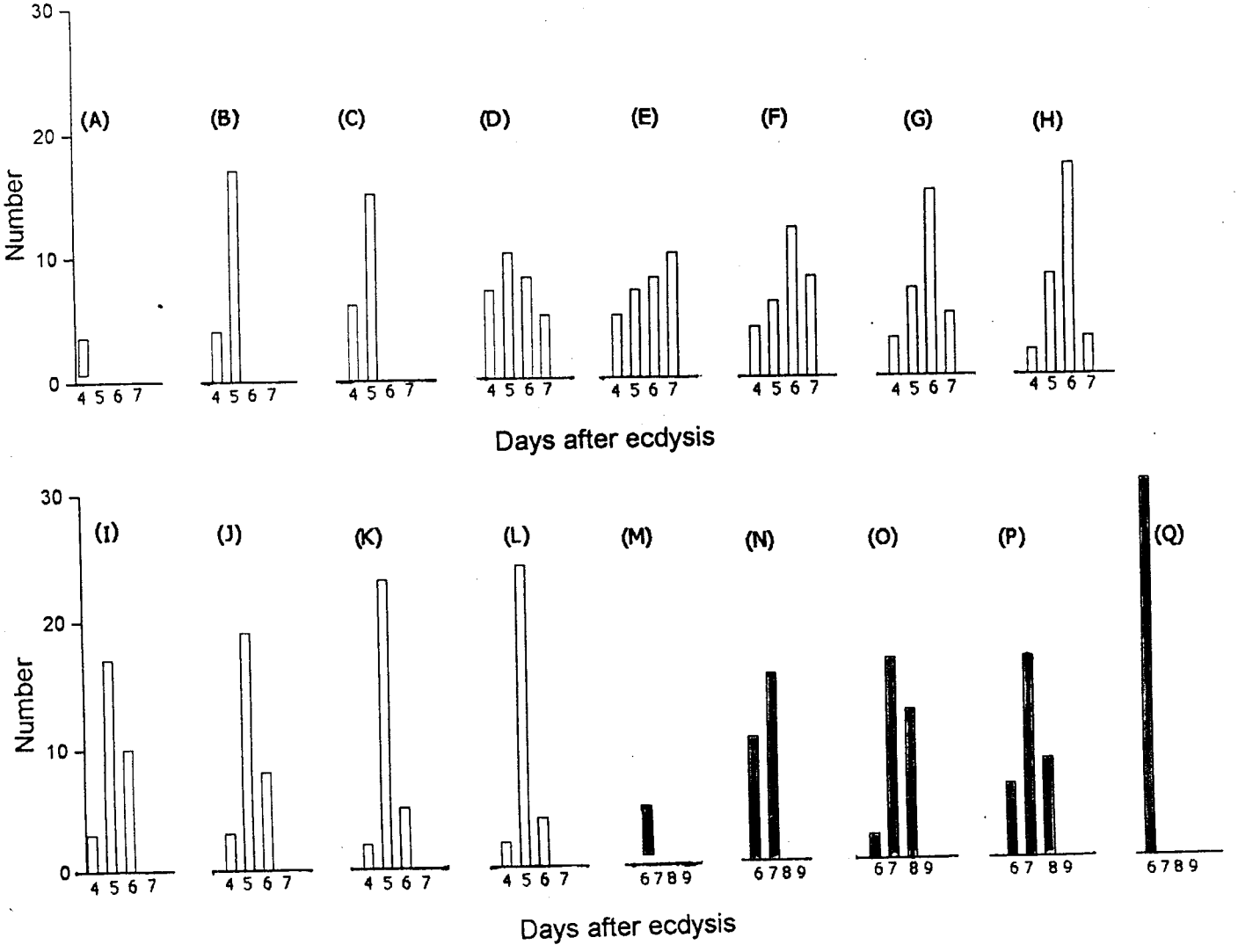


Fig. 10 **The timing of gut purge (open column) and pupation (black) in sixth instar larvae neck-ligated at (A) 24h (B) 30h (C) 36h (D) 42h (E) 48h (F) 54h (G) 60h (H) 66h (I) 72h (J) 78h (K) 84h (L) 90h (M) 96h (N) 102h (O) 108h (P) 114h (Q) 120h (n=30)**

126 h, 132 h and 138 h underwent pupation in 6 ± 0 days. The tentative timings of the two PTTH releases (HCP) are illustrated in Fig. 8.

3.3.3 Effects of ligations of sixth instar larvae on metamorphosis and haemolymph ecdysteroid titres

This study was undertaken to investigate the role of brain factors and prothoracic gland secretions on metamorphosis and haemolymph ecdysteroid titres of *S. mauritia*. In one set of experiments, sixth instar larvae of day 1 and day 4 were 'neck-ligated' to remove the endogenous source of cerebral neurosecretory factors and JH. In another set of experiments, sixth instar larvae of day 1 and day 4 were 'thorax-ligated' to eliminate the endogenous sources of both head factors (cerebral neurosecretory factors and JH) and prothoracic gland secretions. Neck-/thorax-ligated larvae were closely observed for signs of gut purge/pupal cuticle secretion.

(i) Effects of ligations of sixth instar day 1 larvae on metamorphosis and haemolymph ecdysteroid titres

Earlier studies have suggested that the head critical period for gut purge occurred between 24 h and 36 h on day 1 followed by the commitment peak of ecdysteroids on day 3. Therefore in this experiment, sixth instar larvae were neck-/thorax-ligated on day 1 and the effects on metamorphosis and haemolymph ecdysteroid titres were studied. Seventy percent of larvae

Table 1. **Effects of ligations of sixth instar day 1 larvae on metamorphosis**

Nature of ligation	*n*	Days of survival (Mean ± SD)	% of surviving larvae which showed:		
			No change	Gut purge	Pupation
Neck-ligated	25	9 ± 1	30	70	--
Thorax-ligated	25	9 ± 1	30	70	--
Unligated (Control)	15 each	6 ± 0	--	100	100

Table 2. **Effects of ligations of sixth instar day 4 larvae on metamorphosis**

Nature of ligation	*n*	Days of survival (Mean ± SD)	% of surviving larvae which showed:	
			No change	Pupation
Neck-ligated	25	4 ± 1	100	--
Thorax-ligated	25	4 ± 1	100	--
Unligated (Control)	15 each	2 ± 0	--	100

neck-/thorax-ligated on day 1 exhibited gut purge. Although the ligated larvae survived for 9 ± 1 days, none of them pupated while all the control larvae underwent gut purge and became pupae on day 6 (Table 1).

Ecdysteroid titres of experimental and control larvae from days 1-4 are shown in Fig. 11. Hormone titres in the neck-ligated larvae for day 1, day 2, day 3 and day 4 were 8 ± 2 pg/µl, 5 ± 1 pg/µl, 5 ± 1 pg/µl and 6 ± 1 pg/µl haemolymph respectively. Ecdysteroid titres for thorax-ligated larvae on day 1, day 2, day 3 and day 4 were 6 ± 1 pg/µl, 5 ± 0 pg/µl, 4 ± 2 pg/µl and 2 ± 1 pg/µl haemolymph respectively. Hormone titres of larvae kept as controls were 15 ± 2 pg/µl, 26 ± 5 pg/µl, 39 ± 20 pg/µl and 372 ± 13 pg/µl haemolymph on day 1, day 2, day 3 and day 4 respectively. Neck/thorax ligation of day 1 sixth instar larvae induced a significant reduction in haemolymph ecdysteroid titres ($P < 0.1$) on all days when compared to those of control larvae. Control larval titres exhibited an increasing trend from days 1-4.

(ii) Effects of ligations of sixth instar day 4 larvae on metamorphosis and haemolymph ecdysteroid titres

Earlier studies have clearly demonstrated that the head critical period for moulting in post-feeding larvae occurs during day 4 followed by the moulting peak of ecdysteroids on day 5. Hence it was thought worthwhile to investigate the effects of ligation of sixth instar day 4 larvae on larval-pupal

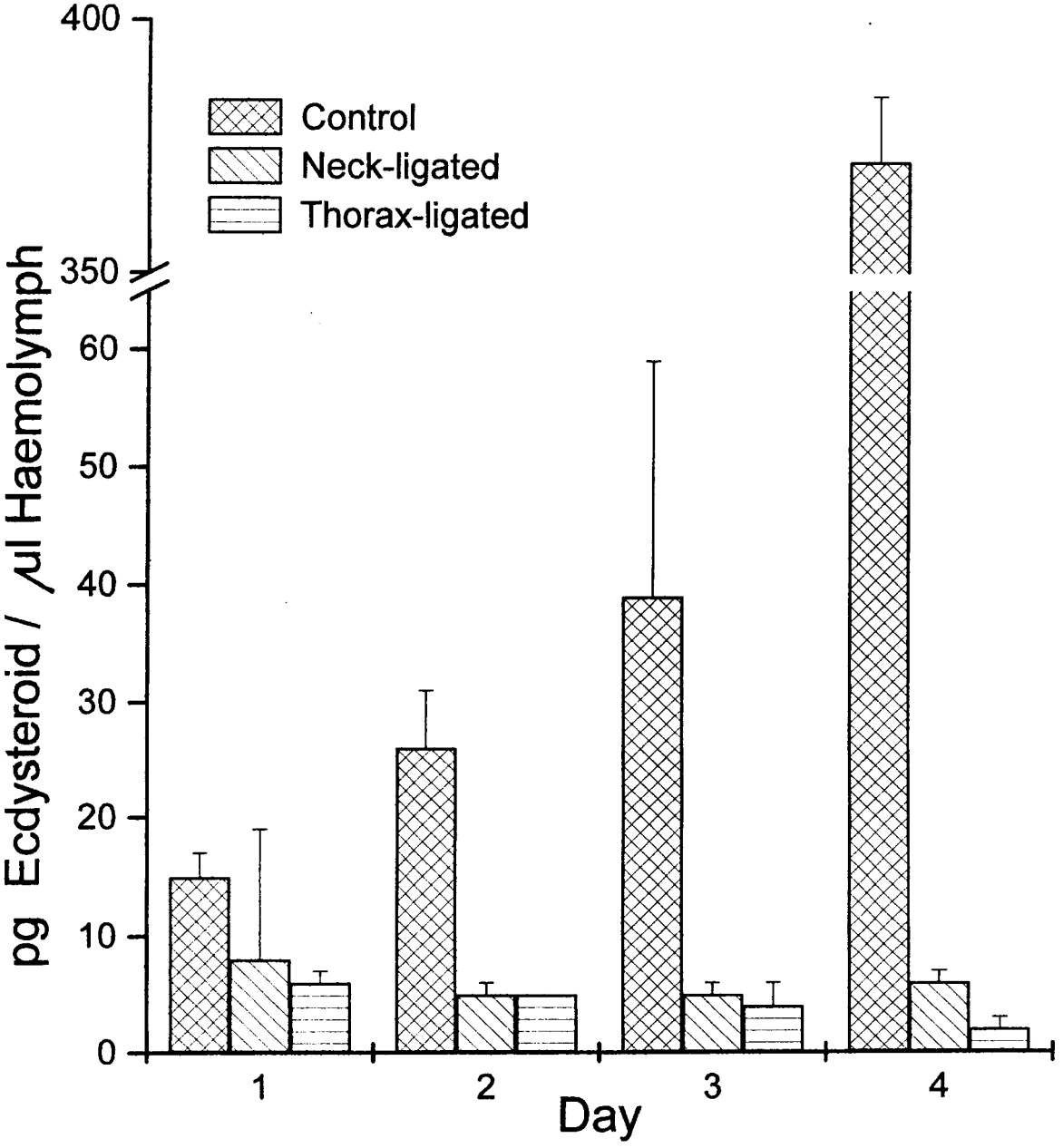


Fig.11 Effects of neck/thorax ligation of day 1 larvae on haemolymph ecdysteroid titres

metamorphosis and haemolymph ecdysteroid titres. Neck/thorax ligation of day 4 larvae resulted in a complete inhibition of pupation. The ligated larvae survived for 4 ± 1 days and died without pupating while control larvae underwent pupation on day 6 (Table 2).

Ecdysteroid titres of experimental and control larvae from days 4-8 are shown in Fig. 12. Hormone titres of neck-ligated larvae were 330 ± 29 pg/µl on day 4, 385 ± 69 pg/µl on day 5, 570 ± 195 pg/µl on day 6, 989 ± 377 pg/µl on day 7 and 1785 ± 453 pg/µl on day 8. Ecdysteroid titres of thorax-ligated larvae were 309 ± 151 pg/µl, 315 ± 119 pg/µl, 124 ± 29 pg/µl, 145 ± 53 pg/µl and 148 ± 8 pg/µl on day 4, day 5, day 6, day 7 and day 8 respectively. Ecdysteroid titres of control unligated larvae on the other hand were 372 ± 13 pg/µl on day 4 and 2524 ± 453 pg/µl on day 5. Neck ligation of day 4 larvae does not prevent the normal increase in haemolymph ecdysteroid titres. Haemolymph ecdysteroid peak on day 8 in the neck-ligated larvae was not significantly different from the ecdysteroid peak on day 5 in the control larvae. Thorax ligation led to a significant reduction ($P < 0.05$) in haemolymph ecdysteroid titres when compared to that of control larvae on day 5. Conversely, titres in the control (unligated) larvae increased significantly ($P < 0.05$) to reach its peak on day 5 and pupation occurred on day 6.

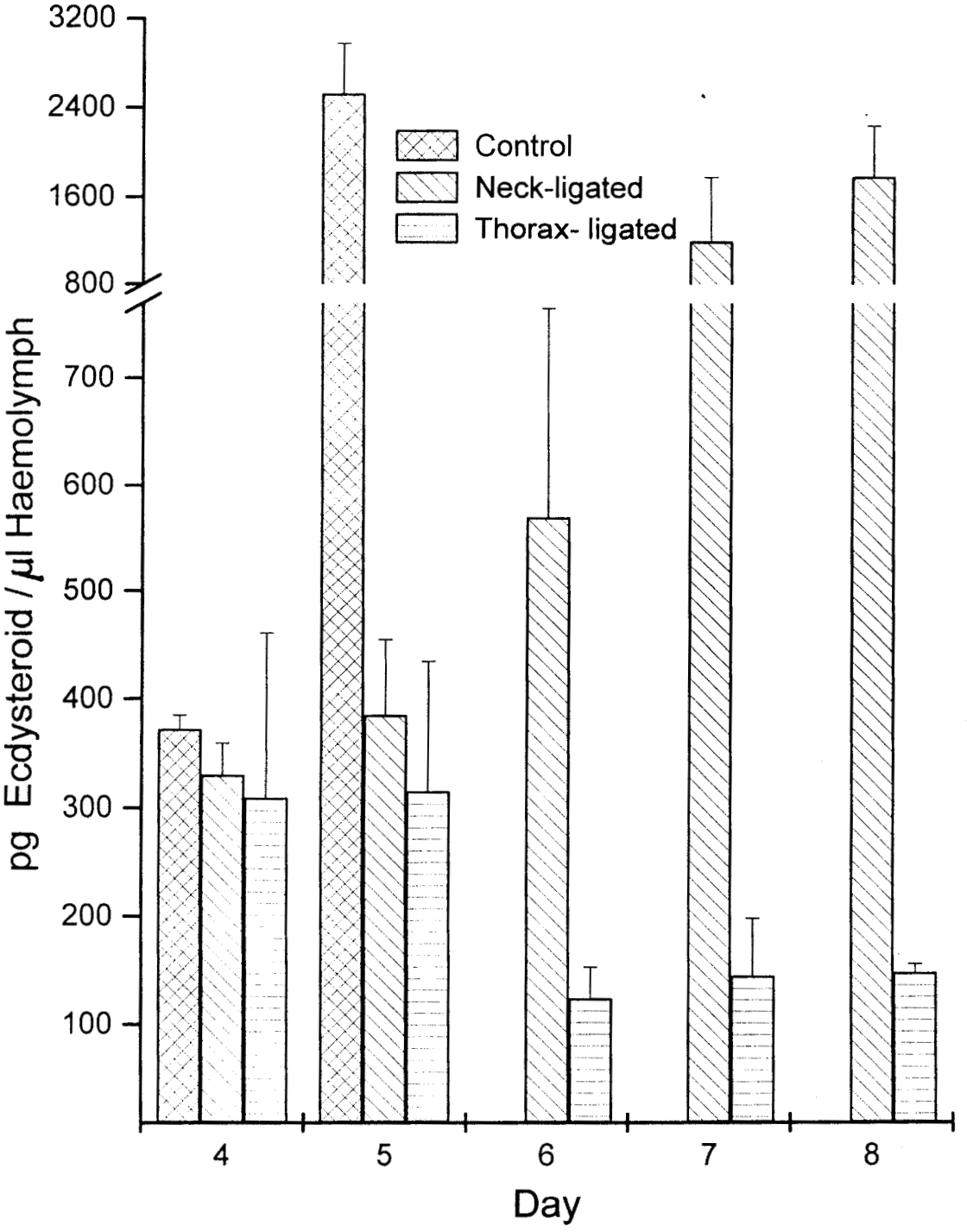


Fig. 12 Effects of neck/thorax ligation of day 4 larvae on haemolymph ecdysteroid titres

Table 3. **Morphogenetic effects of repeated daily treatments of sixth instar larvae with JHA from day 0 onwards**

Dosage	*n*	% mortality	Number of larvae transformed to:	
			Imperfect supernumerary larvae	Pupae
20 µg	25	20	20	--
5 µl Acetone (Control)	15	--	--	15

3.3.4 Effects of treatments of JHA on metamorphosis and haemolymph ecdysteroid titres

Juvenile hormone (JH) has been known to be a necessary factor for the maintenance of larval state and also prevent metamorphosis in insects (Wigglesworth, 1934, Williams, 1961). The transformation of larvae into adults in exopterygote insects as well as the transformation of pupae into adults in endopterygote insects is affected by JH or its analogues. The interplay of ecdysteroids and juvenile hormones in larval insects serve to orchestrate the progression from one developmental state to the next, with ecdysteroids regulating the onset and timing of the moulting cycle and JH directing the quality of the moult (Riddiford, 1994). The present study analyses the effect of JHA treatment on morphogenesis and haemolymph ecdysteroid titres during larval-pupal and pupal-adult development.

(i) Effects of repetitive treatments of unligated sixth instar larvae with JHA on metamorphosis and ecdysteroid titres

Sixth instar day 0 larvae were topically treated with repeated daily dose of 20 μg JHA from day 0 to day 3. Control larvae received an equal volume of acetone. The effects of these treatments on larval development, metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Table 4. **Morphogenetic effects of repeated daily treatments of JHA to sixth instar day 1 ligated larvae**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed :		
			No change	Gut purge	Pupation
Neck-ligated	5 µg	20	100	--	--
Neck-ligated (Control)	5 µl Acetone	10	30	70	--
Thorax-ligated	5 µg	20	100	--	--
Thorax-ligated (Control)	5 µl Acetone	10	30	70	--

Morphogenetic effects

The JHA treated larvae were similar to control larvae in appearance but they fed voraciously and were considerably larger in size. These larvae moulted into imperfect supernumerary larvae within 24 h of termination of hormone treatment, i.e. on day 4. The supernumerary larvae were more or less sluggish, with reduced feeding activity. Head and thoracic region showed pupal characters whereas the integument in general and the abdominal region were larval in appearance. Antennae showed signs of segmentation and were sclerotised. Thoracic legs and the mouth parts were mildly sclerotised especially the labio-hypopharyngeal complex. The paired wing discs on meso- and metathoracic segments were partially tanned. These supernumerary larvae survived for 6-7 days. Mortality rate was less after JHA treatments. Some of the larvae became prepupae but failed to pupate. Control larvae purged their guts and transformed into wandering stage by the end of day 4. These larvae transformed to prepupae at the end of day 5 which pupated after 24 h, on day 6 (Table 3).

Changes in ecdysteroid titres

Ecdysteroid titres of experimental and control larvae from day 1 - day 4 and that of day 0 supernumerary larvae are represented in Fig. 13. Haemolymph ecdysteroid titres of JHA treated larvae measured 14 $\pm$ 3 pg/µl, 36 $\pm$ 8 pg/µl, 28 $\pm$ 19 pg/µl and 67 $\pm$ 19 pg/µl for day 1, day 2, day 3 and day 4

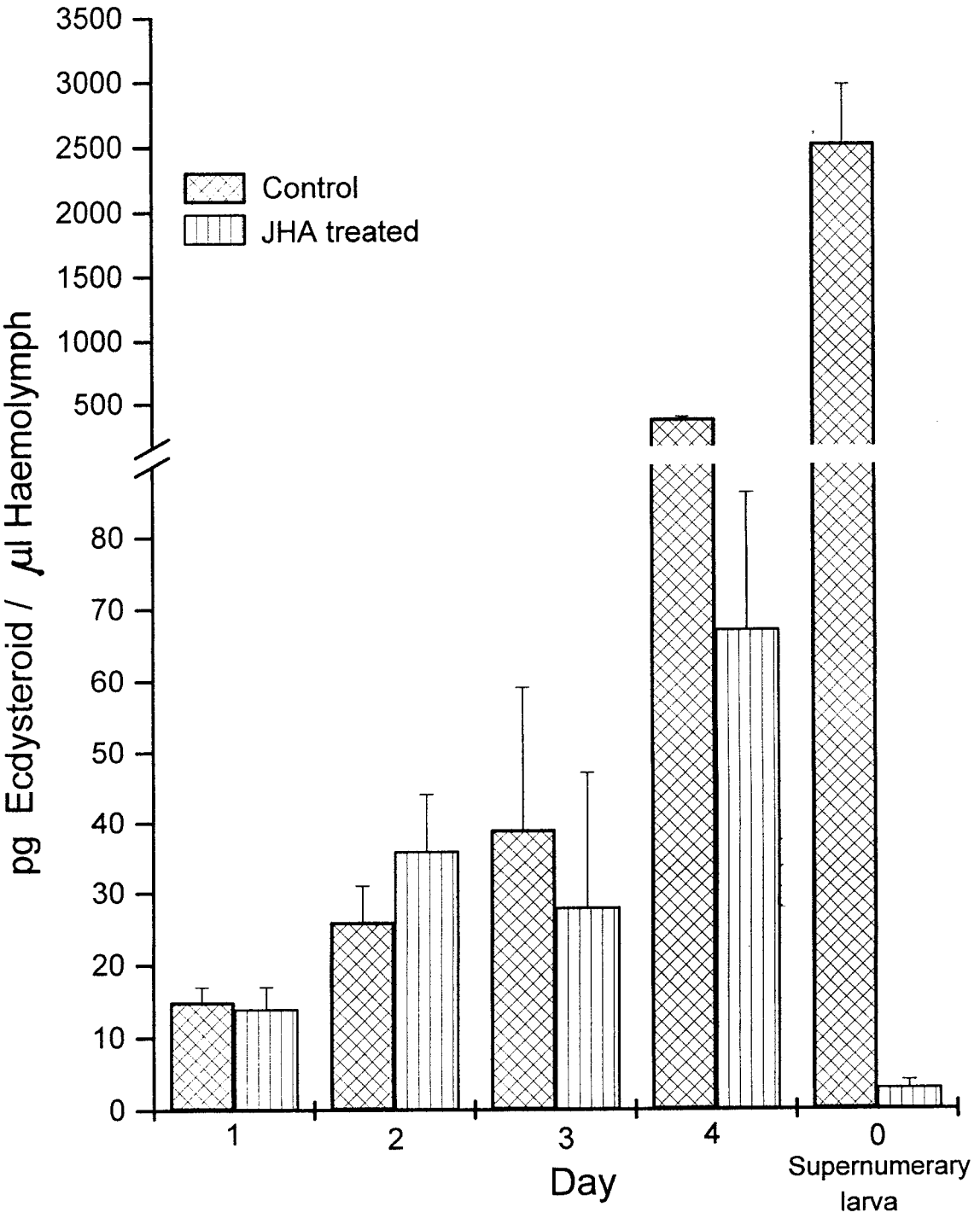


Fig.13 Effects of repetitive treatments of larvae with 20 µg JHA from day 0 onwards on haemolymph ecdysteroid titres

respectively. In the case of day 0 supernumerary larvae the haemolymph ecdysteroid titres measured 2 ± 1 pg/µl. Ecdysteroid titres of control (normal) larvae measured 15 ± 2 pg/µl (day 1), 26 ± 5 pg/µl (day 2), 39 ± 20 pg/µl (day 3), 372 ± 13 pg/µl (day 4) and 2524 ± 453 pg/µl (day 5). Ecdysteroid titres increased gradually to reach its peak on day 4 and decreased to very low titres in the supernumerary larvae (day 0) in the JHA treated larvae. Conversely, titres in the control larvae increased steadily from days 1-5 to reach its peak on day 5. The peak titre of JHA treated larvae was significantly reduced ($P < 0.05$) when compared to that of control larvae.

(ii) Effects of repetitive treatments of ligated sixth instar day 1 larvae with JHA on metamorphosis and ecdysteroid titres

Sixth instar larvae were neck- or thorax-ligated on day 1 and topically treated with a repetitive daily dose of 5 µg JHA for three days. Ligated larvae kept as controls were treated with an equivalent quantity of acetone. The effects of these treatments on metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

The JHA treated larvae did not show the premetamorphic behaviour of gut purge while 70% of the control larvae exhibited gut purge after 4.5 ± 0.5 days. None of the JHA treated or control larvae pupated (Table 4).

Table 5. **Morphogenetic effects of treatments of JHA to sixth instar day 4 ligated larvae**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed:	
			No change	Pupation
Neck-ligated	5 µg	20	20	80
Neck-ligated (Control)	5 µl Acetone	10	100	--
Thorax-ligated	5 µg	20	100	--
Thorax-ligated (Control)	5 µl Acetone	15	100	--

Changes in ecdysteroid titres

Ecdysteroid titres of experimental and control larvae are represented in Fig. 14. Titres of JHA treated neck-ligated and thorax-ligated larvae on day 4 were 4 ± 2 pg/µl and 2 ± 1 pg/µl respectively. In the insects kept as control, the hormone titres on day 4 of the neck-ligated and thorax-ligated larvae were 6 ± 1 pg/µl and 2 ± 1 pg/µl respectively. Ecdysteroid titres of the JHA treated and control larvae were not significantly different.

(iii) Effects of treatments of ligated sixth instar day 4 larvae with JHA on metamorphosis and ecdysteroid titres

Sixth instar larvae were neck- or thorax-ligated on day 4 and topically treated with a single dose of 5 µg JHA. Ligated larvae kept as control received an equivalent volume of acetone. The effects of these treatments on metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

JHA treatment of larvae neck-ligated on day 4 accelerated the moulting process and the larvae underwent pupal development. Eighty percent of ligated larvae treated with JHA transformed into headless pupae on day 7. Neck-ligated day 4 larvae kept as control showed a complete inhibition of pupation. The ligated larvae survived for 4 ± 1 days and died without pupating (Table 5).

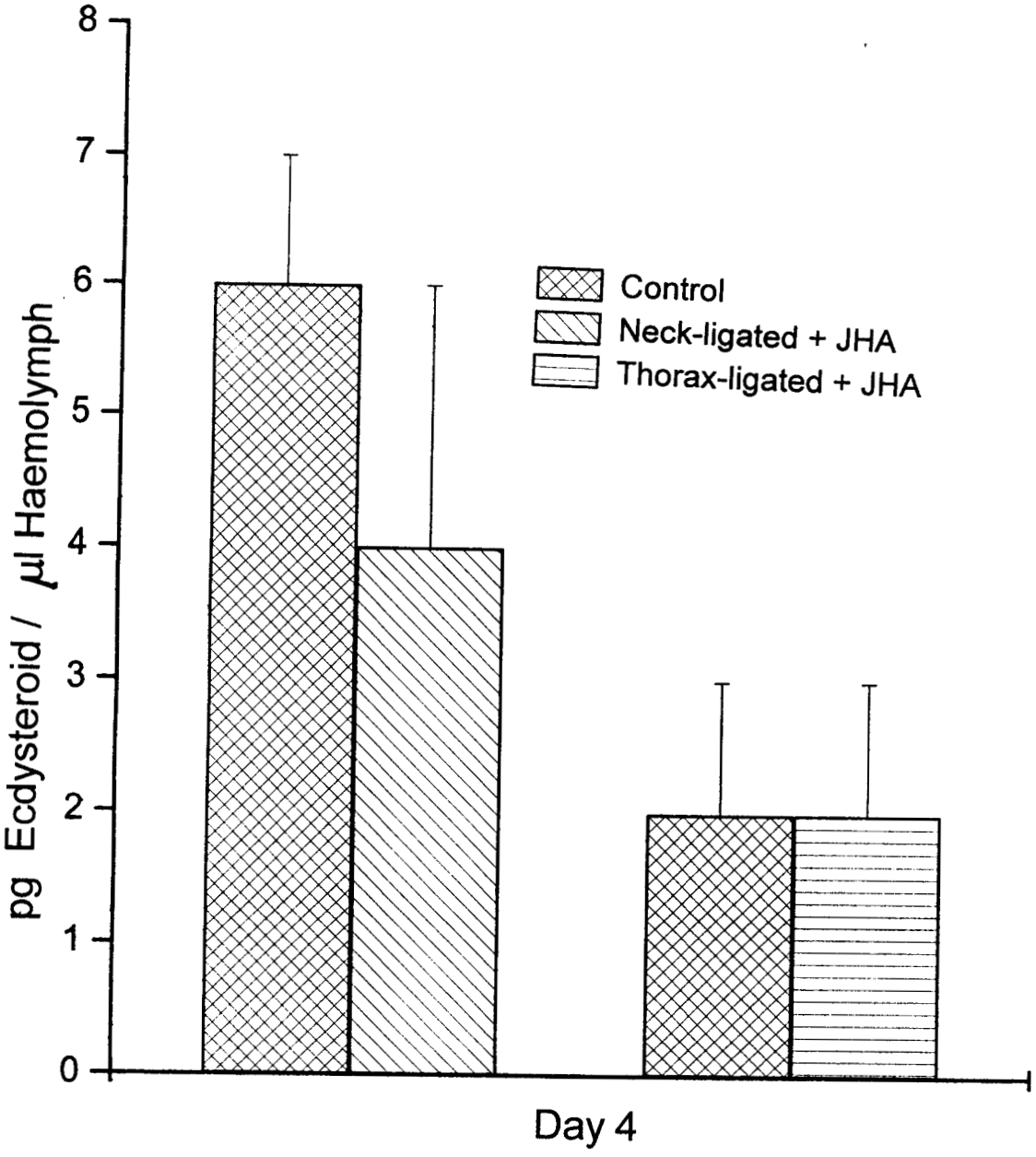


Fig.14 Effects of treatment of JHA to neck/thorax-ligated day 1 larvae on haemolymph ecdysteroid titres

Larvae thorax-ligated on day 4 failed to pupate even after treatment of JHA. The larvae survived for 4 ± 1 days. Thorax-ligated larvae kept as controls also showed a complete inhibition of pupation (Table 5).

Changes in ecdysteroid titres

Ecdysteroid titres of neck-ligated larvae treated with JHA and those of control larvae are represented in Fig. 15. Hormone titres of day 0 headless pupae were 1760 ± 453 pg/µl whereas that of control (neck-ligated) larvae were 989 ± 377 pg/µl on day 7. Titres of the headless pupae were higher than that of the control larvae although the increase was not significant.

Ecdysteroid titres of thorax-ligated larvae treated with JHA and those of control larvae are represented in Fig. 16. Hormone titres of the treated larvae on day 5, day 7 and day 8 were 240 ± 90 pg/µl, 84 ± 14 pg/µl and 89 ± 26 pg/µl respectively. Titres of the control larvae were 315 ± 119 pg/µl, 145 ± 53 pg/µl and 148 ± 8 pg/µl on day 5, day 7 and day 8 respectively. Ecdysteroid titres of the JHA treated and control larvae were not significantly different and both exhibited a declining trend.

