1. INTRODUCTION

The conservation and maintenance of silkworm races in the germplasm station are centred on proper rearing of the silkworms and harvesting the bumper crop by robustness of the larvae without diseases. This requires an understanding of the immune system of each silkworm races and their application as a tool to assess silkworm health (Rodriguez and Le Moullac, 2000; Yao et al., 2006; González-Tokman et al. 2011; Isaac González-Santoyo and Alex Córdoba-Aguilar, 2012) and the value of immune parameters act as biomarkers in ecotoxicology (Galloway and Depledge, 2001).

*Bombyx mori* is a domesticated silkworm and is one of the most economically important insects because of its silk. Sericulture is an agro based cottage industry of many farmers and weavers in countries like China, Japan, India, France, Italy and other in the world. Silkworm species secrete diverse varieties of silk fibres and are belonging to Bombycoid. Among these species *Bombyx mori* of family Bombycidae and wild silk moths are of saturniidae, *Antheraea mylitta* (Indian tropical Tasar silk moths), *Antheraea assama* (Indian golden silk moths), *Antheraea pernyi* (Chinese silk moths), *Antheraea yamamai* (Japanese oak silk moths) and *Philosomia synthia ricini* (Indian castor silk moths). Silk production is mainly dependent on *Bombyx mori; Antheraea assama, Antheraea pernyi* and *Antheraea yamamai* plays important role in the rural economies of many developing countries.

The silkworm is the holometabolous insects. The life cycle of the silkworm is about fifty days with distinct four stages viz., egg, larva, pupa and adult moth (Fig.1.1). The larval are the only stage at which food is ingested. *Bombyx mori* comprises a large number of ecotypes and synthetic inbreed lines, which represent high degree of divergence with respect to geographic
origin, morphological, qualitative and quantitative traits. The silkworm strains inhabiting temperature countries like China, Japan and Korea are bivoltine (two generation per year) and lay diapauses eggs, while strains available in tropical countries like India are multi or polyvoltine silk worms (many generations per year).

The tropical strains are hardy, can withstand adverse eco-climatic conditions but produce very little quantity of good quality silk. Never the less, bivoltine stains are low silk yielder in the tropical environment because of heat, humidity, disease and inadequate rearing conditions. The classical breeding approaches, although increased the silk productivity, have failed to integrate the high yielding traits of temperature strains with low yielding disease resistant tropical strains. About 3000 silkworm genotypes with different geographical origins are been maintained in tropical in temperature countries (Nagaraj and Singh, 1997). It is clear that the ability to mount an efficient and effective immune response should be closely related to fitness (Cotter et al., 2004). Thus, fitness related traits, such as life history characters and nonspecific disease resistance traits are expected to have lower heritabilities than morphometric and other, traits that are only weakly related to fitness. Although there is some support for this idea (Mousseau and Roff, 1987; Kruuk et al., 2000; Merila and Sheldon, 2000), many studies have shown that disease resistance traits often have high heritabilities and high levels of additive genetic variation (Fellowes et al., 1998; Huang et al., 1999; Coltman et al., 2001).

A number of studies have examined correlated responses to selection for resistance to specific parasites and have identified potential trade-offs with life history traits such as developmental rate, competitive ability, pupal weight, adult lifespan, testes size, fecundity and egg viability (Fuxa and Richter, 1989; Boots and Begon, 1993; Groeters et al., 1994; Kraaijeveld
Fig:1.1. Life cycle of the silkworm *Bombyx mori*
and Godfray, 1997; Webster and woolhouse, 1999; Hosken, 2001). Therefore, additive genetic variation in immune parameters could be maintained because high levels can be bought only at the expense of other important functions or traits. Immune function/immunocompetence, is often thought of as a composite trait, it is also conceivable that there are trait-offs within the immune system, with high levels of one component resulting in low levels of another. The upregulation of the humoral response may be at the expense of cellular responses (Gross et al., 1980; Gehad et al., 1999; Johnsen and Zuk, 1999; Gill et al., 2000). In order to reveal potential genetic trade-offs associated with the insect’s innate immune system under LPS stress, the present study used the silkworm Bombyx mori as a model system and examined the quantitative genetics of innate immune function and other life history parameters.

1.01. An insect-pathogen infection model

One of the main factors in the successful examination of biological processes is the selection of a relevant model system. The mammalian infection models of mouse and rat are such since they share high similarity with the human body although their maintenance is expensive and their experimental use is limited by ethical reasons. Therefore, insect models are being developed for e.g., the studies of pathomechanism, because these have the advantages of low cost, genetic and physiological malleability and the lack of ethical problems involved in studying mammals (Silva et al., 2002). On the other hand the observations in insect systems can be instructive for studies in vertebrate-pathogen bacteria systems, because many innate immune mechanisms are conserved throughout the animal kingdom (Michael et al., 2004). For example, in adult Drosophila melanogaster, homologues of the transcription factor NF-κB are activated upon bacterial invasion in a process mediated by the TOLL family of receptors. Similarly to immune cells in mammals which induces the expression of defense genes (Lemaitre et al., 1996;
Preston-Hurlburt and Janeway, 1997). Studying bacterial – insect interactions can also have significance through the development of environment-friendly crop-protection technologies based on insect specific microbial toxins and virulence strategies (Chattopadhyay et al., 2004).

1.02. Silkworm as the host in an infection model system

Silkworm (Bombyx mori, Lepidoptera) has been widely used as a model for insect biochemical and molecular research due to its size and haemolymph volume: the ten vth instar larva reaches 18-31g, and 1–2 mL of haemolymph (with approximately 106 hemocytes) can be collected from it. This species is also easy to rear in the laboratory. Thus it is well suited for studies of hemocytes, haemolymph proteins and their interactions (Silva et al., 2002; Michael et al., 2004). At the same time Bombyx mori larvae are sensitive to bacterial infection, so they are perfect model to study the pathogenic mechanism and the interaction of the bacterial virulence factor(s) and the insect immune system, despite the fact that Bombyx mori lacks the genetics of Drosophila.

1.03. Defense system of insects

Insects have developed efficient mechanisms to eliminate microbial invaders. Innate immunity is common to all insects and serves as the first-line of defense. It consists of recognition of microorganisms by receptors, and rapid effector ten mechanisms that involve phagocytosis, nodule formation, and encapsulation, activation of proteolytic cascades and synthesis of potent antimicrobial peptides. The adaptive immune system, as the second line of defense, is restricted to some 45,000 vertebrate species, and involves the complex repertoire of immune receptors in lymphocytes through somatic gene rearrangement and clonal expansion of activated lymphocytes which endow memory. Since insects lack adaptive immunity, they are
excellent model organisms for studies of the innate immune system (Michael et al., 2004; Hoffman, 2003; Eleftherianos et al., 2006). This system comprises both cellular and humoral reactions (Fig. 1.2.).

Fig. 1.2. Once pathogens gain entry into the hemocoel of the host, they must encounter a complex system of innate defense mechanisms.
Each species of pathogens has a characteristic spectrum of hosts. The exploration of the molecular interactions which determine virulence is important to understand host specificity and might be instrumental in the prevention and treatments of microbial infections. Virulence factors of microorganisms belong to either microbial toxins or microbial enzymes. Secreted enzymes may function as virulence factors which are essential for survival and spread in the host. Among these enzymes, phenoloxidase and carboxyl esterases can neutralize the host’s defense systems by different ways. However, the roles of only few of these enzymes in the pathomechanism have been documented. Other function of secreted enzymes during infection includes invasion of the host and bioconversion of its tissues for nutrient supply of the growing microorganism. Production of inhibitors as a component of the immune response against the bacterial enzymes can provide protection for the host. Enzymes function in defense systems. A defence system consists of a immune enzymes, as well as its natural substrate(s) and its natural inhibitor(s). The full understanding of the physiological role of immune enzymes can be reached only via the knowledge of the whole defense system in which they participate. Since this is very difficult it is not surprising that such systems of neither immune enzymes of the pathogens is known with the exception of the mentioned substrate proteins and several inhibitors of pathogen origin.

