Chapter ~ 5
The present investigation was carried out with the aim to evaluate selected biological, biochemical and histological parameters caused by the exposure of methoxyfenozide in V instar larva of *B. mori*. Although lots of works have been done on these parameters in relation to IGR, reports on haematological and biochemical parameters are scanty in *B. mori*. The behavioural and morphological changes brought about by methoxyfenozide (RH-2485) in V instar larva of *B. mori* like cessation of feeding, restricted movement, loosening of skin, head capsule slippage, symptoms of premature molt, and fluid excretion agree with those of Retnakaran *et al.* (1995), Smagghe *et al.* (2000) and Hussein *et al.* (2005).

According to Pugazhvendan and Soundararajan (2009), penfluron, a chitin inhibitor caused a great reduction in *Chrysocoris purpurens* (Westwood) haemocytes in both sexes after 72 and 98h treatment. Berger *et al.* (2003) reported that IGR possesses both stimulation and inhibition of haemocyte concentration; here methoxyfenozide caused decrease in the THC with increasing hours after exposure to IGR. Anandakumar and Michael (2011) showed that *Bacillus thuringiensis* infected silkworm *B. mori* showed 15% decrease in THC when compared to healthy worms. The effect of chitin synthesis inhibitor on *Dysdercus koenigii* was reported to affect THC (Zade *et al.*, 2012).
Sendi and Salehi (2010) showed that the low doses of methoprene caused THC reduction in general and of plasmatocytes, adipohaemocytes and spherule cells in particular. Methoxyfenozide is found to cause an alteration in THC and the percentage of differential haemocyte count in the larva of silk worm, which may be related to the morbidity in silkworm. The DHC is also found to be affected by this IGR, where the granulocyte population increases during 24h treated larvae than the control larval haemocytes and decreases in other hours after treatment than the control larval haemocytes. Similarly β-ecdysone to S.litura, caused an increase in the prohaemocytes and plasmatocytes (Gupta and Sutherland, 1968), plasmatocyte and granulocyte count alone (Anandakumar and Michael, 2011). The differential haeomocytic count was found to be affected by the insecticidal stress in D.cingulatus (Qamar and Jamal, 2009). The increase in the prohaemocytes in all the treated larva haemocytes shows the worm physiologically trying to produce more cells for their immunological reasons, because when the circulating haemocytes decreases new haemocytes were found to be produced from hematopoietic tissues to balance the lost haemocytes (Marmaras and Lampropoulou, 2009).

In the IGR treated groups the granulocytes were found to discharge their granules out into the haemolymph. Thus the degranulation may lead to increased recruitment of granular cells. Further the granules may be responsible for the chemo tactic attraction of the plasmatocytes as reported by Faraldo and Lello (2003). Such a type of observation was seen among the granular cells of G.mellonella during clotting and phagocytic reaction (Ratcliffe et al., 1985) and wound healing (Mangalika et al., 2010). Similarly, the relative number of prohaemocytes, plasmatocytes and granulocytes was found to increase in P. americana due to genistein treatment (Berger et al., 2003). Even though the plasmatocytes and granular cells are the important
haemocytes in immune responses to pathogens via, phagocytosis (Strand, 2008) the role of granulocytes due to ecdysone agonist is yet to be understood.

An increase in plasmatocytes and reduction in the granular cells was already reported by Ghasemi et al. (2014) due to methoxyfenozide in *Ephestia kuehniella* Zell. The immune system functions in insects depend upon the total haemocytic count. Similarly the plasmatocytes and the granulocytes are the very active haemocytes involved in immune reaction of an insect thus reduction and alteration in these cells challenges the insect survival. During metamorphosis, haemocytes undergo morphological changes by increasing their cell size and transforming their granulocytes into macrogranulocytes. The population of haemocytes also changes with increased number of granulocytes and decreased plasmatocytes (Zhai and Zhao, 2012). Thus the release of granules may be due to the antagonistic effect of methoxyfenozide like ecdysone.

In plasmatocytes the thinning of cell membrane and plugging of cytoplasm out of the cell was observed. Similar observations were also reported by Sendi and Salehi (2010), on methoprene treated *Papilio demoleus* and Hegazi et al. (2000) by chitin synthesis inhibitor on *S. littoralis*. The oenocytes in normal situation are spherical cells with peripheral nucleus and crystalline inclusion without any granules. When any immune challenge occurred nucleus became small and granules appear indicating the crucial role in phenoloxidase (PO) cascade (Strand, 2008; Beckage, 2008). Here in this study the cell size decreased in all the treated groups compared to normal cells. Extreme pathological symptoms were observed in cell membrane, distortion of the cytoplasmic and nuclear membrane and abnormal staining of the haemocytes which
coinside with the observations in *Schistocerca gregaria* due to Nomolt at various concentrations (Teleb, 2011).

Prohemocytes in hematopoietic organs differentiate primarily into plasmatocytes, whereas other hemocyte types differentiate after release into circulation and maintenance of hemocyte populations in circulation depends heavily on continued division of each cell type after differentiation (Yamashita and Iwabuchi, 2001; Lavine and Strand, 2002). Even though the prohaemocytes population increased in methoxyfenozide treatment reduction in total haemocytes may be it affected the cell differentiation. Ghasemi *et al.* (2014) reasoned out that an increase in total number of hemocytes of methoxyfenozide treated larvae may be due to stimulation of haemocyte release from hematopoietic organs as well as increase in mitotic activity of hemocytes, at the same time reduction in THC due to pyriproxyfen treated larvae could be linked to interference of pyriproxyfen on ecdysone biosynthesis. Begum *et al.* (1998) suggested that it may be due to effective utilization of fat reserves during the period decreased respiratory metabolism and also to produce extra energy under stress condition. The reduction in the protein carbohydrate and lipid concentration in the haemolymph and other tissues may also lead to the reduction of haemocytes.