(iv) Effects of treatments of pupae with JHA on metamorphosis and ecdysteroid titres

Newly ecdysed (day 0) pupae were treated with a single dose of 0.5 µg or 1 µg JHA. Control pupae received an equivalent quantity of acetone. The

Table 6. **Morphogenetic effects of treatments of JHA to day 0 pupae**

Dosage	*n*	% mortality	No. of pupae transformed into normal adults
0.5 μg	25	18	--
1 μg	25	20	--
1 μl Acetone (Control)	20	10	18

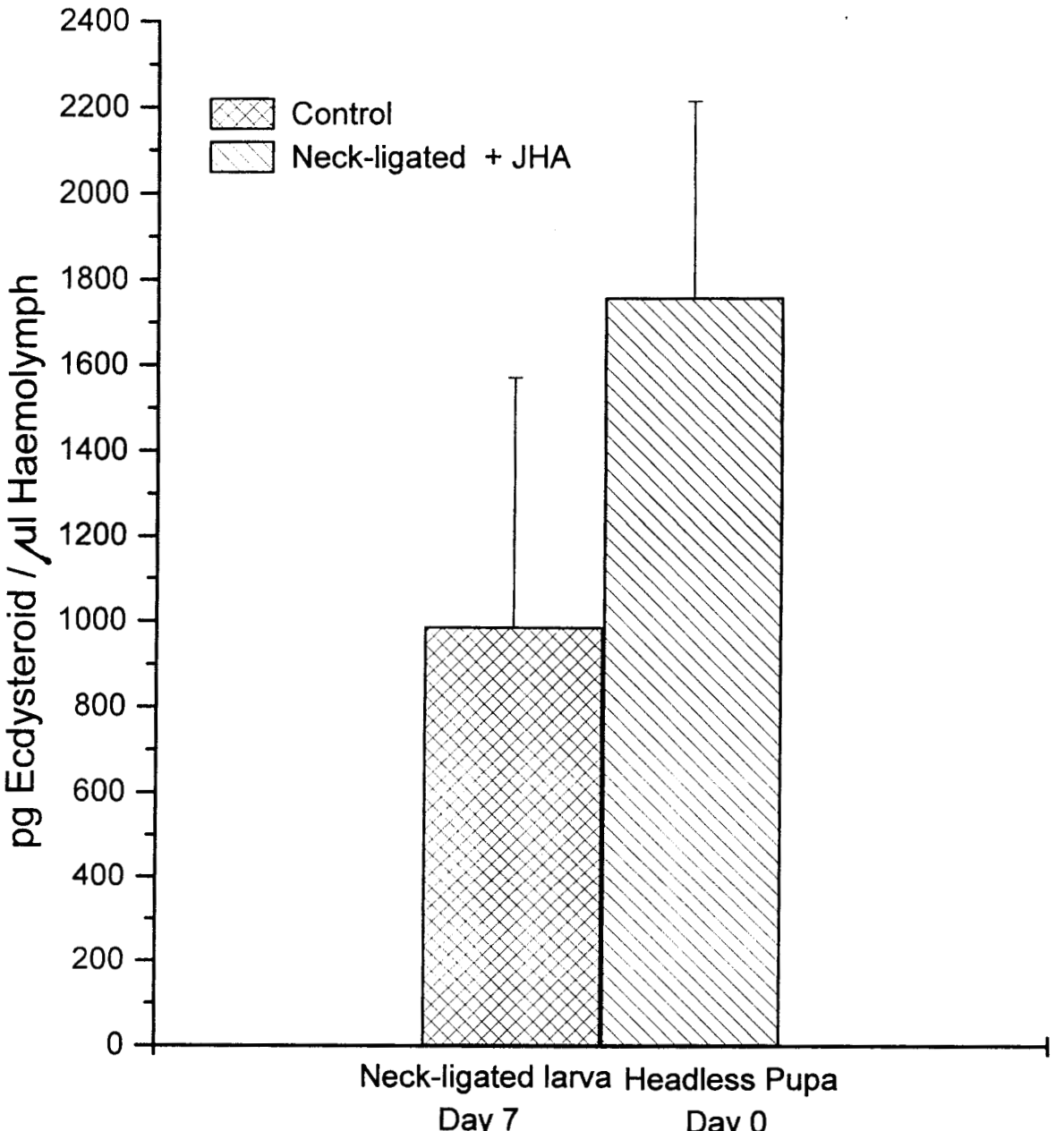


Fig. 15 Effects of treatment of 5 µg JHA to neck-ligated day 4 larvae on haemolymph ecdysteroid titres

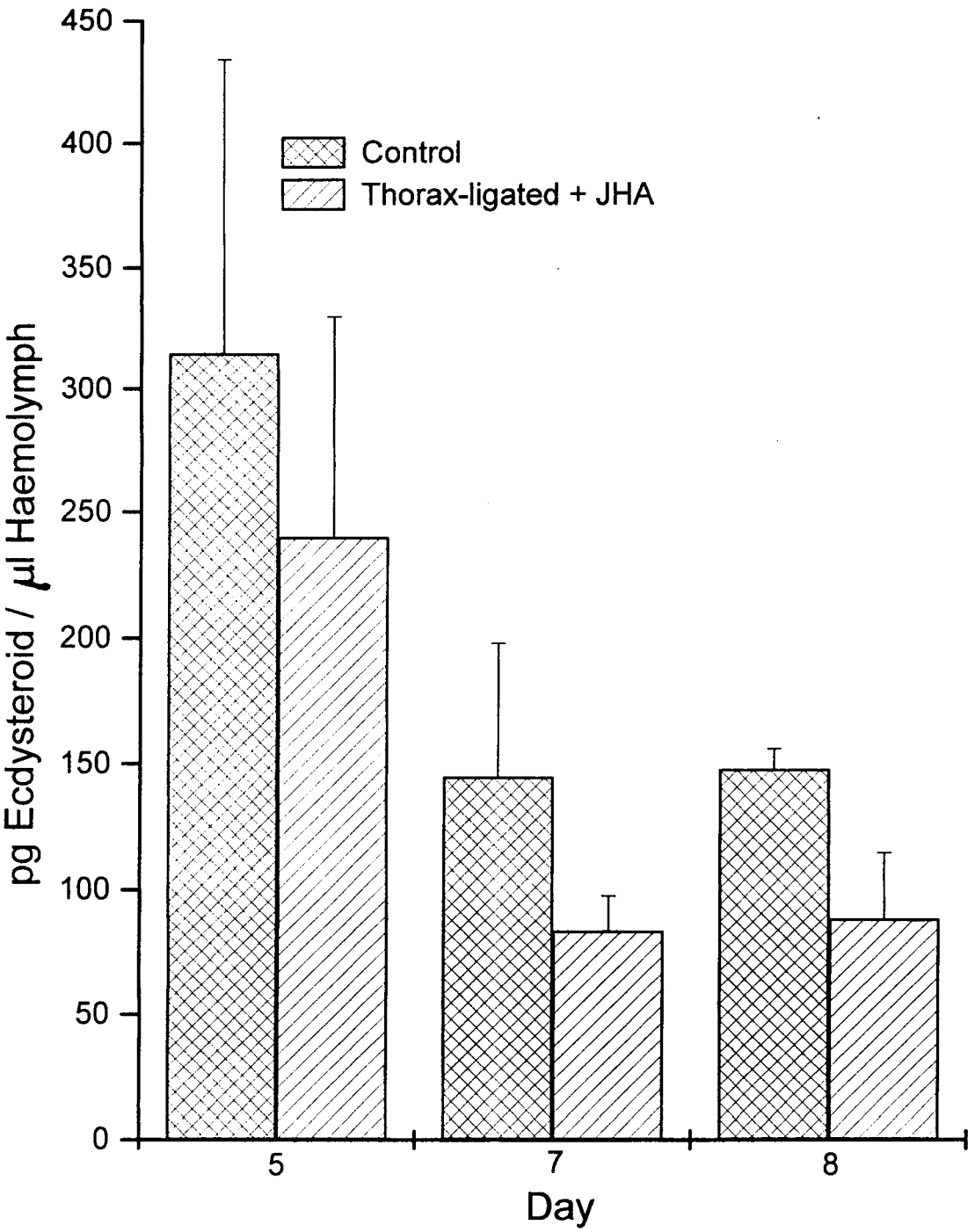


Fig. 16 Effects of treatment of 5 µg JHA to thorax-ligated day 4 larvae on haemolymph ecdysteroid titres

effects of these treatments on pupal development, metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

High mortality was observed in the treated pupae. Surviving pupae showed various abnormalities in pupal-adult development. Failure of emergence or the emergence of abnormal adults were observed. None of them emerged successfully as morphologically normal adults. When the pupal cases of such unemerged pupae were removed, they were found to contain pupal-adult intermediates or adultoids. These adultoids showed normal imaginal differentiation in the head and thorax. However, the wings although developed were unstretched. Moths which emerged from the control pupae were normal and healthy (Table 6).

Changes in ecdysteroid titres

Ecdysteroid titres of experimental and control pupae from days 1 - 6 are shown in Fig. 17. Hormone titres in the pupae treated with 0.5 µg JHA were 4819 ± 221 pg/µl on day 1, 8968 ± 2928 pg/µl on day 2, 14996 ± 1878 pg/µl on day 3, 8231 ± 963 pg/µl on day 4, 1922 ± 129 pg/µl on day 5 and 1833 ± 268 pg/µl on day 6. Titres in the pupae treated with 1 µg JHA were 5107 ± 2191 pg/µl on day 1, 17679 ± 1980 pg/µl on day 2, 10010 ± 1506 pg/µl on day 3, 6831 ± 748 pg/µl on day 4, 4053 ± 916 pg/µl on day 5 and

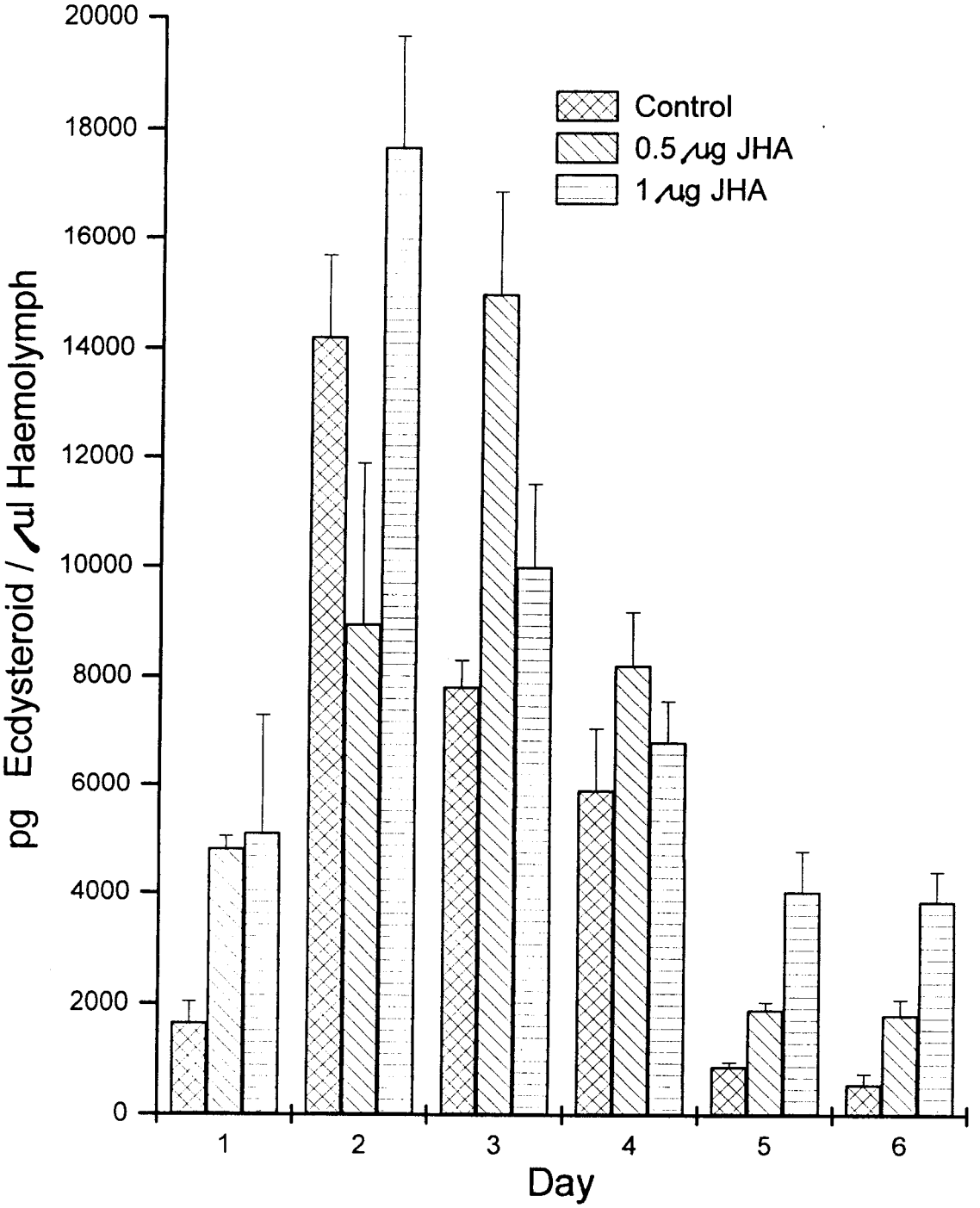


Fig. 17 Effects of treatments of pupae with 0.5 and 1 µg JHA on haemolymph ecdysteroid titres

3881 ± 538 pg/µl on day 6. Ecdysteroid titres of pupae kept as control were 1973 ± 521 pg/µl on day 1, 14221 ± 1458 pg/µl on day 2, 7831 ± 478 pg/µl on day 3, 5915 ± 1159 pg/µl on day 4, 894 ± 74 pg/µl on day 5 and 574 ± 186 pg/µl on day 6. Although the pattern of fluctuation was similar in the JHA treated and control pupae, titres of the 0.5 µg JHA treated pupae were significantly higher ($P < 0.1$) than those of control pupae on day 1, day 5 and day 6 while those of 1 µg JHA treated pupae were higher than those of controls on day 1, day 2, day 3, day 5 and day 6.

3.3.5 Effects of treatments of ecdysteroid agonist RH 5992 on metamorphosis and haemolymph ecdysteroid titres

Ecdysteroids are known to have positive/negative feedback effects on endogenous ecdysteroid biosynthesis (Sakurai and Williams, 1989). Hence it was thought worthwhile to investigate the effects of non-steroidal ecdysone agonist RH 5992 on metamorphosis and haemolymph ecdysteroid titres.

(i) Morphogenetic effects of treatments of unligated sixth instar larvae with RH 5992

Sixth instar larvae on day 0 and day 4 were treated topically with different doses of RH 5992 (2 µg, 5µg). Control larvae received an equivalent volume of acetone. The effects of these treatments on metamorphosis and mortality were observed. The RH 5992 treated day 0 sixth instar larvae

Table 7. **Morphogenetic effects of treatments of RH 5992 to sixth instar day 0 larvae**

Dosage	*n*	% of surviving larvae which showed:			% of larvae showing premature lethal larval moult
		No change	Gut purge	Pupation	
2 µg	10	--	100	--	100
5 µg	10	--	100	--	100
5 µl Acetone (Control)	5	--	100	100	--

Table 8. **Morphogenetic effects of treatments of RH 5992 to sixth instar day 4 larvae**

Dosage	*n*	% of surviving larvae showing abnormal and lethal pupation	% of larvae showing normal pupation
2 µg	10	100	--
5 µg	10	100	--
5 µl Acetone (Control)	5	--	100

showed symptoms of a prematurely induced larval moult. Within 24 h of treatment, the old head capsule had slipped down, revealing a fragile and often unsclerotised new head capsule underneath. Formation of a new larval cuticle was also observed. During this period, larval feeding and weight gain was significantly suppressed and the larvae underwent gut purge prematurely. In some cases loss of haemolymph and extrusion of the hindgut was also seen. Most treated larvae died in their old cuticle shortly afterwards. In some cases, the old cuticle was partially shed, with remnants remaining attached to the new cuticle. Likewise, rupture of the imperfectly formed new cuticle was sometimes observed. The RH 5992 treated larvae never showed pupation or adult emergence. Control larvae purged their guts and transformed into wandering stage by the end of day 4. These larvae underwent normal pupation on day 6 (Table 7).

The post feeding (day 4) sixth instar larvae treated with different doses of RH 5992 underwent abnormal and lethal pupation within 24-48 h. They were unable to ecdyse successfully or to build up a normal pupal cuticle. Control larvae underwent normal pupation on day 6 (Table 8).

(ii) Effects of treatments of sixth instar day 1 ligated larvae with RH 5992

Day 1 sixth instar larvae were neck- or thorax-ligated and treated topically with a single dose of 2 μg or 5 μg RH 5992. Ligated larvae kept as

Table 9. **Morphogenetic effects of treatments of RH 5992 to sixth instar day 1 ligated larvae**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed:		
			No change	Gut purge	Pupation
Neck-ligated	2 µg	20	--	100	--
Neck-ligated	5 µg	20	--	100	--
Neck ligated (Control)	5 µl Acetone	15 each	30	70	--
Thorax-ligated	2 µg	20	--	100	--
Thorax-ligated	5 µg	20	--	100	--
Thorax-ligated (Control)	5 µl Acetone	15 each	30	70	--

control were treated with an equivalent quantity of acetone only. The effects of these treatments on metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

Ligated (day 1) larvae treated with various dosages of RH 5992 survived for 3 ± 1 days while larvae kept as control survived for 9 ± 1 days. However, both failed to pupate. All larvae treated with RH 5992 exhibited the premetamorphic behaviour of gut purge after 2.5 ± 0.5 days. Seventy percent of the control larvae also exhibited gut purge behaviour after 4.5 ± 0.5 days (Table 9). An imperfectly formed new cuticle was also observed beneath the partially shed, ruptured old cuticle.

(a) Changes in ecdysteroid titres of neck-ligated larvae treated with RH 5992

Ecdysteroid titres of neck-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 18. Titres of neck-ligated larvae treated with 2 µg RH 5992 were 5 ± 1 pg/µl, 3 ± 1 pg/µl and 70 ± 21 pg/µl for day 1, day 2 and day 3 respectively. Those of 5 µg RH 5992 treated larvae were 8 ± 1 pg/µl , 8 ± 3 pg/µl and 42 ± 5 pg/µl for day 1, day 2 and day 3 respectively. Ecdysteroid titres of larvae kept as control were 8 ± 2 pg/µl, 5 ± 1 pg/µl and 5 ± 1 pg/µl for day 1, day 2 and day 3 respectively. Hormone

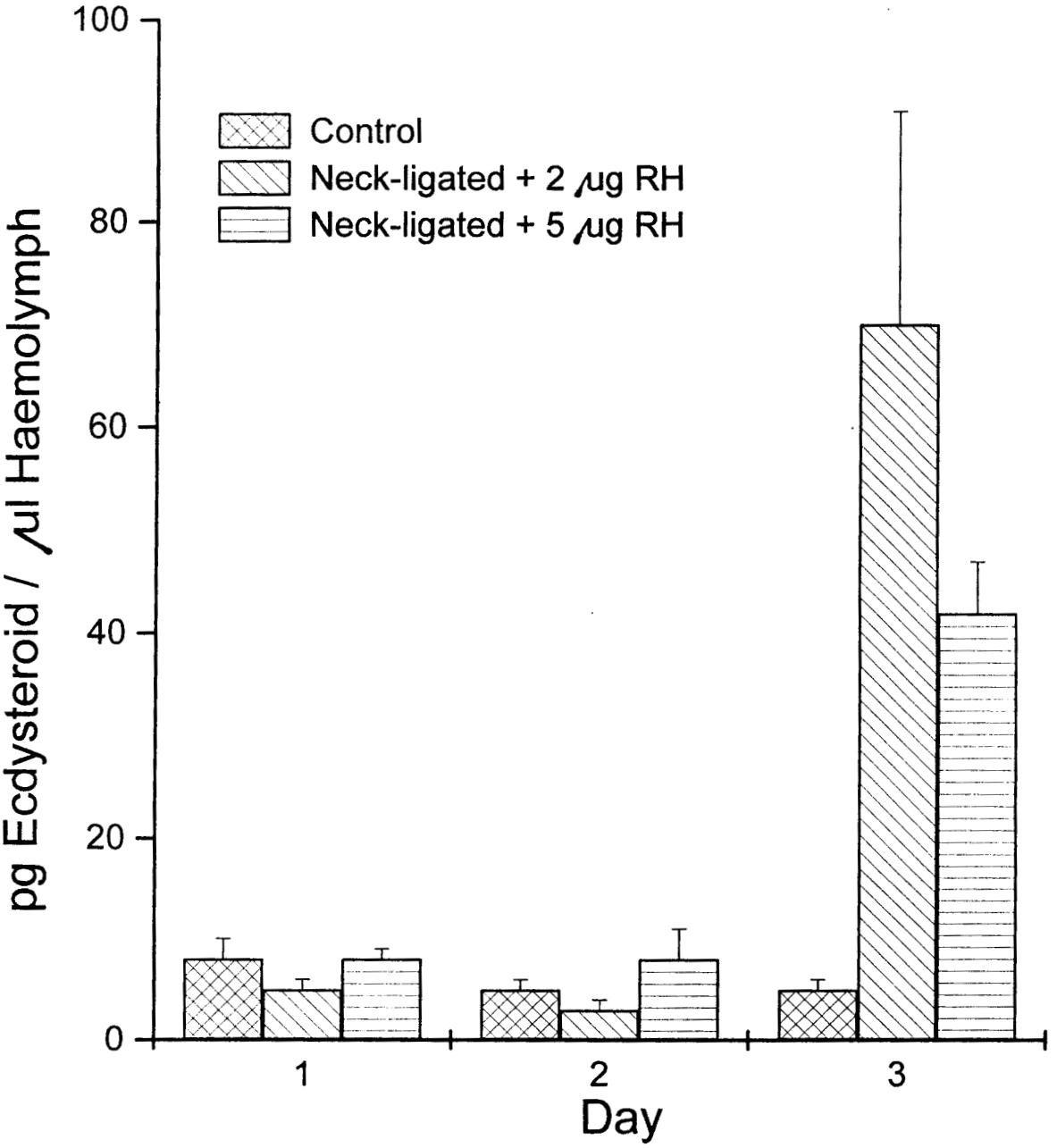


Fig. 18 Effects of treatments of RH 5992 to neck-ligated day larvae on haemolymph ecdysteroid titres

Table 10. **Morphogenetic effects of treatments of RH 5992 to sixth instar day 4 ligated larvae**

Nature of ligation	Dosage	*n*	% of surviving larvae which transformed into:		
			No change	Pupae	Larval-pupal intermediates
Neck-ligated	2 µg	20	20	--	80
Neck-ligated	5 µg	20	20	--	80
Neck-ligated	10 µg	20	20	--	80
Neck-ligated (Control)	5 µl Acetone	15 each	100	--	--
Thorax-ligated	2 µg	20	20	--	80
Thorax-ligated	5 µg	20	20	--	80
Thorax-ligated (Control)	5 µl Acetone	15 each	100	--	--

titres that were reduced following neck ligation decreased further though not significantly in the 2 μg RH 5992 treated larvae but remained unchanged in the 5 μg RH 5992 treated larvae on day 1 and day 2. On day 3 an abrupt and significant increase ($P < 0.05$) in titres occurred both in the 2 μg and 5 μg RH 5992 treated larvae when compared to those of control larvae which remained low on all days.

(b) Changes in ecdysteroid titres of thorax-ligated larvae treated with RH 5992

Ecdysteroid titres of thorax-ligated larvae treated with RH 5992 and that of control larvae are represented in Fig. 19. Titres of thorax-ligated larvae treated with 2 μg RH 5992 were 11 ± 2 pg/μl, 7 ± 3 pg/μl and 3 ± 0 pg/μl for day 1, day 2 and day 3 respectively. Those of 5 μg RH 5992 treated larvae were 8 ± 2 pg/μl, 4 ± 0 pg/μl and 6 ± 2 pg/μl for day 1, day 2 and day 3 respectively. Ecdysteroid titres of larvae kept as control were 6 ± 1 pg/μl, 5 ± 0 pg/μl and 4 ± 2 pg/μl for days 1-3. RH 5992 treatment did not cause a significant change in the titres of the treated larvae when compared to those of the control larvae.

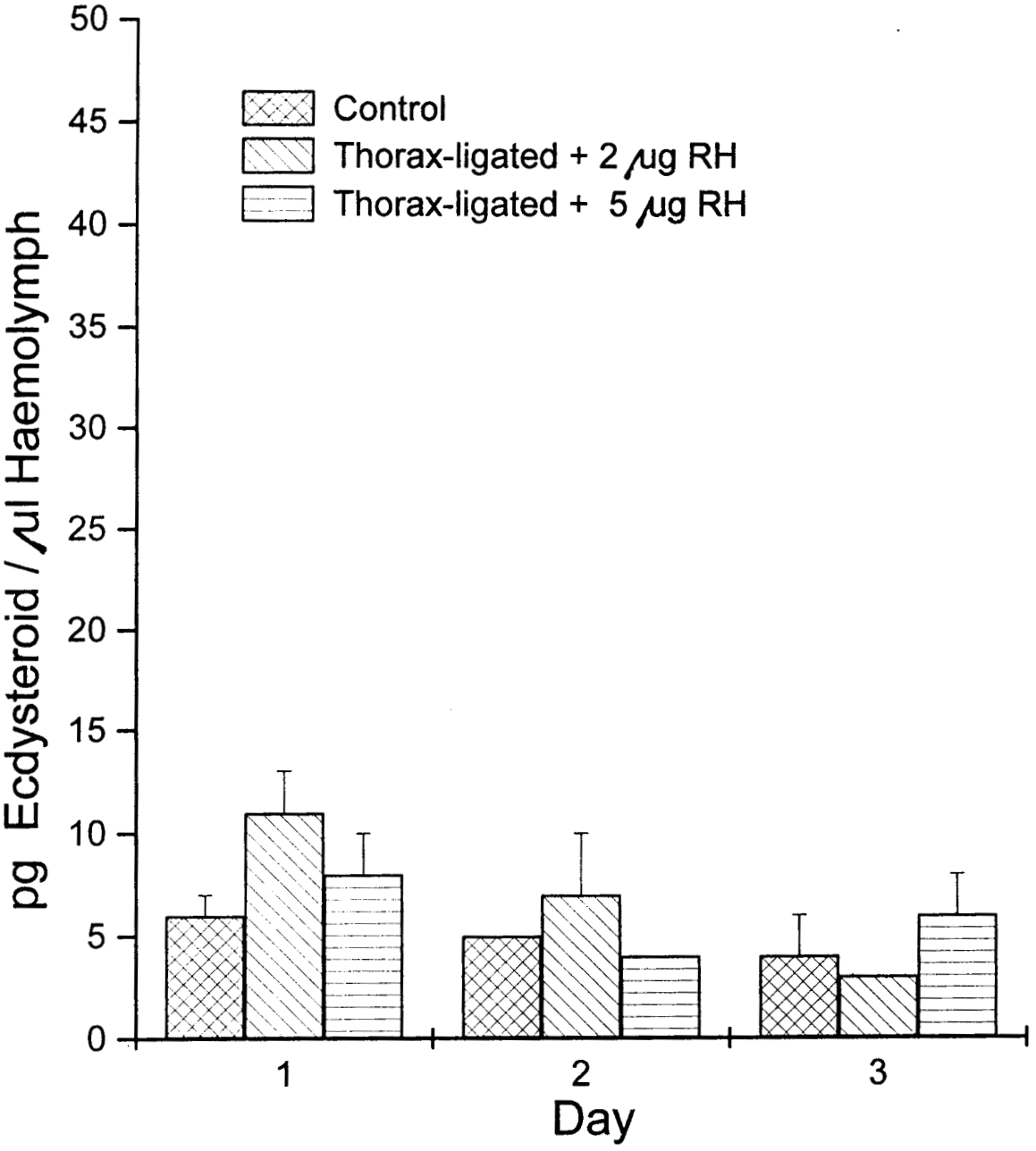


Fig. 19 Effects of treatments of RH 5992 to thorax-ligated day 1 larvae on haemolymph ecdysteroid titres

(iii) Effects of treatments of sixth instar day 4 ligated larvae with RH 5992

Day 4 sixth instar larvae were neck- or thorax-ligated and treated topically with a single dose of 2 μg, 5 μg or 10 μg RH 5992. Ligated larvae kept as control were treated with an equivalent quantity of acetone only. The effects of these treatments on metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

Ligated (day 4) larvae treated with various dosages of RH 5992 transformed into larval-pupal intermediates on day 6. They survived for 3 ± 1 days. These intermediates had thoracic legs which were larval in appearance. Pupal cuticle was seen beneath the old larval one in the abdominal region. The treated larvae were unable to ecdyse successfully or to build up a normal pupal cuticle. Neck-/thorax-ligated day 4 larvae kept as control showed a complete inhibition of pupation, they survived for 4 ± 1 days and then died (Table 10).

(a) Changes in ecdysteroid titres of neck-ligated larvae treated with RH 5992

Ecdysteroid titres of neck-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 20. Titres of neck-ligated

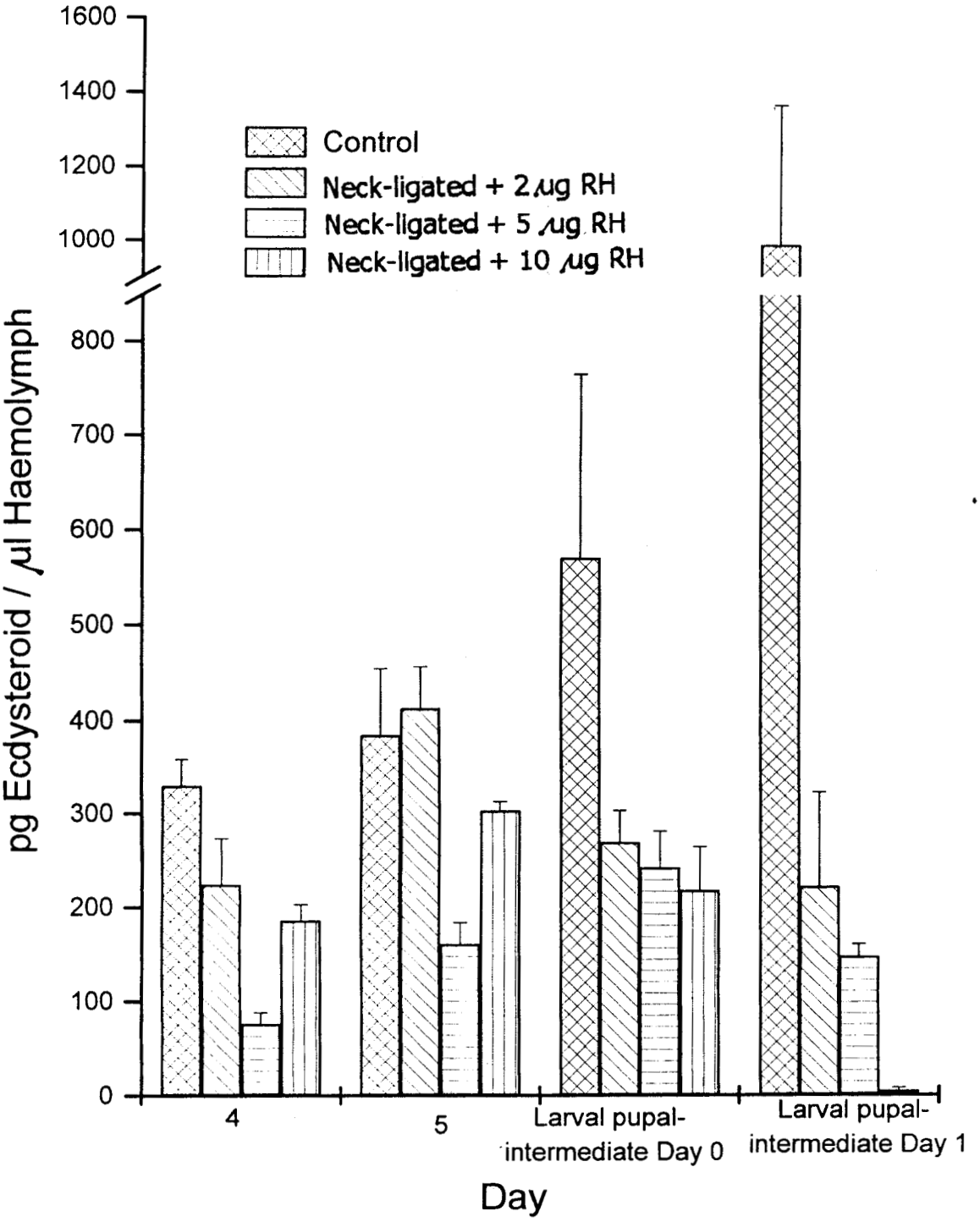


Fig. 20 Effects of treatments of RH 5992 to neck-ligated day 4 larvae on haemolymph ecdysteroid titres

larvae treated with 2 μg RH 5992 were 224 ± 49 pg/μl, 413 ± 43 pg/μl, 269 ± 34 pg/μl and 222 ± 101 pg/μl on days 4 and 5 of sixth instar larval stage and day 0 and day 1 of larval-pupal intermediate stage respectively. Those of 5 μg RH 5992 treated larvae were 76 ± 12 pg/μl, 161 ± 23 pg/μl, 242 ± 39 pg/μl and 148 ± 13 pg/μl on days 4 and 5 of sixth instar larval stage and day 0 and day 1 of larval-pupal intermediate stage respectively. Titres of 10 μg RH 5992 treated larvae were 186 ± 17 pg/μl, 303 ± 10 pg/μl, 218 ± 46 pg/μl and 5 ± 3 pg/μl on day 4 and day 5 of sixth instar larval stage and day 0 and day 1 of larval-pupal intermediate stage respectively. The control larvae had the following titres for days 4 - 7 : 330 ± 29 pg/μl, 385 ± 69 pg/μl, 570 ± 195 pg/μl and 989 ± 377 pg/μl. The hormone titres decreased significantly ($P < 0.1$) on most days following RH 5992 treatment when compared to control larvae which exhibited an increasing trend of ecdysteroids reaching its maximum on day 7.

