MORPHOGENETIC EFFECTS OF INSECT GROWTH REGULATORS ON *SPODOPTERA MAURITIA* BOISD. (LEPIDOPTERA: NOCTUIDAE)

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By

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CERTIFICATE

This is to certify that the thesis entitled "Morphogenetic effects of insect growth regulators on *Spodoptera mauritia Boisd.* (Lepidoptera: Noctuidae)", is an authentic record of research work carried out by Mrs. Sakunthala, C., under my guidance and supervision, from 12-1-1999 to 30-3-2005 as a part time research scholar and from 30-3-2005 to 30-3-2007 as a full-time research fellow under FIP scheme of UGC, X^{th} plan period, in partial fulfillment of the requirement of the Degree of Doctor of Philosophy under the Faculty of Science, University of Calicut. No part of this thesis has been presented previously for any other Degree.

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DECLARATION

I, Sakunthala. C., do hereby declare that the resent work is original an Department of Zoology, University of Calicut, under the guidance of Dr. V. S. K.Nair. I further declare that no part of this thesis has been submitted previously for any other Degree.

Calicut University,
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Insects remain the main enemies of man through their varied roles as pests of agricultural crops and vectors of various diseases. With the increased demand for food in the scenario of increasing human populations, man is compelled to reduce insect populations by any means. A variety of insecticides are used to control the insect pests and prevent crop destruction. Pesticides such as organophosphates and carbamates are in use extensively in agriculture since World War II. The indiscriminate use of these chemicals has led to the deterioration of environmental quality, which in turn has threatened the very existence of humans on earth. The issues of environmental contamination, toxicological problems such as bioaccumulation and biomagnification of pesticide residues in the ecosystem have assumed a global status which demands the utilization of ecofriendly techniques for controlling insect pests. Further damage to non-target organisms by chemical pesticides has led to certain deleterious effects that may result in total disruption of ecosystems. The resurgence of pesticide resistant strains of insects and outbreak of pests of secondary importance raise a lot of other issues that endanger the nature.

With ever increasing world population there is urgent need to develop alternative chemicals with reduced risk to the environment, at the same time controlling the pests effectively. Recently a number of alternative chemicals with minimum side effects on ecosystem were synthesized and evaluated with regard to their usefulness in integrated pest management (IPM) programmes. These compounds are the outcome of basic research on insect hormones and are named as insect growth regulators (IGRs).

IPM programmes aim at application of substances that are specific in their action on target species, biodegradable and non toxic to other non-target organisms. They mostly interfere with the processes of growth, development, metamorphosis and reproduction of only insects only and thus have a very

favourable profile regarding environmental side effects, worker safety and toxicity problems (Altstein *et al*., 1993; Nair, 1993).

1.2 Insect growth regulators based on hormones (IGRs): an overview

1.2.1 Insect hormones

Insects depend on their hormones for a variety of life processes such as growth, development, metamorphosis and reproduction. There are three categories of insect hormones. They are (a) juvenile hormones (JHs) secreted by corpora allata (b) ecdysteroids secreted by prothoracic glands/other tissues and (c) the neurohormones secreted by neurosecretory cells of brain and segmental ganglia. Juvenile hormones (JH) are sesquiterpenoid compounds which regulate growth and metamorphosis by the interaction with ecdysteroids (Williams, 1956; Wigglesworth, 1934, 1936) and in adults they control the reproductive processes (Engelmann, 1970) and a number of physiological processes. Ecdysteroids are responsible for growth, moulting, metamorphosis and reproduction. They initiate the moulting process though the nature of the moult is determined by JH. A high titre of JH leads to larval-larval moult and that of ecdysteroids leads to larval-pupal moult and absence of JH leads to pupal- adult moult. The neuropeptides are crucial in their action controlling a number of life processes such as water balance, lipid and carbohydrate metabolism, muscle contraction, reproduction and also the secretion of other hormones.

In insects, a critical titre of hormones in body fluids is a prime requirement and any interference in the biosynthesis or degradation of hormones will disrupt the hormone dependent physiological processes involved in morphogenesis, reproduction and behaviour. The intensive research in the field of insect endocrinology has highlighted the importance of IGRs based on insect hormones in controlling insect pests. The identification of the chemistry of molting hormones (Karlson *et al*., 1963) and JHs (Roller *et al*., 1967) increased the scope of exploring these possibilities. In the recent years this idea has been

exploited successfully for the development of agonists and antagonists of insect hormones.

1.2.2 Neurohormones as IGRs

Early studies on insect control by endocrine manipulation did not give sufficient importance to neuropeptides that are released from neurosecretory cells of insect central nervous system. These function as "master regulators" of growth, development and reproduction of insects and so demand more attention in pest control programmes. They act as neurotransmitters and neuromodulators and influence the secretion of other hormones such as JH and ecdysteroids. The pentapeptide proctolin was the first neuropeptide to be identified and isolated (Brown, 1975, Brown and Starratt, 1975). Later a number of other peptides such as adipokinetic hormones and myoactive peptides having regulatory roles in different physiological processes were identified from insects.

It was found that the control of insect pests can be brought about by the disruption of synthesis, release or degradation of neurohormones (Masler *et al*., 1993). There are several useful features in a neuroendocrine approach to insect pest control as the protein nature of neurohormones makes them amenable to recombinant DNA technology. However there are certain drawbacks as their uptake through insect cuticle is minimal. Moreover, neuropeptides are susceptible to degradation under field conditions. However, research in this area has suggested other methods for employing a neuropeptide based insect control strategy. These include the synthesis of stable and more lipophilic agonists and antagonists of natural neuro-peptides by molecular modelling. This would minimize the problems of rapid degradation in the field and reduced penetration through the cuticle. Another approach would be to insert insect neurohormone genes to the genomes of crop plants so that transformed plants would produce the hormone and disrupt the physiological balance of phytophagous insects. Transfer of the insect neurohormone genes into target species using insect

specific viruses such as baculovirus vector has proved to be very effective in insect control (Masler *et al*., 1993; Bonning and Hammock, 1996; Shiomi *et al*., 2003).

A number of neuropeptides that regulate JH biosynthesis have been described of which many inhibit JH biosynthesis in corpora allata (Gade *et al*., 1997). A family of neuropeptides (Allatostatins) was identified from crickets and stick insects having the ability to inhibit JH biosynthesis (Lorenz *et al*., 1995). Employing specific inhibitors of the enzymes involved in their biosynthesis one can inhibit the production of these neuropeptides. From desert locust, *Schistocerca gregaria* (Van den Broeck *et al*., 1996) and the mosquito *Aedes aegypti* (Veenstra *et al*., 1997) the genes that code for a precursor polypeptide containing 5 to 15 allatostatins have been isolated.

Insect neurohormones are synthesized as prohormones that are processed to a number of simpler hormonally active peptides. As the inhibition of enzymes that are involved in this also disrupt the hormone balance, manipulations can be brought about here to control insect pests. In insects, secretion of peptide hormones is mediated by biogenic amines such as octopamine. Octopamine is a neurotransmitter which also functions as a neurohormone, producing either a hyperglycemic or hyperlipaemic response *in vivo* and *in vitro* (Orchard and Lange, 1985). The fact that octopamine has a significant role in the release of insect neurohormones can be successfully exploited to design agonists and antagonists that act on aminergic receptors. Another potential aspect that can be manipulated is the hydrolysis of hormones as prolonged or reduced activity will have an undesirable effect. Synthesis of degradative enzymes can be stimulated or inhibited to influence the normal physiological processes of insects. It was found that excess proctolin caused temporary inactivity to set in (Starratt and Steele, 1984). The absence of detailed information about insect neurohormones is the major impediment to the development of a neuroendocrine strategy for insect pest control.

1.2.3 Juvenile Hormone Analogues (JHAs)

Williams (1967) suggested that JH or its analogues could be used as specific control agents against insect pests. The natural JHs are difficult to synthesize and are very expensive for application due to this reason. Another problem is that they are environmentally unstable. Their synthetic analogues (JHAs or Juvenoids) are easier to synthesize, highly economical for use and have great stability in the field. The discovery of aromatic terpenoid esters (Bowers, 1969) initiated the era of studies on JH analogues. Since then, a number of JH analogues were tested for their insecticidal activity (Sehnal, 1976). They were highly effective as IGRs and were environmentally safe due to reduced toxicity for fish, birds and mammals (Grenier and Grenier, 1993, Dhadialla *et al*., 1998). Among the well known juvenoids are the epofenonane (Handgartner *et al*., 1976), hydroprene, kinoprene, methoprene (Henrick *et al*., 1976) and phenoxy carbamate (Peleg, 1982).

Juvenoids have varied effects on pest species such as induction of morphogenetic abnormalities, disruption of physiological processes leading to deleterious effects in reproduction and development. Experiments with *Pyrrhocorris apterus* gave the first example of ovarian dysfunction through injection of JHAs (Masner, 1969). Application of juvenoids to the young pupae and adults resulted in the sterilization of the females (Metwally and Lande, 1972; Metwally *et al*., 1972). Ovicidal effects were obtained from *Hylemnia brassicae* (Van de Veire and de Loof, 1973) and *Laspeyresia pomonella* (Gelbic and Sehnal, 1973). In *Spodoptera mauritia* treatments of pupae with juvenoids resulted in ovarian abnormalities and inhibition of ovarian growth (Sam Mathai and Nair, 1990). Juvenoids induced a distortion of ovarian development in *Blatta germanica* (Maiza *et al*., 2004).

Juvenoids reduced the larval growth and food consumption in *Bombyx mori* (Leonardi *et al*., 1996). In the eastern spruce budworm *Choristoneura*

fumiferana, eggs failed to hatch when the adult females were treated with juvenoids (Hicks *and* Gorden, 1992) and post-embryonic development was affected on treatments at the egg stage (Hicks *and* Gorden, 1994). Juvenoids inhibited larval-pupal transformation and produced giant larvae in stored product insects *Tribolium castaneum, Rhyzopertha dominica* and *Sitsphilus oryzae* (Kostyukovsky *et al*., 2000). Treatments with the juvenoids inhibited larval ecdysis and emergence in *Cotesia congregata* (Beckage *et al*., 2002). Juvenoids were found to prolong developmental time and also increase mortality in the wandering phase in *Plodia interpunctella* (Mohandas *et al*., 2006).

Larval treatments of *Spodoptera mauritia* with juvenoids resulted in the production of extra larval and abnormal pupal forms (Santha and Nair, 1987) whereas pupal treatments led to various types of abnormalities and inhibition of ovarian growth (Sam Mathai and Nair, 1990). In *Sarcophaga ruficornis*, JHA treatments in larvae resulted in three types of morphogenetic forms such as larval-pupal intermediates, pupal-adult intermediates and deformed adults (Mathur *et al*., 1990). Juvenoids applied topically of larvae of *Corcyra cephalonica* harbouring the larvae of parasitoid produce various types of developmental derangements in parasitoid such as development of compound egg chamber, ill developed ovariole, increased or decreased ovariole number (Chanda and Chakravorty, 2001). Exposure to juvenoids inhibited the cell proliferation and protein synthesis in lepidopteran imaginal discs (Oberlander *et al*., 2000; Safarulla *et al*., 2003).

Inhibition of secretory activity of prothoracic glands was observed followed by juvenoid treatments of early last instar larvae of *Spodoptera mauritia* (Balamani and Nair, 1992) whereas treatments at the prepupal stage resulted in stimulation of secretion (Balamani and Nair 1989a, b; 1991). Similar effects were observed in many other Lepidoptera (Hiruma *et al*., 1978b; Cymborowski and Stolarz, 1979, Safranek *et al*., 1980; Gruetzmacher *et al*., 1984). It was proved that the juvenoids such as methoprene can be used

effectively to control the vector of dengue fever, *Aedes aegypti* (Braga *et al*., 2005).

Pyriproxyfen (4-phenoxy phenyl (RS)-2-(2)-pyridiloxy) propyl ether, PPN) the new generation juvenoid, is highly effective in controlling lepidopterans (Smagghe and Degheele, 1994), white flies (Ishaaya *et al*., 1992, 1994), mosquitoes (Kono *et al*., 1997, Kawada *et al*., 1997, Paul *et al*., 2006) bumble bees (Mommaerts *et al*., 2006) and the Californian red scales (Grafton-Cardwell *et al*., 2006). It is slow acting with long residual effects, selective on target species, non-toxic to man and wild life and is excellent for use in IPM programmes (Palumbo, 2001). It had no impact on nontarget organisms (Vythilingam *et al.,* 2005). Topical application of PPN on pupae of *Spodoptera litura* reduced the number of eggs oviposited (Hatakoshi, 1992). PPN disrupt molting, induce defective embryogenesis and morphological deformities in the desert locust, *Schistocerca gregaria* (Vennard *et al*., 1998). No progeny was obtained in *Hyposoter didmator* following PPN treatments (Schneider *et al*., 2003). This compound also causes the development of mosaic and precocious nymphal cuticles in crickets (Erezyilmaz *et al*., 2004). Female California red scale, *Aonidiella aurantii* that survived first instar exposure to PPN experienced reduced fecundity with increasing concentration of the compound. Reduced fecundity was due to reduced survival of the females rather than sterility (Rill *et al*., 2007). PPN suppressed embryogenesis when *Aonidiella aurantii* females were treated with pyriproxyfen (Eliahu *et al*., 2007).

Following treatments of PPN, a complete inhibition of adult emergence was observed in different species of mosquitoes (Lee, 2001; Nayar *et al*., 2002; Yapabandara and Curtis, 2004; Nishiura *et al*., 2005, Sihuincha *et al*., 2005; Seng *et al*., 2006; Darriet and Corbel, 2006; Jambulingam *et al*., 2008; Seccacini *et al*., 2008). It was found to be very effective in controlling dengue vectors due to this ability (Vythilingam *et al*., 2005) and was confirmed to be a potent IGR for mosquitoes (Paul *et al*., 2006, Aiku *et al*., 2006). Similar results

were obtained in cat fleas, *Ctenocephalides felis* (Meola *et al*., 1996; Rajapakse *et al*., 2002) *Chironomus tentans* (Ali *et al*., 1993) and *Callosobruchus maculatus* (Abo-elghar *et al*., 2004). Colonies treated with PPN yielded sexuals with physical abnormalities. Both female and male sexuals of Pharaoh ant, *Monomorium pharaonis* developed bulbous wings, decreased melanization, and died shortly after emergence on treatments with PPN (Lim and Lee, 2005). After treatments of PPN, a gradual accumulation of sterile adultoids followed by population suppression was observed in *Blatta germanica* (Reid and Bennet, 1994). Further, PPN induced a reduction in population development in *Tribolium castaneum* and *T. confusum* (Arthur, 2001). Cuticular penetration of PPN was found to reach 99% in 3 days in adults and the absorption was more than 65% in tissues (Schneider *et al.,* 2008). It has been found to cause a number of changes in the pigment pattern including disappearance of melanisation in the migratory locust, *Locusta migratoria* (Nemec *et al*., 2003).

In spite of the excellent potential of juvenoids as insect control agents, they have a few disadvantages. As they do not disrupt the normal development of insect larvae at the early stages, they are not ideal for use against species in which the early larval instars are responsible for crop damage (Edwards and Menn, 1981). In Lepidoptera, the action of juvenoids is limited to last larval instar and pupal stages as a result of which the application of juvenoids must be timed precisely to get maximum benefit. It was proved that the control of *Corcyra cephalonica* with this could be brought about only when applied to just emerged sixth instar larvae (Jadhav and Bhatia, 2000). Several studies have demonstrated these compounds extend the larval period and feeding activity of insects (Santha and Nair, 1987; Sindhu and Nair, 2004). Another limitation is that in the case of many insect pests, the effects of juvenoids on reproductive biology are not always irreversible. Their low field stability and residual activity demands repeated applications, which lead to huge expenditure. Moreover, JHAs are extensively metabolized to non-toxic degradation products that are

readily excreted (Hammock, 1981). From all these we can assume that the juvenoids have limited scope as potential pest control agents. To worsen the problem, it is reported that many insects have developed resistance to juvenoids (Spindler Barth, 1992; Horowitz and Ishaaya, 1994; Turner and Wilson 1995; Horowitz *et al*., 2002; Cornel *et al*., 2002). These facts have necessitated the development of alternative chemicals for use in pest management programmes.

1.2.4 Anti juvenile hormone agents (AJHAs)

The limited scope of juvenoids in pest control demanded the use of alternative chemicals that are environmentally safe. Compounds which can block or reduce the production of JH were considered as better alternatives as the lack of JH in haemolymph also would derange the hormone dependent processes of morphogenesis and reproduction. These were named as anti JH agents as they will inhibit JH dependent reproductive activities and would induce precocious lethal metamorphosis in treated insects. Two such anti JH substances were extracted from the garden plant (*Ageratum haustonianum*) and were named as precocene I and II. These compounds exert a cytotoxic action on the corpora allata, mediated by an oxidative bioactivation *in vivo*, thus inhibiting JH biosynthesis (Brooks and Mc Caffery, 1990).

Precocenes induce premature metamorphosis and delay in development in the aphid *Myxus persicae* (Hales and Mittler 1981), *Nilaparvata lugens* (Pradeep and Nair, 1989; Bertuso *et al.*, 2002) and *Spodoptera mauritia* (Sam Mathai and Nair, 1984a; Santha and Nair, 1986, 1988, 1991; Santha *et al*., 1987). Precocene treatments of *Leptinotarsa decemlineata* resulted in irreversible diapause (Bowers, 1976, 1982; Bowers *et al*., 1976). A variety of behavioural changes could be induced in the nymphs of *Dermacentor variabilis* by exposing eggs to precocene vapours (Hayes and Oliver, 1981). Administration of precocene II to freshly emerged virgin females of flesh flies *Sarcophaga ruficornis* adversely

affected the development and differentiation of ovarian follicles leading to a number of morphological abnormalities (Kumar and Khan, 2004).

The compounds such as Fluoromevalonolactone (FMev, tetrahydro-4-fluoromethyl-4-hydroxy-2H pyran-2-one), ETB (ethyl-4-[2-(*tert*-butyl carbony-loxy) butoxy] - benzoate) and EMD (ethyl-(E)-3-methyl-2-dodecenoate) showed anti JH activity against lepidopterans (Staal, 1986). FMev treatments inhibited the early steps in JH biosynthesis and were found to be very effective in lepidopteran insects (Quistad *et al.*, 1981 a, b). In *Spodoptera mauritia* the treatments of larvae and pupae using FMev, induced a significant lowering of the production of eggs in the emerged adults and inhibited the ovarian growth and differentiation of the ovarian follicles (Nair and Rajalakshmi, 1989). ETB showed a dose dependent JH agonist and antagonist activity in *Manduca sexta* (Staal, 1977), *Bombyx mori* (Kiguchi, 1984) and in *Spodoptera mauritia* (Balamani and Nair, 1989). Agonist activity was observed at higher doses whereas antagonist activity was observed at doses ranging from 30-100 µg. In *Manduca sexta*, a competitive inhibition of endogenous JH by ETB was reported by Staal (1977). ETB caused the formation of precocious larval-pupal intermediates after the treatments of penultimate larval instars of *Manduca sexta* (Riddiford *et al.*, 1983). EMD caused symptoms of JH deficiency in fairly high doses in many insects. In *Spodoptera mauritia*, treatments of ligated larvae with lower doses of EMD induced formation of larval-pupal intermediates whereas those treated with higher doses moulted into either pupae or larval-pupal intermediates (Balamani and Nair, 1989).

In Lepidoptera, JH esterase hydrolyses JH and regulates its titre in the haemolymph. As the stimulation or inhibition of JH degradation by JH esterase would also modify JH titre in haemolymph and influence the development of an insect, any factor that interferes with the presence of JH esterase is harmful for insect development. Using recombinant DNA technology, the coding sequence of this enzyme can be inserted into a baculovirus, and a derangement of JH titre

can be brought about. It was found that infection of larvae of *Trichoplusia ni* with this recombinant virus induced anti JH effects such as cessation of feeding and mortality (Bonning *et al*., 1997).

1.2.5 Ecdysone Analogues

In insects, ecdysteroids are mostly secreted by the prothoracic glands during larval development, though alternative sites of ecdysteroid production such as ovary, testes, abdominal integument etc. have also been reported in pupae and adults. Initially, the complexity of steroid molecule and the ability of insects to metabolise the hormone at a greater rate have led to little interest in the production of the ecdysteroid analogues as pest management tools. In addition to this, there were the problems of environmental instability and the poor penetration of this hormone through cuticle. Even though numerous ecdysone analogues with immense biological activity have been isolated from plants, their applied value as insect control agents remained unclear (Williams 1968; Ohtaki and Williams, 1970).

There are mainly four groups of ecdysoids. They are (a) Zooecdysoids (b) Phytoecdysoids (c) Synthetic ecdysoids and (d) Non-steroidal ecdysone agonists. Zooecdysoids are substances which are biologically and chemically similar to ecdysone. A methanolic extract of ecdysone like substance from the nematode *Phocanena depressum*, when injected into an early pupa of *Hyalophora cecropia*, was found to accelerate moulting (Novak, 1974). Most important phytoecdysoids are ponasterone, inokosterone and iso-inokosterone. Many phytoecdysoids were reported to be of greater biological activity when compared to naturally occurring ecdysteroids (Ohtaki and Williams, 1970). A number of steroids with moulting hormone activity have been synthesized which are structurally related to ecdysone, lacking only one, two or three hydroxyl groups. 22-isoecdysone was one such synthetic ecdysteroid with moulting hormone activity (Mori, 1968). Synthetic ecdysteroids and their analogues such as

azasteroids have proved to be effective in disrupting the larval moulting process by acting directly on the metabolism of endogenous ecdysteroids (Svoboda *et al.*, 1972). A real breakthrough came in the area of pest control when a nonsteroidal compound code named as RH 5849, was reported to be possessing ecdysone mimetic properties (Wing, 1988). The compound was found to be interfering with feeding activity and inducing a premature lethal molt (Wing *et al*., 1988; Monthean and Potter, 1992).

RH 5849 was found to be very effective against insects belonging to various orders. Topical treatment of second instar larvae of *Leptinotarsa decemlineata* with RH 5849 resulted in premature lethal moult (Darvas *et al*., 1989). Larvae fed on diets treated with different concentrations of RH 5849 shortened the larval and pupal durations and induced a premature lethal molt in *Musca domestica vicina* (Ghoneim *et al*., 1991), *Spodoptera mauritia* (Sakunthala and Nair, 1995) and *Spodoptera littoralis* (Smagghe *et al*., 2001, 2002b). In *Spodoptera litura*, mating success, number of eggs laid by untreated females crossed to treated males, hatching success etc. were found to be reduced by larval exposure to RH 5849 and to another compound tebufenozide with code RH 5992 (Seth *et al*., 2004).

In *Acherontia atropos* and *Lacanobia oleracea* (Blackford and Dinan, 1997) and also in *Ephestia kuehniella* (Hami *et al*., 2005) RH 5992 was found to be more effective than other ecdysone agonists. Malformed cuticle was produced in insect larvae when they ingested tebufenozide (Wing *et al*., 1988, Dhadialla *et al*., 1998). In sugarcane borer *Diatraea saccharalis*, 92% of the larvae died when treated with tebufenozide at 0 hour after ecdysis where as treatment of the larvae at 12 hour after ecdysis resulted in only 30% mortality (Rodriguez *et al*., 2001 a, b). High mortality and malformed larvae were observed after the application of RH 5992 in the oblique-banded leaf roller, *Choristoneura rosaceana* (Waldstein and Reissig, 2001) and also in the cotton leaf worm *Spodoptera littoralis* (Mourad *et al*., 2004). Dose-dependent

mortality, changes in the larval characters and changes in protein profile were observed in *Bombyx mori* (Kumar *et al*., 2000). In codling moth *Cydia pomonella*, significant reduction in fecundity and egg hatch were found to be followed by tebufenozide treatments (Sun and Barrett, 1999; Knight, 2000). Similar results were obtained in and oblique-banded leaf roller, *Choristoneura rosaceana*, *Argyrotaenia velutinana* (Sun *et al*., 2000) and *Grapholita molesta* (Borchert *et al*., 2004; 2005). The ingestion of tebufenozide by larval spruce budworm, *Choristoneura fumiferana* did not impair adult reproduction but the presence of the compound in the environment could inhibit oviposition (Cadogan *et al*., 2002). In tufted apple moth *Platynota idaeusalis*, the treated larvae exhibited higher pupal mortalities, lower pupal weights and generally more deformed adults than untreated larvae (Biddinger *et al*., 2006).

After the discovery of RH 5849 and RH 5992, two other ecdysone agonists were introduced: methoxyfenozide (RH 2485) and halofenozide (RH 0345). These compounds belong to a class of diacyl hydrazines which were selective in action to lepidopteran insects. Methoxyfenozide is an ideal pesticide to be included in integrated pest management programmes because it is non-toxic to mammals and non-target organisms such as birds, fish, honeybees and other pollinators but toxic to target species. It exhibits insecticidal activity against a wide range of caterpillar pests including many members of the family Pyralidae, Tortricidae and Noctuidae (Carlson *et al*., 2001). Methoxyfenozide was harmless to eggs and larvae of *Chrysoperla externa* showing good potential for use in integrated pest management (Schneider *et al*., 2006). Tebufenozide and methoxyfenozide mimic natural ecdysone and induce a premature lethal molt (Dhadialla *et al*., 1998, Nakagawa *et al*., 2000; Carlson *et al*., 2001, Retnakaran *et al*., 2001). The larvae of *Sesamia nonagrioides* deposited two cuticles in a single apolysis and died following methoxyfenozide treatments (Eizaguirre., *et al*., 2007).

Exposure of males of codling moth *Cydia pomonella* (Hoelscher and Barrett, 2003; Barrett, 2008) and oriental fruit moth *Grapholita molesta* (Reinke and Barrett, 2007) to the agonist methoxyfenozide reduced their ability to respond to a calling female. Treated females of *Trichogramma brassicae* showed reduced egg deposition whereas treated males increased the percentage of sterile eggs (Hewa-Kapuge *et al*., 2003). Methoxyfenozide was reported to be toxic to eggs and larvae of codling moth *Cydia pomonella* (Bylemans *et al.*, 2003) and the oriental fruit moth, *Grapholita molesta* (Borchert *et al*., 2004 a, 2005). Larval mortality was observed in *Spodoptera littoralis* on treatment of eggs with methoxyfenozide (Pineda *et al*., 2004) and feeding of third instar larvae with different concentrations of agonist, resulted in pupal mortality and development of deformed adults. Exposure of adults of grape berry moth *Endopiza viteana*, reduced the survival of eggs through to larval and pupal stages (Issacs *et al*., 2005). Oral administration of methoxyfenozide reduced the fecundity and fertility of adults in *Lobesia botrana* whereas the larval treatments resulted in mortality in which the older larvae were more susceptible (Saenz-de-Cabezon Irigaray *et al*., 2005). Oral and residual application of methoxyfenozide reduced the fecundity and fertility of *S. littoralis* (Pineda *et al.*, 2007).

In *Cydia pomonella*, application of methoxyfenozide just prior to egg deposition resulted in reduced fecundity of female moths (Bylemans *et al*., 2003) and confirmed the larvicidal and ovicidal properties of the compound (Charmillot, 2001). Methoxyfenozide was found to be the most effective compound against mosquitoes, *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles gambiae* and the three ecdysteroid agonists RH 5849, tebufenozide and methoxyfenozide were extensively used for the control of mosquito larvae, preventing their ecdysis (Beckage *et al*., 2004). They were also effective for the control of the pest of apple, *Lacanobia subjuncta* (Doerr *et al*., 2004). In *Ephestia kuehniella*, among the three tested compounds RH 5849, tebufenozide and halofenozide, the tebufenozide appeared to be the most potent and all of

them affected the growth of ovaries (Hami *et al*., 2005). Methoxyfenozide was five to ten fold more potent than tebufenozide (Ishaaya *et al*., 1995) and was more active against budworm, bollworm and diamond black moth (Dhadialla *et al*., 1998). It exhibited greater activity than tebufenozide in *C. pomonella* eggs in dose response bioassays (Borchert *et al*., 2004). These diacylhydrazines are considered highly selective with no harm to natural enemies (Dhadialla *et al*., 1998; Smagghe and Degheele, 2002b, 2004) and as such fit well to IPM programmes.

Halofenozide is similar to the other three ecdysteroid agonists described earlier, in initiating a premature lethal moult. It was reported to be more potent and selective against Coleoptera but mildly active on Lepidoptera (Dhadialla *et al*., 1998; Carton *et al*., 2000; Retnakaran *et al*., 2003; Nakagawa, 2005). Topical application of halofenozide on adult beetles of *Aubeonymus mariaefranciscae* and *Leptinotarsa decemlineata* resulted in reduced fecundity and less progeny survival. On the other hand, larval treatments resulted in premature moulting and ecdysis (Farinós *et al*., 1999). Control of japanese beetle *Popillia japonica* was found to be effective with halofenozide applications (Zenger and Gibb, 2001; Mannion *et al*., 2001). Application of the compound on late second instar caused rapid mortality in *Cyclocephalis borealis* (Grewal *et al*., 2001). It was also found to interfere with protein production and vitellogenesis in pupae of *Tenebrio molitor* (Aribi *et al*., 2001; Boukachabia *et al*., 2003). Reduction of larval and pupal period was observed in the mosquito *Culex pipiens* (Boudjelida *et al*., 2002). Topical application of halofenozide or oral feeding of the compound was found to interfere with ovarian development in the termite *Coptotermes formosanus* (Raina *et al*., 2003). Halofenozide reduced the number of oocytes in the German cockroach *Blatta germanica* (Maiza *et al*., 2004).

The nonsteroidal ecdysone agonists act as ecdysone mimics at the molecular level (Wing, 1988) and interact with ecdysteroid receptor-Ultra

spiracle protein complex (EcR-USP). Even though these agonists are not steroids, their action is similar to 20-HE in causing wing disc eversion (Silhacek *et al*., 1990; Smagghe *et al*., 1996) and other physiological processes such as adult development in diapausing pupae (Sielezniew and Cymborowski, 1997). The specific activity and insect selectivity of the nonsteroidal agonists, especially the high toxicity of tebufenozide for Lepidoptera and nonsusceptibility of Coleoptera is related to selective binding in the EcRs (Dhadialla *et al*., 1998; Smagghe et al., 1999). Methoxyfenozide binds with very high affinity to the EcRs in Lepidopteran insects where it functions as a potent agonist of 20-HE (Carlson *et al*., 2001). Experiments using cells or tissues cultured *in vitro* indicate that the bisacyl hydrazines have the same mode of action as that of 20-hydroxyecdysone (Retnakaran *et al*., 1995, Carlson *et al*., 2001; Smagghe *et al*.,2001, 2002b; Nakagawa *et al*., 2002; Mourad *et al.*, 2004; Hu *et al*., 2004; Fujitha and Nakagawa, 2007). As a result of this, the larva undergoes apolysis and head capsule slippage. But these synthetic analogues continue to bind strongly to the ecdysone receptors and remain in place unlike the 20-hydroxy ecdysone which would repress the genes necessary for cuticle elaboration and ecdysis. Developmental arrest brought about at this stage would lead to a precocious incomplete molt that is lethal (Retnakaran *et al*., 2003).

1.2.6 Anti ecdysteroid agents

Compounds with anti ecdysteroidal properties were found to be inducing abnormal growth and development in insects (Svoboda *et al*., 1972). These substances also hold promise as potential as insect control agents. Several azasterols were found to be acting as inhibitors of sterol reductase enzymes and interfering with ecdysteroid production. The fungicide triarimol was found to be effective on pupae of *Sarcophaga bullata* in blocking the development (Matolcsy *et al*., 1975). Many plant compounds are able to modulate insect steroid hormone biosynthesis. These include brassinosteroids, which are polyhydroxylated derivatives of common plant membrane sterols such as

campesterol. They bind competitively to ecdysteroid receptors and inhibit biological responses to the active form of molting hormone (Lehmann *et al.*, 1988). They possess anti ecdysteroidal properties and interfere with moulting and reproduction (Richter and Coolman, 1991; Spindler, 1992; Brosa *et al*., 1994). A brassinosteroid analogue was found to induce immediate mortality and retard larval development in *Aedes aegypti* (Davison *et al*., 2003). Brassinosteroids in different concentrations are found to be inducing high mortality when ingested late in the last instar and inhibit evagination of imaginal wing discs in the cotton leaf worm *Spodoptera littoralis* (Smagghe *et al*., 2002a).

The most efficient natural substance that shows inhibition of molting in insects is azadirachtin, a tetranortriterpenoid extracted from the seeds of neem *Azadirachta indica* (Mordue and Blackwell, 1993). It is highly effective in modifying insect haemolymph ecdysteroid titres due to a blockage of release of the prothoracicotropic hormone from the brain-corpus cardiacum complex. Azadirachtin is a strong antifeedant (Butterworth and Morgan, 1968; Koul *et al*., 2004; Deota and Upadhyay, 2005). It also disrupts growth (Redfern *et al*., 1981, Schluter *et al*., 1985, Koul *et al*., 2004) prevents adult emergence (Saber *et al*., 2004) reduce fecundity (Su and Mulla, 1999) arrest spermatogenic meiosis (Mordue *et al*., 1997) and inhibit DNA synthesis and protein content in the imaginal wing discs (Josephrajkumar and Subrahmanyam, 2002) of various insect species. It was found to be potent in causing 100% mortality in malaria vectors (Nathan *et al*., 2005). Azadirachtin induces morphogenetic effects in various insects, which is due to suppressed ecdysteroid titres (Rembold and Sieber, 1981; Smith and Mitchell, 1988; Jagannadh and Nair, 1992; 1993). Neem extract 'Neemarin' caused prevention of adult emergence and increased the larval and pupal mortality in mosquitoes (Vatandoost and Vaziri, 2004). Neem products reduced larval and pupal survival, adult emergence, pupal weight, adult wing length and fecundity on the subsequent generation of blow fly and house fly (Siriwattanarungsee *et al*., 2008). Azadirachtin and neem seed

oil significantly increased nymphal mortality and reduced survival to adulthood in *Aphis glycines* and *Harmonia axyridis* (Kraiss and Cullen, 2008).

1.3 Objectives of the present investigation

From the review it is evident that IGRs based on hormones appear to be effective tools for pest control though the problem of insects developing resistance to them is observed in a few cases. Recent advances in recombinant DNA technology and research in the field of neuroendocrinology of insects has brought about a number of promising substances that are 'reduced risk' alternatives for chemical pesticides. Pest control with environment safety is a fact that can be realized in the near future through introduction of these new compounds into agrochemical industry. The present investigation is an extension of the work being carried out with these compounds in our laboratory during the past few years (Sam Mathai and Nair, 1983,1984 a,b,c,d; Santha and Nair,1986; Sam Mathai,1987; Santha and Nair, 1987,1988; Santha, 1988; Nair and Rajalakshmi,1989; Balamani and Nair, 1989 a, b; Pradeep and Nair,1989; Sam Mathai *et al*.,1989; Balamani, 1990; Sam Mathai and Nair,1990; Balamani and Nair, 1992 a,b; Jagannadh, 1992; Jagannadh and Nair 1992, 1993; Venugopalan *et al* ., 1994; Sakunthala and Nair, 1995; Benny and Nair, 1999; Safarulla *et al* ., 2003; Sindhu and Nair, 2004; Pradeep and Nair, 2005).

The biological activities of the compounds Methoxyfenozide, Halofenozide and Pyriproxyfen have demonstrated the tremendous potential of the compounds in pest management programmes. IGRs such as pyriproxyfen, tebufenozide and methoxyfenozide were proved to cause no acute toxicity on the beneficial insect *Bombus terrestris* (Mommaerts *et al*., 2006). The effects of halofenozide on lepidopteran insects are not thoroughly investigated. Hence, it was thought worthwhile to investigate the effects of pyriproxyfen, methoxyfenozide and halofenozide on embryogenesis, postembryonic

development, metamorphosis and some aspects of reproductive biology in *Spodoptera mauritia* Boisd (Lepidoptera : Noctuidae).

The understanding of the effects of hormone analogues on reproductive biology is of fundamental importance for their effective use as insect control agents. Further, the SDS-PAGE pattern of wing disc proteins and also the changes in their profile following treatments with hormone agonists PPN, methoxyfenozide and halofenozide were also studied. It is hoped that the results of the present study will help in a better understanding on the effects of these IGRs on the postembryonic development, metamorphosis and reproductive activities of *S. mauritia* and the potential of these hormone agonists as reduced risk alternatives in pest control programmes.

Chapter I deals with a detailed review of importance of IGRs in insect control, hormone based IGRs and their effects on various insect pests.

Chapter II provides a basic information on the pest status and a detailed account of the rearing, biology and maintenance of *S. mauritia* Boisd. (Lepidoptera: Noctuidae) under laboratory conditions.

Chapter III deals with the morphogenetic effects of treatments of the hormone agonists PPN, methoxyfenozide and halofenozide on eggs, larvae and pupae of *S. mauritia.*

Chapter IV deals with the morphogenetic effects of the hormone agonists on the reproductive structures of *S. mauritia.*

Chapter V examines the SDS-PAGE pattern of wing disc proteins and also the changes in their profile followed by treatments with hormone agonists PPN, methoxyfenozide and halofenozide.

2.1 Pest status

Spodoptera mauritia Boisd. (Lepidoptera: Noctuidae) popularly known as the rice swarming caterpillar is a major sporadic pest of paddy, *Oryza sativa* in our state. The larvae feed on the leaves voraciously and destroy the rice plants by defoliating them. Early stages of caterpillar are so small and inconspicuous that they escape notice. In the caterpillar stage they migrate from field to field in large swarms of thousands which gives them the name army worm. During off-season periods, they migrate to the alternate host *Ischaemum aristatum* and come back on the nursery stages of paddy. The early growing stages of paddy are most susceptible to the attack of caterpillar. The seedlings attacked by the pest appear as though grazed by cattle. The caterpillars feed from the margin of leaves and leave behind only the midribs. Heavy loss of the crop will be the result when the outbreak is severe. It is estimated that the loss in yield caused by larval infestation of *S. mauritia* range from 10 to 20%. The *Spodoptera* is an important insect species used in various areas of research such as endocrine studies, physiological and also biochemical studies.

2.2 Rearing and maintenance of the larvae

The adult moths of the insect were collected at night using fluorescent lights. They were kept in glass beakers covered with musline cloth and were fed with a dilute solution of honey. They were allowed to lay eggs on the cloth (Pl. I: Fig.1). Larvae hatched out after 3-4 days. The larvae were reared in glass chimneys and were fed with fresh leaves of young paddy plants or leaves of the grass *Ischaemum aristatum.* When the larvae grew in size, they were kept in large plastic troughs with enough space for free movement. Care was taken to avoid extreme light and moisture as it may lead to mass death of the larvae. During dry season the cloth covering of troughs were wetted occasionally. The total larval period was found to range from17 to 19 days and consisted of 6 larval instars. The fully grown sixth instar larvae pupated in the soil. The pupae

were kept in beakers for adult emergence. The female pupae took 7 days and the male pupae 8 days to moult into adults.

2.3 Biology of *S.mauritia*

The *S.mauritia* larvae under laboratory conditions underwent six larval instars and developed at a uniform rate.

(1) First instar larva

The hatched out first instar larvae were characterised by the presence of a large, black head shield and light green coloured body. Setigerous, small, wart-like dark pigmented tubercles were present all over the body in a cross-wise row. They were found to be hanging by means of silken threads to the grass supplied. The larvae had a characteristic leaping movement. They measured about 1mm in length and 0.5 mm in width and the larval period was found to be 2-3 days.

(2) Second instar larva

In the second instar larvae, three white longitudinal stripes were found on the dorsal surface of the body. They extend from the prothorax to the last abdominal segment. Body was green coloured and two pairs of white, longitudinal, lateral stripes were also present of which one was very prominent. On each segment setigerous, small, wart-like dark tubercles were present. As in the case of first instar larvae, second instar also descended by means of silken threads. Larvae measured about 2.5 mm in length and 0.5 mm in width. The larval period extended to 2-3 days.

(3)Third instar larva

The third instar larvae had three white longitudinal stripes on the dorsal side and two pairs on the lateral side of the body. A pair of characteristic reddish black supraspiracular stripes extending from anterior to posterior end of the body was also seen. The cervical and anal shields had three markings in continuation

with the longitudinal stripes. The newly moulted larvae measured 6 mm in length and 1 mm in width. The duration of third instar was 2-3 days.

(4) Fourth instar larva

In the fourth instar larva, the three dorsal longitudinal stripes became dull white and the body became grayish in colour. The two lateral reddish black stripes were present, one on each side of the body. The dorsal part was paler than the supraspiracular area overlaid with strands. Black intermittent dots appeared on each segment dorsolaterally which broadened towards the later stages of instar. The newly moulted larva measured about 11.5 mm in length and 2 mm in width. The larval period extended up to 2-3 days.

(5) Fifth instar larva

The fifth instar larvae were black in colour (Pl. I: Fig. 2). The larvae were characterized by double row of black, triangular markings present on the dorsolateral side bordered with narrow white stripes. The triangular markings became more prominent than the fourth instar larval stage. Three dorsal longitudinal stripes were also present. The paired supraspiracular stripes became transparent and pink in colour. The larvae fed voraciously and grew quickly. The integument became partially transparent as a result of which the internal structures such as testes were visible. Larva measured about 22 mm in length and 4.5 mm in width. The fifth instar larval period was 3±0 days.

(5) Sixth instar larva

The sixth instar larvae were grayish black in colour. The newly moulted larvae (Pl. I: Fig.3a) were characterised by the conspicuous triangular markings on the dorsal side. They measured about 30 mm in length and 6 mm in width. The larvae fed voraciously during the first three days after ecdysis and attained the maximum size and body weight. On the third day, they measured about 35

mm in length and 6 mm in width (Pl. I: Fig.3b). They stopped feeding, emptied their guts and entered the wandering stage by day 4.

The wandering larva measured about 25.5 mm in length and 5 mm in width (Pl. I: Fig.4a). This stage extended up to 24 h. They got transformed in to prepupae by day 5. The prepupal stage was characterized by a highly wrinkled larva which was very small in size (Pl. I: Fig.4b). It measured about 20 mm in length and 5 mm in width. Pre-pupa underwent the larval-pupal apolysis after 24 h.

(5) Pupal instar

The pupae were of the obtect type, black in colour and measured 16 mm in length and 5 mm in width (Pl. I: Fig.5). Adults emerged out from female pupae after 7 days and from male pupae after 8 days. The adult moths survived for 7-8 days.

(6) Adults

The adults of *S. mauritia* were found to be medium sized moths which exhibited sexual dimorphism. They measured about 15 mm in length and possessed a wing span of 30-35 mm. The males were dark greyish in colour with white markings on the forewings and also large tufts of hair on the forelegs (Pl. I: Fig.6b). The females lacked the white markings on forewings and the tufts of hairs (Pl. I: Fig.6a). Both the males and females possessed a conspicuous black spot one each forewing.