Methoxyfenozide was shown to have an excitatory effect on phenoloxidase activity in adult (Zibaee *et al*., 2012). The oozing fluid which turns black may be due to phenoloxidase action in methoxyfenozide treatment, which is the key component of immune system in insects (González-Santoyo and Córdoba-Aguilar, 2012). ProPOs are polypeptides, with a total weight of 50–60 and 70–80 kDa in their active and inactive forms (Ashida and Brey, 1997), which are one of the storage proteins (Burmester, 2002) and they occur mostly in haemocytes (Cerenius and So”derha˚ll, 2004). So the
involvement of haemocytes in association with haemocytes in defense mechanism may be understandable from the evidence of storage proteins reduction.

The macro molecules are concerned with the regulation of all biochemical events in the organism (Harper et al., 1993). In the silkworm B. mori the V instar is the most active feeding period during which the larvae accumulate large quantity of bio molecules in various tissues and are endowed with unique biochemical adaptation to conserve nutritional resources, cocoons spinning, metamorphosis and reproduction (Hugar and Kaliwal, 1998). Methoxyfenozide prevented the larvae from feeding, so decrease of biochemical compounds could be due to starvation stress. It was reported that the feeding activity of the silkworm larva was affected by Juvenile hormone analogue (JHA) (Sakurai and Imokawa, 1988) this is one of the stresses that could decrease some of the biochemical compounds in the larvae. Etebari and Matindoost (2004a and 2004b) demonstrated that even starvation could cause the reduction of many biochemical compounds of haemolymph such as glucose.

Proteins, being the key organic constituents, could be expected to play a role in the compensatory mechanisms of silkworm during stress. Methoxyfenozide was found to influence the protein concentration as well as the protein band thickness. Reduction of proteins in all the tissues like haemolymph, fat body, silk gland and gut was observed due to methoxyfenozide, but otherwise it was rare to find the loss of any protein band. It was reported that pyriproxyfen did not affect the protein band pattern in treated insects, although it affected the amount of protein concentrations (Aribi et al., 2006). Also it was reported that JH (Juvenile hormone) and the JHA fenoxycarb and pyriproxyfen induced an inhibition of larval haemolymph protein synthesis in Locusta migratoria (De Kort and Koopmanshap, 1991).
A significant decrease in total protein was observed in fifth instar larva when exposed to pyriproxyfen residue (Etebari et al., 2007). Monconduit and Mauchamp (1998) showed that the total protein of haemolymph in IV day of V instar larvae decreased due to fenoxycarb treatment. Channamadhavni and Sangam (2011) have shown that there was a decrease in protein content in the fat body of *Tribolium confusum* (Duval) treated with RH-5849.

At 72h after exposure to methoxyfenozide the gut protein showed the appearance of 29 kDa proteins, which was not present in control larva. 29kDa protein may be a proteinaceous factor involved in the uptake of chymotrypsin inhibitor (CI-8) into the midgut from the haemolymph. The tissue distribution of CI-8 changes on metamorphosis from larva to pupa. CI-8 is synthesized in fat body, secreted into haemolymph during the feeding stage, accumulated on the basal side of the midgut epithelium of a mature larva, and sequestered into the fat body after the onset of the spinning stage (Shirai et al., 2000). CI-8 has also been reported to inhibit 35kDa protease from the digestive juice of the larval midgut and involved in mid gut remodeling (Jiang et al., 2000; Ueno et al., 2006). The appearance of 29kDa protein in the methoxyfenozide treatment shows the advancement of larva towards pupal stage.

The amount of protein in *Tribolium castaneum* decreased due to application of sublethal concentrations of flufenoxuron (Salokhe et al., 2006). Schmidt et al. (1998) showed that treatment of *S. littoralis* and *Agrotis ipsilon* with azadirachtin decreased protein of hemolymph. This could be due to the break-down of protein into amino acids, so with the entrance of these amino acids to TCA cycle as a keto acid, will help to supply energy for the insect. So, protein depletion in tissues may constitute a physiological mechanism and might play a role in compensatory mechanisms under
insecticidal stress to provide intermediates to the krebs cycle by retaining free amino acid content in hemolymph (Nath et al., 1997).

The reduction in the protein content was also reported by pyriproxyfen (El-Sherif, 1995) and lufenuron (Bakr et al., 2007) against S. gregaria; pyriproxyfen (Shaurub et al., 1998), and tebufenozide (Abd El-Mageed, 2008) against S. littoralis; pyriproxyfen against A. ipsilon (El-Sheikh, 2002); and by pyriproxyfen and hexaflumuron against Parasarcophaga aegyptiaca (Assar, 2004) and Guneidy et al. (2011) stated that the chitin synthesis inhibitor (lufenuron) decreased the total protein in M. domestica. In the present study methoxyfenozide treatment in silkworm larvae has reduced the protein content in the gut and silk gland significantly compared to control. However reduction in the protein content may be due to inhibition DNA and RNA synthesis and this may reflect decrease in enzymatic activities as explained by Mitlin et al. (1977) where, the IGR diflubenzuron was found to inhibit protein synthesis, the first sign of cell death.