(b) Changes in ecdysteroid titres of thorax-ligated larvae treated with RH 5992

Ecdysteroid titres of thorax-ligated larvae treated with RH 5992 and that of control larvae are represented in Fig. 21. Titres of thorax-ligated larvae treated with 2 μg RH 5992 were 328 ± 90 pg/μl, 90 ± 20 pg/μl, 165 ± 31 pg/μl and 113 ± 33 pg/μl on day 4 and day 5 of sixth instar larval stage and day 0 and day 1 of larval-pupal intermediate stage respectively.

Table 11. **Effects of treatments of RH 5992 to day 0 pupae**

Dosage	*n*	% Mortality	% of adult emergence	% of unemerged adultoids
5 µg	25	20	20	60
10 µg	25	20	20	60
5 µl Acetone (Control)	20	10	90	--

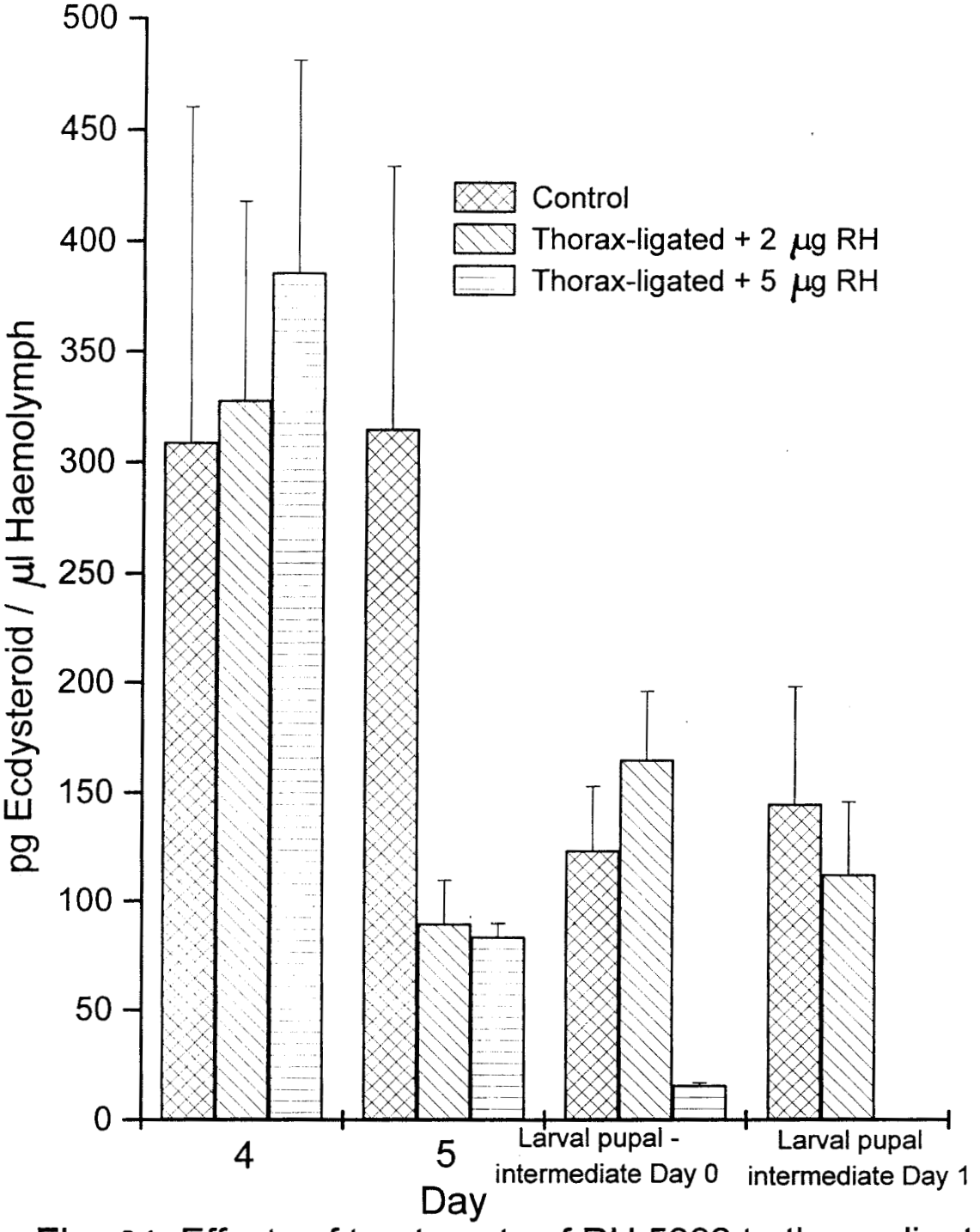


Fig. 21 Effects of treatments of RH 5992 to thorax-ligated day 4 larvae on haemolymph ecdysteroid titres

Those of 5 μg RH 5992 treated larvae were 386 ± 95 pg/μl, 84 ± 6 pg/μl and 16 ± 1 pg/μl on day 4 and day 5 of sixth instar larval stage and day 0 of larval-pupal intermediate stage respectively. The control larvae were 309 ± 151 pg/μl, 315 ± 119 pg/μl, 124 ± 29 pg/μl and 145 ± 53 pg/μl on day 4, day 5, day 6 and day 7 respectively. The hormone titres decreased considerably both in the RH 5992 treated and control larvae and there was no significant difference on most days between the treated and control larval titres.

(iv) Effects of treatment of pupae with RH 5992

Newly ecdysed (day 0) pupae were treated topically with a single dose of 5 μg or 10 μg RH 5992. Control pupae received an equivalent quantity of acetone. The effects of these treatments on pupal development, metamorphosis and haemolymph ecdysteroid titres were carefully studied.

Morphogenetic effects

All the treated pupae survived the pupal period like the control pupae. Mortality was observed towards the end of the pupal period. Most of the treated pupae died on or before the day of adult emergence but a few emerged as normal adults. The pupal period of the RH 5992 treated pupae was found slightly prolonged (9 ± 1 days) whereas adults emerged from the control pupae on day 8 (Table 11).

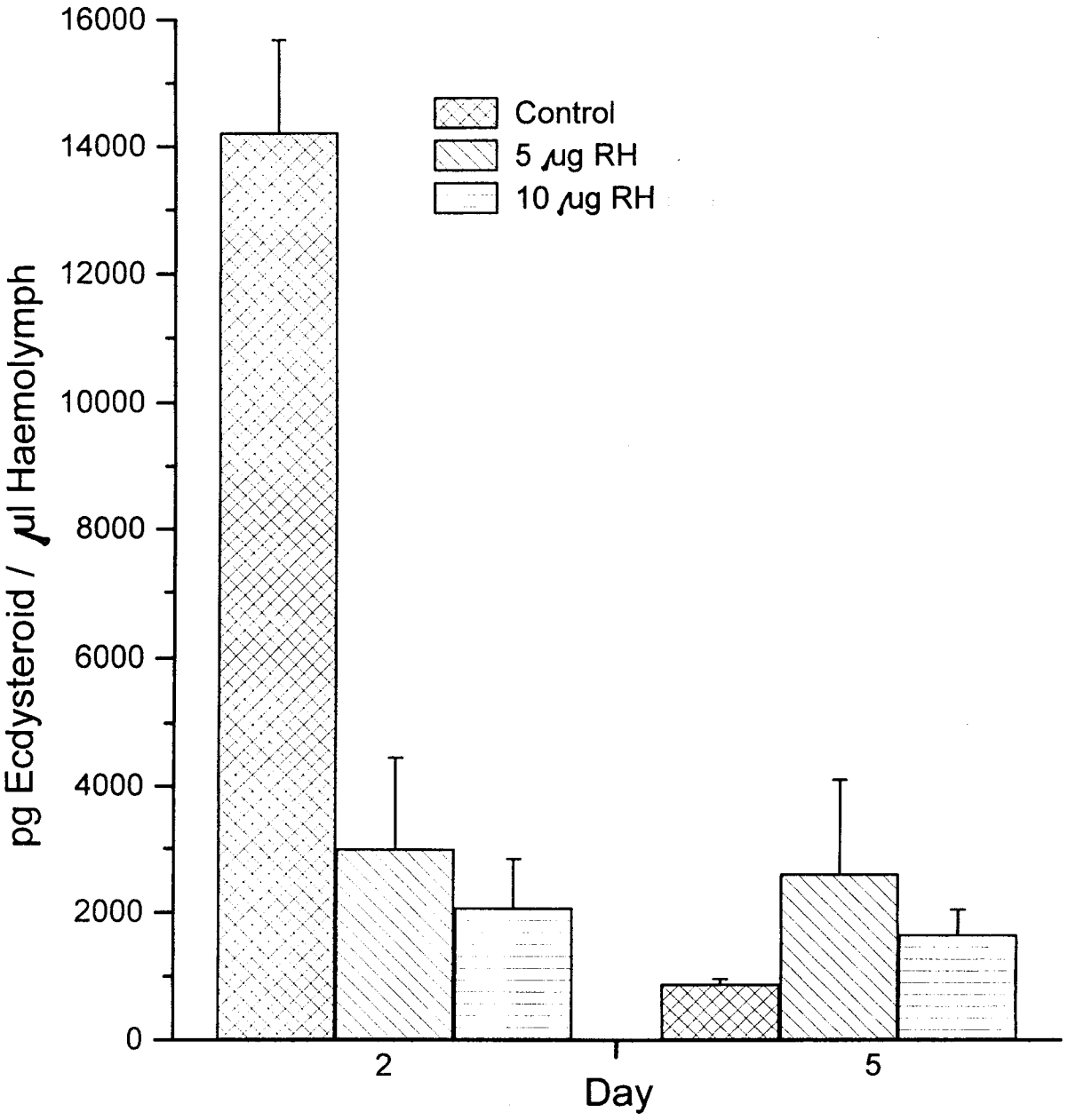


Fig. 22 Effects of treatments of pupae with 5 µg and 10 µg RH 5992 on haemolymph ecdysteroid titres

Changes in ecdysteroid titres

Ecdysteroid titres of pupae treated with RH 5992 and those of control pupae are represented in Fig. 22. Titres of pupae treated with 5 μg RH 5992 were 3007 ± 1458 pg/μl and 2629 ± 1499 pg/μl on day 2 and day 5 respectively. Titres of pupae treated with 10 μg RH 5992 were 2087 ± 761 pg/μl and 1674 ± 390 pg/μl on day 1 and day 5 respectively. Ecdysteroid titres of control pupae on day 2 and day 5 were 14221 ± 1458 pg/μl and 894 ± 74 pg/μl respectively. Ecdysteroid titres in the treated pupae decreased significantly ($P < 0.05$) on day 2 when compared to the control pupae which had the highest titre on the same day. On day 5, the treated pupae had titres significantly higher ($P < 0.1$) than those of the control pupae.

3.4 DISCUSSION

Developmental profile of ecdysteroids during larval development and metamorphosis

The primary source of ecdysteroids in most immature insects is the prothoracic glands (Chino *et al.*, 1974; King *et al.*, 1974). They produce a prohormone, ecdysone which is converted to the actual hormone, 20-hydroxyecdysone in the peripheral tissues (King, 1972). Both ecdysone, 20-hydroxyecdysone and their hormonally active metabolites are inactivated,

biochemically and/or depleted by excretion (Koolman, 1978). Their content, i.e. ecdysteroid titre in the body obviously depends on the balance between the rate of ecdysone secretion and the rate of ecdysone metabolism. Information regarding the ecdysteroid profile of insects is vital to the understanding of endocrine regulation and interaction during postembryonic development. Because of vast differences in the time sequence of developmental events occurring in insects, ecdysteroid titres obtained from one species may not accurately represent the same event found in another species. Consequently, ecdysteroid titre determinations for individual species are essential if critical evaluation of titre changes and inference of its significance are required. Hence the ecdysteroid titre changes during the period between last larval instar and adult of *S. mauritia* has been determined. The relationships between the ecdysteroid titres and developmental events during larval development and metamorphosis are also analysed.

Haemolymph ecdysteroid profile during larval-pupal development

Changes in ecdysteroid titres during development of insects have been determined for a number of lepidopteran species (Burdette, 1962; Shaaya and Karlson, 1965a; Kaplanis, *et al.*, 1966a; Hanaoka and Ohnishi, 1974; Bollenbacher *et al.*, 1975, 1978; Calvez *et al.*, 1976; Lagaeux *et al.*, 1976; Dean *et al.*, 1980; Fujishita *et al.*, 1982; Loeb, 1982; Gelman and Woods, 1983; Kiguchi, 1983; Gelman and Brents, 1984; Yin and Chaw, 1984; Kiguchi *et al.*, 1985; Okuda *et*

al., 1985; Smith, 1985; Wolfgang and Riddiford, 1986; Kelly *et al.*, 1986; Sakurai *et al.*, 1998).

Haemolymph ecdysteroid profile of last larval stadium of *S. mauritia* is consistent with previous determinations in other lepidopteran insects. Ecdysteroid titres reach a small peak on day 3 and form a significant peak on day 5 during larval-pupal development. Histological and ultrastructural studies of prothoracic glands of *S. mauritia* also suggest two phases of secretory activity during last instar larval development (Balamani and Nair, 1994, 1997). Biphasic secretion of ecdysone during larval-pupal development has also been reported in *Manduca sexta* (Bollenbacher *et al.*, 1975), *Samia cynthia* (Calvez *et al.*, 1976; Fujishita *et al.*, 1982), *Galleria mellonella* (Bollenbacher *et al.*, 1978), *Calpodes ethlius* (Dean *et al.*, 1980), *Heliothis virescens* (Loeb, 1982), *Ephestia kuhniella* (Kallenborn and Mosbacher, 1983), *Ostrinia nubilalis* (Gelman and Brents, 1984), *Bombyx mori* (Okuda *et al.*, 1985), *Trichoplusia ni* (Jones *et al.*, 1986) and *Lymantria dispar* (Kelly *et al.*, 1986).

The first small peak was observed on day 3 of the sixth instar larvae. This peak is interpreted as a signal for the change in developmental commitment (reprogramming) causing the epidermal cells to synthesise a pupal cuticle when the high ecdysteroid titre peak preceding the pupal moult occurs (Riddiford, 1976; Bollenbacher *et al.*, 1981). This commitment peak

causes the termination of feeding and the induction of both physiological (gut purge) and behavioural (wandering) syndromes which occur 24 h after the small surge (Truman and Riddiford, 1974; Fujishita *et al.*, 1982). Histological and ultrastructural observation of prothoracic glands of *S. mauritia* also reveal secretory activity on day 3 of last larval instar (Balamani and Nair, 1994, 1997).

The ecdysteroid titres increased exponentially on day 5 of the sixth instar to form a significant peak. Prothoracic glands of *S. mauritia* also showed high secretory activity on day 5 of the last larval instar (Balamani and Nair, 1994, 1997). A similar surge of ecdysteroid titre was observed in all the other lepidopteran insects listed earlier. This ecdysteroid peak was interpreted to be responsible for the initiation of apolysis and pharate pupal development (Riddiford, 1976). Daily sampling of haemolymph did not reveal smaller peaks which have been reported in other insects (Dean *et al.*, 1980; Agui and Hiruma, 1982; Gelman and Brents, 1984; Sakurai *et al.*, 1998). However six hour sampling of haemolymph revealed the presence of additional peaks.

Haemolymph ecdysteroid titres at six hour intervals during larval-pupal development

With 6 h sampling of haemolymph, five significant ecdysteroid peaks were detected during larval-pupal development. Only four peaks have been

reported so far in *C. ethlius* (Dean *et al.*, 1980), *M. brassicae* (Agui and Hiruma, 1982), *O. nubilalis* (Gelman and Brents, 1984), *M. sexta* (Wolfgang and Riddiford, 1986). In *B. mori*, a small increase in the haemolymph ecdysteroid titre appeared prior to gut purge afterwhich the titre increased with daily fluctuations (Sakurai *et al.*, 1998). A comparison of the times of peak titres revealed that in each insect, peaks occurred at different times within the last instar. In *S. mauritia,* two small peaks occurred before the reprogramming peak as is observed in *C. ethlius* (Dean *et al.*, 1980) and *M. sexta* (Wolfgang and Riddiford, 1986) and two after the reprogramming peak. In *M. brassicae* (Agui and Hiruma, 1982) two small peaks occurred after the reprogramming peak and in *O. nubilalis* (Gelman and Brents, 1984) one small peak occurred before and one after the reprogramming peak. In *B. mori*, there was a single ecdysteroid peak prior to gut purge after which daily ecdysteroid surges occurred (Sakurai *et al.*, 1998). In *S. mauritia*, two small peaks Peak I and Peak II appeared at 18 h and 36 h respectively. Peak III which appeared at 60 h is probably associated with the change of epidermal commitment from larval to pupal (reprogramming peak) type. The reprogramming peak, typically occurs at 40-60% of the last instar, assuming that pharate pupal formation was initiated very soon after the premoult ecdysteroid peak (132 h in *S. mauritia*, 168 h in *C. ethlius,* 192 h in *M. brassicae*, 126 h in *O. nubilalis*). The major ecdysteroid peak, peak IV which occurred at 114 h may be considered the "moulting peak" of

ecdysteroids. A much smaller peak, peak V appeared at 126 h whose function could not be ascertained. A peak at this stage has not been reported so far.

The two subtle peaks in *S. mauritia*, peak I and peak II may be associated with the initiation of nucleolar activity, RNA synthesis and organelle formation in the fat body and epidermis. It may also be correlated with fat body DNA synthesis, polyploidy and the initiation of low rate of lipid synthesis as reported during the intermoult peak in *C. ethlius* (Dean *et al.*, 1980). The low haemolymph ecdysteroid concentration has recently been suggested to possess an important role in the development of *Bombyx* wing discs (Kawasaki, 1995). The reprogramming peak (Peak III) described earlier is believed to be responsible for the cessation of feeding and initiation of wandering. It also correlates with the initiation of epidermal DNA synthesis and mitosis, and with the progressive determination of pupal characteristics (Change in commitment, reprogramming). This ecdysteroid peak may also be involved in the massive intermoult synthesis in the epidermis and the fat body. Gut purge occurred on day 4 (96 h) in *S. mauritia* which was approximately 42 h after the appearance of the commitment peak. The premoult peak (Peak IV) was the largest peak and occurred at the pharate pupal stage promoting successful larval-pupal transformation (Riddiford, 1980). The peak appeared soon after gut purge (18 h) and the larvae pupated on day 6, 18 h after the appearance of the prepupal peak. The shoulder at

102 h and the relatively high standard error for 114 h could be explained by the lack of synchrony in pupal ecdysis. The function of peak V which appeared at 126 h could not be ascertained. Ecdysteroids have also been implicated in initiating testis fusion and torsion in *E. kuhniella* (Nowock, 1972) and in influencing the progress of spermatogenesis which will be discussed in a later section.

The daily fluctuation in ecdysteroid titres seen during postembryonic development is presumably brought about by the changing secretory activity of the prothoracic glands (Birkenbeil *et al.*, 1979; Glitho *et al.*, 1979; Sedlak *et al.*, 1983). The cellular changes in prothoracic glands are followed by a major increase haemolymph ecdysteroids in *T. molitor* (Glitho *et al.*, 1979), *M. sexta* (Sedlak *et al.*, 1983) and *S. littoralis* (Zimowska *et al.*, 1985). Fluctuations in the haemolymph ecdysteroid titre in last instar larvae of *M. sexta* (Bollenbacher *et al.*, 1975; Ciancio *et al.*, 1986), *L. dispar* (Kelly *et al.*, 1986) and *B. mori* (Okuda *et al.*, 1985) correlated temporally with fluctuations in the *in vitro* production of ecdysone by prothoracic glands from the last instar larvae of these species. Earlier studies on the histology and ultrastructure of prothoracic gland cells during sixth instar larval development of *S. mauritia* suggested a biphasic secretion of ecdysone (Balamani and Nair, 1994, 1997). The first phase of activity occurred in day 3 larvae and the second activity phase on day 5 during the late prepupal stage.

The findings corresponded well with the present determination in the appearance of the commitment and premoult peaks in the ecdysteroid titre determined during the sixth larval stadium. However additional peaks although much smaller, have also been determined which may be caused by low levels of activity of the prothoracic glands.

A possible cause for the sporadic prothoracic gland activity is the daily release of PTTH as reported in the last instar larvae of *B. mori* (Mizoguchi, 1995) and *R. prolixus* (Vafopoulou and Steel, 1996). It has been suggested that PTTH release after JH decline in the haemolymph may be the reason for increase of the basal secretory activity of the prothoracic glands (Rountree and Bollenbacher, 1986; Sakurai *et al.*, 1989) and the glands with higher basal secretory activity produce more ecdysone when stimulated by PTTH. Thus the daily fluctuation seen in the ecdysteroid titres may be due to daily PTTH releases in *S. mauritia.*

Haemolymph ecdysteroid profile during pupal-adult development

Ecdysteroid concentration of pupal haemolymph of *S. mauritia* showed a rapid increase between day 1 and day 2, peaking on day 2. A single large ecdysteroid peak has also been reported in pupae and pharate adults of *Pieris* (Lafont *et al.*, 1975), *B. mori* (Calvez *et al.*, 1976), *G. mellonella* (Bollenbacher *et al.*, 1978) and *C. ethlius* (Dean *et al.*, 1980). The peak comprises the first third to fifth of the pupal stadium. The position of the peak may be correlated

with the time needed for the very extensive remodelling necessary for adult morphogenesis. The amount of ecdysteroids found in pupal and pharate adult stages of *S. mauritia* were several times (5.5x) greater than the amount detected in the post-feeding larval stage. The higher amount of ecdysteroid synthesis in the pupal and pharate adult stages reflect a greater need of ecdysteroid titres during this stage of development. Ecdysteroid titres of *S. mauritia* decreased gradually from days 3 - 7. Daily sampling of haemolymph did not detect smaller peaks associated with cuticle deposition in pharate adults and other developmental events. However, the overall pattern is similar to that reported for other insects (Dean *et al.*, 1980, Gelman and Brents, 1984).

It is well known that in insects prothoracic glands are the primary source of ecdysteroids. However, pupal-adult development occurs after the degeneration of prothoracic glands in *S. mauritia* (Balamani and Nair, 1994, 1997). It has been reported that prothoracic gland degeneration occurs due to programmed cell death during normal pupal-adult development of *M. sexta* (Dai and Gilbert, 1997). The ecdysteroid titres of *S. mauritia* decreased gradually during pupal-adult metamorphosis from day 3 to day 7 after the peak on day 2. This may be because prothoracic gland degeneration during metamorphosis is a gradual rather than abrupt process (Dai and Gilbert, 1997). The temporal response of individual gland cells to the programmed cell

death signal may be different and the chronology of apoptosis may be tissue- or gland-specific even in the same animal (Dai and Gilbert, 1998).

One cannot rule out the possibility that ecdysteroids synthesised during last instar larval development are stored in an inactive and undetectable form by conjugate formation (Delbecque *et al.*, 1988). This might be especially true in an embryo or pupa which is a closed system living on its reserves. At appropriate times during embryonic/postembryonic development, these conjugates may be hydrolysed to yield biologically active ecdysteroids which probably promotes pupal-adult transformation (Hoffmann *et al.*, 1980). It is possible that in *S. mauritia* in the absence of the prothoracic glands, pupal-adult metamorphosis is affected by a similar mechanism.

Endocrine significance of critical periods during larval-pupal development

The central role of PTTH in insect growth and development is well documented (for review see Gilbert *et al.*, 1996). PTTH stimulates the synthesis of ecdysteroids or their immediate precursors by the prothoracic glands. Knowledge of the times during development when PTTH is released is crucial to understanding the endocrine regulation of development. 'Neck ligation' has been employed here to tentatively define "critical periods" of PTTH release during larval-pupal development. These critical periods were determined by percent of neck-ligated larvae pupating or showing other

prodromal signs of pupation. The validity of the results of the timing of PTTH release was confirmed by comparing with the ecdysteroid titres of the insect determined during sixth instar larval stadium.

The present studies demonstrated that in *S. mauritia*, neck ligation of sixth instar larvae at 30 h, 36 h, 42 h, 48 h, 54 h, 60 h, 66 h, 72 h, 78 h, 84 h and 90 h after ecdysis had no effect on gut purge. Gut purge although slightly delayed, occurred in the usual manner in the ligated larvae. But larvae neck-ligated at 6 h, 12 h, 18 h and 24 h did not exhibit gut purge behaviour. This may be because the first release of PTTH occurred during the scotophase between 24 h and 36 h after ecdysis to the sixth larval stadium. This PTTH secretion probably activated the prothoracic glands to release the commitment peak of ecdysteroids (Peak III) on day 2 (60 h) described earlier. Gut purge was induced 36 h after the commitment peak in *S. mauritia*.

Post-feeding larvae neck-ligated at 96 h did not exhibit pupal cuticle secretion. On the other hand, larvae neck-ligated at 102 h, 108 h, 114 h, 120 h, 126 h and 132 h after ecdysis showed normal larval-pupal transformation. These findings suggest that the second release of PTTH necessary for the increase in ecdysteroids needed for pupation took place 96 h after ecdysis but prior to 102 h. Concomitantly, ecdysteroid titres rose at 114 h (Peak IV) on day 5. This is identified as the moulting peak of

ecdysteroids. Thus two head critical periods for PTTH release have been identified, one for gut purge and the other for pupal moult.

In *S. mauritia* the time interval between the possible head critical periods for PTTH release and the ecdysteroid titres is 1-2 days in the case of the first release of ecdysteroids whereas it is nearly 12 h in the case of the second peak of ecdysteroids at the prepupal stage. Similar results are observed in *Samia cynthia ricini* (Fujishita and Ishizaki, 1982). In *M. sexta* (Truman and Riddiford, 1974) and *O. nubilalis* (Gelman and Hayes, 1982) an interval of 10 h separate the two critical periods. A wide separation in the magnitude of days for the critical period of ecdysone secretion from that of PTTH release is observed in other lepidopterans.

Direct measurement of haemolymph PTTH concentration in *Bombyx* showed that PTTH secretion occurs three times on different days before gut purge and two in the prepupal period (Shirai *et al.*, 1993). Similarly in *R. prolixus* an *in vitro* assay revealed that PTTH is released into the haemolymph three times (Vafopoulou and Steel, 1996). Moreover, a direct measurement of haemolymph PTTH concentration in *Bombyx* larval haemolymph by time resolved fluoroimmunoassay showed that PTTH is released everyday (Mizoguchi, 1995). Sakurai *et al.* (1998) suggested that HCP in *B. mori* signifies a change in the responsiveness of the prothoracic glands to PTTH so that daily PTTH releases may result in daily ecdysteroid

surges. Hence, HCP was regarded as the turning point of all developmental events after which metamorphic events occurred in a progressive, sequential pattern. Thus, additional releases of PTTH may be causing the additional ecdysteroid peaks in *S. mauritia*. However, in the course of this study it was found that the two HCPs particularly was essential for the activation of prothoracic glands and PTTH if released at other times might be important to synchronise the developmental rhythm by stimulating the prothoracic glands.

Effect of ligation on ecdysteroid titres

Role of brain factors and prothoracic gland secretions in the endocrine regulation of metamorphosis and haemolymph ecdysteroid titres were further analysed by neck/thorax ligation of day 1 and day 4 sixth instar larvae. Although the commitment peak failed to appear in the neck-/thorax-ligated day 1 larvae and the haemolymph ecdysteroid titres of the ligated larvae showed lower values, 70% of the ligated larvae underwent gut purge after a slight delay. In the last instar larvae of lepidopterans, it is the commitment peak of ecdysteroids which induces gut purge (Truman and Riddiford, 1974; Fujishita *et al.*, 1982). In the light of earlier studies on the HCP in *S. mauritia*, the first pulse of PTTH may have already been released in the ligated larvae. Prevention of further stimulation of prothoracic glands by PTTH in the neck-ligated larvae and the absence of prothoracic glands in the thorax-ligated larvae would have caused the decrease in ecdysteroid titres

following ligation. The promotion of gut purge in the ligated larvae suggest that the HCP may alter the tissue responsiveness to ecdysteroids (Sakurai *et al.*, 1998) so that even low titres of ecdysteroids can bring about gut purge. Hence it may be assumed that the increased titre of ecdysteroids (so-called commitment peak) found in day 3 larvae serve as pacemakers which synchronise developmental events during metamorphosis. It may also be possible that JH released before the ligation has already disappeared and hence the tissue response to ecdysteroids is not modified by endogenous JH titres. The ligated larvae however do not pupate, therefore these low titres may not be sufficient to induce pupation.

Neck/thorax ligation of day 4 larvae prevented pupation while the control (normal) larvae pupated on day 6. However neck ligation did not prevent the increase in ecdysteroid titres in the haemolymph. Titres of the ligated larvae on day 8 were comparable with the premoult peak on day 5 of normal larvae. The failure of the ligated larvae to undergo pupation even at these high titres suggest that increased titre of ecdysteroids alone will not induce pupation. The absence of JH in the ligated larvae may also prevent pupation. JH is known to enhance the metamorphic response of tissues to ecdysteroids and/or alter the sensitivity of these tissues to ecdysteroids (Denlinger, 1979). Moreover, determination of JH titres during last instar larval development of several lepidopteran species revealed the occurrence of a

premoult peak of JH in the prepupal stage (Jones *et al.*, 1990; Edwards *et al.*, 1995). There is also suggestive evidence to show that prepupal increase of JH has a morphogenetic role in promoting development of normal pupal morphology (Safranek *et al.*, 1980; Jones and Hammock, 1985; Balamani and Nair, 1989a,b). Earlier studies evaluating the HCP of *S. mauritia* have suggested that the second HCP occurs 96 h after ecdysis (day 4) to the sixth instar. Thus PTTH may already be released in the ligated larvae. It may be recalled that haemolymph ecdysteroid titre in the day 4 larvae is significantly high. Hence, the increase in titres after neck ligation may be due to a positive feedback effect of the initially secreted ecdysteroids (Sakurai and Williams, 1989) on the prothoracic glands after initial activation by PTTH. Ecdysteroid titres in the thorax-ligated larvae however decreased considerably following ligation. This may be due to the absence of the prothoracic glands in these ligated larvae which is known to be the major source of haemolymph ecdysteroids (Chino *et al.*, 1974; King *et al.*, 1974).

Role of JH in the endocrine control of metamorphosis

The interplay of ecdysteroids and juvenile hormones in larval insects serve to orchestrate the progression from one developmental stage to the next, with ecdysteroids regulating the onset and timing of the moulting cycle and JH regulating the quality of the moult (Riddiford 1985, 1994; Sehnal, 1985). During the last larval stadium of holometabolous insects such as Lepidoptera,

a reduction of the haemolymph JH levels is a necessary step in the initiation of metamorphosis (Riddiford, 1980; Sehnal, 1985). However, increase in JH levels associated with the wandering stage of last instar larvae has been shown to be critical for the success of larval-pupal ecdysis (Shooley *et al.*, 1976; Hsiao and Hsiao, 1977; Bean *et al.* 1982; Jones and Hammock, 1985; Balamani and Nair, 1991; Edwards *et al.*, 1995). There is very little published information on JH levels in lepidopterous pupae. The accepted view is that JH is largely absent in the pupa in order that development of adult structures may proceed. However, increased levels of JH has been reported in pharate adults and adults (Edwards *et al.*, 1995).

Inhibitory effects of JH on ecdysteroid titres

Repetitive treatments of high dosage of JHA to early sixth instar larvae of *S. mauritia* interfere with larval development and metamorphosis. The treated larvae moulted into imperfect supernumerary larvae within 24 h of termination of hormone treatment. These were larval in appearance except for a few pupal characteristics especially the mouth parts, antennae and thoracic legs which were sclerotised. The paired wing discs were also mildly sclerotised. These findings confirm and supplement earlier observations in *S. mauritia* (Santha and Nair, 1987; Balamani and Nair, 1991; Venugopalan, 1995). Similar results were observed with treatments of JHA to last instar *Galleria* larvae (Slama and Mala, 1984) and *S. litura* (Hatakoshi *et al.*, 1986).

The commitment peak of ecdysteroids did not appear in the JHA treated *S. mauritia* larvae. The large premoult peak preceding larval-pupal moult was also missing. A much smaller peak appeared one day earlier (day 4) to the premoult peak (day 5) of the control larvae and the treated larvae moulted into supernumerary larvae on the next day. The induction of the formation of supernumerary larvae may be caused due to suppression of normal haemolymph ecdysteroid titres by the JHA. JHA also caused ecdysteroid titres to appear 12 - 24 h earlier than normal. It seems that prothoracic glands of treated larvae become active earlier than those of controls. JH apparently accelerates the release of PTTH, which in turn stimulates the prothoracic glands (Sehnal *et al.*, 1981). It has also been reported that juvenile hormone or its analogue, administered externally exerts its morphogenetic effects along with other environmental stimuli by either inhibiting or accelerating moulting processes through interference with the prothoracic glands and brain (Slama, 1971; Gilbert *et al.*, 1980; Sehnal, 1983). JH converts the metamorphic cycle into a larval one by modifying the growth and metabolism of prothoracic glands (Slama and Mala, 1984). In holometabolous insects, the commitment for a larval-pupal moult is induced by 20-hydroxyecdysone in the absence of JH (Riddiford, 1996). Therefore, persistence of JH or JHA during that time may have resulted in the formation of supernumerary larvae.