1.04. Immune recognition

Recognition of pathogens is the first step of the immune response which is mediated by pattern recognition proteins (PRP) such as Hemolin (HEM), Peptidoglycan recognition protein (PGRP), Immuclectin-2 (IML-2), Lipidbinding protein (LBP), Pattern recognition serine proteinase (PRSP) and β-1,3-glucan recognition proteins (βGRP-1 and βGRP-2) (Michael et al., 2004; Eleftherianos et al., 2007). These proteins bind to conserved pathogen-associated molecular pattern (PAMP) molecules such as peptidoglycan, lipopolysaccharide, lipoteic acid and β-1,3-
glucan present on the surface of bacteria and fungi but not on the host cells, and trigger – after a shorter or longer process of signal transduction - a protective response directly or indirectly through the induction of antimicrobial genes (Eleftherianos et al., 2007; Ling et al., 2006) (Fig. 1.3.).

![Diagram of immune responses](image)

Fig. 1.3. Illustration of pathogenicity in insects under LPS

1.05. *E. coli* as the ideal pathogen in an infection model system

*E. coli* is Gram negative bacterium species in the family of Enterobacteriaceae. It is highly virulent and toxic against insects. Such properties are very important in studies of infection mechanisms because they offer a stable way of generating infection. Also the infection can be relatively easily investigated in the laboratory on Lepidopteran host models (Silva et al., 2002). This bacterium is also cause diseases in human. As revealed by genomic analysis, they share not
only the chromosomal backbone of Enterobacteriaceae, but also many putative mobile regions encoding virulence factors and proteins of unknown function (Duchaud et al., 2003). *E. coli* strains are intensively studied for their strong virulence and interesting symbiotic life. Silkworms are very sensitive to microbial infections. Hence, the present study aimed at analyzing the effect of bacterial endotoxin viz., Lipopolysaccharide (LPS) as used as a biotic stress to investigate the bio-molecular changes in selected silkworm races.

1.06. Lipopolysaccharide (LPS) and its direct role in antibacterial resistance

Endotoxin is a lipopolysaccharide (LPS) and its molecular weight is >100,000 Daltons. LPS is composed of two major parts, the hydrophobic lipid A portion and the hydrophilic polysaccharide portion (commonly called the "O" region) (Fig. 1.4.). The lipid A portion of the molecule has been shown to be responsible for numerous in vivo and in vitro effects of endotoxin. LPS stimulate the immune responses (Yu and Kanost, 2004) and enhances the cellular immune reactions (Foukas et al., 1998; Soldatos et al., 2003). However, it has been reported that commercial LPSs contain enough peptidoglycan (PGN) to activate antimicrobial peptide (Dziarski and Gupta, 2006). Low activities of endotoxin stimulate the immune response and higher activities can lead to septic shock.

Lipopolysaccharide (LPS) is the major molecular component of the outer membrane of Gram negative bacteria and serves as a physical barrier providing the bacteria protection from its surroundings (Raetz and Whitfield, 2002; Nikaido, 1989). LPS is also recognized by the immune system as a marker for the detection of bacterial pathogen invasion, responsible for the development of inflammatory response, and in extreme cases to endotoxic shock (Mathias Rauchhaus and Stefan Anker, 2000; Yosef Rosenfeld and Yechiel Shai, 2006). Because of these functions, the interaction of LPS with LPS binding
Fig. 1.4. Structure of bacterial endotoxin - Lipopolysaccharide
molecules (LBP) attracts great attention. One example of such molecules is antimicrobial peptides (AMPs). These are larger epertoire of gene encoded peptides produced by living organisms of all types, which serve as part of the innate immunity protecting them from pathogen invasion. AMPs are known to interact with LPS with high affinities. The biophysical properties of AMPs and their mode of interaction with LPS determine their biological function, susceptibility of bacteria to them, as well as the ability of LPS to activate the immunesystem. Negatively charged molecules of LPS consist of a preserved lipophilic component lipidA and polysaccharides, or oligosaccharides linked to this membrane anchored domain. The saccharide portion is diverse in length and composition amongst the different Gram-negative bacteria species (Alexander and Rietschel, 2001). The outer membrane of Gram negative bacteria (cell wall) is a symmetric membrane (Fig.1.4.). LPS covers more than 90% of the cell surface in its outer leaflet, whereas phospholipids that are located in the inner leaf let, have a composition similar to that of the cytoplasmic inner membrane. This unique membrane serves as a physical barrier, providing the bacteria protection from antibacterial agents (Nikaido, 1989; Hancock and Diamond, 2000; Papo and Shai, 2005).

This assumption is supported by the findings that’s one antimicrobial peptides are active against one bacterial strain but not against others, although the inner membranes of these bacteria have similar phospholipid compositions (Hancock and Diamond, 2000). This is because of the differences in the LPS composition of the various strains (Nikaido, 1994). Furthermore, different sensitivities were found for some antibacterial peptides of deep rough bacteria, compared to smooth phenotypes that are diverse in the length of LPS saccharidic portion (Allende and McIntosh, 2003). Gram negative bacteria outer membrane serves as the first barrier encountered by peptides and they need to transverse it in order to reach the inner cytoplasmic membrane.
Initially, the peptides interact with the LPS exterior and competitively displace the divalent cations that partially neutralize the LPS negative charge (Hancock and Scott, 2000). Rana et al. (1991), studied the effect of LPS structure on the interaction between magainin 2 and *Salmonella typhimurium* outer membrane. It was found that the susceptibility of Gram negative cells to magainin 2 is associated with factors that facilitate the transport of the peptide across the outer membrane, such as the magnitude and location of LPS charge, the concentration of LPS in the outer membrane, outer membrane molecular architecture, and the presence or absence of the O-antigen side chain. Other studies point to the tight packing of the lipid acylchains of LPS bilayers (Papo and Shai, 2005; Allende and McIntosh, 2003), to the variations in the chemical structure of LPS, e.g., in the composition of the sugar head group (Gutsmann et al., 2000), and to the highly charged O-specific sugar side chains (Gutsmann et al., 2005), as the causes for the bacteria resistance towards antibacterial peptides.

Recently this peptide was found to modulate the inflammatory response to LPS/endotoxin and other Agonists of TLR by a complex mechanism that involves multiple. It seems that antimicrobial peptides protect the host against microbial pathogens by multifaceted mechanism with several points of intervention. A model outlining the major events in induction of sepsis by bacteria and the points at which cationic peptides are proposed to intervene is presented in Fig. 1.5.

The biophysical properties and the mode of action of AMPs with LPS determine their biological function, the susceptibility of bacteria to them, the ability of LPS to activate the immune system, as well as the potential of AMPs to interfere with this activation and to neutralize LPS-induced endotoxic shock. The 3-D structure of AMP and their properties are shown in (Fig. 1.6.) From a therapeutic point of view, although antimicrobial peptides were found
to be potential candidates for treatment against endotoxic shock and bacterial infection both in vitro and in vivo, still no peptide is available for clinical use mainly because of toxicity and stability problems.