On the contrary significant increase in total protein content was reported in III instar larva of M. domestica treated with Applaud was reported by Assar et al. (2010). Similarly Dimilin and Altosid were reported to cause an increase in protein content (Bakr, 1986). In this study also the protein bands were found to increase gradually after methoxyfenozide application on the first day as well as in the control. Both the storage proteins (72kDa-76kDa), 30kDa proteins, vitellogenin (28kDa and 42kDa) were found to increase gradually in both control and treated. These results may be related with ecdysteroidal function of methoxyfenozide, which leads the larva towards pupa stage. The inhibitory effect of juvenile hormone analogue methoprene was also observed by Uranli et al. (2011) on B. mori larva.
The variation in the number of protein bands and specificity of protein bands during various days of fifth instar development could be ascribed to the fact that the proteins in the haemolymph vary quantitatively during the development of silkworm. It has been reported that haemolymph proteins fluctuate during the development of *B. mori* (Heller, 1924). The appearance of the common bands throughout V instar observed in the present study is concomitant with earlier work of Vanishree *et al.* (1999) and Janarthanan *et al.* (1998), who reported 30kDa, 29kDa, 35kDa, 42kDa and 82kDa protein bands were found throughout fifth instar. In the present study, the presence of thick protein bands near the region 29kDa is assumed to be *B. mori* serum protein (BmLSP), (Fujiwara and Yamashita,1990) and the BmLSP occurs as a major protein throughout the early instars until the initial day of last instar and gets decreased towards spinning stage.

Disappearance of protein bands indicates either the non-production or utilization or the degradation of haemolymph proteins to maintain amino acid concentration in the haemolymph. The hydrolysis of proteins might have occurred during the larval period to form amino acids, which in turn, might be utilized to form silk proteins. This hypothesis is in agreement with the results of Beadle and Show (1950) and Wigglesworth (1965) who reported the hydrolysis of proteins during the larval life of *B. mori* for the maintenance of amino acid concentration in the haemolymph. Neilsen and Mills (1968) have postulated that the mid gut and fat body manufacture some of the haemolymph proteins. Such proteins could be reabsorbed or hydrolyzed to form amino acids during the life cycle, leading to disappearance of protein bands in the haemolymph; or they may be changed into one or more of the other protein components of the haemolymph, resulting in the appearance of new bands.
However, any change in the protein pattern during the development can be considered as being directly determined by the gene function, which reflects the alteration in the metabolism of the developing organism (Pasteur and Kastritsis, 1971). 30kDa proteins bands were found to be reduced in the treated haemolymph as well as in the fat body tissue. The function of 30kDa suggested that they involve in the inhibition of apoptosis (Kim et al., 2003; Park et al., 2003) and play a role in self defence (Ujita et al., 2005). Such a kind of reaction could have happened in silkgland also, because programmed cell death was found to be triggered by insect steroid hormone 20 hydroxy ecdysone in the anterior silk gland of B.mori. It was found to be mediated by two distinct processes, one through nuclear hormone receptors and the other independent from de novo gene expression (Terashima et al., 2000), because RH-2485 was found to have higher binding affinity for the nuclear ecdysone receptor complex than 20E (Minakuchi et al., 2003).

The changes in haemolymph pool of nutrients certainly affect silk gland development and link it to food digestion and reserve mobilisation (Sehnal and Akai, 1990). Since haemolymph is the immediate environment of the organs in the silk worm, the metabolic activity and development are affected by the haemolymph (Nakayama et al., 1990). A decrease in the level of protein in the silk gland may be due to the reduction of proteins (or) other bio reserves to be supplied to the silk gland due to the impact of IGR.

The carbohydrates, proteins and lipids play an important role in the biochemical process underlying growth and development of insects (Ito and Horie, 1959; Wyatt, 1961 and 1967). Glycogen serves as the major food reserve in insects (Kilby, 1963). Wyatt and Kalf (1956 and 1957) reported that trehalose is the major blood carbohydrate
in insects. Simex and Kotrik (1986) have reported that the glycogen content in the fat body, body wall and silk gland and the free carbohydrates in the haemolymph changed significantly during last larval instar and metamorphosis in silkworms. Carbohydrates are the major components in the food of all the living organisms which either directly (or) indirectly used as the source of energy for all viral activity.

Topical application of halofenozide was found to get distributed in the different body tissues of gut as reported by Chebira et al. (2006). Hence the topically applied methoxyfenozide have caused biochemical changes in the gut as well as in the silk gland. The carbohydrate content was found to decrease in gut and silk gland in all the treated groups with 1µg/larva [AI]/ liters irrespective of the duration of exposure. The reduction in glucose content in the present study is similar to the result reported by El-Kordy (1985) using triflumaron and diflubenzuron in and Assar et al. (2010), using applauds against M. domestica. The results are also in accordance with the earlier works of Ranjit and Dash, (1994) in mosquitoes, Abdel-Aal (2004) in S.littoralis and El-Sheikh (2002) in A. ipsilon.

The reduction of many biochemical compounds of the haemolymph such as glucose was also demonstrated by Etebari et al. (2007). Considering the glucose content there was a decreasing effect due to diflubenzuron treatment reported by Ishaaya and Casida (1974). JHA decreased the total glucose (or) carbohydrate content in Chrysocoris stollii (Saha et al., 1985) and diflubenzuron, pyriproxyfen and flufenoxuron in S. littoralis (Farag, 2001; Abdel- Aal, 2004). Shekari et al. (2008) also showed similar results on Xanthogaleruca luteola Mull in the level of glucose. Similar results were also observed due to chemical pesticides: Radhakrishna and Devi (1992) showed that treatment of silkworm larvae with organophosphorus decreased the amount
of glucose and Nath (2003) represented that lethal and sub lethal doses of ethion and fenitrothion decreased the amount of glucose and trehalose of silkworm. The result of the present study also agrees with the above earlier reports.

The lipids are necessary as an alternate source of energy in living creatures such as insects. Insects obtain lipids from the food sources or synthesis them within the bodies (Gilbert, 1967). It has been reported that the lipid accumulation is more likely to be related to lack of juvenile hormone (Hill and Ezzat, 1974). Hence, the lipid turnover in insects is regulated by neuroendocrine controlled feedback loops (Downer, 1985).

In the present study on V instar larva of *B. mori* haemolymph and fat body lipid content distribution varied with IGR (RH-2485) treatment. Haemolymph and gut lipid content of the larva had revealed a reducing trend after treatment with a concentration of 1µg/larva [AL/ litre] of methoxyfenozide. Similar effects were also observed in desert locust *S.gregaria* in different tissues of nymph and adult treated with pyriproxyfen and tebufenozide (Hamadah *et al.*, 2012).