The moths mated 24 h after emergence which took place during night. Egg laying was found to commence from 24 h after mating. Eggs were laid in masses of 100-500 each and the maximum number ranged between 700-1000. The egg mass was covered with buff coloured silken hairs. The larvae were hatched out in 2-3 days. On the whole, the egg period extended to 3 days, larval period 19-23 days and pupal period 7-8 days.

2.4 Experimental animals

The larvae, pupae and adults used for various experiments were obtained from the laboratory stock culture reared and maintained as explained above. The age of larvae/ pupae/adults were abbreviated to day n where day 0 indicates the day of ecdysis to this stage. The newly ecdysed larvae/pupae/adults were considered as those belonging to day 0, 24 h old as day 1 old and so on. For the isolation of newly ecdysed larvae from the basic culture, the slipped head capsule and pale colour were taken as identifying markers. The newly ecdysed pupae could be easily isolated as they possess pale cream colour. Sexing was possible only in the late fifth instar stage when the testes become visible through the transparent cuticle of male larvae. Exuviae were counted on every day to determine the intermoult duration. Treated larvae were carefully observed for the percentage of mortality during each instar, the morphogenetic abnormalities and the feeding behaviour. Slight variations in the intermoult duration were of common occurrence in the laboratory stock culture. In order to avoid discrepancies, larvae from the same egg mass were taken for related experiments.

2.5. General experimental techniques

2.5.1 Chemicals

The JH agonist, PPN was obtained as a research sample from Valent Corp., USA. The compound was dissolved and diluted in acetone to obtain the required concentrations. The ecdysone agonists methoxyfenozide and halofenozide were obtained as gift from Dr. G. C. Unnithan, Arizona University, USA. Methoxyfenozide was dissolved in distilled water and halofenozide was dissolved in acetone and diluted to obtain different concentrations. For the various treatment procedures, measured quantities of these compounds were used utilizing a Hamilton microsyringe. The control larvae were treated with an

equal volume of acetone/distilled water in order to minimize the interference of the solvent in the test results.

2.5.2 Treatments

2.5.2.1 Treatment of eggs

Newly laid egg masses were treated with the three different doses (0.1 µg, 1 µg, 10 µg) of the hormone agonists, PPN, methoxyfenozide and halofenozide. Eggs kept as controls were treated with equal volumes of solvents i.e., acetone in the case of PPN/halofenozide treatments and distilled water in the case of methoxyfenozide treatments. The treated and control egg masses were kept in separate beakers. The eggs were observed daily to check the hatchability. The effects of the treatments of the compounds on embryogenesis, and postembryonic development were studied. The larvae hatched out of the treated egg masses and the egg masses kept as control were fed and maintained as that of the stock culture. They were inspected daily for mortality, abnormal moulting behaviours and changes in the duration of larval/pupal instars.

2.5.2.2 Treatments of first and second instar larvae

The first and second instar day 0 larvae were obtained from laboratory stock culture. They were treated by contact method with three different doses (0.01µg, 0.1 µg, 1 µg) of the compounds PPN/methoxyfenozide/halofenozide such as dissolved in acetone/distilled water by the contact method. In this method, the inner surface of the Petridish of 5 x 1.7 cm size was coated with the required concentration of the compound dissolved in the solvent. About 70-80 larvae were exposed to the compound for 30 minutes in each experiment. The larvae kept as controls were exposed to equal volumes of acetone/distilled water coated petridish. After treatments, the treated and control larvae were transferred to clean vessels and reared as in the case of laboratory culture. They were

inspected daily for mortality, abnormal moults, and changes in the duration of larval/pupal instars.

2.5.2.3 Treatments of third, fourth, fifth and sixth instar larvae

The third, fourth, fifth and sixth instar larvae of different age groups were isolated from laboratory stock culture. They were topically treated using a 10 µl Hamilton microsyringe with single doses of five different concentrations of PPN/methoxyfenozide/halofenozide (0.01 µg, 0.1 µg, 1 µg, 10 µg, 20 µg) dissolved in 5 µl acetone/distilled water. The larvae kept as controls received equal volumes of solvents alone. They were kept in separate beakers and were reared as in the case of stock colony.

2.5.2.4 Treatments of pupae

The newly ecdysed day 0 unpigmented and tanned pupae were subjected to topical treatments of five different concentrations of the compounds PPN / methoxyfenozide/halofenozide such as 0.01µg, 0.1 µg, 1 µg, 10 µg and 20 µg dissolved in 5 µl acetone/distilled water. The compound was applied topically on the abdominal region of newly ecdysed day 0 pupae using a 10 µl Hamilton microsyringe. The control pupae received equal volumes of solvent only. Experimental and control pupae were kept in separate beakers covered with muslin cloth. The pupae were checked daily for mortality and morphological abnormalities.

2.5.3 Surgical techniques

The treated and control pupae were kept in a wax bottomed petridish. They were pinned with the dorsal side facing up in a wax-lined petridish. The dissections were carried out in insect ringer solution (NaCl - 0.65 gm, KCl - 0.025 gm, CaCl - 0.03 gm, $NaHCO_3$ - 0.025 gm in 100 ml distilled water), using sterilized instruments. A longitudinal cut was made on the dorsal side of the

larva/pupa on the abdominal region using fine forceps and needle. The right and left cuticular flaps were then pinned laterally on to the wax tray. The alimentary canal was detached from both ends and removed to expose the reproductive system. The testes were seen as paired lateral organs in larvae and a single median organ in pupae. Ovaries were seen as long strands when the alimentary canal was removed. Testes/ovaries were dissected out from both treated and control larvae and examined for the morphogenetic abnormalities.

2.5.4 Histological techniques

After dissections the testes/ovaries were washed thoroughly in Insect Ringer solution and fixed overnight in Bouin's fluid. The tissues were washed for a long time in distilled water, dehydrated in grades of ethanol, cleared in methyl benzoate, infiltrated and embedded in paraffin wax. Five micron thick sections were cut in a Reichert Precision Rotary Microtome. Sections were stained using Harris haematoxylin - eosin, observed and photographed under Carl Zeiss Research microscope provided with Axovision software for image analysis.

2.5.5 Statistical analysis of data

The findings were represented as histograms. Standard parametric procedures, ANOVA and paired *t*-test were employed to compare group means. The values are represented as the mean ± standard error (SE).

3.1 Introduction

Insect metamorphosis is characterized by a sequence of morphological changes which are regulated and controlled by haemolymph titres of several hormones. The major hormones: the neurohormones secreted by neurosecretory cells of brain and ganglia, juvenile hormone (JH) secreted by the corpora allata and the ecdysteroids secreted by the prothoracic glands, all have been implicated in promoting moulting and metamorphosis of insects. The interplay of ecdysteroids and JHs is responsible for the management of progression from one developmental stage to the next, in which the ecdysteroids regulate the timing and the JH regulates the quality of moult (Sehnal, 1985; Riddiford, 1970 b, 1971, 1985). A high titre of JH leads to larval-larval moult, low titre of JH leads to larval-pupal moult and absence of JH leads to pupal- adult moult. Maintenance of a critical titre of major hormones, (*i.e*., JH and ecdysteroids) is very essential for insects to complete their normal development and metamorphosis. Any disruption in this leads to abnormalities in moulting and metamorphosis of insects.

Insect growth regulators (IGRs) belong to a class of compounds naturally occurring or synthetically prepared which interfere with normal metamorphosis and reproduction of insects. Among the different IGRs those which directly or indirectly influence the endogenous titre of hormones have immense potential in insect control strategies. JHAs, anti JH agents and the moult accelerating compounds such as ecdysone analogues/agonists belong to this category of IGRs. These compounds have great potential in IPM programmes as insect control agents (Nair, 1993). JHAs mimic the physiological effects of the JH and disrupt the normal developmental processes of insects if administered in the larval or nymphal stage (Beckage, 1999). Among the JHAs, Pyriproxyfen (PPN) is found to be effective against a variety of arthropods. It is a pyridine based pesticide which was introduced to the United States in 1996 to protect cotton

crops. PPN is highly effective in controlling lepidopteran insects (Smagghe and Degheele, 1994). Topical application of PPN on pupae of *Spodoptera litura* reduced the number of eggs oviposited (Hatakoshi, 1992). Pyriproxyfen disrupt molting; induce defective embryogenesis and morphological deformities in the desert locust, *Schistocerca gregaria* (Vennard *et al*., 1998).

The ecdysone analogues/agonists act primarily by inducing a premature lethal moult (Wing *et al*., 1988; Aller and Ramsay, 1988; Silhacek *et al*., 1990; Smagghe and Degheele, 1993, 1994; Sakunthala and Nair, 1995). The fact that exogenous administration of ecdysteroids initiates moulting and metamorphosis in insects and the changes are due to the fluctuations in the haemolymph titres of moulting hormones was not supported by experimental evidences until 1960s. The synthesis of nonsteroidal ecdysone agonists led to a breakthrough in the area of pest control. Nonsteroidal ecdysone agonists such as RH 5849, tebufenozide, methoxyfenozide and halofenozide have been extensively used for controlling several species of insects. These compounds act as ecdysone mimics at the molecular level and interact with ecdysteroid receptor complex (Wing, 1988). Methoxyfenozide is an ideal pesticide to be included in IPM management programmes because it is non-toxic to mammals and non-target organisms such as birds, fish, honeybees and other pollinators (Palumbo, 2001). Halofenozide is similar in action to the other three ecdysteroid agonists, in initiating a premature lethal moult. It is reported to be more effective on Coleoptera (Dhadialla *et al*., 1998; Carton *et al*., 2000; Retnakaran *et al*., 2003; Nakagawa, 2005). Topical application of halofenozide on adult beetles of *Aubeonymus mariaefranciscae* and *Leptinotarsa decemlineata* resulted in reduced fecundity and less progeny survival (Farinós *et al*., 1999). These agonists are not steroids but their action is similar to 20-hydroxyecdysone (20-HE) in causing wing disc eversion (Silhacek *et al*., 1990; Smagghe *et al*., 1996) and other physiological processes such as adult development in diapausing pupae (Sielezniew and Cymborowski, 1997).

The present study deals with the effects of hormone agonists PPN, methoxyfenozide and halofenozide on the development and metamorphosis of *S. mauritia*. Different developmental stages of *S. mauritia* were treated with these compounds and the effects of these treatments on the embryogenesis, postembryonic development and the metamorphosis were studied.

3.2 Materials and methods

3.2.1 Experimental animals: (See Chapter 2).

3.2.2 Chemicals: (See Chapter 2).

3.2.3 Treatment of eggs/larvae /pupae (See chapter 2)

3.3 Results

3.3.1 Effects of hormone agonists on egg hatchability and postembryonic development.

PPN treatments of eggs with the doses 0.1 µg, 1 µg and 10µg of hormone agonists resulted in varied effects. When the eggs were treated with the juvenile hormone agonist PPN no marked effect on hatchability was observed with the lowest dose. Treatments with 0.1 µg of PPN led 96.84% of the eggs to hatch out into larvae (Table 1, Fig. 7). They were normal in every respect to the larvae hatched out from control eggs but 41.57% of mortality was observed in the first instar stage itself. Treatments with 1 µg of PPN led only 31.6% of eggs to hatch out and 50.9% of these larvae died in the first instar stage. With the highest dose of 10 µg only 8.15% of hatchability was observed and the larvae were normal in appearance. Of these, 55.17% of larvae died in the first instar stage and a major percentage of the remaining larvae died in the succeeding instars.

The surviving larvae after PPN treatments fed more actively than the control larvae and grew bigger in size. Abnormally large sized larvae were seen

in all the instars which developed into large prepupae. Pupae formed from eggs kept as control that received an equivalent volume of acetone were normal in every respect (Pl. II: Fig.8). Treatments with 0.1 μg and 1 μg of PPN led to formation of 15% and 22.86% of larval-pupal intermediates respectively. The dose 10 μg led to formation of 34% larval-pupal intermediates. The head region, mouth parts and thorax of these larval-pupal intermediates were larval in appearance. The cuticle of the thoracic tergum showed the three yellowish longitudinal stripes, a pair of supraspiracular stripes and the black intermittent dots characteristic of normal sixth instar larvae (Pl. II: Fig. 9). All the three pairs of larval thoracic legs were retained but the abdominal region was entirely pupal in appearance. The pupal integument was highly sclerotised and the abdominal prolegs were completely disappeared. These intermediates survived for 3-4 days and died without undergoing further changes.

Pupation rate was very low and the pupae were deformed. Only 21.47% and 6.9% of pupation was observed after treatments with 0.1 μg and 1 μg of PPN respectively. The deformities were more profound in pupae formed from 1 μg treatment (Pl. II: Figs. 10, 11) than those of 0.1 μg treatment (Pl. II: Figs. 12, 13). Abnormal constrictions were seen between head and thorax and the pupal proboscis was malformed at the thoracic region ventrally (Pl. II: Fig. 10). They were very large in size when compared to the pupae formed from eggs kept as controls (Pl. II: Figs. 12, 13). The ventral regions of some pupae were found to be very soft with the larval cuticle retained there (Pl. II: Fig. 13). Some of the pupae had the larval head capsule retained at the anterior region and the larval abdominal prolegs were found to be attached to the posterior tip of the pupal case (Pl. II: Fig. 14). Their pupal cases were not attached ventrally at the thoracic region thus exposing the inner structures. Here the larval legs were found to be attached to the pupal case (Pl. II: Fig. 15). These large pupae survived for 10-13 days and died.

Methoxyfenozide treatments of eggs with the above mentioned doses resulted in no marked effect on hatchability (Table 1, Fig. 7). The larvae, which hatched out from the treated eggs were similar to the control larvae in every respect. But majority of them died in the first instar itself and the rest of them died in the second instar. None of them developed normally. Lower doses such as 0.1 µg and 1 µg induced mortality of 79.86% and 87.11% of first instar larvae. Higher doses resulted in higher mortality in the first instar larval stage and 90.78% of the larvae died in the first instar larval stage itself with the dose 10 µg. The remaining larvae died in the second instar stage.

Halofenozide treatments of eggs with all the doses mentioned resulted in no marked effect on hatchability (Table 1, Fig.7). All the eggs hatched out into normal looking larvae after 0.1 µg and 1 µg treatments where as 10 µg treatments resulted in 96.66 % hatchability and 10 % mortality at the first instar stage. The larvae developed normally and 78.53 % pupation was observed after 0.1µg treatments. Pupation lessened in a dose dependent manner and 1µg and 10µg treatments led to 55.38% and 30% pupation respectively. The pupae were normal in appearance but none of them emerged as adults.

3.3.2 Effects of hormone agonists on the larval-larval development and metamorphosis of *S. mauritia*

3.3.2.1 Effects of hormone agonists on first and second instar larvae

Treatments of first instar larvae with 0.01 µg of PPN led to 19.07% larval mortality (Table 2, Fig.16). Treatments with 0.1 and 1 µg of PPN resulted in 51.68% and 67.96% mortality respectively. The second instar larvae showed 17.78 %, 48.14% and 62.09% mortality within 24 h of treatments with doses 0.01 µg, 0.1 µg and 1 µg of PPN respectively. A considerable number of the surviving larvae died during the succeeding instars. The remaining larvae fed more actively and became larger in size when compared to controls.

Other important features noted after treatments were the extension of larval period in the succeeding instars and a significant delay of moulting. The first instar larvae treated with 0.01 µg PPN took 2.56 ± 0.04 days to complete third instar while the control larvae took 2.0 ± 0 days for development. These larvae took 2.38 ± 0.4 days in fourth instar, 3.32 ± 0.2 days in the fifth instar and 6.29 ± 0.3 days in the sixth instar. The control larvae took 2 ± 0 days in fourth instar, 3 ± 0 days in fifth instar and 6 ± 0 days in sixth instar. Treatments with 0.1 µg and 1 µg resulted in similar effects and dose dependent variations were not observed in the extension of larval period. The sixth instar larval stadium of treated larvae showed a slight extension of larval period (Table 2, Fig. 17). Second instar larvae treated with 0.01 µg PPN took 2.59 ± 0.25 days in the third instar, 2.46 ± 0.16 days in the fourth instar, 3.33 ± 0.2 days in the fifth instar and 6.36 ± 0.4 days in the sixth instar larval stage. All these values were found to be significantly different ($P < 0.001$, *t* - test) when compared to controls. The effects were similar in treatments with the doses 0.1 µg and 1 µg and no marked difference was noticed depending on the concentration of PPN.

Pupation was observed in 8.22% of first instar larvae and 10.6% of second instar larvae when treated with 1 µg of PPN. The prepupae which failed to pupate formed large larval-pupal intermediates. They were morphologically similar to those formed from the prepupae of treated eggs. They survived for 10-13 days and then died without emerging into adults. Methoxyfenozide treatments resulted in premature lethal moult in treated larvae. Treatments with 0.01µg resulted in mortality of 30% of first instar larvae and 25.38% of second instar larvae within 24 h (Table 3, Fig. 18). Remaining larvae died in the succeeding instars and none of them survived up to pupation. The treatments with 0.1 µg led to mortality of 50% of first instar larvae and 42.85% of second instar larvae. Treatments with 1µg resulted in mortality of 66.67% of first instar larvae and 53.68% of second instar larvae within 24 h of treatment. All the larvae died with a fluid exuding out which turned black on exposure to air.

Halofenozide treatments with the lowest dose 0.01µg resulted in premature lethal moult of 10% of first instar larvae (Table 3, Fig. 18). A considerable number of the treated larvae died in the succeeding instars. The remaining larvae developed normally and 50% of them pupated after 0.01 µg treatments. The larvae which failed to pupate formed larval-pupal intermediates and 30% - 42.14% of larval-pupal intermediates were formed in treatments of first instar. Treatments with the doses such as 0.1 µg, 1 µg elicited the symptoms of premature lethal moult and mortality in 20% and 35.7% of first instar larvae and 11.76% and 26.67% of second instar larvae respectively. The surviving larvae of all these treatments developed normally. Pupation was very low in 0.1µg and 1µg treatments and a considerable number of larval-pupal intermediates were formed in all these treatments. Only 14% of first instar larvae pupated in 1µg treatments and 42.14 % of the larvae formed larval-pupal intermediates where as 20% of second instar larvae pupated and 35% formed larval-pupal intermediates with the same dose. Treatments of second instar larvae with 0.01 µg and 0.1µg of halofenozide led to formation of 20% and 25.88% of larval-pupal intermediates and 55.33% and 35.29% pupation respectively.

3.3.2.2 Effects of treatments of hormone agonists on third instar larvae

PPN treatments with the lower doses such as 0.01 µg and 0.1 µg resulted in 8% and 10% of larvae showing mortality within 24 h where as the higher concentrations such as 10 µg and 20 µg resulted in 30% and 36% mortality (Table 4, Fig. 19). A major percent of the remaining larvae died in the succeeding instars. The surviving larvae fed more actively and became larger in size when compared to larvae kept as controls. They formed large prepupae which either moulted into large sized pupae or larval-pupal intermediates. A dose dependent increase was observed in the formation of larval-pupal intermediates (Table 4, Fig. 19). The pupae did not succeed in adult emergence

and died after 10-13 days of survival. When the pupal cuticle was dissected out, defective adults/adultoids with various degrees of morphological abnormalities were found inside. They had developed adult features but the wings appeared to be small and crumpled. A significant ($P < 0.001$, *t* - test) extension of larval period was observed in the treated stadium as well as in succeeding instars as observed earlier (Table 5, Fig. 20). The larvae treated with 0.01 µg of PPN had a larval period of 2.69 ± 0.13 days in the third instar, 2.59 ± 0.09 days in fourth instar, 3.62 ± 0.11 days in fifth instar and 6.43 ± 0.3 days in sixth instar. The effects were similar with the other treated dosages and dose dependent variations were not noticed in the extension of larval period (Table 5, Fig. 20).

Metoxyfenozide treatments of third instar larvae resulted in premature lethal moult of a considerable percentage of the treated larvae (Table 4, Fig. 19). Treatments of the day 0 larvae with the doses 0.01 µg, 0.1 µg and 1 µg resulted in premature lethal moult and dose dependent mortality. Higher doses of 10µg and 20µg elicited the moulting response in 82% and 86% of the larvae respectively. The larvae showing moulting symptoms had pharate head capsule as a white collar like structure (Pl. III: Fig. 21). They failed to slip off the head capsule (Pl. III: Fig. 22). The larvae failed to move successfully and exhibited wriggling movements (Pl. III: Fig. 23). They stopped feeding and became shrunken in appearance and died with the old head capsule and ruptured cuticle remaining attached to the body (Pl. III, Fig. 24).

Halofenozide treatments of the larvae with the lowest dose 0.01 µg resulted in no premature lethal moult (Table 4, Fig. 19). The larvae underwent normal development and 76% of them pupated where as 24% formed larval-pupal intermediates. Premature lethal molt and mortality were observed in 12% and 32% of larvae after treatments with 0.1 µg and 1µg respectively. The remaining larvae underwent normal development and 52% of them pupated and 36% formed larval-pupal intermediates after 0.1 µg treatments. Treatments with

1µg resulted in pupation of 22% of larvae where as 40% of them failed to pupate and formed larval-pupal intermediates. Treatments with 10 µg and 20µg elicited moulting response in 50% and 56% of the larvae which died within two days. The surviving larvae developed normally but failed to pupate and formed larval-pupal intermediates. They survived only upto day 5 and then died. The control larvae developed normally.

3.3.2.3 Effects of treatments of hormone agonists on fourth instar larvae

PPN treatments of fourth instar day 0 larvae with 0.01 µg of PPN led to 5% mortality within 24 h (Table 6, Fig. 29) where as day 1 larvae did not show mortality with this dose (Table 10, Fig. 33). The dose of 10 µg induced mortality in 20% and 15% of day 0 and day 1 larvae respectively. The highest dose of 20 µg induced mortality in 36.67% and 20% in day 0 and day 1 larvae respectively. Subsequently considerable number of the remaining larvae died. Surviving larvae fed actively and became large sized in all the succeeding instars as seen in earlier treatments. Pupation decreased with the increase in concentration where as formation of larval-pupal intermediates increased correspondingly. Treatments of day 0 and day 1 larvae with 0.01 µg of PPN resulted in formation of 11.67% and 20% larval-pupal intermediates and 38.33% and 31.67% pupation respectively. Larvae treated with higher doses of 10µg and 20µg did not pupate and moulted into larval-pupal intermediates. Treatments of day 1 larvae with doses of 10 µg and 20 µg resulted in formation of 41.67% and 63.33% larval-pupal intermediates and very low percent of pupation (Table 10, Fig. 33).

PPN treatments induced a significant ($P < 0.001$, *t* - test) extension of larval period in the treated stadium as well as in the succeeding instars (Table 7, Fig. 30). The fourth instar day 0 larvae treated with 0.01 µg of PPN took 2.87 ± 0.12 days in the fourth instar, 3.79 ± 0.24 days in fifth instar and 6.92 ± 0.15 days in sixth instar. The treatments of day 1 larvae with 0.01 µg PPN led to an extension of larval period of 2.76 ± 0.15 days in the fourth instar, 3.92

± 0.16 days in the fifth instar and 6.78 ± 0.14 days in the sixth instar. The effects were similar in treatments with the other doses mentioned above.

An increase in their length was observed in the treated stadia as well as in the succeeding instars following PPN treatments (Table 8, Fig. 31). Larvae treated with dose 0.01 µg showed a length of 16.8 ± 2.5 mm in fourth instar stage (control larvae had a length of 11.54 ± 0.05 mm), 29.23 ± 2.4 mm in the fifth instar stage (control larvae measured 22.06 ± 0.25 mm), 40.7 ± 1.5 mm during feeding stage of sixth instar (control larvae measured 30.02 ± 0.24 mm), 35.9 ± 2.6 mm in the wandering stage (control larvae measured 25.5 ± 0.15 mm), 27.6 ± 1.2 mm at the prepupal stage (control prepupae 20.04 ± 0.12 mm), 24.3 ± 0.98 mm at the pupal stage while the control pupae were 16.1 ± 0.13 mm long. All these values were found to be significantly different ($P < 0.001$, *t* - test) when compared to controls. The treatments with all the other doses gave similar results and dose dependent variations were not observed (Table 8, Fig. 31).

PPN treated larvae also showed a significant ($P < 0.001$, *t* - test) increase in width corresponding to the observed increase in length (Table 9, Fig. 32). The fourth instar day 0 larvae treated with 0.01 µg of PPN had a width of 3.65 ± 0.17 mm, the fifth instar larvae had 5.72 ± 0.3 mm, sixth instar feeding stage had 7.84 ± 0.13 mm, wandering stage had 6.62 ± 0.2 mm, the prepupae had 6.4 ± 0.14 mm and pupae had 6.29 ± 0.16 mm width. The control larvae had 2.08 ± 0.16 mm width in fourth instar, 4.5 ± 0.14 mm in fifth instar, 6.04 ± 0.12 mm in sixth instar feeding stage, 5.05 ± 0.13 mm in the wandering stage and 5.03 ± 0.15 mm width in the prepupal stage. The control pupae had 5.01 ± 0.12 mm width.

Methoxyfenozide treatments of fourth instar day 0 and day 1 larvae led to premature lethal moult (Table 6, Fig. 29; Table 10, Fig. 33) and cause many morphological abnormalities. The pharate head capsule of treated larvae had a white collar like structure (Pl. III: Figs. 25, 26). The larvae did not succeed in

completing the moult and remnants of old cuticle were found attached to the body (Pl. III: Fig. 27). The old cuticle was found to be tightly wrapped around the body at certain places which made it uneasy for the larvae to move and feed (Pl. III: Fig. 28). All these larvae died without further development. The dosages 0.1µg and 1 µg resulted in 63.33% and 86.67% of the day 0 larvae exhibiting moulting symptoms. Treatments with higher doses 10 µg and 20 µg, induced premature lethal moult within 24 h. Moulting fluid was found exuding out which turned black on exposure to air. Cessation of feeding was observed and the treated larvae failed to move normally. They exhibited a sort of wriggling movements and died within 24 h. None of them survived up to pupation.

Halofenozide treatments resulted in similar effects but the lower doses such as 0.01 µg and 0.1 µg were found to be ineffective in inducing lethal moult and mortality. Treatments with 1µg resulted in premature lethal moult of 23.33% of day 0 larvae (Table 6, Fig. 29) and 20% of day 1 larvae (Table 10, Fig. 33). The higher doses such as 10 µg and 20 µg induced premature lethal moult in 46.67% and 60% of the day 0 larvae and 33.83% and 50% of day 1 larvae. The surviving day 0 and day1 larvae underwent normal development and metamorphosis. Treatments of day 0 larvae with 0.01µg and 0.1µg led to 73.33% and 60% pupation whereas only 26.67% pupated after 1µg treatments. Treatments of day 1 larvae with 0.01µg and 0.1µg resulted in 60% and 48.33% of pupation respectively. No pupation was observed after treatments with doses 10 µg and 20 µg in both day 0 and day 1 larvae. The doses ranging from 0.01µg to 20 µg led to formation of 18.33% - 38.33% larval-pupal intermediates after treatments of day 0 larvae and 23.33% - 56.67% larval-pupal intermediates after treatments of day 1 larvae. Both the pupae and the larval-pupal intermediates were large sized and defective in morphology as described in earlier treatments. The larval-pupal intermediates survived up to day 5 and died. The pupae survived for 10 - 13 days and died with out emerging as adults.

3.3.2.4 Effects of treatments of hormone agonists on fifth instar larvae.

PPN treatments of day 0 larvae with lower doses 0.01 µg and 0.1 µg resulted in 6.67% and 8.33% mortality where as methoxyfenozide treatments with the same doses resulted in 56.67% and 78.33% mortality (Table 11, Fig. 34). Halofenozide treatments of day 0 with 0.01 µg resulted in no mortality in the treated larvae whereas 0.1 µg treatments induced 35% mortality. PPN treatments with the higher doses 10 µg and 20 µg resulted in 20% and 30% mortality. Treatments with these doses of methoxyfenozide led to 100% mortality in the treated larvae. Halofenozide treatments with 10 µg and 20 µg resulted in 51.67 % and 55 % mortality in the treated larvae. All these larvae exhibited moulting symptoms but failed to complete the process. Moulting fluid exuded out which turned black on exposure to air. The larvae died with the old head capsule and remnants of old cuticle retained on the body.

The fifth day 0 larvae which survived PPN treatments fed actively and grew bigger in size. The sixth instar larvae formed from the treated fifth instar also exhibited mortality and delayed development. Gut purge activity was delayed by 3 - 4 h. The large sized wandering larvae got transformed into large sized prepupae. These prepupae moulted into large sized pupae and large larval-pupal intermediates (Table 11, Fig. 34). A slight increase in pupation rate was observed in the treated larvae depending upon the progress in the stadia at the time of receiving treatment. But treatments with high doses reduced the rate of pupation. Pupal mortality was observed to be very high after PPN treatments of fifth instar day 0 larvae and only 2-3 pupae succeeded in adult emergence in each set. Some of the emerged adults exhibited very small wings (Pl. IV: Fig. 35). The adults that emerged from treated fifth instar day 1 larvae also exhibited morphological abnormalities. Some of them showed twisted wings (Pl. IV: Fig. 36). In certain others wings were found to be unstretched (Pl. IV: Fig. 37). These moths exhibited scarce hair on their abdomen. It was also

observed that some of the pupae formed from treated larvae failed to perform the pre-eclosion movements while emerging. They retained the pupal case at the posterior part of the body and failed to emerge successfully. The anterior part of the body and wings were exposed but the abdomen was not visible outside (Pl. IV: Fig. 38).

An extension of larval period was observed after PPN treatments of day 0, day 1 and day 2 larvae (Table 12, Fig. 39). The day 0 larvae treated with 0.01 µg PPN took 3.88 ± 0.45 days to complete the fifth instar and 6.54 ± 0.72 days in the sixth instar. The treated day 1 larvae took 3.46 ± 0.14 days in fifth instar and 6.53 ± 0.26 in sixth instar. The larvae treated on day 2 took 3.38 ± 0.35 days in fifth instar and 6. 64 ± 0.3 days in sixth instar. All these values were significantly different ($P < 0.001$, *t* - test) when compared to the controls. Similar results were obtained after treatments with the other doses.

A significant ($P < 0.001$, *t* - test) increase in length of the larvae were observed in PPN treatments irrespective of the doses applied (Table 13, Fig. 40). The fifth instar day 0 larvae treated with 0.01µg showed an increase in length of 29.6 ± 1.32 mm on day 2 (control larvae measured 22.08 ± 0.2 mm), sixth instar feeding larvae had 42.8 ± 1.2 mm (control larvae measured 30.2 ± 0.5 mm), wandering stage had 37.48 ± 1.3 mm (control larvae measured 25.52 ± 0.2 mm), prepupae measured 32.5 ± 1.14 mm length (control prepupae measured 20.4 ± 0.35 mm), pupae measured 27.58 ± 1.2 mm (control pupae measured 16.3 ± 0.12 mm). Results were similar on treatments with other doses of the compound.

There was an increase in width of the PPN treated larvae corresponding to the increase in length (Table 14, Fig. 41). The fifth instar day 0 larvae treated with 0.01 µg of PPN had a width of 4.67 ± 0.19 mm (control larvae had a width of 4.5 ± 0.12 mm), sixth instar day 3 measured 6.46 ± 0.13 mm (control larvae

measured had 6.08 ± 0.2 mm), wandering stage measured 5.64 ± 0.13 mm (control larvae measured 5.09 ± 0.1 mm), the prepupae had 5.46 ± 0.1mm width (control prepupae measured 5.06 ± 0.2 mm) and pupae had 5.35 ± 0.2 mm width (control pupae measured 5.02 ± 0.13 mm). Similar results were obtained with the other doses. All these values were significantly different ($P < 0.001$, *t* - test) when compared to controls.

PPN treatments of day 1 larvae with the doses 0.01 µg, 0.1 µg, 1 µg, 10 µg and 20 µg induced very less mortality when compared to ecdysone agonists treatments. Methoxyfenozide induced premature lethal molting in 62% and 85% of the larvae with 0.01 µg and 0.1 µg respectively (Table 15, Fig. 42). Halofenozide elicited no moulting response. Treatments with the doses 1µg and 10µg of methoxyfenozide resulted in 90% and 95% of larvae showing lethal moult respectively. Halofenozide elicited moulting response in 30% and 48% of larvae with these doses. The dose 20µg of methoxyfenozide led to lethal moult of 97.5% of larvae whereas halofenozide led to lethal moult of 62% of larvae.

The surviving larvae of PPN treatments fed voraciously and grew bigger in size. Treatments with the dose 0.01 µg resulted in 40% pupation. Increase in dose decreased pupation and induced formation of larval-pupal intermediates. Treatments with 20 µg PPN led to formation of 52% larval-pupal intermediates and only 18% pupation. Methoxyfenozide treated day 1 larvae did not survive beyond 3 days. Treatments with 0.01µg halofenozide resulted in formation of 24% of larval-pupal intermediates and 26% of pupation. PPN induced no mortality in fifth instar day 2 larvae when treated with 0.01 µg and 0.1 µg of the compound. Methoxyfenozide treatments with these doses induced premature lethal moult in 90% and 96.67% of the treated larvae respectively (Table 16, Fig. 43). The doses 1 µg, 10 µg and 20 µg of methoxyfenozide induced lethal moult in all the treated larvae. PPN treatments with these doses led to mortality in only 3.33%, 5% and 10% of the larvae respectively. Halofenozide did not induce

moulting with the treated doses 0.01 µg and 0.1 µg. The doses 10 µg and 20 µg induced moulting and mortality in 31.67% and 41.67% of the larvae respectively. A major number of the larvae did not survive upto pupation in all these treatments.

Treatments with 0.01 µg and 0.1 µg of halofenozide led to 30% and 18.33% pupation. Only 8.33% of the larvae pupated after treatments with the dose 10 µg whereas no pupation was observed after treatments with the highest dose of 20 µg. PPN treatments with the doses 0.01 µg to 20 µg resulted in pupation of only 26.67 to 11.67% of the larvae respectively in a decreasing order with the increase in dose. The larvae which failed to pupate formed larval-pupal intermediates. The pupae and larval-pupal intermediates formed from PPN treatments were large in size as seen in earlier treatments. The mortality was high in pupae from PPN treatments and only 1-2 emerged in each set. They showed abnormalities as seen in the adults emerged from pupae of treated fifth day 0 larvae. Methoxyfenozide treatments resulted in mortality of all the treated larvae whereas halofenozide showed adult survival and pupation though a lesser percentage (Table 16, Fig.43). Larval-pupal intermediates were also formed in halofenozide treatments which were of normal size when compared to those formed from PPN treatments. Pupal mortality was high and only 1 - 4 pupae emerged into adults. The adults emerged were normal in appearance and resembled those emerged from larvae kept as controls.

3.3.2.5 Effects of treatments on sixth instar day 0, day1and day2 larvae

PPN treatments with the doses ranging from 0.01 µg - 20 µg elicited moulting response in 40 - 58.33 % of day 0 larvae; 45 - 55 % of day 1 larvae and 43.33 - 60 % of day 2 larvae (Table 17, Fig. 44). Though moulting was initiated they could not complete it successfully. The pharate head capsule was seen as a white collar like structure behind the old head capsule in them (Pl. V:

Figs. 45 - 50). The larvae which showed symptoms of moulting exhibited the phenomenon of rectal prolapse (Pl.V: Figs. 48, 51, 52) which is protruding out of thin walled rectum. It was fluid filled and swollen in appearance. The fluid was found to be discharged from it (Pl. V: Fig. 51). Larval movements resulted in the rupture of this area. The surviving larvae fed voraciously and grew bigger in size and formed large sized larval-pupal intermediates.

An extension of larval period was observed in the treated day 0, day 1 and day 2 larvae (Table 17, Fig. 53). Day 0 larvae treated with 0.01 µg of PPN took 6.76 ± 0.3 days to complete sixth instar where as 0.01 µg treated day 1 larvae and day 2 larvae took 6.72 ± 0.3 days and 6.62 ± 0.2 days. The larvae kept as controls took 6 ± 0 days to complete sixth instar. All these values are found to be significantly different ($P < 0.001$, *t* - test) when compared to the control larvae. Treatments with all the other concentrations mentioned earlier resulted in an extension of larval period. Dose / age dependent differences were not noticed in the extension of larval period in any of these treatments.

The treated day 0 larvae showed an increase in length when compared to the larvae kept as controls (Table 19, Fig. 54). Sixth instar day 3 larvae formed from day 0 larvae treated with 0.01 µg had a length of 49.11 ± 1.68 mm (the control larvae measured 30.09 ± 0.23 mm); wandering stage had a length of 39.06 ± 1.52 mm (control larvae measured 25.55 ± 0.29 mm); prepupae had a length of 33.7 ± 1.9 mm (prepupae formed from control larvae measured 20.08 ± 0.28 mm); pupae had 31.82 ± 1.8 mm (control pupae measured 16.2 ± 0.3 mm). All these values were found to be significantly different ($P < 0.001$, *t* - test) when compared to controls.

There was an increase in width in the treated larvae corresponding to the increase in length (Table 19, Fig. 55). The sixth instar day 3 larvae formed from 0.01 µg treated day 0 larvae had 8.12 ± 0.37 mm width (control larvae had

6.2 ± 0.2 mm width); wandering stage had a width of 7.13 ± 0.3 mm (width of control larvae was 5.08 ± 0.2 mm); the prepupae had 6.97 ± 0.43 mm width (width of control prepupae was 5.06 ± 0.15 mm) and pupae had a width of 6.88 ± 0.4 mm (width of control pupae was 5.01 ± 0.2 mm). All these values are found to be significantly different ($P < 0.001$, *t* - test) when compared to controls.

Treatments of sixth instar day 0, day 1 and day 2 larvae with various doses of ecdysone agonists resulted in lethal moult of the treated larvae (Table 20, Fig. 56). Methoxyfenozide was more effective when compared to halofenozide. Mortality decreased with the progress in the larval stadia and maximum mortality was observed after treatments on day 0, day 1 and day 2. Higher doses induced premature lethal moult and mortality of a larger percent of treated larvae. The larvae treated with the lowest dose of 0.01 µg of methoxyfenozide led to lethal moult in 75% of day 0 larvae, 65% of day 1 larvae and 60% of day 2 larvae. The dose 0.1 µg induced premature lethal moult in 86.67% of day 0 larvae and 85% of day 1 larvae respectively. Only 70% of the day 2 larvae showed lethal moult with this dose. Higher doses such as 10 µg elicited moulting response in 95% of day 0 larvae and 88.33% of day 1 larvae respectively. All the treated day 0 and day 1 larvae showed lethal moult with the dose of 20 µg. Halofenozide treatments with the higher doses such as 10 µg and 20 µg induced a higher percent of lethal moult and mortality in day 0 larvae. The effect was found to be decreased with the progress in the larval stadia.

3.3.2.6 Effects of treatments on sixth instar day 3, day 4 and day 5 larvae

PPN treatments of day 3, day 4 and day 5 larvae elicited moulting response and mortality though very less when compared to the response in treatments on earlier days (Table 18, Fig. 44). Treatments with the doses ranging from 0.01 µg - 20 µg led to lethal moult in 13.33% to 33.33 % of day 3

larvae, 15% to 35% of day 4 larvae and 11.67 % to 36.67 % of day 5 larvae. A higher percentage of larval-pupal intermediates were formed from these treated larvae. The percentage of their formation decreased with the increase in PPN concentration. Treatments with 0.01 µg PPN induced 65% of day 3 larvae, 68.33% of day 4 larvae and 70% of day 5 larvae to form larval - pupal intermediates. Treatments with 20 µg led to formation of 55 % of larval-pupal intermediates from treated day 3 larvae, 56.67% from day 4 larvae and 53.33% from day 5 larvae. They survived upto day 5 and then died. A few percent of the treated larvae which exhibited shrinking also died after surviving for 4-5 days.

PPN treatments with all the above mentioned doses on day 3 resulted in moulting of a few percent of treated larvae to form imperfect superlarvae. They resembled normal day 3 larvae but were very large in size and possessed sclerotised antennae and sclerotised thoracic appendages (Pl. VI: Figs. 57, 58). The mouth parts of the superlarvae were highly sclerotised and showed pupal characters. The antennae were long and sclerotised. The thoracic legs were not sclerotised and the body was larval in appearance. Larvae treated on day 4 *i.e.*, the wandering stage (Pl. VI: Fig. 59) and the wandering larvae formed from the treatments on day 0 (Pl. VI: Fig. 60) were similar in the sclerotisation of their mouth parts, antennae and thoracic legs though the second set of larvae differed in their large size. Some of the larval-pupal intermediates formed from the treatments with lower doses showed the prolapsed rectum attached to the posterior pupal case and the moulting mark retained at the anterior larval head (Pl. VI: Fig. 61). A large number of the remaining larvae of all these treatments exhibited a sort of shrinking thus becoming extremely small in size (Pl. VI: Figs. 62, 63, 64). They showed sclerotised antennae, mouth parts and thoracic legs. The shrinking was moderate after treatments with lower doses (Pl. VI: Fig. 62) where as maximum shrinking was observed after treatments with the highest dose of 20 µg (Pl. VI: Figs. 63, 64).

The late last instar larvae showed lethal moult on treatments of various doses of ecdysone agonists in which 31.67 % of the treated day 3 larvae, 23.33% of day 4 larvae and 21.67% of day 5 larvae showed signs of lethal moult with the lowest dose i.e., 0.01 µg of methoxyfenozide (Table 20, Fig.56). The doses 0.1 µg and 1 µg induced premature lethal moult in 46.67% and 50% of the day 3 larvae; 36.67% and 45% of day 4 larvae; 26.67% and 40% of day 5 larvae respectively. Higher doses such as 10 µg and 20 µg elicited molting in 55% and 60% of day 3 larvae; 50% and 55% of day 4 larvae; 46.67% and 50 % of day 5 larvae respectively. These larvae showed rectal prolapse. Moulting fluid was seen to exude out which turned black on exposure to air.

The sixth instar larvae of various age groups which showed premature lethal moult in treatments with methoxyfenozide exhibited the pharate head capsule as a white collar like structure (Pl. VII: Figs. 65, 67, 68, 69). The control larvae did not show any morphological abnormality (Pl. VII: Fig. 66). A large number of the treated larvae showed rectal prolapse. Moulting fluid was found to be exuding out which turned black on exposure to air. These larvae were black in colour which died with the body soaked in the fluid (Pl. VII: Fig. 70). The larval cuticle was found to be sac like and fluffy as though filled with fluid (Pl. VII: Fig. 71). Day 4 and day 5 larvae showed moulting mark and mortality though at a lesser percentage when compared to the earlier treatments. The treated prepupae showed rectal prolapse and a fluid discharge from the prolapsed rectum (Pl. VII: Fig. 72).

Methoxyfenozide treatments of day 3 larvae with the lowest dose of 0.01µg resulted in formation of 25% of larval-pupal intermediates (Table 21, Fig. 73) and 43.33% of pupation (Table 22, Fig. 74). Doses such as 0.1 µg, 1 µg and 10 µg induced production of 30%, 28.33% and 25% of larval pupal intermediates from the treated day 3 larvae. Only 23.33%, 21.67% and 20% of day 3 larvae underwent pupation. Increase in concentration of methoxyfenozide

reduced the percentages of pupation and induced the formation of larval-pupal intermediates. Only 6.67-20% of pupae formed from these treatments emerged into normal looking adults (Table 23, Fig. 75).