1.07. Insect haemolymph

Insect blood (haemolymph) has the following properties: a) it accumulates carbon dioxide, b) transfers nutritive elements to cells and tissues, c) offers hydraulic support for the body shape, d) adds plasticity to wings of newly emerged adults, e) regulates heating during insect flight and f) protects the organism against microbial invaders and parasitoids (Nation, 2008). Coagulation of haemolymph initiates wound repairs and limits the entry of microorganisms into the hemocoel (Haine et al., 2007). The insect haemolymph consists from two major components: the plasma (e.g. the fluid phase of the tissue) and the cellular fraction (hemocytes) (Ribeiro and Brehélin, 2006; Wyatt, 1961). Proteomic analysis for the composition of phytophagous lepidopteran caterpillar haemolymph has been reported for Manduca sexta and Bombyx mori (Furusawa et al., 2008; Li et al., 2006b). Here in the plasma components will be discussed followed by the cellular phase, both of which participate in the two immune states: i.e. innate immunity and induced immunity.
Fig. 1.5. A cartoon outlining the major events in induction of sepsis by bacteria and the points at which cationic peptides are proposed to intervene. Antibiotics can stimulate the release of endotoxin (LPS) which binds to LPS-binding protein (LBP) present in the blood, and transfers it to CD14 receptor on the surface of immune cells. This complex initiates intracellular signaling reactions, which mediate the production of inflammatory cytokines (Hancock and Diamond, 2000). AMPs inhibit this event by several mechanisms: (A) by direct binding to LPS, making it unavailable to LBP; (B) by competing with LPS for binding to the TLR signaling complex; (C) by inhibiting NFκB translocation into the nucleus (Mookherjee et al., 2006); (D) by altering inflammatory genes expression through direct triggering of the MAPK pathways; and (E) by direct killing of microbes either via disruption of their membranes or by reacting with internal molecules.
Fig. 1.6. 3D homology modeling of silkworm antibacterial proteins families by using PHYMOL program. A) Cercopin, B) Moricin, C) Enbocin, D) Lysozyme, E) Gloverin, F) Lebocin, G) Attacin, H) Carboxyl, I) Phenoloxidase, J) Hemolin, K) Coagulogen, L) Anti-LPS factor
## Table 1.1: Major antibacterial proteins in lepidopteran

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>References</th>
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| Attacins | **Properties:** Attacins (20kDa) in the hemolymph, are peptides secreted from the fat body.  
**Function:** Proteolytic digestion of attacin E can produce attacin F. Gene analysis of attacins in *Hyalophora cecropia* shows that the protein carries an NF-κB nuclear factor binding site. Attacins are effective against non-pathogenic bacterial species, and facilitate the function of other antimicrobial peptides like lysozyme and cecropins. Against *E.coli*, attacins play an inhibitory role on the synthesis of outer membrane proteins. Attacins bind to *E. coli* lipopolysaccharide.  
**Lepidopteran sources:** Attacins E and F from *Hyalophora cecropia*. | Boman *et al.*, 1985  
Boman *et al.*, 1991  
Carlsson *et al.*, 1991  
Carlsson *et al.*, 1998  
Engström *et al.*, 1984  
Gunne and Steiner, 1993  
Sun *et al.*, 1991 |
| Cecropins | **Properties:** Cecropins (~4 kDa) are cationic peptides, with antibacterial, antifungal, antiprotozoan and anticancer properties. Their molecule structure is C-amidated peptide with two linear α helices separated by a hinge. The antimicrobial effect is expressed by disruption of membrane permeability and pore formation on the outer surface of the microorganism.  
**Function:** Antibacterial assays of lepidopteran cecropins show an increased activity against non-pathogenic bacteria by adhering and forming pores through interaction with the bacteria cell membrane.  
**Lepidoptera sources:** Cecropin A from *Hyalophora cecropia*; Cecropin B from *Hyalophora cecropia* and *Antheraea pernyi*; Cecropin D & E from *Bombyx mori*, *Agrius convolvuli*; Hinnavin II from *Artogeia rapae*. | Andra *et al.*, 2001  
Boman *et al.*, 1985  
Bulet and Stocklin, 2005  
Cociancich *et al.*, 1994  
Ekengren and Hultmark, 1999  
Hui *et al.*, 2002  
Ji and Kim, 2004  
Lee *et al.*, 1999b  
Lee *et al.*, 2004  
Merrifield *et al.*, 1994  
Sato and Feix, 2006  
Yoe *et al.*, 2006 |
| Defensins | **Properties:** Defensins are cationic peptides (~5 kDa) with antibacterial, antifungal and antiplasmodial properties within insect hemolymph. They are usually larger than cecropins. Defensins have 33-46 residues, and three to four internal disulfide bridges.  
**Function:** Most studies on Lepidoptera describe the antifungal activity of the molecules. However, antibacterial defensin activity spectra have been reported using Gram-positive microbes in *Mamestra brassicae* cell line.  
**References** and *Dimarcq,* 2007  
Bergin *et al.*, 2006  
Bulet and Stocklin, 2005  
Lamberty *et al.*, 2001  
Mandrioli *et al.*, 2003  
Reddy *et al.*, 2004  
Shahabuddin *et al.*, 1998 | Andres and Dimarcq, 2007  
Bergin *et al.*, 2006  
Bulet and Stocklin, 2005  
Lamberty *et al.*, 2001  
Mandrioli *et al.*, 2003  
Reddy *et al.*, 2004  
Shahabuddin *et al.*, 1998 |
<table>
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<th><strong>Lepidopteran sources</strong>: Spodopterican from <em>Spodoptera frugiperda</em>; Gallerimycin from <em>G.mellonella</em> and <em>S. frugiperda</em>; Heliomycin from <em>Heliothis virescens</em>.</th>
<th>Volkoff <em>et al.</em>, 2003 Yamauchi, 2001</th>
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<tr>
<td><strong>Gloverin</strong></td>
<td>Properties: Gloverins (~14 kDa) are peptides rich in glycine (16-20%). Function: They are active against Gram-negative bacteria but are not active against Gram-positive bacteria. Gloverin exhibits antibacterial activity similar to attacins. While interacting with LPS, they form an α-helical structure. <strong>Lepidopteran sources</strong>: Gloverin from <em>H. gloveri</em>, <em>Helicoverpa armigera</em> and gloverins 1-4 from <em>B.mori</em>.</td>
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<tr>
<td><strong>Lebocin</strong></td>
<td>Properties: Proline-rich peptides with 32 amino acid residues express antibacterial activity against nonpathogenic <em>E. coli</em> in <em>B. mori</em> and <em>B.thuringiensis</em> upregulates lebocin synthesis in Mexican strains of Trichoplusia ni. Function: Relprotein regulates the expression of lebocin in <em>B. mori</em>. Its main source of synthesis is the fat body. <strong>Lepidopteran sources</strong>: Lebocins 1 – 5 from <em>B.mori</em>, lebocin from <em>T.ni</em>.</td>
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<tr>
<td><strong>Lysozymes and lysozyme-like proteins</strong></td>
<td>Properties: Lysozyme (~14 kDa) is a bacteriolytic enzyme which hydrolyzes the bond between N-acetylglucosamine and muramic acid of peptidoglycan. Function: During bacterial lysis, fragments of free peptidoglycan disperse throughout the hemolymph inducing a series of non-self responses. Lysozyme activity in the hemolymph increases not only in the presence of bacteria but also in the presence of fungi. Lysozyme levels drop when parasitoids with polydnaviruses invade the lepidopteran hemocoel. Lysozyme species in lepidopteran species have similar molecular weights (~16kDa) and isoelectric points; however, the latter differs from the chicken lysozyme. Gene silencing of lysozyme leads to an increased expression of phenoloxidase in mosquitoes. RNA inhibition of lysozyme in <em>M. sexta</em> did not reverse responses to <em>E. coli</em>. There are lysozyme-like proteins which lack muramidase activity but are active against</td>
</tr>
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| Moricin | **Properties:** It is a 42-residue peptide (4 kDa), highly cationic, containing one aspartate, two arginine, and nine lysine residues.  
**Function:** Limits the growth of *E. coli*, *S. aureus*, *P. aeruginosa*, *B. thuringiensis*, *Micrococcus luteus*, *Enterococcus faecium*. Transcription of *M. sexta* moricin is upregulated in hemocytes and the fat body in the presence of bacteria.  
**Lepidopteran sources:** Moricin from *B. mori*, *S. littura* and *M. sexta.* | Dai *et al.*, 2008  
Hara and Yamakawa, 1996  
Oizumi *et al.*, 2005 |
| Viresin | **Properties and Function:** Viresin (12kDa) exhibits antibacterial activity against *E. coli* and *M. luteus*.  
**Lepidopteran sources:** Viresin from *H. virescens.* | Chung and Ourth, 2000 |