In addition, diofenolan treatments remarkably reduced the lipid content along the pupal stage of the house fly *M. domestica* except the last day at which pupae gained more lipids (*Amer et al.*, 2005). Lipid levels in the haemolymph of silkworm *B. mori* larvae declined throughout the experimental period but elevated initially in the fat body and then lowered (*Etebari et al.*, 2007). Changes in total lipid content were significantly reduced as compared to control at different time intervals especially in gut and the silk gland. The reason for decreased total lipid content may be due to its conversion to protein to substitute the reduction in protein content or to supplement energy (*Faten et al.*, 2011).
Lipid content in the whole body of *P. interpunctella* larvae was reduced as a response to the action of 20-hydroxyecdysone and azadirachtin (Rharrabe *et al.*, 2008) and to pyriproxyfen (Ghasemi *et al.*, 2010). Pyriproxyfen treatments resulted in decreasing lipid content in hemolymph and fat body of *Eurygaster integriceps* nymphs (Zibaee *et al.*, 2011). However, increase in haemolymph lipid content was found in 72h. Similarly, the gut also showed an increase in lipid content at 24h as well as in 96h. A similar result was also observed in *R. ferrugineus* by Ghoneim *et al.* (2003), where lufenuron and difenolan treatments resulted in decreasing lipids at the start and towards later stages of development, the gut lipid there found to increase.

Methoxyfenozide was also found to show a significant increase among the mid gut lipid in this study. The IGRs pronouncedly interfered with not only the synthesis of lipids but also their mobilization as promoted to convert into other metabolites or fatty acids. This suggestion may be supported by the increasing cholesterol in the mid gut brush border membrane of silkworm *B. mori* larva after treatment with fenoxycarb (JHA) (Leonardi *et al.*, 2001) or in the hemolymph 120h post-treatment of silkworm larvae with pyriproxyfen (JHA) (Etebari *et al.*, 2007). According to Tanani *et al.* (2012) the lipid increase after treatment with pyriproxyfen, tebufenozide or lufenuron may be attributed to the accumulation of carbohydrates which might lead to an inverse effect in their conversion rate to lipid as a reverse material. Juvenile hormone and ecdysteroids act on the formation and possibly also on the release of fat body lipids (Zera and Zhao, 2004).

However, Hamadah *et al.* (2012) showed the gut lipid concentration decreased due to the inhibitory effect of IGR. In *Gryllus firmus* decrease in-vivo biosynthesis of total lipid and triglycerides increases the absolute and relative biosynthesis of
phospholipids. Further an increase of oxidation of fatty acids and decrease in-vitro specific activities of six lipogenic enzymes and a transaminase was observed after methoprene application (Zera and Zhao, 2004). This proves that ecdysone hormone alters the biochemical pathway in insects by increasing as well as decreasing the concentration of lipids in spite of difference in nature of tissues and duration of exposure in the silkworm *B.mori*.

Different studies showed that IGR’s have a direct effect on nutritional indices of treated larvae (Miranda *et al.*, 2003). Thus, the biochemical changes in some metabolites related to feeding was obvious after major alteration in nutritional indices, after topical application of JHA (Miranda *et al.*, 2002). The most important reason in non-spinning syndrome is interruption in concentration of ecdysone and juvenile hormone.

Methoxyfenozide caused prevention of feeding in the silkworm larvae, leading to lack of supply of nutrients and affected the biological components of the organisms. It was reported that the feeding activity of the silkworm larva was affected by Juvenile hormone (Sakurai and Imokawa, 1988). This stress could have reduced the biochemical compounds in the larva. Etebari and Matindoost (2004a) demonstrated that even starvation could cause the reduction of many biochemical compounds of haemolymph such as glucose. This study makes it clear that tissues such as silk gland and gut responded to IGR treatment in a way that lead to the death of the animal.

Proteases are a group of proteolytic enzymes that hydrolyse proteins into amino acids (Chen, 1971). Protease activity has been reported in silkworm and other insects (Bharathi and Miao, 2003). Protease activity levels recorded a continuous increase throughout the fifth instar development. Enzyme activity was observed to be increased
in haemolymph. The presence of non-intestinal protease activity in silkworm tissues is attributed to its role in proteolysis, characterizing insect metamorphosis (Chen, 1971). In this study the IGR, RH-2485 has induced positively the proteolytic enzymes in the haemolymph of silk worm *B. mori*.

Similarly, there was significant reduction in the activities of protease (maximum of 82%) amylase (maximum of 90%), and lipase (maximum of 92%) in the IGR treatment. Digestive enzyme activity considerably decreased when the insects fed on seeds treated with all IGRs, compared to control treatment. Digestive enzyme activities significantly decreased with IGR treatment (Khatter and Abuldahb, 2011). It was also reported that the combination of Btk and botanical insecticides caused a two-fold decrease in enzyme activity even at reduced concentration (Gamal and Abuldahab, 2012).

The increase in the protease I and II as well as the proteolytic enzymes in the haemolymph and the fat body may be associated with the immunological function of this insect. Proteases are a major group of hydrolytic enzymes in insects and are not only involved in digestive processes but also in the activation of proenzymes and liberation of physiologically active peptides, complement activation, and inflammation processes (Neurath, 1984). The insect may utilize this enzyme for their survival, because the enzyme was found to involve in immune response in Diptera and Lepidopterans (Ashida and Yamazaki, 1990).