The increase in concentration of the compound induced higher mortality which considerably reduced the number of larvae undergoing transformation in to larval-pupal intermediates and also pupae. A large number of the treated prepupae exhibited shrinking of body as seen in PPN treatments. The lower doses led to moderate shrinking (Pl. VIII: Fig. 76) where as higher doses induced maximum shrinking of the treated prepupae (Pl. VIII: Figs. 77, 78, 79). The larval legs, mouth parts and antennae were only partially sclerotised unlike the PPN treatments. The pupae formed from treatments were normal in appearance (Pl. VIII: Fig. 80) where as the larval-pupal intermediates possessed larval thoracic legs and pupal abdomen (Pl. VIII: Fig. 81). Only 10 -23.33% and 6.67-15% pupae succeeded in emerging into normal looking adults in day 4 and day 5 treatments respectively (Table 23, Fig. 75).

The treatments of day 0, day 1 and day 2 sixth instar larvae with 0.01 µg of halofenozide did not result in premature lethal moult (Table 20, Fig. 56). The treatments of day 0 larvae with the higher doses of halofenozide elicited symptoms of lethal moult (Pl. VIII: Fig. 82 A) and the affected larvae showed partial sclerotisation of antennae and larval legs (Pl. VIII: Fig. 82 B). The day 0 larvae treated with 0.01 µg of halofenozide showed 90 % of pupation but only 20 - 55 % of pupae emerged into adults (Table 23, Fig. 75) which were normal in appearance (Pl. VIII: Fig. 83). The results were similar in treatments with the other doses.

Day 1 larvae treated with halofenozide exhibited the pharate head capsule as a white collar like structure as observed in earlier treatments (Pl. IX: Fig. 84) where as the control larvae treated with acetone were normal in every respect (Pl. IX: Fig. 85). The surviving day 1 and day 2 larvae developed

normally and progressed to the succeeding instars. A major percent of them pupated successfully though the rate of pupation decreased with the increase in dose and also the progress in larval stadia. The pupae were of normal size as in the case of methoxyfenozide treatments. Adults emerged normally and 18.33 - 56.67% pupae from treated day 1 larvae and 20 - 60% pupae from day 2 larvae succeeded in the process (Table 23, Fig. 75). Prepupae which failed to pupate formed larval-pupal intermediates which survived for 7-8 days and then died.

The treatments of day 3 larvae with 0.01 µg of halofenozide did not result in lethal moult and mortality where as the doses 0.1 µg - 20 µg elicited moulting response in treated larvae (Pl. IX: Fig. 86). Halofenozide treatments of day 4 larvae with 0.1µg - 20 µg resulted in lethal moult (Pl. IX: Fig. 87) and 10-35% of larvae died within 24 h. A higher percent of larval-pupal intermediates were formed after treatments with all these doses. Eighty percent of treated day 3 larvae and 71.67 % of day 4 larvae pupated after 0.01 µg treatments. Of these, 16.67- 41.67 % pupae from treated day 3 and 18.33 - 50 % pupae from treated day 4 succeeded in emerging into normal looking adults. The percentage of pupation decreased with increase in dose.

The halofenozide treatments of day 5 larvae (prepupae) with lower doses such as 0.01 µg induced no mortality (Table 20, Fig. 56) and 58.33% pupated (Table 21, Fig. 73) where as 41.67% formed larval-pupal intermediates (Table 22, Fig. 74). The effects were similar with the other treatments also. A few of the treated prepupae showed rectal prolapse (Pl. IX: Fig. 88). A major percent of treated prepupae showed shrinking of body and partial sclerotisation of antennae (Pl. IX: Fig. 89). Shrinking was not severe with the compound as observed in earlier treatments. The treated day 4 larvae (Pl. IX: Fig. 90) and prepupae (Pl. IX: Fig. 91) which showed symptoms of moulting exhibited rectal prolapse irrespective of the doses applied as observed in larvae and pupae treated

with PPN and methoxyfenozide. This ecdysone agonist was found to be effective only at higher concentrations in *S. mauritia*.

3.3.3 Effects of hormone agonists on pupal-adult transformation

PPN treatments of pupae resulted in age/dose dependent mortality within 24 h. The newly ecdysed pupae on treatments with 0.01 µg of PPN showed 70% mortality in comparison to the tanned pupae which showed only 45% of mortality (Table 24, Fig. 92). The doses 0.1 µg - 1 µg induced mortality in 80 - 100% of newly ecdysed pupae and 50 - 80 % of tanned pupae. None of them survived beyond day 4. When the pupal cases of unemerged pupae were removed, pupal - adult intermediates / adultoids were found inside. They had very small crumpled wings which were unstretched and twisted.

Methoxyfenozide treatments of pupae with the lower dose 0.01µg induced 27.5% of mortality of newly ecdysed pupae and 17.5% mortality of tanned pupae (Table 25, Fig. 93). Halofenozide treatments with 0.01 µg and 0.1 µg resulted in no mortality in tanned pupae. Adults emerged after treatments of pupae with lower doses of ecdysone agonists were morphologically similar to those emerged from control pupae. But they laid very few eggs when kept with the males emerged from the treated pupae. The eggs were very few in number. They were not attached to the substratum and were scattered in appearance. None of these eggs hatched.

Methoxyfenozide treatments with higher dose 10 µg induced mortality in 47.5% of newly ecdysed unpigmented pupae and 40% of tanned pupae. The highest dose 20 µg induced 52.5% mortality in newly ecdysed pupae and 45% mortality in tanned pupae. Halofenozide treatments with 10 µg induced mortality in 32.5% of newly ecdysed unpigmented pupae and 17. 5% of tanned pupae. Treatments with 20 µg led to mortality of 40% of unpigmented pupae and 22.5% of tanned pupae. Higher doses decreased adult emergence and the adults

emerged from these pupae when kept with males of the same group did not lay eggs. No differences were noticed in the period of pupation. Females took 7 days and males took 8 days to complete development as in the case of pupae kept as controls. The unemerged pupae when dissected out on day 7 (females) and on day 8 (males) showed adultoids inside them. The adultoids were characterized by the presence of bifurcated proboscis and unstretched wings. External genitalia were developed but were deformed. Eye pigmentation resembled that of compound eye of normal adults.

3.4 Discussion

3.4.1 Hormone interactions in the development and metamorphosis of insects: a brief preview

Experiments with many Lepidoptera indicate that two major hormonal factors regulate prothoracic gland activity: the prothoracicotopic hormone (PTTH) and JH secreted by corpora allata. In *Manduca sexta*, PTTH serves to activate ecdysone secretion from prothoracic glands (Smith, 1993). Depending on the developmental state, JH either inhibits or stimulates prothoracic glands (Cymborowski and Stolarz, 1979; Hiruma and Agui, 1982; Balamani and Nair, 1991). Humoral regulation of PTTH synthesis and release in Lepidoptera involves JH (Williams, 1961; Yagi and Fukaya, 1974; Nijhout and Williams, 1974). In several insects JH appears to be a feed back inhibitor of PTTH release, inducing and/or maintaining larval diapauses (Yin and Chippendale, 1973; Takeda, 1978; Chippendale and Yin, 1979; Sieber and Benz, 1980b; Chippendale and Turunen, 1981; Eizaguirre, 2005).

Larval-larval moulting in insects is controlled by the prothoracic glands as they secrete ecdysteroids. Sehnal *et al.*, (1981) suggest that the rate of ecdysone production depends on a balance between PTTH and JH. Their evidences along with other studies (Nijhout, 1975; Hiruma *et al.*, 1978b;

Safranek *et al.,* 1980) indicate that JH may exert a direct restraining action on prothoracic glands. High titres of JH inhibit prothoracic gland activity early in the last larval instar while late in the instar JH influence is reversed resulting in activation of prothoracic gland function in *S. littoralis* (Cymborowski and Stolarz, 1979; Cymborowski and Zimowska, 1984; Zimowska, *et al*., 1985). Similar results were obtained in *Mamestra brassicae* (Hiruma *et al*., 1978a; Hiruma and Agui, 1982), *Manduca sexta* (Safranek *et al*., 1980), *Laspeyresia pomonella* (Sieber and Benz, 1980b) and *Spodoptera mauritia* (Balamani and Nair,1991). Safranek *et al*., (1980) have demonstrated that the switch over from inhibition to activation may occur spontaneously but that it is accelerated by exposure of prothoracic glands to PTTH or ecdysone. Further experiments in *Manduca sexta* larvae indicated that the JH activation of prothoracic glands may be mediated by a prothoracic gland stimulatory factor which is induced in the fat body by JH. This tropic factor along with PTTH stimulates them to produce the large peak in ecdysteroids needed for pupation (Gruetzmacher *et al*., 1984).

In Lepidoptera, the initiation of larval-pupal metamorphosis depends upon a decline in the high titre of JH present during last larval-larval ecdysis to very low or undetectable levels by the end of feeding period. This drop in JH titre permits a biphasic secretion of ecdysone (Calvez *et al*., 1976; Lafont *et al*., 1977; Maroy and Tarnoy, 1978; Bollenbacher *et al*., 1981; Edwards *et al.,* 1995). A decline in the JH titre is necessary for the release of PTTH from the brain which in turn activates the prothoracic glands to synthesize ecdysone resulting in the first increase in ecdysteroid titre. The first ecdysteroid surge is believed to be responsible for the cessation of feeding and initiation of wandering stage and also for induction of a change in the commitment of epidermal cells from larval to pupal type (Riddiford, 1985). This commitment peak of ecdysteroids also induces gut purge (Truman and Riddiford, 1974; Fujishita *et al.,* 1982).

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). Studies have demonstrated that this increase of JH along with the second release of PTTH activates the prothoracic glands to secrete large amount of ecdysteroids needed for larval-pupal transformation and moulting (Cymborowski and Stolarz, 1979; Hiruma, 1980; Sieber and Benz, 1980a; Balamani and Nair, 1991). It was also suggested that the 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). JH may also enhance the response of ecdysteroids and/or alter the sensitivity of tissues to ecdysteroids (Denlinger, 1979). This release of ecdysone promotes apolysis and pupal cuticle formation and the completion of the process of transformation into pupa (Riddiford, 1985). During larval-pupal transformation in most Lepidoptera, 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). Earlier studies have demonstrated that the absence of JH in the pupal stage of Lepidoptera is a prerequisite for both the initiation and completion of prothoracic gland degeneration and also the development of adult structures. The ecdysteroid titre drop to a negligible quantity towards the end of pupal phase for the eclosion hormone to be released (Truman, 1971; Riddiford, 1985). During pharate adult stage, ecdysteroids increase to a major peak in the haemolymph and this increase promotes pupal-adult metamorphosis.

3.4.2 Toxicity of hormone agonists

3.4.2.1 Toxicity of JH agonist PPN

In *S.mauritia*, PPN was found to induce dose dependent mortality in treated insects. With lower concentrations, the mortality induced was low where as the higher doses induced higher mortality. Treatments of eggs with high doses

of PPN resulted in a reduction in hatchability as observed in egg treatments of *Chrysoperla rufilabris (Chen and Liu,* 2002). This result is similar to the earlier observations in which the application of JH analogues to the females or eggs prevented hatching in *Pyrrhocoris apterus* (Slama and Williams, 1966), *Hyalophora cecropia* (Riddiford and Williams, 1967), *Choristoneura fumiferana* (Hicks and Gordon, 1994) and *Plodia interpunctella* (Mohandass *et al.*, 2006). When the adult females were exposed to PPN, egg hatch was retarded in the predatory coccinellids *Chilocorus circumdatus* and *Cryptolaemus montrouzieri* (Smith *et al.*, 1999).

Ovicidal activity of PPN was exhibited in *Cydia pomonella* (Canela *et al.*, 2000) and *Plutella xylostella* (Oouchi, 2005) when the eggs earlier than 24h were exposed to the compound. PPN had a lethal effect on 1-2 day old eggs of glassy-winged sharpshooter *Homalodisca coagulata* (Prabhakar and Toscano, 2007). No progeny was obtained in *Hyposoter didmator* following PPN treatments (Schneider *et al.*, 2003). PPN caused direct mortality of treated first instar nymphs (>68%) in *Aphis glycines* (Richardson and Lagos, 2007) and higher concentrations of PPN reduced the survival of first and fourth instar nymphs. PPN suppressed embryogenesis on treatments of females of *Aonidiella aurantii* (Eliahu et al., 2007). When the untreated adults of *Bemisia tabaci* were exposed to PPN treated adults, high mortality of eggs was observed (Nakamura, 2007). Present studies suggest that high concentrations of PPN can act as an ovicide in *S.mauritia*.

The present observations reveal that majority of the larvae which hatched out from treated eggs showed a high rate of mortality during postembryonic development. Only very few of them underwent larval-pupal metamorphosis. This is similar to the earlier observations on *Hyalophora cecropia* (Riddiford and Williams, 1967; Willis, 1969), *Oncopeltus fasciatus* and *Pyrrhocoris apterus* (Riddiford, 1970a). In all these cases the hatched out larvae appeared to

be normal when the eggs were treated with JHA but they suffered high mortality during larval period. It was seen in earlier studies that in the presence of JHA, the fertilized egg cell is unable to develop or it develops only to a certain critical stage (Slama et *al.* 1974).

High rate of mortality was observed in larval treatments also. The larvicidal activity of PPN was observed in the fourth and fifth instar larvae of the predatory coccinellids *Chilocorus circumdatus* and *Cryptolaemus montrouzieri* (Smith *et al.*, 1999). Direct mortality was observed in the larvae of *Culex pipiens* (El-Shazly and Refaie, 2002). Pupal mortality was significantly higher than larval and adult mortality following PPN treatments of *Aedes aegypti* (Resende and Gama, 2006). PPN was proved to be highly toxic to the predatory bug *Podisus maculiventris* and caused severe deformities at ecdysis independent of the method of exposure (De Clercq *et al*., 1998). Topical application of low JH doses to early embryos resulted in larval abnormalities while high doses of hormone induced embryonic mortality in *Manduca sexta* (Orth *et al*., 2003). Treatments of adult females of *Aonidiella aurantii* with higher doses of PPN resulted in embryonic mortality (Rill *et al*., 2007; Eliahu *et al*., 2007).

In *S. mauritia*, in all the tested concentrations of PPN, the effects were age/dose dependent. Early instars exhibited comparatively higher mortality than the later stages of the stadia. Studies on California red scale *Aonidiella aurantii* (Rill *et al*., 2007) have shown that the first instar larvae exposed to PPN responded with mortality during the first and second molts where as the second instars were more tolerant of PPN. The age related toxicity may be the effect of several factors like post ecdyseal changes in the integument which modify the rate of penetration of the compound. PPN penetration was found to be fast and high after larval treatments of *Chrysoperla carnea*, with 80% penetrated when analyzed after one hour (Medina *et al*., 2003). Cuticular penetration of PPN was found to reach 99% in 3 days in adults of *Hyposoter didymator* and the

absorption was more than 65% in body tissues which was very high when compared to ecdysone agonists applied (Schneider *et al.*, 2008).

A physiological basis for the toxicity and morphogenetic effects has been suggested by linking these effects with interference with the expression or action of certain genes, particularly the *Broad-Complex* (*BR-C*) transcription factor gene, that direct metamorphic change (Wilson, 2004). Misexpressed *BR-C* leads to improper expression of one or more downstream effector genes controlled by *BR-C* gene products, resulting in abnormal developmental and physiological changes that disrupt metamorphosis.

3.4.2.2 Toxicity of ecdysone agonists methoxyfenozide and halofenozide

The ecdysone agonists methoxyfenozide and halofenozide did not affect hatchability in *S.mauritia.* But the hatched out larvae of methoxyfenozide treatments died before completing the second instar. Similar results were obtained after treatments of the nonsteroidal ecdysone agonists on other Lepidoptera. More than 90% of eggs died when egg masses of *Ostrinia nubilalis* were dipped in a solution of 100 ppm methoxyfenozide or tebufenozide in acetone (Trisyono and Chippendale, 1997). The egg mortality exceeded 95% following tebufenozide treatments in codling moth *Cydia pomonella*, (Knight, 2000) and evidences are there for the larvicidal and ovicidal properties of methoxyfenozide (Charmillot, 2001; Bylemans et *al*., 2003). Methoxyfenozide induced egg mortality when applied on the eggs of less than 1 day old and larvicidal effect was exhibited on treatments of older larvae of *Lobesia botrana* (Saenz-de-Cabezon Irigaray, 2005). Larval mortality was observed in *Spodoptera littoralis* on treatment of eggs with methoxyfenozide and pupal mortality was observed on feeding of third instar larvae with different concentrations of agonist (Pineda *et al*., 2004). Methoxyfenozide and halofenozide were found to be equally toxic to the multicoloured Asian lady

beetle *Harmonia axyridis* (Carton *et al*., 2003). Methoxyfenozide exhibited greater toxicity than tebufenozide against the eggs of Codling moth, *Cydia pomonella* and the Oriental fruit moth, *Grapholita molesta* (Borchet *et al*., 2004b).

Topical application of halofenozide on adults of Colarado potato beetle, *Leptinotarsa decemlineata* severely affected the fecundity and treatments of *Aubeonymus mariaefranciscae* drastically decreased the progeny survival and more than 80% of first instar larvae died after egg hatching due to premature moulting (Farinós *et al*., 1999). It was proved to be highly toxic to Japanese beetle, *Popillia japonica* and Oriental beetle, *Exomala orientalis* (Cowles *et al*., 1999). Halofenozide treatments of late second instar of *Cyclocephalis borealis* caused rapid mortality (Grewal *et al*., 2001). Topical application of halofenozide on female adult beetles of mealworm, *Tenebrio molitor* reduced the egg viability by 68% (Taibi *et al*., 2003). The compound halofenozide exhibited insecticidal activity In Mediterranean flour moth *Ephestia kuehniella*, (Hami *et al*., 2005).

Methoxyfenozide negatively affected the reproduction of *Spodoptera littoralis* adults because of its presence in the adult body and accumulation in the laid eggs (Pineda *et al*., 2006). Experimental studies on *Chironomus tentans* proved that the increased toxicity of the nonsteroidal ecdysone agonists has a high correlation to the affinity of these compounds for EcR complex (Smagghe *et al*., 2002b). It can be inferred that methoxyfenozide can be successfully employed as a control agent in *S. mauritia* due to its larvicidal properties. Halofenozide was found to be effective only at higher concentrations in *S. mauritia*. It was reported to be highly effective on coleoptera but mildly active on Lepidoptera (Retnakaran *et al*., 2003; Nakagawa, 2005). This might be due to the selective binding of the compound on EcRs as seen in the case of tebufenozide which is selectively toxic to Lepidoptera and ineffective on Coleoptera (Smagghe *et al*., 1996).

3.4.3 Effects on embryogenesis and postembryonic development

3.4.3.1 Effects of JH agonist PPN

In the present study, treatments of eggs of *S.mauritia* with higher doses of PPN considerably inhibited the hatchability of eggs. Fenoxycarb, the precursor molecule of PPN and PPN were shown to suppress egg hatch in *Cacopsylla pyricola* (Higbee *et al*., 1995). PPN suppressed egg hatch and adult formation in *Bemisia tabaci* (Ishaaya and Horowitz, 1992) and *Haematobia irritans* (Bull *et al.*, 1993). Fenoxycarb induced the same effect in *Chrysoperla rufilabris* (Liu and Chen, 2001). A number of studies on various insect species have proved that application of JH or its analogues to eggs or egg laying females of insects interrupt embryogenesis and cause serious developmental disorders (Slama and Williams, 1966; Riddiford and Williams, 1967; Novak, 1969; Riddiford, 1969, 1970 a,b, 1972; Hunt and Shappirio, 1973; Rohdendorf and Sehnal, 1973; Smith and Arking, 1975; Negishi *et al*., 1976; Enslee and Riddiford, 1977; Srivastava and Shukla, 1987; Itaya, 1987; Langley *et al*., 1990; Koehler and Patterson, 1991; Hicks and Gordon, 1994; Horowitz et al., 1999; Lee, 2001; Mohandass *et al.*, 2006, Prabhaker and Toscano, 2007). PPN induced defective embryogenesis and morphological deformities in the desert locust, *Schistocerca gregaria* (Vennard *et al*., 1998). No progeny was obtained in *Hyposoter didmator* following PPN treatments (Schneider *et al.*, 2003). The females of California red scale, *Aonidiella aurantii* survived first instar exposure to PPN but experienced reduced fecundity with increasing concentration of the compound (Rill *et al*., 2007). The compound also suppressed embryogenesis (Eliahu *et al*., 2007).

Surviving larvae developed normally but a large percentage of them failed to pupate and formed larval-pupal intermediates. Studies of Riddiford (1969, 1970 a, b; 1971) have shown that a large percent of the larvae which

hatched out from JHA treated eggs of *Oncopeltus fasciatus* developed normally upto the last larval instar but instead of undergoing normal larval-pupal metamorphosis they exhibited extra larval moults. Supernumerary moults were observed in various insects following JHA treatments (Grenier and Grenier, 1993; Quennedey *et al*., 1995; Dedos and Fugo, 1999b). The results of the present experiments on *S. mauritia* suggest that the treatment of eggs with PPN might have selectively interfered with the embryonic programming of the corpus allatum so that it could not cease secretion as a prelude to metamorphosis as observed in the case of other JHAs by Riddiford and Truman (1972). This might have led to the failure of treated larvae to undergo normal metamorphosis and instead led to supernumerary moults.

3.4.3.2 Effects of ecdysone agonists methoxyfenozide and halofenozide

The results of the present study demonstrate that in *S.mauritia,* treatments of eggs with 1 µg, 10 µg and 15 µg of methoxyfenozide had no effect on hatchability of eggs but the compound induced mortality in the hatched out larvae. None of them survived beyond the second instar. Mortality had occurred only after hatching and not a single embryo died within the egg. Similar results were obtained after methoxyfenozide treatments of egg masses of *S. littoralis* (Pineda *et al.,* 2004). Halofenozide induced no mortality at all in egg treatments. The apparent insensitivity of *S.mauritia* embryos to methoxyfenozide and halofenozide suggests that either the compounds may not be able to penetrate through the chorion or that the target cells of embryos are not yet competent to react to the compound morphogenetically. Halofenozide was similar to other ecdysone agonists in initiating a premature lethal moult and was reported to be selectively toxic to Coleoptera (Cowles *et al*., 1999; Nakagawa, 2005; Potter, 2008). The eggs and hatched out larvae of *S. mauritia* exhibited insensitivity to the compound. It was found to be effective only at higher doses.

The studies on the mode of action of nonsteroidal ecdysone agonists have shown that they act directly on the target tissues at the level of ecdysone receptors (Wing *et al.,* 1988, Wurtz *et al.,* 2000). The synchronization of morphogenetic events controlled by hormones is critically dependent upon an adequate concentration of receptors in the appropriate tissues. The mortality occurring shortly after hatching strongly indicate that the target cells become susceptible only after hatching. Presumably, traces of the compound penetrating through the chorion before hatching or contamination by the compound residues that remain in the container or on chorion may be responsible for the lethal effects on first instar larvae.

3.4.4 Effects on larval-larval moulting

3.4.4.1 Effects of PPN

3.4.4.1.1 Extension of larval period

Treatments of PPN on *S. mauritia* resulted in an extension of larval period in the treated stage and also in the subsequent larval instars. Similar results were obtained from the treatments with JH analogues on *Tribolium castaneum* (Ishaaya and Yablonski, 1976), *Laspeyresia pomonella* (Sieber and Benz, 1978), *Manduca sexta* (Watson and Bollenbacher, 1988), *Chelonus blackburni* (Chanda and Chakravorty, 2001) and *Bombyx mori* (Miranda *et al.,* 2002; Etebari *et al*., 2007). Juvenoids were found to prolong developmental time and also increase mortality in the wandering phase in *Plodia interpunctella* (Mohandas *et al*., 2006). JH is responsible for the maintenance of larval state and it prevents metamorphosis in insects (Wigglesworth, 1934; Williams, 1961). It converts the metamorphic cycle into a larval one by modifying the growth and metabolism of treated larvae (Slama and Mala, 1984). The ecdysteroids released at the end of each instar is responsible for moulting and they cause the epidermal cells to synthesise a new larval/pupal/adult cuticle (Riddiford, 1980). The studies

on *Manduca sexta* (Watson and Bollenbacher, 1988) and *Bombyx mori* (Sakurai *et al.*, 1989; Gu *et al.*, 1997) 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.

PPN was found to suppress the release of ecdysteroids and promote the feeding activity. Topical application of PPN delayed the pupal ecdysteroid peak by 4 days in the honey bee *Apis mellifera* (Zufelato *et al.*,2000). PPN decreased ecdysteroid production in mealworm pupae *Tenebrio molitor* (Aribi *et al.*, 2001) and in *in vitro* pupal integument explant cultures; PPN caused a significant inhibition of ecdysteroid amounts and of the thickness of new cuticle secreted (Aribi *et al.*, 2006). The decrease in ecdysteroid production might have prolonged the feeding period in PPN treated larvae. A juvenile hormone response element and two binding proteins are identified in *Drosophila melanogaster* and the honey bee, *Apis mellifera* and the enzyme assays showed that these two proteins interact with each other as well as with EcR, ultraspiracle (USP) and methoprene-tolerant protein (Li *et al.*, 2007). It was suggested that these proteins play important roles in cross-talk between JH and ecdysteroids.

3.4.4.1.2 Increase in size of larvae/pupae/adult

The larvae, pupae and adults of *S. mauritia* arising from the treatments with PPN on egg mass and different instars were larger in size. An increase in length and a corresponding increase in width were observed in them. This response was observed earlier in studies with *Galleria mellonella* and *Spodoptera littoralis* (Sehnal, 1976). The juvenoids inhibited larval-pupal transformation and produced giant larvae in stored product insects *Tribolium castaneum, Rhyzopertha dominica* and *Sitsphilus oryzae* (Kostyukovsky *et al.*, 2000). In our earlier studies, topical treatments of JHA showed an increase in

most of the nutritional parameters such as approximate digestibility, efficiency of conversion of ingested food, consumption index and growth rate (Sindhu and Nair, 2004). The activities of digestive enzymes amylase, invertase, trehalase and protease increased significantly in JHA treated larvae. The increased size of larvae might be due to this increased activity of enzymes which would have increased the food consumption and assimilation in treated larvae. The extension of larval period and the increased feeding by the larvae under the influence of JH agonist PPN might have increased the size of larvae and consequently the size of pupae and adults.

3.4.4.1.3 Delayed effects

PPN treatments of the eggs and larvae of *S. mauritia* caused delayed effects on metamorphosis. Following egg treatments with PPN, the surviving larvae developed normally but a large percentage of them failed to undergo normal pupation. These larvae underwent defective moulting into larval-pupal intermediates or developed into adultoids. Studies of Riddiford (1969, 1970 a, b; 1971) have shown that a large percent of the larvae which hatched out from JHA treated eggs of *Oncopeltus fasciatus* developed normally up to the last larval instar but instead of undergoing normal larval-pupal metamorphosis they exhibited extra larval moults. There are two explanations for this phenomenon. One is that the embryonic brain or corpora allata might have been directly affected by the hormone analogue so that secretion of JH was abnormally prolonged and other is that the applied hormone might have persisted throughout growth until metamorphosis (Willis and Lawrence, 1970). It was shown by transplantation experiments that the corpus allatum of the treated insects failed to stop secreting the hormone at the onset of late larval instar.

Studies utilizing certain haemolymph binding proteins in imaginal discs of *Manduca sexta* indicated that these proteins retarded the uptake of JH by

tissues and also protect them from degradation by tissue enzymes (Hammock *et al*., 1975). The delayed effects on metamorphosis after exposure of insect embryos to JH or any of its analogues are produced by the interference of JHA with the programming of the embryonic corpora allata (Riddiford and Truman, 1972). Present experiments on *S. mauritia* suggest that the treatment of eggs with PPN might have selectively interfered with the embryonic programming of the corpus allatum so that it did not cease secretion as a prelude to metamorphosis as observed by in the case of other JHAs.

When the earlier larval instars were treated with the compound the effects such as mortality, increased feeding activity, an extension of larval period, the developmental deformities etc. were observed in the later instars also. Similar delay in development was observed on PPN treatments of immature larvae of *Chrysoperla rufilabris* (Chen and Liu, 2002). Treatments with fenoxycarb, the JHA from which PPN was synthesized gave the same results (Liu and Chen, 2001). Juvenoids were reported to induce delayed effects between different developmental stages of insects (Slama, 1971, Safranek *et al*., 1980) and such effects could be either due to the persistence of the compound through the instars and due to the effects of the compound on the secretory activity of prothoracic glands. Many authors have explained that degradation of JH mimics takes place rapidly (Gilbert *et al*., 2000: Kamita *et al*., 2003). But it was observed that the action of PPN has a delay of approximately 24 h after treatments (Edwards *et al*., 1993; Steigenga *et al*., 2006). This proves that the compound is not metabolized immediately. PPN resistant strains of housefly larvae show high levels of cytochrome P 450 in gut and fat body than PPN susceptible strains (Zhang *et al*., 1998). Studies indicate that cytochrome P 450 inhibitors decrease *in vitro* metabolism of PPN. This suggests the existence of some mechanism which inhibits the action of cytochrome P 450 in PPN treated eggs and larvae and prevents the metabolism of PPN. This mechanism along with the presence

of haemolymph JH binding proteins in *S.mauritia*, as seen in *Blattella germanica* (Sevala *et al*., 1997) might have led to persistence of PPN in haemolymph through instars escaping action of JH esterases.

3.4.4.2 Effects of ecdysone agonists methoxyfenozide and halofenozide on larval-larval moulting

3.4.4.2.1 Premature lethal moulting

The initiation of premature moult in the treated larvae was found to be the most remarkable effect of the treatments of day 0 larvae of *S.mauritia* with different doses of methoxyfenozide and halofenozide. The larvae showed the slippage of head capsule down over the mandible which is an overt morphological marker for the accompanying epidermal apolysis. They underwent a premature moult though not successful in completing it. Halofenozide treatments reduced the length of larval and pupal period when the treatments were made on newly moulted fourth instar larvae of mosquito, *Culex pipiens* (Boudjelida, *et al.,* 2002). Methoxyfenozide and halofenozide treatments were successful in initiating a premature lethal moult in larvae of *S. littoralis* (Smagghe *et al.,* 2001). Similar results were obtained from the larval treatments of *Lobesia botrana* with methoxyfenozide which resulted in larval mortality and the older larvae were more susceptible (Saenz-de-Cabezon Irigaray *et al*., 2005). In the multicoloured Asian lady beetle *Harmonia axyridis*, the ecdysone agonists methoxyfenozide and halofenozide caused premature induction of larval moulting, cessation of feeding and incomplete pupation (Carton *et al* ., 2003). The remnants of old unshed cuticle were visible through the black colouration in Colarado potato beetle, *Leptinotarsa decemlineata* (Smagghe and Degheele, 1993; Carton *et al.,* 2000). Larval treatments with methoxyfenozide disturbed the development and reproduction of the hairy rose beetle, *Tropinota squalida* (Hussein et al., 2004).

Initiation of premature moulting and apolysis but failure to complete the moult was observed in the mosquitoes *Aedes aegypti, Culex quinquefasciatus* and *Anopheles gambiae* following treatments with ecdysone agonists (Beckage *et al.,* 2004). Halofenozide is similar to the other ecdysteroid agonists in initiating a premature lethal moult. These findings confirm the similar results obtained earlier with ecdysone agonist RH 5849 in *Manduca sexta* (Wing, 1988; Wing *et al*., 1988) and other insect species (Darvas *et al*., 1992; Monthean and Potter, 1992). Moulting is the result of the expression of a cascade of genes that is sequentially both up and down regulated by the moulting hormone, 20-HE. Nonsteroidal ecdysone agonists act like 20-HE at the molecular level by binding with the ecdysone receptor complex (EcR) and transactivating a succession of molt initiating transcription factors (Retnakaran *et al*., 1995, 2003; Dhadialla *et al*., 1998; Smagghe *et al*., 1999, 2001, 2002b; Carlson *et al*., 2001; Nakagawa *et al*., 2002; Mourad *et al.,* 2004; Hu *et al*., 2004; Fujitha and Nakagawa, 2007). This in turn induces the expression of a group of moult related genes. As a result of the expression of these up-regulated genes, the larva undergoes apolysis and head capsule slippage. But unlike 20-HE which is cleared at this juncture, allowing the down-regulated genes to be expressed, these synthetic analogues bind strongly to the receptors, remain in place and repress all the down-regulatory genes such as those necessary for cuticle elaboration, sclerotisation and ecdysis resulting in a developmental arrest at this state (Retnakaran *et al.,* 2003). As a consequence of this, the treated larva undergoes a precocious incomplete lethal moult.

3.4.4.2.2 Reduced feeding and decrease in weight

It was observed that after the application of ecdysone agonists, the treated larvae of *S.mauritia* exhibited reduced feeding activity and reduced growth rate. The feeding stages of Lepidoptera show minimum ecdysteroid content as a rule and exogenous ecdysteroids applied during the feeding stage always act as

strong antifeedants (Slama, 1982). The nonsteroidal ecdysone agonists interfered with feeding activity and induced a premature lethal molt in many lepidopteran insects (Wing *et al*., 1988; Monthean and Potter, 1992; Sakunthala and Nair, 1995). Cessation of feeding and decrease in weight were observed in *Diatraea grandiosella* (Trisyono and Chippendale, 1998) and *S. littoralis* after larval treatments with methoxyfenozide (Pineda *et al*., 2007). Treatments with tebufenozide induced reduction in larval weight in *Mythimna unipuncta* and *S. littoralis* (Gobbi *et al*., 2000). The well known basic feature of ecdysone action is that the endogenous ecdysteroid peaks generally occur in a non-feeding stage when the larva enters metamorphic phase. Hence the ecdysonergic properties of methoxyfenozide and halofenozide are responsible for the antifeedant effects and lack of weight gain exhibited by the treated larvae of *S. mauritia*.

3.4.5 Effects on larval-pupal moulting

3.4.5.1 Effects of PPN

The present study showed that the effects of PPN treatments on the larval-pupal moulting of *S.mauritia* were dose/age-dependent. The initiation of premature moult in the treated sixth instar larvae was the most remarkable effect observed in the treatments. The larvae showed the slippage of head capsule down over the mandibles but failed to complete the accompanying epidermal apolysis. Our earlier studies have shown that JHA treatments of sixth instar day 2 and day 3 larvae of *S. mauritia* resulted in supernumerary moults (Santha, 1987; Balamani and Nair, 1992; Venugopalan, 1995) though the effect was somewhat reduced in day 2 larvae.

Application of PPN to the last instar larvae of *S. litura* and *Manduca sexta* induced moulting of larvae into supernumerary larvae (Hatakoshi *et al*., 1986, 1988). Similar results were obtained from the studies on German

cockroach *Blatella germanica* (Reid *et al*., 1994), *Spodoptera exigua* (Smagghe and Degheele,1994), *Locusta migratoria migratorioides* and *Schistocerca gregaria* (Pener *et al*., 1997). PPN and a new series of 3-pyridyl ethers were found to induce precocious metamorphosis in the larvae of silkworm *Bombyx mori* (Kuwano *et al*., 1999). Brains of these larvae were presumed to be activated to secrete prothoracicotropic hormone when a high dose of PPN is introduced. PPN is a derivative of the JHA fenoxycarb in which a part of aliphatic chain is replaced by pyridyl oxyethylene and as a consequence both of them exhibit similarity in action. When fenoxycarb was topically applied to the third or fourth instar larvae of *Bombyx mori*, an extra larval moult was induced (Kamimura and Kiuchi, 2002). The fifth instar period was shortened to 5 days and the extra sixth instar ranged from 8 to more than 20 days depending on the dose applied. The titres of active moulting hormone 20-HE and its precursor ecdysone had similar titres in the sixth instar whereas ecdysone was much less than 20-HE in the extra moulting larvae. These results suggest that fenoxycarb triggered the extra larval moult by inducing an additional larval molt type of ecdysteroid surge before the last larval instar (Kamimura and Kiuchi, 2002). PPN might have induced a similar surge of ecdysteroids in the early days of last larval instar which was sufficient for eliciting a moulting response in the treated larvae.

Treatments on day 3 larvae of *S. mauritia* resulted in supernumerary moult. This finding is consistent with earlier observations in *S.mauritia* (Santha, 1988; Venugopalan, 1995). Day 3 larvae when treated with JHA undergo supernumerary moults forming large sized larvae with sclerotised mouth parts, larval legs and antennae. This is observed in many Lepidopteran insects (Koçak and Kilinçer, 1997). When first and fourth instar nymphs of *Aphys glycines* were treated with PPN, supernumerary moulted nymphs with 1-3 extra moults were formed (Richardson and Lagos, 2007). Larvae of *S. litura* treated on day 1 or day 2 with JHA moulted in to supernumerary larvae (Wu and

Lu, 2008). The persistence of PPN during this critical period might have changed the type of moult from larval-pupal to larval-larval type producing supernumerary larvae as JH/JHA can determine the quality of moult. During the last larval instar stadium of holometabolous insects such as Lepidoptera, a reduction of the haemolymph JH levels is a necessary step in the initiation of larval-pupal metamorphosis (Riddiford, 1980; Sehnal, 1985).

The extension of feeding period was observed in a greater percent of last instar larvae when PPN treatments were made up to day 2. None of the sixth instar day 0 and day 1 larvae pupated following PPN treatments. These findings are consistent with earlier observations in *S.mauritia* that the larvae failed to pupate on treatments of day 0 and day 1with JHA and the thoracic appendages and mouthparts showed pupal development irrespective of the dose and day of treatment of JHA (Santha and Nair, 1987). JHA treatment to final instar larvae of Lepidoptera before they attain maximal body weight was found to prolong the feeding period (Safranek *et al.,* 1980; Watson and Bollenbacher, 1988; Chanda and Chakravorty, 2001; Miranda *et al.,* 2002; Etebari *et al*., 2007). As seen earlier, the timing of moult is determined by ecdysteroids (Riddiford, 1985, 1994). The prolongation of feeding period might be due to the suppressing effect of PPN on the secretory activity of prothoracic glands, necessary for further changes in the direction of pupation. Acquiring the competence to respond to ecdysteroids or PTTH is of prime impotance in the process of larval-pupal metamorphosis. It was observed that JHA inhibited prothoracic gland competency to generate cAMP and promote ecdysteroidogenesis in early last instar larvae of *Bombyx mori* (Gu *et al*., 1997). A similar response of PPN might have prolonged the feeding period in PPN treated larvae of *S. mauritia.*

PPN prevented pupation in *Chilocorus circumdatus* and *Cryptolaemus montrouzieri* (Smith *et al*., 1999). Induction of dauer/permanent larvae was observed in *Bombyx mori* after application of fenoxycarb, the precursor

compound of PPN (Dedos and Fugo, 1999b). There are evidences that JH acts during the early stages of the last larval instar to suppress both the secretory activity of prothoracic glands and also the acquisition of competence to respond to PTTH in *Bombyx mori* (Sakurai *et al*., 1989; Watson and Bollenbacher, 1988; Gu *et al*., 1997) and *Manduca sexta* (Watson and Bollenbacher, 1988). The failure of pupation in the larvae treated on early days of the last larval instar may be due to the suppressing effect of PPN on the secretory activity of prothoracic glands which otherwise stimulate the secretion of ecdysone through the production of PTTH. The cells of these larvae would not have acquired the competence to respond to PTTH or ecdysteroids to pupate.

PPN treatments on sixth instar day 4 and day 5 larvae led to development of larval-pupal intermediates with larval and pupal characteristics in the body. In *Sarcophaga ruficornis*, JHA treatments in larvae resulted in three types of morphogenic forms such as larval-pupal intermediates, pupal-adult intermediates and deformed adults (Mathur *et al*., 1990). In *S.mauritia* it seems that reprogramming of general epidermal cells from larval to pupal type occurs by the end of day 3 following the first release of ecdysteroids. A second longer and larger release of ecdysone occurs some time later in the prepupal stage which promotes apolysis and pupal cuticle formation and the completion of the process of transformation into pupa (Riddiford, 1985). Our earlier studies have shown that the commitment peak of ecdysteroids did not appear in the JHA treated *S.mauritia* larvae and the large premoult peak preceding larval-pupal moult was also missing (Mona, 2001). The epidermal cells normally respond to JHA treatment by secreting another larval cuticle but here pupal cuticle is formed. This could be due to the pupal commitment of epidermal cells that has already occurred by reprogramming larval to pupal type.

The production of larval type cuticle as a result of PPN treatments in certain parts of the body might be due to the failure in switch over as

ecdysteroids are not alone in the system. The larval specific genes are not permanently repressed due to the presence of PPN in the haemolymph. Application of JHAs to early last instar lepidopteran larvae resulted in development of supernumerary instars whereas treatments of later stages would result in abnormal pupation and development of larval-pupal mosaics or intermediates (Koçak and Kilinçer, 1997).

3.4.5.2 Effects of ecdysone agonists

The effects of the ecdysone agonists methoxyfenozide and halofenozide on the larval-pupal transformation of *S.mauritia* were found to be age-dependent which vary depending upon the difference in commitment of tissues. It was observed that sixth instar day 0 and day 1 larvae treated with various doses of these compounds showed moulting symptoms. Earlier studies have suggested that during larval-pupal transformation of *S.mauritia*, the JH level gets reduced to undetectable levels and the first peak of ecdysteroids occurs by day 3 (Santha and Nair, 1987). Since the target cells and tissues of treated insects have not been exposed to the first peak of ecdysteroids which occurs on day 3, they have not changed their commitment from larval type to pupal type at the time of treatment with the ecdysone agonists. Hence the treated larvae moulted into supernumerary larvae and failed to undergo pupation. Similar results were obtained from studies on multicoloured Asian lady beetle *Harmonia axyridis* (Carton *et al*., 2003) in which methoxyfenozide and halofenozide treatments caused premature induction of moulting and incomplete pupation. Day 2 larvae when subjected to treatments with various doses of ecdysone agonists exhibited an acceleration of the ecdysteroid dependent gut purge activity which is consistent with the ecdysonergic properties of these compounds.

Treatments of day 3, day 4 and day 5 larvae with methoxyfenozide and halofenozide induced a large percentage of the larvae to produce larval-pupal

intermediates. The surge of ecdysteroids on day 3 is responsible for the change in programming of the commitment from larval to pupal type. The target tissues of day 4 and day 5 larvae have already been exposed to the first release of ecdysteroids on day 3 which is responsible for the change in the commitment of epidermal cells from larval to pupal type. The second peak of ecdysteroids on day 5 initiates the pupal moult and here the commitment of epidermal cells to pupate remains unaffected (Oberlander, 1985). But here the high titre of nonsteroidal ecdysone agonists along with the endogenous ecdysteroid titres interfered in their normal moulting to pupae and formed a large proportion of larval-pupal intermediates. The failure of a considerable percentage of treated day 4 and day 5 larvae to complete larval-pupal transformation and ecdysis might be due to the high concentration of ecdysonergic compounds circulating in the haemolymph.

