INTERACTIONS BETWEEN LARVAL *Malacosoma disstria* (LEPIDOPTERA: LASIOCAMPIDAE) HEMOLYMPH AND SELECTED ANTIGENS

By

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ABSTRACT

The tent caterpillar, *Malacosoma disstria*, a major pest insect of North American arboreal forests, has not been extensively studied in terms of the innate blood cell (hemocyte) responses to bacterial antigens. Herein, physico-chemical factors and signaling enzymes affecting the adhesion of fifth instar larval *M. disstria* hemocytes to slides were determined. Maximum adhesion of the hemocyte types, the granular cells and plasmatocytes, occurred at pH 6.0-7.0 and 6.0, respectively, in phosphate-buffered saline (PBS). Activated protein kinases A and C inhibited hemocyte adhesion. Dead, Gram-negative, entomopathogenic *Xenorhabdus nematophila* and non-pathogenic Gram-positive *Bacillus subtilis* exhibited differential binding to different hemocyte types altering patterns in phenoloxidase and lysozyme activities.

The plasma protein, apolipophorin-III, limited hemocyte responses. However, initial binding of the protein to *X. nematophila* and *B. subtilis* increased granular cell levels and lowered plasmatocytes levels with bacteria. Injections of the protein spontaneously activated phenoloxidase. The protein delayed bacterial removal from (blood) hemolymph i*n vivo.*

Surface antigens of *X. nematophila* and *B. subtilis* participated in hemocyte reactions. *X.nematophila* endotoxin and its lipid A moiety elevated damaged hemocyte levels limiting *X. nematophila* removal from the hemolymph. Endotoxin and lipid A inhibited phenoloxidase activation but enhanced enzyme activity. *B. subtilis* lipoteichoic acid elicited nodulation and enhanced phenoloxidase activation and/or activity. Apolipophorin-III neutralized the effects of the three surface antigens on hemocytes and prophenoloxidase until the antigens exceeded a critical threshold.

Selected *M. disstria* hemocyte cell lines were characterized in terms of the optimum growth temperature, morphology and basal extracellular enzyme profiles and protein release during their interaction with selected antigens. Dead *B. subtilis* in PBS affected secreted enzymes and total protein

discharge. Md66 and Md108 cell lines in PBS without or with *B. subtilis* did not discharge detectable amounts of lysozyme and phenoloxidase.

RÉSUMÉ

La livrée des forêts, *Malacosoma disstria*, un insecte qui est une peste majeure des forêts de l'Amérique du Nord, n'a pas été examinée de façon approfondie dans les réponses de ses cellules du sang (hémocytes) face aux antigènes bactériens. Dans cette étude, les facteurs physico-chimiques et la signalisation enzymatique affectant l'adhésion sur lamelle des hémocytes de la larve du cinquième stade *M. disstria* ont été étudiés.

L' adhésion maximale des cellules sanguines, (plus précisément) des cellules granulaires et des plasmocytes a lieu respectivement à un pH 6.0-7.0 et 6.0 dans une solution phosphate saline (PBS). L'activation des protéines kinases A et C entraîne une inhibition de l'adhésion des cellules sanguines.

L'utilisation de l' inactif entomopathogène *Xenorhabdus nematophila* bactérie Gram-négative et du *Bacillus subtilis,* une bactérie Gram-positive non-pathogène montre une liaison distincte aux différentes cellules sanguines à laquelle on observe une modification du patron des activités de la phénoloxydase et de la lysozyme.

La protéine plasmatique, apolipoprotéine-III, restrinct la réponse immunitaire des hémocytes. Cependant, la liaison initiale de la protéine aux *X.nematophila* et *B. subtilis* augmente les niveaux des cellules granulaires et diminue les niveaux des plasmocytes en présence des bactéries. *In vivo*, la protéine suspend le déplacement bactérien de l'hémolymphe.

Les antigènes de surface de *X. nematophila* et *B. subtilis* participent aux réactions des hémocytes. L'endotoxine *X.nematophila* et sa fraction lipide A altèrent les niveaux des hémocytes limitant le déplacement de *X. nematophila* de l'hémolymphe. L'endotoxine et la fraction lipid A inhibent l'activation de la phénoloxydase mais induisent l'activité de l'enzyme.