The increase in protease activity may also be associated with the immune response involved in the survival of the insect because protease in the haemolymph of *Rhodnius* was found to be involved in defense mechanism (Feder et al., 1998) during bacterial infection. Serine proteases that lead to proteolytic activation of BmPP (*B. mori*...
paralytic peptide) in the hemolymph enhanced the immunity against bacterial infection. The enhancement of host immunity against invading pathogens by active BmPP is because of the activation of hemocytic phagocytosis and up-regulation of expression of AMP genes through activation of p38 MAP kinase signaling (Ishii et al., 2010). Johnson et al. (1990) made a study of protease activity in the mid guts of larvae of susceptible and resistant strains of P. interpunctella and results indicated that resistance was not due to obvious changes in larval mid gut protease activity.

Two serine proteases were also found in hemolymph, which shared 66% and 67% similarities to serine protease homologs (SPHs) purified from the plasma of M. sexta larvae. The SPHs of M. sexta are reported to bind to pattern recognition receptor and function as mediators to recruit prophenoloxidase and prophenoloxidase-activating protease to the site of infection (Yu et al., 2003). Prophenoloxidase has long been considered a key enzyme in melanization, which is considered to be an effective defense against microorganisms and without prophenoloxidase, the melanization of insects is almost completely inhibited (Shiao et al., 2001).

Alteration of protease activity due to IGR treatment in the haemolymph was also expressed in fat body tissue in this V instar larva. Because haemolymph seems to act as a transitory repository of biochemical constituents, from which tissues retrieve them depending on their need. Thus, a dynamic biochemical exchange mechanism like that of liver and plasma seems to operate in silkworm and other insects that facilitate the exchange of substances between fat body and haemolymph (Nagata and Kobayashi, 1990; Ravikumar and Sarangi, 2004).

The protease activity levels were recorded as a continuous increase throughout the V instar development in B.mori (Bharathi and Miao, 2003). Protease I activity was
found to be enhanced by the IGR treated groups compared to control in gut of V instar *B. mori* larva. A similar result was also observed in the midgut protease activity in all the groups of herbal drug treatment which increased significantly (Vitthalrao and Sucheta, 2012). IGRs affect serine proteases under the alkaline conditions in the intestinal fluid. The damage to the mid gut caused a decrease in digestive enzyme activity (Smirle *et al.*, 1996; Senthil Nathan *et al.*, 2005a). IGRs caused a two-fold decrease in enzyme activity even at reduced concentration (Khatter and Abuldahb, 2011), a significant decrease in the activities of protease was observed in *Callosobruchus maculatus*, and the digestive enzymes activities significantly decreased with increasing concentrations of IGR. Digestive enzymes protease in the *A. aegypti* larvae are affected by botanical insecticides and bacterial toxins individually and in combination (Gamal and Abuldahab, 2012).

Ashida and Yamazaki, (1990) has showed that the enzyme involved in the immune response are serine protease in Diptera and Lepidoptera. It is also possible that bacterial infection altering the fat body cell membrane can in same way influence the liberation into the haemolymph of two metallo Proteases. The occurrence of metallo protease was associated with bacterial infection in *R. prolixus* (Feder *et al.*, 1998). Protease activity in methoprene treated *B. mori* V instars larvae muscle and silkgland showed an increasing trend over the corresponding experimental group (Mamatha *et al.*, 2008). And with the increase in growth there is an increase in the protease activity of *B. mori* larval tissue (Magadum and Hooli, 1989). The increasing enzymes or increase in membrane related transport function may be for the purpose of energy generation that contributes for the secretion of silk protein (Reddy *et al.*, 1997; Towle, 1984).
IGRs affect serine proteases under the alkaline conditions in the intestinal fluid. The damage to the mid gut caused a decrease in digestive enzyme activity (Eguchi et al., 1972; Mathavan et al., 1989; Smirle et al., 1996; Senthil Nathan et al., 2005b). Here, methoxyfenozide also caused damages to the mid gut in *B. mori* larva, which in turn could be the reason for the reduction of protease secretion. It is evident that exposure to IGRs may interfere with the production of certain types of proteins in adult where the diet plays significant effects on several enzyme activities found in the late instar larvae and adult in *C. maculatus*. This activity is apparently strongest during pupation. Pupae were very susceptible after larval exposure of hydroprene (Khatter and Abuldahb, 2011).

The acid phosphatase is a set of hydrolytic enzymes that hydrolyze phosphomonoesters under the alkaline condition. The activity of these enzymes is related to the physiological condition of silkworms and reflects the absorption, digestion and positive transportation of nutrients in the midgut. Different stress and disease causes considerable decrease in the activity of ACP (Miao, 2002). In this study the ACP activity decreased significantly in the haemolymph and it cannot recover its normal level after 48h treatment. Similar results were also seen when *Melia azedarach* seed extract was applied on rice leaf folder (Senthil Nathan, 2006). The larvae mid gut enzyme the activity of ACP decreased dramatically due to neem limonoids treatment (Senthil Nathan et al., 2005a) ACP activity in all tissues examined. JHA treatments was found to increase ACP activities in all tissues, on contrast 20-hydroxy ecdysone did not increase activity in any tissue (Yi and Adams, 2001).

Here in this study the acid phosphatase activity was also found to be enhanced by methoxyfenozide treatment in the gut which agrees with the earlier report of Assar
et al. (2010). An increase in ACP activity was reported due to IGRs, such as, JHA and ecdysterone on Chrysocoris stollii (Saha et al., 1986). Since the mid gut cells were already disturbed by the action of ecdysone analogue the secretion of ACPase was also found to be affected and led to imbalance the carbohybrate concentration in the haemolymph.

Among insects the acid phosphatase is released from the midgut cells, in the blood and midgut tissue which are strongly related to silk protein synthesis, digestion and absorption in silkworm larva (Miao et al., 2004). It is analogous to mammalian glucose-6-phosphatase which maintains the blood glucose homeostasis (Faulkner, 1955). Ecdysone is responsible for increase in the number of lysosomes (Radford and Misch, 1971) and of the activity of acid phosphatase (Van Pelt-Verkuil, 1979). This indicates that the increased activity of acid phosphatase in the gut of B.mori may be due to increased number of lysosomes as suggested by Assar et al. (2010) in M.domestica.