### 1.08. Insect haemolymph plasma

Plasma pigmentation, composition and pH are highly variable with the insect species instar, diet and in the case of infection, microbial species (Klowden, 2007; Shelby and Popham, 2007). The inorganic components of haemolymph plasma consist mainly of sodium, potassium, calcium and magnesium (Wyatt, 1961). Organic substances found in plasma include free amino acids, organic acids, carbohydrates, proteins, enzymes and lipoproteins (Ryan and vander Horst, 2000; Wyatt *et al.*, 1956; Wyatt and Pan 1971). The plasma components provide the osmotic pressure required to support the circulating hemocytes (Wittig, 1962). In terms of immunity, plasma components such as phenoloxidase (Tong *et al.*, 2005) and lysozyme (Wilson and Ratcliffe 2000) and the lipoprotein shuttle for lipid/energy transfer (Weers and Ryan, 2006) are the most interesting aspects for study due to their involvement in cell-free and hemocyte mediated immunological properties and their relation to microbial pathogenicity.
1.09. Larval hemocytes in lepidopteran species

Lepidoptera larval hemocytes are classified based on their function, morphology (Price and Ratcliffe, 1974), ultrastructure (Neuwirth, 1973), antigenic properties (Gardiner and Strand, 1999), and staining properties (Ling et al., 2003). The following cell types are recognized: a) prohemocytes, b) plasmatocytes, c) granular cells, d) spherulocytes and e) oenocytoids (Fig.1.2.). Their role in immunity, depending on insect species and stage, is to recognize the presence of foreign objects within the haemolymph, encode antimicrobial peptides and adhere to and/or discharge onto the antigen surfaces (Beckage, 2008). Some hemocyte types, e.g. plasmatocytes, are produced by hematopoetic organs and their dissociation and dispersion in the haemolymph begins when adhesion molecules such as integrins, cover the outer membrane of hemocytes (Nardi et al., 2003).

1.10. Prohemocytes

Prohemocytes are round cells, comprising approximately 5% of the total hemocyte counts in many species of lepidopteran larvae (Chapman, 1998; Jones, 1962). These cells have a large central nucleus, ribosomes, mitochondria and sparse endoplasmatic reticulum (Butt and Shields, 1996) and are considered to be multipotent insect stem cells (Beaulaton 1979; Corley and Lavine 2006). Prohemocytes may differentiate into plasmatocytes, granular cells and spherulocytes (Ling et al., 2005a; Yamashita and Iwabuchi, 2001). This class of hemocytes is able to function as phagocytes for hemocyte debris (Ling et al., 2005a). In tissue culture systems, larval prohemocytes are small and resemble fibroblast-like cells (Kurtti and Brooks, 1970). In vitro, with use of serum-free culture medium, 40% of fresh haemolymph lepidopteran prohemocytes continue to produce prohemocytes through cell division (Yamashita and Iwabuchi, 2001).
1.11. Plasmatocytes

Plasmatocytes constitute 30% - 50% of the total hemocyte population of insects in general (Chapman, 1998). They are motile and variable in shape ranging from ameboid to stellate forms with extremely expandable pseudopodia (Arnold and Hinks, 1974). The cytoplasm contains Golgi bodies, mitochondria, ribosomes, membrane-bound vesicles, phagocytic vacuoles and smooth and rough endoplasmic reticulum (Butt and Shields, 1996). Their total size and their nucleus size in lepidopteran caterpillars are approximately 20-40 μm and 5-10μm, respectively (Nardi et al., 2006). A least nine different proteins that occur on Lepidoptera plasmatocytes can be used to distinguish this class of hemocytes from the others (Gardiner and Strand, 1999). For most insect species, plasmatocytes can adhere to glass, phagocytose small particles, and participate in nodulation and encapsulate large foreign objects in vitro (Weisner, 1991; Zakarian, 2002). Hyperphagocytic plasmatocytes detected in M. sexta exist as a plasmatocyte subpopulation that engulfs bacteria in the haemolymph; their contribution to the total hemocyte count is low (~1%) (Dean et al., 2004a, b).

1.12. Granular cells

Granular cells comprise approximately 30-50% of the total hemocyte counts (Falleiros et al., 2003). Cell shape is spherical (diameter 7-9Åm) and the cytoplasm contains a plethora of granules (diameter 350-1000 nm each) (Gardiner and Strand, 1999; Kaya and Tanada, 1993), a central nucleus, Golgi bodies, mitochondria, multivesicular bodies, smooth and rough endoplasmatic reticulum (Butt and Shields, 1996). Seven anti-hemocyte monoclonal antibodies are known to bind specifically to granular cells and can be used in cell sorting techniques (Gardiner and Strand, 1999). Larval hemocytes may differentiate into other types; in silkworm,
*B. mori*, granular cells are regarded as the transient stage between prohemocytes and spherulocytes (Yamashita and Iwabuchi, 2001; Ling *et al*., 2005a). Granular cells are involved in nodulation and encapsulation of foreign objects within the haemolymph, the hemocytes degranulating adhesive materials about the particulate (Kaya and Tanada, 1993; Pech and Strand, 1996; Rowley and Ratcliffe, 1976). Apoptosis of the granular cells, which is caused by factors released from the plasmatocytes during nodulation, limits their attack on foreign objects (Pech and Strand, 2000).

### 1.13. Spherulocytes

Spherulocytes (size 6-11 μm), which comprise 20-30% of the hemocyte counts, contain large spherical inclusions that occupy much of the cytoplasm almost hiding the small nucleus (3–4 μm) in the center (Butt and Shields, 1996; Brehelin *et al*., 1978; Falleiros *et al*., 2003; Gupta, 1991). In the silkworm *B. mori*, these cells are derived through differentiation from prohemocytes and granulocytes (Yamashita and Iwabuchi, 2001). Spherulocytes of *M. disstria* and *Helothis virescens* contain heparin analogs, glycosaminoglycans with anticoagulant and wound healing properties similar to those found in vertebrate mast cells (Cook *et al*., 1985, Fallon and Sun, 2001; Neuwirth 1973). Lepidopteran spherulocytes express antibacterial lysozyme when exposed to *E. coli* (Lavine *et al*., 2005). In *B. mori*, it is not conclusive that phenoloxidase occurs in these cells (Ling *et al*., 2005b).