In *S. mauritia*, the prothoracic glands show maximum secretory activity on day 4 and day 5 of the last larval instar (Balamani, 1990; Balamani and Nair, 1994, 1997). Exogenous application of nonsteroidal ecdysone agonists on the larvae of these age groups results in hyperecdysonism. Lower doses did not affect the morphogenesis adversely but promoted pupation. Higher doses exerted a lethal effect and interfered with the normal moulting. In the normal conditions, the ecdysteroids are released gradually but in the treated larvae a high dose of the compound is introduced in a single application. As a consequence of this large amounts of ecdysonergic compounds circulate in the haemolymph and cause the telescoping of various morphogenetic events abruptly instead of promoting their sequential occurrence. This can lead to a disturbance of the regular co-ordinated processes which result in abnormal metamorphosis.

The specific activity and insect selectivity of the nonsteroidal agonists, especially the high toxicity of tebufenozide on Lepidoptera and nonsusceptibility of Coleoptera is related to selective binding of the compound on the EcRs

(Dhadialla *et al*., 1998; Smagghe *et al*., 1999; Wurtz *et al*., 2000; Retnakaran *et al.*, 2003). Methoxyfenozide binds with very high affinity to the EcRs in Lepidopteran insects where it functions as a potent agonist of 20-HE (Carlson *et al*., 2001). As a result of this, the larva undergoes apolysis and head capsule slippage. But these synthetic analogues were reported to bind strongly to the receptors and remain in place and repress the other genes necessary for cuticle elaboration, sclerotisation and ecdysis to be expressed (Retnakaran *et al.*, 2003) unlike 20-HE. Developmental arrest brought about at this stage would lead to a precocious incomplete molt that is lethal.

3.4.6 Effects on pupal-adult development

3.4.6.1 Effects of PPN

In the present study single treatments of newly ecdysed pupae of *S.mauritia* with different doses of PPN induced age/dose dependent mortality. Similar results were obtained from the experiments on *Chrysoperla rufilabris* (Liu and Chen, 2001). It was observed that mortality at the pupal stage was significantly higher than the larval and adult mortality in mosquitoes for all doses employed (Resende and Gama, 2006). Our earlier studies have shown that treatments of newly ecdysed pupae of *S.mauritia* with JHA considerably increased endogenous ecdysteroid titres (Mona, 2001). The disruption of endogenous ecdysteroid titre may eventually lead to production of toxic effects. This might have caused pupal mortality in all the treated doses.

The compound also distorted pupal-adult transformation to varying degrees. None of the treated pupae emerged successfully. Adultoids with crumpled wings were found inside the pupal case when the unemerged pupae were dissected. Formation of pupal-adult intermediates were observed in many Lepidoptera after treatments of JHA (Srivastava and Gilbert, 1969; Daoud and Sehnal, 1974; Srivastava and Prasad, 1982; Santha and Nair, 1988; Chanda and

Chakravorty, 2001). In insects, the pupal-adult metamorphosis has been demonstrated to be under the control of ecdysteroids, juvenile hormones (JH) and neuropeptides (Gilbert *et al.*, 1996). The prothoracic glands degenerate during eclosion (Gilbert, 1962) by undergoing programmed cell death or apoptosis (Dai and Gilbert, 1997). In normal conditions ecdysteroid titre drop to a negligible quantity towards the end of pupal phase for the eclosion hormone to be released (Truman, 1971; Riddiford, 1985). Ecdysteroids increase to a major peak in the haemolymph during pharate adult stage and this increase in the ecdysteroids titre promote pupal-adult metamorphosis. Further, during the normal pupal-adult metamorphosis, the absence of JH is a prerequisite for both the initiation and completion of prothoracic gland degeneration.

Earlier studies show that absence of JH in the pupal stage of lepidoptera is required for promoting the development of adult structures. So, treatment of pupae with juvenoids blocks their transformation into adults and this explains the development of adultoids in PPN treated pupae. This morphogenetic action of juvenoids was observed in *Sarcophaga bullata* (Fraenkel and Hsiao, 1968; Srivastava and Gilbert, 1969; Bhaskaran, 1972), *Tenebrio molitor* (Critchley and Campion, 1971), *Trogoderma granarium*, *Careydon gonagra* (Metwally and Sehnal, 1973), *Ceratitis capitata* (Daoud and Sehnal, 1974), *Ephestia kuhniella* (Tan, 1975), *Cylas formicarius* (Ram *et al*., 1980) and also in *Spodoptera mauritia* (Santha and Nair, 1987,1988).

Application of PPN to pupae resulted in complete inhibition of adult emergence in all the treated insect species. Though PPN did not prevent the earlier processes of moulting with lower doses, it prevented the final step of moulting i.e., ecdysis. Adult emergence was inhibited in the PPN treated pupae of *Plutella xylostella* (Oouchi, 2005), the mosquitoes *Aedes togoi* (Lee, 2001), *Anopheles culicifacies* and *A. subpictus* (Yapabandara and Curtis, 2004), *Aedes aegypti* (Sihuincha *et al*., 2005; Seng *et al*., 2006; Darriet and Corbel, 2006),

and *Culex quinquefasciatus* (Nayar *et al.*, 2002; Jambulingam *et al* ., 2008; Seccacini *et al*., 2008). The studies in honey bee *Apis mellifera* (Zufelato *et al.*, 2000) have proved that the application of PPN to the unpigmented pupae led to a delay of ecdysteroid peak by 4 days. The enzyme immunoassay measurements after the topical treatments of newly ecdysed pupae of *Tenebrio molitor* with PPN showed a decrease in ecdysteroid titre (Aribi *et al*., 2001, 2006).

The failure of emergence of JHA treated insects was suggested to be due to the inhibition of synthesis or release of eclosion hormone (Truman, 1971). The eclosion hormone is released by a drop in the ecdysteroid level after a peak level, prior to ecdysis (Riddiford, 1985). It may be suggested that in *S.mauritia*, PPN application may have shifted the ecdysteroid peak towards the late pupal phase as seen in the case of *Apis mellifera* (Zufelato *et al*., 2000) and this might have blocked the release of eclosion hormone. Treatments with the JHA fenoxycarb before and after pupal ecdysis of *Bombyx mori* disturbed adult eclosion and the insects were unable to escape from the pupal exuviae (Dedos and Fugo, 1999a). Occurrence of permanent pupae was also observed in *Bombyx mori* after fenoxycarb applications (Dedos and Fugo, 2002). Since the treated pupae of *S. mauritia* failed to perform eclosion movements with the result that the pupal case remained attached to the body of adult insects, it may be suggested that PPN influence some mechanism responsible for eclosion.

Ecdysteroids are the principal endocrine signaling molecules which trigger apolysis and promote the new cuticle formation (Quennedey *et al*., 1983). Studies on the mode of action of ecdysteroids indicate that the ecdysone receptor-ultra spiracle protein complex (EcR-USP) binds to the ecdysteroid response element to modulate gene transcription (Yao *et al*., 1993; Henrich, 2005). USP has been shown to bind JH and juvenoids and act as a modulator of JH action (Jones and Sharp, 1997; Mu and Leblanc, 2004; Goodman and Granger, 2005). As a consequence, JH blocks the ability of ecdysteroids to

induce the expression of some genes in insects (Cherbas *et al*., 1989; Cottam and Milner, 1998). This JH modulator action of hormone-receptor might have led to the failure of PPN treated pupae to emerge successfully in the present experiments. Juvenoids possibly act as anti-ecdysteroids through a molecular mechanism involving inhibition of ecdysteroid signaling (Tuberty *et al*., 2005).

The treatments of PPN on *S. mauritia* resulted in varied response of tissues which suggests that the sensitivity of different tissues to PPN is different. Some of the adultoids obtained from the unemerged pupae had very few hairs developed in the abdominal region. This effect of PPN on the imaginal differentiation of abdominal cells is consistent with the earlier observations of other workers with other JH analogues in *Sarcophaga bullata* (Bhaskaran, 1972) and *S. mauritia* (Santha and Nair, 1988). The present studies have also shown that treatments of newly ecdysed pupae had the morphogenetic effects to the maximum when compared to the treatments of tanned pupae. The differential effects of the compound on pupal-adult development after treatments show that the critical time for the onset of transformation of different parts or organs is different. Earlier observations had also shown that the critical periods for the differentiation of different organs of pupa occur at different times (Gilbert and Scheiderman, 1960; Srivastava and Gilbert 1969; Metwally and Sehnal, 1973; Slama *et al*., 1974; Willis, 1974).

3.4.6.2 Effects of methoxyfenozide and halofenozide

The effects of pupal treatments with lower doses of methoxyfenozide and halofenozide were age/dose dependent. Methoxyfenozide treatments with the lower doses resulted in mortality of a few percentages of treated pupae and the rest of them emerged into normal looking adults whereas lower doses of halofenozide did not interfere with the adult emergence. The peaks of expression of most of the ecdysone-regulated genes coincided with the rise in ecdysteroid

levels during the last larval and pupal stages of *Aedes aegypti* whereas in the early pupal stage the expression of ecdysone regulated genes was different (Margam *et al*., 2006). The time of application was reported to be important for ecdysteroid analogues/agonists to exert their effects on target tissues (Pszcolkowski *et al*., 1998). The differences in tissue distribution and protein levels of EcR-B1 during the programmed cell death of anterior silk glands of *Bombyx mori* indicated that the receptors play a major role in the modulation and function of ecdysone activity (Goncu and Parlak, 2008, 2009). The lack of adequate number of ecdysone receptors in the target tissues of pupa at the time of treatment might explain why lower doses of ecdysone agonists had minimum influence on pupal-adult metamorphosis. These observations are comparable to the lack of sensitivity shown by eggs.

The females emerged from the pupae treated with lower doses of ecdysone agonists laid very few eggs when kept with the males emerged from the treated pupae. The eggs were scattered in appearance and were not attached to the substratum. Methoxyfenozide treatments reduced fecundity and fertility in *Lobesia botrana* (Saenz-de-Cabezon Irigaray *et al*., 2005); *Cydia pomonella* (Sun and Barrett, 1999; Knight, 2000; Bylemans *et al*., 2003); *Spodoptera litura* (Seth *et al*., 2004); oblique-banded leaf roller, *Choristoneura rosaceana*, *Argyrotaenia velutinana* (Sun *et al*., 2000) and *Grapholita molesta* (Borchert *et al*., 2004a; 2005).Treatments with higher doses of ecdysone agonists increased pupal mortality. There are evidences that the ecdysone agonists exhibited insecticidal activity in pupal treatments of Mediterranean flour moth *Ephestia kuehniella* (Hami *et al*., 2005). It also resulted in failure of pupae to emerge into normal adults. Adultoids were found inside the unemerged pupae when the pupal cuticles were removed. Inhibitory effects of ecdysteroids on cell division and differentiation after application of high doses was reported in *Drosophila* cell lines (Wyss, 1976). The formation of adultoids in *S. mauritia* might be due to the high titre of methoxyfenozide prevailing in the haemolymph which would have

exerted inhibitory effects on differentiation leading to the altered development. It was proved from earlier studies that a drop in the ecdysteroid titre is a prerequisite for the release of eclosion hormone (Truman, 1971; Riddiford, 1985) which is necessary for adult emergence.

Due to high specificity of action against Lepidoptera methoxyfenozide is considered as environmentally friendly compound (Palli and Retnakaran, 2001). The foregoing observations clearly indicate that ecdysone agonists have a specific mode of action that is mainly connected with ecdysis. It appears that they induced no direct toxic effect as conventional insecticides. At sub lethal doses they show similarity to the other IGRs in causing developmental retardation and deformities, while at lethal doses the effect was mainly connected with ecdysis. This characteristic together with low toxicity towards warm blooded animals may render them quite safe for use in pest control strategies unlike the conventional broad spectrum insecticides.

3.4.7 Morphogenetic effects of hormone agonists

3.4.7.1 Morphogenetic effects of PPN

The compound PPN induced moulting symptoms of various degrees in treated sixth instar larvae of *S. mauritia*. A majority of the treated larvae including wandering larvae and prepupae exhibited moulting mark in all the treatments. PPN induced the production of superlarvae from treated day 3 larvae with a number of pupal characteristics such as sclerotised mouth parts, antennae and thoracic legs. This finding confirms and supplements the results of earlier experiments in last larval instars of *Galleria mellonella* (Slama and Mala, 1984), *S. mauritia* (Santha and Nair, 1987; Balamani and Nair, 1991; Venugopalan, 1995; Mona, 2001) and *S. litura* (Hatakoshi *et al*., 1986, Wu and Hu, 2008). Application of JHAs to early last instar larvae result in development of supernumerary instars whereas treatments of later stages lead to abnormal

pupation and development of larval-pupal mosaics or larval-pupal intermediates (Koçak and Kilinçer, 1997).

The pupae formed from PPN treatments of larvae had their pupal cases not joined ventrally at the thoracic region thus exposing the inner structures; the larval legs were found to be retained, attached to the pupal case. This is consistent with the earlier observations that PPN treatments of the nymphs of the direct developing cricket after the first moult resulted in failure to complete dorsal closure and also precocious formation of nymphal characters (Erezyilmaz *et al.*, 2004). It has been reported that JH or its analogues, administered externally exert their morphogenetic effects along with other environmental stimuli by either inhibiting or accelerating moulting process through interference with the prothoracic glands and brain (Slama, 1971; Gilbert *et al.*, 1980; Sehnal, 1983). The action of JH is either inhibiting or stimulating depending on the developmental state (Cymborowski and Stolarz, 1979; Hiruma and Agui, 1982). The failure to complete moulting/defective pupal cuticle formation observed in treated larvae might be the consequence of a high titre of JH analogue PPN in the haemolymph which would have led to deleterious effects in morphogenesis. PPN might have interfered in prothoracic gland activity thus inhibiting the processes of pupal cuticle formation in the treated larvae.

Extreme shrinkage of larval body was observed in PPN treated last instar larvae of *S. mauritia*. Fenoxycarb, the precursor molecule of PPN at high doses was reported to affect important nutritional parameters which suggest a reduced efficiency in the utilization of ingested food (Leonardi *et al.*, 1998). When last instar larvae of silkworm were treated with JHA, feeding activity declined (Sakurai and Imokawa, 1998) and larvae remained in the larval stage for more than two weeks. PPN prevented larval feeding by antifeedent characteristics (Etebari *et al.*, 2007). PPN might have elicited a similar response in the treated early last instar feeding larvae thus preventing utilization of ingested food and

the larval growth.Another possibility is the decline in muscle volume following PPN treatments as observed in JHA treatments. Topical application of synthetic JHAs induced highly significant muscle volume decline in *Ips confuses* (Borden and Slater, 1968), *Ips paraconfuses* (Unnithan and Nair, 1977) and the cricket, *Gryllus firmus* (Zera and Cisper, 2001). In *Manduca sexta*, the endocrine event which triggers the pupal development was a fall in the blood titre of JH followed by two subsequent elevations of blood ecdysteroids. The commitment pulse was found to cause both qualitative and quantitative changes in the sensitivity of the larval muscles and motor neurons (Weeks and Truman, 1985). After the commitment pulse, exposure to ecdysteroids in presence of JH caused regression and death of larval cells (Weeks and Truman, 1986, Weeks *et al*., 1993). Juvenoids reduced the larval growth in *Bombyx mori* (Leonardi *et al*., 1996). Ultra structure studies of the fourth larval instar of *Culex pipiens* treated with PPN revealed the destruction of procuticle lamellae, formation of cuticular vacuoles, deformed mitochondria and destruction of nuclear envelops and the epidermal layer and an increase in electron-dense lysosome like bodies (El-shazly and Refaie, 2002). PPN treatments of silkworm larvae after 4^{th} moult showed that in 24 h after the application, the amounts of glucose, urea, uric acid, cholesterol, total protein, alanine amino transferase, aspartate aminotransferase and alkaline phosphatase showed a decrease (Etebari *et al*., 2007). Fenoxycarb, the precursor molecule of PPN enhanced dose dependently the activity of larval muscle and silk gland protease and also certain other enzymes in *Bombyx mori* indicating an upsurge in the oxidative metabolism of larval tissues (Mamatha *et al*., 2008). The shrinkage of the larval body observed in treated last instar larvae might be due to a similar response of PPN.

3.4.7.2 Morphogenetic effects of methoxyfenozide and halofenozide

The treated larvae of various age groups showed premature lethal moult after treatments with methoxyfenozide and halofenozide. They exhibited the

pharate head capsule as a white collar like structure. Moulting fluid was found to be exuding out which turned black on exposure to air. These larvae were black in colour which died with the body soaked in the fluid. The larval cuticle was found to be sac like and fluffy as though filled with fluid. Methoxyfenozide and halofenozide treatments were successful in initiating a premature lethal moult and mortality in larvae of *S. littoralis* (Smagghe *et al.*, 2001), *Lobesia botrana* (Saenz-de-Cabezon Irigaray *et al.*, 2005), *Harmonia axyridis* (Carton *et al.*, 2003). The remnants of old unshed cuticle were visible through the black colouration in Colarado potato beetle, *Leptinotarsa decemlineata* (Smagghe and Degheele, 1993; Carton *et al.*, 2000). Larval treatments with methoxyfenozide disturbed the development and reproduction of the hairy rose beetle, *Tropinota squalida* (Hussein et al., 2004).

A large number of the treated larvae showed rectal prolapse. The treated prepupae showed rectal prolapse and a fluid discharge from the prolapsed rectum. The phenomenon of rectal prolapse was observed in a few of the sixth instar larvae moulted from the treated fifth instar and also the sixth instar larvae which received treatments on various days of the larval stadia. This phenomenon was observed by some earlier workers after injection of high doses of ecdysone to the last instar larvae of *Bombyx mori* (Kurisu and Manabe, 1976), *Spodoptera litura* (Negishi *et al.*, 1976), Colarado potato beetle, *Leptinotarsa decemlineata* (Smagghe and Degheele, 1993; Carton *et al.*, 2000) and Asian lady beetle *Harmonia axyridis* (Carton *et al.*, 2003). But they could not give any explanation to this interesting phenomenon. It may be pointed out that the rectal prolapse is observed only in the last larval instar which is destined to undergo larval-pupal metamorphosis. The ecdysone agonists methoxyfenozide and halofenozide might have caused a hyperstimulation of cuticle forming processes in the rectal epithelia resulting in rectal prolapse. Since the rectal epithelial cells are ectodermal in origin, it is possible that these cells also undergo a similar switchover in commitment during pupal development; In support of this, a few

in vitro and *in vivo* studies have shown that in *Manduca sexta* and *Hyalophora cecropia*, the rectal epithelium exposed to ecdysteroids undergoes considerable expansion and a high degree of differentiation resulting in rectal dialation and prolapse (Judy, 1969; Judy and Gilbert, 1970; Judy and Marks, 1971).

Methoxyfenozide treatments of late last instar larvae resulted in formation of larval-pupal intermediates and also a few pupae. The surge of ecdysteroids on day 3 is responsible for the change in commitment from larval to pupal type. Second peak of ecdysteroids on day 5 initiates the pupal moult and the commitment of epidermal cells to pupate remains unaffected (Oberlander, 1985). The prothoracic glands show maximum secretory activity on day 4 and day 5 of the last larval instar in *S. mauritia*, (Balamani, 1990; Balamani and Nair, 1994). A high titre of nonsteroidal ecdysone agonists occur in last instar larvae after the exogenous application which interferes with the normal pupation and produce a large proportion of larval-pupal intermediates. Treatment of pupae with higher doses of methoxyfenozide induced development of adultoids in them. The hyperecdysonism resulting from the abrupt administration of ecdysone agonists might be the reason for this as a decrease in ecdysteroid titre is a prerequisite for the release of eclosion hormone (Truman, 1971; Riddiford, 1985).

Shrinkage of larval body was observed in treatments of last instar larvae of *S. mauritia* with ecdysone agonists. In *Manduca sexta* a fall in the JH titre followed by two subsequent elevations of ecdysteroids promote pupal development. This commitment pulse was found to cause both qualitative and quantitative changes in the sensitivity of the larval muscles and motor neurons (Weeks and Truman, 1985). After the commitment pulse, exposure to ecdysteroids in presence of JH caused regression and death of larval cells (Weeks and Truman, 1986). The shrinkage of the larval body observed with ecdysone agonists might be due to degeneration of muscles induced by exposure to excess titre of ecdysone agonists after the commitment peak.

3.5 Summary

1. Different developmental stages of *S. mauritia* were treated by contact method and also by topical application of various doses of a juvenile hormone agonist (JHA) PPN, and two ecdysone agonists methoxyfenozide and halofenozide. The effects of these compounds on the embryogenesis, postembryonic development and metamorphosis were studied.

2. Treatments of newly laid egg mass with PPN resulted in dose dependent embryonic lethality. Mortality was observed in the subsequent larval development also. The larvae, pupae and adults developed from treated eggs were larger in size when compared to controls. Methoxyfenozide and halofenozide treatments had no noticeable effect on egg hatchability. But the hatched out larvae died in methoxyfenozide treatments where as mortality was not observed after halofenozide treatments.

3. Treatments of day 0 first and second instar larvae with 0.01, 0.1 and 1µg of PPN/methoxyfenozide/halofenozide were carried out by contact method. This resulted in dose dependent high rate of mortality within 24 h in the case of PPN and methoxyfenozide. Halofenozide induced mortality only at higher doses. The surviving larvae also showed high rate of mortality during their subsequent larval development. Only very few percentage of these larvae pupated. The pupae developed from treated larvae did not emerge as normal adults but died after surviving for 10-13 days.

4. Topical applications of PPN on day 0 third, fourth and fifth instar larvae produced high mortality and extension of pupal-adult period. The treated larvae showed a significant delay in larval development and metamorphosis. Methoxyfenozide induced premature lethal moult in the treated larvae and halofenozide was found to be effective at higher doses.

5. Treatments of various ages of sixth instar larvae with different doses of PPN, methoxyfenozide and halofenozide induced age-dependent effects on larval period and metamorphosis. Extension of larval period was observed after PPN treatments of day 0, day 1and day 2 larvae. The treatments of day 3, day 4 and day 5 larvae showed no marked difference in the timing of larval-pupal moulting. These results are similar to the earlier observations that when the final instar larvae were treated with JHA before they attain their maximal body weight, the feeding period was extended. Methoxyfenozide and halofenozide treatments of day 0 and day 1 larvae resulted in moulting symptoms; treatments of day 3 resulted in moulting into imperfect superlarvae and treatments of day 4 and day 5 led to production of a large number of larval-pupal intermediates.

6. No pupation was observed in larvae treated with PPN on different days of sixth instar. Only larval-pupal intermediates were formed from them. The larvae treated on all days exhibited shrinking of body to very small size. Treatments of methoxyfenozide resulted in high mortality and production of larval-pupal intermediates. Body shrinkage was observed in treatments with ecdysone agonists also. Halofenozide treatments resulted in a higher rate of pupation and production of larval - pupal intermediates. Very few of them emerged into adults.

7. Treatments of pupae of *S.mauritia* with different doses of PPN inhibited their transformation into adults. Higher doses induced 100% mortality. Lower doses not only inhibited ecdysis but also induced the formation of adultoids. Treatments with ecdysone agonists induced mortality at higher doses. Lower doses led to pupation and adult emergence in a considerable percentage of treated insects. The emerged females on mating with the males emerged from the treated pupae laid very few eggs which were scattered and were not attached to the substratum. None of them hatched.

4. 1. Introduction

The understanding of the effects of IGRs on reproductive biology is of fundamental importance in the Integrated Pest Management (IPM) programmes in the context of potential use of these compounds as insect control agents. Insect metamorphosis is characterized by a sequence of morphological changes which are regulated and controlled by haemolymph titres of several hormones. The brain of insects synthesizes the brain hormone or the prothoracicotropic hormone (PTTH) which stimulates the prothoracic glands to synthesise and secrete moulting hormone (MH). MH initiates the moulting process though the nature of the moult is determined by JH. A high titre of JH leads to larval-larval moult and that of MH leads to larval-pupal moult and absence of JH leads to pupal- adult moult.

Moult affecting IGRs usually interfere with the proper formation and hardening of a new cuticle or interfere with the initiation of moult. The fact that the fluctuations in the haemolymph titres of MH are responsible for the moulting and metamorphosis in insects was not supported by experimental evidences earlier. It was proved by experiments on *Bombyx mori* (Burdette, 1962; Shaaya and Karlson, 1965) and *Calliphora erythrocephala* (Fraenkel and Zdarek, 1970) that the MH titres varied during larval-larval, larval-pupal, and pupal-adult development. The major IGRs are JHAs / anti JH agents and the moult accelerating compounds such as ecdysone analogues/agonists. These compounds have great potential in IPM programmes as insect control agents (Nair, 1993).

Among the JHAs, Pyriproxyfen (PPN) is found to be very effective in controlling the lepidopteran insects (Smagghe and Degheele, 1994). Topical application of PPN on pupae of *Spodoptera litura* reduced the number of eggs oviposited (Hatakoshi, 1992). Pyriproxyfen induce defective embryogenesis in the desert locust, *Schistocerca gregaria* (Vennard *et al*., 1998, Horowitz *et al*.,

1999; Mohandas *et al*., 2006) and *Aionidiella aurantii* (Eliahu *et al*., 2007). Ecdysone agonists such as RH 5849, tebufenozide, halofenozide and methoxyfenozide control the insects successfully. Tebufenozide significantly affected reproductive parameters in adults such as fecundity, fertility, egg laying period and embryonic duration of development in Mediterranean flour moth, *Ephestia kuehniella* (Khebbeb *et al*., 2008).

The reproduction and development in insects occurs under the influence of hormones and a critical titre of JH and ecdysteroids in haemolymph is necessary for their development and metamorphosis. The increase or deficiency of hormones result in severe deformities in the reproductive organs and also the eggs/sperms developed. This leads to incapability of mating and prevent normal fertilisation. The sperm development in insects occurs in spermatocytes in which germ cells develop more or less synchronously surrounded by a capsule of somatic cells (Chapman, 1998). During the spermatogonial stage, cysts begin to be formed and a fully grown cyst is a hollow sphere of 32-64 germ cells surrounded by an envelope. In most insects, the spermatogonia and spermatocytes develop in the nymphal and pupal stages and the testes of the imago contains only spermatids and spermatozoa. Spermatogenesis is initiated with the formation of the primary spermatogonia which develop into spermatocytes. They undergo meiotic divisions and form spermatids which then differentiate into spermatozoa. Maturation divisions that occur in the larva give rise to eupyrene sperm bundles while those that occur in the pupa give rise to apyrene sperm bundles (Numata and Hidaka, 1980).

The production of a mature egg involves various cellular mechanisms for the synthesis of the reserves of RNA containing molecules. In insects such as Apterygota, Orthoptera etc., oocyte nucleus is the source of all of the RNA contained within the mature egg. In Lepidoptera, Hymenoptera, Diptera and some Coleoptera a cluster of cells called nurse cells or the trophocytes which

originate from germinal tissue produce nearly all of the RNA required for the growing oocyte and mature egg. Oocyte maturation occurs in two stages in which the first phase is a period of slow growth and the second is a period of rapid growth and massive accumulation of yolk in the oocyte.

Development of germ cells in insects is controlled by hormones released by corpora allata (Fukuda, 1944), ecdysteroids (Handler and Postlethwait, 1977) and also brain hormones (Loeb et al., 1985, 1986). Spermatogonial mitotic divisions occur throughout the early instars but spermatogenesis begins in the last larval or pupal instar when ecdysteroids stimulate the process under a low JH titre (Engelmann, 1970). Wigglesworth (1936) concluded that JH is not necessary for spermatogenesis. But studies on *Bombyx mori* (Takeuchi, 1969; Yagi and Fukushima, 1975), *Dysdercus cingulatus* (Ambika and Prabhu, 1978) and many other insects (Dhadialla *et al*., 1998) showed that JH or its analogues can induce defective spermatogenesis or alter/inhibit the process. On the other hand, ecdysteroids were known to promote spermatogenesis in *Bombyx mori* (Takeuchi, 1969; Kawamura *et al*., 2003), *Hyalophora cecropia* (Kambysellis and Williams, 1971, 1972), *Monema flavescens* (Takeda, 1972), *Rhodnius prolixus* (Dumser and Davey, 1975) and also in many other insects (Happ, 1992). Brain hormone is believed to be involved in the testis ecdysone production and the brain removal in *Heliothis virescens* larvae inhibited spermatocyte elongation (Loeb *et al*., 1985).

Oogenesis is controlled by JH which plays a central role in vitellogenesis both in the biosynthesis and their incorporation into growing oocytes. It is necessary for yolk deposition and egg maturation (Wigglesworth, 1936; Kaiser, 1949; Thomsen, 1952, Thomsen *et al*., 1980; Mundall and Engelmann, 1977; Pan, 1977; Sorge *et al*., 2000). Ecdysteroids are known to stimulate vitellogenin synthesis and uptake of vitellogenin to ovary in various insects (Tsuchida *et al*., 1987; Dhadialla and Raikhel, 1992; Sun *et al.*, 2003; Ogihara *et al.*, 2007). Both JH and ecdysteroids stimulate yolk protein synthesis in fat body and ovary in

Drosophila melanogaster (Bownes *et al*., 1993; Handler and Postlethwait, 2005). JH initiates only early stages of vitellogenesis as well as ecdysteroid synthesis in ovaries whereas 20-HE plays the major role in the control of oogenesis (Richard *et al.,* 2001 a, b). The increase in 20-HE titre led to changes in the level of JH, the phenomenon being mediated by the neuropeptides dopamine, thus controlling the fertility in *Drosophila virilis* (Rauschenbach *et al*., 2004, 2007).

There are a number of evidences for the regulatory role exerted by the neurohormones on the process of vitellogenesis. The neurosecretory cells of pars intercerebralis exert a direct action on ovarian development in *Calliphora erythrocephala* (Thomsen, 1952) *Musca domestica* (Lea, 1975) and *Locusta migratoria* (MčCaffery, 1976). In some insects the action of pars intercerebralis is exerted by inducing the release of ovarian ecdysone which triggers the fat body to produce yolk proteins (Thomsen *et al.*, 1980). Factors from the head are proved to be essential for the corpora allata activation and the egg maturation in mosquitoes (Hernández-Martínez et al., 2007). The present study deals with the effects of hormone agonists on the reproductive biology of *S. mauritia*. The sixth instar larvae and pupae of *S. mauritia* were treated with the compounds and the effects of these treatments on the process of reproduction were studied.

4.2. Materials and methods

4.2.1 Experimental animals

The larvae and pupae used for various experiments were obtained from laboratory stock culture reared and maintained as described earlier (Chapter 2).

4.2.2 Chemicals

The JH agonist, PPN and the ecdysone agonist halofenozide were dissolved and diluted in acetone to obtain the required concentrations. Ecdysone

agonist methoxyfenozide was dissolved in distilled water and diluted to obtain different concentrations. For the various treatment procedures, measured quantities of these compounds were used utilizing a Hamilton micro syringe.

4.2.3 Treatment of larvae and pupae

Sixth instar day 0 larvae and newly ecdysed pupae were topically treated with three different doses of PPN/methoxyfenozide/halofenozide such as 0.1 µg, 1µg and 10µg. Fifth instar day 0 and day 1 larvae were also treated in studies with PPN as the sixth instar larvae showed failure of pupation. The compound was applied topically on the abdominal region of larvae/ newly ecdysed pupae using a 10 µl Hamilton microsyringe. The control larvae/pupae received equal volumes of solvent only. The experimental and control larvae were maintained as in the case of laboratory stock culture. Experimental and control pupae were kept in separate beakers covered with muslin cloth. The effects of the treatments of the compounds on reproductive structures were studied.

4.2.4 Surgical techniques (See Chapter 2)

4.2.5 Histological techniques (See Chapter 2)

4.3 Results

4.3.1 Effects of hormone agonists on morphogenesis of testes

The reproductive system of adult male *S. mauritia* consists of a single median testis, a pair of seminal vesicles, vasa deferentia, accessory sex glands, and ductus ejaculatorius duplex (Pl. X: Fig 94). In the larvae of *S.mauritia* testes are paired organs, ellipsoidal in shape situated dorsolateral to the alimentary canal. Each testes lobe is made up of four follicles. In the pharate pupal stage testes are seen to fuse into a single median structure located in the mid dorsal line. The testis becomes sphere like and this shape is maintained throughout

pupal and adult period. The epithelial strands are lost before the formation of fused testes.

PPN treatments of larvae resulted in severe morphogenetic abnormalities of testes. The fifth instar day 0 larvae treated with the dose 5 μg showed failure to complete fusion of testes lobes when the testes were dissected out from the large sized pupae on day 2 (Pl. X: Fig. 95). Various morphological abnormalities were observed in testes dissected out from day 2 pupae which were formed from fifth instar larvae treated on day 1 with 5 μg of PPN (Pl. X: Fig. 96). The sixth instar larvae treated on day 0 with 0.01 μg of PPN formed larval-pupal intermediates. The testis of these larval-pupal intermediates did not show the proper sphere shape and was irregular in outline with abnormal depressions on the surface (Pl. X: Fig. 97). PPN treatments of newly ecdysed pupae with the doses 0.01 μg, 5 μg and 10 μg of PPN did not induce much morphological abnormalities on pupal testis. Testis was small in size (Pl. X: Fig. 98) when compared to the normal pupal testes (Pl. X: Fig. 99). Treatments of larvae/pupae with the above mentioned doses of ecdysone agonists did not induce morphological abnormalities and the testis was normal in appearance.

4.3.2 Effects of hormone agonists on histology of testes

The testes in *S. mauritia* is encapsulated and covered with a sheath of 3 membranes. The larval testis is bound by tunica externa and tunica interna. Four follicles of each testes are covered with membrana communis which is a thin connective tissue layer made up of irregularly shaped cells. The thickness of these cells decreases with the age. In the pharate pupal stage the membrane communis is seen as a very thin layer inside which the testes fuse. The tunica externa develop over the membrana communis to cover the whole testis.

In the late pupal stage, the four follicular nature of testes is lost. The testis is divided into eight longitudinal compartments by a membrane which resembles

capsula lobuli. Spermatogonial cysts are found at the periphery of the testicular lumen and sperm bundles and elongating spermatids are seen in the middle region of testes. In the eupyrene spermatozoa, the nuclei are seen in a band at the head of the bundle whereas in the apyrene spermatozoa the nuclei are granular in shape and scattered in the central zone or along the length of sperm bundle. Fully formed sperm bundles appear for the first time in the pupal period. Maturation divisions that occur in the larva give rise to eupyrene sperm while those that occur in the pupa give rise to apyrene sperm. The eupyrene spermatozoa which develop from larval spermatocytes are considered to be the functional sperms and the apyrene sperms which originate from pupal spermatocytes degenerate.

The results of the present study demonstrate that treatments of larvae with PPN inhibited spermatogenesis. The normal development of spermatogonia, spermatocytes, spermatids, eupyrene and apyrene sperm bundles were retarded in topical treatments of different doses of PPN such as 0.01 µg, 5 µg and 10 µg. Masses of disintegrating cysts was seen in sections. The histological preparations of testis dissected out on day 3 from 10 µg PPN treated sixth instar day 0 larvae showed the presence of interfollicular septa (Pl. XI: Fig. 100). The germinal cysts could not be distinguished spermatogonial or spermatocyte cysts and the interior of testes gave the appearance of an unorganized mass. Wide spaces were present in testicular tissue (Pl. XI: Fig. 101). The sections of testes of control day 3 larvae showed well developed spermatogonial and spermatocyte cysts (Pl. XI: Fig 102). Treatments of sixth instar day 0 larvae with high doses of methoxyfenozide such as 5 µg and 10 µg resulted in a large percentage of larvae undergoing lethal moult and mortality. The larvae died with the moulting fluid exuding out which turned black on exposure to air. The testes dissected out from the day 3 larvae treated on day 0 with 0.01 µg of methoxyfenozide showed an acceleration of spermatogenesis. Histological preparations of testis showed the presence of well developed spermatocysts and spermatids and a few sperm

bundles (Pl.XI: Fig. 103). Treatments of sixth instar day 0 larvae with higher doses 5 µg and 10 µg of halofenozide did not induce considerable changes in the spermatogenesis.

Fully formed sperm bundles can be easily recognized in pupal testes of untreated *S.mauritia*. The pupae formed from the PPN treatments of fifth instar day 0 and day 1 larvae with the doses 0.01 µg, 5 µg and 10 µg did not emerge into adults. Adultoids were found inside when the pupal cuticle of these unemerged pupae were removed. The histological preparations of testis of these adultoids dissected on day 7 showed disintegrating germ cells which appeared to have stopped proliferation and differentiation (Pl. XI: Fig.104). The coalescing lobes of testis were visible in sections but the processes of fusion and torsion were not completed. Treatments of newly ecdysed pupae with 0.01 µg and 5 µg of PPN also resulted in retardation of spermatogenesis. The cells appeared to have stopped differentiation and were found scattered showing lack of cohesion between them (Pl. XI: Fig. 105). PPN treatments with 10 µg PPN resulted in a total disintegration of germinal cysts. The spermatogenesis was completely suppressed and the necrotic nature of tissues was visible by the presence of darkly stained bodies (Pl. XI: Figs. 106, 107).

Treatments of newly ecdysed pupae with 0.01 µg of methoxyfenozide resulted in an acceleration of spermatogenesis. Histological preparations of testis dissected out on day 3 showed a large number of sperm bundles (Pl. XII: Fig. 108). Control pupal testis dissected on day 3 showed spermatocyte cysts and a few spermatids and sperm bundles (Pl. XII: Fig. 109). The testis of day 3 pupae which were treated on day 0 with 0.01 µg of halofenozide was similar to the testis of control pupae. A few spermatogonial cells, spermatids and also a few sperm bundles were observed in histological preparations (Pl. XII: Fig. 110).

The treatments with 5 µg and 10 µg of methoxyfenozide increased pupal mortality. The unemerged pupae had adultoids inside them when the pupal cuticle was removed. The testis of adultoids formed from 5 µg treatments exhibited malformed cells and also degenerated sperm bundles when the dissections were made on day 6 (Pl. XII: Fig. 111). The testis of control pupae on day 6 showed spermatocysts, spermatids and a large number of eupyrene and apyrene sperm bundles (Pl. XII: Fig. 112). The testis of adultoids from 10 µg treatments of newly ecdysed pupae also showed the presence of a large number of malformed sperm bundles (Pl. XII: Fig. 113). Wide gaps were seen between sperm bundles showing lack of cohesion between them. Halofenozide treatments with 0.01 µg and 5 µg did not induce any marked change in spermatogenesis. Treatments of the newly ecdysed pupae with 10 µg of halofenozide induced formation of adultoids. Testis dissected out from the adultoids showed malformation of the spermatids and sperm bundles (Pl. XII: Figs. 114, 115).

4.3.3 Effects of hormone agonists on morphogenesis of ovary

Adult female reproductive system of *S. mauritia* consists of a pair of ovaries, oviduct, bursa copulatrix, spermatheca with its spermathecal gland, paired accessory glands and vagina (Pl. XIII: Fig. 116). Each pair of ovary comprises four ovarioles which are continuous with the lateral oviducts by means of a short pedicel. The paired lateral oviducts fuse to form the median oviduct. Bursa copulatrix is a pouch like structure. Seminal duct arise from the anterior end of bursa copulatrix and join at point of attachment of the spermathecal duct. Accessory sex glands are found under the spermatheca as a pair of elongated tubular glands. Ovarioles are hardly visible during larval period. The ovariole growth and follicle differentiation take place during pupal-adult period.

Treatments of newly ecdysed pupae of *S. mauritia* with 0.01 µg, 5 µg and 10 µg of PPN induced severe abnormalities in the morphogenesis of ovaries. The

ovarioles were reduced in size and large gaps were seen in between the apparently degenerating follicles. Methoxyfenozide treatments of pupae with the lower dose 0.01 µg resulted in normal development of ovarioles (Pl. XIV: Fig. 124). But treatments of newly ecdysed pupae with 5 µg and 10 µg of the compound induced deleterious effects on morphogenesis. The ovarioles did not develop properly and the follicles were arranged loosely in a bead-like fashion along the length of ovarioles. Halofenozide did not induce any marked abnormalities in the morphogenesis of ovarioles.

4.3.4 Effects of hormone agonists on histology of ovary

The treatments of sixth instar day 0 larvae with the different doses of PPN such as 0.01 µg, 5 µg and 10 µg induced no considerable changes in the ovarian development when compared to controls. But the treatments of newly ecdysed pupae with the different doses of PPN such as 0.01 µg, 5 µg and 10 µg induced various abnormalities in the ovarian development. Histological preparations of the ovaries dissected out from the adultoids formed from pupae treated on day 0 with 0.01 µg of PPN showed many abnormalities when compared to the ovary of control adult (Pl. XIII: Fig. 118). Ovarian development was retarded and the follicles were found to be degenerating. They could be easily distinguished by their opaque nature and dark pink colour (Pl. XIII: Fig. 117).

Higher doses of PPN produced more pronounced effects on ovarian growth. When the ovaries of adultoids formed from 5 µg PPN treated pupae were dissected out, many follicles were found to be fused together and arranged in 2-3 rows forming compound egg chambers (Pl. XIII: Figs. 119, 120). None of the follicles have completed yolk deposition. Along the length of ovarioles, blank spaces without any follicles were observed. The follicles did not have their characteristic round or spheroid shape and were triangular and had other abnormal shapes. The oocyte area was drastically reduced when compared to the

total area of trophocytes. Ooplasm did not exhibit signs of vitellogenesis and yolk deposition. Treatments with 10 µg of PPN also induced similar effects and compound egg chambers were frequently observed (Pl. XIII: Figs. 121, 122). The egg chambers were seen to undergo progressive disorganization and disintegration (Pl. XIII: Fig. 123).

The treatments of newly ecdysed pupae with different doses of methoxyfenozide such as 0.01 µg, 5 µg and 10 µg induced dose dependent effects. There was an acceleration of oogenesis after treatments with the lowest dose 0.01 µg and well developed follicles were observed in histological preparations (Pl. XIV: Fig. 125). The yolk content of follicles was clearly visible in sections. But the follicles did not develop further with the nurse cells and oocytes showing signs of degeneration (Pl. XIV: Fig. 126). One significant feature of many of the follicles was that the follicular epithelial cells were lacking in some or were loosely arranged while in others they appear to have slipped off from the surface of follicles. The follicular epithelial cells were of the columnar type as seen in the case of normal oocytes.

After treatments with 5 µg of methoxyfenozide, the ovarioles and the follicles were well developed and the oocytes showed presence of large amount of yolk granules in them which indicated occurrence of normal vitellogenesis (Pl. XIV: Fig. 127). But the follicles did not develop further and exhibited degeneration (Pl. XIV: Fig. 128). The highest dose 10 µg induced abnormal development and the follicles were found to be degenerating as seen earlier (Pl. XIV: 129).