Repetitive treatments of *S. mauritia* with JHA also seems to affect the growth, differentiation and developmental competence of imaginal structures resulting in the formation of imperfect superlarvae. This may be because of alterations in the haemolymph titre of ecdysteroids leading to unco-ordinated development of different tissues since different tissues may have different hormonal requirements for further development (Riddiford, 1981).

In neck-ligated sixth instar larvae of *S. mauritia*, ecdysteroid titres that were reduced following neck ligation were further reduced after JHA treatment. However, titres in the thorax-ligated larvae were similar for treated and control larvae. From previous studies, head critical period for gut purge is tentatively proposed to occur during the period from 24 h to 36 h after ecdysis in *S. mauritia*. It follows that PTTH is already released in the ligated larvae. Hence application of JHA is not expected to have any effect on gut purge. However, JHA treated, ligated larvae did not exhibit gut purge whereas 70% of the control larvae exhibited the premetamorphic behaviour of gut purge.

The ecdysteroid data support the concept that JH can directly inhibit the secretory activity of prothoracic glands (Cymborowski and Stolarz, 1979; Safranek *et al.*, 1980). In *M. sexta* JH affects the phophorylation of the 34 KDa proteins which regulate a rate-limiting step in the ecdysone biosynthetic pathway (Rountree *et al.*, 1987). Hydroprene suppressed the

steroidogenic competence of prothoracic glands in neck-ligated larvae during the phagoperiod suggesting that the hormone acted directly on prothoracic glands in *M. sexta* (Watson and Bollenbacher,1988). In *B. mori* JH inhibits PTTH and also ecdysone release from prothoracic glands (Sakurai, 1984). Further studies in *B. mori* have shown that JH acts during the early stages of the instar to suppress both the secretory activity of prothoracic glands and also the acquisition of competence to respond to PTTH (Sakurai *et al.*, 1989). From neck ligation experiments it has been established that JH is able to suppress ecdysteroid production. This strengthens the observation that JH inhibits ecdysteroid production independent of the brain (Safranek *et al.*, 1980; Sehnal *et al.*, 1981; Sakurai, 1983). However, ecdysteroid titres were not significantly different in the neck-ligated JHA treated larvae compared to the control larvae and were similar in the thorax-ligated, JHA treated and control larvae. Hence prevention of gut purge by decreased ecdysteroid titres alone in the JHA treated larvae does not seem plausible. Neck/thorax ligation of day 1 larvae have shown that even low levels of ecdysteroids could cause gut purge. After the HCP, sensitivity of tissues to ecdysteroids increase (Sakurai *et al.*, 1998) hence this change. But JHA may have an inhibitory effect at the receptor level preventing gut purge although ecdysteroid titres are comparable in both the JHA treated and control larvae. In this case, JHA may also be acting at the level of other target tissues responsible for gut purge.

It is generally accepted that JH is present at relatively high levels, throughout all but the final larval stadium. The presence of JH in larval insects serves to maintain the larval state, and prevent metamorphosis. JH shows high titre early in the last larval instar which then declines to undetectable levels by the end of the feeding period (Bollenbacher *et al.*, 1981; Baker *et al.*, 1987; Jones *et al.*, 1990; Edwards *et al.*, 1995). In the last larval instar, the absence of JH by the end of the phagoperiod enables the process of metamorphosis to ensue. The subsequent release of ecdysteroids cause gut purge and wandering behaviour.

Stimulatory effects of JH on ecdysteroid titres

Increased levels of JH has been associated with the prepupal stage of several lepidopteran species (Baker *et al.*, 1987; Jones *et al.*, 1990; Edwards *et al.*, 1995). JH titres reportedly increase approximately 24 h prior to pupal ecdysis (Edwards *et al.*, 1995). It has also been reported that after gut purge, JH or analogues actually seem to accelerate prepupal development by elevating ecdysteroid titres (Cymborowski and Stolarz, 1979; Hiruma, 1980; Safranek *et al.*, 1980).

During the course of this study, it was found that neck-ligated day 4 sixth instar larvae treated with JHA underwent larval-pupal ecdysis while thorax-ligated larvae treated with JHA and control larvae (neck- and thorax-ligated) remained unchanged. The JHA treated neck-ligated day 4 larvae

transformed into headless pupae. Earlier studies have shown that PTTH may already be released at this time. Ecdysteroid titres of the headless pupae (day 0) were higher than control larvae on day 7 but slightly lower than that of the neck-ligated larvae on day 8. Ecdysteroid titres of the JHA treated, thorax-ligated and control larvae were low. This suggested that the presence of prothoracic glands and hence a critical titre of ecdysteroids is essential for JH to bring about pupation. Induction of pupation in the JHA treated neck-ligated larvae is consistent with the observations of Masner *et al.* (1975), Mitsui and Riddiford (1978) and other authors that JH can lower the hormonal requirements of epidermis for cuticle secretion. This may be the reason for the induction of pupation in the JHA treated, neck-ligated larvae and the failure of pupation in the control larvae at similar titres. Increased levels of JH has also been reported immediately prior to the moult in anti-penultimate and penultimate instar larvae and also in pharate adults (Edwards *et al.*, 1995) of the tomato moth *Lacanobia oleracea*. Hence it may be assumed that these pre-ecdysial increases may be intimately associated with the promotion of morphogenesis in addition to the stimulation of ecdysteroid production by the prothoracic glands. During larval-pupal transformation in most Lepidoptera, a brief burst of JH occurs in the prepupal phase of development (Bean *et al.*, 1982; Baker *et al.*, 1987; Jones *et al.*, 1990; Edwards *et al.*, 1995). Several studies have demonstrated that this increase of JH along with the second release of PTTH activates the prothoracic glands to

their maximal rate of ecdysone secretion needed for moulting and metamorphosis (Cymborowski and Stolarz, 1979; Hiruma, 1980; Sieber and Benz, 1980; Balamani and Nair, 1991). Further, experiments have indicated that JH activation of prothoracic glands may be mediated by a prothoracic gland stimulatory factor which is induced in the fat body by JH (Gruetzmacher *et al.*, 1984b). There is also suggestive evidence to show that prepupal increase of JH has a morphogenetic role in promoting the development of normal pupal morphology (Safranek *et al.*, 1980; Jones and Hammock, 1985; Balamani and Nair, 1991). Treatments of last instar larvae of *S. mauritia* during prepupal phase with anti-JH agents induced the production of larval-pupal intermediates (Santha and Nair, 1986; Nair and Rajalekshmi, 1989). It has also been suggested that the presence of a critical titre of JH in the prepupal phase is needed for ecdysteroids to exert their effects on imaginal structures (Balamani and Nair, 1991). JH may also enhance the response of ecdysteroids and/or alter the sensitivity of tissues to ecdysteroids (Denlinger, 1979).

In Lepidoptera, prothoracic glands degenerate during or after eclosion to the adult form (Gilbert, 1962) by undergoing programmed cell death or apoptosis (Dai and Gilbert, 1997). However, treatment of JHA to newly ecdysed pupae of *S. mauritia* increased the levels of ecdysteroid titres when compared to controls. The increase in titres appeared to be a dose dependent

phenomenon as titres increased with higher dosage of JHA. It has been reported that in paurometabolous insects, the degeneration of prothoracic glands is prevented by application of JHA suggesting that either the presence of JHA acts to maintain prothoracic glands (Smith and Nijhout, 1983) or the absence of JH programmes the glands for degeneration (Lanzrein, 1975). JH may also have a tropic effect on the prothoracic glands in addition to preventing its degeneration (Sakurai and Gilbert, 1990). The premoult peak of ecdysteroids appeared on day 3 in the 0.5 μg JHA treated pupae while the peak appeared on day 2 in the 1 μg JHA treated and control pupae and titres decreased gradually thereafter. Increased levels of ecdysteroids on most days in the JHA treated pupae may be due to increased rates of synthesis elicited by JHA treatment in the prothoracic glands. In *M. sexta*, administration of JH prevented apoptosis of the prothoracic glands even 11 days after JH injection into young pupae. The prothoracic glands remained intact and their ability to synthesise ecdysteroids was maintained at a fairly active level as ascertained by RIA after *in vitro* incubation (Dai and Gilbert, 1998). Thus in addition to its maintenance function, JH acts as a tropic factor by stimulating ecdysteroidogenesis in the prothoracic glands of various insects (Sakurai and Gilbert, 1990). The present experiment confirms this tropic function in *Spodoptera*. JH may stimulate PTTH secretion from the brain retrocerebral complex as well, since the ecdysteroid biosynthetic capacity of JH-injected *M. sexta* pupae having an intact brain was significantly higher than that of

JH-injected brainless pupae (Dai and Gilbert, 1998). While JH plays a role in maintaining the glands, prothoracic gland degeneration itself is stimulated by ecdysone in the absence of JH (Smith and Nijhout, 1983).

The presence of ecdysteroid receptor (ER) complex in the prothoracic glands is well accepted (Bidmon and Sliter, 1990; Talbot *et al.*, 1993) and that the sensitivity of the prothoracic glands to ecdysteroids is dependent upon the ER concentration. It was hence suggested that JH could maintain the glands by interfering with the increase in ER concentration elicited by circulating ecdysteroids. Thus it may be possible that in the absence of JH, the prothoracic glands initiate and modulate adult development by synthesising and secreting large quantities of ecdysteroids and in that way also elicit their self destruction, i.e. it is a suicidal event for the glands (Dai and Gilbert, 1997). The composite data suggest that JH can both maintain and stimulate the prothoracic glands. Therefore during normal pupal-adult metamorphosis, the absence of JH is a prerequisite for both the initiation and completion of prothoracic gland degeneration. Also, the generally accepted view is that JH is largely absent in the pupa in order that development of adult structures may proceed.

JHA treatment was found to interfere with transformation of pupae into adults. Slama *et al.* (1974) reported that application of different doses of the juvenoids during the critical period evokes the formation of intermediate

forms between the previous and subsequent developmental stages. The failure of emergence of JHA treated insects may be due to the inhibition of synthesis or release of eclosion hormone (Truman, 1971). Since eclosion hormone is released by a drop in the ecdysteroid level after a peak level, prior to ecdysis (Riddiford, 1985). In this respect, high ecdysteroid titres caused by the prothoracicotropic action of JHA, may cause an inhibition of eclosion hormone release in *S. mauritia*. The development of pupal-adult intermediates may be due to differential sensitivity of different tissues to ecdysteroids.

Effects of ecdysteroid agonist RH 5992 on metamorphosis of sixth instar larval stadium

In the course of this study, it was found that RH 5992 acts primarily by inducing a premature, lethal moult. This mode of action has been confirmed by several authors by applying RH 5847, a similar ecdysteroid agonist to larvae of several Lepidoptera: *Spodoptera eridania* (Aller and Ramsay, 1988) *M. sexta* (Wing *et al.*, 1988), *B. mori* (Kiuchi, 1990) *Plodia interpunctella* (Silhacek *et al.*, 1990), *S. littoralis* (Smagghe and Degheele, 1992), *S. frugiperda* (Monthean and Potter, 1992), *Plutella xylostella* and *P. brassicae* (Darvas *et al.*, 1992), *S. exempta* and *S. exigua* (Smagghe and Degheele, 1993[a,b]) and *S. exempta, S. littoralis, S. exigua, M. brassicae, G. mellonella* (Smagghe and Degheele, 1994[a,b]) and *S. mauritia* (Sakunthala and Nair, 1995). In some

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coleopteran species, RH 5849 caused a variety of effects on moulting and behaviour (Aller and Ramsay, 1988; Darvas *et al.*, 1992; Smagghe and Degheele, 1993, 1994). RH 5992 also induced precocious, lethal moulting in Lepidoptera: *S. exempta, M. brassicae, S. littoralis, S. exigua* and *G. mellonella* (Smagghe and Degheele, 1994b) and *Choristoneura fumiferana* (Retnakaran *et al.*, 1997). However, RH 5992 had no effect on larval instars of Coleoptera, Heteroptera and Orthoptera (Smagghe and Degheele, 1994b).

Treatment of RH 5992 to newly moulted sixth instar *S. mauritia* larvae induced an incomplete larval moult resulting in the formation of a new untanned head capsule in 48 h. Since the treatment was before the commitment peak of ecdysteroids, the newly secreted cuticle was larval. Acceleration of gut purge behaviour also occurred in these larvae. Gut purge has been demonstrated to be an ecdysteroid dependent phenomenon (Truman and Riddiford, 1974; Fujishita *et al.*, 1982). Hence the treated RH 5992 may have stimulated this behaviour. When post-feeding (day 4) larvae were treated with RH 5992, the newly secreted cuticle was pupal. This may be because the treatment was done after the commitment peak of ecdysteroids when the epidermal cells are already committed to the pupal programme. Ultrastructural studies on treated larvae also showed that ecdysteroid agonists caused a forced, untimely synthesis of a new, however incomplete cuticle (Smagghe *et al.*, 1994). Treatment of RH 5992 to post-feeding larvae

resulted in the formation of larval-pupal intermediates. The larval-pupal intermediates have an unsclerotised pupal cuticle in the abdominal region and thoracic tergum beneath the old cuticle whereas the thoracic legs were larval in appearance. Since thoracic legs develop from imaginal discs, they may have different hormonal requirements from the epidermal cells. Injection of ecdysterone to post-feeding ligated larvae of *S. mauritia* also induced the formation of larval-pupal intermediates (Balamani and Nair, 1991).

It was observed that feeding and weight gain ceased simultaneously in treated larvae after the induction of premature moulting. This was also the case in normal *S. mauritia* larvae where cessation of feeding and fresh weight gain occurred prior to pupation. Similar effects on growth and development have been reported in other insects, either during normal development, or when treated with natural ecdysteroids (Kiguchi and Agui, 1981; Sehnal *et al.*, 1981; Kubo *et al.*, 1983). For non-steroidal ecdysteroid agonists, the observed effects agree with those of Wing *et al.* (1988) who reported that RH 5849 caused precocious moulting and cessation of feeding in *M. sexta* larvae. Likewise, Silhacek *et al.* (1990), Darvas *et al.* (1992) and Smagghe and Degheele (1994a,b) reported suppression of weight gain in various insect species treated with RH 5849 and RH 5992.

Positive feedback effect of RH 5992

Treatment of RH 5992 induced gut purge and precocious lethal moulting in both neck-ligated and thorax-ligated day 1 larvae. Induction of gut purge and moulting in thorax-ligated larvae which lacked a biosynthetic source of ecdysteroids strengthens the assumption that RH 5992 and other ecdysteroid agonists behave as a bonafide ecdysone in Lepidoptera. Moreover, Smagghe and Degheele (1992) have reported that ecdysteroid agonists (RH 5849) stimulate epidermal cells to undergo apolysis and synthesise a new cuticle *via* binding of RH 5849 to ER of epidermal cells. They also demonstrated by using electrophoretic methods that malformation of the new larval cuticle was due to the disappearance of several cuticular and haemolymph proteins in the treated larvae. Also, RH 5849 and RH 5992 showed receptor binding activity, ecdysteroid responsive gene expression activity and induction of morphological changes in *Drosophila* KC cells corresponding to their *in vivo* potencies (Mikitani, 1996). Acceleration of gut purge behaviour may also be due to a similar mechanism.

Ecdysteroid titres of treated neck-ligated larvae decreased marginally on day 1 and day 2 but an abrupt increase was observed on day 3 when compared to control titres which showed a decreasing trend. The reduction in titres on day 1 and day 2 in RH 5992 treated and control larvae may be due to the absence of cephalic endocrine structures secreting PTTH which is known

to be the major neurohormone regulating ecdysteroid production in insects (Sakurai and Gilbert, 1990). The abrupt increase on day 3 may be due to positive feedback effect of RH 5992 on the prothoracic glands. The prothoracic glands of the ligated larvae are relatively inactive and it has been postulated that the nature of the feedback (positive or negative) is likely to depend on the secretory history of the glands (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989). Since prothoracic glands have also been known to possess ecdysteroid receptors (Bidmon and Sliter, 1990; Talbot *et al.*, 1993), it is possible that receptor induction allows the prothoracic glands to respond to ecdysteroids leading to a rise in ecdysteroid production by prothoracic glands and hence the haemolymph ecdysteroid titres. A major contributing factor to the efficacy of RH 5992 is its superior absorption and metabolic stability in Lepidoptera or their tissues (Retnakaran *et al.*, 1995). This effect did not manifest itself on day 1 and day 2 because larvae exposed to RH 5992 after ligation still contains a high JH titre and JH can directly inhibit the secretory activity of prothoracic glands (Cymborowski and Stolarz 1979; Safranek *et al.*, 1980). Synthesis of a larval cuticle following RH 5992 treatment during early sixth larval stadium also indicates that JH titre is high during this time.

Thorax-ligated larvae treated with RH 5992 did not show a significant difference in ecdysteroid titres when compared to control larval titres. The

ligated larvae lacked cephalic and thoracic endocrine structures and hence the reduction in titres in both the treated and control larvae.

Negative feedback effect of RH 5992

Treatment of RH 5992 induced abnormal and lethal pupation both in neck-ligated and thorax-ligated day 4 larvae 48 h after treatment. Larval-pupal intermediates with unsclerotised pupal cuticle in the abdominal region and thoracic tergum, larval thoracic legs and fused testis were formed. A similar phenomenon has been reported by Friedlander and Brown (1995) causing reinitiation of pupal development in isolated abdomens of diapausing *Cydia pomonella* larvae treated with RH 5992. Synthesis of pupal cuticle and testicular fusion, which are pupal characteristics were also observed in the treated isolated abdomens. Treatment of RH 5849 and RH 5992 inducing larval-pupal intermediates has also been reported in other Lepidoptera: *S. exempta, S. exigua, S. littoralis, M. brassicae* and *G. mellonella* (Smagghe and Deheele, 1994a, b).

Pupal cuticle synthesis in the treated, thorax-ligated larvae which lacked prothoracic glands further strengthened the ER-activation concept. The newly synthesised cuticle was pupal because the epidermal cells were already committed to pupal phase as has been described previously. Testicular fusion was also observed in all the treated larvae while the control

larvae failed to exhibit any of the above mentioned changes in morphogenesis and died as prepupae.

Treatment of RH 5992 to neck-ligated larvae led to a decrease in haemolymph ecdysteroid titres on all days following treatment both in the sixth instar larvae and the larval-pupal intermediates. Conversely, titres showed an increasing trend in the control larvae. The decline in titres may be due to a negative feedback effect of RH 5992 on prothoracic glands in the neck-ligated larvae. The prothoracic glands of *S. mauritia* of day 3 has been reported to be moderately active (Balamani and Nair, 1994, 1997). Haemolymph ecdysteroid titre determination of *S. mauritia* reported earlier has also suggested active secretory activity of prothoracic glands during day 3 – day 4. That the nature of the feedback effect depends on the secretory history of the prothoracic glands has already been discussed. Hence, it follows that the reduction in ecdysteroid titres may be due to a negative feedback effect. Thus a change in sensitivity of the prothoracic glands cells to RH 5992 is observed, the feedback effect being positive in the day 1 ligated larvae and negative in day 4 ligated larvae.

Treatment of RH 5992 to thorax-ligated day 4 larvae led to a decreasing trend of titres. Control larvae also exhibited a similar trend of declining ecdysteroid titres. This may be due to the absence of prothoracic glands in both treated and control larvae. Moreover, other ecdysteroid secreting tissues

in the abdomen may be refractory to the action of RH 5992 at this stage due to negative feedback effect.

In *S. mauritia* treatment of RH 5992 to newly ecdysed pupae caused high mortality in the treated pupae towards the end of the pupal period. The pupal period of the treated pupae was also found to be prolonged. Ecdysteroid titres were found to be significantly reduced in both the dosages treated when compared to the control pupae. They also did not exhibit the moulting peak on day 2. On day 5, titres were lower than that on day 2 in the treated pupae but significantly higher than those of control pupae. RH 5992 may have a negative feedback effect on the prothoracic glands (as observed in late sixth instar larvae) preventing the premoult ecdysteroid peak on day 2 and in the process preventing apoptosis.

Beydon and Lafont (1983) have reported a similar phenomenon in *P. brassicae* pupae where 20-hydroxyecdysone acts on ecdysteroid production as a negative-feedback regulator. 20-hydroxyecdysone also suppressed ecdysone production by the prothoracic glands *in vitro* (Scott, 1982). Prothoracic glands possess ecdysteroid receptors (Bidmon and Sliter, 1990; Talbot *et al.*, 1993) and the nature of the feedback effect depends on the secretory history of the glands (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989). Ecdysteroid titres of the normal pupae of *S. mauritia* show that prothoracic glands of day 0 pupae are moderately

active, hence the negative feedback effect. RH 5992 however induces pupal-adult metamorphosis *via* ER activation. Hence pupal-adult metamorphosis could be promoted by the treated RH 5992 which serves as the "moulting peak" for the development of imaginal structures in the absence of endogenous premoult ecdysteroid peak. The high levels of RH 5992 also prevents ecdysis by inhibiting the release of eclosion hormone since eclosion hormone is released by a drop in the ecdysteroid level after a peak titre, prior to ecdysis (Riddiford, 1985).

3.5 SUMMARY

1. The haemolymph ecdysteroid profile of *S. mauritia* was determined during the sixth (final) larval instar and pupal stage by means of RIA. Effects of hormones and hormone mimics on haemolymph ecdysteroid titres were studied by means of ligation and treatments of a JHA (hydroprene) and a non-steroidal ecdysteroid agonist (RH 5992). Control insects were treated with acetone.

2. Haemolymph ecdysteroid profile of sixth instar larvae revealed two peaks, the commitment peak on day 3 and the moulting peak on day 5 when daily sampling was done. Six hourly sampling of sixth instar larval haemolymph revealed five peaks. In addition to the commitment and moulting peaks of ecdysteroids, two small peaks appeared before the commitment peak and one small peak after the moulting peak. The commitment peak induces gut purge and other prodromes of pupation while the moulting peak promotes pupation. Daily sampling of pupal haemolymph revealed a single large peak on day 2 which promotes pupal-adult metamorphosis.

3. The timings of PTTH release during larval-pupal development were studied by observing the percentage of neck-ligated larvae pupating or showing other prodromal signs of pupation. Sixth instar larvae were neck-ligated at 6 h intervals from the time of ecdysis and the effect on two ecdysteroid-dependent developmental processes i.e., gut purge and pupal cuticle secretion were observed. This study suggested that the first release of PTTH occurs during the scotophase between 24 h and 36 h after ecdysis. The second release of PTTH probably occurs after 96 h of ecdysis but prior to 102 h. The first release of PTTH probably stimulates the secretion of the commitment peak and the second release of PTTH, the moulting peak of ecdysteroids.

4. Influence of brain factors and prothoracic gland secretions on haemolymph ecdysteroid titres were studied by neck/thorax ligation of day 1 and day 4 sixth instar larvae. Haemolymph ecdysteroid titres were reduced significantly following ligation on day 1 but 70% of the ligated larvae purged their gut after a slight delay even at these reduced ecdysteroid titres. Following neck ligation on day 4, titres increased gradually to reach a peak on day 8 which was not significantly different from the ecdysteroid titre peak on day 5 (prepupae) of the control larvae. But thorax ligation led to a significant decrease in titres of the haemolymph. However, both neck- and thorax-ligated larvae failed to undergo pupation.

5. The influence of JH on haemolymph ecdysteroid titres was investigated by topical application of JHA to unligated day 0 larvae and neck-/thorax-ligated day 1 and day 4 sixth instar larvae and intact pupae of *S. mauritia*. The effects of these treatments on larval-pupal and pupal-adult transformations were observed.

6. Repetitive treatments of 20 µg JHA to intact sixth instar larvae induced an imperfect supernumerary larval moult on day 5. The commitment peak failed to appear and a much smaller premoult peak appeared on day 4 in the treated larvae. The commitment peak appeared on day 3 and a larger premoult peak appeared on day 5 in the control larvae. JHA treatment probably promoted PTTH release 24 h earlier than in controls and also suppressed prothoracic gland secretion of ecdysteroids.

7. Repetitive treatments of 5 µg JHA to neck-/thorax-ligated day 1 larvae prevented gut purge in the treated larvae while 70% of the control larvae purged their gut. Haemolymph ecdysteroid titres of JHA treated, neck-ligated larvae were slightly lower than control while titres were equal in the thorax-ligated and control larvae. Ecdysteroid titres of JHA treated and control larvae were not significantly different, yet the prevention of gut purge in the treated larvae suggested that JH in addition to suppressing the prothoracic glands also alters tissue sensitivity to ecdysteroids.

8. Single treatment of JHA to neck-/thorax-ligated day 4 larvae promoted pupation in the neck-ligated larvae but failed to induce pupation in the thorax-ligated larvae. Control larvae failed to undergo pupation in both the neck- and thorax-ligated larvae. Ecdysteroid titres of the headless pupae were higher than those of the control larvae on the same day. The presence of JH may be essential for normal pupal morphogenesis. JH may also stimulate higher rates of ecdysteroid synthesis by the prothoracic glands at this stage. Thorax-ligated, JHA treated larvae exhibited ecdysteroid titres lower than that of control larvae. The failure of pupation in the JHA treated (thorax-ligated) larvae may be due to the absence of the prothoracic glands.

9. JHA treatment to pupae induced formation of adultoids which failed to emerge. Haemolymph ecdysteroid titres increased considerably following JHA treatment. However, the pattern of fluctuation was similar in the treated and control pupae. JHA prevents apoptosis (programmed cell death) of the pupal prothoracic glands and also has a tropic effect on the glands, stimulating ecdysteroidogenesis. The increased titres maintained during the latter half of pupal development

probably prevented release of eclosion hormone resulting in the failure of emergence of adults in the treated pupae.

10. The effects of ecdysone agonist RH 5992 on larval development and metamorphosis and haemolymph ecdysteroid titres was investigated by topical application of RH 5992 on unligated larvae and neck-/thorax-ligated day 1 and day 4 sixth instar larvae and intact pupae of *S. mauritia.*

11. Treatment of RH 5992 to intact sixth instar day 0 larvae induced a premature, lethal larval moult and acceleration of gut purge in the treated larvae. The newly synthesised cuticle was larval since the treatment was before the commitment peak of ecdysteroids. Treatment of RH 5992 to intact day 4 larvae induced the formation of larval-pupal intermediates. The control larvae underwent normal pupation on day 6.

12. Treatment of RH 5992 to neck-/thorax-ligated sixth instar day 1 larvae with different dosages of RH 5992 caused a precocious lethal larval moult in the ligated larvae. All the treated larvae exhibited gut purge precociously while only 70% of the control larvae purged their gut that too after some delay. Ecdysteroid titres in the RH 5992 treated neck-ligated larvae decreased on days 1 and 2 but increased abruptly on day 3 when compared to control titres which showed a decreasing trend on all days. Titres of the RH 5992 treated and control thorax-ligated larvae were similar. Induction of a moult at low titres indicated that RH 5992 promotes a moult at the receptor level without an increase in haemolymph ecdysteroid titres. Increase in titres on day 3 may be due to a positive feedback effect of RH 5992 on prothoracic glands. In the absence of prothoracic glands in the thorax-ligated larvae, such a feedback effect may not have occurred.

13. Treatment of different dosages of RH 5992 to neck-/thorax-ligated sixth instar day 4 larvae induced an abnormal and lethal pupation. The larvae transformed into larval-pupal intermediates. Control larvae failed to exhibit any change and died as larvae. Ecdysteroid titres of the treated, neck-ligated larvae decreased on all days while control titres increased to reach a peak on day 8. The reduction in titres in the treated larvae may be due to negative feedback effect of RH 5992 on prothoracic glands. Ecdysteroid titres of the thorax-ligated larvae also decreased following treatment as in the case of control larvae. Induction of pupation in the treated larvae even at low titres of ecdysteroids further demonstrates that RH 5992 acts at the receptor level.

14. RH 5992 treatment to pupae induced formation of adultoids. The pupal period got extended following treatment and mortality occurred towards the end of the pupal period. The moulting peak failed to appear in the treated pupae while control pupae exhibited a single large premoult peak on day 2. On day 5, titres of the treated pupae decreased slightly but was higher than that of the control pupae. Reduction in titres on day 2 in the treated pupae may be due to negative feedback effect of RH 5992 which inturn may prevent apoptosis. Hence, the basal secretory activity of the prothoracic glands was maintained even on day 5 in the treated pupae. This may cause the higher titre in the treated pupae on day 5. The relatively high ecdysteroid titres and retention of RH 5992 in the tissues during the latter half of pupal stage may have prevented release of eclosion hormone and hence the mortality towards the end of pupal period.

CHAPTER 4

DEVELOPMENTAL PROFILE OF TESTICULAR ECDYSTEROIDS AND THE EFFECTS OF HORMONE MIMICS

4.1 INTRODUCTION

Ecdysteroids have been reported in the testes of many lepidopteran species (Loeb *et al.*, 1982, 1984, 1988; Shimizu *et al.*, 1985; Gelman *et al.*, 1989; Jarvis *et al.*, 1994). Earlier studies revealed that ecdysteroid secretion in testes occurred at discrete periods during the last larval instar and pupal stadium of various Lepidoptera. Adult testes have also been reported to contain ecdysteroids. Synthesis of ecdysteroids is suggested to be induced by a brain peptide, testis ecdysiotropin (Loeb *et al.*, 1986, 1987). Testes from larvae (final instar) and pupae released large amounts of ecdysteroids *in vitro* (Loeb *et al.*, 1982, 1984; Shimizu *et al.*, 1985). Testicular metabolism of ecdysteroids has been investigated by incubating the testes with [^{3}H] ecdysone which was found to be converted mostly to 20-hydroxyecdysone and traces of other ecdysones (Gelman *et al.*, 1985; Loeb *et al.*, 1988; Loeb and Woods, 1989). Comparison of ecdysteroids in the testes and haemolymph of *H. virescens* (Loeb *et al.*, 1982), *O. nubilalis* (Gelman *et al.*, 1988a) and *S. littoralis* (Jarvis *et al.*, 1994) revealed that the pattern of fluctuation of ecdysteroids is similar for total levels but differs in the timing of their fluctuations and in their content.

A vast amount of literature has accumulated implicating hormones in the regulation of spermatogenesis (reviewed by Dumser, 1980; Koeppe *et al.*, 1985). JH has been implicated both as an inhibitor (Fukuda, 1944; Nowock,

1973; Leviatan and Friedlander, 1979; Deb and Chakravorty, 1981) and stimulator (Nishitsutsuji-Uwo, 1961; Yagi, 1975; Shinyaeva, 1981) of testicular development. The stimulatory effects of ecdysteroids on spermatogenesis has also been well documented in several insect species both in *in vitro* and *in vivo* experiments (Nishitsutsuji-Uwo, 1961; Yagi *et al.*, 1969; Dumser and Davey, 1975; Dumser, 1980; Gelman and Borkovec, 1986).

The present work investigates the fluctuations in testicular ecdysteroids of *S. mauritia* during the immature (larva, pupa) and adult stages of development. Role of head factors and prothoracic gland secretions in influencing testicular ecdysteroid content has been analysed by ligation techniques as described earlier. Effects of treatments of a JHA (hydroprene) and an ecdysteroid agonist (RH 5992) on testicular ecdysteroid titres have also been investigated. The endogenous testicular ecdysteroid levels during the sixth (final) instar larval and pupal stages are compared with those in the haemolymph. This information, it was thought would facilitate correlation of timing of testicular ecdysteroid peaks with developmental stage in *S. mauritia* and thus uncover some clues on the physiological importance of testicular ecdysteroid content. Testicular ecdysteroid titres have also been correlated with known stages of spermatogenesis in *S. mauritia* (Venugopalan *et al.*, 1994) to ascertain the role of testicular ecdysteroids in regulating testicular development and spermatogenesis.

4.2 MATERIALS AND METHODS

Animals

Larvae, pupae and adults of *S. mauritia* were taken from the laboratory stock culture reared and maintained as described previously. The present studies were conducted on sixth (final) instar larvae, pupae and adults of various ages.

Ligations

Ligations were done as described earlier (see page 45).

Chemicals

Chemicals were obtained from various sources as mentioned earlier (see page 46).

Treatments

Larvae and pupae were treated with different dosages of chemicals as described previously (see page 46).