L'acide lipotéichoïque de *B. subtilis* entraîne la formation de nodules et induit l'activation de la phénoloxydase et /ou son activité. L'apolipophorine-III neutralise les effets des trois antigènes de surface sur les

hémocytes et la prophénoloxydase jusqu'à ce que les antigènes excèdent un seuil critique.

Les lignées cellulaires d' hémocytes de *M. disstria* ont été caractérisées selon une température de croissance optimale, leur morphologie, les profils enzymatiques extracellulaires basals et les protéines relarguées pendant leur interaction avec l'antigène sélectionné. Les *B. subtilis* inactivés dans le PBS affectent les enzymes secréteés et les protéines totales relarguées. Les lignées cellulaires Md66 et Md108 dans le PBS en absence ou présence de *B. subtilis* ne libèrent pas de quantités détectables de lysozyme et phénoloxydase.

SUGGESTED SHORT TITLE

Innate immune responses of *Malacosoma disstria* hemolymph to selected antigens

CONTRIBUTIONS OF AUTHORS

The author of this Thesis has held discussions with and received guidance from Professors Gary B. Dunphy and Donald F. Niven.

Cory Brooks assisted in the experiments pertaining to *M. disstria* hemocytes and protein kinases.

Vlad Gulii conducted preliminary experiments on quantities of hemolymph samples being used in the monolayer assays with buffers.

Robert Zakarian and Cory Brooks provided support in the laboratory and assisted in the isolation and preparation of apolipophorin-III.

Jason Lapointe assisted in the determinations of total protein and phenoloxidase in the experiments described in Chapter 5.

In all other instances, the experimental designs and collection of data were carried out by the author.

Professors G.B. Dunphy, D.F. Niven and C.A. Mandato corrected and edited the manuscripts.

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ITHACA

As you set out for Ithaca
hope your road is a long one,
full of adventure, full of discovery.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbours you're seeing for the first time.

Keep Ithaca always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.

Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaca to make you rich.

Ithaca gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaca won't have fooled you.
Wise as you will have become, so full of experience,
you 'll have understood by then what these Ithakas mean.

Constantinos P. Kavafis (1863 - 1933)

This work is dedicated to my family and my friends in Greece
who I missed so much all these years.

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LIST OF ABBREVIATIONS

Abbreviations of chemicals, target molecules and pathways

ALP: Apolipophorin-III

cAMP: cyclic adenosine monophosphate

cGMP-dependent protein kinase: Cyclic guanosine monophosphate-dependent protein kinase

DSCAM: Down syndrome cell adhesion molecule

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

FBS: Fetal bovine serum

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

JNK: c-Jun N-terminal kinase

IMD pathway: Immunodeficiency pathway

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MAP Kinase: Mitogen-activated protein kinase

PKA: Protein kinase A

PKC: Protein kinase C

PBS: Phosphate-buffered saline

PIPES: Piperazine-N,N′-bis(2-ethanesulfonic acid)