Treatments with the lower doses of halofenozide such as 0.01 µg did not induce any change in the development of ovarian follicles and oogenesis. The treatments with 5 µg induced a slight acceleration in ovarian development when the ovaries were dissected out from adultoids on day 7. The follicles had a

syncytial appearance. The nurse-cell/oocyte syncytium of adultoids was fully developed and they were in the previtelline stage (Pl. XIV: Fig. 130). The ooplasm was finely granular with a large number of yolk granules and large cells arranged towards the peripheral region as seen in developing oocytes. Highly basophilic granules were seen in the follicular area. The nutritive cords were distinct in the histological preparations. The treatments with 10 µg induced the formation of adultoids, the ovaries of which showed many abnormalities in development (Pl. XIV: Fig. 131). The follicle cells were squamous in shape as seen in the earlier developmental stage of the insect and space was seen in between the oocytes and follicle cells. The trophocytes were found to be degenerating. The ovaries seemed to have commenced their development normally but showed signs of degeneration without completing the maturation process.

4.4 Discussion

4.4.1 Hormone interactions in the reproductive processes of insects: a brief preview

4.4.1.1 Hormone interactions on testis development and spermatogenesis

The role of JH or its analogues on development of testis and the process of spermatogenesis in insects is contradictory. Slama *et al*., (1974) believe that the effect of corpora allata on the male reproductive system is limited only to stimulation of differentiation/function of accessory sex glands. Wigglesworth (1936) found that mature sperms can be produced in the absence of JH in *Rhodnius prolixus.* JHA was found to inhibit the ecdysone stimulated gonial mitosis in *Rhodnius prolixus* (Dumser and Davey, 1974). In *Periplaneta*, ecdysone accelerated testis development but JH inhibited it (Blaine and Dixon, 1976). JH prevented spermatogenesis or induced defective sperm production in *S.littoralis* (Metwally and Gelbic, 1974) and cause sperm lysis in *Bombyx mori*

(Takeuchi, 1969; Yagi and Fukushima, 1975) and *Lasperesia pomonella* (Friedlander and Benz, 1982). Treatments of *Blatella germanica* with PPN resulted in spermatogenesis disorders and a decrease in the number of spermatocytes and spermatozoa (Fathpour *et al.*, 2007).

Ecdysteroids are known to control the fusion of the paired testes as well as the development of the rest of the genital tract in the pupae of *Heliothis virescens*, *Lymantria dispar* and *Spodoptera mauritia* (Loeb *et al.*,1986, Benny and Nair, 1995). They stimulate the maturation and differentiation of male germ cells in *Samia cynthia* (Schmidt and Williams, 1953) and role of 20-HE in promoting spermatogenesis has been well demonstrated in *Bombyx mori* (Takeuchi, 1969). Ecdysteroids were found to stimulate the process of spermatogenesis under a low JH titre (Engelmann, 1970). There are evidences for the role of ecdysteroids in promoting spermatogenesis in many insect species such as *Hyalophora cecropia* (Kambysellis and Williams, 1971, 1972), *Mamestra brassicae* (Takeda, 1972); *Ephestia kuhniella* (Nowock, 1972, 1973) *Spodoptera litura* (Fukushima and Yagi, 1975) and *Rhodnius prolixus* (Dumser and Davey, 1975). JH has an inhibitory effect on spermatogenesis whereas ecdysone accelerates it (Blaine and Dixon, 1976). In the last instar larvae of Lepidopterans the first peak of ecdysteroids promotes the differentiation of eupyrene sperm bundles. The second surge of ecdysteroids at the prepupal stage accelerates spermatogenesis. Further, ecdysteroids synthesized by larval/pupal testis stimulate the development of apyrene sperm (Hoffmann and Behrens, 1982; Loeb *et al*., 1982; Gelman *et al*., 1989).

4.4.1.2 Hormone interactions on ovarian development and oogenesis

There are two major types of oogenesis within the class Insecta, the distinction being based primarily on the cellular mechanisms used for the synthesis of the reserves of RNA containing organelles. The first type is panoistic ovary in which the oocyte nucleus is the source of all of the RNA

contained within the mature egg. The other type is the meroistic ovary which is characterized by the presence of a cluster of cells, the nurse cells or the trophocytes. They have their origin from germinal tissue but do not form part of the mature egg. These cells produce nearly all of the RNA required for both the oocyte and the mature egg. This type of ovary is seen in Lepidoptera. Oocyte maturation has two stages: the first stage is characterized by relatively slow growth of oocytes and trophocytes which grow approximately at the same rate. The second stage is vitellogenesis, characterized by rapid growth and massive accumulation of yolk in the oocyte.

The evidence for the gonadotropic role of corpora allata or JH activity was first provided by Wigglesworth (1936). Extirpation of corpora allata or the neurosecretory cells of the brain effectively blocks yolk deposition. Neurosecretory cells of brain alone control egg maturation in certain species of insects. *In vitro* and *in vivo* investigations have shown that in *Tenebrio molitor* vitellarium differentiation and previtellogenic growth of oocytes are under the control of neurohormones (Mordue, 1965; Laverdue, 1972). Considerable evidences have been obtained from a number of insect species for the regulatory role of neurohormones in various aspects of vitellogenesis. In many insects destruction of neurosecretory cells of the pars intercerebralis causes inhibition of vitellogenesis (Raabe, 1986). Immunochemical analysis of the distribution of the new ovary maturating neurohormone has revealed that it is produced by type-B neurosecretory cells of the pars intercerebralis and it has primary direct function is gonadotropic role in females (Richard and Girardie, 1992). Biogenic amines as neurohormones are found to regulate the reproductive function in *Drosophila* (Rauschenbach *et al*., 2004, 2007).

Considerable evidence has been obtained for the regulatory effect of neurohormones on ovarian development in insects. Implantation of corpora allata reversed the disorders in ovarian development induced by the removal of neurosecretory cells in pars intercerebralis region of brain (Sroka and Gilbert,

1971; Baher, 1973; Barth and Sroka, 1975). A direct action of neurosecretory cells on ovarian development was reported from *Locusta migratoria* (Girardie, 1966; MčCaffery, 1976), *Sarcophaga bullata* (Wilkens, 1968) and *Musca domestica* (Lea, 1975). Various gonadotropic peptides are reported to exert their gonadotropic effects on ovarian development and oocyte maturation in insects (Kuczer *et al*., 2007).

In insects, JH plays a major role in vitellogenesis and the incorporation of yolk into growing oocytes. Wigglesworth (1936) found that JH is necessary for successful yolk deposition and egg maturation in *Rhodnius prolixus.* Similar results were obtained from studies on *Pieris brassicae* (Kaiser, 1949), *Calliphora erytrocephala* (Thomsen, 1952, Thomsen et al., 1980), *Triatoma protracta* (Mundall and Engelmann, 1977) and *Spodoptera frugiperda* (Sorge *et al*., 2000). Topical treatment of JH analogue methoprene and 20-HE was found to exert a promoting influence on vitellogenesis in *Drosophila* (Jowett and Postlethwait, 1981; Handler and Postlethwait, 1977, 2005), *Aedes aegpti* (Borovsky *et al*., 2005) and *Antheraea yamamaz* (Gong-yin *et al*., 2008).

There are observations which suggest that the role of JH is not the same in all insects. Allatectomy did not prevent egg maturation and oviposition in pupae of cecropia silkworm (Williams, 1952; Pan, 1977). Secretion of follicle cell protein is found to be independent of corpora allata activity in *Manduca sexta* (Nijhout and Riddiford, 1974). The rise in JH elicited choriogenesis and egg laying in codling moth *Cydia pomonella* (Webb *et al*., 1999). JH was found to participate both in the control of insect development and the establishment of reproductive maturity in *Drosophila* and during sexual maturation JH provided a signal to the ovary that led to the production of several maternally inherited mRNAs (Dubrovsky *et al*., 2002).

Ecdysteroids are known to stimulate vitellogenin synthesis and uptake of vitellogenin to ovary in certain insects. Previtellogenic growth of oocyte is

affected by 20-HE in *Tenebrio molitor* (Laverdue, 1970) and *Galleria mellonella* (Shibuya and Yagi, 1972). The administration of 20-HE to brainless pupae of *Bombyx mori* stimulated vitellogenin production (Tsuchida *et al*., 1987). In *Lymantria dispar*, a low titre of JH is required for vitellogenin production and treatment of JHA suppressed the process (Fescemeyer *et al.*, 1992). In A*edes aegypti*, ecdysteroids induce vitellogenin production (Dhadialla and Raikhel, 1992) whereas both JH and ecdysteroids stimulate yolk protein synthesis in fat body and ovary in *Drosophila melanogaster* (Bownes *et al*., 1993; Richard *et al.*, 2001a, b; Handler and Postlethwait, 2005). Tebufenozide and methoxyfenozide significantly increased the vitellogenin level in the haemolymph but did not affect their deposition in the oocytes in *Cydia pomonella* (Sun *et al.*, 2003). Topical application of ecdysteroids accelerated vitellogenin synthesis and induced egg maturation in the soft tick *Ornithodoros moubata* (Ogihara *et al.*, 2007).

4.4.2 Effects of hormone agonists

4.4.2.1 Effects of hormone agonists in the spermatogenesis of *S. mauritia*

The present study demonstrated that the treatment of larvae with PPN led to inhibition of spermatogenesis. The testes lobes of treated sixth instar larvae failed to complete fusion and the coalescing lobes of testes were visible in histological preparations. The treatments of larvae of *S.mauritia* with PPN might have led to inhibitory effects on ecdysteroid biosynthesis. The regulatory effect of JHA early in the last larval instar inhibits the secretory activity of prothoracic glands in *S. mauritia* (Balamani and Nair, 1992). The observed failure of fusion of testes lobes in the sixth instar larvae of *S. mauritia* in the present study is in accordance with our earlier results which suggested the involvement of ecdysteroids in fusion of testes lobes in *S. mauritia* (Benny and Nair, 1999). Similar results were obtained from studies on *Ephestia kuhniella* (Nowock, 1972, 1973).

Takeuchi (1969) have verified the acceleratory effect of ecdysone on germ cell numbers and their differentiation and the inhibitory effect of JH in *Bombyx mori*. He suggests that in larval instars germ cells differentiate to form large pool of secondary spematocytes. The increase in the rate of cell divisions in the spermatogonial cells of *Rhodnius prolixus* which occurs in the last larval instars requires ecdysone in the absence of JH (Dumser and Davey, 1974). Presence of a high titre of JH agonist PPN through the exogenous application at the last larval instar thus prevented the normal sequence of spermatogenesis in *S. mauritia.*

In *S.mauritia* treatments of pupae with different doses of PPN was found to interfere with the spermatogenesis. Treatments of day 0 pupae with a single dose of 0.01 μg of PPN resulted in an inhibition of further progress in spermatogenesis and in the necrosis of already formed sperm bundles. The present results support the earlier observations that JHA inhibits the ecdysone stimulated gonial mitosis in *Rhodnius prolixus* (Dumser and Davey, 1974) and prevents spermatogenesis or induce defective sperm production in *S.littoralis* (Metwally and Gelbic, 1974), *Corcyra cephalonica* (Deb and Chakravorty, 1981) and cause sperm lysis in *Bombyx mori* (Takeuchi, 1969; Yagi and Fukushima, 1975) and *Lasperesia pomonella* (Friedlander and Benz, 1982). Treatments of *Blatella germanica* with PPN resulted in spermatogenesis disorders and a decrease in the number of spermatocytes and spermatozoa (Fathpour *et al.*, 2007).

The inhibitory effects of PPN might be due to the altered behaviour of the germ cells on exposure to a high titre of JHA which is otherwise absent in pupa. This might be due to the direct effect of the high titre of JH agonist circulating in the haemolymph on the germ cells or due to PPN induced ecdysteroid deficiency as observed in experiments with other JHAs (Balamani and Nair, 1992). Studies on the red cotton bug *Dysdercus cingulatus* have proved that JH or its analogues

can disrupt or completely inhibit spermatogenesis (Ambika and Prabhu, 1978; Leviatan and Friedlander, 1979). When the JH was excluded from the media in *in vitro* studies, spermatids were found to develop normally in codling moth *Cydia pomonella,* (Friedlander and Benz, 1982). JH is not necessary for spermatogenesis and mature sperm could be produced in *Manduca sexta* in the absence of JH as observed by Wigglesworth (1936). Exogenous application of JHA disturbed sperm production in *Leptocoris coimbatorensis* to varying degrees (Kaur *et al*., 1987). Studies in *Bombyx mori* (Takeuchi, 1969; Yagi and Fukushima, 1975) and *S. litura* (Yagi and Kurumochi, 1976) have proved that high JH titre disturbs spermatogenesis.

The present studies revealed that the lower doses of methoxyfenozide accelerated spermatogenesis in *S. mauritia* whereas the higher doses distorted the testis development and induced malformation of sperm bundles. A considerable number of sperm bundles were observed in histological preparations of testes dissected out on day 3 from sixth instar larvae treated on day 0 with the lowest dose 0.01µg of compound. Our earlier studies have proved that in *S. mauritia*, the fully formed sperm bundles appear for the first time in the pupal period (Venugopalan *et al.,* 1994). The predominant type of cysts present during the feeding period of sixth instar stadia (from day 0 to day 3) were the spermatogonial cysts and spermatocyte cysts and the sperm bundles appear for the first time in the prepupal phase on day 4/day 5 of last instar larvae of *S. mauritia* (Benny, 2000). Ecdysteroids are known to increase apyrene sperm production in *Bombyx mori* (Kawamura *et al*., 2003). Presence of well developed sperm bundles in the histological preparations of methoxyfenozide treated sixth instar day 3 larval testis has confirmed the stimulatory effect of lower doses of the ecdysone agonist methoxyfenozide on the process of spermatogenesis.

There are evidences for the role of ecdysteroids in promoting spermatogenesis in many insect species such as *Samia cynthia* (Schmidt and

Williams, 1953), *Bombyx mori* (Takeuchi, 1969), *Hyalophora cecropia* (Kambysellis and Williams, 1971, 1972), *Mamestra brassicae* (Takeda, 1972); *Ephestia kuhniella* (Nowock, 1972, 1973) *Spodoptera litura* (Fukushima and Yagi, 1975) and *Rhodnius prolixus* (Dumser and Davey, 1975). This explains the acceleration in the development of sperm bundles in the methoxyfenozide and halofenozide treated pupal testes of *S. mauritia*. It is well established that a high titre of ecdysone in the absence of JH induces the production of a large number of spermatozoa in the late last instar larval period of Lepidopterans.

The highest dose, 10 μg of methoxyfenozide/ halofenozide was found to induce malformation of sperm bundles in the pupal testis of *S.mauritia*. Several studies have shown that testis contains considerable quantities of ecdysteroids secreted by testes sheath (Koolman *et al*., 1979; Loeb *et al*., 1982; Gelman *et al*., 1985, 1989). Exogenous application of a high dose of these two ecdysone agonists might have led to alterations in their haemolymph titre. Alterations in the haemolymph titre of ecdysteroids may lead to lack of co-ordination in the development of tissues as different tissues require different quantities of ecdysteroids for their further development (Fristrom, 1981; Riddiford, 1980, 1981). A high titre of ecdysteroids in haemolymph leads to hyperecdysonism. This possibly explains the aggregation of spermatocyte cysts and presence of malformed spermatogonial and spermatocyte cysts in certain regions of testes showing lack of cohesion between them. In *S.litura*, dose-dependent reductions occurred in the production of eupyrene and apyrene spermatozoa in adult testes, and in the number of spermatozoa released from testes into male reproductive tract followed by treatments with ecdysone agonists (Seth *et al.*, 2004).

Observations on *Spodoptera mauritia* reveal significant morphological changes in the spermatocysts during development of the insects and these changes are directly related to the process of differentiation of spermatocysts into mature spermatozoa, like in other lepidopterans (Laifook, 1982; Kasuga

et al., 1985; Scheepens and Wysoki, 1985; Osanai *et al.,* 1986). The rate at which meiotic prophase proceeds during spermatogenesis determines the regular and irregular divisions of lepidopteran spermatocytes resulting in the formation of either eupyrene or apyrene spermatozoa (Friedlander and Hauschteck Jungen, 1986). Bownes and Rembold (1987) report that generally pupae have high ecdysteroid titre and low JH titre. Our earlier studies have shown the existence of a high ecdysteroid titre in the pupae of *S. mauritia* (Mona, 2001). The high endogenous titre of ecdysteroids circulating in the haemolymph after treatments of ecdysone agonists might have interfered with the normal differentiation of eupyrene and apyrene sperm bundles leading to their disintegration and degeneration.

4.4.2.2 Effects of hormone agonists in the oogenesis of *S. mauritia.*

The treatments of sixth instar day 0 larvae with PPN did not affect the ovarian development in *S. mauritia*. From the studies on the normal ovarian development of *S. mauritia*, it was observed that the ovarian growth and previtellogenic differentiation of follicles take place during the pupal period and a completely mature chorionated egg is formed at about 60 h after adult emergence. The reason for the failure of PPN to interfere in ovarian development after larval treatments might be due to the inability to carry forward an effective concentration of PPN to the pupal stage.

Pupal treatments with the lowest dose 0.01 µg of PPN inhibited ovarian development and induced various developmental abnormalities in *S. mauritia*. Similar results were obtained from experimental studies using JHA on *Corcyra cephalonica* (Deb and Chakravorty, 1981). In cat flea *Ctenocephalides felis*, PPN induced the formation of large autophagic vacuoles in the maturing oocytes leading to partial reabsorption of yolk, degeneration of nucleus and lysis of follicular epithelium (Meola *et al.*, 1996). Degeneration of ovarian follicle cells was observed in *Blatella germanica* after PPN treatments (Fathpour *et al.,*

2007). The inhibitory effect of PPN is more conspicuous in pupae as this JH agonist was applied when the endogenous titre of JH was minimum or absent. This observations agree to the earlier observations that the application of the JHA disrupted ovarian development (Sam Mathai and Nair, 1990; Maiza *et al.*, 2004; Aiku *et al*., 2006).

Many authors have suggested that the action of JH mimics takes place rapidly (Gilbert *et al*., 2000; Kamita *et al*., 2003) but the action of PPN was found to have a delay of about 24 h (Edwards *et al.,* 1993). This might be the reason for the blocking of ecdysteroid peak on day 2 even though PPN was applied on day 0. In insects the JH titre is usually controlled by JH specific esterases and some other mechanisms (Gade *et al.,* 1997; Gilbert *et al*., 2000; Kamita *et al*., 2003). However PPN may disrupt the regulatory mechanisms which result in high JH titres in the haemolymph. The JH or its analogues in the haemolymph might inhibit the development of testes and ovary in two ways. The first mechanism is by the direct blocking of cell division and differentiation. This might have resulted in the deleterious effects of the compound on the ovarian development and oogenesis. The toxicity of JH can be correlated with the concentration of unbound hormone (Orth *et al.,* 2003).

Another possibility is that the JH analogues or agonists can induce abnormalities by stimulating ecdysteroid production. Treatments of newly ecdysed pupae with JHA were found to increase endogenous ecdysteroid titres in *S. mauritia* (Mona, 2001). The abnormally high ecdysteroid titres thus produced might have caused serious abnormalities in the ovarian development of treated insects. A critical endogenous titre of ecdysteroids in the haemolymph is crucial for the proper development of ovaries in *Cydia pomonella* (Sun *et al*., 2003) and *Popillia japonica* (Berghiche *et al*., 2008).

One remarkable feature of the present study is that a high titre of PPN permits differentiation of follicles upto a particular stage. The formation of

compound egg chambers in ovarioles of PPN treated pupae of *S.mauritia* take place only during late stages of ovarioles differentiation. The trophocytes were found to be well developed in these egg chambers which suggest that the high titre of JH during pupal period permits the previtellogenic differentiation of follicles to a certain extent. We have seen that JH is required for ovarian differentiation during pupal period. The abnormalities in the treatments with high doses of PPN were induced at a later stage in the differentiation. This might be due to the delayed effects of PPN as observed in the other morphogenetic effects in the present study. JHA hydroprene was found to induce development of compound egg chambers in *S. Mauritia* (Sam Mathai and Nair, 1987) and *Achaea janata* (Nair and Muraleedharan, 1998). Juvenoid application was reported to induce development of compound egg chambers and ill-developed ovarioles in the wasps *Chelonus blackburni* (Chanda and Chakravorty, 2001) and *Ropalidia marginata* (Agrahari and Gadagkar, 2003). These compound egg chambers were found to degenerate later.

The histological preparations of PPN treated ovaries of adultoids showed that none of the follicles have completed yolk deposition. This result is similar to the earlier observations that the high doses of PPN inhibited vitellogenin synthesis in *Apis mellifera* (Pinto *et al.,* 2000). PPN was found to decrease haemolymph protein concentration (Aribi *et al.*, 2001). In *Lymantria dispar*, a low titre of JH is required for vitellogenin production and treatment of JHA suppressed the process (Fescemeyer *et al* ., 1992). Both JH and ecdysteroids are needed in *Drosophila melanogaster* to stimulate yolk protein synthesis in fat body and ovary (Bownes *et al*., 1993; Richard *et al.,* 2001 a, b; Handler and Postlethwait, 2005). In our earlier studies it was found that the pre-adult exposure of ovarian tissue to JHA suppressed mitotic divisions of germinal cells and induced abnormalities in the later stages in *Nilaparvata lugens* (Pradeep and Nair, 2005). Failure of the treated larvae to proceed with vitellogenesis and yolk deposition observed in the present studies can be assumed as the consequence of

the fairly high titre of JH agonist PPN circulating in the haemolymph. The high titre of PPN might have interfered with the normal ecdysteroid titre and inhibited the vitellogenin synthesis though the earlier division phase was not affected much. Specific haemolymph titres of both these hormones are necessary for successful completion of vitellogenesis and yolk deposition.

The present studies revealed that the pupal treatments with lowest dose 0.01 µg of methoxyfenozide induced an acceleration of oogenesis. The doses 5 µg and 10 µg distorted the development though the follicles started the division phase of the development normally. Exposure of the adults of *Schistocerca gregaria* to lower doses of methoxyfenozide accelerated oogenesis but the higher doses reduced the viability of embryos (Wang and Sehnal, 2002). The failure of attachment of eggs to the substratum might be due to the adverse effect of the compound on accessory sex glands which produce the cementing substance. The treatments of pupae with halofenozide accelerated the ovarian development at 5 µg and suppressed the development of ovaries with the highest dose of 10 µg. The lowest dose 0.01 µg was found to be ineffective on ovarian development. Halofenozide treatments of *Leptinotarsa decemlineata* resulted in cessation of oviposition due to distorted ovaries, detrimental oocyte growth and reduced yolk protein synthesis and incorporation into eggs (Farinós *et al.*, 1999). In *Tenebrio molitor*, halofenozide treatments have been reported to affect growth and development of ovaries (Soltani-Mazouni *et al.*, 2001). Decrease in number of ovarioles was observed in ovaries of termite *Coptotermes formosanus* following halofenozide treatments (Raina *et al.*, 2003). The females formed from 0.01 µg methoxyfenozide treated pupae showed a considerable reduction in fecundity. The eggs were scattered in appearance and were not attached to the substratum in a normal manner. The reduction in fecundity was observed in *Diatraea saccharalis* following tebufenozide treatments (Rodriguez *et al.*, 2001a, b) and also in *Spodoptera littoralis* in similar experiments with ecdysone agonists (Pineda *et al*., 2007).

Treatments of adult females of mealworm *Tenebrio molitor* (Taibi *et al.*, 2003) and the cockroach *Blatta germanica* (Maiza *et al.*, 2004) with halofenozide resulted in reduced size and volume of oocytes. The ecdysone agonists RH 5849, tebufenozide and halofenozide were found to affect the growth of ovaries (Hami *et al*., 2005). The exogenous application of the high dose 10 µg of halofenozide was reported to increase the haemolymph titre of ecdysone like compounds in the pupae of *Tenebrio molitor* (Aribi *et al.*, 2001; Berghiche *et al.*, 2003) and in the mosquito *Culex pipiens* (Boudjelida *et al* ., 2002). A similar increase in the haemolymph titre of ecdysone agonists might have occurred in *S. mauritia* resulting in altered development. The presence of yolk granules in the histological sections of ovaries suggests the occurrence of normal vitellogenesis in the pupae treated with ecdysone agonists. Ecdysteroids were known to stimulate vitellogenin synthesis and uptake of vitellogenin in ovary in many insects such as *Tenebrio molitor* (Laverdue, 1970), *Galleria mellonella* (Shibuya and Yagi, 1972) and A*edes aegypti* (Dhadialla and Raikhel, 1992). Tebufenozide and methoxyfenozide were found to increase the vitellogenin level in the haemolymph but did not affect their deposition in the oocytes in *Cydia pomonella* (Sun *et al.*, 2003). Topical application of ecdysteroids accelerated vitellogenin synthesis and induced egg maturation in the soft tick *Ornithodoros moubata* (Ogihara *et al.*, 2007) and in oak silk worm, *Anthraea yamamaz* (Gong-yin *et al*., 2008).

The experiments using *Drosophila* cell lines have proved that low levels of ecdysone stimulate cell divisions and differentiation whereas high levels had an inhibitory effect (Wyss, 1976). Acceleration in ovarian development with the lower doses of ecdysone agonists and inhibition with the higher doses observed in present experiments is in agreement with this finding. The ecdysone agonists bind strongly to receptors and remain in place unlike 20-HE and repress genes necessary for further development (Retnakaran *et al*., 2003). This might have led to deformities in the ovarian tissues after treatments with ecdysone agonists.

4.5 Summary

1. The fifth and sixth instar larvae and newly ecdysed pupae of *S. mauritia* were topically treated by various doses of a juvenile hormone agonist (JHA) PPN, and two ecdysone agonists methoxyfenozide and halofenozide. The effects of these compounds on the morphogenesis of reproductive structures were studied.

2. PPN treatments of fifth instar larvae led to pupation though very low. No pupation was observed in sixth instar day 0 larvae treated with PPN and only larval-pupal intermediates were formed. Treatments of sixth instar larvae with methoxyfenozide resulted in high mortality and production of larval-pupal intermediates. Halofenozide treatments resulted in a higher rate of pupation and production of larval - pupal intermediates.

3. The testis of pupae/larval-pupal intermediates formed from PPN treated fifth/sixth instar larvae showed severe morphological abnormalities. The testis of adultoids formed from pupae treated on day 0 showed only size difference. Testis of control pupae was normal in appearance.

4. The treatments of fifth and sixth instar larvae with different doses of PPN inhibited spermatogenesis. Large masses of undifferentiated cells were seen in sections of testis of sixth instar day 3 larvae. Wide gaps were also seen in between the undifferentiated cells showing lack of cohesion among cells. Treatments of the sixth instar day 0 larvae with low doses of methoxyfenozide induced acceleration of spermatogenesis. Halofenozide showed no marked effects on spermatogenesis with the treated doses.

5. The PPN treatments of newly ecdysed pupae led to complete inhibition of adult emergence. Adultoids were found inside the unemerged pupae when the pupal cuticle was removed. Treatments with ecdysone agonists induced

pupal mortality at higher doses and formation of adultoids inside the unemerged pupae. Lower doses led to pupation and adult emergence.

6. The treatments of newly ecdysed pupae with PPN resulted in the formation of an unorganized mass of cells in the testes which appeared to have stopped proliferation and differentiation. Treatments with 0.01 µg methoxyfenozide resulted in an acceleration of spermatogenesis. Treatments of newly ecdysed pupae with 5 µg and 10 µg of methoxyfenozide led to malformation of sperm bundles. The testis of control pupae on day 6 showed spermatocysts, spermatids and a large number of eupyrene and apyrene sperm bundles.

7. Halofenozide treatments of sixth instar day 0 larvae with the doses 0.01 µg, 5 µg and 10 µg did not induce considerable changes in the spermatogenesis. The treatments of newly ecdysed pupae with the doses 0.01 µg also did not induce any change in the spermatogenesis. Treatments of pupae with 5 µg induced a slight acceleration in spermatogenesis whereas 10 µg of halofenozide induced malformation of the spermatids and sperm bundles.

8. Treatments of newly ecdysed pupae of *S. mauritia* with the different doses of PPN such as 0.01 µg, 5 µg and 10 µg led to abnormaities in the ovaries. The ovarioles were reduced in size and the follicles were not arranged in a linear fashion.

9. Treatments of newly ecdysed pupae with 0.01 µg of halofenozide and methoxyfenozide led to normal development of ovarioles. But the treatments with the higher doses 5 µg and 10 µg of methoxyfenozide induced deleterious effects on ovarian development. The females laid very few eggs on 0.01µg treatments. The eggs were not attached to the substratum as in the case of control larvae and were scattered in appearance. Treatments with 5 µg of halofenozide induced a slight acceleration in oogenesis and 10 µg induced abnormalities in the ovarian development.

5.1 Introduction

5.1.1 Insect imaginal discs: a brief preview

In the larval stages of holometabolous insects, latent adult structures are organized into a sort of specific packets of undifferentiated primordial cells called ‘imaginal discs’ They are destined to differentiate into particular adult structures such as wing, antennae, genitalia, leg etc. They can be identified on the basis of their shape, size, and location within the insect and are enclosed within a cellular epithelium called peripodial membrane.

The developmental significance of imaginal discs was first recognized by Weismann (1864) during the course of his studies on Diptera. In the late final larval instar or early prepupal stage, the discs exhibit accelerated growth, during which they reach the approximate size they will be in the pupa and adult (Oberlander, 1985). The dramatic metamorphic changes that we observe in holometabolous insects is the result of patterned cell proliferation and cell movement that occur during the morphogenesis of imaginal discs.

The anatomy and developmental profile of imaginal discs differ in various orders of holometabolous insects (Oberlander, 1985; Svacha, 1992). The *Drosophila* imaginal discs were well studied where as the physiological control of imaginal disc development of other insects still remain unrevealed as very little studies were conducted so far. Although *Drosophila* larvae are very small, their imaginal discs are a prominent feature to locate and dissect out easily. The imaginal discs of insects appear to differ from those of *Drosophila* and other higher dipterans in almost every morphological respect (Eassa, 1953; Svacha, 1992). It is interesting to note that in lepidopteran larvae which are larger in size when compared to *Drosophila* larvae, the imaginal discs are less obvious due to the huge amount of fat body and body wall muscle. The wing discs of Lepidoptera form discrete epidermal placodes that evaginate and become completely encased by peripodial membrane (Cals, 1968; Nijhout, 1991).

5.1.2 Hormonal control of disc development

Imaginal discs were recognized to be an important system for the investigation of hormonal action for the first time by Vogt (1942) and Bodenstein (1943). Various reports have suggested that the hormones ecdysone (Oberlander, 1972a), JH (Kurushima and Ohtaki, 1975) or low levels of 20-HE (Bulliere and Bulliere, 1977) may be needed for proliferation in immature wing discs. The wing discs of mature larvae begin their metamorphosis by everting and unfolding to produce the shape of the adult structure into which they will differentiate. This process known as evagination is an intrinsic response of discs to hormones and is induced by ecdysteroids. There have been numerous *in vitro* studies which reveal that the presence of a suitable titre of 20-HE/other ecdysteroids is a prerequisite for evagination to occur (Oberlander and Lynn, 1982).

5.1.3 Role of juvenile hormone

The action of JH on the development of imaginal discs has received less attention due to the rapid metabolism of JH in the system as observed in the experiments on *Drosophila melanogaster* (Chihara *et al*., 1972), *Manduca sexta* (Hammock *et al*., 1975) and *Plodia interpunctella* (Oberlander and Silhacek, 1976). This makes it difficult to determine whether JH or any of its metabolites exert any influence on disc development. However, there are evidences that precocious development of the imaginal discs can be brought about by transplanting them into a JH free medium. An interesting fact is that the imaginal discs, when exposed to JH for a long period, remain in an undifferentiated state. Thus, it can be stated that the JH is important in maintaining the "status quo" during insect development and also in the prevention of metamorphosis of imaginal discs (Willis, 1974). JH was found to suppress the initiation and progression of the signaling that transforms immature wing discs or primordia into a fluffy patterned imaginal wings (Truman and Riddiford, 2007).

5.1.4 Role of ecdysteroids

Ecdysterone is the major hormone that is involved in the regulation of imaginal disc development as observed in a variety of *in vitro* and *in vivo* experiments (Oberlander, 1972 b, c). Ecdysone regulatory pathway was found to control morphogenesis of imaginal tissues in *Drosophila* (D'Avino and Thummel, 2000). The competence to respond to ecdysterone is not observed in the early stages and is acquired later in development (Mindek and Nothiger, 1973; Obara *et al*., 2002; Koyama *et al*., 2004a, 2004b). It was found that the metamorphosis of the disc is induced when the prothoracic gland secrets a prohormone, ecdysone, that is then converted in to the actual hormone, 20-HE by peripheral tissues (Gilbert and King, 1973). The wing imaginal discs show two different developmental responses to 20-HE in conjunction with cell cycle and the responses depend on the developmental stage and the concentration to which is exposed (Koyama *et al*., 2004a). It is the active hormone stimulating the development of imaginal discs in *Sarcophaga bullata* (Chen and Hodgetts,1974), *Plodia interpunctella* (Siaussat *et al*., 2004, 2008) and *Bombyx mori* (Koyama *et al*., 2004a). The hormone 20-HE was reported to be more active than ecdysone in stimulating evagination (Chihara *et al.*, 1972) and in promoting differentiation (Milner and Sang, 1974). When the potency of ecdysone agonists were examined, methoxyfenozide was found to be more potent in comparison to RH-5992 and RH -5849 (Smagghe *et al*., 2002b). RH-5992 was found to be more potent than 20-hydroxyecdysone in lepidopteran cell line (Auzoux-Bordenave *et al.*, 2005).

5.1.5 Protein synthesis in imaginal discs

Protein synthesis in holometabolous insects increases in response to the hormones, mainly ecdysteroids. The cells of imaginal discs undergo rapid changes in their morphology and biosynthetic activity during metamorphosis which is very significant for their development (Doctor and Fristrom, 1985).

Evidences of the hormonal control of differentiation of imaginal discs confirm that both steroid hormones and JH affect the transcription process. The primary response of imaginal discs to 20-HE is at the level of transcription. The incorporation of label into disc proteins is stimulated by 20-HE in *Sarcophaga peregrina* and rapid reduction in incorporation results from the removal of the hormone from the culture medium (Ohmori and Ohtaki, 1973). JH is reported to inhibit protein synthesis in imaginal discs (Williams, 1961) and blocks ecdysone-induced evagination (Fristrom *et al*., 1976b).

The patterns of protein synthesized during the last larval instar of *Plodia interpunctella* remained quantitatively the same with some stage-specific quantitative differences (Oberlander and Leach, 1978). The effect of hormone 20-HE on the synthesis of disc specific proteins have been demonstrated in wing discs of the larvae of *Galleria mellonella* (Miller and Oberlander, 1981). A combined synergistic effect of JH and 20-HE was observed on protein expression of butterfly imaginal wing discs (Cieslak *et al.*, 2007). It was found that JH apparently does not inhibit the synthesis of the ecdysterone - inducible proteins, although wing disc development is inhibited to various extents by JH in *Drosophila melanogaster* (Sin, 1988). The present study investigates the SDS-PAGE pattern of wing disc proteins and also the changes in their profile followed by treatments with hormone agonists PPN, methoxyfenozide and halofenozide.

5. 2 Materials and methods

5.2.1 Experimental animals

The sixth instar day 5 larvae (prepupae) were used for the treatments of the present study. Experimental and control insects were fed and maintained in a similar manner as in the case of laboratory stock culture.

5.2.2 Chemicals (See Chapter 2).

5.2.3 Treatments

The sixth instar day 5 larvae were topically treated with single doses of 10µg of PPN, halofenozide or methoxyfenozide, using a Hamilton microsyringe. Larvae kept as controls received equal volumes of the solvent acetone/distilled water as is the case may be. The larvae were fed and maintained as described previously (Chapter 2). Wing discs from the treated and control larvae were dissected out after 20 h, before the onset of pupation.

5.2.4 Surgical techniques

The treated and control day 5 larvae were anaesthetized in specimen tubes containing cotton soaked in diethyl ether at the bottom, 20 h after treatment. The larvae were dissected in cold insect saline under a Wild stereo zoom dissection microscope as described previously (See chapter 2). Pairs of fore wing and hind wing imaginal discs were identified by the presence of distinct tracheolar mass at their base and were dissected out.

5.2.5 Preparation of tissues for protein analysis

Wing discs were dissected out in cold insect saline under a dissection microscope. Other tissues and adhering fat bodies in contact with the discs were carefully removed. About 30-40 discs of larvae were used to make the samples, with tissue weight ranging from 15-25 mg. Acid soluble components and pigments in the disc tissue were washed off as per schedule of Mittermayer *et al.* (1964). The tissues were first homogenized in 50 µl of distilled water utilizing a glass homogenizer. The homogenate was centrifuged at 2000 rpm for 10 minutes, decanted the supernatant and stored at 0^{o}C until use. Total proteins were estimated by the modified Lowry protein assay as described by Sandermann and Stromiger (1972).

The supernatant was taken in test tubes (20-30 µl) to which 1 ml of working solution (freshly prepared by mixing 25 ml of 2% Na_2CO_3, 0.02% of Na K tartarate, 0.1 M NaOH and 1% SDS) and 1 ml of 0.05 % CuSO4 were added. This mixture is allowed to stand for 15 minutes at room temperature and 0.1ml of 1N Folin reagent was added and vortexed the tubes immediately. This solution was allowed to stand for 30 minutes at room temperature and read at 650 nm against a reagent blank in a Shimadzu UV Spectrophotometer. Bovine serum albumin (Sigma Chemical Co., St. Louis, USA) was used as the standard. The results were expressed in µg per mg of tissue.

5.2.6 Preparation of samples for electrophoresis

The wing discs of sixth instar control and treated larvae were dissected out and washed thoroughly in insect saline. The tissues were homogenized in distilled water by using a glass homogenizer. The homogenate was then centrifuged at 2000 rpm for 10 minutes and the resulting aqueous supernatant was kept at 0^oC until use.

5.2.7 Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE)

The aqueous protein samples were subjected to SDS-PAGE using 10% acrylamide slab gel as described by Laemmli (1970). Electrophoresis was carried out using vertical slab gel in 8 x 7 cm length and 1mm thickness in a mini model vertical electrophoresis unit (Bangalore Genei Pvt Ltd., Peenyaa, Bangalore). The 10% separating gel was overlayered by a 3% spacer gel. Both these gels were prepared from stock solution of 30% by weight of acrylamide and 0.8% by weight of N, N'-methylene-bis-acrylamide. The 10% acrylamide gel was prepared by mixing:

(a) 6 ml of acrylamide (stock solution).

(b) 11ml of buffer solution (0.614 M Tris - base adjusted pH 6.8 with HCl

containing 0.164% SDS w/v).

(C) 0.9 ml of ammonium persulphate (15 mg/ml and 0.02 ml of TEMED.

The spacer gel was prepared by mixing:

(a) 1 ml of acrylamide (Stock solution).

(b) 8.5 ml of buffer (0.147 M Tris, pH adjusted to 6.8 with HCl containing 0.108% of SDS w/v.

(c) 0.5 ml of ammonium persulphate (15 mg/ml).

(d) 0.01 ml of TEMED.

Protein sample containing 1% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol was heated at 100^{o}C in a water bath for two minutes and cooled before loading on to the gel. Equal amounts of tissue were loaded in all the wells of the gel. Bromophenol blue was used as a tracking dye. SDS-PAGE was carried out using a buffer system (Chamber buffer, pH 8.3) containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS at a constant current of 25 mA. After electrophoresis, gel was fixed in 50% methanol containing 0.075% formaldehyde for 45 minutes. The gel was stained to visualize the separated protein bands by soaking in Coomassie Brilliant Blue (R-250) staining solution (44 ml methanol; 44 ml distilled water; 12 ml glacial acetic acid containing 0.125 gm Coomassie Brilliant Blue) for 45 minutes. Destaining was accomplished by transferring gels to a destaining solution (Methanol - 25 ml; Acetic acid - 37.5 ml; distilled water - 437.5 ml). The staining intensities of protein bands were analysed.

Myosine (Rabbit muscle) (205 kDa), Phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumen (43kDa), Carbonic anhydrase (29 kDa), Soyabean Trypsin Inhibitor (20.1kDa), Lysozyme (14.3 kDa), Aprotinin (6.5 kDa) and Insulin (3kDa) were used as molecular weight standards. Mobility of sample protein was compared to the mobility of standards in 10% acrylamide

concentration. Molecular weight was calculated from a plot of log molecular weight versus the relative mobility of the standards.

5.3 Results

5.3.1 Normal growth and differentiation of imaginal discs in *S. mauritia*

During early stages of sixth instar development of *S.mauritia*, the imaginal discs appear as very small pouches of tissues which can be easily identified by the presence of large tracheole masses at their bases. They become more visible from day 3 onwards. The paired fore and hind wing discs are located on the dorso-lateral regions of meso and metathoracic segments respectively. The evagination occurs during the transformation of larva from wandering to prepupal stage. The evaginated wing discs of day 5 larvae are thin, transparent and membranous structures. In addition, the evaginated forewing discs are very large and much folded. Due to shortening of the length of larva on day 5 the fore and hind wing discs get close to each other. The wing discs of late last instar larvae begin their metamorphosis by everting and unfolding to produce the shape of the adult structure into which they will differentiate. This process is known as evagination. Any indication of tanning process was not observed during sixth instar larval development.

5.3.2 Effects of hormone agonists on morphogenesis of imaginal discs

The paired wing discs of meso and metathoracic segments of the PPN treated larvae were found to be partially tanned and sclerotised (Pl. XV: Fig. 132). The wing discs were found to be partially differentiated and uneverted. Treatments of day 5 larvae with the ecdysone agonists methoxyfenozide and halofenozide resulted in well developed and everted wing discs (Pl. XV: Figs. 134, 135). They were similar in appearance to the wing discs of control day 5 larvae though a slight increase in size was observed (Pl. XV: Fig. 133).

5.3.3 Electrophoretic profile of wing disc proteins

The wing discs of the control sixth instar day 5 larvae and the larvae treated with hormone agonists PPN, methoxyfenozide and halofenozide were dissected out and the aqueous protein samples were subjected to the electrophoretic analysis. Lanes 1-4 (Fig. 136) represent the electrophorogram of wing disc proteins of treated and control day 5 larvae. Lane 1 and lane 2 represent the electrophoretic profile of wing disc proteins of halofenozide treated and methoxyfenozide treated larvae respectively. Lane 3 represents the electrophoretic profile of wing disc proteins of control day 5 larvae. Lane 4 represents the electrophoretic profile of wing disc proteins of PPN treated larvae and lane 5 represents the molecular weight markers.

5.3.3.1 Pattern of wing disc proteins in sixth instar day 5 control larvae

Lane 3 represents the electrophoretic profile of day 5 control larvae. The predominant wing disc proteins expressed were DP 1 (239.9 kDa), DP 2 (229.1 kDa), DP3 (166 kDa), DP 4 (138 kDa), DP 5 (131.8 kDa), DP 6 (109.6 kDa), DP 7 (91.20 kDa), DP 8 (83.18 kDa), DP 9 (72.44 kDa), DP 10 (66.07), DP 11 (60.26 kDa), DP 12 (59.53 kDa), DP 13 (52.68 kDa), DP14 (47.05 kDa), DP 15 (37.24 kDa), DP 16 (36.31kDa), DP 17 (32.43 kDa), DP 18 (29.77 kDa), DP 19 (28.62 kDa), DP 20 (25.83 kDa), DP 21 (23.84 kDa) and DP 22 (14.89 kDa). Of these, DP 1, DP 12, DP13, DP 14 and DP 15 exhibited high staining intensity.