Presence of amylase enzyme was reported in the gut and the haemolymph (Ngernyuang et al., 2011). In this study not only the haemolymph and the gut showed the amylase activity they were also observed in silk gland and fat body in the V instar larva. Amylase enzyme was found to be responsible for inducing hardiness among the multivoltine races (Kumar et al., 2013). An increase in the amylase activity was observed in certain days after IGR treatment but also reduction in the amylase activity was observed. The reduction in the carbohydrate concentration in almost all the four tissues was studied due to methoxyfenozide treatment. Due to starvation, there is raw material reduction and amylase function is impaired and carbohydrate utilization failed and survival of the larvae was at a threat. It was also suggested by Wyatt (1967) that amylase may be involved in the degradation of fat body glycogen.
Change in ACP activities after treatment with methoxyfenozide indicates the changing physiological activity as reported by Senthil Nathan et al. (2004) due to neem limonoids toxin. Methoxyfenozide was found to influence almost all the four tissues in silkworm larva since reduction in the concentrations of haemolymph is noted. Such a kind of reduction was noted in the midgut of Cnaphalocrocis medinalis, and it was found to be due to the toxic effects of neem derivatives on membrane permeability, especially of the gut epithelium (Smirle et al., 1996; Senthil Nathan et al., 2004, 2005a).

Here the activity of the enzymes was not found to be affected, because methoxyfenozide enhanced the activity of LDH in case of fat body, silk gland as well as in the gut. The higher LDH activity in control insects may probably due to consumption as well as utilization of large quantities of food. LDH is involved in the production of energy, being particularly important when a considerable amount of additional energy is required immediately (Diamantino et al., 2001; Senthil Nathan et al., 2005a). Maiza et al. (2013) in Blattella germanica, showed that the increase in LDH activity in the control may be related to carbohydrate metabolism and he also found that treatment of spinosad and indoxacarb, increased the LDH activity and he explained that this chemical stress induced by these insecticides. In order to survive the animals LDH activity might have increased, otherwise a stimulatory effect due to ecdysteroidal activity for the metamorphosis might have took place in V instar larva.

However, confusion among the juvenile and ecdysone hormone have disrupted the normal enzymatic function in B. mori. Because, factors like weather condition, feeding habit, diet, chemicals, may affect the enzymatic and non enzymatic compound of insect body (Zibaee et al., 2008). But this decline may result from a greater
degradation or a lower synthesis of digestive proteases produced by a quantitative 
decrease of the feed intake when larvae are near the next molt stage (Broadway and 
Duffey, 1985).

The mid gut histology of V instar larva was found to affect the architecture of 
the mid gut tissue itself, the effect was such that it was similar to the effect caused by 
insecticides, where insecticides caused vacuolization of the mid gut epithelium after the 
treatment with arsenites (Rizvi and Khan, 1973) and organophosphates (Deshmukh and 
Tembhare, 1998) Carbaryl and γ – BHC (Deshmukh et al., 2009). Apical swelling and 
blebbing of large cytoplasmic vesicles in the columnar cell and lysis of the gut 
epithelium was also reported (Blackburn et al., 1998). Similarly in this study also the 
vesicle formation and swelling were found in the mid gut.

The mid gut epithelium was affected by treatment with Tannic acid in aquatic 
diptera larva was reported by Muramorosch (2002). The methoxyfenozide treated 
larval mid gut in 24, 36 and 48h showed the damaged epithelial cells. It was also shown 
by Rharrabe et al. (2009) where the ingestion of 20-hydroxy ecdysone on 
P. interpanctella caused severe cytotoxicity on the midgut epithelial cells.

The ecdysone agonist tebufenozide exerted several histopathological effects on 
the mid-gut of last instar hoppers. The majority of epithelial cells lost their normal 
arithmetic (Ghoneim et al., 2008). A similar effect was observed in the 
methoxyfenozide treated groups where the epithelial cells lost their original nature and 
they appeared spongy especially after 24h. The epithelial cells became spongy in nature 
which was also reported by Iizuka (1973) when B.mori was fed with B. thuringiensis.
Treatment of plant metabolite saponin in *S. littoralis* also showed histopathological alterations such as loss of lining with dislocation of chitinous cuticular intima away from the epithelial lining, increasing the space between the epithelial cells (Adel and Sammour, 2012). Fraxinellone was found to destroy the peritrophic membrane of the midgut of larvae of *M. Separata* (Lu *et al*., 2010). This indicates that proper digestion and absorption of food components is possible only to a reduced degree. In this way, insects may lose their appetite and feeding inhibition may occur, which may lead to the death of the animal.

Methoprene was found to destruct the larval midgut by interfering with the expression of genes involved in 20E action resulting in a block in midgut remodeling and death during pupal stage (Uwo *et al*., 2002). May be the ecdysone analogue methoxyfenozide could have done the same function. In the destruction and death of the animal and this effect were same irrespective of mode of application (Parthasarathy and Palli, 2007). The neem seeds extract negatively affected the physiological and biological parameters of *Anticarsia gemmatalis* which was reported by Almeida *et al.* (2014), where, the cells of the midgut epithelium of larvae showed swelling, basal membrane detachment and complete disruption.