Dissection of testes lobes

Sixth instar larvae, pupae and adults of *S. mauritia* were anaesthetised in specimen tubes containing a wad of cotton soaked in diethyl ether at the bottom. The anaesthetised insects were taken out after 5 minutes and pinned

dorsal side up in a wax lined petridish. The dissections were carried out in insect Ringer solution (Ephrussi and Beadle, 1936). After dorsal incision, the cuticle was cut open along the mid-dorsal line upto the hind edge of the head capsule, and pinned back exposing the internal anatomy. The testis was taken out using fine forceps, cleaned of extraneous tissue and rinsed in insect Ringer solution to remove adherent haemolymph.

Preparation of tissue samples for RIA

Tissue samples were prepared by the procedure described earlier (see page 49) and RIA done as described earlier.

4.3 RESULTS

4.3.1 Testicular ecdysteroid titres during larval-pupal-adult development

The larvae of *S. mauritia* pass through six instars before they pupate. Sixth instar larvae purge their gut and exhibit wandering behaviour on the fourth day of the sixth (final) instar. Pupation occurs on day 6 and the adult males emerge after eight days.

Testes of *S. mauritia* are a pair of kidney shaped organs attached to the body wall on either side of the mid-dorsal line in the fifth abdominal segment. The testicular volume increases gradually during the fifth (penultimate) instar and reaches a maximum on day 3 sixth instar larvae, when it measures

4.3 ± 0.9 mm^3. During the pharate pupal stage, the paired testes fuse in the mid-dorsal line to form a single median, spherical structure. The fused testis shows a remarkable increase in volume from early pupal stage and reaches its maximum size in day 2 pupae when it measures 10.5 ± 0.86 mm^3. The testis volume decreases from day 2 onwards and is least in the adult stage. The compartmentalisation of the testicular lumen into four follicles is also lost in the late pupal stages.

The present study was undertaken to determine testicular ecdysteroid titres of larvae, pupae and adults of *S. mauritia*. Testicular ecdysteroids were determined by means of RIA and compared with known stages of spermatogenesis. Results are expressed as pg 20-hydroxyecdysone equivalents/testes pair.

(i) Testicular ecdysteroid titres during sixth instar larval stadium

Testicular ecdysteroid titres for sixth instar larvae of *S. mauritia* are shown in Fig. 23. In the newly moulted (white head capsule) sixth instar larvae (day 0), the titres were 145 ± 31 pg which increased to 151 ± 29 pg on day 1. On day 2 hormone titres got reduced to 120 ± 7 pg. Ecdysteroid titres started to rise steadily on day 3 (225 ± 59 pg). The increasing trend continued on day 4 (460 ± 37 pg). The paired testes fused on day 5, the prepupal stage. An increase in ecdysteroid titres were also observed on day 5 (594 ± 153 pg). The testicular ecdysteroid titres increased greatly towards the late sixth

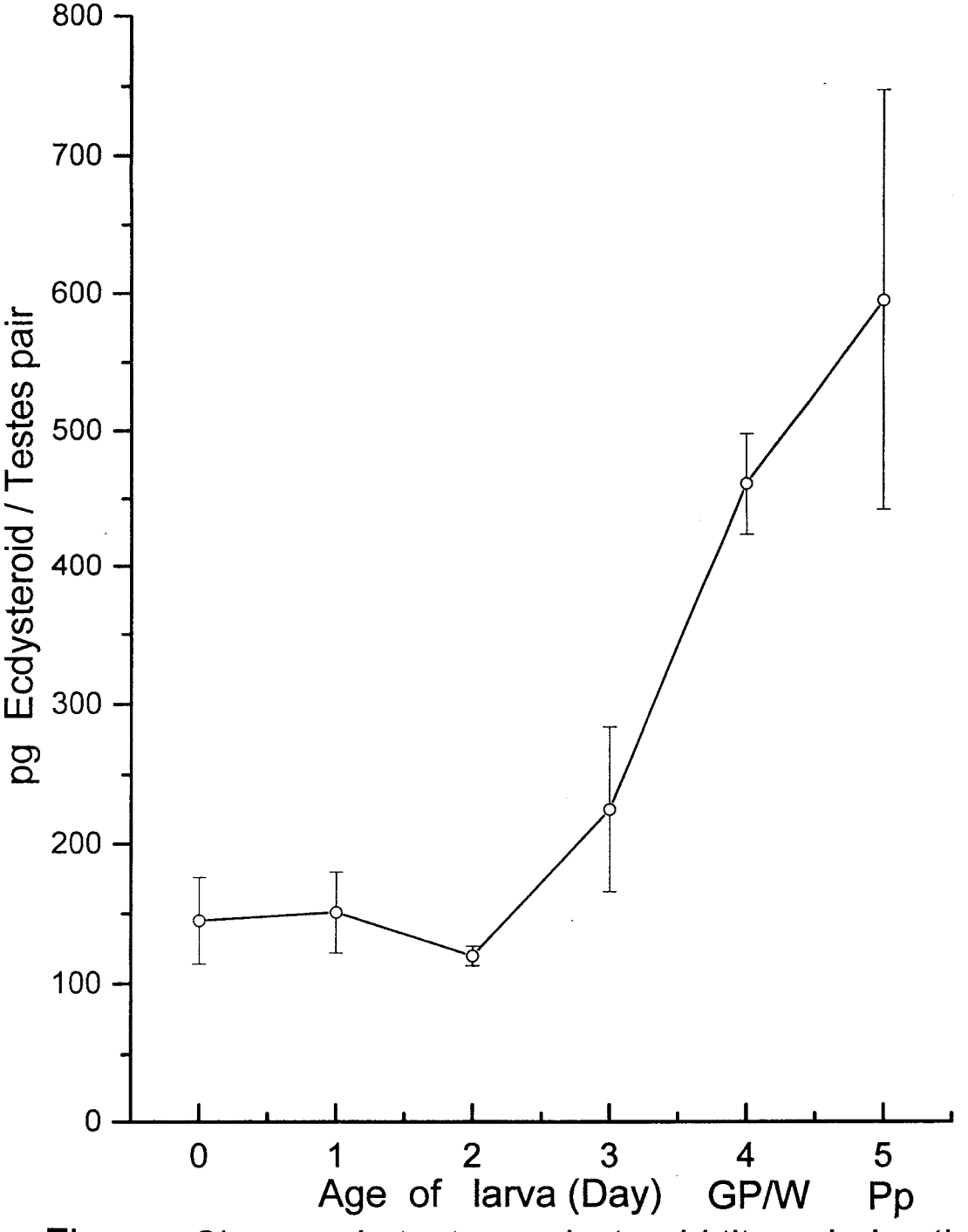

Fig. 23 Changes in testes ecdysteroid titres during the sixth instar larval development

GP – Gut purge ; W – Wandering ; Pp – Prepupa

Table12. **Effects of ligations of sixth instar day 1 larvae on metamorphosis and testicular fusion**

Nature of ligation	*n*	Days of survival (Mean ± SD)	% of surviving larvae which showed:			Nature of testes lobes
			No change	Gut purge	Pupation	
Neck-ligated	25	9 ± 1	30	70	--	Separate
Thorax-ligated	25	9 ± 1	30	70	--	Separate
Unligated (Control)	15 each	6 ± 0	--	100	100	Fused

instar larval stage and formed a significant peak ($P < 0.05$) on day 5, following testis fusion.

(ii) Testicular ecdysteroid titres during pupal stadium

Ecdysteroid titres of the testes during the post-pupal stage are represented in Fig. 24. In the newly moulted pupae (day 0), the hormone titres were low (851 $\pm$ 226 pg). Ecdysteroid titres increased considerably to reach its highest value on day 1 (2642 $\pm$ 192 pg). Titres declined steadily on day 2 (2106 $\pm$ 335 pg), day 3 (1913 $\pm$ 446 pg), day 4 (1452 $\pm$ 534 pg), day 5 (773 $\pm$ 139 pg), day 6 (320 $\pm$ 68 pg) and day 7 (269 $\pm$ 43 pg). The testicular ecdysteroid titres increased significantly ($P < 0.05$, relative to titres on day 0 and day 6) in the early pupal stage and then declined gradually, reaching a minimum on day 7.

(iii) Testicular ecdysteroid titres during adult stadium

Testicular ecdysteroid titres of day 0 adults were 17 $\pm$ 12 pg. Hormone titres reduced considerably in the adult.

4.3.2 Effects of ligations of sixth instar larvae on testicular ecdysteroid titres

This study was undertaken to investigate the role of brain factors and prothoracic gland secretions on testicular ecdysteroid titres of *S. mauritia*. In one set of experiments, sixth instar day 1 or day 4 larvae were 'neck-ligated' to

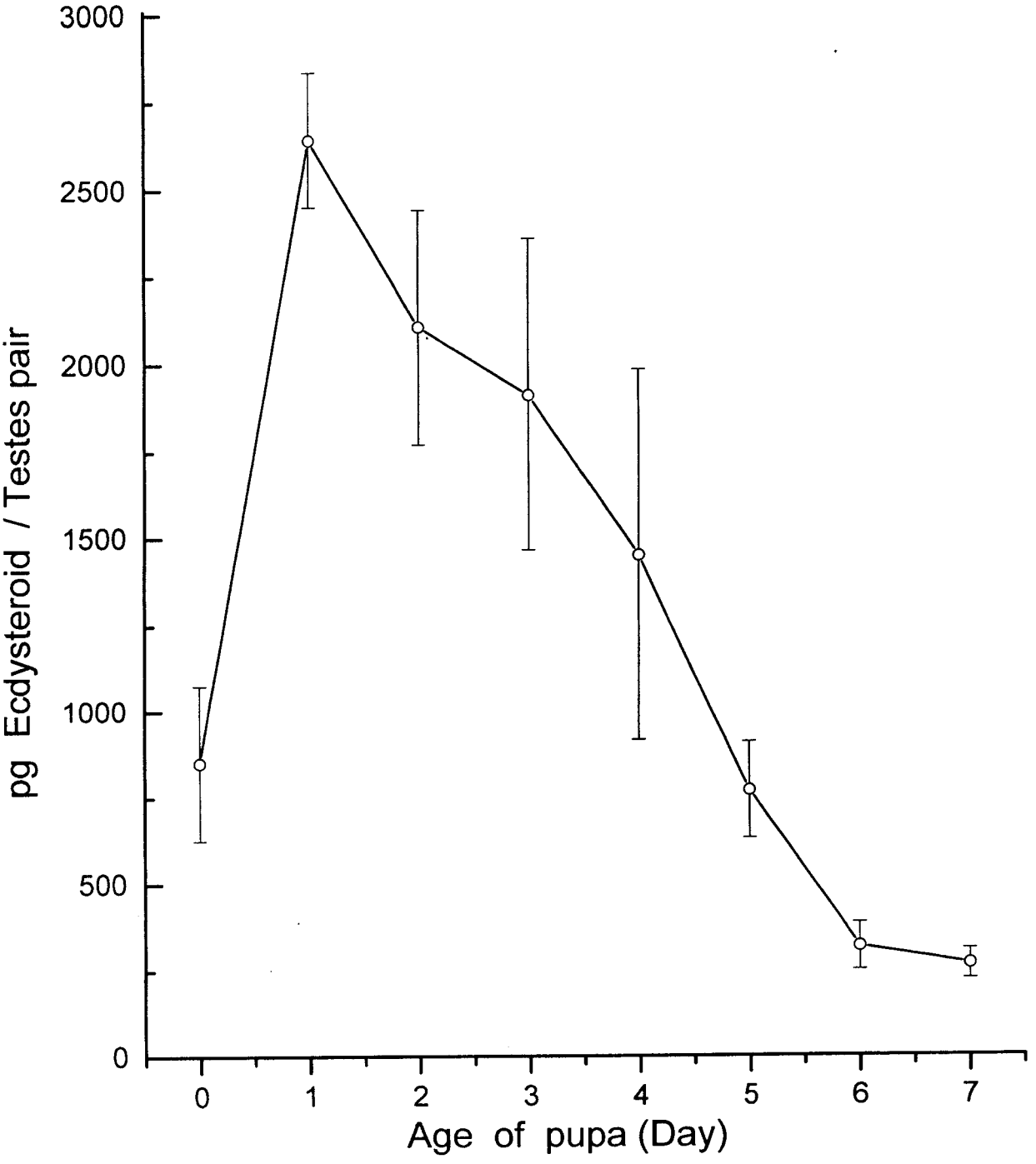

Fig. 24 Changes in testes ecdysteroid titres during pupal development

Table 13. **Effects of ligations of sixth instar day 4 larvae on metamorphosis and testicular fusion**

Nature of ligation	*n*	Days of survival (Mean ± SD)	% of surviving larvae which showed:		Nature of testes lobes
			No change	Pupation	
Neck-ligated	25	4 ± 1	100	--	Separate
Thorax-ligated	25	4 ± 1	100	--	Separate
Unligated (Control)	15 each	2 ± 0	--	100	Fused

remove the endogenous source of cerebral neurosecretory factors and JH. In another set of experiments, sixth instar day 1 or day 4 larvae were 'thorax-ligated' to eliminate the endogenous sources of both head factors (cerebral neurosecretory factors and JH) and prothoracic gland secretions.

(i) Effects of ligations of sixth instar day 1 larvae on testicular ecdysteroid titres

The present study was undertaken to determine the effect of neck/thorax ligation of day 1 larvae on testicular ecdysteroid titres. Seventy percent of the ligated larvae exhibited gut purge, however none of them underwent pupation although the larvae survived for 9 ± 1 days. On the other hand, all the control larvae exhibited gut purge and became pupae on day 6. The testes of the ligated larvae did not undergo fusion while the testes of the control larvae underwent fusion on day 5 (Table 12).

Ecdysteroid titres of experimental and control larvae are shown in Fig. 25. Hormone titres of the neck-ligated larvae were 130 ± 47 pg, 148 ± 20 pg, 53 ± 17 pg and 45 ± 4 pg for day 1, day 2, day 3 and day 4 respectively. Those of thorax-ligated larvae were 96 ± 26 pg, 33 ± 19 pg, 32 ± 16 pg and 0 pg for day 1, day 2, day 3 and day 4 respectively. Hormone titres of larvae kept as control were 151 ± 29 pg, 120 ± 7 pg, 225 ± 59 pg and 460 ± 37 pg for day 1, day 2, day 3 and day 4 respectively. Ligation of sixth instar day 1 larvae caused a significant reduction ($P < 0.05$) in testicular

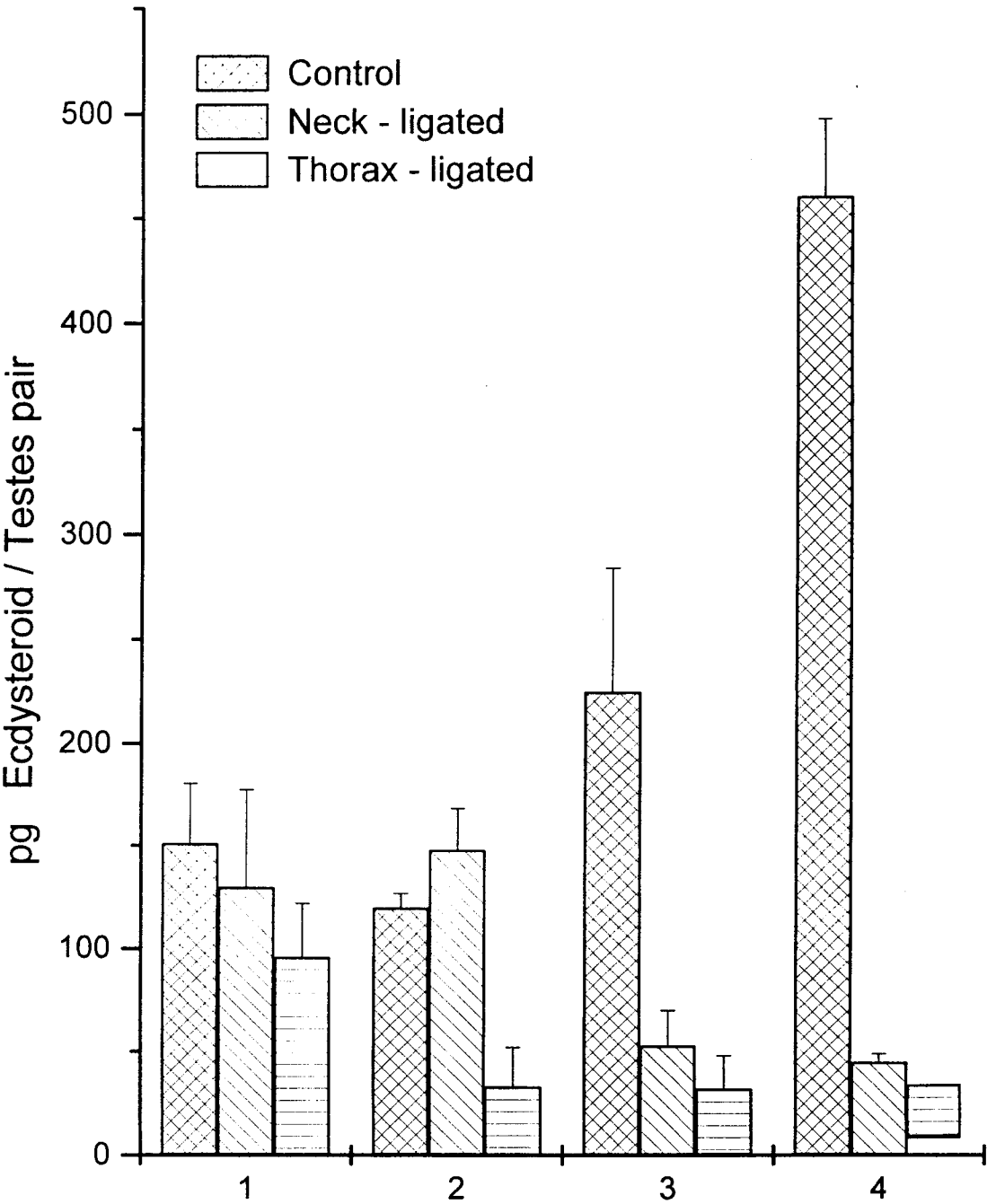

Fig. 25 Effects of neck/thorax ligation of day 1 larvae on testes ecdsteroid titres

ecdysteroid titres, on day 3 and day 4 in the neck-ligated larvae and on day 2, day 3 and day 4 in the thorax-ligated larvae. In the control larvae, hormone titres exhibited an increasing trend on all days.

(ii) Effects of ligations of sixth instar day 4 larvae on testicular ecdysteroid titres

The present study was undertaken to determine the effects of neck/thorax ligation of day 4 larvae on testes ecdysteroid titres. The ligated larvae survived for 4 ± 1 days and died without pupating. The testes of the ligated larvae remained separate and did not undergo fusion. On the other hand, the control larvae pupated on day 6 and their testes underwent fusion on day 5 (Table 13).

Ecdysteroid titres of experimental and control larvae are shown in Fig. 26. Hormone titres of the neck-ligated larvae were 434 ± 62 pg, 468 ± 55 pg, 511 ± 114 pg, 562 ± 169 pg and 712 ± 84 pg on day 4, day 5, day 6, day 7 and day 8 respectively. Those of thorax-ligated larvae were 399 ± 43 pg, 346 ± 86 pg, 185 ± 20 pg, 161 ± 20 pg and 209 ± 19 pg on day 4, day 5, day 6, day 7 and day 8 respectively. Hormone titres of larvae kept as control showed testicular ecdysteroid content of 460 ± 37 pg and 594 ± 153 pg on day 4 and day 5 respectively. Ecdysteroid titres in the neck-ligated larvae increased gradually to form a peak on day 8. Testicular ecdysteroid peak on day 8 was not significantly different from the peak titre on day 5 in the control larvae.

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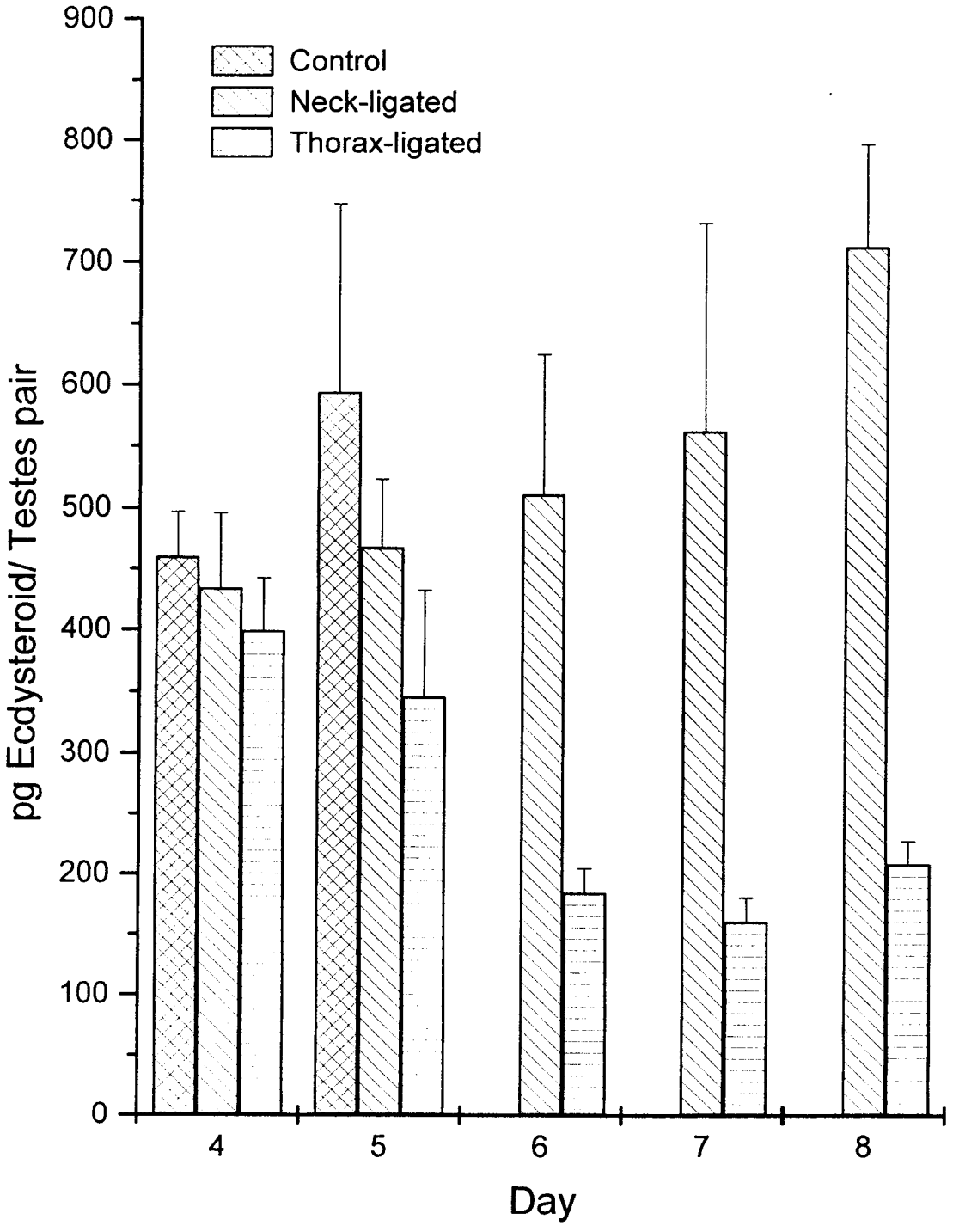

Fig. 26 Effects of neck/thorax ligation of day 4 larvae on testes ecdysteroid titres

Table 14. **Effects of repeated daily treatments of JHA to sixth instar larvae from day 0 onwards on metamorphosis and testicular fusion**

Dosage	*n*	% mortality	Number of larvae transformed to:		Nature of testes lobes
			Imperfect supernumerary larvae	Pupae	
20 µg	25	20	20	--	Separate
5 µl Acetone (Control)	15	--	--	15	Fused

On the other hand thorax ligation led to a significant reduction ($P < 0.05$) in testicular ecdysteroid titres on day 8 when compared to control larval titres on day 5. Control larval titres on the other hand, increased on day 4 and day 5.

4.3.3 Effects of treatments of JHA on testicular ecdysteroid titres

Previous studies on *S. mauritia* suggested that JHA has an inhibitory or stimulatory effect on metamorphosis and haemolymph ecdysteroid titres depending on the stage of development of the insect. The present investigation was undertaken in order to find out whether JHA has a similar effect on the testicular ecdysteroid titres of *S. mauritia* during larval-pupal and pupal-adult development.

(i) Effects of repetitive treatments of unligated sixth instar larvae with JHA

Sixth instar larvae were topically treated with repeated daily dose of 20 μg JHA from day 0 to day 3. Control larvae received an equivalent volume of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied. The treated larvae moulted into imperfect supernumerary larvae within 24 h of termination of hormone treatment, i.e. on day 4. The supernumerary larvae survived for 6-7 days. However, the control larvae underwent pupation on day 6. The testes remained separate in the JHA treated larvae whereas testes became united on day 5 in the control

Table 15. **Effects of repeated daily treatment of JHA to sixth instar day 1 ligated larvae on metamorphosis and testicular fusion**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed:			Nature of testes lobes
			No change	Gut purge	Pupation	
Neck-ligated	5 µg	20	100	--	--	Separate
Neck-ligated (Control)	5 µl Acetone	10	30	70	--	Separate
Thorax-ligated	5 µg	20	100	--	--	Separate
Thorax-ligated (Control)	5 µl Acetone	10	30	70	--	Separate

larvae. The testes volumes also were much lower in the treated larvae when compared to that of the control larvae (Table 14).

Ecdysteroid titres of experimental and control larvae are represented in Fig. 27. Hormone titres of JHA treated larvae were 84 ± 31 pg, 150 ± 26 pg, 155 ± 33 pg, 269 ± 77 pg and 207 ± 66 pg for day 1, day 2, day 3, day 4 sixth instar larval stage and day 0 supernumerary larval stage respectively. Testicular ecdysteroid titres of larvae kept as control were 151 ± 29 pg, 120 ± 7 pg, 225 ± 59 pg, 460 ± 37 pg and 594 ± 153 pg on day 1, day 2, day 3, day 4 and day 5 respectively. Ecdysteroid titres increased gradually to reach its peak on day 4 and decreased in the supernumerary larvae (day 0). Titres in the control larvae increased steadily to reach its peak on day 5. The peak titre of JHA treated larvae was significantly lower ($P < 0.1$) than that of the control larvae.

(ii) Effects of repetitive treatments of ligated sixth instar day 1 larvae with JHA

Sixth instar larvae were neck- or thorax-ligated on day 1 and topically treated with a repetitive daily dose of 5 μg JHA for three days. Ligated larvae kept as control were treated with an equivalent volume of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied. The JHA treated larvae did not show the premetamorphic behaviour of gut purge while 70% of the control larvae exhibited gut purge after 4.5 ± 0.5 days.

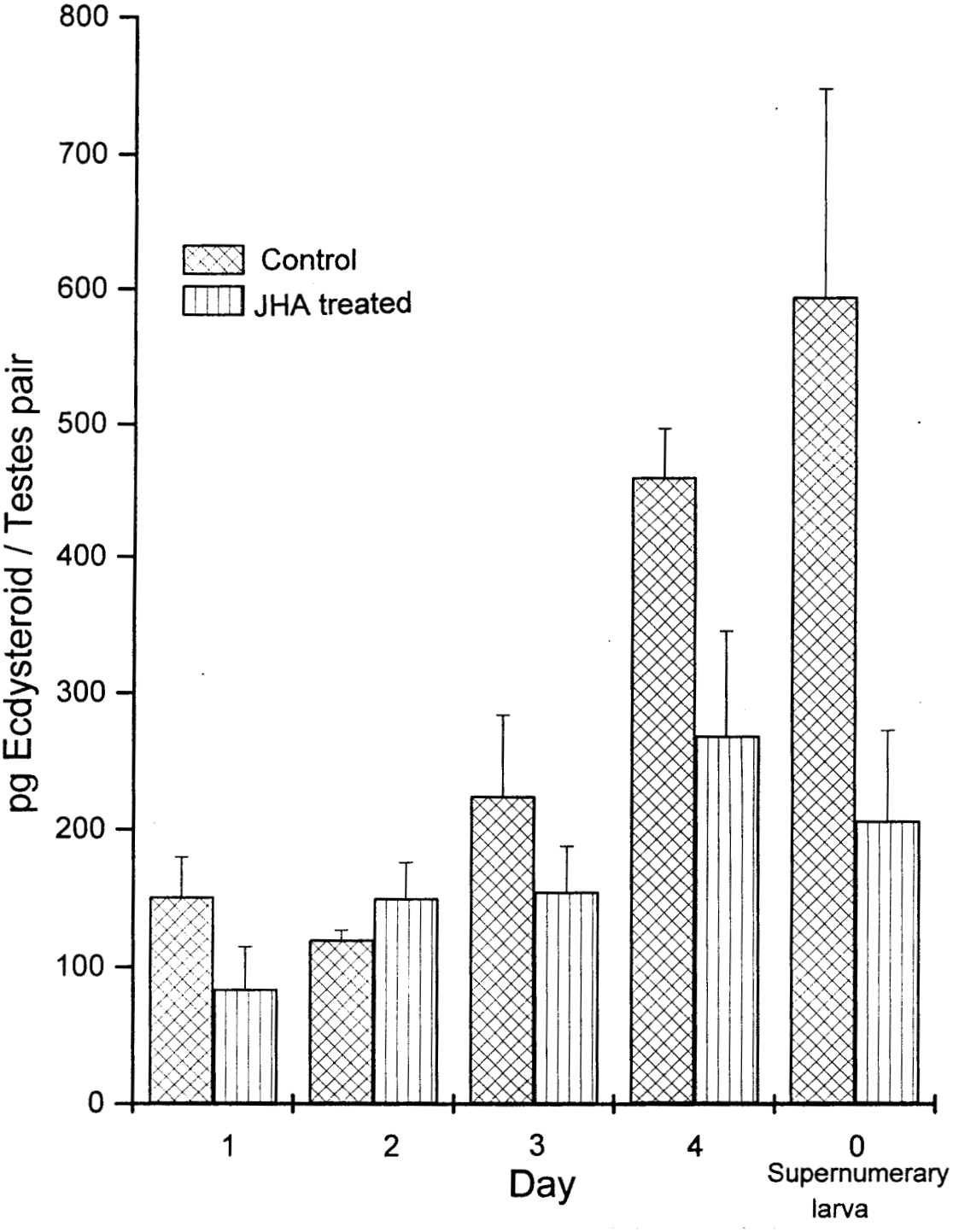

Fig. 27 Effects of repetitive treatments of larvae with 20 μg JHA from day 0 onwards on testes ecdysteroid titres

Table 16. **Effects of treatments of JHA to sixth instar day 4 ligated larvae on metamorphosis and testicular fusion**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed:		Nature of testes lobes
			No change	Pupation	
Neck-ligated	5 µg	20	20	80	Fused
Neck-ligated (Control)	5 µl Acetone	10	100	--	Separate
Thorax-ligated	5 µg	20	100	--	Separate
Thorax-ligated (Control)	5 µl Acetone	15	100	--	Separate

None of the JHA treated or control larvae pupated. Testes remained separate in the JHA treated and control larvae (Table 15). Testes volumes decreased in the JHA treated larvae when compared to the control larvae.

Ecdysteroid titres of the experimental and control larvae are represented in Fig. 28. The ecdysteroid titres of the JHA treated, neck-ligated and thorax-ligated larvae were 17 ± 2 pg and 0 pg respectively on day 4. In the control larvae, the hormone titres of neck-ligated and thorax-ligated larvae were 45 ± 4 pg and 0 pg respectively for the same day. Titres of the JHA treated, neck-ligated larvae were significantly lower ($P < 0.05$) than those of the control larvae whereas titres of both JHA treated, thorax-ligated larvae and control larvae were reduced to zero.

(iii) Effects of treatments of ligated sixth instar day 4 larvae with JHA

Sixth instar day 4 larvae were neck- or thorax-ligated and topically treated with a single dose of 5 μg JHA. Ligated larvae kept as control were treated with an equivalent volume of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied.