5.3.3.2 Pattern of wing disc proteins in PPN treated larvae

The analysis of SDS-PAGE pattern of wing disc proteins from PPN treated day 5 larvae and acetone treated control larvae showed significant differences. The lane 4 represents the electrophoretic profile of wing disc proteins of PPN treated larvae. When compared to control day 5 larvae, the expression of wing disc proteins of PPN treated larvae appeared to be highly

reduced or completely absent. The disc peptides DP 3, DP 4, DP 7, DP 8 and DP 14 were completely absent in PPN treated larvae. The peptides DP 15, DP 17, DP 19 and DP 21 showed less intensity in staining whereas the peptide bands DP1 and DP2 were prominent. DP 9 and DP 10 showed moderate staining intensity.

5.3.3.3 Pattern of wing disc proteins in methoxyfenozide treated larvae

The lane 2 represents the electrophoretic pattern of wing disc protein following methoxyfenozide treatments. Analysis of peptide bands indicated that all the bands were present though the expression of some of the proteins was slightly reduced. Expression of the peptide DP 14 was considerably reduced. The peptides DP 3, DP 6 and DP 19 showed very less staining intensity. The peptides DP 1, DP 2, DP 12 and DP 13 showed high intensity of staining. DP 16, DP 17 and DP18 showed a slight increase in their concentration.

5.3.3.4 Pattern of wing disc proteins in halofenozide treated larvae

The lane 1 represents the electrophorogram of wing disc proteins following halofenozide treatments. The pattern of expression was similar in both treated and control larvae though the bands showed variation in staining intensities when compared to the electrophorogram of wing disc proteins of control insects. The peptides DP 3, DP 6, DP 16 and DP 19 showed a slight reduction in staining intensity. The peptides DP 1, DP 12, DP 13 and DP 21 showed high intensity of staining. All the other bands were of moderate staining intensity.

5.3.4 Effects of hormone agonists on total protein of imaginal discs

The concentration of protein in PPN treated sixth instar day 5 larvae showed a considerable decrease when compared to the controls. Amount of protein in the control day 5 larvae was 73.57 µg/mg of tissue whereas protein content obtained from wing discs of larvae treated with PPN was only 56.49

µg/mg of tissue (Fig. 137). The concentration of protein in wing discs of sixth instar day 5 larvae treated with methoxyfenozide / halofenozide showed an increase in protein content when compared to controls. The protein content obtained from wing discs of methoxyfenozide treated larvae was 83.47 µg/mg of tissue and the amount of protein obtained from wing discs of halofenozide treated larvae was 79.38 µg/mg of tissue.

5.4 Discussion

5.4.1 Effects of PPN on wing disc development

The present study revealed that the wing discs of 10 µg PPN treated larvae were uneverted, partially differentiated, tanned and mildly sclerotised. This result is similar to our earlier observations on *S. mauritia*. The wing discs of PPN treated larvae were highly tanned but uneverted as seen in JHA treatments (Safarulla *et al*., 2003). JH application to the final instar larvae of many lepidopteran insects have resulted in the formation of supernumerary larvae with tanned, partially differentiated and uneverted pupal wing discs (Sehnal and Schneiderman, 1973; Oberlander and Silhacek, 1976; Riddiford, 1980; Slama and Mala, 1984; Hatakoshi *et al*., 1986; Okuda *et al.*, 1986; Santha and Nair, 1987; Balamani and Nair, 1991; Venugopalan, 1995).

It was observed that JH inhibits the development of imaginal discs in *Drosophila* (Chihara *et al*., 1972; Chihara and Fristrom, 1973). In *Plodia interpunctella*, the JH containing diet prevented the growth of wing discs and their acquisition of competence to metamorphose (Oberlander and Silhacek, 1976). Application of PPN during the fourth instar molt delayed and reduced the levels of mRNAs / transcription factors in the wing discs of *Manduca sexta* but did not completely prevent the appearance of these factors in this tissue (Zhou *et al*., 1998 a, b). The wing discs were partially tanned in PPN treatments. This could be due to the accumulation of certain proteins needed for tanning from haemolymph as observed in holometabolous insects (Silvert, 1985). Another

possibility is that of precocious pigmentation resulting from the removal of inhibition on prophenoloxidase activation by the PPN induced low ecdysteroid titre as observed in honey bee *Apis mellifera* (Zufelato *et al*., 2000; Santos *et al*., 2001).

The imaginal cells respond to 20-HE by a proliferative arrest followed by morphological differentiation. *In vitro* studies on *Plodia interpunctella* have demonstrated that JH was able to prevent 20-HE induced differentiation of imaginal wing cells (Siaussat *et al*., 2004). JH may enhance the response of ecdysteroids and/or alter the sensitivity of tissues to ecdysteroids (Denlinger, 1979). High doses of JH prevent the wing discs from developing to an ecdysteroid responsive state (Oberlander, 1985). JH suppressed the initiation and progression of the signaling that transforms immature discs or primordia into a fluffy patterned imaginal disc (Truman and Riddiford, 2007). In *Manduca sexta* JH was found to inhibit the intrinsic signaling needed for imaginal disc morphogenesis (Truman *et al*., 2006, 2007).

The uneverted imaginal discs in the present study have demonstrated the JH activity of PPN suppresses the development of the imaginal tissue. In the sixth instar larvae of *S. mauritia*, which were treated on day 5, the first peak of ecdysteroids on day 3 had already occurred. The second surge of ecdysteroids might not have occurred by the interference of high titre of PPN. The present results can be explained as the deleterious effects of the high dose of PPN on the mitosis of wing discs and their competence to respond to 20-HE.

5.4.2 Effects of ecdysone agonists on wing disc development

The present study revealed that the wing discs of sixth instar day 5 larvae treated with 10 µg of the ecdysone agonists, methoxyfenozide and halofenozide were everted and were similar to the discs of control larvae in size and shape. These observations point to the fact that ecdysteroids promote wing

disc development. Ecdysterone is the major hormone that is involved in the regulation of imaginal disc development as observed in a variety of *in vitro* and *in vivo* experiments (Oberlander, 1972 a, b,c). 20-HE is the active hormone stimulating the development of imaginal discs in *Mamestra brassicae* (Agui and Fukaya,1973), *Sarcophaga bullata* (Chen and Hodgetts,1974), *Plodia interpunctella* (Siaussat *et al*., 2004, 2008) and *Bombyx mori* (Koyama *et al*., 2004a). Non-steroidal ecdysone agonists RH 5849 and RH 5992 were found to be similar to 20-HE in inducing imaginal disc proliferation (Farkaš and Sláma, 1999). RH-5992 was more potent than 20-HE in Lepidoptera (Smagghe *et al*., 1996; Auzoux-Bordenave *et al.*, 2005).

The low haemolymph titres of ecdysteroids have recently been suggested to possess an important role in the development of *Bombyx mori* wing discs (Kawasaki, 1995) and they showed dynamic morphogenesis in 4-5 days under the control of ecdysteroids (Kawasaki *et al*., 2004). The experimental data suggests that the control mechanism of cell cycle in *Bombyx mori* was altered in the cells which were already committed to develop into pupae (Koyama *et al.*, 2004b). As a result a high ecdysteroid titre which induces a stationary moult in young instars does not affect the cell division of imaginal disc cells which are uncommitted. This explains the normal evagination which was observed in the wing discs of day 5 larvae of *S. mauritia* treated with ecdysone agonists. The ecdysone agonists are known to have an action similar to that of 20-HE in binding to the ecdysteroid receptors and elicit a biological response similar to that of ecdysone in Lepidoptera (Smagghe *et al*., 1996).

5.4.3 Effects of hormone agonists on wing disc protein content and protein patterns.

There was a considerable decrease in the protein content observed in the wing discs of PPN treated day 5 larvae when compared to controls. Juvenile hormone is reported to inhibit protein synthesis in imaginal discs (Williams,

1961, Fristrom et al., 1976b). The patterns of protein synthesized during the last larval instar of *Plodia interpunctella* remained quantitatively the same with some stage-specific quantitative differences (Oberlander and Leach, 1978). This effect can be attributed to the disruption in JH esterase activity of imaginal discs by the high titre of PPN. It is possible that the high titre of PPN might have interfered at the gene level thus preventing the expression of those genes which are responsible for the expression of the missing peptides either direct or through the action of ecdysteroid titres as observed in earlier studies with JH (Zhou *et al*., 1998 a,b).

Electrophoretic studies of proteins in wing discs of control insects showed 22 protein components differing in their molecular weights and charge properties. The variation in the staining intensity of the protein bands reflects alteration in the apparent concentration of these compounds. The major peptide components were DP 1 (239.9 kDa), DP 2 (229.1 kDa), DP3 (166 kDa), DP 4 (138 kDa), DP 5 (131.8 kDa), DP 6 (109.6 kDa), DP 7 (91.20 kDa), DP 8 (83.18 kDa), DP 9 (72.44 kDa), DP 10 (66.07 kDa), DP 11 (60.26 kDa), DP 12 (59.53 kDa), DP 13 (52.68 kDa), DP14 (47.05 kDa), DP 15 (37.24 kDa), DP 16 (36.31kDa), DP 17 (32.43 kDa), DP 18 (29.77 kDa), DP 19 (28.62 kDa), DP 20 (25.83 kDa), DP 21 (23.84 kDa) and DP 22 (14.89 kDa). During prepupal period imaginal discs respond to ecdysteroids by undergoing two developmental responses, that include morphogenesis to form the basic structure of the adult appendages (evagination) and differentiation to form pupal cuticle. A continued protein synthesis is required for evagination and discs require a number of specific proteins for the deposition of cuticle (Fristrom and Yund, 1976 a; Fristrom *et al.*, 1977; Fristrom, 1981). The high level of certain peptides produced may be important for differentiation of wing discs, their evagination and cuticle deposition.

Analysis of protein components in the wing discs of PPN treated day 5 larvae showed reduction in the expression of many of the major bands that were

predominant in the wing discs of day 5 control larvae. The observed low amount of these major peptides and the absence of other peptides (DP 3, DP 4, DP 7, DP 8 and DP 14) indicate that high titre of PPN might have an inhibitory effect on wing disc protein synthesis. Our ealier studies have shown that JHA treated larvae of *S. mauritia* showed a reduction in the expression of many major proteins that were predominant in the wing discs of control larvae (Safarulla *et al*., 2003). In *Plodia interpunctella*, JH induced inactivation of genes involved in the synthesis of proteins needed for the 20-HE mediated gene expression (Siaussat *et al*., 2004). JH suppressed imaginal disc formation in *Manduca sexta* (Truman *et al*., 2006). The reduction in the expression of protein bands in PPN treated larvae might be the result of interference of this JH agonist on 20-HE mediated gene expression. Exogenous JHA was proved to inhibit growth of imaginal discs during the last larval instar of *Precis coenia* (Kremen and Nijhout, 1998, 1989) and also in *in vitro* studies of Lepidopteran imaginal disc cell line (Oberlander *et al*., 2000). The failure of eversion observed in the wing discs of PPN treated larvae might be the result of interference of the agonist in the expression of certain genes that are JH responsive. The peptides DP 1, DP 2, DP 12 and DP 13 were prominent in wing discs of PPN treated larvae. This result points to the fact that the expressions of certain peptides were not affected by the treatments of JHAs or that the genes responsible for their expression are not dependent on the changes in the haemolymph JH titre.

The results of present investigation reveal that treatments of ecdysone agonists on day 5 larvae of *S.mauritia* had an accelerating effect on protein synthesis. The fact that methoxyfenozide and halofenozide treatments show a slight increase in the protein level demonstrates that the ecdysone agonists had a stimulatory effect on protein synthesis. Ecdysterone was reported to stimulate protein synthesis in imaginal discs of *Calliphora vicina* (Scheller *et al*., 1978). High ecdysteroid titre during prepupal stage has a stimulatory effect on protein synthesis in the imaginal discs of many lepidopteran larvae (Patel and

Madhavan, 1969; Kurushima and Ohtaki, 1975; Oberlander and silhacek, 1976, Oberlander *et al*., 1973; Lafont *et al.*, 1977; Williams, 1980; Meyer *et al.*, 1980). The increased protein content observed in the present studies might be the consequence of the stimulatory effect of the ecdysone agonists methoxyfenozide and halofenozide on protein synthesis in wing discs of the day 5 larvae of *S. mauritia*. The electrophoretic profile of wing disc proteins of the larvae treated with ecdysone agonists have shown that the pattern of expression of proteins in the treated wing discs was similar to that of the control larvae. The peptides DP1 and DP2 showed an increase in their concentration in both the methoxyfenozide and halofenozide treatments. The peptides DP 3, DP 6 and DP 19 showed a slight reduction in expression whereas DP 14 showed a considerable reduction in expression in methoxyfenozide treatments. Another interesting feature observed was that the peptides DP 1, DP 12 and DP 13 showed similarity in intensity of staining in both the control and larvae treated with hormone agonists.

These observations clearly demonstrate that ecdysone agonists have a stimulatory effect on some of the expressed genes in the wing discs. The intensity of staining of certain bands indicates that the genes which are not normally stimulated by the 20-HE in the wing discs of control larvae are stimulated by ecdysone agonists. The expression of these genes may be under the control of ecdysteroids as observed in the protein synthesis in the wing discs of *Drosophila* (Mandaron, 1973, 1976), *Sarcophaga bullata* (Chen and Hodgetts, 1974), *Galleria mellonella* (Benson and Oberlander, 1974; Miller and Oberlander, 1981). The similarity observed in staining intensities of some of the peptide bands from the wing discs of control and treated day 5 larvae suggests that the genes responsible for their expression are ecdysone regulated as observed in *Drosophila melanogaster* (Russel and Ashburner, 1996). The differences observed in the pattern and intensity among them might be due to the fact that ecdysone and its agonists use the same receptors but show maximum effectiveness at different concentrations (Cottam and Milner, 1998)

5.5 Summary

1. The sixth instar day 5 larvae (prepupae) were topically treated with a single dose of 10 µg of PPN / methoxyfenozide / halofenozide. The effects of the compounds on wing disc protein synthesis and development of wing discs were studied. The wig discs of PPN treated larvae were uneverted, partially tanned and sclerotised. Treatments with ecdysone agonists resulted in the formation of wing discs which were everted and were similar to those of controls.
2. The failure of wing disc evertion indicates that the JH activity of PPN suppresses the development of the imaginal tissue. The tanning of wing discs of PPN treated larvae might be the result of accumulation of certain haemolymph proteins which are needed for tanning. Another possibility is that of precocious pigmentation resulting from the removal of inhibition on prophenoloxidase activation by the PPN induced low ecdysteroid titre

3. The concentration of protein in wing discs of PPN treated larvae was found to decrease when compared to controls. Amount of protein in the control day 5 larvae was found to be 73.57 µg/mg of tissue whereas wing discs of larvae treated with PPN had protein content of 56.49 µg/mg of tissue. This points to the fact that PPN has inhibitory effect on protein synthesis.

4. The concentration of protein in wing discs of sixth instar day 5 larvae treated with ecdysone agonists showed an increase in protein content when compared to controls. Protein content in wing discs of methoxyfenozide treated larvae was 83.47 µg/mg of tissue and halofenozide treated larvae was 79.38 µg/mg of tissue. This suggests that ecdysone agonists have a stimulatory effect on protein synthesis in the imaginal wing discs of *S.mauritia.*

5. The predominant wing disc proteins expressed on day 5 control larvae were DP 1 (239.9 kDa); DP 2 (229.1 kDa); DP3 (166 kDa); DP 4 (138 kDa); DP 5 (131.8 kDa); DP 6 (109.6 kDa); DP 7 (91.20 kDa); DP 8 (83.18 kDa); DP 9 (72.44 kDa); DP 10 (66.07 kDa); DP 11 (60.26 kDa); DP 12 (59.53 kDa); DP 13 (52.68 kDa); DP14 (47.05 kDa); DP 15 (37.24 kDa); DP 16 (36.31kDa); DP 17 (32.43 kDa); DP 18 (29.77 kDa); DP 19 (28.62 kDa); DP 20 (25.83 kDa); DP 21 (23.84 kDa) and DP 22 (14.89 kDa).

6. When compared to control day 5 larvae, the expression of wing disc proteins of PPN treated larvae appeared to be highly reduced or completely absent. The disc peptides DP 3, DP 4, DP 7, DP 8 and DP 14 were completely absent in PPN treated larvae. The peptides DP 15, DP 17, DP 19 and DP 21 showed less intensity of staining whereas the peptide bands DP1 and DP2 were prominent. DP 9 and DP 10 showed moderate intensity of staining.

7. The expression of wing disc proteins of methoxyfenozide treated larvae indicated that some of the proteins showed reduced expression. The peptides DP 1, DP 2, DP 12 and DP 13 showed high intensity of staining. DP 16, DP 17 and DP 18 showed a slight increase in their concentration. Halofenozide treatments resulted in reduced expression of peptides DP 3, DP 6, DP 16 and DP 19. The peptides DP 1, DP 12, DP 13 and DP 21 showed high staining intensity. All the other bands showed moderate intensity of staining.

8. Methoxyfenozide and halofenozide showed similarity in the expression of protein bands. This was similar to the expression patterns of proteins in the control larvae though they differed in staining intensity. The intensity of staining of certain bands indicates that the genes which are not normally stimulated by the 20-HE in the wing discs of control larvae are stimulated by ecdysone agonists. The expression of these genes may be under the control of ecdysteroids.

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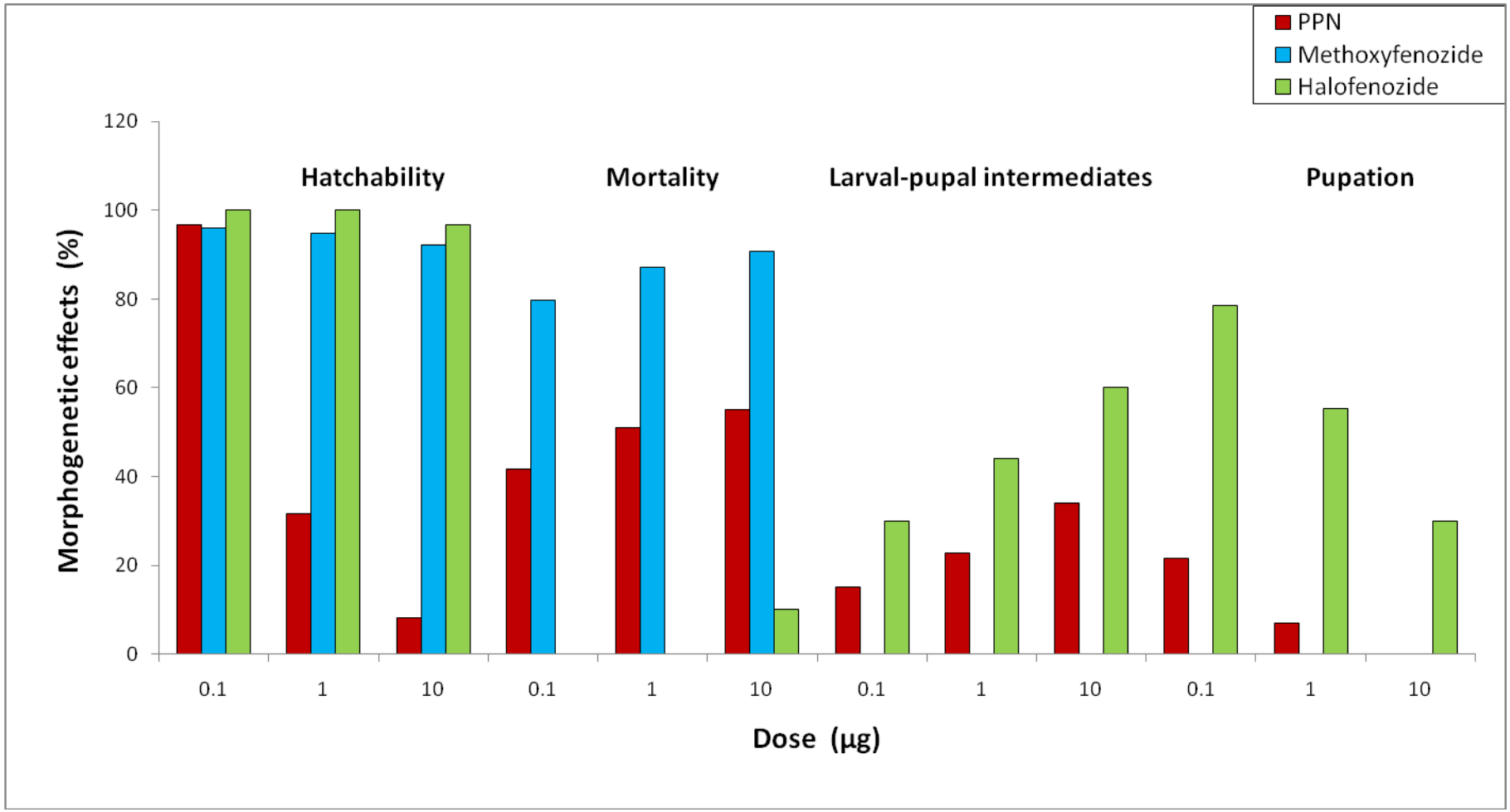

Fig. 7. Morphogenetic effects of hormone agonists on treatments of eggs.

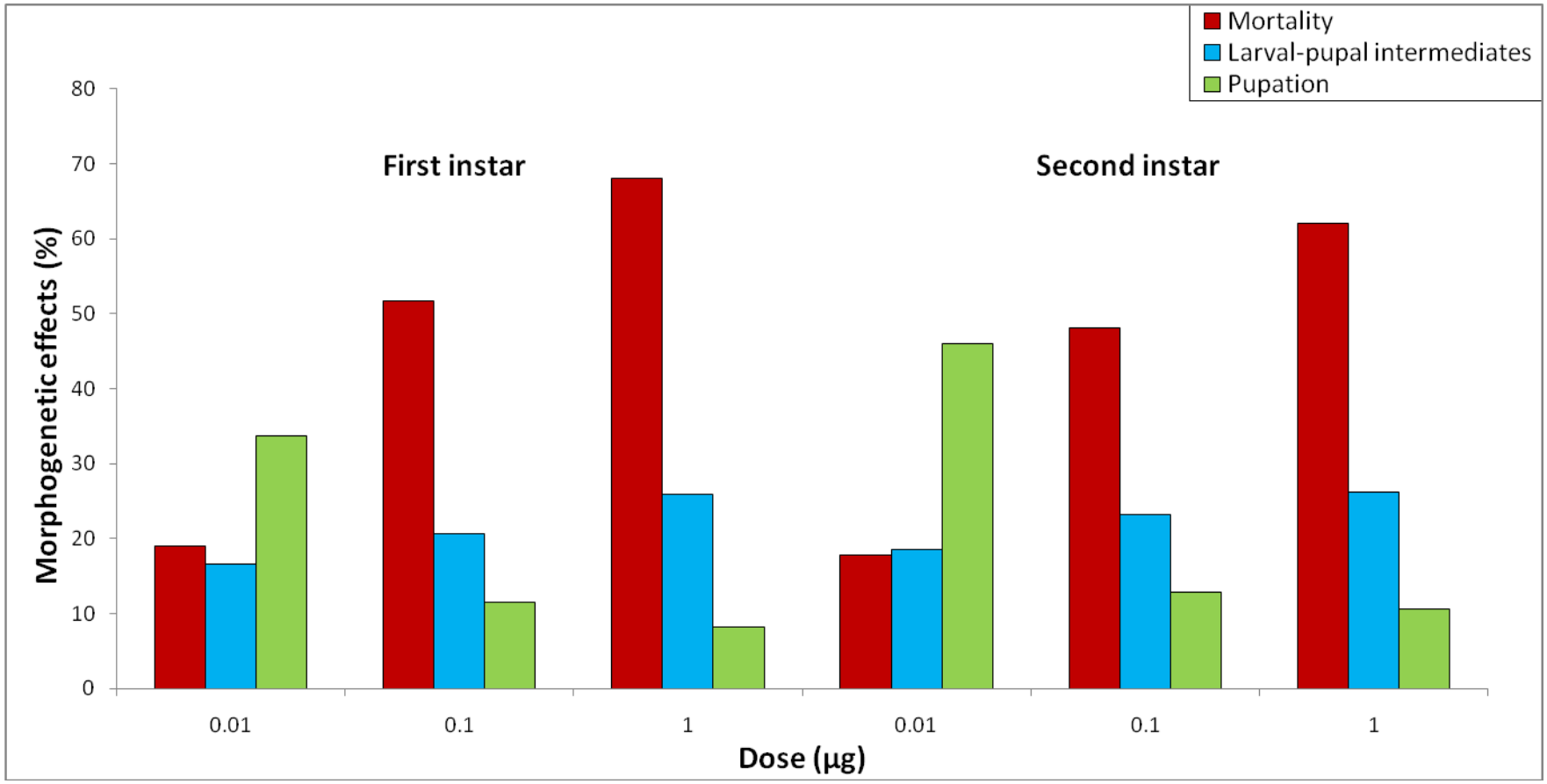

Fig. 16. Morphogenetic effects following PPN treatments on first and second instar day 0 larvae.

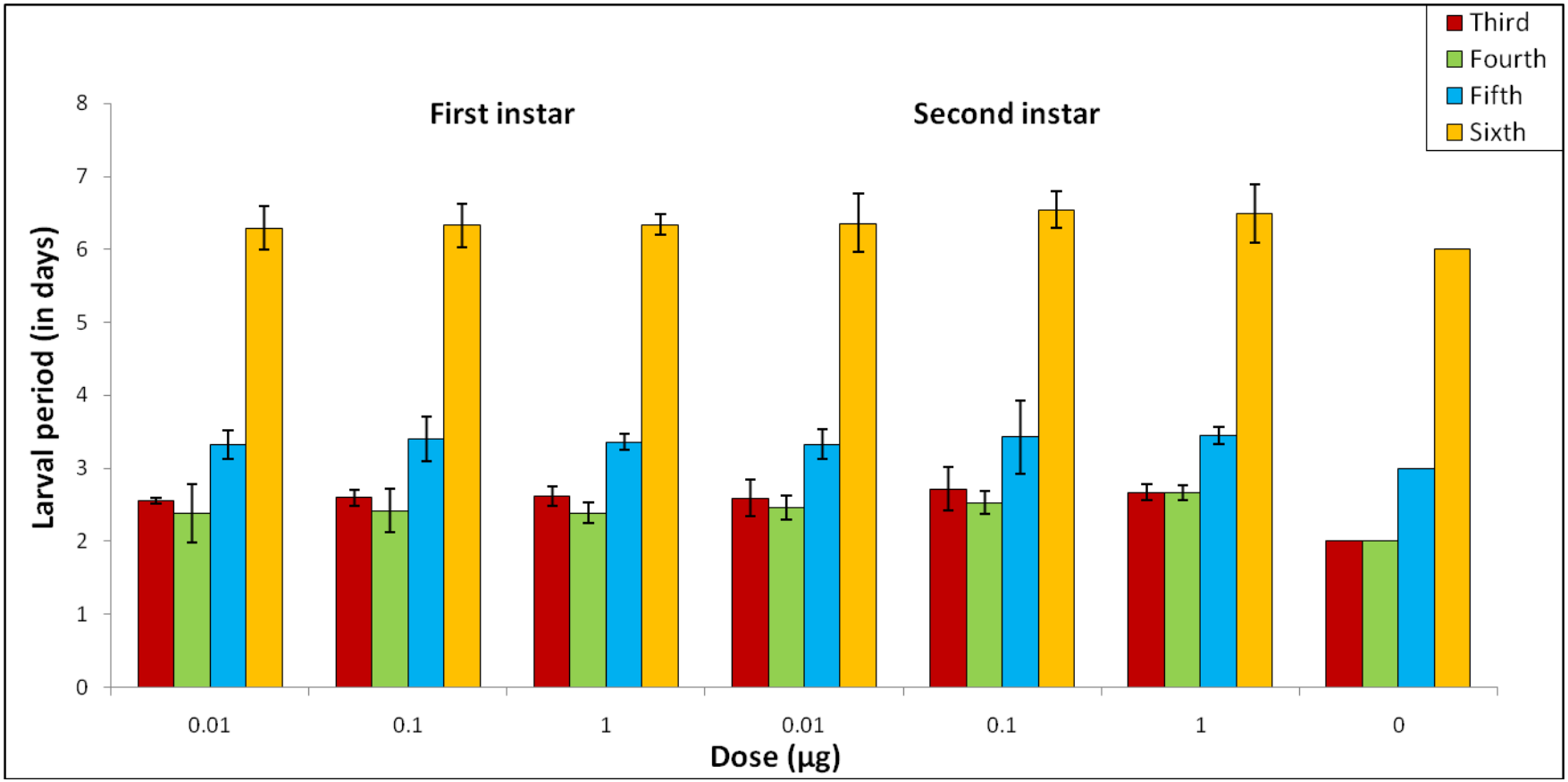

Fig. 17. Effects of PPN treatments of first and second instar larvae on larval period.

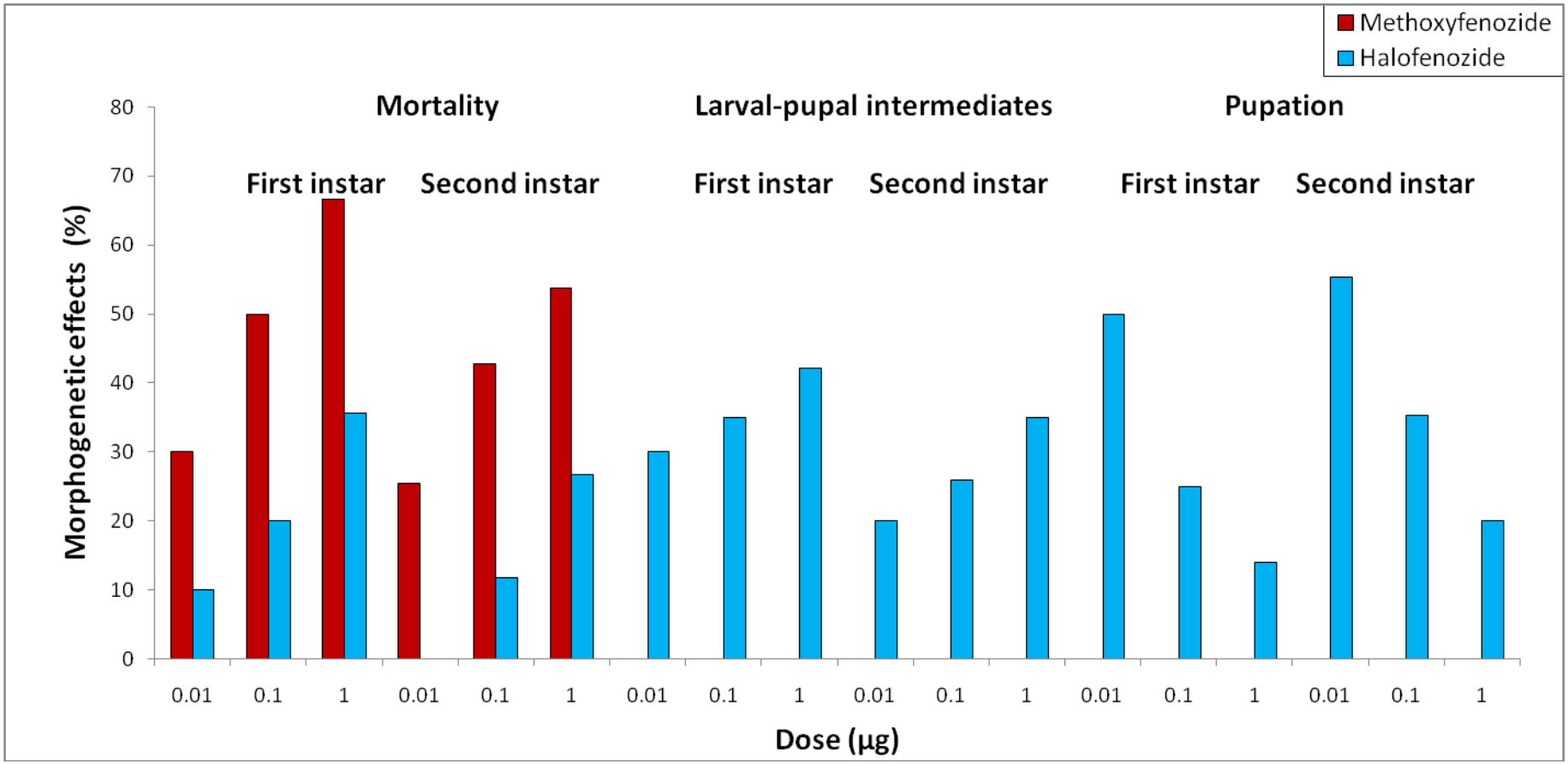

Fig. 18. Morphogenetic effects of ecdysone agonists on first and second instar day 0 larvae.

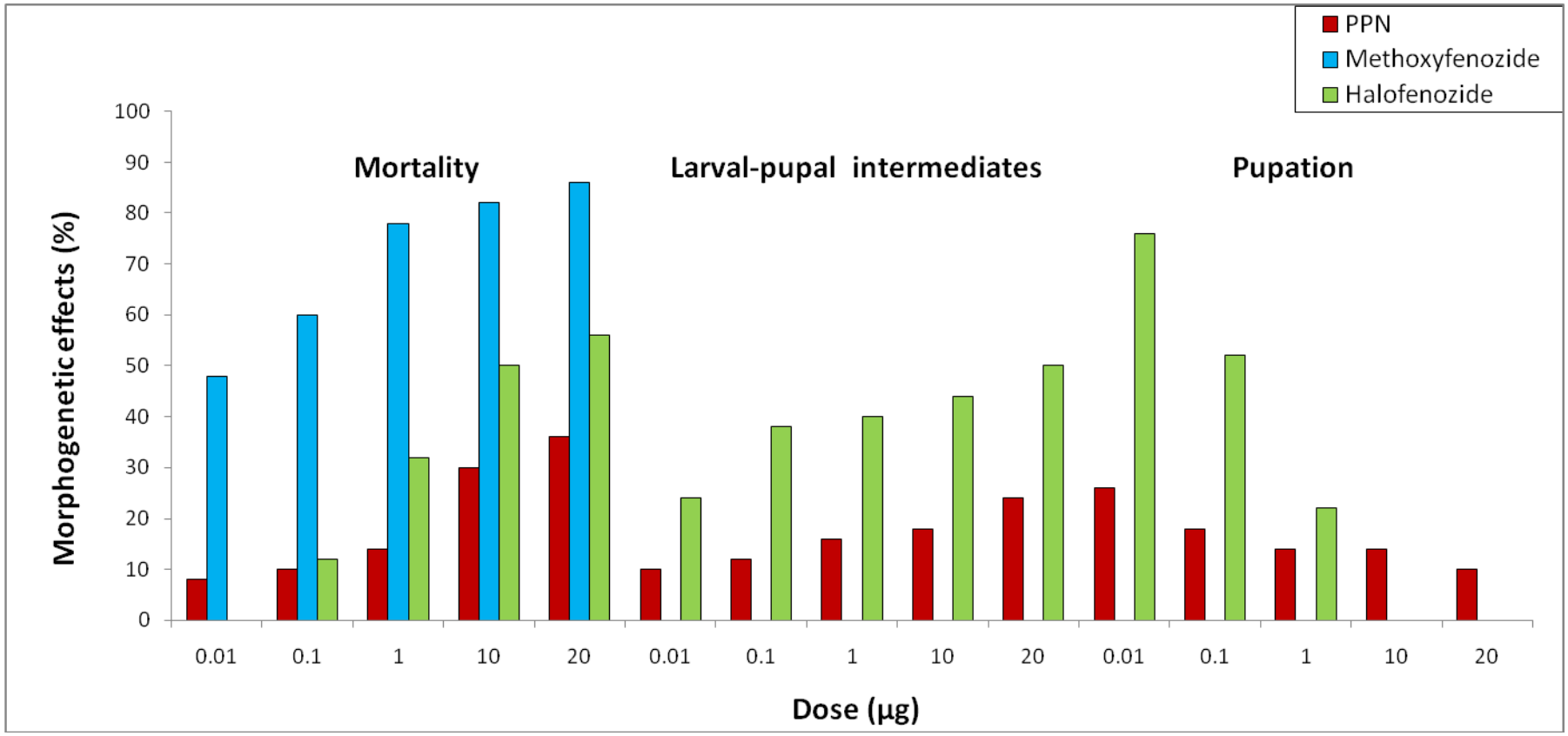

Fig. 19. Morphogenetic effects of hormone agonists on third instar day 0 larvae.

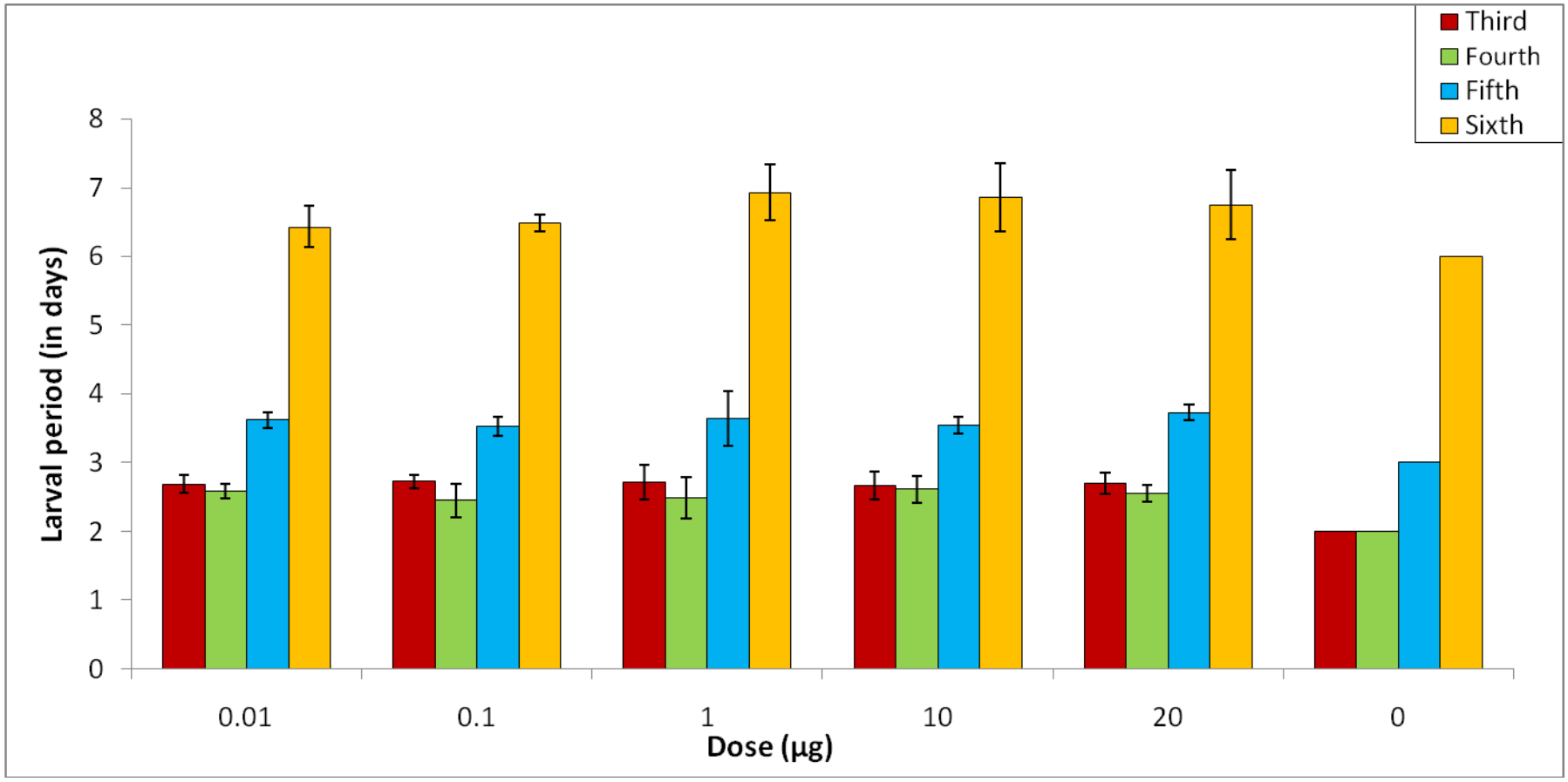

Fig. 20. Effects of PPN treatments of third instar day 0 larvae on the larval period.

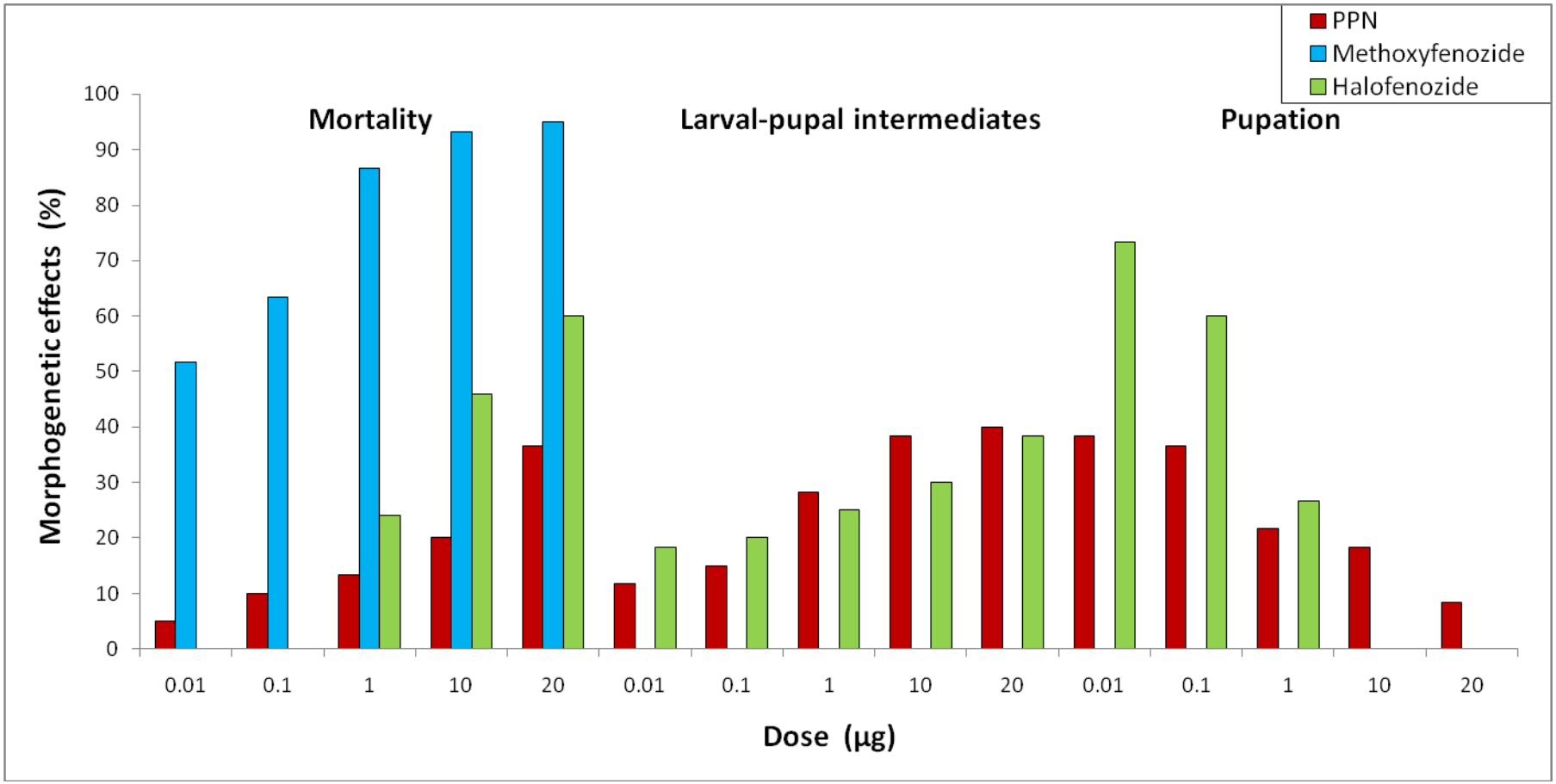

Fig. 29. Morphogenetic effects of hormone agonists following treatments on fourth instar day 0 larvae.