Granulocytes play a key role in the histolysis during metamorphosis (Yang *et al*., 2007; Zhao *et al*., 2005). Exposure of *B. mori* larvae to methoxyfenozide after 48h resulted in the accumulation of granulocytes inside the lumen as well as near the epithelial cells. This indicated that IGR may force the animal to go for metamorphosis, as haemocytes were thought to accumulate in the midgut during larval and pupal metamorphosis. Granulocytes also involved in the secretion of basal membrane components of Lepidoptera (Nardi *et al*., 2001). Shinohara *et al.* (2008) reported that granulocytes were seen in midgut lumen of *B. mori* after occurrence of apoptotic
bodies. Reports of previous studies (Adachi et al., 2005; Shinohara et al., 2008) and the present observation inferred that the round shaped cells might be granulocytes and they might be involved both in phagocytosis of apoptotic bodies and rearrangement of pupal basal membrane.

But this is in contrast to Wang et al. (2007), who showed RH-2485 upregulated the gene hm17g which is responsible for sustanance of larval midgut during larval feeding and protecting the midgut from histolysis and remodeling in Helicoverpa. In B.mori after 12h with RH-2485 treatment the nucleus size of mid gut columnar cells underwent enlargement which may be due to the upregulative action of RH-2485. But after 24h the IGR caused major histolysis.

Shrinkage and even complete degeneration of the nuclei in insects treated with organophosphorous insecticide was reported by Mistra (1981). In this study also a similar type of nuclear degeneration was observed in 48h treated groups as the nucleus was not prominent. So the entire function of secretion and absorption of the mid gut cells were affected altogether.

The Bacillus sphaericus brought cell hypertropy in A. aegypti, A.albitarsis and C.quinque fasciatus as well as increased the apocrine secretion. The basophilic vesicles were filled with material of unknown nature (Oliveira, 2009). The RH-2485 treated midgut epithelial cells became loosely arranged and may be considered as a defensive mechanism and a similar observation where the midgut cells of C.cephalonica larva infected with B. thuringiensis showed a loose arrangement of epithelial cells and separated from each other (Chiang et al., 1986). Sutter and Raum (1967) showed that the gut epithelium loosened from basement membrane and vacuolation of goblet cells due to α-endotoxin of B. entomocidus.
Similarly mucus like vesicles were found both in control and treated larvae but these mucus secretions were found to be more intense in RH-2485 treated groups. This may be a self defense against degeneration. In *C. cephalonica* a mucous layer covered the surface of the epithelial cells of the midgut to protect the new cells from attack of *B. thuringiensis* and this defense process in the midgut epithelium prolonged through the life-span of infected larvae (Chiang *et al.*, 1986). Such a type of secretion was also observed by Oliveira (2009) in the posterior region of the gut both in control and treated larvae, and was intense in bacteria-exposed larvae of mosquitoes. Whereas, Sutter and Raun (1967) explained that the cells of the gut epithelium contained lipid like inclusions which were normally present in healthy cells and disappeared, in insects fed with crystal-forming bacteria in *Ostrinia nubilalis* Hubner. Pinheiro *et al.* (2008) studied that the mucus secretion is a glycoprotein produced exclusively by columnar cells. So the ecdysone agonist methoxyfenozide could have stimulated the columnar cells to defend against destruction of the midgut in *B. mori* larvae.

The important energy source lipids regulated by neuro endocrine functions (Downer, 1985) were also found to be influenced by methoxyfenozide. Mulye and Gordon (1993) have shown that lipid synthesis and catabolism in the fat body were severely impaired in JHA treated budworms. Etebari *et al.* (2007) reported that lipids in the haemolymph were depleted throughout the experimental period and lipid concentration in the fat body was elevated initially and then lowered. A similar result was also observed in this study. It was suggested that methoxyfenozide caused an alteration in the pattern of the relative proportions of the categories of lipids synthesized. Leonardi *et al.* (2001) also represented that topical application of fenoxycarb could increase cholesterol in midgut brush border membrane of silkworm larvae.
Different studies showed that IGRs have a direct effect on nutritional indices of treated larvae (Miranda et al., 2003) Thus, the biochemical changes in some metabolites related to feeding was obvious after major alteration in nutritional indices. These changes in different compounds may be the main reason for reduction in economical parameters of silkworm after topical application of JHA that was reported by Miranda et al. (2002), whereas the most important reason for non-spinning syndrome is interruption in concentration of ecdysone and juvenile hormone. It has been reported that at doses exceeding 100 pg/larva, the fenoxycarb affects important nutritional parameters, suggesting a reduced efficiency in the utilization of ingested food (Assal, 1994).

Endocrine conditions during the fifth larval stage differ from those of other larval instars and are important for larval to pupal metamorphosis. The ecdysone levels during the first 3 days of the last instar are undetectable, but juvenile hormone is still found in the hemolymph (Sakurai et al., 1998). Disappearance of juvenile hormone on day 3 is required for recovery of the prothoracic gland activity for secretion be detected in the hemolymph (Sakurai et al., 1989) after day 5 of the fifth instar combination of ecdysone and juvenile hormone stimulates the total protein level in the haemolymph after day 5.

The influence of IGR, methoxyfenozide on the endocrine function is evidenced by a relative decrease in the protein bands of 72 and 76kDa. It resulted in the ecdysone agonist action and the larvae prepared them for pupal remodeling. It has been shown that a juvenile hormone analogue methoprene application to the fourth instar larvae of B. mori caused the increase of the hemolymph protein concentrations and this result
arose due to preventing sequestration of the storage proteins by the fat body (Kajiura and Yamashita, 1992; Uranli, 2011).

The anterior silk glands (ASGs) of the silkworm, *B. mori*, begin to undergo programmed cell death (PCD) in response to a high haemolymph concentration of ecdysteroid, which induced pupal metamorphosis (Terashima *et al.*, 2000). The biologically active form of ecdysone, 20-hydroxyecdysone (20E), binds to a functional nuclear ecdysone receptor consisting of an ecdysone receptor (EcR) and its heterodimeric partner, Ultraspiracle (USP) thereby controls the transcriptional activity of target genes (Riddiford *et al.*, 2000). Thereby, the hormone ecdysone agonist methoxyfenozide influencing the destruction of the silk gland is evident from the results of biochemical changes. 72h treated larvae exhibited a protein banding pattern of 96h control which advanced the silk gland to function. It might be due to the fact that the glands begin to undergo programmed cell death (PCD), in response to a high hemolymph ecdysteroid concentration, which induces pupal metamorphosis (Terashima *et al.*, 2000).