Eighty percent of the neck-ligated day 4 larvae treated with JHA transformed into headless pupae on day 7 while neck-ligated day 4 larvae kept as control showed a complete inhibition of pupation. The ligated larvae survived for 4 ± 1 days and died without pupating. Testes of the headless

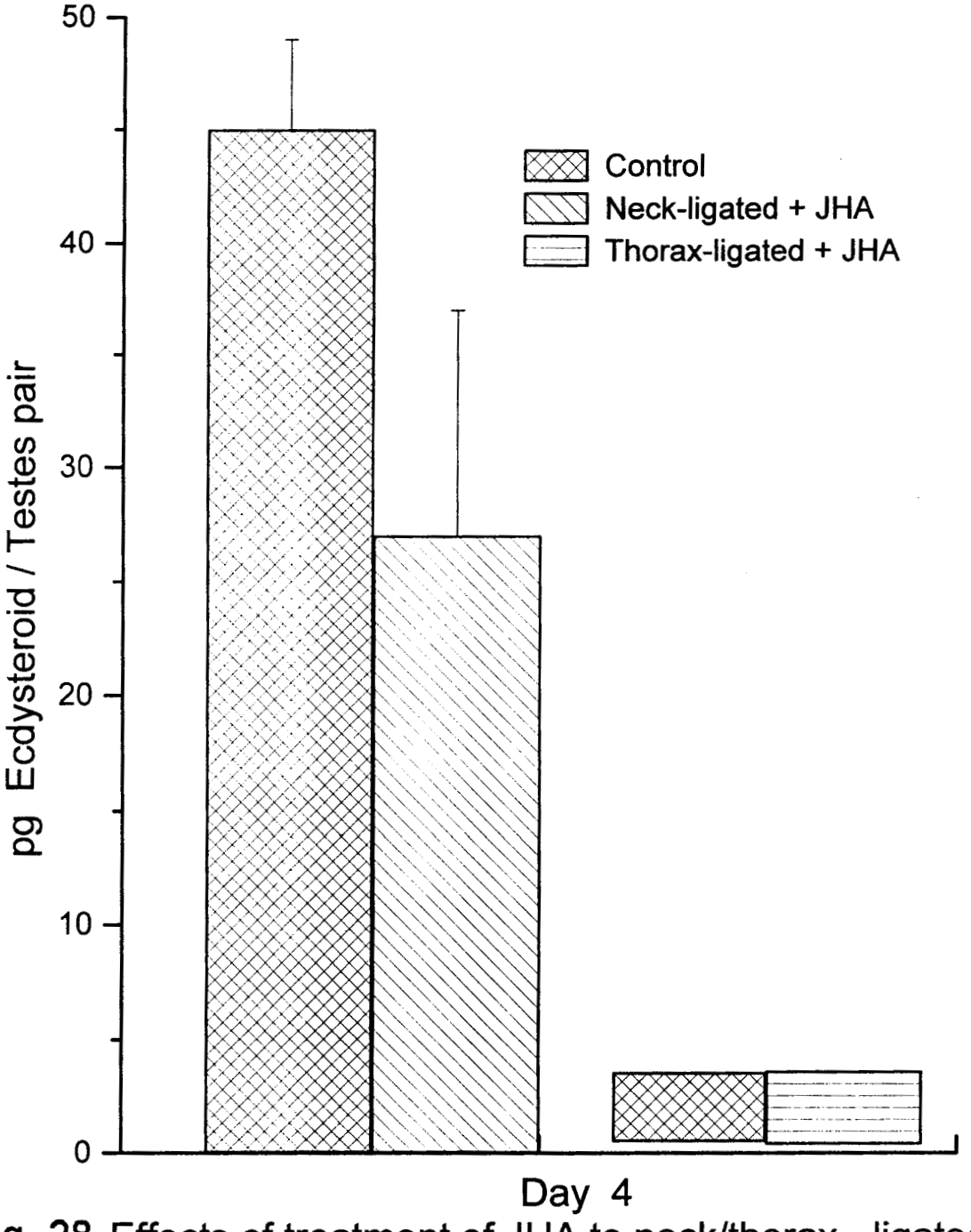

Fig. 28 Effects of treatment of JHA to neck/thorax - ligated day 1 larvae on testes ecdysteroid titres

pupae were fused while those of the control larvae remained separate. Larvae thorax-ligated on day 4 failed to pupate even after treatment of JHA. These larvae survived for 4 ± 1 days. Control larvae also showed a complete inhibition of pupation. The testes of both the JHA treated and control larvae remained separate (Table 16).

(a) Changes in ecdysteroid titres of neck-ligated larvae treated with JHA

Ecdysteroid titres of experimental and control larvae are represented in Fig. 29. Testicular ecdysteroid titres of day 0 headless pupae were 1031 ± 160 pg while those of control (neck-ligated on day 4) larvae were 562 ± 169 pg. Titres of headless pupae were significantly higher ($P < 0.1$) than that of control larvae (day 7).

(b) Changes in ecdysteroid titres of thorax-ligated larvae treated with JHA

Ecdysteroid titres of experimental and control larvae are represented in Fig. 30. Testicular ecdysteroid titres of treated larvae were 106 ± 8 pg, 411 ± 131 pg and 181 ± 4 pg on day 5, day 7 and day 8 respectively. Titres of control larvae were 346 ± 86 pg, 161 ± 20 pg and 209 ± 19 pg on day 5, day 7 and day 8 respectively. Hormone titres decreased both in the JHA treated and

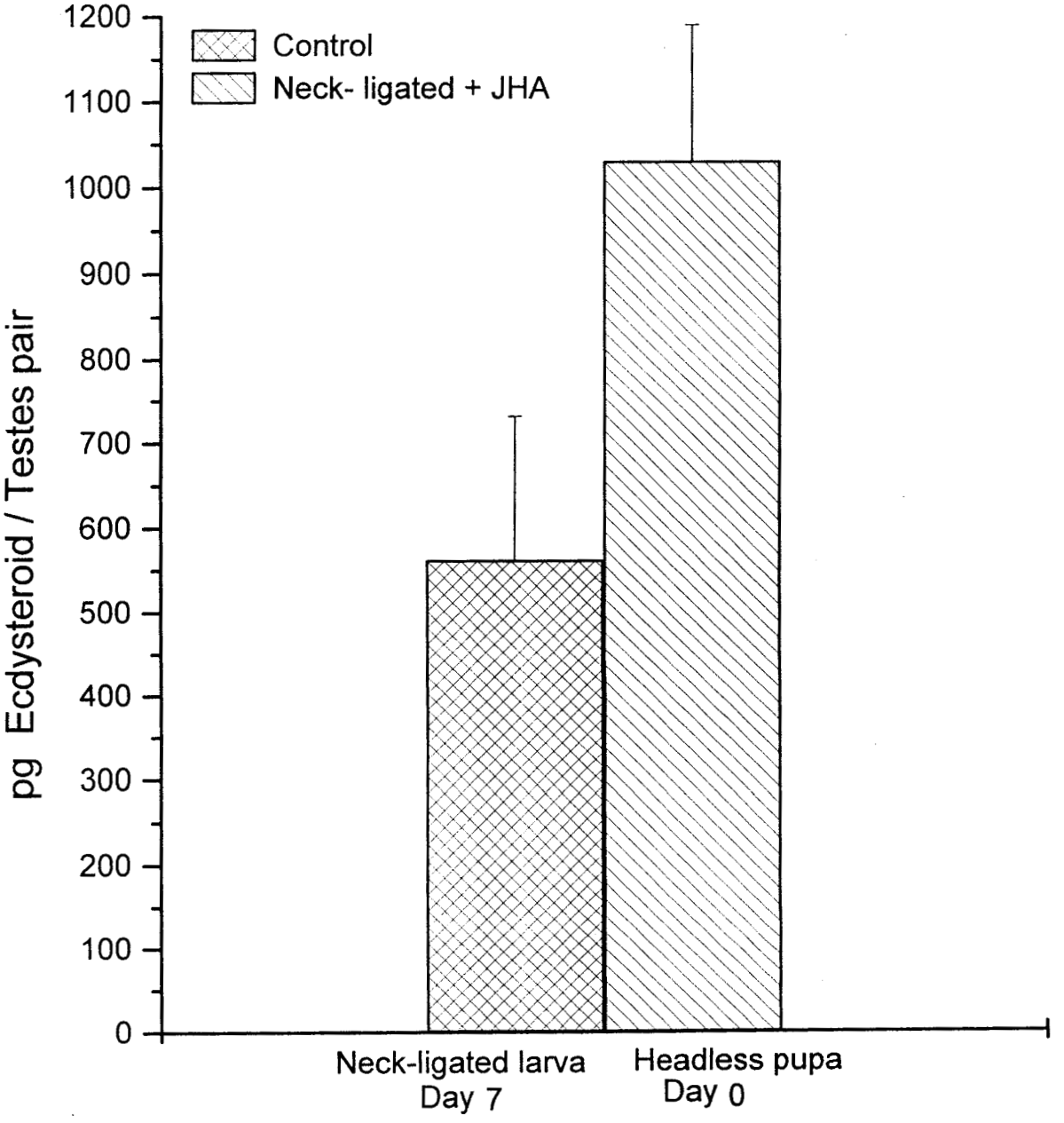

Fig. 29 Effects of treatment of 5 μg JHA to neck-ligated day 4 larvae on testes ecdysteroid titres

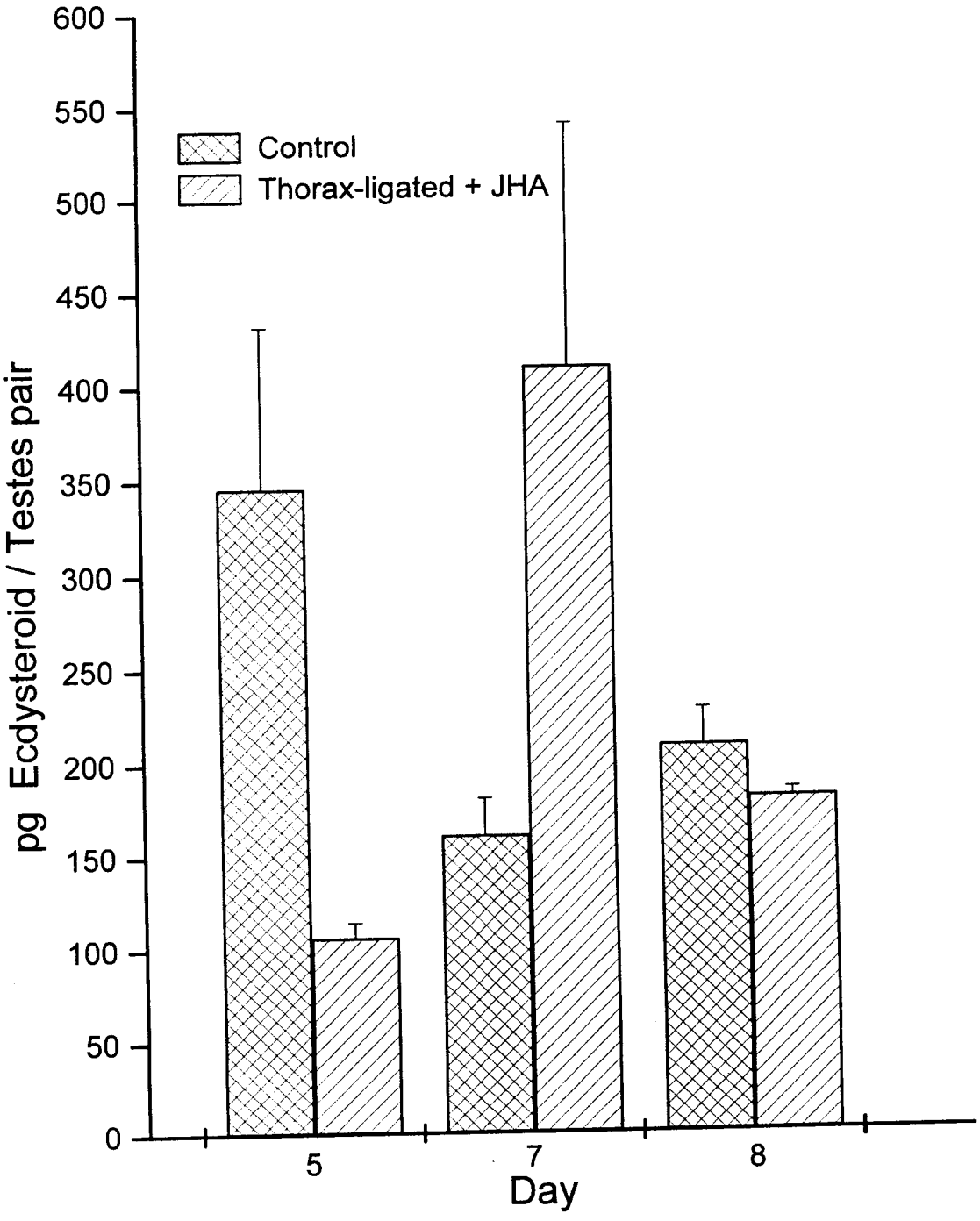

Fig. 30 Effects of treatment of 5 μg JHA to thorax-ligated day 4 larvae on testes ecdysteroid titres

control larvae. There was no significant difference between ecdysteroid titres of the treated and control larvae on day 8.

(iv) Effects of treatments of pupae with JHA on testicular ecdysteroid titres

Newly ecdysed day 0 pupae were treated with a single dose of 0.5 μg or 1 μg JHA. Control pupae received an equivalent volume of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied. High mortality was observed in the treated pupae. Surviving pupae showed various abnormalities in pupal-adult development and none of them succesfully emerged as morphologically normal adults. When the pupal case of such unemerged pupae were removed they were found to be pupal-adult intermediates or adultoids. Conversely, moths which emerged from the control pupae were normal and healthy.

Ecdysteroid titres of experimental and control pupae are shown in Fig. 31. Hormone titres of pupae treated with 0.5 μg JHA were 1112 ± 257 pg, 2547 ± 656 pg, 2937 ± 451 pg, 1963 ± 137 pg, 1396 ± 305 pg and 780 ± 194 pg on day 1, day 2, day 3, day 4, day 5 and day 6 respectively. Titres of pupae treated with 1 μg JHA were 5852 ± 1424 pg, 4124 ± 1066 pg, 4370 ± 933 pg, 1917 ± 556 pg, 2607 ± 149 pg and 1285 ± 197 pg on day 1, day 2, day 3, day 4, day 5 and day 6 respectively. Ecdysteroid titres of pupae kept as controls were 2642 ± 192 pg, 2106 ± 335 pg, 1913 ± 446 pg, 1452 ± 534 pg, 773 ± 139 pg

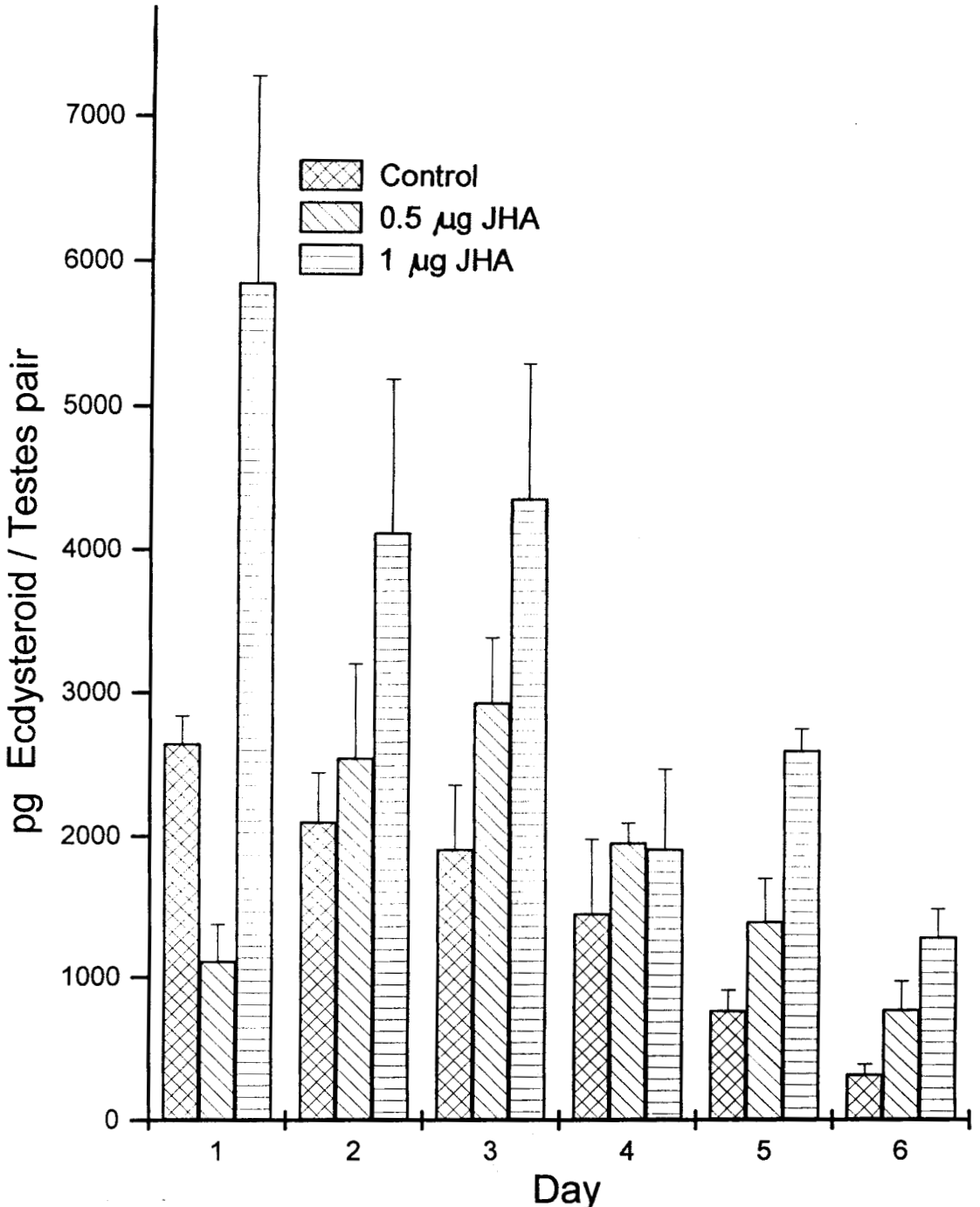

Fig. 31 Effects of treatments of pupae with 0.5 and 1 µg JHA on testes ecdysteroid titres

Table 17. **Effects of treatments of RH 5992 to sixth instar day 1 ligated larvae on metamorphosis and testicular fusion**

Nature of ligation	Dosage	*n*	% of surviving larvae which showed :			Nature of testes lobes
			No change	Gut purge	Pupation	
Neck-ligated	2 µg	20	--	100	--	Separate
Neck-ligated	5 µg	20	--	100	--	Separate
Neck-ligated (Control)	5 µl Acetone	15 each	30	70	--	Separate
Thorax-ligated	2 µg	20	--	100	--	Separate
Thorax-ligated	5 µg	20	--	100	--	Separate
Thorax-ligated (Control)	5 µl Acetone	15 each	30	70	--	Separate

and 320 ± 68 pg day on 1, day 2, day 3, day 4, day 5, and day 6 respectively. Ecdysteroid titres were significantly higher ($P < 0.1$) in the 0.5 µg JHA treated pupae on day 5 and day 6 and almost on all days in the 1 µg JHA treated pupae when compared to control titres. Testicular ecdysteroid titres increased greatly in the early pupal stage and then declined both in the JHA treated and control pupae.

4.3.4 Effects of treatments of ecdysteroid agonist RH 5992 on testicular ecdysteroid titres

Previous studies on *S. mauritia* suggested that RH 5992 induces a precocious moult in the treated larvae. However, depending on the stage of development, RH 5992 had a positive or negative feedback effect on the haemolymph ecdysteroid titres of the treated animals. The present investigation was undertaken in order to find out whether RH 5992 has a similar effect on the testicular ecdysteroid titres of *S. mauritia* during larval-pupal and pupal-adult development.

(i) Effects of treatments of ligated sixth instar day 1 larvae with RH 5992

Sixth instar day 1 larvae were neck- or thorax-ligated and treated topically with a single dose of 2 µg or 5 µg RH 5992. Ligated larvae kept as controls were treated with an equivalent volume of acetone. The effects of

these treatments on testicular ecdysteroids were carefully studied. Ligated larvae treated with RH 5992 survived for 3 ± 1 days while larvae kept as control survived for 9 ± 1 days. RH 5992 treatment induced gut purge in all the treated larvae whilel only 70% of the control larvae underwent gut purge. The testis remained separate both in the treated and control larvae (Table 17). The testes volume increased slightly following RH 5992 treatment when compared to control larvae.

(a) Changes in ecdysteroid titres of neck-ligated larvae treated with RH 5992

Ecdysteroid titres of neck-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 32. Titres of neck-ligated larvae treated with 2 μg RH 5992 were 74 ± 11 pg, 60 ± 32 pg and 58 ± 27 pg on day 1, day 2 and day 3 respectively. Those of 5 μg RH 5992 treated larvae were 56 ± 5 pg, 83 ± 23 pg and 174 ± 40 pg on day 1, day 2 and day 3 respectively. Ecdysteroid titres of larvae kept as controls on day 1, day 2 and day 3 were 130 ± 47 pg, 148 ± 20 pg and 53 ± 17 pg respectively. Testicular ecdysteroid litres were significantly lower ($P < 0.1$) in the RH 5992 treated larvae on day 1 and day 2 when compared to control larvae but an abrupt but significant increase ($P < 0.05$) in titres occurred in the 5 μg RH 5992 treated larvae on day 3.

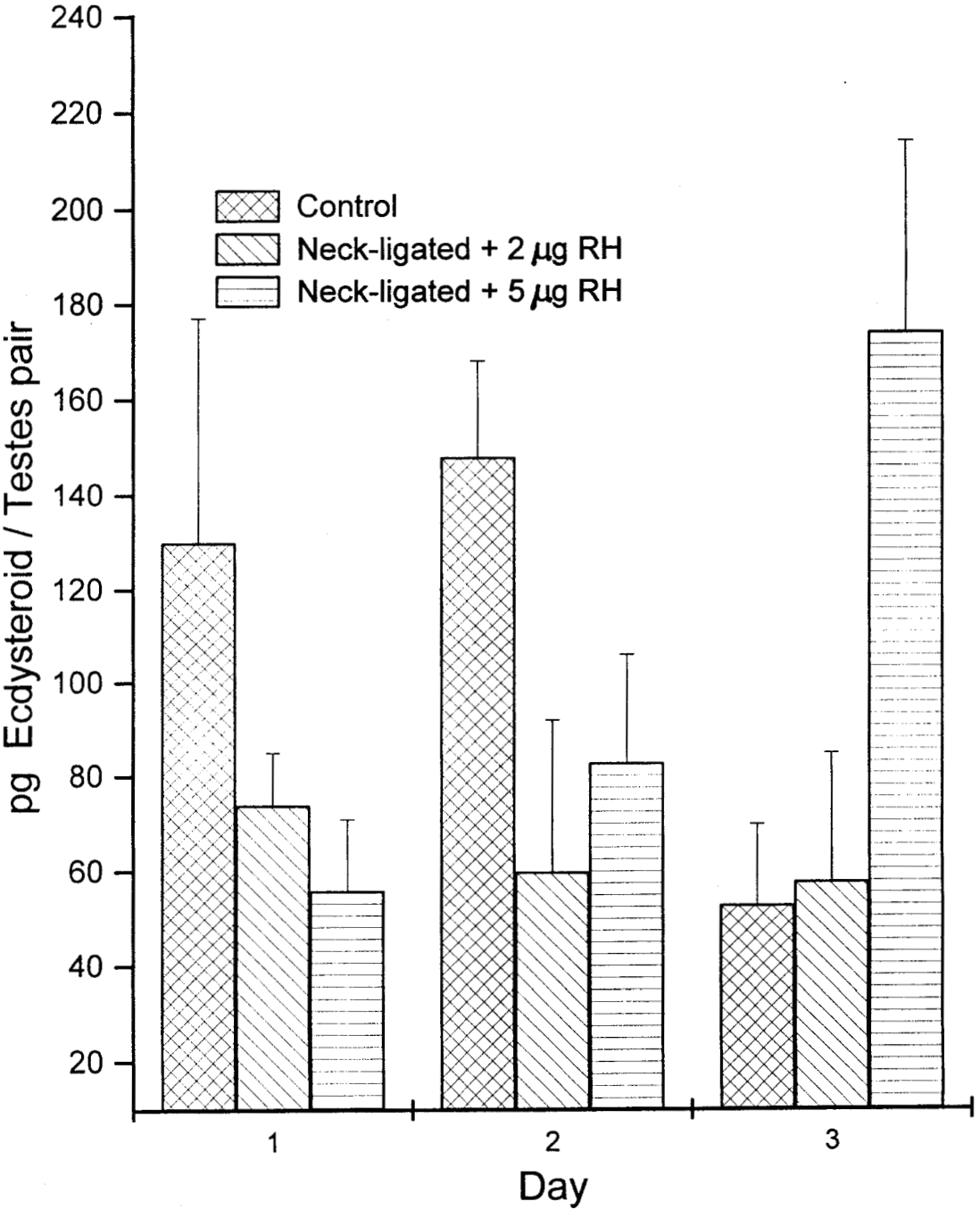

Fig. 32 Effects of treatments of RH 5992 to neck-ligated day 1 larvae on testes ecdysteroid titres

Table 18. **Effects of treatments of RH 5992 to sixth instar day 4 ligated larvae on metamorphosis and testicular fusion**

Nature of ligation	Dosage	*n*	% of surviving larvae which transformed into:			Nature of testes lobes
			No change	Pupae	Larval-pupal intermediates	
Neck-ligated	2 µg	20	20	--	80	Fused
Neck-ligated	5 µg	20	20	--	80	Fused
Neck ligated	10 µg	20	20	--	80	Fused
Neck-ligated (Control)	5 µl Acetone	15 each	100	--	--	Separate
Thorax-ligated	2 µg	20	20	--	80	Fused
Thorax-ligated	5 µg	20	20	--	80	Fused
Thorax-ligated (Control)	5 µl Acetone	15 each	100	--	--	Separate

(b) Changes in ecdysteroid titres of thorax-ligated larvae treated with RH 5992

Ecdysteroid titres of thorax-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 33. Titres of thorax-ligated larvae treated with 2 μg RH 5992 were 61 ± 15 pg, 19 ± 8 pg and 23 ± 23 pg on day 1, day 2 and day 3 respectively. Those of 5 μg RH 5992 treated larvae were 75 ± 19 pg, 55 ± 20 pg and 28 ± 9 pg on day 1, day 2 and day 3 respectively. Ecdysteroid titres of larvae kept as control (on day 1, day 2 and day 3) were 96 ± 26 pg, 33 ± 19 pg and 32 ± 16 pg respectively. Hormone titres of both 2 μg and 5 μg RH 5992 treated larvae were lower than those of control larvae for most days. However, the difference in titres of RH 5992 treated and control larvae were not significant.

(ii) Effects of treatments of ligated sixth instar day 4 larvae with RH 5992

Sixth instar day 4 larvae were neck-/thorax-ligated and treated with a single dose of 2 μg or 5 μg or 10 μg RH 5992. Ligated larvae kept as control were treated with an equivalent quantity of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied. Ligated larvae treated with different doses of RH 5992 transformed into larval-pupal intermediates on day 6. They survived for 3 ± 1 days. Neck-/thorax-ligated day 4 larvae kept as control showed a complete inhibition of pupation, they

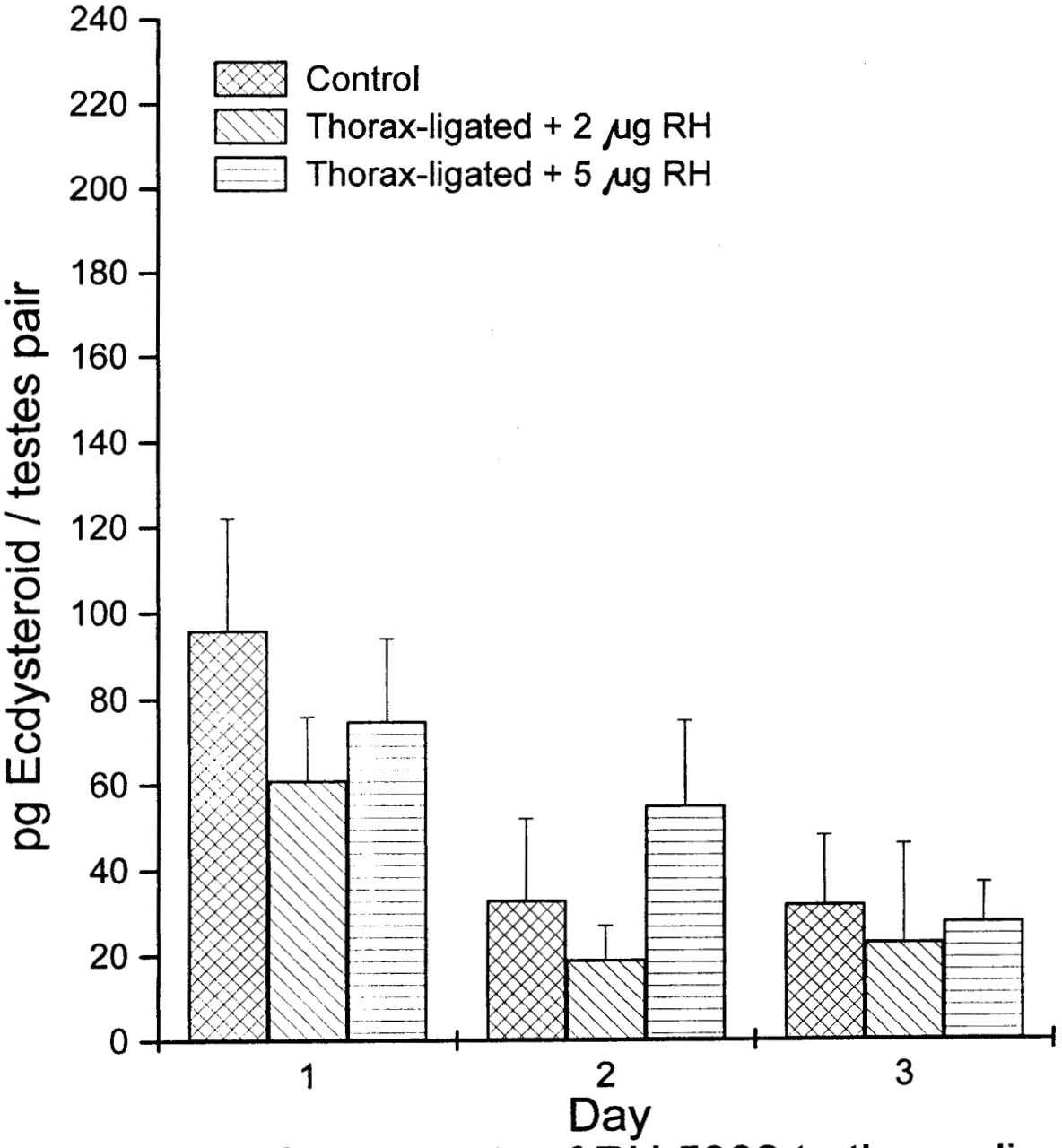

Fig. 33 Effects of treatments of RH 5992 to thorax-ligated day 1 larvae on testes ecdysteroid titres

survived for 4 ± 1 days and then died. Testes of RH 5992 treated larvae underwent fusion while those of the control larvae remained separate (Table 18).

(a) Changes in ecdysteroid titres of neck-ligated larvae treated with RH 5992

Ecdysteroid titres of neck-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 34. Titres of neck-ligated larvae treated with 2 μg RH 5992 were 350 ± 43 pg and 942 ± 252 pg on day 4 and day 5 of sixth instar larval stage and 378 ± 43 pg and 437 ± 83 pg on day 0 and day 1 of larval-pupal intermediate stage respectively. Those of 5 μg RH 5992 treated larvae were 355 ± 45 pg and 288 ± 50 pg on day 4 and day 5 of sixth instar larval stage and 325 ± 14 pg and 179 ± 34 pg on day 0 and day 1 of larval-pupal intermediate stage respectively. Titres of 10 μg RH 5992 treated larvae were 442 ± 39 pg and 453 ± 62 pg on day 4 and day 5 of sixth instar larval stage and 198 ± 57 pg and 154 ± 12 pg on day 0 and day 1 of larval-pupal intermediate stage respectively. Ecdysteroid titres of the neck-ligated control larvae were 434 ± 62 pg, 468 ± 55 pg, 511 ± 114 pg and 562 ± 169 pg on day 4, day 5, day 6, and day 7 respectively. Testes ecdysteroid titres declined (not all are significant at $P < 0.1$) in the RH 5992 treated larvae on all days but for a few exceptions. Control larvae exhibited an increasing trend in the titre of ecdysteroids reaching its maximum on day 7.