### 1.14. Oenocytoids

Oenocytoids are spherical cells (diameter 20-35 Åm) and represent 1- 10% of the total hemocyte population (Butt and Shields, 1996). These cells can also be identified with non-plasmatocyte-cross reacting, anti-hemocyte, monoclonal antibodies (Gardiner and Strand, 1999). Western
blotting and indirect immunofluorescence have been used to show that the zymogenic component of the melanizing pathway, prophenoloxidase, is present in the cell membrane of oenocytoids (Feng and Fu, 2004). Lepidopteran oenocytoids express antibacterial lysozyme in the presence of *E. coli* (Lavine *et al.*, 2005).

### 1.15 Innate immunity

The response is the elimination of activities beginning with non-self recognition of foreign objects by humoral proteins reacting with hemocyte receptors or by the antigens reacting with the hemocyte receptors (Ohta *et al.*, 2006), followed by signal transduction and the actual hemocytic response (Williams, 2007). Induced immunity is not well documented in insects; it represents the synthesis of antimicrobial proteins which may be consistutive or synthesized de novo from the fat body, hemocytes or other tissues (Bulet *et al.*, 1999) in response to direct antigenic stimulus (Gandhe *et al.*, 2006; Eleftherianos *et al.*, 2007) or indirectly by antigens processed by the hemocytes (Lamprou *et al.*, 2005). Many of the induced proteins belong to the immunoglobulin superfamily including hemolin with antibacterial and lipopolysaccharide-binding properties (Eleftherianos *et al.*, 2007) and Down syndrome adhesion molecules (DSCAM) with multiple immunoglobulin domains. The latter protein found in *B. mori* (Watson *et al.*, 2005), *D. melanogaster, Anopheles gambiae, Apis melifera* (Graveley *et al.*, 2004) are implicated in hemocyte innate responses against bacteria (Dong *et al.*, 2006; Zipursky *et al.*, 2006).

### 1.16 Innate immunity within insect haemolymph

In general, the innate non-self cellular response of many lepidopteran larvae consists of phagocytosis, nodulation and encapsulation, which are the products of the interaction of cellular
and humoral factors (Gillespie et al., 1997). Humoral responses involve the binding of pattern recognition molecules to the foreign object (Yu et al., 2002b), the activation of the melanizing enzyme phenoloxidase (Brivio et al., 2002) and, the formation of melanotic capsules around microorganisms (Boguñá et al., 2007) with the subsequent induction of antimicrobial peptides (Leclerc and Reichhart, 2004). Salient humoral elements include the opsonic, melanizing phenoloxidase system (Cotter and Wilson, 2002), lysozyme (a cationic, constitutive plasma protein) (Lockey and Ourth, 1996; Wilson and Ratcliffe, 2000), C-type lectins (Watanabe et al., 2006), β-1,3-glucan-binding proteins (Fabrick et al., 2003), hemolin (Bao et al., 2007), peptidoglycan-binding proteins (Steiner, 2004) and apolipophorin-III (Halwani et al., 2000), many of which facilitate the binding of microorganisms to hemocytes.

Cellular responses include phagocytosis (Ling and Yu, 2006a), nodulation (Tunaz, 2006) and cellular encapsulation (Hu et al., 2003) which may be mediated by plasma factors and cell membrane receptors (Ohta et al., 2006). Physicochemical properties of antigens affect the adhesion of insect hemocytes to foreign material (Lavine and Strand, 2001). Decline in cationic charge increased the adhesion of P. luminescens to G. mellonella hemocytes independently of bacterial hydrophobicity (Dunphy, 1995) whereas neither electrostatic charge nor hydrophobicity influenced X.nematophila adhesion to these hemocytes (Maxwell et al., 1995). Both charge and wet ability of polystyrene plates and beads increase adhesion by P.americana hemocytes than did S. gregaria (Tackle and Lackie, 1987). In most cases regarding the adhesion of insect hemocytes, emphasis is placed on hemocyte receptors linking to extracellular matrix proteins. These receptor proteins include those in families of integrins (Levin et al., 2005), lectins (Okazaki et al., 2006), collagen (Yasothornsrikul et al., 1997) and immunoglobulin-like containing molecules (Watson et al., 2005).
1.17. Phagocytosis

Phagocytosis is a mechanism for cells to internalize particulates (Garcia-Garcia, 2005) which, in the case of insect hemocytes, can be either microorganisms or apoptotic cell debris (Ling and Yu, 2006b). The whole process is divided in steps: a) recognition, b) attachment, c) signal transduction, d) pseudopodia formation and e) ingestion (Gillespie et al., 1997). Recognition molecules in plasma can trigger phagocytosis. These molecules include lectins in *Blaberus discoidalis* (Wilson et al., 1999). Lectins are carbohydrate binding recognition molecules in the haemolymph (Yu and Kanost, 2004) which bind antigens to hemocytes inducing phagocytic activities (Yu et al., 2005). C-type lectins on *B. mori* hemocytes enhance non-self responses (Ohta et al., 2006). In insects, thioester containing proteins analogous to macroglobulin and complement C3 enhance in phagocytosis of bacteria by the dipteran hemocytes (Cherry and Silverman, 2006). Down syndrome cell adhesion molecules, in their soluble form in plasma enhance also hemocytic phagocytosis of bacteria (Kurtz and Armitage, 2006). Both the soluble and hemocyte bound form of hemolin (Bettencourt et al., 1999) increases the phagocytic ability of larval *M. sexta* hemocytes towards *E. coli* (Eleftherianos et al., 2007) and the hemolin binding to lipopolysaccharide linking the bacteria to the hemocytes (Daffre and Faye, 1997). Foreign recognition is not only defined by lepidopteran humoral pattern recognition proteins binding to receptors (Beckage, 2008; Ohta et al., 2006) but also by hemocyte receptors that directly react with the antigens. This includes RGD-integrin receptors on plasmatocytes (Levin et al., 2005) and granular cells (Pech and Strand, 1996) which bind to both collagen type IV or their fragments generated by metalloproteinases released by invading bacteria (Altincicek et al., 2007), and bind to discharged granular cell extracellular matrix proteins (Lavine and Strand, 2002).
Independently of Toll protein family function, the Imd pathway also senses the presence of Gram-negative bacteria in insect hemocytes (Wang and Ligoxygakis, 2006). Despite the homology of the Imd pathway found in lepidopteran fat body cells (Gandhe et al., 2006) with Diptera fat body and hemocytes (Hultmark and Borge-Renberg, 2007), the homology of Imd proteins in lepidopteran hemocytes is unknown (Ao et al., 2008b). Peptidoglycan recognition proteins, which were found on hemocytes and soluble in the haemolymph, are able to activate both Toll and Imd pathways (Dziarski and Gupta, 2006). These cations possibly play a significant role in phagocytic non-self responses.

1.18. Encapsulation

Encapsulation is divided into two main categories: a) humoral encapsulation which results in a capsule of melanin around foreign objects in the haemolymph of Diptera (Götz and Vey, 1974) with low hemocyte counts (Kaya and Tanada, 1993) and b) cellular encapsulation which occurs extensively in Lepidoptera (Pech and Strand, 1996). Cellular encapsulation involves hemocytes forming a multilayered capsule around antigens (Gillespie et al., 1997) such as nematodes in G.mellonella (Mastore and Brivio, 2008), fungi in G.mellonella (Dunphy et al., 2003), all antigens too large for phagocytosis. In Lepidoptera, the capsule has two main contributing hemocytes types: the granular cells and the plasmatocytes (Schmit and Ratcliffe, 1977). The formation of the capsule is a two step process initiated when granular cells contact the foreign object discharging matrix proteins and cytoplasmic material (Pech and Strand, 1996). Thereafter, the plasmatocytes are attracted to the granular cell-antigen coagulum by chemotaxis (Levin et al., 2005). Aggregation of hemocytes around the foreign object is due to plasma factors, like lectins (Ling and Yu 2006b; Yu and Kanost, 2004). Noduler, an immune protein found in the silkmoth, Antheraea mylitta, and upregulates immune responses by enhancing
cellular nodulation of *E. coli* and *S. aureus* and may apply to encapsulation because the overall hemocyte mechanisms are similar (Gandhe et al., 2007b).