PMA: Phorbol-myristate-acetate

Abbreviations of organisms

A. gambiae: Mosquito, *Anopheles gambiae* (Diptera)

A. convolvuli: Convolvulus Hawk-moth, *Agrius convolvuli* (Lepidoptera)

A. melifera: Bee, *Apis melifera* (Hymenoptera)

A. mylitta: Tasar silkworm, *Antheraea mylitta* (Lepidoptera)

A. pernyi: Chinese (Oak) Tussah Moth, *Antheraea pernyi* (Lepidoptera)

A. rapae: Cabbage White Butterfly, *Artogeia rapae* (Lepidoptera)

A. segetum: Turnip Moth, *Agrotis segetum* (Lepidoptera)

B. discoidalis: Discoid cockroach, *Blaberus discoidalis* (Blattodea)

B. glabrata: Fresh water snail, *Biomphalaria glabrata*

B. mori: Silkworm, *Bombyx mori* (Lepidoptera)

B. subtilis: Bacterium, *Bacillus subtilis*

B. thuringiensis: Bacterium, *Bacillus thuringiensis*

C. capitata: Medfly, *Ceratitis capitata* (Diptera)

C. fumiferana: Spruce budworm, *Choristoneura fumiferana* (Lepidoptera)

C. gigas: Pacific oyster, *Crassostrea gigas*

C. madrasensis: Bivalve, *Crassotrea madrasensis*

C. virginica: Eastern oyster, *Crassostrea virginica*

D. melanogaster: Fruit fly, *Drosophila melanogaster* (Diptera)

E. acreae: Salt marsh caterpillar, *Estigmene acreae* (Lepidoptera)

E. aulicae: Entomopathogenic fungi, *Entomophaga aulicae*

E. coli: Bacterium, *Escherichia coli*

E. declarata: Moth, *Euxoa declarata* (Lepidoptera)

E. foetida: Earthworm, *Eisenia foetida*

E. lindoense: Trematode, *Echinostoma lindoense*

F. gastropachae: Entomopathogenic fungus, *Furia gastropachae*

G. domesticus: Domestic chicken, *Gallus domesticus*

G. mellonella: Wax moth, *Galleria mellonella* (Lepidoptera)

G. portentosa: Madagascar hissing cockroach, *Gromphadorhina portentosa* (Blattodea)

***H. americanus**:* American lobster, *Homarus americanus*

H. armigera: Cotton Bollworm, *Helicoverpa armigera* (Lepidoptera)

***H. cecropia**:* Cecropia moth, *Hyalophora cecropia* (Lepidoptera)

***H. cunea**:* Fall webworm, *Hyphantria cunea* (Lepidoptera)

H. gloveri: Glover's Silk Moth, *Hyalophora gloveri* (Lepidoptera)

***H. virescens**:* Tobacco budworm, *Heliothis virescens* (Lepidoptera)

***K. oxytoca**:* Bacterium, *Klebsiella oxytoca* (Enterobacteriaceae)

L. dispar: Gypsy moth, *Lymantria dispar* (Lepidoptera)

***L. grandis**:* Sheep crab, *Loxorhynchus grandis*

***L. fiscellaria fiscellaria**:* Hemlock looper, *Lambdina fiscellaria fiscellaria*

***L. oleracea**:* Tomato moth, *Lecanobia oleracea* (Lepidoptera)

***L. polyphemus**: Horseshoe crab, Limulus polyphemus*

M. anisopliae: *Entomopathogenic fungi, Metarhizium anisopliae*

***M. brassicae**:* Cabbage moth, *Mamestra brassicae* (Lepidoptera)

M. disstria: Forest tent caterpillar, *Malacosoma disstria* (Lepidoptera)

***M. luteus**:* Bacterium, *Micrococcus luteus (=M. lysodeikticus)*

***M. lysodeikticus**:* Bacterium, *Micrococcus lysodeiktus (= M. luteus)*

***M. roseus**:* Bacterium, *Micrococcus roseus*

M. sexta: Tobacco hornworm, *Manduca sexta* (Lepidoptera)

N. rileyi: Entomopathogenic fungus, *Nomurea rileyi*

***N. bullata**:* Grey flesh fly, *Neobellieria bullata* (Diptera)

***P. aeruginosa**:* Bacterium, *Pseudomonas aeruginosa*

P. americana: American cockroach, *Periplaneta Americana* (Blattodea)

***P. hypochondriaca**:* Endoparasitic wasp, *Pimpla hypochondriaca* (Hymenoptera)

P. includens: Soybean looper, *Pseudoplusia includens* (Lepidoptera)

***P. interpunctella**:* Indianmeal moth, *Plodia interpunctella* (Lepidoptera)

***P. interruptus**:* California spiny lobster, *Panulirus interruptus*

P. leniusculus: Signal crayfish, *Pacifastacus leniusculus*

***P. luminescens**:* Bacterium, *Photorhabdus luminescens*

P. unipuncta: *True armyworm, Pseudaletia unipuncta* (Lepidoptera)

R. prolixus: Blood sucking bug, *Rhodnius prolixus* (Hemiptera)