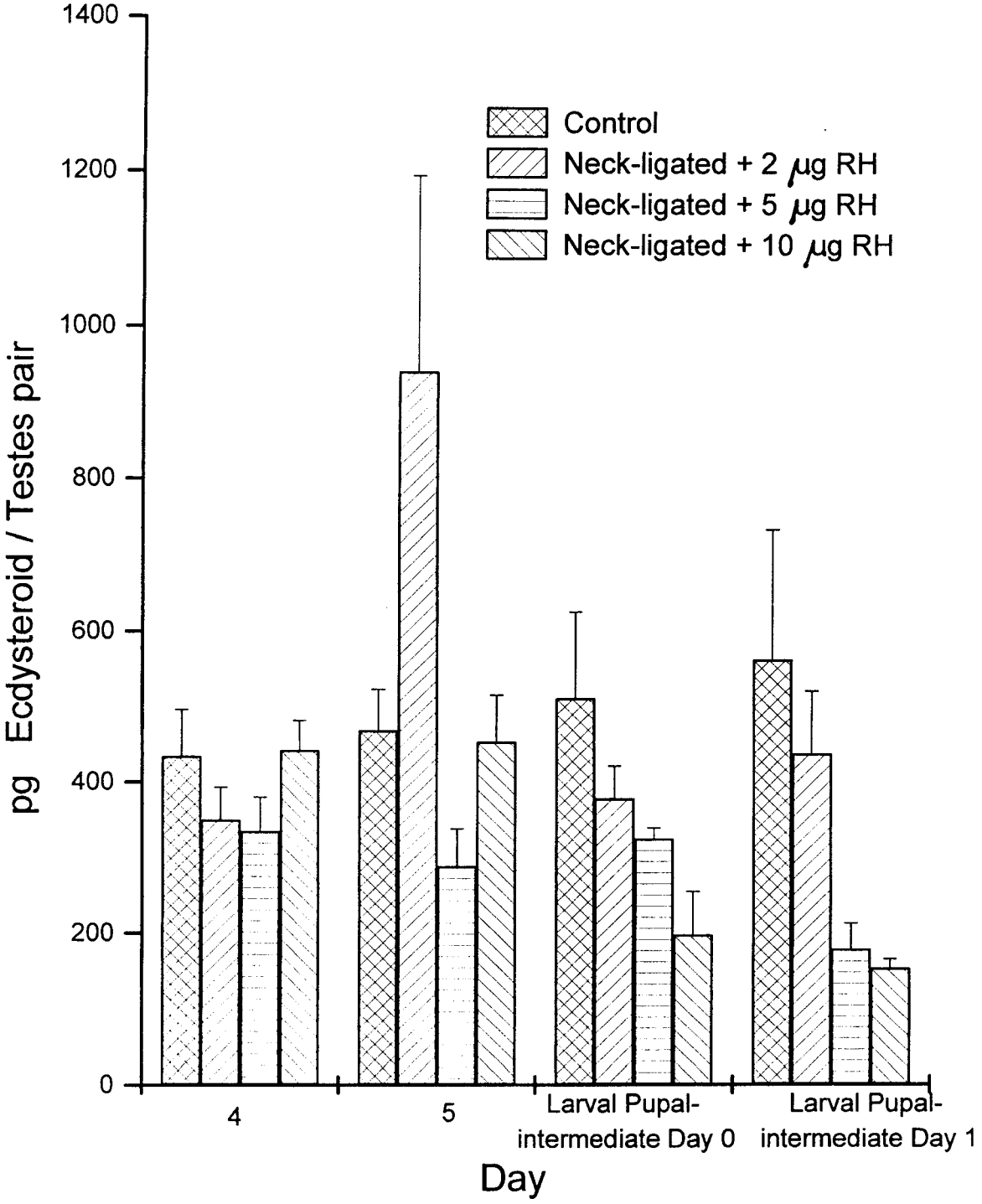

Fig. 34 Effects of treatments of RH 5992 to neck-ligated day 4 larvae on testes ecdysteroid titres

(b) Changes in ecdysteroid titres of thorax-ligated larvae treated with RH 5992

Ecdysteroid titres of thorax-ligated larvae treated with RH 5992 and those of control larvae are represented in Fig. 35. Titres of thorax-ligated larvae treated with 2 μg RH 5992 were 554 ± 86 pg and 171 ± 47 pg on day 4 and day 5 of sixth instar larval stage and 124 ± 41 pg on day 0 of larval-pupal intermediate stage respectively. Those of 5 μg RH 5992 treated larvae were 430 ± 54 pg, 188 ± 23 pg on day 4 and day 5 of sixth instar larval stage and 140 ± 16 pg on day 0 of larval-pupal intermediate stage respectively. Ecdysteroid titres of control larvae were 399 ± 43 pg, 346 ± 86 pg and 185 ± 20 pg on day 4, day 5 and day 6 respectively. Testicular ecdysteroid titres decreased in the RH 5992 treated and control larvae, however titres were lower but not significantly in the RH 5992 treated larvae.

(iii) Effects of treatments of pupae with RH 5992

Newly ecdysed day 0 pupae were treated with a single dose of 5 μg or 10 μg RH 5992. Control pupae received an equivalent volume of acetone. The effects of these treatments on testicular ecdysteroid titres were carefully studied.

Most of the treated pupae survived for 9 ± 1 days but failed to emerge. Removal of the pupal cases of such unemerged pupae revealed the presence of

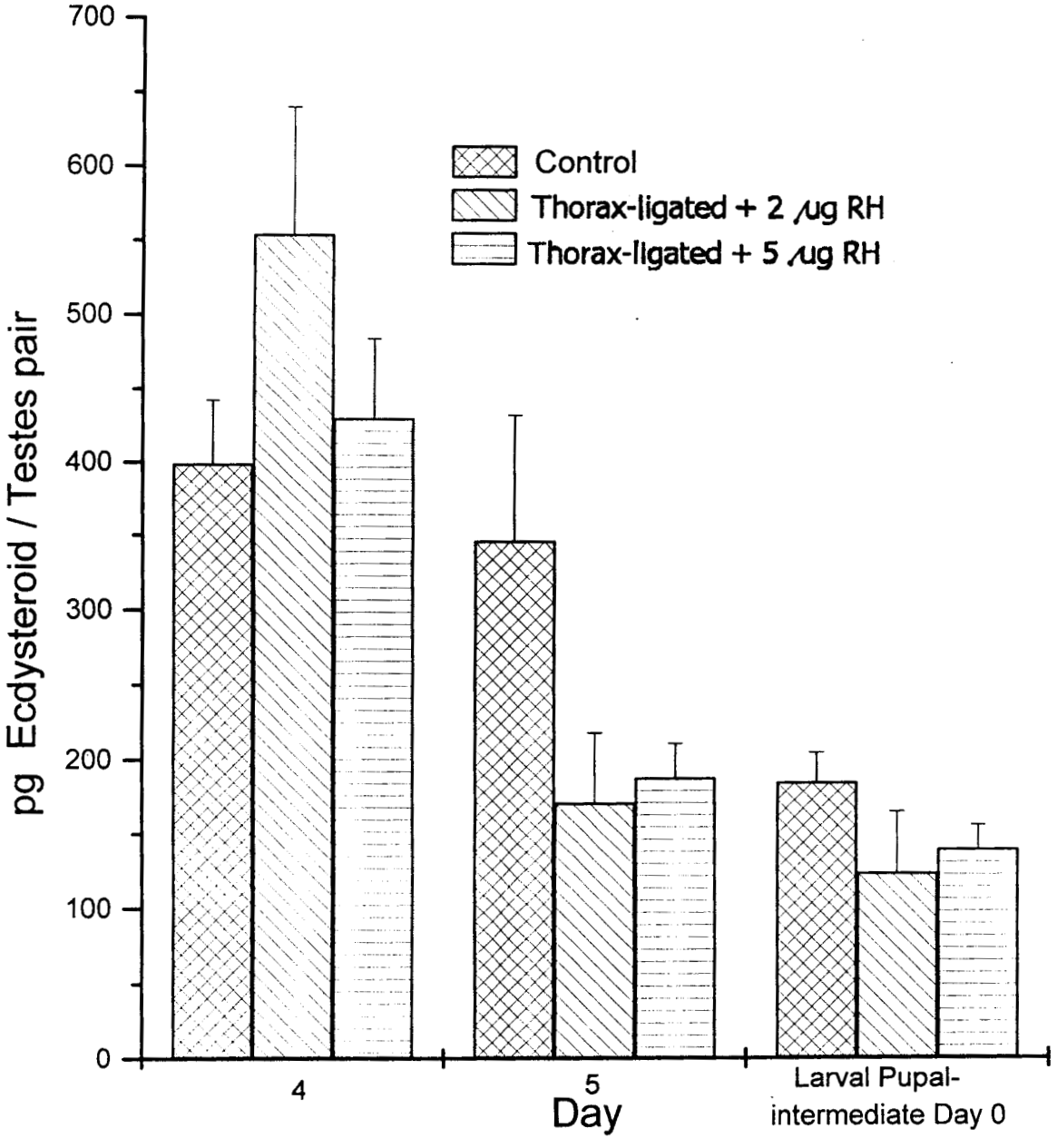

Fig. 35 Effects of treatments of RH 5992 to thorax-ligated day 4 larvae on testes ecdysteroid titres

adultoids. High mortality was observed towards the end of the pupal period. Most of the pupae died on or before the day of adult emergence. The pupal period of the RH 5992 treated pupae was found to be slightly prolonged (9 ± 1 days). Conversely, moths which emerged from the control pupae were normal and healthy.

Ecdysteroid titres of experimental and control pupae on day 2 and day 5 are represented in Fig. 36. Hormone titres of the pupae treated with 5 µg RH 5992 were 2090 ± 335 pg and 780 ± 70 pg on day 2 and day 5 respectively. Titres of 10 µg RH 5992 treated pupae were 1373 ± 604 pg and 774 ± 42 pg on day 2 and day 5 respectively. Ecdysteroid titres of the control pupae were 2106 ± 335 pg and 773 ± 139 pg on day 2 and day 5 respectively. Ecdysteroid titres of treated and control pupae did not show significant difference on day 2 and day 5.

4.3.5 Comparison of testicular and haemolymph ecdysteroid titres during larval-pupal and pupal-adult development

(i) Testicular ecdysteroid profile Vs Haemolymph ecdysteroid profile during sixth instar larval stadium

Testicular and haemolymph ecdysteroid titres for sixth instar larvae of *S. mauritia* are shown in Fig. 37. In the newly moulted (white head capsule) larvae (day 0) the testicular ecdysteroid titres were 145 ± 31 pg and increased

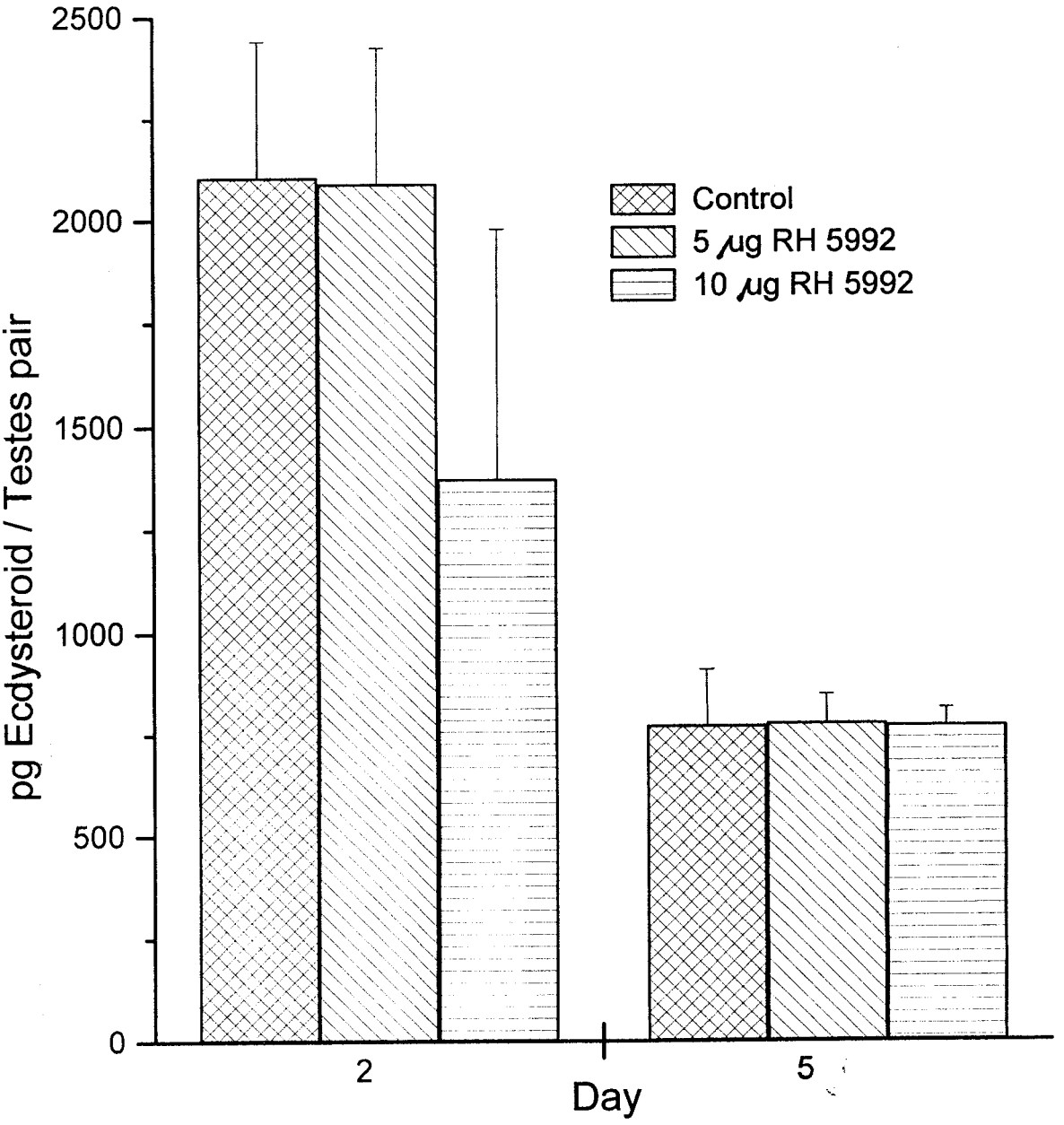

Fig. 36 Effects of treatments of pupae with 5 µg and 10 µg RH 5992 on Testes ecdysteroid titres

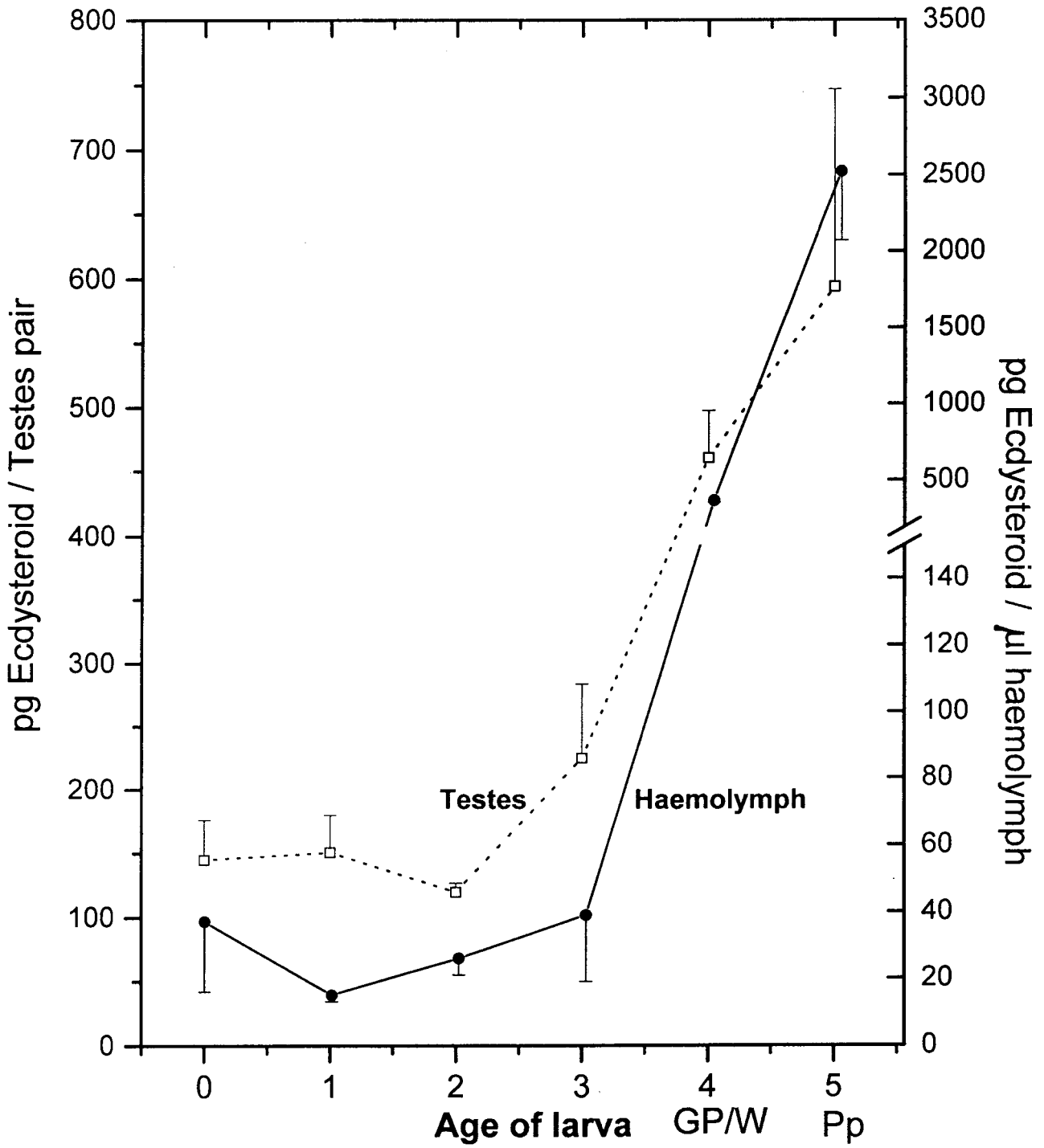

Fig. 37 Changes in testes and haemolymph ecdysteroid titres during sixth instar larval development

GP – Gut purge ; W – Wandering ; Pp – Prepupa

to 151 ± 29 pg on day 1. On day 2, hormone titres decreased (120 ± 7 pg) and increased on day 3 (225 ± 59 pg). The increasing trend continued on day 4 (460 ± 37 pg). Fusion of the paired testes occurred on day 5, the prepupal stage. Testicular ecdysteroid titres increased greatly on day 5 to form the highest titre (594 ± 153 pg) during the sixth larval stadium. In the newly moulted (white head capsule) sixth instar larvae (day 0), titres were 37 ± 21 pg. Hormone titres decreased on day 1 (15 ± 2 pg) and increased slightly on day 2 (26 ± 5 pg) and day 3 (39 ± 20 pg). Haemolymph titres increased rapidly on day 4 (372 ± 13 pg) and formed the highest peak on day 5 (2524 ± 453 pg). Ecdysteroid titres of the testes and haemolymph exhibited a minor increase on day 3 and a significant peak on day 5.

(ii) Testicular ecdysteroid profile Vs Haemolymph ecdysteroid profile during pupal stadium

Testicular and haemolymph ecdysteroid titres of pupae stadium of *S. mauritia* are shown in Fig. 38. In the newly moulted pupae (day 0), hormone titres were low (851 ± 226 pg). Titres increased rapidly to reach its maximum on day 1 (2642 ± 192 pg). Testes ecdysteroids then declined steadily on day 2 (2106 ± 335 pg), day 3 (1913 ± 446 pg), day 4 (1452 ± 534 pg), day 5 (773 ± 139 pg), day 6 (320 ± 68 pg) and day 7 (269 ± 43 pg). In the newly moulted pupae, titres were low (686 ± 63 pg), which increased gradually to 1973 ± 521 pg on day 1. Hormone titres increased abruptly at this stage to

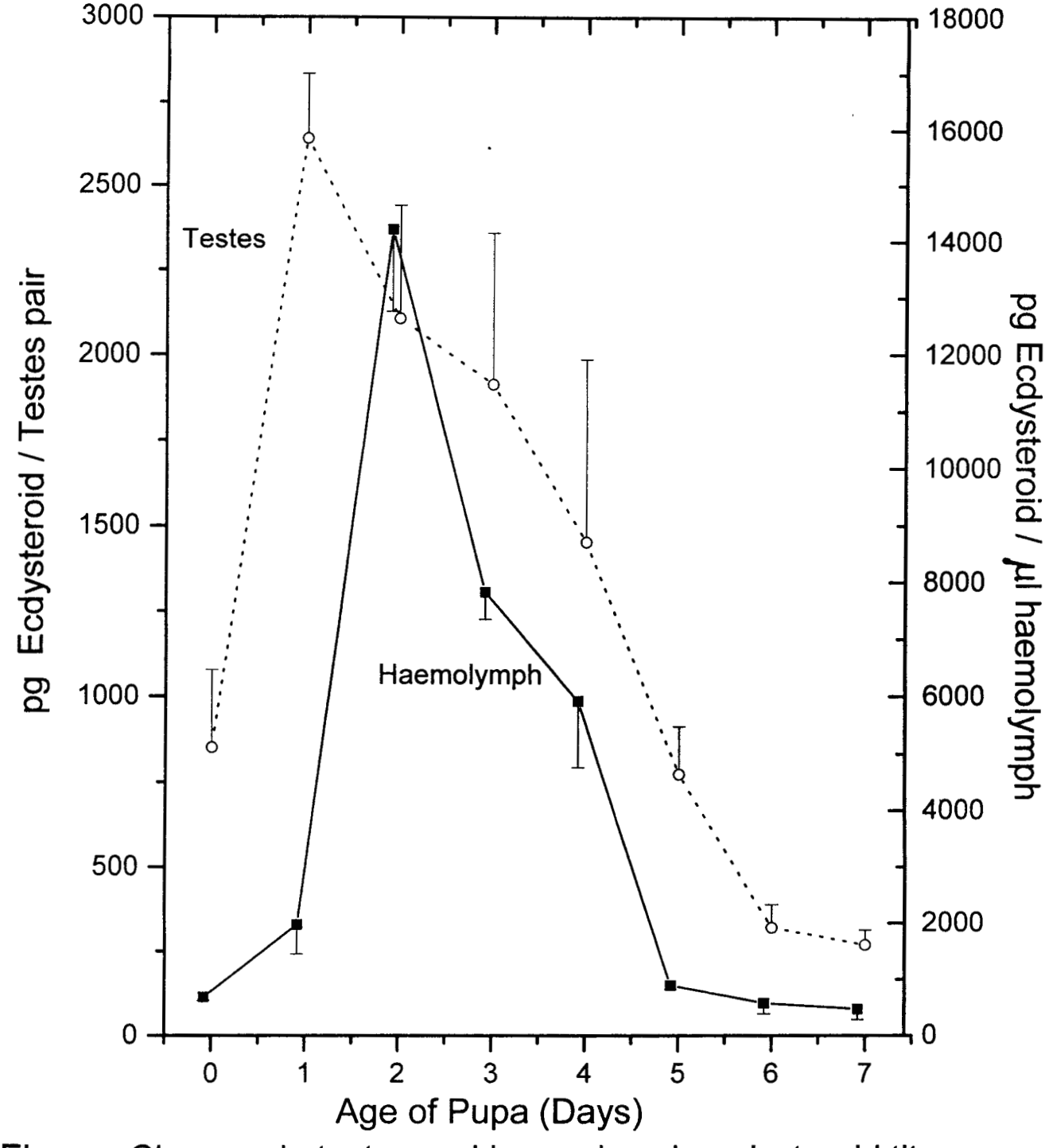

Fig. 38 Changes in testes and haemolymph ecdysteroid titres during the pupal instar development

reach its maximum (14221 ± 1458 pg). Titres then declined on day 3 (7831 ± 478 pg), day 4 (5915 ± 1159 pg), day 5 (894 ± 74 pg), day 6 (574 ± 186 pg) and day 7 (467 ± 185 pg). Testicular and haemolymph ecdysteroid titres increased significantly during the early pupal stage. Testicular titres formed a peak on day 1 while haemolymph titres formed a peak on day 2. Titres declined gradually thereafter in both the haemolymph and testes to reach their minimum on day 7.

4.4 DISCUSSION

In *S. mauritia*, the timetable of testicular development and spermatogenesis (Venugopalan *et al.*, 1994) is synchronised with the somatic development of the animal as has been reported for other insects. In insects, spermatogenesis can be sub-divided into three consecutive phases. During the first phase, the germ cells undergo mitotic proliferation and form clusters containing a species specific, defenitive number of spermatogonia. The second phase begins with the transformation of each spermatogonium into a primary spermatocyte which in turn produces four spermatids through two successive meiotic divisions. During the last phase of spermatogenesis (= spermiogenesis), the spermatids differentiate into mature spermatozoa (Dumser, 1980). Histological studies of spermatogenesis have revealed that spermatogenesis of *S. mauritia* conform to the general pattern reported for other lepidopterans (Venugopalan *et al.*, 1994). Testes of *S. mauritia* grow in

size gradually throughout the sixth (final) larval instar and reach its maximum volume in day 2 pupae and decrease gradually thereafter. Testes of the fifth (penultimate) instar larvae is mainly composed of spermatogonial cells which undergo repeated mitotic divisions towards the later stages of the instar. In the early sixth instar larvae, the spermatogonia aggregate into cysts (spermatocysts) in which mitotic divisions occur resulting in the formation of spermatocytes. These spermatocytes undergo meiotic division to form spermatids which appear on day 2 of the sixth instar larvae. The differentiation of eupyrene and apyrene sperm bundles is initiated in the prepupal phase (day 4 and day 5) of the sixth instar. Also, the paired testes undergo fusion along the mid-dorsal line to form a single median structure in the pharate pupal (day 5) phase. The fused testis undergoes torsion in the young pupa. Fully differentiated eupyrene and apyrene sperm bundles also appear for the first time during the early pupal period. Maximum number of eupyrene and apyrene sperm bundles is found in day 6 pupae. The spermatogonial and spermatocyte cysts disappear during the late pupal period and are no longer visible in the testis of the adults. In the adult testis, sperm bundles both eupyrene and apyrene are found distributed throughout the lumen of the testis. The number of sperm bundles decrease towards adult eclosion (Venugopalan *et al.*, 1994).

Ecdysteroid synthesis by the testis

Ecdysteroids have been reported in adults of *D. melanogaster* (Hodgetts *et al.*, 1977) and *C. vicina* where they are localised primarily in the testes (Koolman *et al.*, 1979). Subsequent investigations on testicular ecdysteroids in various lepidopteran species have largely been confined to the immature stages, although the testis of adult *H. virescens* have also been shown to contain ecdysteroids (Loeb *et al.*, 1984). In the testes of last instar larvae of *O. nubilalis*, 20-hydroxyecdysone and various high polarity ecdysteroids were detected, while in pupae and pharate adults, ecdysone was additionally detected (Gelman *et al.*, 1988a,b). Ecdysteroids have also been reported in the testes of *L. separata* (Shimizu *et al.*, 1989) and *B. mori* (Fugo *et al.*, 1995). Testes of *H. virescens* and *L. dispar* secrete ecdysteroids *in vitro* during mid- and late-last instar larval stage and mid to late developmental period during the pupal stage (Loeb *et al.*, 1982, 1984, 1988). Testes of *O. nubilalis* was also found to synthesise ecdysteroids *in vitro* especially during late last larval instar (Gelman *et al.*, 1989). However, exogenous edcysteroids is needed to initiate (Loeb *et al.*, 1988) or boost (Loeb *et al.*, 1986) endogenous ecdysteroid production in the testes *in vitro*. Testes of *M. brassicae* and *S. littoralis* have also been found to spontaneously secrete ecdysteroids *in vitro*. When pupal testes of diapausing *M. brassicae* were incubated *in vitro*, 2-deoxyecdysone, ecdysone and 20-hydroxyecdysone were found in the culture medium when

analysed by HPLC-RIA (Shimizu *et al.*, 1985). HPLC-RIA analysis of larval testes ecdysteroids of *S. littoralis* also yielded similar results (Jarvis *et al.*, 1994). Immunocytochemical techniques and dissections have implicated the testis sheath as the site of synthesis of ecdysteroids in the testis (Loeb, 1986). The results of the present study also shows that ecdysteroids are clearly present in the testes of *S. mauritia.* Testicular ecdysteroid titres also exhibit significant fluctuations during larval-pupal-adult development.

Testicular ecdysteroid profile during larval-pupal development

The testes of *S. mauritia* grow in size throughout the last larval instar and attain maximum volume in day 3 larvae. Histological studies on spermatogenesis of *S. mauritia* have shown that spermatogonial cells undergo multiplication and proliferation from day 0 - day 3 of sixth larval instar. Further, the first phase of spermiogenesis, the formation of spermatids, occur during day 2 and day 3 of sixth larval instar (Venugopalan *et al.*, 1994). The testicular ecdysteroid titres of the sixth instar larvae of *S. mauritia* exhibited fluctuations concomitant with the developmental phase of the testicular cyst cells. Titres which were at a base level on day 0 and day 1 decreased on day 2 and formed a peak on day 3. This titre peak may be promoting meiotic division and hence the formation of spermatids. The proliferation of gonial cells may be occurring at low levels of ecdysteroids observed on day 0 and day 1. The reduction in titres on day 2 may be a cue for the gonial cells to

undergo meiotic division for the formation of spermatids. The increase in titres on day 3 may further promote spermatid formation. Benny and Nair (unpublished data) reported that the DNA and RNA content of the testes of *S. mauritia* increased from day 0 to day 3 and the protein content also increased gradually during the sixth larval instar.

In *S. mauritia*, mid-last instar production of ecdysteroids in the testes was observed one day after the change from mitotic to meiotic division in the testicular gonial cells. Testicular ecdysteroid titres of *H. virescens* also increased during mid-last instar larval period which coincided with the change from mitotic to meiotic division (Loeb and Birnbaum, 1981), increase in testes size and eupyrene cyst maturation (Loeb *et al.*, 1984). The *Heliothis* testes contained and released a spectrum of at least 12 different ecdysteroids (Loeb *et al.*, 1982). Testicular ecdysteroids have also been reported to stimulate meiosis and elongation of eupyrene spermatocysts *in vivo* in *O. nubilalis* (Gelman and Borkovec, 1986). Ecdysteroids have been reported to stimulate mitotic and/or meiotic division during early stages of spermatogenesis (Yagi *et al.*, 1969; Kambysellis and Williams, 1971b, 1972; Takeda, 1972; Dumser and Davey, 1975). The present determination suggests that synthetic periods of the testes may affect or be affected by internal changes of spermatocyst development. Testes ecdysteroids may regulate maturation of male genital

tissues by induction of growth factors secreted by the testis itself and the fat bodies (Loeb and Hakim, 1991; Loeb, 1991b, 1994).

The differentiation of eupyrene and apyrene sperm bundles is initiated in the prepupal phase (day 4 and day 5) of the sixth larval instar of *S. mauritia* (Venugopalan *et al.*, 1994). The fusion of the paired testes occurs in the pharate pupal stage (day 5). The present study reveals that testicular ecdysteroid titres increased on day 4 and reaches a peak on day 5. This increase in titres on day 4 and day 5 may be stimulating the differentiation of eupyrene and apyrene sperm bundles. Testicular fusion may also be under the influence of the testes ecdysteroid peak on day 5. Testicular ecdysteroids may also be influencing RNA content of the testes of *S. mauritia* which declined on day 4 but increased dramatically on day 5. Testicular sheath peptides were also reported to increase on day 5 (Benny and Nair, unpublished results). Ecdysteroid content of the *Heliothis* testes also exhibited a peak in late-last instar larvae, one day after the start of spermatocyst elongation (Loeb *et al.*, 1984). The role of ecdysteroids in stimulating the maturation and differentiation of male germ cells has been suspected since the early work of Schmidt and Williams (1953) in *S. cynthia*. Also, the role of ecdysteroids in promoting spermatogenesis has been well documented *in vivo* (Takeuchi, 1969; Dumser and Davey, 1975) and *in vitro* (Yagi *et al.*, 1969; Kambysellis and Williams, 1971b, 1972; Takeda, 1972;

Fukushima and Yagi, 1975) observations. Ecdysteroids have also been reported to stimulate apyrene meiosis and elongation in *O. nubilalis* (Gelman *et al.*, 1988a,b). That ecdysteroids are involved in testicular fusion has also been reported by a few authors (Nowock, 1972, 1973; Loeb *et al.*, 1984; Benny and Nair, 1999). A peak in testicular ecdysteroids at the time of fusion of the paired testes has also been reported in *O. nubilalis* (Gelman *et al.*, 1988a,b) and *B. mori* (Fugo *et al.*, 1995). Deficiency of ecdysteroids in the testes caused by heat treatment of larvae induced male sterility during the wandering and pharate pupal stage of *B. mori* (Fugo *et al.*, 1995).