The size of the cellular capsule around the foreign object in the haemolymph is regulated by apoptosis of granular cells at the periphery of the capsule induced by plasmatocytes (Pech and Strand, 2000). Lepidopteran hemocytes contribute also to encapsulation of foreign surfaces through the RGD binding motifs on integrins (Lavine and Strand, 2002; Zhuang et al., 2008). Integrins besides physically binding to extracellular matrix proteins (Bokel and Brown, 2002), may regulate lepidopteran hemocytic encapsulation.

### 1.19. Nodulation

Nodulation is a cellular response of the haemolymph against bacteria (Miller et al., 1994) that exceed a critical level and cannot be readily handled by phagocytosis alone (Kavanagh and Reeves, 2004; Ratcliffe and Gagen, 1976). Granular cells adhere to the antigen forming an antigen coagulum about which the plasmatocytes adhere (Lavine and Strand, 2002) resulting in the rapid removal of bacteria from the haemolymph (Da Silva et al., 2000). Nodulation limits feeding and proliferation of invading microorganisms by limiting oxygen to the microbes and reduces all the microbial metabolic processes (Da Silva et al., 2000).

### 1.20. Humoral immunity

Humoral immunity in lepidopteran insect haemolymph is based on plasma proteins such as apolipoprotein-III; the phenoloxidase system and antimicrobial peptides (Table 1.1). Humoral immunity is classified into two main categories: a) innate humoral immunity, where antimicrobial peptides and other types of constitutive plasma proteins contribute to innate immunity mechanisms such as phagocytosis, encapsulation and nodulation and phenoloxidase
activation and b) induced humoral immunity, where antimicrobial proteins are synthesized de novo in the fat body, hemocytes or any other tissues (Bulet et al., 1999) in the presence of antigens (Gandhe et al., 2006; Eleftherianos et al., 2007; Lamrou et al., 2005).

1.21. Apolipophorin-III

The existence of apolipophorin-III (ALP) within the haemolymph was reported for at least 19 different insect species (Weers and Ryan, 2006). ALP exists in two forms: a) the lipid bound form where, in conjunction with apolipophorin I and II, it forms a lipophorin complex with enhanced lipid carrying capacity and b) the lipid free form (Kahalley et al., 1999). ALP is active in programmed cell death mechanisms in muscle and neural cells during molting in Lepidoptera (Sun et al., 1995) and has multifaceted immunological properties (Halwani et al., 2001) which compete with lipid transport (Adamo et al., 2008). ALP binds to bacterial lipopolysaccharide and to lipid A inactivating endotoxin toxicity and the inhibition of phenoloxidase activation (Pratt and Weers, 2004; Leon et al., 2006a). The core carbohydrate region on E. coli lipopolysaccharide is involved in the binding event to ALP (Leon et al., 2006b). The interaction of ALP is not restricted only to surface antigens of Gram-negative bacteria; it binds to surface antigens of Gram-positive bacteria (Halwani et al., 2000) and fungal -glucans (Brown and Gordon, 2005) providing host protection against a large spectrum of pathogens.

1.22. Phenoloxidase System

The active form of proPO, phenoloxidase (PO; monophenol, dihydroxyphenylalanine: oxido reductase; EC 1.14.18.1), also known as tyrosinase, catalyzes two successive reactions: hydroxylation of a monophenol to o-diphenol (monophenoloxidase activity) and the oxidation of the o-diphenol to o-quinone (diphenoloxidase activity) (Söderhäll and Cerenius, 1998; Decker
and Tuczek, 2000). Phenoloxidases are a group of copper containing enzymes, responsible for the production of antimicrobial melanin and free radicals (Shelby and Popham, 2007) in insects (Marmaras et al., 1996). Categorized in two major phenoloxidase groups: the laccase type is found only in the integument (Arakane et al., 2005, Sugumaran et al., 1992) and resists cuticular invasion by fungi (Gillespie et al., 2000) whereas the tyrosinase types found in plasma, hemocytes and integument (Zhao et al., 2007). Phenoloxidase mediated insect immunity was shown in Fig.1.7.

The role of the plasma enzyme is to produce cytotoxic quinones through oxidation of phenolic compounds with the subsequent formation of melanin around the invading microorganism (Nappi and Christensen, 2005; Xue et al., 2006). In non-challenged haemolymph, phenoloxidase is expressed in a non active form, the prophenoloxidase (Lee et al., 1999a). In Lepidoptera, prohemocytes, plasmatocytes, granular cells and oenocytoids are capable of phenoloxidase production (Shrestha and Kim, 2008). Serine proteinases and prophenoloxidase activating proteinases mediate the conversion to phenoloxidase in a complex cascading reaction in the presence of microorganisms (Zhao et al., 2007). Ling and Yu et al., (2005) show that prophenoloxidase is bound to lepidopteran hemocytes and in presence of foreign particles, extensive melanization of the cells occurs.

The presence of microbial surface antigens such as LPS (other than X.nematophila LPS) and its lipid A moiety, β1,3-glucan, peptidoglycan and lipoteichoic acids in lepidopteran haemolymph increase phenoloxidase activity (Kanost et al., 2004). Phenoloxidase increases hemocyte attachment to foreign objects, enhancing encapsulation and/or nodulation (Eleftherianos et al., 2008). In contrast, suppression of phenoloxidase activity within the haemolymph is one of the survival strategies of entopathogenic nematodes (Brivio et al., 2004),
and in some cases, entomopathogenic bacteria like *X. nematophila* (Da Silva *et al.*, 2000). Hydrophobic antioxidants in haemolymph of *G. mellonella* increase phenoloxidase activity against *B. subtilis* (Dunphy *et al.*, 2007). Cases of lepidopteran larvae with increased phenoloxidase in haemolymph samples, in absence of microbial antigen or parasitoid, have been attributed to environmental pollution with heavy metals (Van Ooik *et al.*, 2007) which may reflect subsequent alterations in host immunity. Prophenoloxidase activity can be controlled at the genetic level by down regulation which occurs in *C. fumiferana* infected with polydnaviruses (Doucet *et al.*, 2008).

The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial gram positive and gram negative, fungal, and viral agents. Phenoloxidase requires a complex system of activation and inhibition that involves various cell types, PO zymogens, inhibitor enzymes, and signaling molecules. Research in evolutionary ecology has studied the costs of PO in terms of resource use and pleiotropic relations with other key traits and functions. These studies indicate that PO is a costly trait, whose production and maintenance have fitness costs for hosts (Isaac González-Santoyo and Alex Córdoba-Aguilar, 2012). It was reviewed that, the existing evidence testing this prediction by filtering studies in which different sources of stress were studied in relation to their effect on animal condition. These studies appear in Table 1. From this review, there is clear evidence that individuals in better condition produce higher levels of proPO and/or PO. However, the enzyme is also involved in function other than immunity, and the interactions and regulation of phenoloxidase, its metabolites and substrates are not understood (Ashida and Brey, 1997). Therefore, it seems important to establish the relationship between phenoloxidase
activity and resistance to various patho gens prior to its wide spread use as a marker of resistance to disease (Adamo, 2004).