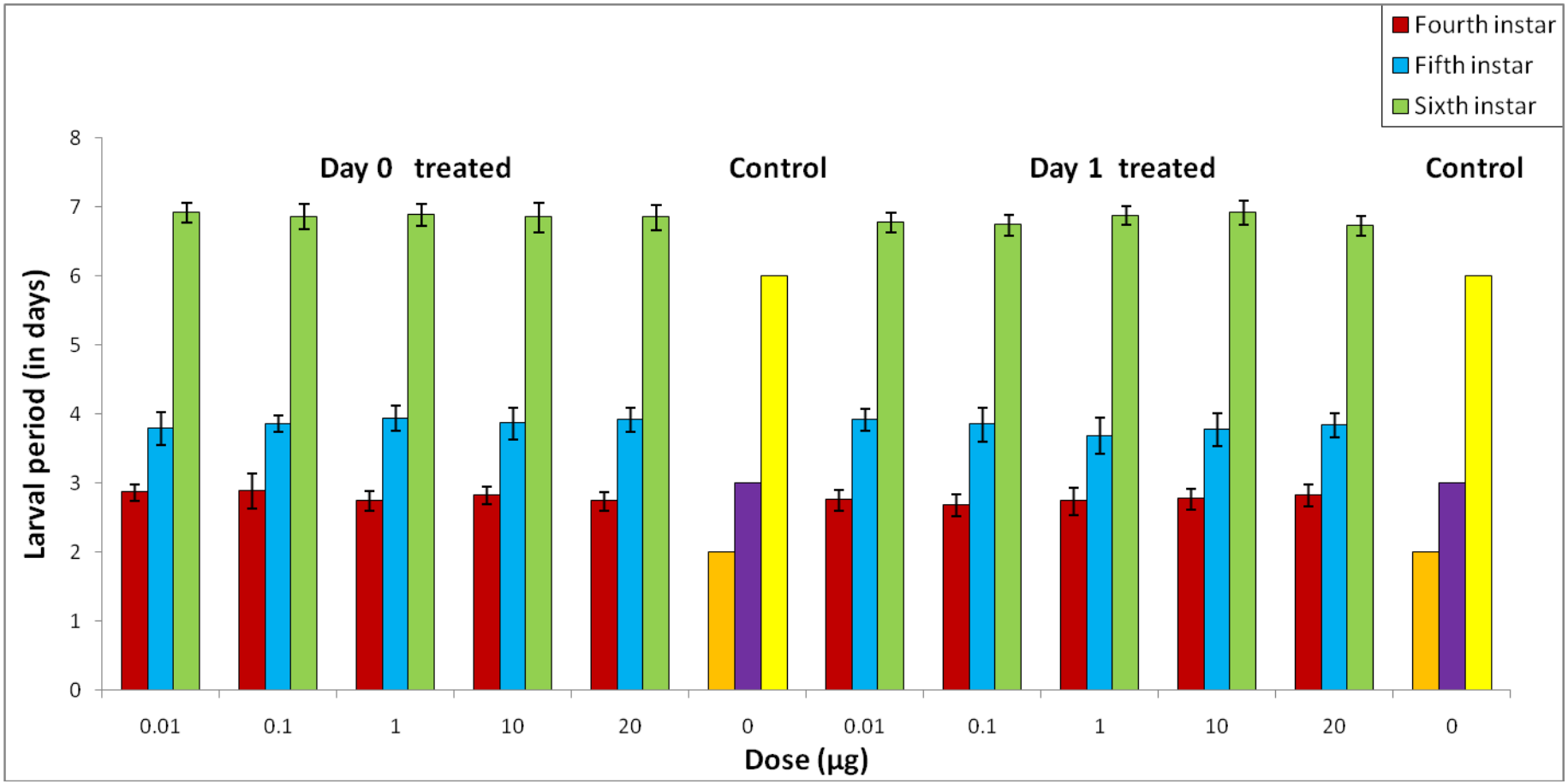

Fig. 30. Larval period in PPN treated fourth instar day 0 and day 1 larvae

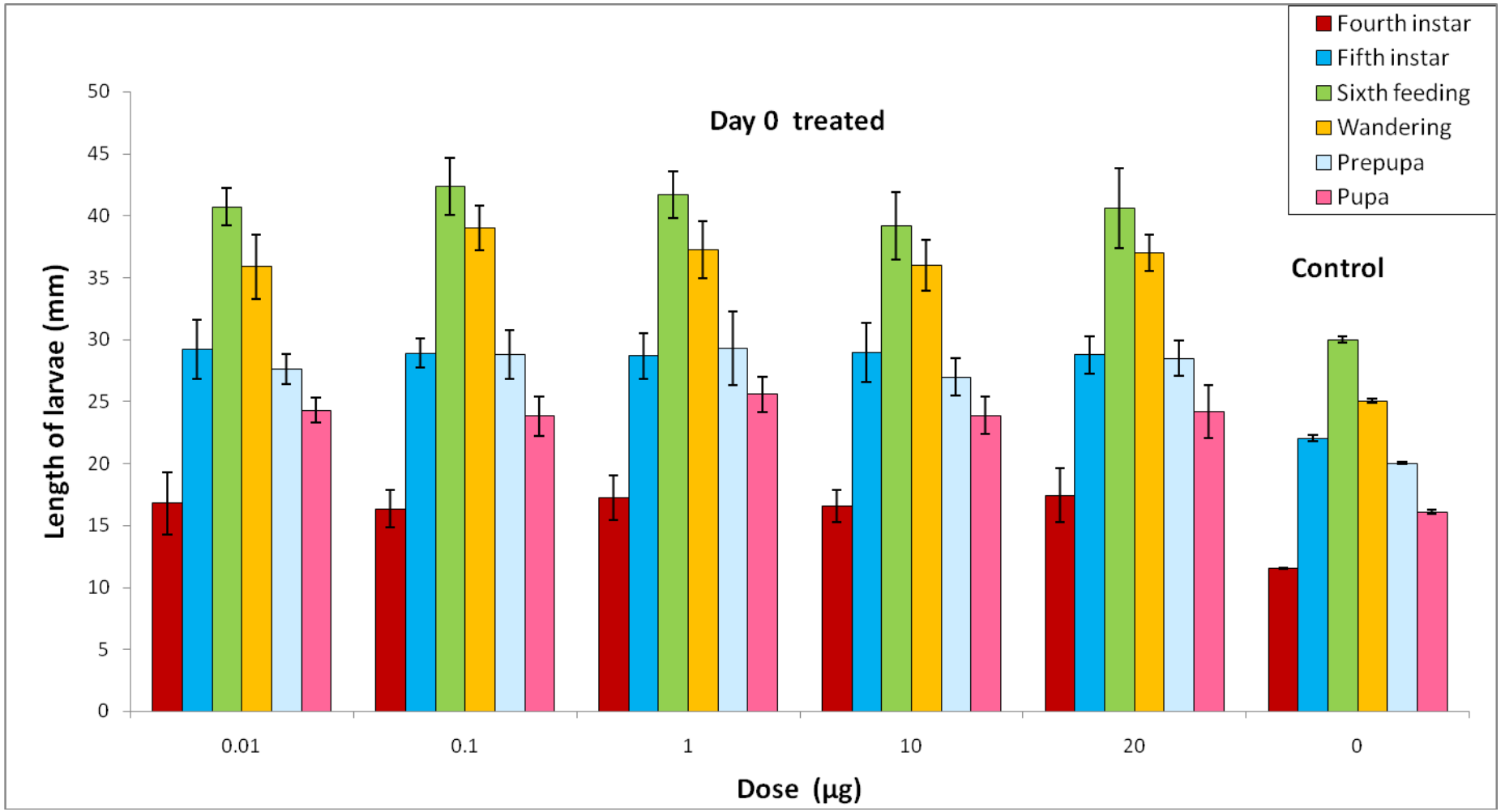

Fig. 31. Effects of PPN treatments of fourth instar day 0 larvae on larval length.

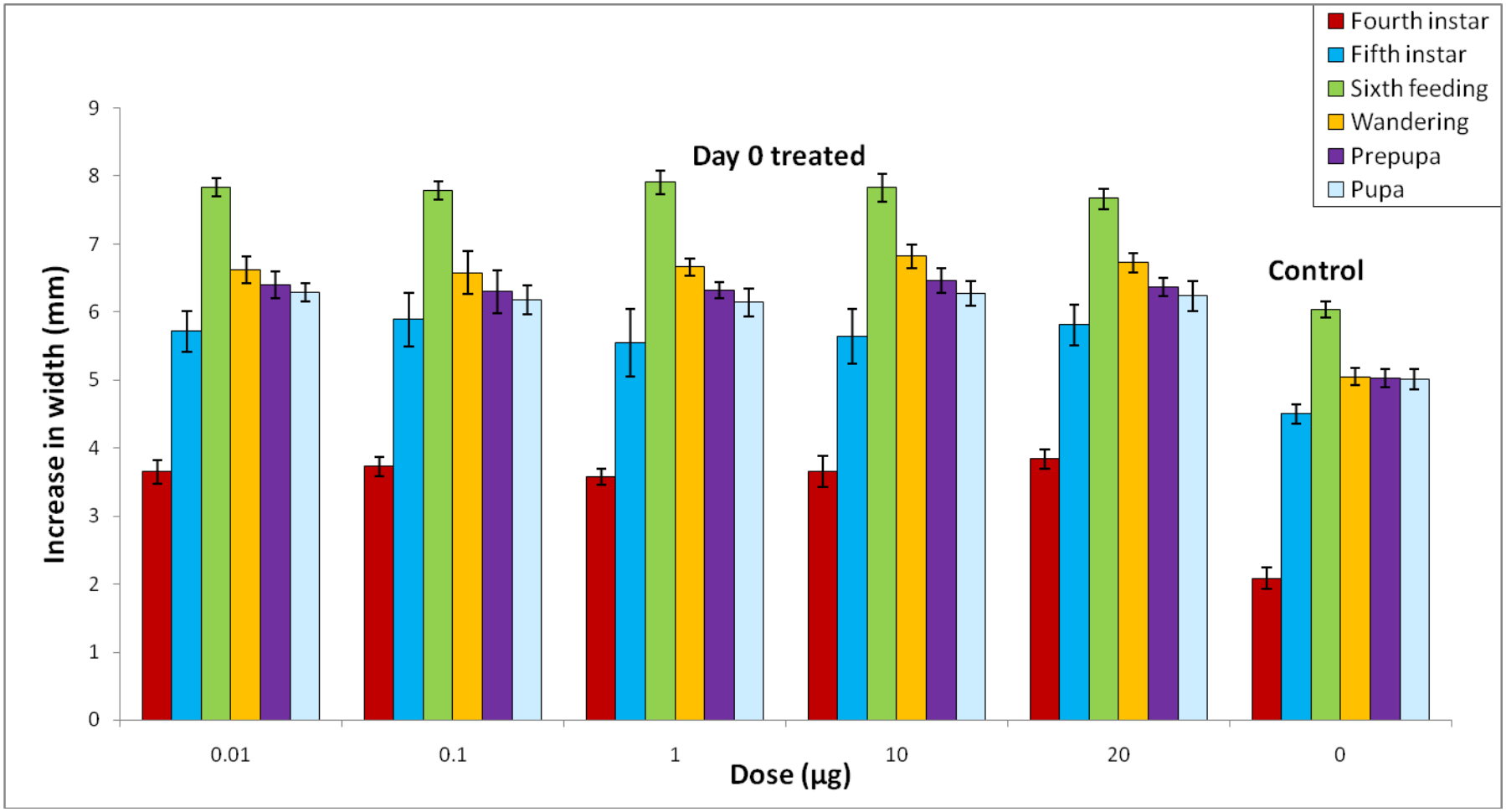

Fig. 32. Width of larvae following treatments of PPN on fourth instar day 0 larvae

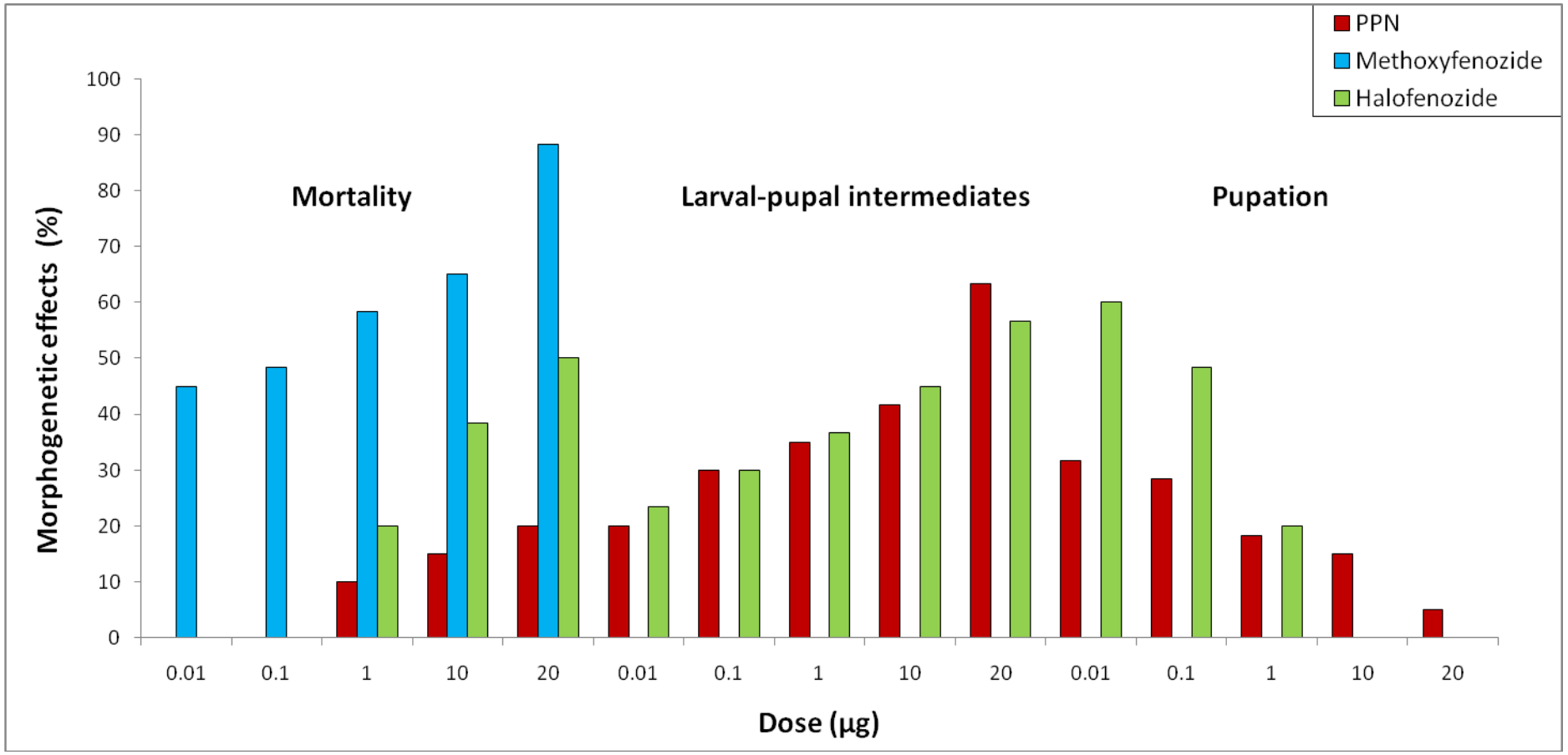

Fig. 33. Morphogenetic effects of hormone agonists following treatments of fourth day 1 larvae

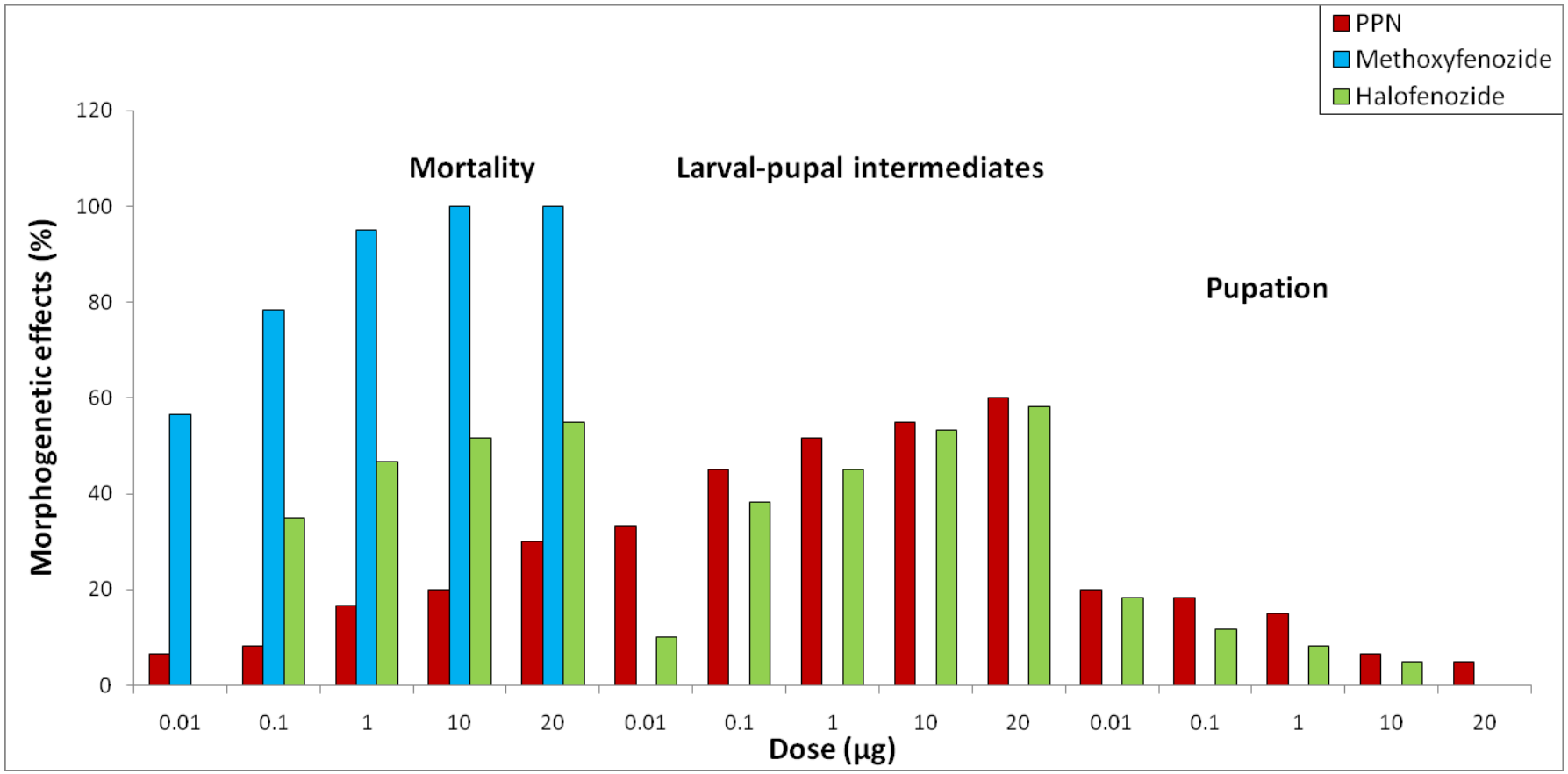

Fig.34. Morphogenetic effects of hormone agonists following treatments of fifth instar day 0 larvae

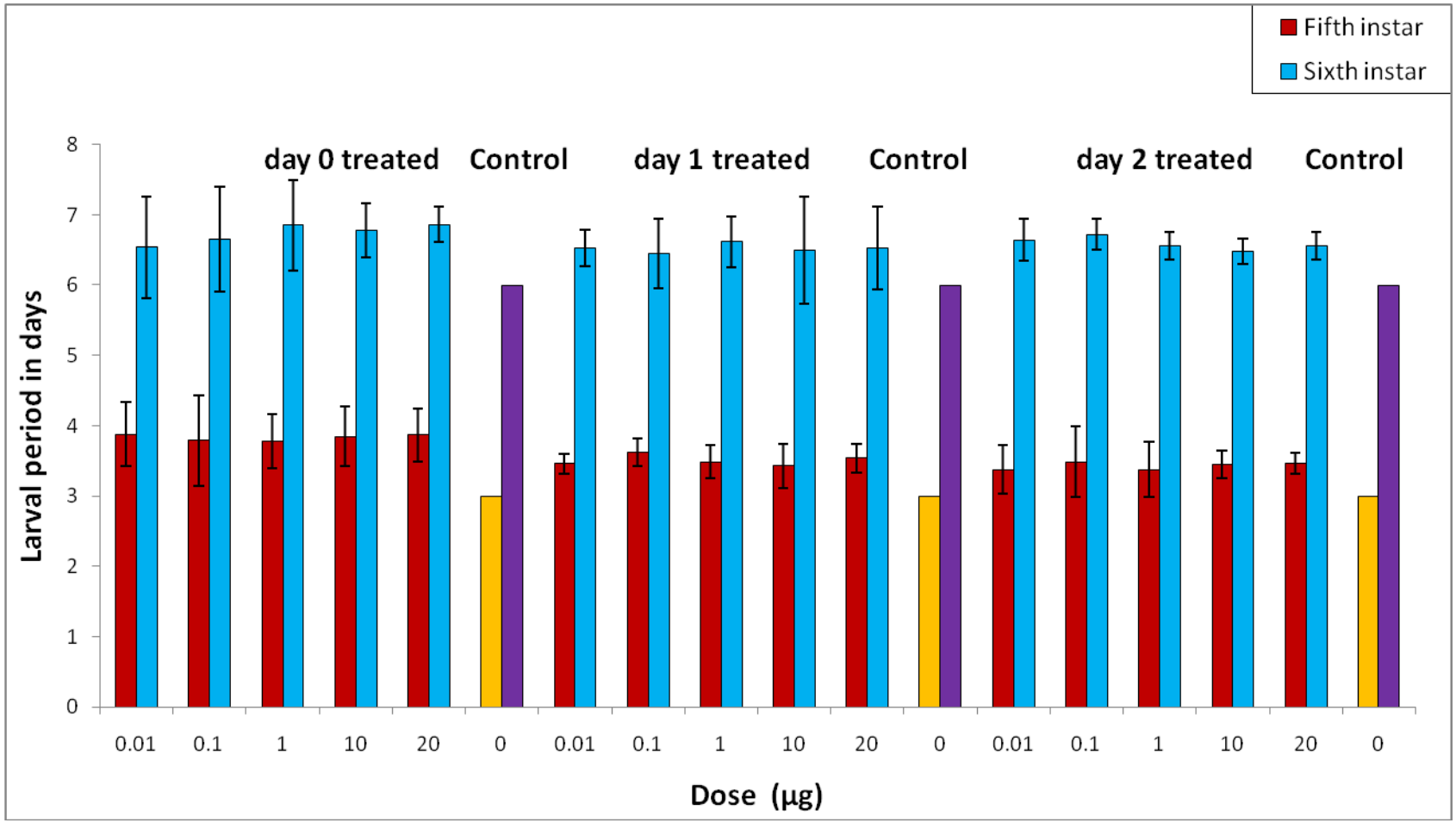

Fig. 39. Larval period following PPN treatments on fifth instar day 0, day1 and day 2 larvae

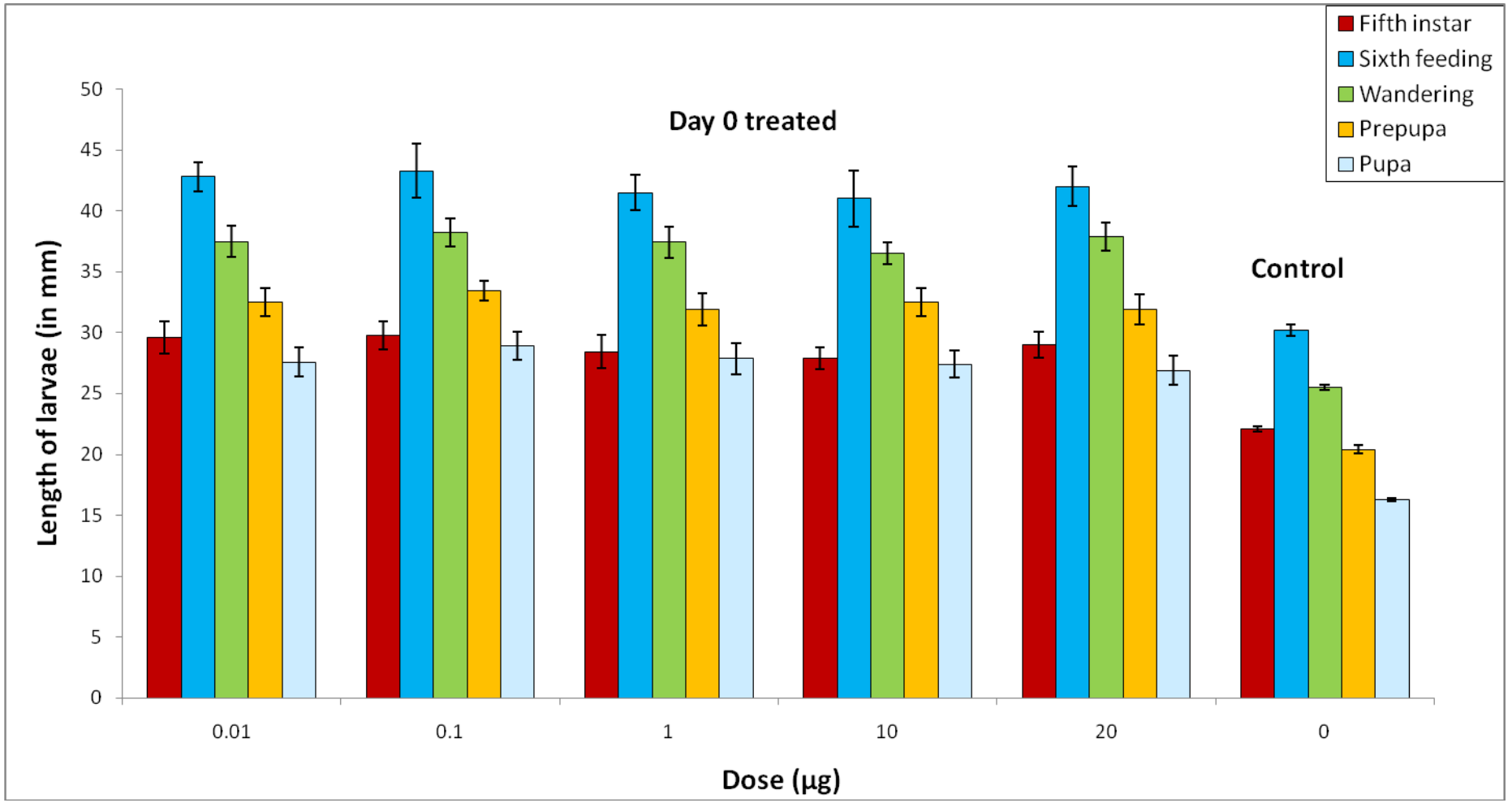

Fig. 40. Effects of PPN treatments of fifth instar day 0 larvae on larval length.

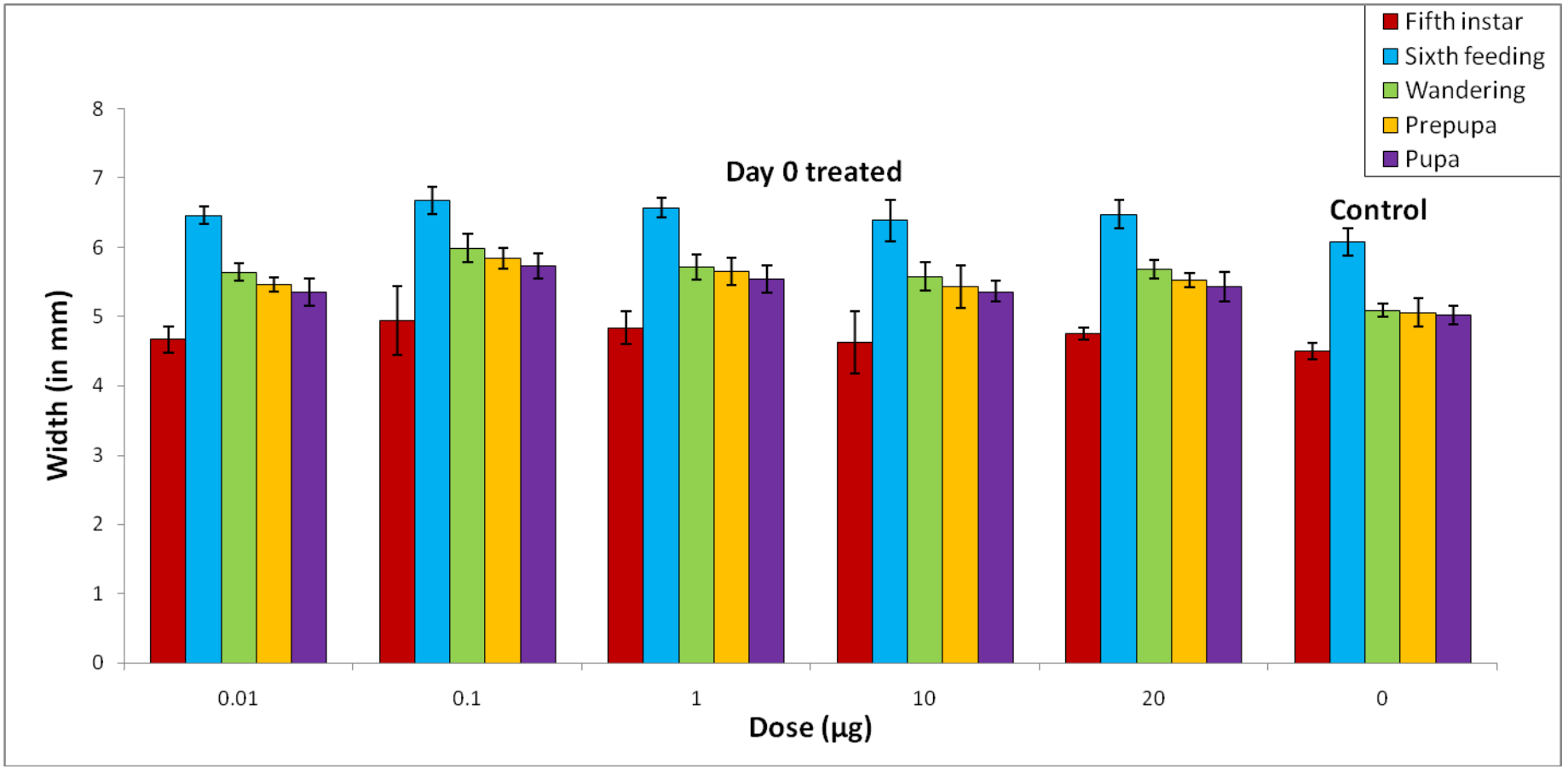

Fig. 41. Width of larvae following PPN treatments on fifth instar day 0 larvae.

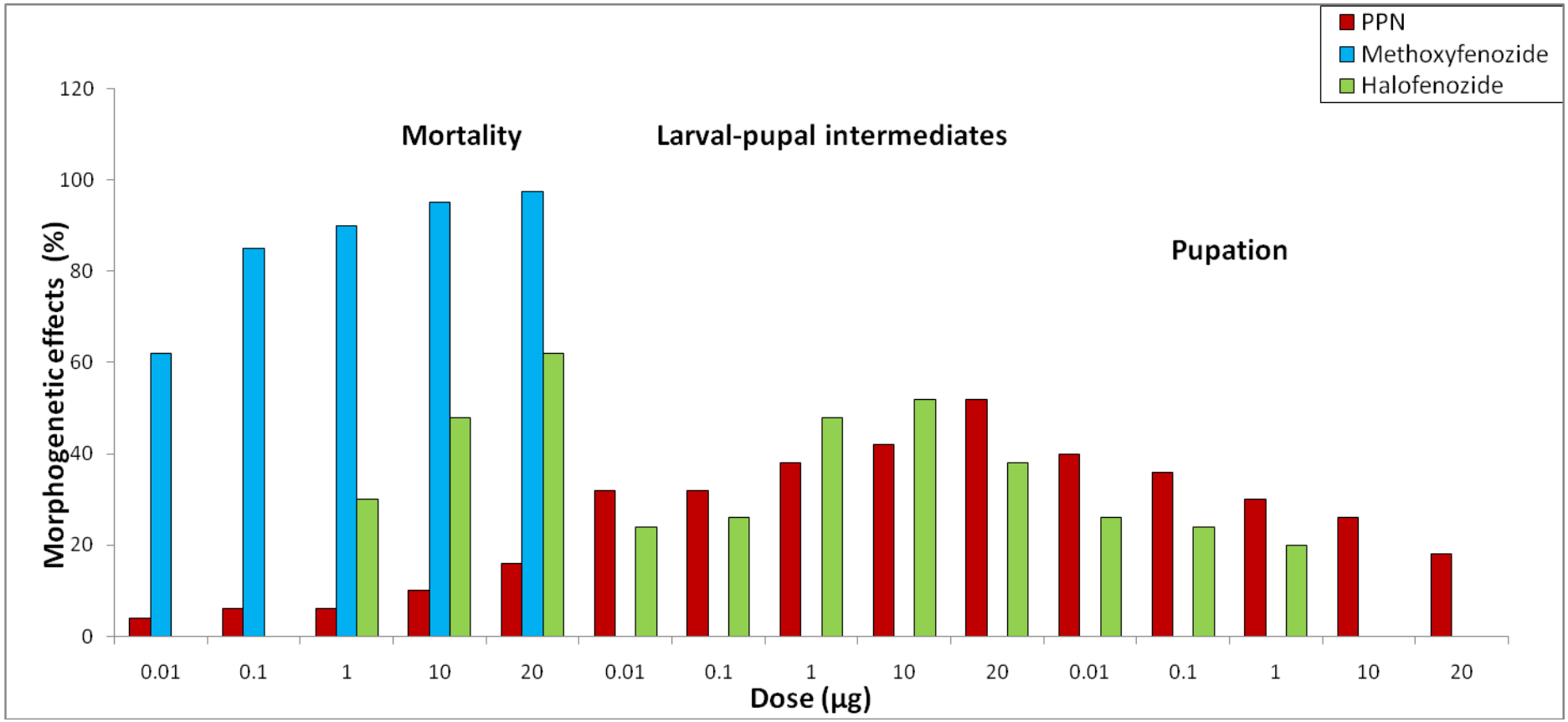

Fig. 42. Morphogenetic effects of hormone agonists following treatments on fifth instar day 1 larvae.

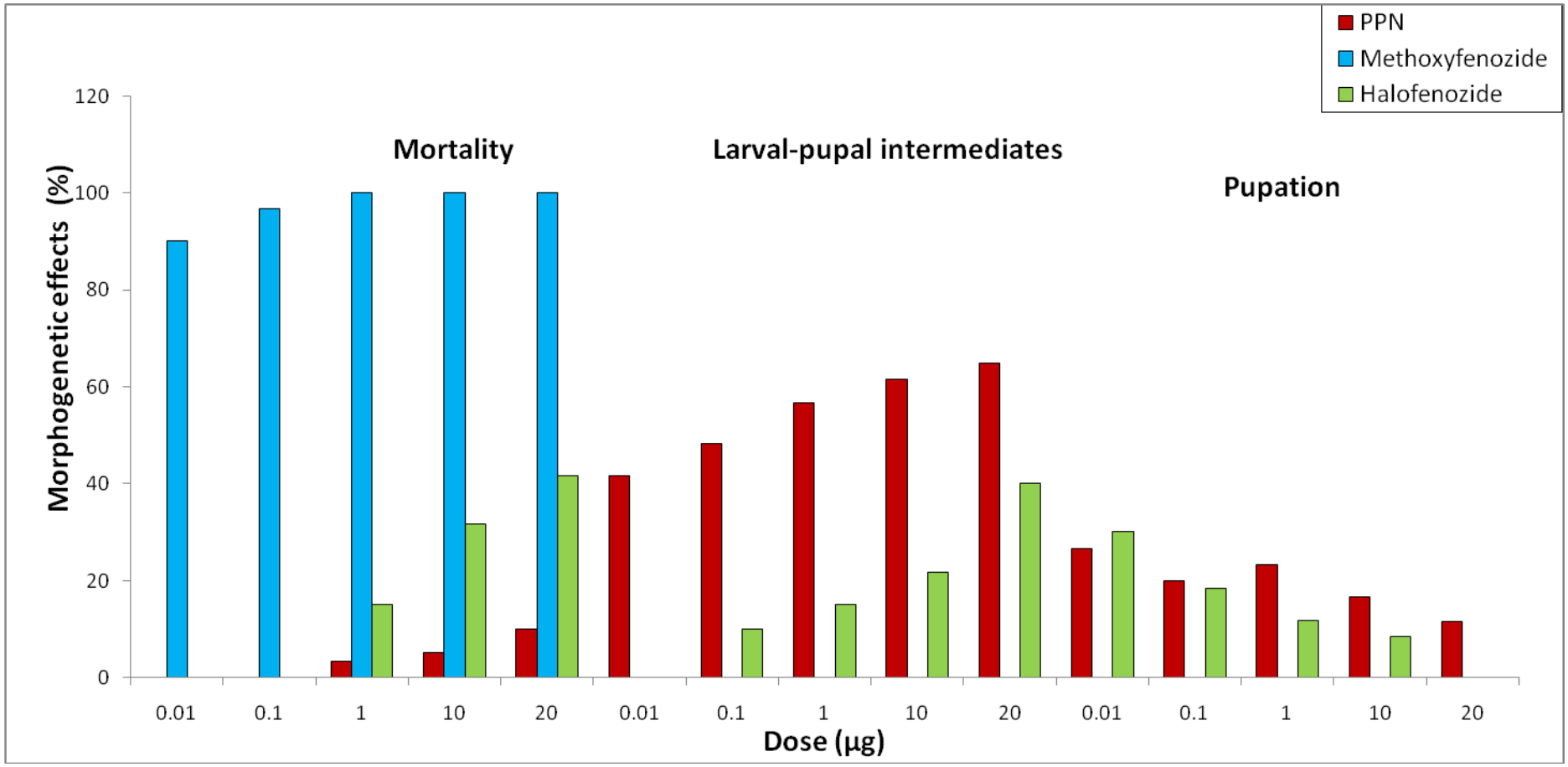

Fig. 43. Morphogenetic effects of hormone agonists on fifth instar day 2 larvae.