***P. temperata* subsp. *temperata*:** Bacterium, *Photorhabdus temperata* subsp. *temperata*

S. aureus: Bacterium, *Staphylococcus aureus*

S. carpocapsae: Entomopathogenic nematode, *Steinernema carpocapsae*

S. exigua: Beet armyworm, *Spodoptera exigua* (Lepidoptera)

S. frugiperda: Fall armyworm, *Spodoptera frugiperda* (Lepidoptera)

S. gregaria: Desert locust, *Schistocerca gregaria* (Orthoptera)

S. invicta: Red imported fire ant, *Solenopsis invicta* (Hymenoptera)

S. mansoni: Trematode parasite, *Schistosoma mansoni*

S. postica: Eusocial bee, *Scaptotrigona postica* (Hymenoptera)

T.ni: Cabbage looper, *Trichoplusia ni* (Lepidoptera)

T. rangeli: Parasitic protozoan, *Trypanosoma rangeli*

T. tridentatus: Japanese horseshoe crab, *Tachypleus tridentatus*

X. nematophila: Bacterium, *Xenorhabdus nematophila*

CHAPTER 1

INTRODUCTION
AND
LITERATURE REVIEW

INTRODUCTION

There are few well-defined models of the hemocytic antimicrobial systems of the Lepidoptera, the salient examples being the economic pest insects *Manduca sexta* (Ling and Yu, 2006) and *Galleria mellonella* (Dunphy, 1995) and beneficial *Bombyx mori* (Lee *et al*., 2007), all of which occupy different niches and have nuance differences in their antibacterial systems (Gillespie *et al*., 1997; Lavine and Strand, 2002). *Malacosoma disstria* was chosen as a model because, unlike the previously considered insects, it is a major gregarious native pest of economically important North American deciduous trees (Fitzgerald, 1995; Furniss and Carolin, 1997). The larval stage of *M. disstria* was chosen because it does the most immediate damage to trees. Furthermore, *M. disstria* larvae have large hemolymph volume per individual and numerous circulating hemocytes. In addition, *M. disstria* larval hemocytes cell lines are also available for comparison with fresh hemolymph samples providing valuable information about hemocyte function in different microenvironments.

In this study two bacterial species were used to challenge hemocytes *in vivo* and *in vitro*: *Xenorhabdus nematophila* (Enterobacteriaceae) and *Bacillus subtilis* (Bacilliaceae). *X. nematophila* is a Gram-negative bacterium that is carried as a symbiotic microorganism in the digestive tract of entomopathogenic nematodes of the genus *Steinernema* (Akhurst and Dunphy, 1993; Cowles and Goodrich-Blair, 2008). Initiation of symbiosis between the nematode and the bacterium occurs when *X. nematophila* colonizes the bacterial receptacle in the intestinal vesicle of the infective stage of the nematode (Martens and Goodrich-Blair, 2005). *X. nematophila* in insect hemolymph interferes with host defences such as the antimicrobial melanizing system of prophenoloxidase (Da Silva *et al*., 2000) and releases lipopolysaccharide (LPS) from the outer membrane (Brillard *et al*., 2001). *Bacillus subtilis* is a Gram-positive, non-pathogenic bacterium that triggers the

immune responses in the hemolymph (Da Silva *et al.*, 2000). Lipoteichoic acid (LTA) is one of the major components of the *B. subtilis* surface (Sonenshein *et al.*, 2002) which activates lepidopteran innate humoral and cellular immunity (Beckage, 2008). Interaction of both bacterial species and their surface antigens with *M. disstria* larval hemocytes is addressed in this thesis.

Lipid transport in insects from the fat body through the hemolymph to other tissues is supported by a lipophorin shuttle (Weers and Ryan, 2006). Apolipophorin III plays a role increasing the loading of the transferred lipids on the shuttle. Apolipophorin III also plays a role in insect innate cellular (blood cells) and humoral antibacterial immunity (Halwani, 1997). Detection of apolipophorin-III in the hemolymph of *M. disstria* and its role in immunity to bacteria and their surface antigens are also examined herein.