Fig.1.7. Insect innate immunity based on PO. Abbreviations: LPS, lipopolysaccharide; HEM, hemolin; IML-2, immunlectin-2; PGRP, peptidoglycan recognition protein; PRSP, pattern recognition serine proteinase; βGRP-1, βGRP-2, β-1,3-glucan recognition protein-1 and -2; proPAPs, zymogen of prophenoloxidase- activating proteinase; PAP, prophenoloxidase-activating proteinase; proSPHs, precursor of serine proteinase homologues; SPHs, serine proteinase homologues (SPH-1 and SPH-2); ProPO, prophenoloxidase; PO, phenoloxidase.
Table 1.2. Phenoloxidase as an indicator of strength against pathogens

<table>
<thead>
<tr>
<th>Species</th>
<th>Immune challenge</th>
<th>Relation PO/ proPO Activity and pathogen Defense</th>
<th>Basis of relation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Spodoptera exempta</em></td>
<td>Contaminated diet with nuclear polyhedrosis virus (NPV)</td>
<td>Positive</td>
<td>Larvae with higher survival after an NPV infection showed higher PO activity in hemolymph</td>
<td>Reeson <em>et al.</em>, 1998</td>
</tr>
<tr>
<td><em>Calopteryx splendens xantostoma</em></td>
<td>Eugregarine parasite burden</td>
<td>Negative</td>
<td>Males with higher capacity to fight off eugregarines had lower PO levels</td>
<td>Siva-Jothy, 2000</td>
</tr>
<tr>
<td><em>Gryllustexensis</em></td>
<td>Inoculation with <em>Serratia marcescens, Serratia liquefaciens</em>, and <em>Bacillus cereus</em></td>
<td>Non-significant</td>
<td>Total PO levels did not predict male survival after three bacterial challenges</td>
<td>Adamo, 2004</td>
</tr>
<tr>
<td><em>Daphniamagna</em></td>
<td>Infection with one bacterial species (<em>Pasteurin aramosa</em>) and three microsporidia Species(<em>Octospore abayeri, Ordospora colligate, Glugoides intestinalis</em>)</td>
<td>Non-significant</td>
<td>PO activity in hemolymph did not predict immunocompetence in any immune-challenged subjects</td>
<td>Mucklow <em>et al.</em>, 2004</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Natural infection with fungus <em>Beauveria bassiana</em> and Challenge with a needle Contaminated with the yeast <em>Candida albicans</em>, the gram) bacteria <em>Agrobacterium tumefaciens</em> and <em>Escherichia coli</em> or the gram+bacteria <em>Enterococcus faecalis</em> and <em>Staphylococcus aureus</em></td>
<td>Non-significant</td>
<td>Drosophila mutants that fail to Activate PO in the hemolymph following microbial challenge were as resistant to infection as wild-type flies</td>
<td>Leclerc <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>Species</td>
<td>Event Description</td>
<td>Result</td>
<td>Description</td>
<td>Reference</td>
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</tr>
<tr>
<td><em>Hetaerina americana</em></td>
<td>Inoculation with <em>S.marcescens</em></td>
<td>Positive</td>
<td>Territorial males subjected to bacterial inoculation and nylon mono filament implants had higher PO activity and survival for longer than non-territorial males</td>
<td>Contreras-Garduno <em>et al.</em>, 2007</td>
</tr>
<tr>
<td><em>Litopenaeus vannamei</em></td>
<td>Challenge with white spot syndrome virus</td>
<td>Negative</td>
<td>Down regulation of proPO</td>
<td>Ai <em>et al.</em>, 2008</td>
</tr>
<tr>
<td><em>Plodia interpunctella</em></td>
<td>Oral inoculation and direct intra haemocoelic injection of naturally occurring Granulosis virus (PiGV)</td>
<td>Non-significant</td>
<td>No elevated haemolymph PO activity during the early stage of infection. Haemolymph PO activity increased in the susceptible (infected) larvae only at a later stage of the infection</td>
<td>Saejeng <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Hetaerina vulnerata</em></td>
<td>Inoculation with <em>S.marcescens</em></td>
<td>Non-significant</td>
<td>Males subjected to bacterial Inoculation had higher PO Activity after 24h, but survival was not related</td>
<td>González-Santoyo <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Daphnia magna</em></td>
<td>Spores of <em>P.ramosa</em></td>
<td>Positive</td>
<td>PO levels in infected hosts did predict spore load</td>
<td>Pauwels <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>Challenged with intra haemolymph and midgut injection of LPS (<em>E.coli</em>) bacterial endotoxin</td>
<td>Positive</td>
<td>PO level increased in resistant silkworm races.</td>
<td>Present study</td>
</tr>
</tbody>
</table>
1.23. Role of esterase in insects

Esterases are a group of hydrolytic enzymes occurring in multiple forms with broad substrate specificity. Heterogeneity of esterases from several species of insects has been demonstrated employing electrophoretic techniques and they are shown to be tissue specific (Markert and Hunter, 1965). In insect, esterases perform both physiological and defensive function and are found in both soluble and membrane bound form. All the groups of esterase hydrolyze the esters present in biological material of all organisms and splits ether bonds of carbonic acids in artificial substrates. A systemic classification of these enzymes remains to be established and multiple systems are currently used. According to the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature, esterases are located within subgroup 1 hydrolases (Enzyme commission 3.1,EC 3.1), which is further categorized into subtypes based on the different types of ester bonds hydrolyzed. CaE are defined as EC 3.1.1.1 in this system as they hydrolyze carboxylic esters (IUMB 1992). The classification proposed by Aldridge groups esterases into three types (A, B and C) based on the nature of their interaction with organophosphorus insecticides such as paraoxon (Aldridge, 1953a and b).

This has been widely recognized and used by those studying esterases due to its easy of use and lack of their nomenclature systems. Enzymes that hydrolyze organophosphorus compounds (EC 3.1.1.2), including those containing an acylated cysteine in their active sites (EC 3.1.8.1), are termed α-esterases (Reiner et al., 1993; Walker, 1993). Esterases that are progressively inhibited by organophosphorus compounds, such as paraoxon, and have an active site serine residue (EC 3.1.1.1) are called β-esterases, (Yan et al., 1994) and those which are resistant to organophosphates but do not degrade them are C- esterases (acetylesterase or EC 3.1.1.6). While this classification system is easy to use, it is lacking in utility as it does not
provide a unique classification for each esterase. Apart from Aldridge’s general taxonomy of esterases, different classifications have been proposed and adopted to describe the esterases in a particular species or group of closely related species. For example, others groups classified esterases based on the substrate and inhibitor specificites, esterases are generally classified in to three types; carboxyl esterases (CE), arylesterases (ArE), and choloineesterases (ChE) (Mounter and Wittaker, 1953; Vedbrat and Whitt, 1974) and with mobility of esterase on electrophoretic gels, such as aphid esterases E1-E7 (Devonshire, 1975). Also in insect esterase are classified according to different substrates (α and β) as a hydrolyzing substrate. α-Napthyl acetate hydrolyzing esterase are α-Est and β-Napthyl acetate are β-Est.

Both hydrolyzing esterases are referred as non specific (Cocharane, 2006). The use of nucleotide homology to classify mammalian carboxylesterase isozyme into one of four families: CES1, CES2, CES3, or CES4 (Satoh and Hosokawa, 1998) novel approach may provide a satisfactory systematic nomenclature, especially as additional sequence data become available. Carboxylesterase implies the isozymes which act vigorously on various simple esters of low molecular weight, but slightly on Phenolic esters (Clements, 1967) and has an important role in the synthesis and transport of cuticular wax (Sudderuddin and Tan, 1973; Ahamad and Forgash, 1976). ArE is small group of lysozymes which function in the degradation of aromatic esters including phenolic esters (Clements, 1967; Sudderuddin and Tan, 1973). ChE is the important regulatory enzyme, responsible for controlling the natural transmission on synapses, where it acts to hydrolyse the excitory of neuro transmitter acetylcholine (Silver, 1974).