Testicular ecdysteroid profile during pupal-adult development

In *S. mauritia*, fully formed eupyrene and apyrene sperm bundles appeared for the first time during the early pupal period. A major increase in sperm bundles and torsion of the fused testis also occurred in the pupa (Venugopalan *et al.*, 1994). These changes in testicular development and spermatogenesis coincided with the single large peak of testicular ecdysteroids observed on day 1 of pupal development in *S. mauritia*. Ecdysteroid titres decreased gradually during the rest of the pupal stadium. The testis volume also reaches a maximum in day 2 pupa which then decreases gradually. Histological studies of spermatogenesis of *S. mauritia* revealed that maximum number of eupyrene and apyrene sperm bundles occurs in day 6 pupa (Venugopalan *et al.*, 1994). However, testicular ecdysteroid titres were on a

downward trend during this time probably signalling the impending moult. Hence, increase in sperm bundles may be promoted by the decreasing titre of ecdysteroids thus preparing for adult eclosion. Thus reproductive maturity may be achieved during pupal stadium in response to the decreasing titre of ecdysteroids. Testicular DNA content also increases considerably during the latter half of the pupal stadium while RNA content of the testis increases during the first half of the pupal period of *S. mauritia* (Benny, 2001) when the testicular ecdysteroid titres were fairly high. Testicular proteins also increases dramatically during the pupal stage reaching a maximum in day 6 pupa (Benny, 2001). Increased titres of testicular ecdysteroids during the first half of pupal stadium has also been reported in *H. virescens* (Loeb *et al.*, 1984) and *O. nubilalis*. In *Ostrinia*, this increase in the titre corresponds to the time when apyrene spermatocysts are elongating (Gelman *et al.*, 1988[a,b]). Testicular ecdysteroids also play an important role in the induction of development of male genital tract of lepidopteran pupae (Nowock, 1972, 1973; Leclerq - Smekens, 1974). Ecdysteroids also interact with haemolymph macromolecular factor to induce spermatocyte differentiation (Kambysellis and Williams, 1971b, 1972) and influence the development of apyrene sperms (Hoffmann and Behrens, 1982; Loeb *et al.*, 1982; Gelman *et al.*, 1989). Ecdysteroids are also believed to be necessary for resumption of spermatogenesis after diapause (Dumser, 1980).

Testicular ecdysteroid titres during adult development

The adults of *S. mauritia* have a very short life span, hence spermatogenesis is completed in the pupal stage itself. In the adult testis, both eupyrene and apyrene sperm bundles were distributed throughout the lumen of the testis. However, the early germinal cysts were completely absent (Venugopalan *et al.*, 1994). Testicular ecdysteroid titres of adults (day 0) although significant were considerably lower than those of mid-last instar larvae and pupae of *S. mauritia.* Testes of adult *H. virescens* were also capable of synthesising ecdysteroids *in vitro* (Loeb *et al.*, 1984). Large amounts of ecdysteroids were found in the testes of adult male blowfly *C. vicina* (Koolman *et al.*, 1979). It has been reported that sexual receptivity of adult male mosquitoes is affected by ecdysteroid titres (Beach, 1980). Thorson and Riemann (1982) have shown that photoperiodically controlled release of eupyrene, but not apyrene sperms from lepidopteran testes into the vas deferens is inhibited by injection of 20-hydroxyecdysone. A decline in haemolymph ecdysteroid titres is also essential for the initiation of sperm release from the testes of *L. dispar* (Giebultowicz *et al.*, 1990). Hence it is possible that ecdysteroid release by the adult testes of insects mediate bursts of sperm release and sexual behaviour.

Data obtained in the present study suggests that testicular ecdysteroid titres in *S. mauritia* are correlated with testicular development and

spermatogenesis. The testicular ecdysteroid profile of *S. mauritia* compares well with testicular titres determined for other lepidopteran species. Hence, it may be concluded that testicular development and spermatogenesis in *S. mauritia* and possibly other insect species are influenced greatly if not solely by testicular ecdysteroid titres.

Comparison between testicular and haemolymph ecdysteroid profile during larval-pupal-adult development

In *S. mauritia*, fluctuations of testicular and haemolymph ecdysteroid titres appear more or less at the same time during the different stages of development. Testicular ecdysteroids exhibited a minor peak during mid-last instar larval period (day 3) which coincided with the formation of spermatids in the testis (Venugopalan *et al.*, 1994). The commitment peak of ecdysteroids in the haemolymph also appeared during this time. This peak was observed at 60 h when 6 h sampling of haemolymph was done and on day 3 when sampling was done on a daily basis. Testicular ecdysteroid titres increased further on day 4 and formed a significant peak on day 5. The differentiation of eupyrene and apyrene sperm bundles was initiated during this phase (day 4 and day 5) of the sixth instar (Venugopalan *et al,* 1994). Haemolymph ecdysteroid titres also exhibited a similar trend, titres increased on day 4 and formed the moulting peak on day 5. Fusion of the paired testes lobes also occurred during the pharate pupal stage (day 5).

During the pupal stadium, testicular ecdysteroids exhibited a single large peak on day 1 after which titres decreased gradually during the subsequent days. Fully formed eupyrene and apyrene sperm bundles appeared for the first time and a major increase in their number also occurred at this time. In *S. mauritia*, maximum number of eupyrene and apyrene sperm bundles occurred in day 6 pupae (Venugopalan *et al.*, 1994). Torsion of the fused testis occurred in the young pupa. Testicular volume reaches a maximum on day 2 of the pupal stage and decreases gradually thereafter. Haemolymph ecdysteroid titres of *S. mauritia* also exhibited an increase in the first half of the pupal stadium and decreased in the latter half. The decrease in haemolymph titres has been demonstrated to be caused by apoptosis of the prothoracic glands during pupal-adult development (Dai and Gilbert, 1997). Whether an apoptotic event occurs in the steroid secreting cells of the testes during pupal-adult development has not been reported in any insect. In the absence of an apoptotic event during pupal-adult testicular development, the pattern of ecdysteroid fluctuations in the testes may be a mere reflection of the haemolymph ecdysteroid titres. Thus testicular ecdysteroids may have been sequestered from the haemolymph ecdysteroid pool. It is also possible that the testes may be a target tissue for the haemolymph ecdysteroids. This facilitates synchronous somatic and testicular development.

Testicular ecdysteroid titres could not be compared with those of haemolymph in the adult stadium as enough blood could not be drawn from the adults to determine haemolymph ecdysteroid titres. However, no ecdysteroids were detected in the haemolymph of adult male blowfly *C. vicina* by Koolman *et al.* (1979) although large amounts of such compounds were found in the testes. Testicular ecdysteroid titres of day 0 adults of *S. mauritia* were much lower than mid-instar larvae and pupae. Spermatogenesis in *S. mauritia* is completed in the pupal stage itself. Hence it is possible that in *S. mauritia* testicular ecdysteroids in the adult may have been sequestered from the haemolymph during earlier stages of development.

Thus in *S. mauritia* the total ecdysteroid content of the testes reflect that of the haemolymph during larval and pupal development. Similar observations have also been made in *H. virescens* (Loeb, 1982), *O. nubilalis* (Gelman *et al.*, 1988a,b) and pupae of *M. sexta* (Friedlander and Reynolds, 1992). However titre levels in the haemolymph were much greater than those of the testes in all the insects examined. Ecdysteroid composition of the testes and haemolymph at identical times of development also differed greatly (Gelman *et al.*, 1988a,b; Jarvis *et al.*, 1994). This in itself does not rule out the possibility of sequestration of haemolymph ecdysteroids by the testes. It is always possible that differential uptake of ecdysone and 20-hydroxyecdysone from the haemolymph could occur in the testes thus explaining the observed

differences. Thus the present data suggests that spermatogenesis and testicular development of *S. mauritia* and possibly other insects are controlled by both haemolymph and testicular ecdysteroids. It could not be conclusively proved that sequestration of haemolymph ecdysteroids occur in the testes, but there is definitely an undeniable reflection of haemolymph ecdysteroid content in the testicular ecdysteroid levels.

Effects of ligations on testicular ecdysteroid profile

Testicular ecdysteroid titres were reduced considerably following neck ligation on day 1 and titres in the thorax-ligated larvae were reduced to nil four days after ligation. On the other hand, unligated (normal) control larvae exhibited an increasing trend of ecdysteroids reaching a maximum on day 4. The reduction in titres following ligation may be due to the lack of testes ecdysiotropins from the brain which (may) stimulate synthesis and release of ecdysteroids by the testes of immature insects (Loeb *et al.*, 1987; Loeb, 1994b). Although both neck- and thorax-ligated larvae lacked the brain, the suspected source of testis ecdysiotropin, testes of the neck-ligated larvae contained significant amounts of ecdysteroids while titres in the thorax-ligated larvae were reduced to nil. Therefore, the total absence of ecdysteroids in the testes of thorax-ligated larvae may be due to the absence of prothoracic glands. Previous studies demonstrated that haemolymph ecdysteroid titres of *S. mauritia* also decreased following neck and thorax ligation, more so in

thorax-ligated larvae. Thus the presence of prothoracic glands and/or haemolymph ecdysteroid titres may be essential for the synthesis of testicular ecdysteroids.

Spermatogenesis in *S. mauritia* was completely blocked in the ligated larvae and the testes contained an undifferentiated mass of tissue (Venugopalan, 1995). As mentioned earlier, the commitment peak of ecdysteroids in the haemolymph and consequently the testicular ecdysteroid peak on day 3 may be promoting maturation of male germ cells. The absence of this peak would have caused the disintegration of the cells.

Earlier studies on *S. mauritia* have demonstrated that neck/thorax ligation of day 4 larvae prevented pupation. Testes lobes also do not undergo fusion in these larvae. However neck-ligation on day 4 does not prevent the increase in testicular ecdysteroid titres. Same was the case with haemolymph ecdysteroid titres of *S. mauritia* following neck ligation on day 4. But thorax ligation led to decreased titres both in the testes and haemolymph ecdysteroid titres. Reduction in haemolymph ecdysteroid titres following thorax ligation was due to the absence of prothoracic glands. Thorax-ligated larvae contained the testes, yet titres reduced in the ligated larvae. Hence the testes ecdysteroid titres may be dependent on haemolymph titre or the presence of prothoracic glands as has been previously suggested.

Spermatogenesis was arrested following neck/thorax ligation on day 4 in *S. mauritia*, the whole testes appeared to be an unorganised mass and spermatocysts were not clearly distinguishable (Venugopalan, 1995). In the neck-ligated larvae, although ecdysteroid titres increased following ligation, the rise in titre is not rapid as in the control (unligated) larvae. The titres in the neck-ligated larvae increased gradually and reached a peak 4 ± 1 days after ligation while titres in the control larvae peaked on day 5. The ecdysteroid titre peak of neck-ligated larvae although delayed was comparable to the peak titre of control larvae. The differentiation of sperm bundles during day 4 - day 5 may be triggered by the ecdysteroid peak on day 5, the absence of which may cause arrest of spermatogenesis in the neck-ligated larvae. The absence of JH in the ligated larvae may also arrest spermatogenesis since JH is reported to promote spermatogenesis during late last larval stage (Yagi, 1975; Nishitsutsuji-Uwo, 1975; Nishitsutsuji-Uwo, 1961; Shinyaeva, 1981; Venugopalan, 1995). The role of ecdysteroids in promoting testicular development has already been addressed in an earlier section. The reduced ecdysteroid titres coupled with the absence of JH in the thorax-ligated larvae may be the cause of disintegration of the testicular tissue. This may also be the reason for the testes lobes not undergoing fusion in the control larvae. The absence of JH which is secreted from the corpora allata in the ligated larvae may further prevent testicular fusion and spermatogenesis. This aspect will be discussed in a later section. Meola and Loeb (1995) suggested

that testis ecdysiotropins may also be involved in the fusion of the paired testis and maturation of the male reproductive system. Hence, the absence of testis ecdysiotropins may also be the reason for the failure of testicular fusion in the ligated larvae.

Effects of JHA on testicular ecdysteroid profile

Inhibitory effects of JHA on testicular ecdysteroid titres

Previous studies have demonstrated that repetitive treatments of unligated sixth instar larvae of *S. mauritia* with JHA affect the growth, differentiation and developmental competence of imaginal structures resulting in the production of imperfect superlarvae. The testes of these larvae remained separate. Testicular ecdysteroid titres of the treated larvae did not exhibit a peak on day 3 unlike the control (normal) larvae. From earlier studies, it is assumed that this peak is responsible for the change from mitotic to meiotic division of the gonial cells of the testes leading to the formation of spermatids. Venugopalan (1995) reported that JHA treatment interfered with normal testicular development and spermatogenesis during last instar larval development in *S. mauritia*. The volume of the testes of the treated larvae also exhibited a progressive decline during the post-treatment days. From the present study, it follows that the absence of the testicular ecdysteroid peak on day 3 may be the cause for the arrest of development of gonial cells and hence the lack of spermatid cysts and sperm bundles in the supernumerary larvae.

The major ecdysteroid peak which appeared at the pharate pupal stage (day 5) in the control larvae was also missing in the JHA treated larvae. Instead, a much smaller peak appeared on day 4, one day prior to the supernumerary moult. This may also contribute to the developmental arrest of spermatogenesis and prevention of fusion of testes lobes of the treated larvae. Earlier studies have revealed that the commitment peak and the large premoult peak of haemolymph ecdysteroids failed to appear following JHA treatment to last stage larvae. Haemolymph ecdysteroid titres of the imperfect supernumerary larvae also exhibited a small peak on day 4, prior to the supernumerary moult. Thus the ecdysteroid titres of the testes and haemolymph showed a similar pattern of fluctuation following JHA treatment. It further demonstrates that JHA may be inhibiting spermatogenesis and fusion of testes lobes by inhibiting ecdysteroid production by the prothoracic glands and/or the testes. Similar profile of ecdysteroids in the testes and haemolymph suggests that the testes sequesters haemolymph ecdysteroids. The role of JH and its analogues in inhibiting the secretory activity of the prothoracic glands has already been addressed in the previous chapter. Hence, it is also possible that steroid secreting cells of the testes are inhibited in a similar way by JHA.

Previous studies have shown that neck-/thorax-ligated day 1 larvae treated with JHA remained unchanged. Their testes also did not undergo

fusion. Testicular ecdysteroid titres of the neck-ligated, JHA treated larvae were much lower than that of the control larvae. Titres in the thorax-ligated larvae treated with JHA were reduced to nil. Control larval titres were also reduced to nil. Haemolymph ecdysteroid titres of neck-ligated, JHA treated *S. mauritia* larvae also showed low titres. Titres in the thorax-ligated, treated larvae were further reduced. The lower testicular ecdysteroid titres in the JHA treated, neck-ligated larvae suggests an inhibitory action of JHA on biosynthesis of testicular ecdysteroids. This may be due to direct inhibition of testicular synthesis of ecdysteroids by JHA or just a reflection of the reduced haemolymph titres following JHA treatment. In the thorax-ligated, control larvae, absence of prothoracic glands may have caused total absence of ecdysteroids in the testes. Therefore, JHA treatment did not alter that status.

Histological studies of spermatogenesis of *S. mauritia* revealed a complete inhibition of spermatogenesis following ligation coupled with JHA treatment to day 1 larvae. The volume of the testes of the treated larvae were lower than that of the control larvae. The testes of the treated larvae was a mass of disintegrating cells. The testes of the control larvae also contained an undifferentiated mass of tissue (Venugopalan, 1995). Testicular DNA, RNA and proteins of *S. mauritia* were also considerably reduced following JHA treatment to ligated day 1 larvae (Benny, 2001). From the present studies, it

may be assumed that the disintegration of germ cells in the ligated and JHA treated larvae may be due to the reduced ecdysteroid titres in the haemolymph and the testes. Inhibitory effects of JHA on macromolecular synthesis of the testes may also be due to the reduced testicular ecdysteroid titres which in turn may have been affected by the low ecdysteroid titres in the haemolymph of the JHA treated larvae.

Stimulatory effects of JHA on testicular ecdysteroid titres

Previous studies have shown that neck-ligated day 4 larvae of *S. mauritia* treated with JHA transformed into headless pupae while control (larvae neck-ligated on day 4, untreated), failed to pupate. However, treatment of JHA to thorax-ligated day 4 larvae did not bring about pupation in the treated larvae. Control larvae (larvae thorax-ligated on day 4, untreated) also failed to pupate. Testes lobes of the headless pupae were fused while those of the thorax-ligated, treated larvae and those of controls failed to undergo fusion.

Testicular ecdysteroid titres of the headless pupae were much higher than those of the control larvae (day 7). However, it may be recalled that testicular ecdysteroid titres of neck-ligated, post feeding larvae on day 8 were of the same range as that of the normal prepupal testicular ecdysteroid titres, but the testes did not undergo fusion. This shows that JH has an important role in testicular fusion. Histological studies of the testes of the headless pupa

demonstrated that spermatogenesis had progressed and also revealed the presence of apyrene sperm bundles. On the other hand, in the testes of the control larvae, spermatocysts were not clearly distinguishable and the whole testes was an unorganised mass (Venugopalan, 1995). The DNA, RNA and protein content of the testes of the headless pupae also showed very high values compared to controls (Benny, 2001). Results of the present studies suggest that JH or its analogue modifies the response of the target tissues to ecdysteroids and hence may act at the receptor level since delayed increase in ecdysteroid titres in the absence of JH in the control larvae did not cause reinitiation of spermatogenesis but JH treatment did.

The present results suggest that the prepupal increase in JH has a critical role to play not only in pupation but also in promoting spermatogenesis. The role of testis ecdysiotropins in the fusion of testes lobes (Meola and Loeb, 1995) has already been discussed. Testis ecdysiotropins may already be released in the day 4 larvae and it may act in conjunction with JH and ecdysteroids. Earlier studies have shown that haemolymph ecdysteroid titres of headless pupae were higher than those of control (day 7). Thus the testes and haemolymph ecdysteroid titres were similarly affected by JHA treatment.

Testicular ecdysteroid titres of the thorax-ligated JHA treated larvae did not differ much from the control larvae. Titres were found to decrease in

both the treated and control larvae. Administration of JHA to these larvae did not bring about pupation and testicular fusion in these larvae. This may be due to the decreased titres of ecdysteroids in the testes following ligation. This shows that successful larval-pupal transformation and spermatogenesis requires a critical titre of ecdysteroids and JH in the haemolymph and testes. The absence of either may affect metamorphosis and spermatogenesis.

Earlier studies on *S. mauritia* have shown that treatment of JHA to pupae caused high mortality in the treated pupae. The surviving pupae showed various abnormalities in pupal-adult development. Testicular ecdysteroid titres increased considerably in the JHA treated pupae. Increased dosage of JHA elicited higher titres of ecdysteroids. Hence titres were greater in the 1 μg JHA treated pupae than in the 0.5 μg JHA treated pupae. Hormone titres increased significantly in the first half of the pupal stage and declined gradually in the latter half both in the treated and control pupae. However ecdysteroid peaks appeared two days later (day 3) in the JHA treated pupae while the peak appeared on day 1 in the control pupae. Earlier studies have shown that haemolymph ecdysteroid titres of pupae also showed considerable increase following JHA treatment and here too the increase in titres was directly proportional to the dosage of JHA. Displacement of peak titres also occurred in the haemolymph where the peak appeared one day later (day 2) in the 0.5 μg JHA treated pupae. Haemolymph titre peaks of control

and 1 μg JHA treated pupae however occurred on day 1. Thus the haemolymph ecdysteroid titres also increased during the first half of the pupal stadium and declined in the latter half both in the JHA treated and control pupae.

Pupal-adult development normally occur in the absence of JH. Also, a decline in the JH titre during the final larval instar is necessary for the initiation of metamorphosis (Wigglesworth, 1955; Gilbert, 1962). It has already been discussed that the prothoracic glands undergo apoptosis during pupal-adult development (Dai and Gilbert, 1997). JHA treatment prevents apoptosis (Dai and Gilbert, 1998) and causes an increase in ecdysteroid titres in the haemolymph. Whether apoptosis occurs in the steroid secreting cells of the testes is not known. However, the reduction in titres during the latter half of (normal) pupal development and increased synthetic rates following JHA treatment point to such a possibility. It may also be possible that these fluctuations in the testicular titres are caused by fluctuations in the haemolymph titres.

JHA treatment also caused developmental arrest of spermatogenesis and necrosis of already formed sperm bundles of *S. mauritia* pupae (Venugopalan, 1995). From the present study it may be assumed that lysis of the fully formed sperm bundles may be caused by the unusually high ecdysteroid titres following JHA treatment. It may be recalled that a

significant increase in sperm bundles occurred during the latter half (day 6) of pupal development when the ecdysteroid titres were on a downward trend. But JHA treatment maintained abnormally high ecdysteroid titres even during the latter half of pupal development which may be responsible for the arrest of spermatogenesis and necrosis of already formed sperm bundles. Further, JH is normally not present during pupal development and its occurrence may directly cause disturbances in testicular development.

Effects of ecdysteroid agonist RH 5992 on testicular ecdysteroid profile

Effects of RH 5992 on ligated sixth instar larvae

Non-steroidal ecdysteroid agonists (RH 5849, RH 5992) possess strong ecdysonergic activities, inducing a premature, lethal moult in the treated larvae of many lepidopterans and other insect species (Wing *et al.*, 1988; Monthean and Potter, 1992; Smagghe and Degheele, 1994; Sakunthala and Nair, 1995). The present study also demonstrated that RH 5992 induced a precocious moult in larvae of *S. mauritia*. Treatment of different dosages of RH 5992 to neck-/thorax-ligated day 1 larvae induced a decrease in testicular ecdysteroid titres on day 1 and day 2. An abrupt increase in titres occurred on day 3 in the neck-ligated larvae treated with 5 μg RH 5992 while those in others continued to decrease. Titres decreased in the control larvae also. However the decrease in titres was more pronounced in the thorax-ligated

larvae. Histological studies revealed that the treatment of ecdysteroid agonist to neck-/thorax-ligated day 1 larvae accelerated spermatogenesis (Venugopalan, 1995). The presence of well differentiated spermatogonial, spermatocytal and spermatidal cysts and eupyrene sperm bundles were observed in the testes of the treated larvae while spermatogenesis was completely arrested in the control larvae. The testes lobes were separate in the treated and control larvae but volumes of testes of the treated larvae were higher than those of the control larvae. Testicular ecdysteroid titres of the RH 5992 treated larvae were lower than those of the control larvae on most days, yet the acceleration of spermatogenesis suggested that RH 5992 acted at the level of ecdysteroid receptors (ER). The prothoracic glands have been reported to possess ER (Bidmon and Sliter, 1990; Talbot *et al.*, 1993). It is possible that the testes sheath may also possess ER through which RH 5992 and haemolymph ecdysteroids regulate testicular ecdysteroid titres and spermatogenesis. Thus, the testes may act as a target tissue for haemolymph ecdysteroid titres or ecdysteroid mimics. It has been reported that non-steroidal ecdysteroid agonists cause precocious moulting in treated larvae by binding to the ER of epidermal cells (Smagghe and Degheele, 1992) without causing an increase in haemolymph ecdysteroid titres. A similar mode of action may be operating in the testes of the treated larvae which causes the acceleration of spermatogenesis without an increase in testicular ecdysteroid titres. It is also possible that the decrease in titres of testicular ecdysteroids

may be a reflection of the decline in the haemolymph ecdysteroid titres in the RH 5992 treated larvae. The spontaneous increase in testicular ecdysteroid titres in some of the treated larvae may also be a reflection of the haemolymph ecdysteroid titres or due to a positive feedback effect of RH 5992 on the steroid secreting cells of the testis sheath. Ecdysteroids and ecdysteroid agonists have been reported to have such a feedback effect on prothoracic glands of insects (Williams, 1952; Siew and Gilbert, 1971; Sakurai and Williams, 1989).

Previous studies showed that treatments of different dosages of RH 5992 to neck-/thorax-ligated day 4 larvae induced the development of larval-pupal intermediates. The testes of these larvae underwent fusion while control larvae remained unchanged and the testes lobes failed to fuse. Testicular ecdysteroid titres were found to decrease following treatment of RH 5992 in the neck-ligated and thorax-ligated larvae but for a few exceptions. However, titres in the control larvae increased gradually in the case of the neck-ligated ones but decreased further in the thorax-ligated larvae. Here too, histological studies revealed that treatment of ecdysteroid agonist to neck-/thorax-ligated day 4 larvae accelerated spermatogenesis (Venugopalan, 1995). Eupyrene sperm bundles were observed in the treated larvae but apyrene sperm bundles were conspicuously absent. Control larval testes contained spermatocysts which were not clearly distinguishable and the whole testes was an unorganised mass. Similar results were obtained with

treatment of RH 5992 to *C. pomonella* which induced spermatogenesis reinitiation in isolated abdomens of diapausing codling moth larvae (Friedlander and Brown, 1995). Testicular ecdysteroid titres of neck-ligated, control larvae (day 7) were almost the same range as the normal prepupal (day 5) testicular titres. However, spermatogenesis was arrested and the testes remained separate in the control larvae. On the other hand, RH 5992 treatment accelerated spermatogenesis and testis fusion both in the neck- and thorax-ligated day 4 larvae even at low testicular ecdysteroid titres. This suggested that RH 5992 stimulates testicular development and spermatogenesis at the level of the ER of the testis as has been previously discussed. Induction of larval-pupal intermediate formation by RH 5992 treatment also occurred at low haemolymph ecdysteroid titres. It has been suggested that RH 5992 caused spermatogenesis renewal in isolated abdomens of diapausing *C. pomonella* larvae by attaching to and saturating, a specific number of receptors (Friedlander and Brown, 1995). Hence, spermatogenesis renewal may not have occurred in the control (neck-ligated) larvae because the "ecdysteroid peak" for saturation of the ER of the testes was delayed and not available during the prepupal period (day 5). In the absence of the required peak titre of ecdysteroids, the germinal cysts would have degenerated. Hence, it may be postulated that a critical titre of ecdysteroids during a specific period of time in the testes and/or haemolymph

is essential for normal progression of spermatogenesis without which the germinal cysts would undergo degeneration.

Effects of RH 5992 on pupae

Treatment of pupae with different dosages of RH 5992 induced the development of adultoids in *S. mauritia* as has been previously reported. However histological studies of the testes of RH 5992 treated *S. mauritia* pupae failed to reveal any abnormalities in testicular development or spermatogenesis (Manogem and Nair, unpublished results). Testicular ecdysteroids also did not show any variation after RH 5992 treatment in the pupae. Testicular ecdysteroid titres were similar in the treated and control pupae on the days examined (day 2 and day 5). Haemolymph ecdysteroid titres however did not exhibit similar titres in the RH 5992 treated and control pupae. This is the first instance where testicular and haemolymph ecdysteroid titres differed in *S. mauritia.* It has been suggested that RH 5992 causes acceleration of spermatogenesis at the level of ecdysteroid receptors. RH 5992 attaches to and saturates a specific number of receptors and surplus of RH 5992 do not disrupt spermatogenesis and physiological function (Friedlander and Brown, 1995). Hence, RH 5992 treatment does not have any effect on spermatogenesis or testicular ecdysteroid titres during the pupal stage of *S. mauritia.*

4.5 SUMMARY

1. Testicular ecdysteroid profile of *S. mauritia* during the sixth (final) larval instar, pupal and adult stadia was determined by means of RIA. Effects of hormones and hormone mimics on the ecdysteroid content of the testes were studied by means of ligation and treatments of a JHA (hydroprene) and a non-steroidal ecdysteroid agonist (RH 5992). Control insects were treated with acetone. The function of testicular ecdysteroid titres was analysed by correlating it with known developmental stages of spermatogenesis in *S. mauritia.*

2. Testicular ecdysteroid profile of sixth instar larvae revealed two peaks, a small peak on day 3 and a large peak on day 5. The paired larval testes fused to form a single median organ on day 5 of the sixth larval instar. Ecdysteroid titres of the pupal testes exhibited a single large peak on day 1 of the pupal stadium. Ecdysteroid titres decreased considerably in the newly emerged adult.

3. Influence of brain factors and prothoracic gland secretions on testicular ecdysteroid titres were studied by means of neck/thorax ligation of day 1 and day 4 sixth instar larvae. The paired larval testes failed to undergo fusion in the neck-/thorax-ligated day 1 and day 4 larvae. Testicular ecdysteroid titres of the neck-/thorax-ligated day 1 larvae were significantly reduced compared to the control larvae. Titres of the neck-ligated day 4 larvae increased gradually to reach a peak on day 8 which was not significantly different from the ecdysteroid titre peak on day 5 (prepupae) of the control larvae while thorax ligation of day 4 larvae led to a significant decrease in testicular ecdysteroid titres.

4. The influence of JH on testicular ecdysteroid titres were investigated by topical application of JHA to unligated day 0 larvae and neck-/thorax-ligated day 1 and day 4 sixth instar larvae and freshly ecdysed intact pupae of *S. mauritia.*

5. Repetitive treatments of 20 µg JHA to intact sixth instar larvae induced a supernumerary larval moult on day 5. The testes of the supernumerary larvae remained separate. JHA treated larvae exhibited a single peak on day 4 which was significantly lower than the testicular ecdysteroid peak on day 5 of the control larvae. The smaller peak observed on day 3 of the control larvae also failed to appear in the JHA treated larvae.

6. Repetitive treatments of 5 µg JHA to neck-/thorax-ligated day 1 larvae prevented metamorphic changes in the treated larvae. The testes

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6. Repetitive treatments of 5 µg JHA to neck-/thorax-ligated day 1 larvae prevented metamorphic changes in the treated larvae. The testes

remained separate in the JHA treated and control larvae and testes volumes decreased in the treated larvae. Testicular ecdysteroid titres of the neck-ligated, JHA treated larvae decreased significantly when compared to those of control larvae while titres of both thorax-ligated, JHA treated and control larvae were reduced to nil.

7. Treatment (single) of 5 μg JHA to neck-ligated day 4 larvae induced the formation of headless pupae with fused testes lobes while control larvae failed to pupate and the testes lobes remained separate. However, thorax-ligated, JHA treated and control larvae failed to pupate and the testes lobes remained separate. Testicular ecdysteroid titres of the headless pupae were significantly higher than those of control larvae. But testicular titres of the thorax-ligated, JHA treated and control larvae showed a decreasing trend and were not significantly different from each other.

8. Treatment of different dosages of JHA (0.5 μg or 1 μg) to newly ecdysed pupae induced the formation of adultoids which failed to emerge. Testicular ecdysteroid titres of JHA treated pupae were significantly higher than those of control pupae on most days. Ecdysteroid titres increased greatly in the early pupal stage and declined in the latter period both in the JHA treated and control pupae. JHA appears to have a tropic effect on testicular ecdysteroid titres.

9. The influence of RH 5992 on testicular ecdysteroid titres was investigated by topical application of RH 5992 to neck-/thorax-ligated day 1 and day 4 sixth instar larvae and intact pupae of *S. mauritia.*

10. Treatment of RH 5992 (2 μg or 5 μg) to neck-/thorax-ligated day 1 larvae induced a precocious and lethal moult in the ligated larvae. The testes lobes remained separate in the treated and control larvae. However, the testes volumes increased in the treated larvae. Testicular ecdysteroid titres decreased further in the RH 5992 treated larvae but a significant but abrupt increase in titres occurred on day 3 in the 5 μg RH 5992 treated neck-ligated larvae when compared to titres of the control larvae. Testicular ecdysteroid titres of RH 5992 treated, thorax-ligated larvae were lower than those of controls on most days. The abrupt rise in titres which occurred in some of the treated larvae were not significant. RH 5992 acts at the (ecdysteroid) receptor level without a rise in ecdysteroid titres and hence the low titres on day 1 and day 2. The increase in titres on day 3 may be due to positive feedback effect of RH 5992 on steroid secreting cells of the testes or a reflection of the fluctuating haemolymph titres.

11. Treatment of RH 5992 (2 μg or 5 μg or 10 μg) to neck-/thorax-ligated day 4 larvae induced the development of larval-pupal intermediates with fused testes lobes. The control larvae remained unchanged and the testes lobes failed to undergo fusion. Testicular ecdysteroid titres of larval-pupal intermediates were significantly lower than those of controls in 5 μg and 10 μg RH 5992 treated larvae. Titres of the RH 5992 treated larvae were lower than those of the control larvae on most days while control larval titres exhibited an increasing trend reaching its maximum on day 7. Testicular ecdysteroid titres of the thorax-ligated, RH 5992 treated and control larvae decreased considerably. Testicular titres although lower in the RH 5992 treated larvae were not significantly different from those of controls. The decrease in ecdysteroid titres may be due to negative feedback effect of RH 5992 on the steroid secreting cells of the testes or a reflection of the fluctuating haemolymph titres.

12. RH 5992 treatment to pupae induced formation of adultoids. The pupal period got extended following treatment and mortality occurred towards the end of the pupal period. However testicular ecdysteroid titres of treated and control pupae did not show a significant difference on day 2 and day 5. Hence RH 5992 may not have any effect on the testicular ecdysteroid content of pupae.

13. Ecdysteroid contents of the testes and haemolymph were compared during larval-pupal and pupal-adult development. Testicular ecdysteroid levels during larval-pupal development increased on day 3 and showed a significant peak on day 5. Haemolymph ecdysteroid titres during larval-pupal development also exhibited a similar pattern of fluctuation. During pupal-adult development, testicular ecdysteroid titres exhibited a single large peak on day 1 and haemolymph titres peaked on day 2. Testicular and haemolymph ecdysteroid titres exhibited a single large and significant peak in the first half of pupal development and decreased in the latter half. Thus, pattern of fluctuation of testicular and haemolymph ecdysteroids were similar which suggest that testicular ecdysteroids may be sequestered from the haemolymph ecdysteroid pool. It is also possible that testicular and haemolymph ecdysteroid titres may be affected by other hormones in a similar manner.

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