In Chapter 1, there is an extensive description of insect hemolymph function against microorganisms with the emphasis mainly on lepidopteran species. In Chapter 2, hemocytes of *M. disstria* are examined in terms of their properties for optimum buffer composition and pH and signal transduction factors affecting adhesion to glass slides. As outlined in Chapter 3, using the optimum buffer condition for *M. disstria* hemocytes, interaction between dead bacteria *X. nematophila* and *Bacillus subtilis* and hemocytes *in vitro* was studied. Additionally, *in vivo* examination of *M. disstria* hemocyte responses to the bacterial were also assessed and compared with the *in vitro* ones. Because of the nature of the challenge e.g. dead microorganisms, it was assumed that surface antigens from the bacteria induce host immune responses; therefore the interactions with the bacterial surface antigens, LPS and LTA respectively, were studied in Chapter 4. In all previous research chapters of this work, native hemolymph samples were used which were not free of hemolymph plasma, underlying a possible effect on direct recognition of antigens by hemocytes. In Chapter 5 the use of *M. disstria* hemocyte cell lines to explore the properties of hemocytes grown in a plasma free environment is discussed. Cell type frequencies, cell blebbing, enzyme secretion, lysozyme production

and temperature-related cell adhesion and growth were parameters examined to determine to what extent the properties of hemocytes from the cell lines and from hemolymph samples resembled each other. Adherent and non-adherent cell lines were also challenged with *B. subtilis* and the effects of the challenge on the cells determined. The final section of the Thesis includes a general discussion of the research chapters and lists of the contributions to original knowledge.

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LITERATURE REVIEW

Malacosoma disstria

The forest tent caterpillar *Malacosoma disstria* (Lepidoptera: Lasiocampidae) is a major forest tree defoliator in North America with population outbreaks each 6 to 16 years , the duration of each outbreak ranging from 2 to 6 years (Batzer *et al*., 1995; Baltzer and Morris 1978; Sippel 1962). Egg masses, which overwinter in clusters of 100 – 200 eggs, are covered with the foam cement like material, spurmaline (Anderson 1960; Drooz, 1985; Hodson and Weinmann, 1945; Meeker, 1997). The first larval stage emerges from the eggs in May and continues development through five instars until mid June (Muggli and Muller, 1980). The body color of the larvae is dark gray to black, with symmetrical lateral pale blue yellow lines on both sides. Larvae have a whitish hairy appearance and their final body length size in the last instars reaches 50-65 mm (Fitzgerald, 1995). Pupation involves the formation of yellowish silken cocoon, from where brown colored adults will emerge (Meeker 1997). The life cycle, which takes 12 months, is synchronized with the appearance of new vegetation on the tree-hosts (Krauss, 2006).

Sugar maple (*Acer saccharum*), birch (*Betula sp*.), oak (*Quercus laevis*), and trembling aspen (*Populus tremuloides*) leaves are the preferred feeding sources and pupation sites (Panzuto *et al*., 2001). Lorenzetti (1993) observed plant host preferences for *M. disstria* based on chemical composition of the leaves. Meals of sun leaf extracts are preferred as food source compared to shade leaf extracts, the former causing faster growth rates (Despland and Noseworthy, 2006). *M. disstria* larvae express social behavior in terms of searching for food and thermoregulation. For the former parameter, silk trails and pheromone trails in conjunction with chemosensors in their maxillary palpi contribute to this behavior (Colasurdo, 2006; Fitzgerald, 1995; Roessingh *et al*., 1988).

Host trees respond to such herbivory with the production of the toxic substrates (Barbehenn and Martin, 1994). Despite the tannins and other phenolic compounds of the leaves, the digestive tract of *M. disstria* larvae is able to avoid subsequent formation of harmful peroxides and maintain its immunity *(*Barbehenn and Martin, 1994; Barbehenn *et al.*, 2005).