Among the esterases, carboxylesterases and acetylcholine esterases have been associated with defense and pesticide resistance in arthropods. In B. microplus, the resistance mechanisms associated with esterase detoxification has been investigated by several authors. Baxter and
Barker (2002) have demonstrated the relationship between the resistance to organophosphates and enhanced AchE activity in Australian *B. microplus* ticks and Jamroz *et al.*, (2000) have shown increased carboxylesterase activity in resistant pyrethroid Mexican strain of *B. microplus* ticks (Hernandez *et al.*, 2000, 2002). Among insect species, carboxylesterases of the *Myzus persiae* aphid (Devonshire, 1977; Devonshire and Sawicki, 1979), mosquito (Georghiou and Pasteur, 1980; Kettermann *et al.*, 1993), housefly (Oppenoorth, 1965) and tobacco cutworm (Bull and Whiten, 1972) have been studied extensively because of their involvement in resistance to organophosphates or other kinds of insecticides.

In silkworm, protein and enzyme polymorphism was widely discussed in relation to the comparative biochemistry, ecology, physiology, and genetics and in breeding (Sankina *et al.*, 1974; 1975; Konicheva *et al.*, 1975; Eguchi and Iwamoto, 1975). Esterase has been studied extensively in silkworm, the six midgut esterase types have been studied in relation to races and esterase type (Yoshitikae, 1963). Eguchi and Sugimoto in 1964 reported five different esterase bands in the haemolymph of silkworms with gradual changes in the enzymatic activity of each esterase band during ontogeny. Types and inheritance of haemolymph esterase in silkworm was described. They stated that four fundamental types of esterase were controlled by co-dominant alleles Bes A, Bes B, and Bes O about 70% of Japanese, Chinese and European races are belong to A type and 20% to O-type, while B types is only found in the Chinese races (Eguchi *et al.*, 1965).

The specific expression of non specific esterase was determined at different stages and observed during ontogenesis of the silkworm in silk gland and fat body (Stoykova, 2001). The role of esterases and their contribution by the male into female genital tract during mating in silkworm *B. mori* is studied. The amount of esterase contributed to females from male during
mating was found to be higher in univoltine and bivoltine than the multivoltines and also involved in the fitness parameters of fecundity and viability (Krishnaswami and Umakanth, 1997). Nasirillaev et al. (1977) found the 3 to 11 esterase bands in the diapausing eggs of the different races, while Egorova et al. (1977) reported 10 esterase bands. The stage specific esterases from larvae, pupae and adults of silkworm races showed ten fraction of esterase enzymes and are designated or controlled by five loci Bes A, Bes B, Bes C, Bes O and Bes E (Stoykova et al., 2003).

The carboxyl esterase act as immune defense molecules against bacteria also degrade insecticides and continuous exposure to insecticides over several generations has resulted in the selection of carboxylesterase that specialize in insecticide degradation. Carboxyl esterase isozymes have been reported in mammal macrophages and monocytes, cells involved in the immune system. Studies on induction of carboxylesterase isozymes in *Bombyx mori* by *E. coli* infection showed an inducible proteins or isoforms of enzymes viz., Carboxyl esterase (CEs) – Est-1 and Est-2 – induced by the injection of *E. coli* or LPS. They were found with a similar other known bacterially inducible proteins and CEs clearly differed from noninducible CEs (Est-3, 4 and 5 which were visualized in the haemoloymps of silkworm. These results suggest that Est-1 and Est-2 are novel CEs that are inducible either by bacterial or bacterial ligands (LPS) injections into the silkworms. (Shiotsuki and Kato, 1999). Hence, a study focusing on investigations of induced isozymes in the haemolymph of silkworm races/breeds challenged with LPS would be of great help to make use of them as biochemical markers for associating the immunity of different silkworm races of the *Bombyx mori*.

Assessment of an animal’s relative disease resistance is critical for many questions in ecological immunology. Invertebrate model systems have become increasingly popular in
ecological immunology research (Rolff and Siva-Jothy, 2003) and various methods have been used to estimate their ability to resist disease. In insects, the measurement of PO enzyme activity in the humoral compounds has often been used to estimate disease resistance (Rantala et al., 2002). Silk constitutes an important source of revenue and employment in many developing countries. However, infectious diseases have affected the profitability of the silk industry. The growth and silk yield of silkworms have been affected by both biotic and abiotic factors.

Hemocytes in the haemolymph of the insects play a major role in the defense system. These are circulating cells found in the haemolymph of insects. As the melanization of the cuticle occurs mostly in larvae living in high-density populations, in which the risk of pathogenesis is greatest, there must be a fitness cost associated with its expression (Wilson et al., 1999). Thus, the present study examined the genetic variance and covariance within and between a number of immune function traits (including cuticular melanization) and life history traits, in order to identify potential genetic trade-offs constraining immune function expression. The illustration of LPS mediated responses and the role of esterase and phenoloxidase enzyme was shown in Fig. 1.8.

In regard to humoral and cellular parameters, the haemolymph and midgut was analysed by key biomolecules of enzymes esterase and phenoloxidase. Esterase and phenoloxidase (PO) activity have been considered as potential markers (Takahiro Shiotoku, and Yusuke Kato, 1999; Arai et al., 2000; Rodríguez and Le, 2000; Adamo, 2004; Pang et al., 2009). Concerning humoral parameters, the antibacterial activity of plasma and the concentration of plasma proteins can be considered as criteria of health status. PO and Antibacterial immune proteins have been well characterized and it can be considered as an environmental marker (Rodriguez and Le Moullac, 2000). However, a clear understanding of the genetic basis and variability in the expression of
quantitative and qualitative genetic traits under biotic stress is an important step for the selection of potential disease tolerance parental resources for breeding programs.

For evaluation of cellular and humoral parameters of the immune response of silkworm *Bombyx mori*, the development of simplified procedures has played a vital role for the development of immunoassays. The quantification of different cellular and humoral parameters of the immune response of silkworm will give a clue for silkworm health status. Among the available tools, Esterase and phenoloxidase (PO) activity quantification (Herna´ndez-Lo´pez et al., 1996; Aquacop et al., 1997) and antibacterial activity measurement (Sung et al., 1996) and determination of plasma protein concentration, and specific antibodies against several humoral proteins (Rodr´ıguez et al., 1995; Vargas-Al- bores et al., 1996), the prophenoloxidase activating system (ProPO-AS) is an integral part of the constitutive innate immune response in insects, the products of which are commonly assayed to assess an individual’s ability to respond to immune challenges (Alice et al., 2010).

LPS is also recognized by the immune system as a marker for the detection of bacterial pathogen invasion, responsible for the development of inflammatory response, and in extreme cases to endotoxic shock (Yosef Rosenfeld and Yechie Shai, 2006). In the present study, the selected silkworm races were screened for their immunity and the degree of tolerance through LPS (bacterial endotoxin from *E.coli*) and their role in biomolecular changes associated with hardiness of the races were analyzed by bio-molecular techniques and aimed to explore the role of the immune enzymes, antibacterial proteins, induced isoforms of a silkworm in *E.coli* bacterial endotoxin viz.LPS infection mechanism. Hence, it is proposed to study the various key biomolecules involved in immunity and their association with genetic hardiness in each
silkworm races and the use of hardy parental races/breeds available from the silkworm genetic resources for silkworm crop improvement.
Fig. 1.8. Role of enzymes in response to production of immunity