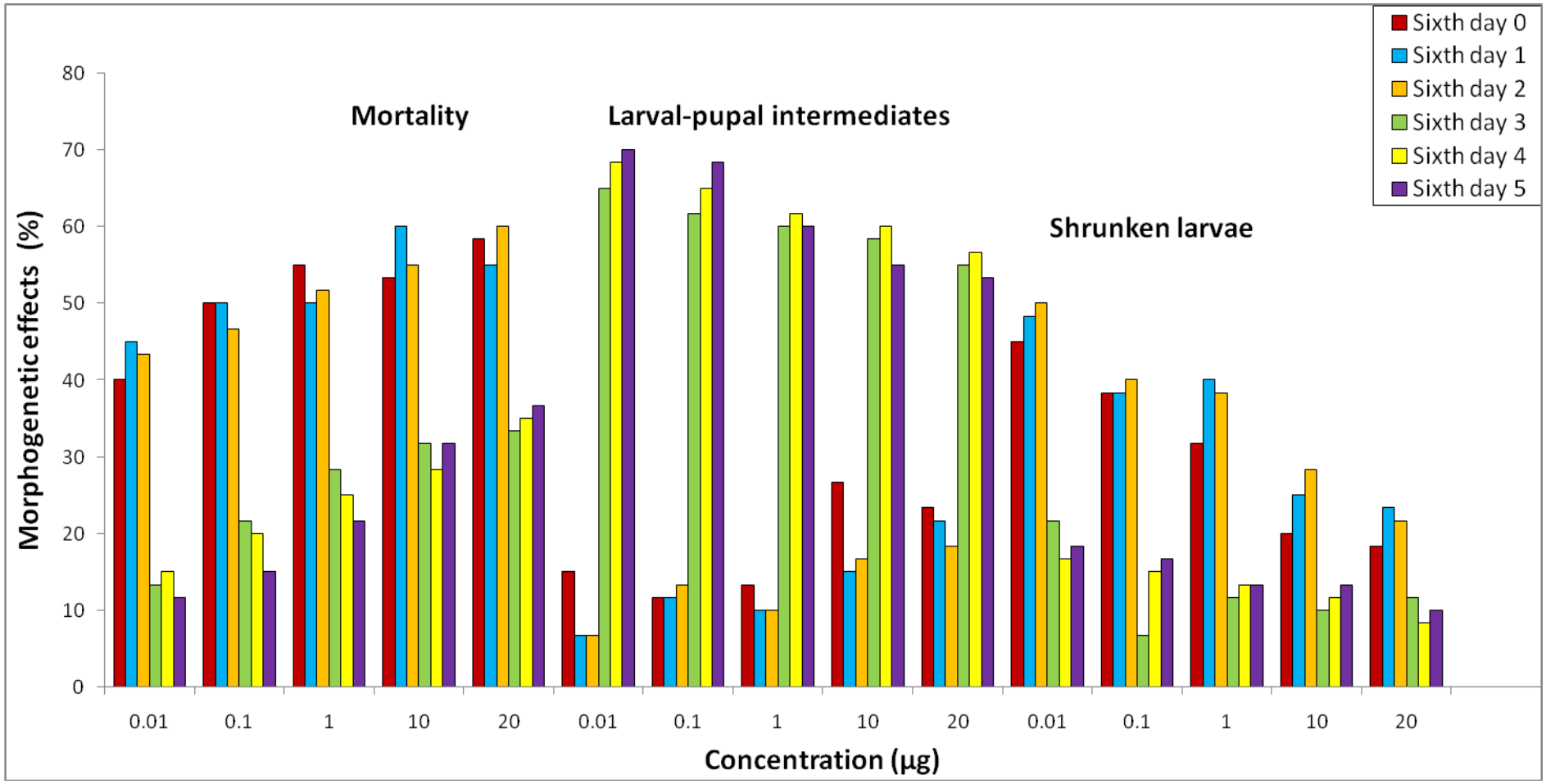

Fig. 44. Morphogenetic effects of PPN following treatments on variously aged sixth instar larvae

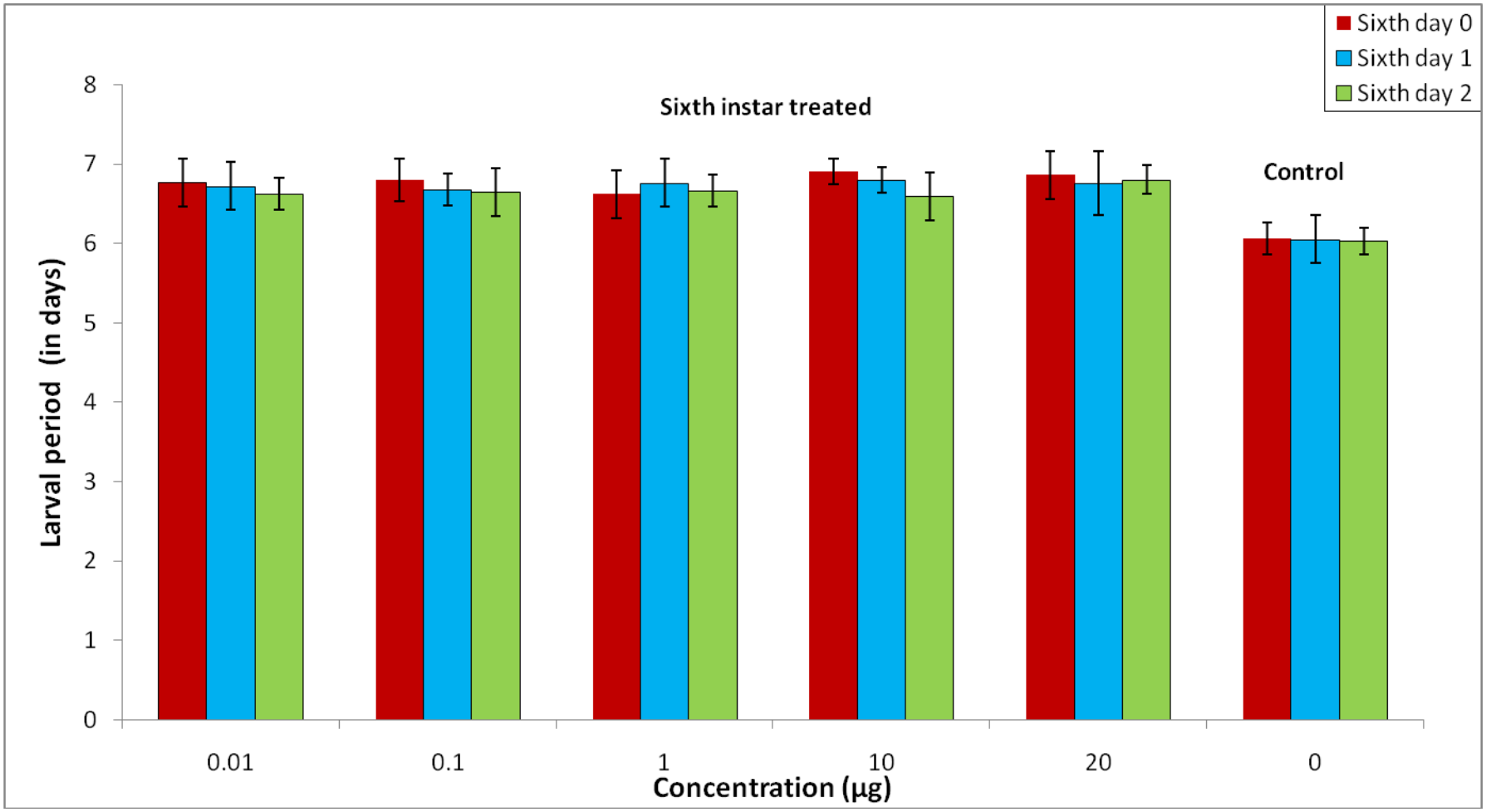

Fig. 53. Effects of PPN on larval period following treatments on sixth instar day 0, day 1 and day 2 larvae.

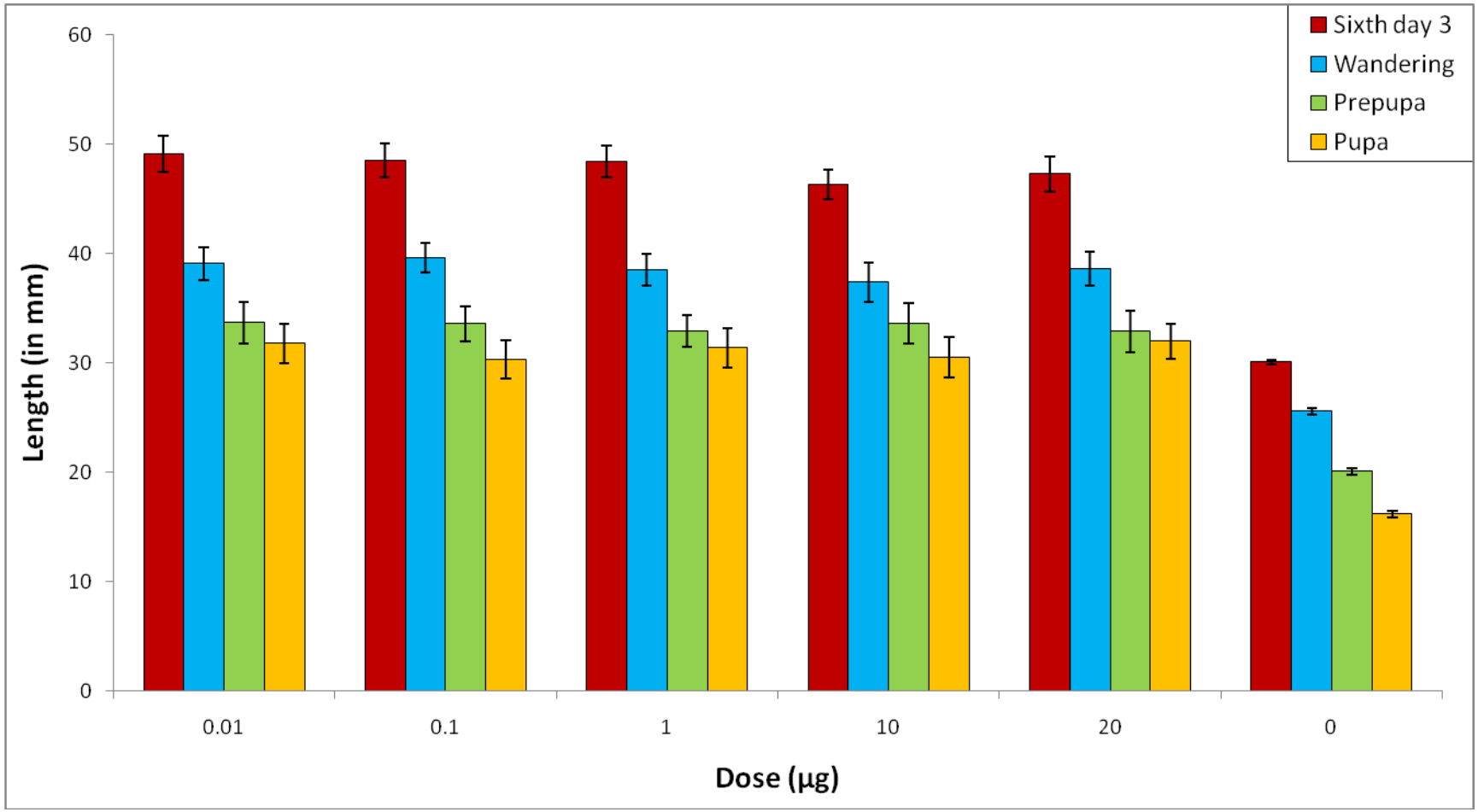

Fig. 54. Effects of PPN on larval length following treatments of sixth instar day 0 larvae

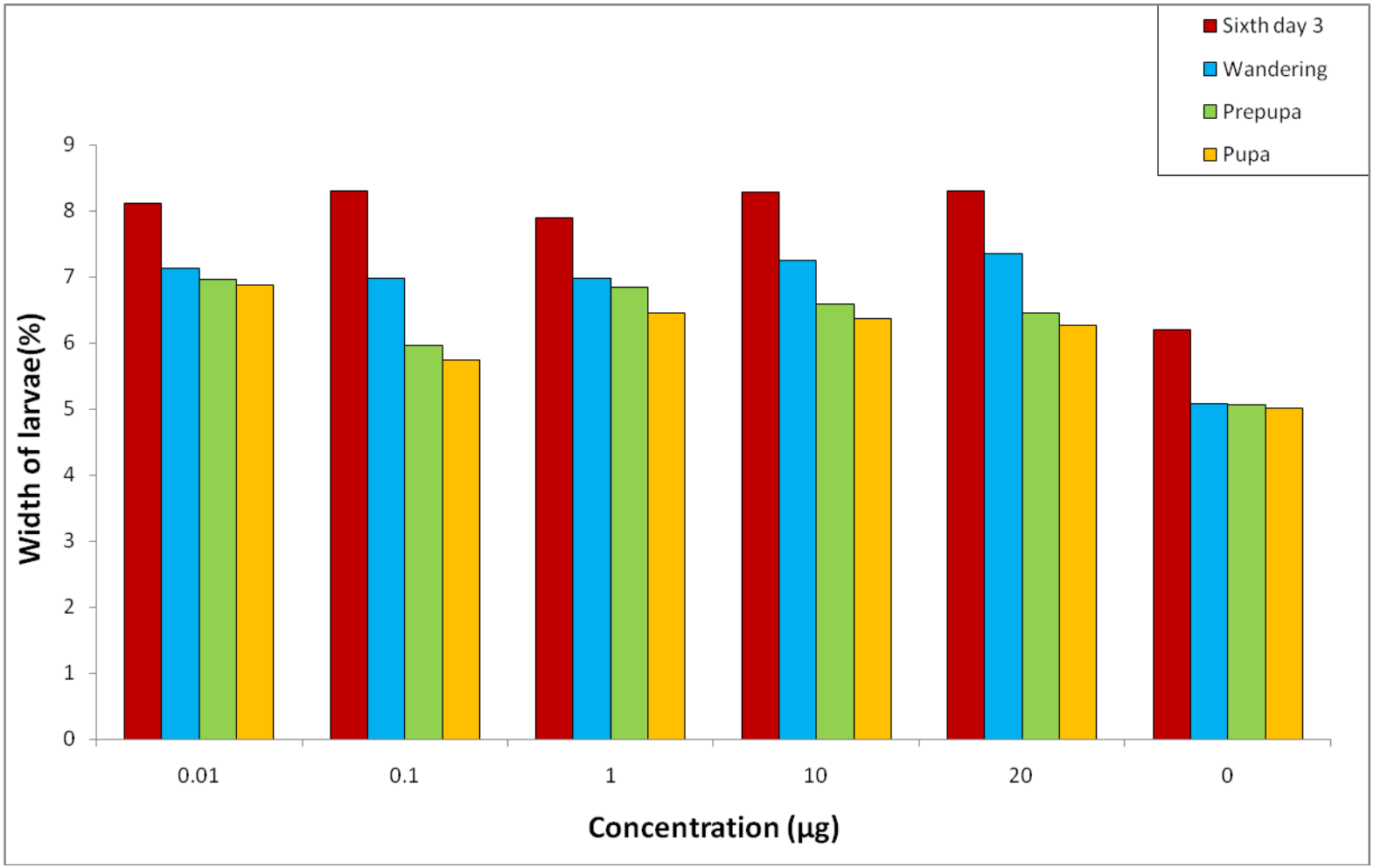

Fig. 35. Increase in width of larvae followed by PPN treatments on sixth instar day 0 larvae

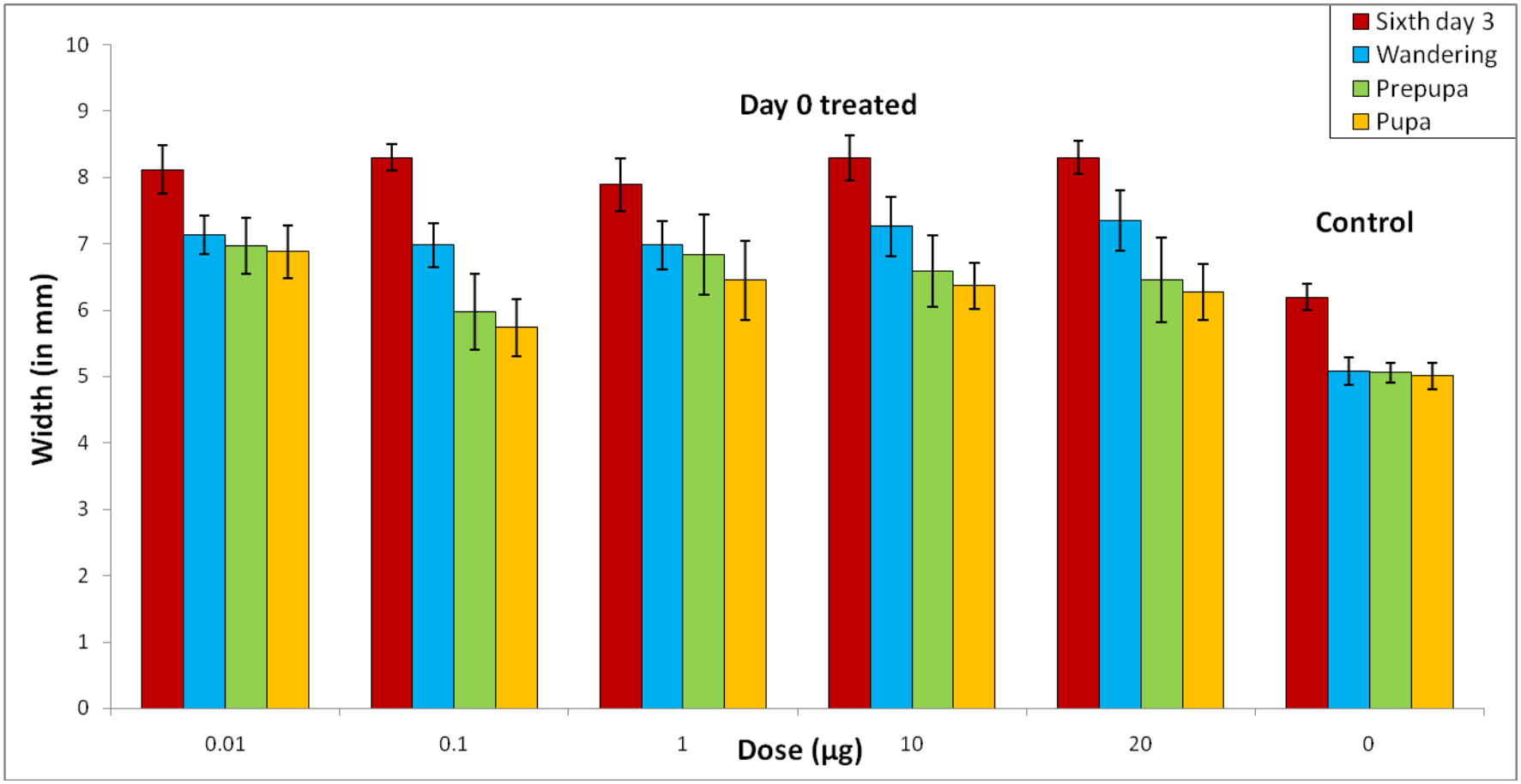

Fig. 55. Effects of PPN on larval width following treatments of sixth instar day 0 larvae.

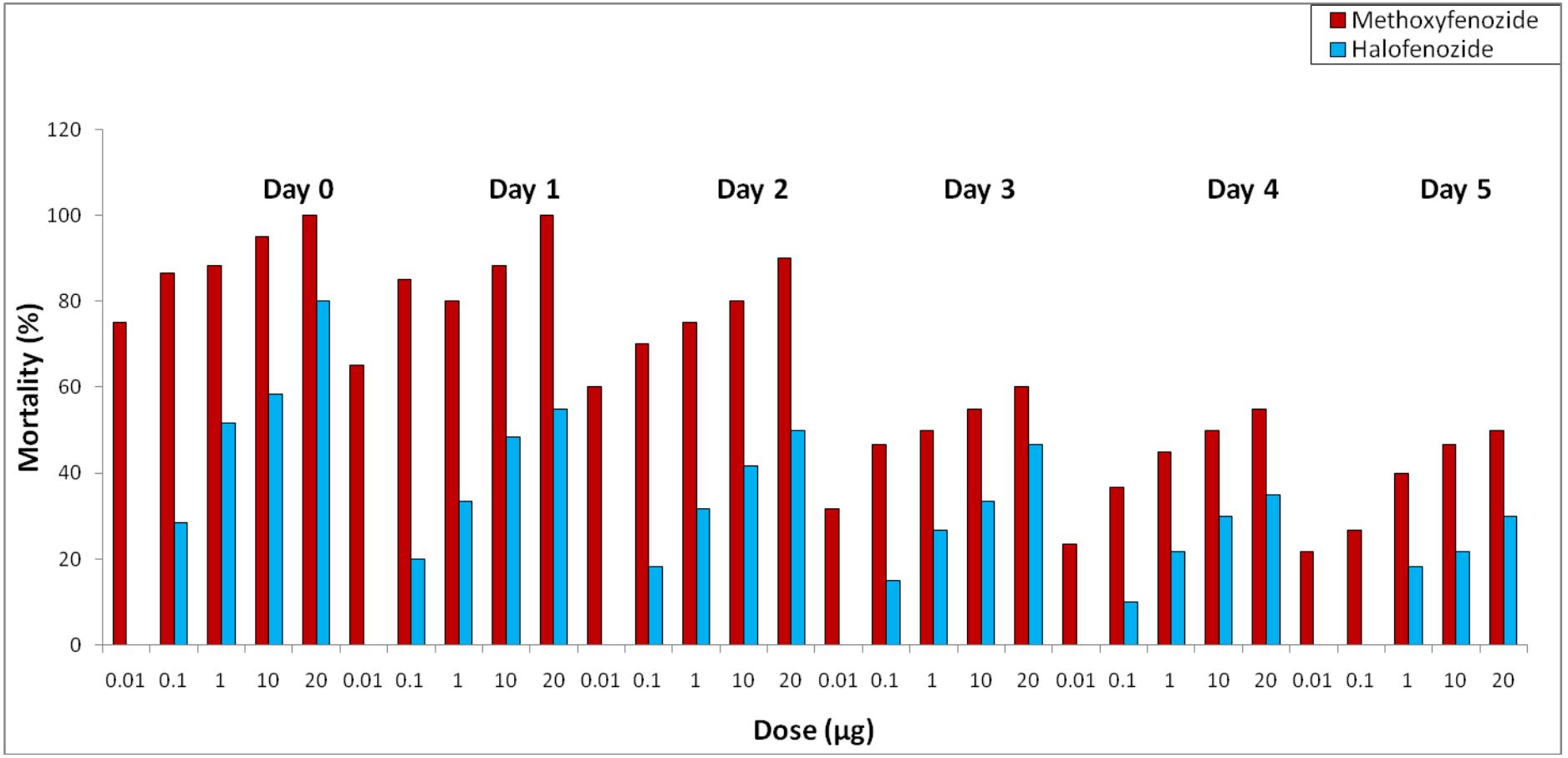

Fig. 56. Mortality induced by treatments of ecdysone agonists on variously aged sixth instar larvae

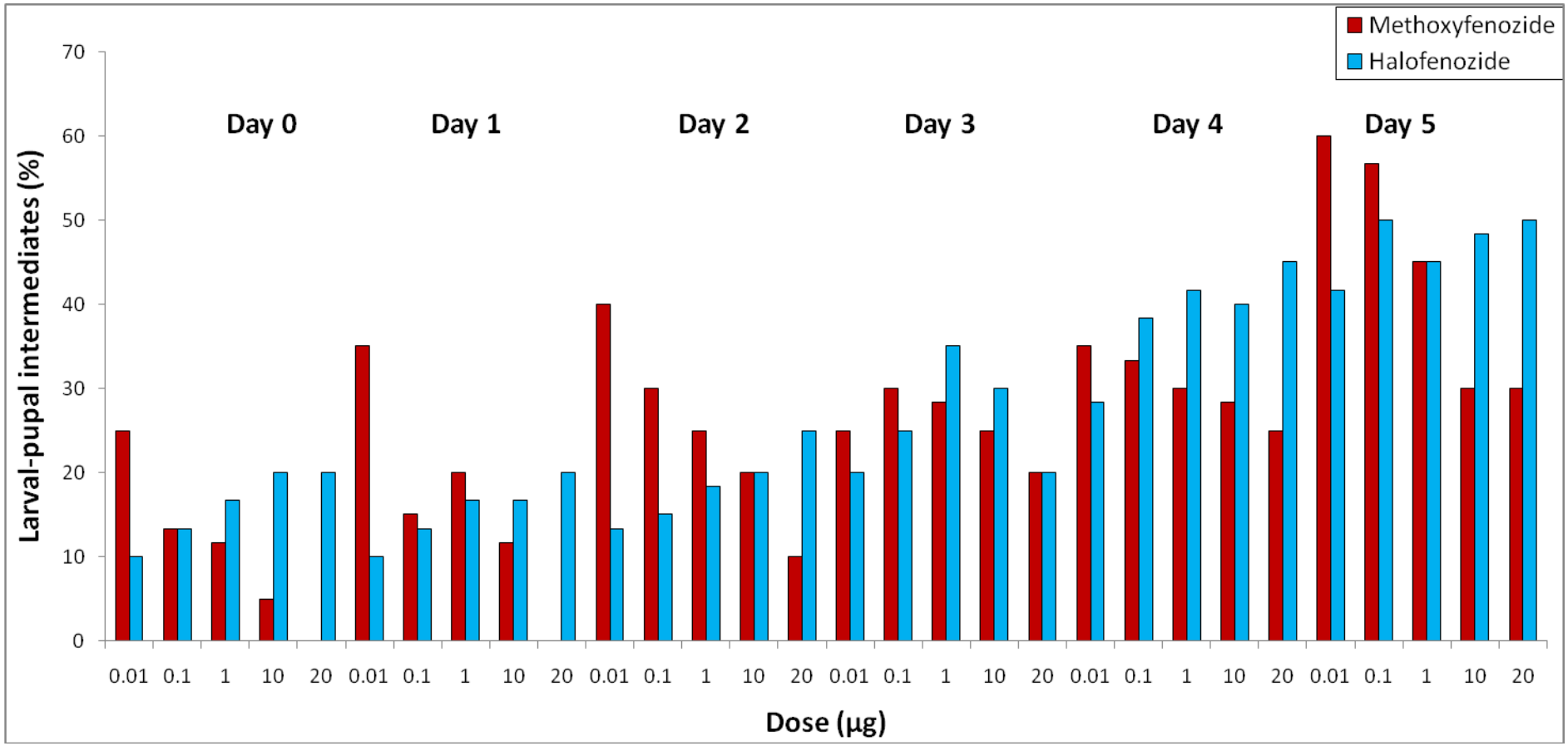

Fig. 73. Formation of larval-pupal intermediates after treatments of ecdysone agonists on variously aged sixth instar larvae.

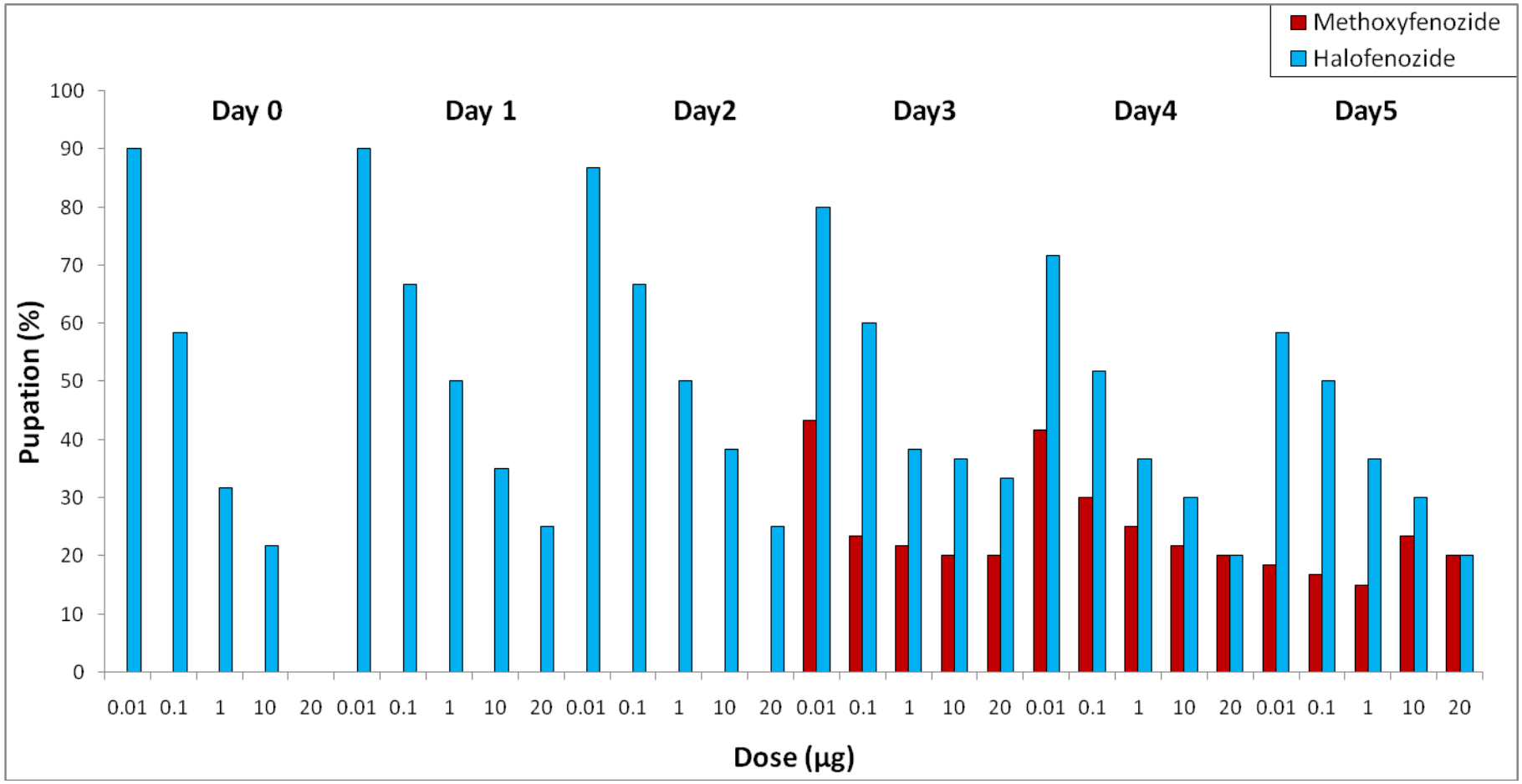

Fig. 74. Effects of ecdysone agonists on pupation of variously aged sixth instar larvae

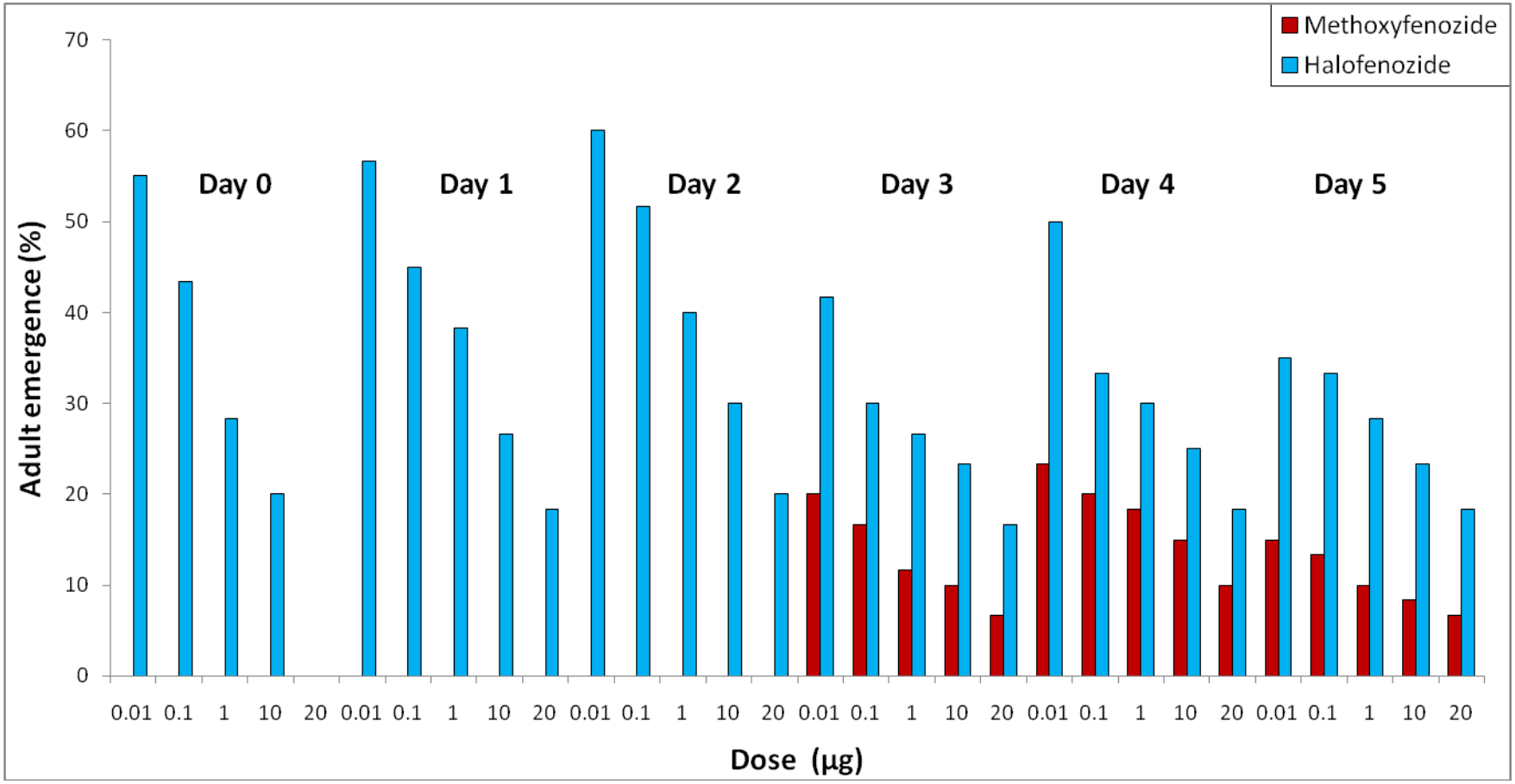

Fig. 75. Effects of ecdysone agonists on adult emergence following treatments on variously aged sixth instar larvae

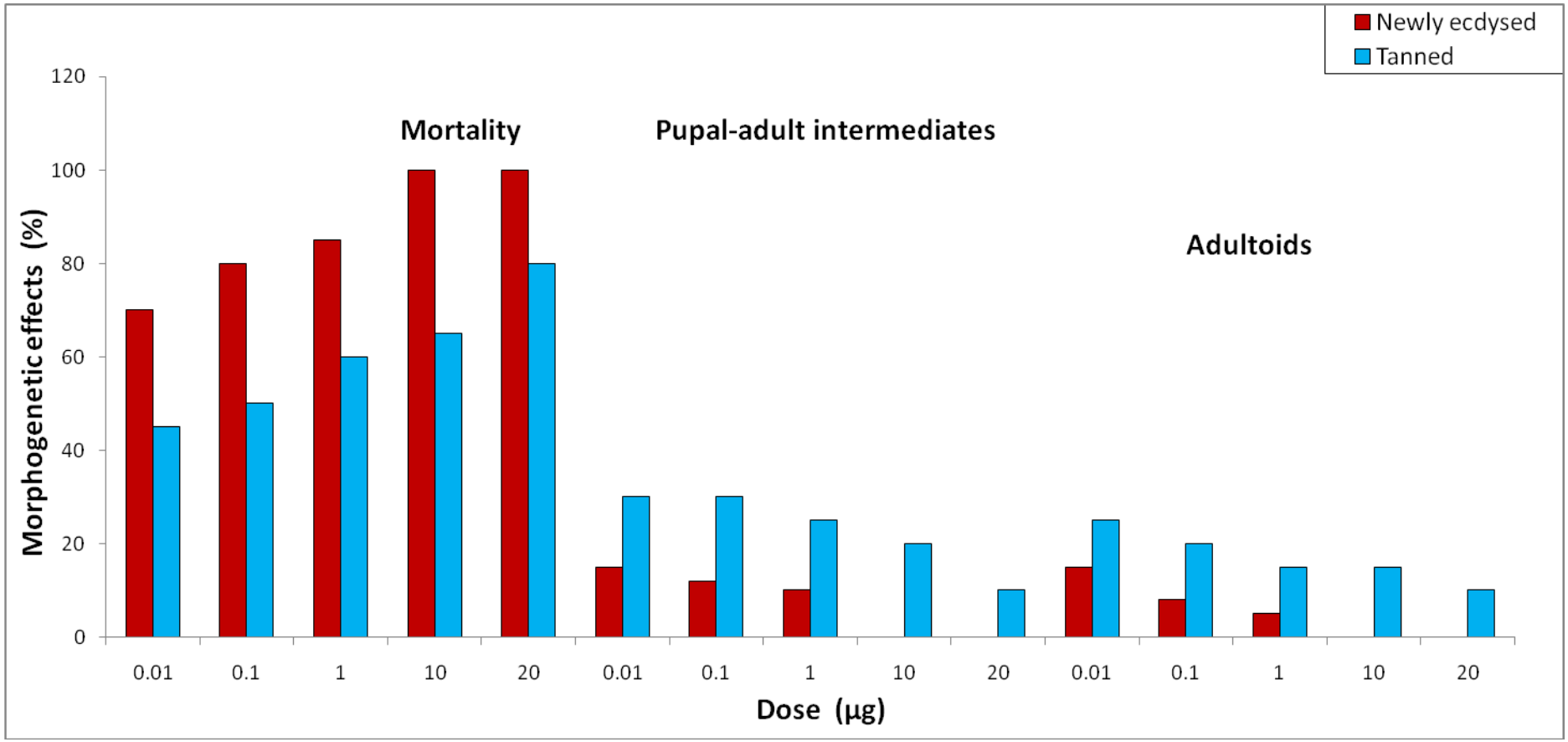

Fig. 92. Effects of PPN treatments on pupal-adult transformation

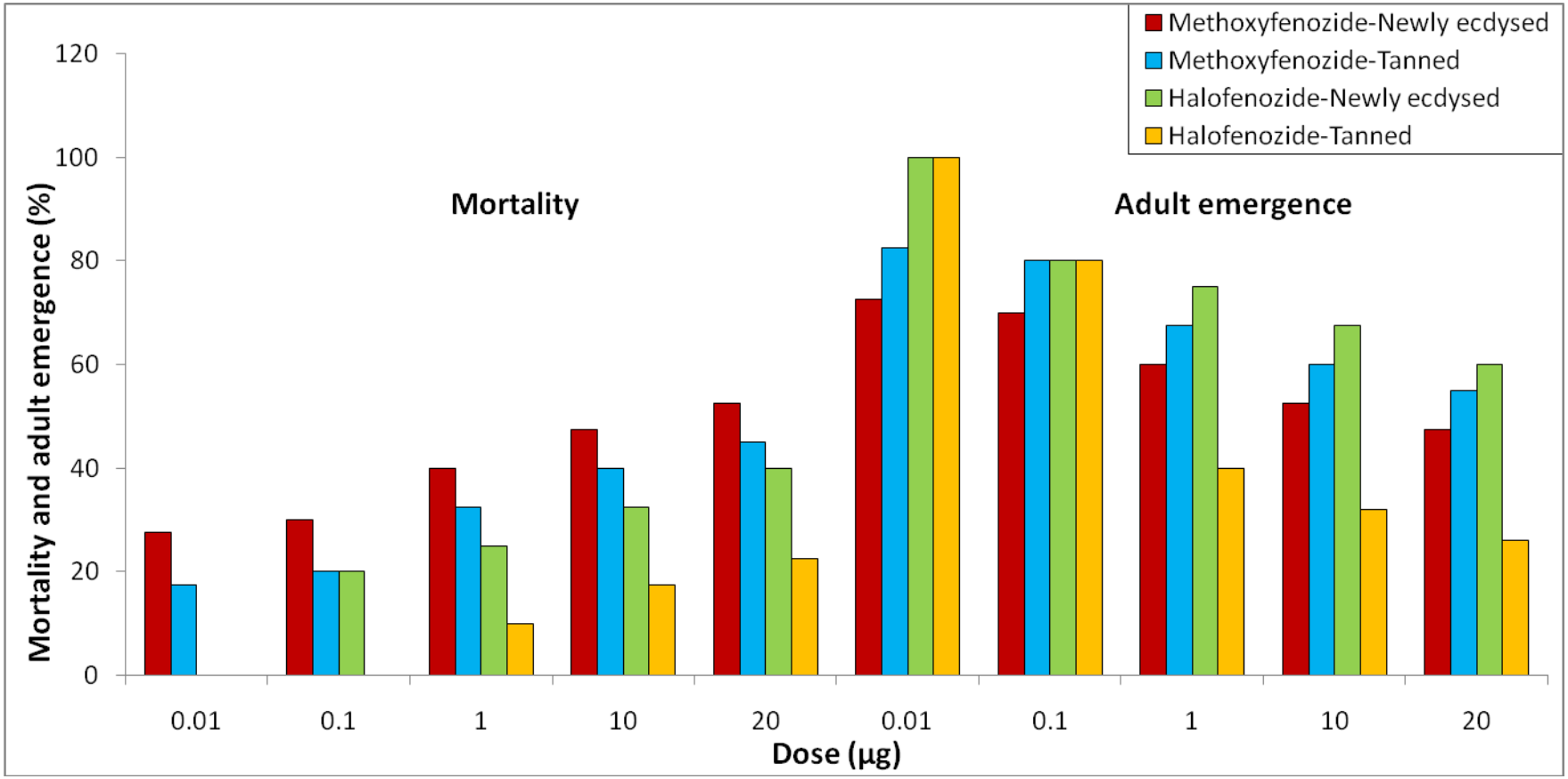

Fig.93. Effects of treatments of ecdysone agonists on pupal mortality and adult emergence

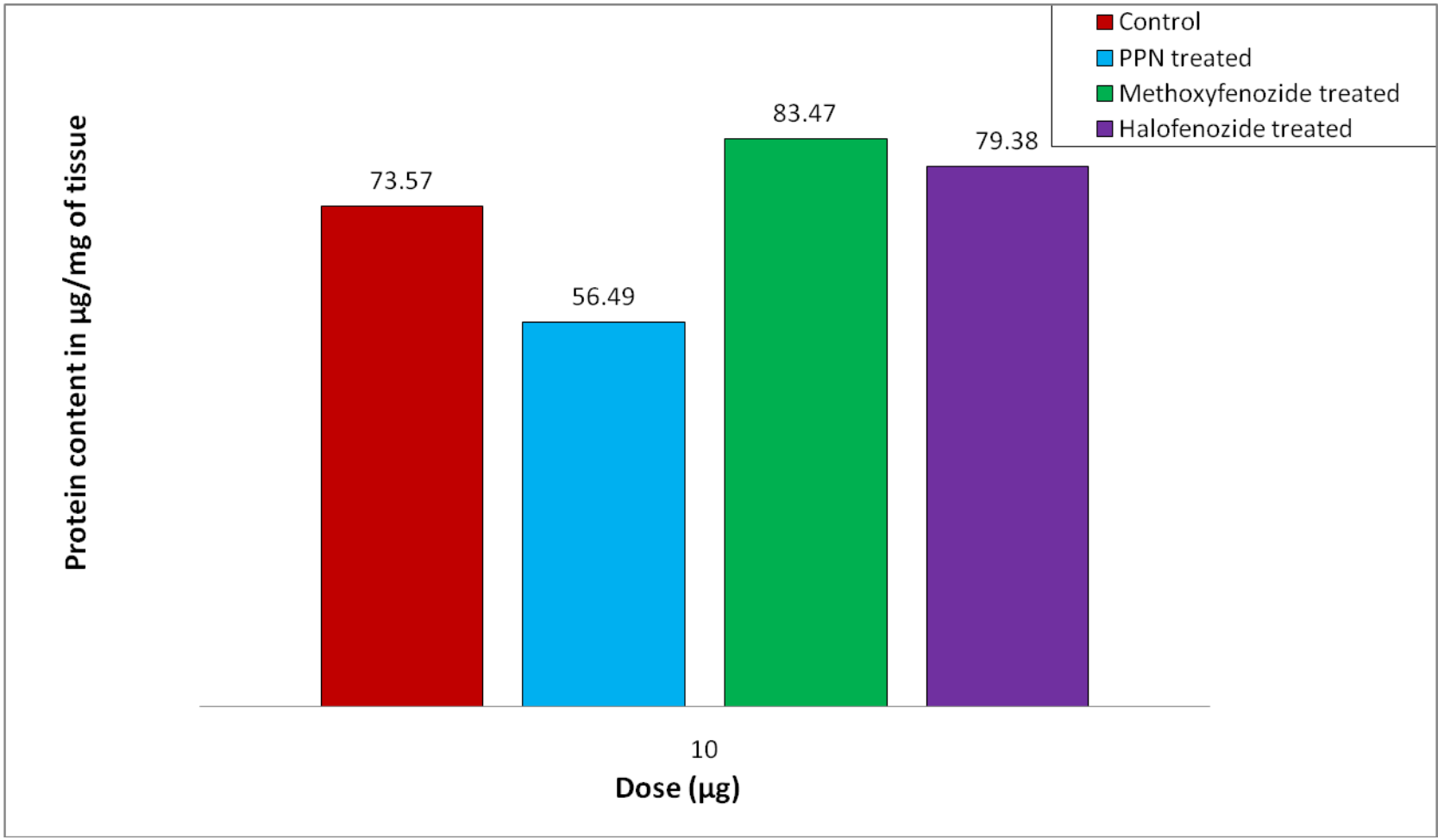

Figure 137. Protein content in the wing discs of sixth instar day 5 cotrol and treated larvae