A dose of 1µg/larva (AI)/litre of methoxyfenozide led to cessation of feeding, that altered many biochemical activities. The cellular integrity of the haemocytes as well as the mid gut cells were changed. Even the symbiotic associations of gut microbes were found to alter due to starvation. These conditions lead to challenge the life of V instar larva, even though the animal tried to survive.

Storage protein 2 (SP2) is not only an important source of energy for the growth and development of silkworm but also has inhibitory effects on cell apoptosis (Yu *et al.*, 2013). Inhibition of apoptosis by 30 kDa protein of silkworm haemolymph
was also reported by Kim et al. (2003). Reduction in the SP2 protein in the haemolymph can be an evidence to prove that apoptosis takes place in the gut.

A wide variety of gram positive and gram negative bacteria inhabit the gut of silkworm *B. mori*. The normal gut flora like *Proteus, E. coli, Pseudomonas, Erwinia, Citrobacter*, were found among the gut of control larva and also reported in normal gut of *B. mori* (Anand et al., 2010). The gut microbiota involvement in endosymbiots and in host nutrition was studied in insects (Tanada and Kaya, 1993). Visotto et al. (2009) elucidated the role of gut bacterium in the digestion and development of *Anticarsia gemmatalis*. Many reports have been published regarding bacteria of the digestive tract of insect. *C. freundii* and *Pseudomonas* sp were found in the digestive tract of the ground beetle *Poecilus chalcites* (Lehman et al., 2008). *Bacillus* species were found in the diverticulum (Gusmao et al., 2007). *Aeromonas* was identified with xylenase activity in the intestine of *Samia cynthia* pryeri (Roy et al., 2003). *Erwinia* was pectinolytic and proteus was cellulolyic and xylanolytic (Anand et al., 2010). The gut microbial role in the nutrition of silkworm was reported by Sekar et al. (2010). So the role of normal gut flora in digesting the mulberry leaf content was understood.

The total cultivable bacterial count was found to decrease in the experimental groups compared to the control. This may be due to the reason that the gut harbours the microbes through food particles they take, because higher heterotrophic bacterial population was reported by Kalpana (1994) in the fourth and fifth instar larva. No micro-organism was isolated or detected in the gut of first instars in grasshopper (Chapman, 1990), reported that the alimentary canal of hatched larva was sterile and the bacterial floral number and species increase throughout life. Similar observation was also observed in *Frankliniella occidentalis* by De vres et al. (2001). Similarly
B. cereus, B. Subtilus, Lactococcus lactis, Staphylococcus, Enterocet, E.coli and Klebsiella were reported to be present in the mulberry leaves. So the reason for the reduction in the total bacterial count may be due to decrease in the food intake, influenced by the IGR.

Even though cellulolytic bacteria are present in the isolate of starvation the absence of citrobacter which is the xylanase activating bacteria and pectinae activating Erwinia, suggest the important role of these microbes which are not required further. The role of bacteria in synthesizing of digestive enzyme is not required, and the substrate needed for the survival of this organism is denied. Because, the microorganism isolated from food plants were also found in the isolate from the gut regions of insect that consumed them (Ademolu and Idowu, 2011). But in contrast interestingly Dillon et al. (2010) reported that starvation increases the gut micro flora in desert locusts.

In this study the decrease in total colonial count is also associated with change in the colonisation by another type of bacteria. Because the control gut showed the presence of ten isolates of bacteria and in case of IGR treated most of the negative stained bacteria were not found to be dominate. However starvation due to treatment enhanced some of the gram positive colonies like Corynebacterium, Clostridium, Streptococcus and the Bacillus species like B. cereus and the gram negative bacterial colony of Enterobacter.

The presence of streptococcus may be associated with some diseased condition also. Because it was reported that starvation in insect are more prone to diseases (Dillon et al., 2010). The domination of streptococcus in the gut of the starved larva raises the question whether they are responsible causing diseases in the organism. Antimicrobial
peptides genes were found to be enhanced due to *staphylococcus aureus* and *E.coli* in *B. mori* was reported by Wu *et al.* (2010). The animals defend themselves against such kind of pathogens through immune mechanisms controlled by antimicrobial genes which are active during spinning and at pre-pupa stage, where active feeding stops. But disappearance of *pseudomonas* also challenges the larval survival. The contribution of gut microbes including *pseudomonas* was demonstrated to be associated with the defenses in insect (Indira Gandhi, 2007, 2008) and it was interesting to note the presence of *pseudomonas* colony from the control larval isolates.

A complete change in the bacterial colonies proves that the bacteria present during active feeding are associated with the digestion of the food material in the larva. During starvation the presence of *Staphylococcus* and absence of *Pseudomonas* may affect the larval survival and it may challenge the immune system of insects. *Bacillus* the lipase producing bacteria dominated the gut colony of silk worm (Feng *et al.*, 2011). So the role of lipase producing bacteria during the condition of starvation is understood. It may help the animal to utilize stored food materials from their fat body as well as their role in disease resistance (Ponnuval *et al.*, 2003). It is the diet that plays a significant role on the gut bacterial community, especially during the conditions like emergency, especially during starvation. Similarly the bacteria without getting the raw material for their purpose may have turned to become pathogen and started degrading the gut as well and cause the death of the animal. Learning the role of bacteria in the development of silkworm will be helpful in probiotic studies. Further studies have to be done to identify the bacterial strains up to species level and to ascertain its identification through subsequent molecular identification influence by insect growth regulators.