Because of the major economic importance of *M. disstria* many pest control strategies have been considered including DDT (Fitzgerald, 1995), parasitoid release (Eggen, 1987), pheromones (Palaniswamy *et al.*, 1983), and treatment with microbial insecticides including the bacterium *Bacillus thuringiensis* (Krywienczyk and Angus, 1969), nucleopolyhedroviruses (Broome *et al.*, 1974), and the entomopathogenic fungus *Erynia crustosa* (MacLeod and Tyrrell, 1979; Perry and Fleming, 1989). Many of the control agents interact with the hemolymph which is capable of responding to foreign materials (see section: Insect immunity). The interaction of selected immune factors with designated antigens that are either part of or unrelated to microbial insecticides are part of the Thesis. The antigens included glass slides, the entomopathogenic bacterium, *Xenorhabdus nematophila*, the non-pathogenic bacterium, *Bacillus subtilis,* and the bacterial surface components, i.e. *X. nematophila* lipopolysaccharide (LPS) and *B. subtilis* lipoteichoic acid (LTA).

Xenorhabdus nematophila

Xenorhabdus nematophila (Enterobacteriaceae) is a Gram-negative, facultatively aerobic, non-sporulating bacterium. It is carried within a vescicle in the digestive tract of entomopathogenic nematodes of many species of *Steinernema* (Martens and Goodrich-Blair, 2005) and released into the hemolymph within 2-4 hours post infection time (Gaugler and Kaya, 1990). The nematode bacterial complex, used in pest control, effectively kills the target insect by releasing *X. nematophila* in the hemolymph (Morton and Del Pino, 2008). The injected bacteria alone in laboratory assays (Mahar *et al.*,

2004) are extremely lethal to most insect species with host death occuring from bacterial toxaemia (Boemare, 2002; Forst *et al.*, 1997). Akhurst and Boemare (1990) suggested that 100 bacterial cells are able to provoke larval death in Lepidoptera. The doubling time of bacteria growth in insect blood (hemolymph) varies from 2.5 to 3 hours (Nealson *et al.*, 1990).

Phenotypic variation has been observed between bacterial cells isolated from nematodes and those constantly subcultured in the laboratory; these variants are referred to as phase I and II, respectively (Boemare, 2002). Highly virulent phase I is characterized by the ability of the pertrichously flagellate motile bacterium (Boemare and Akhurst 1988; Givaudan *et al.*, 1995) to produce essential nutrients for the nematodes, non toxic intracellular inclusions (Couche and Gregson, 1987; Forst and Clarke, 2002; Goetsch *et al.*, 2006; Park *et al.*, 2006a), proteases (Smigielski *et al.*, 1994) and antibiotics (Chen *et al.*, 1996; Gaugler and Kaya, 1990; Ji *et al.*, 2004; Li *et al.*, 1997; Li *et al.*, 1998; Maxwell *et al.*, 1994; McInerney *et al.*, 1991; Ribero *et al.*, 2003; Thaler *et al.*, 1995; Xu, 1998) for the survival of the latter (Akhurst and Boemare, 1990). Phase II, which is lethal also for insects, lacks or has less of phase I attributes (Akhurst, 1982; Boemare, 2002; Forst and Clarke, 2002). Despite these differences, virulence of some of *X. nematophila* strains appears to be unaffected by the phase (Akhurst, 1980; Dunphy and Webster, 1984); however other strains express lower levels of virulence in phase II (Jarosz *et al.*, 1991; Voglyi *et al.*, 1998).

Because phase I is the form usually released from the infective nematode into the hemolymph (Voglyi *et al.*, 1998) subsequent reviewed material will deal with this phase only. *X. nematophila* releases enzymes such as proteases (with suppression ability on antibacterial cecropin A), insecticidal chitinases and lecithinase and other lipases which have no entomotoxic effects in lepidopteran and orthopteran hosts (Bowen *et al.*, 2003; Caldas *et al.*, 2002; Chen *et al.*, 1996; Duchaud *et al.*, 2003; Morgan *et al.*, 2001; Park and Forst, 2006; Thaler *et al.*, 1998).

Fifty per cent of the surface area of the outer membrane of phase I *X. nematophila* is high in porins, proteins that permit the transport of small molecules (e.g. toxins, antibiotics) across the membrane (Forst *et al.*, 1995; Forst *et al.*, 1997). A variety of additional outer membrane proteins of unknown function are also produced during the stationary phase (e.g. OpnB) (Forst and Clarke, 2002; Volgyi *et al.*, 1998). The induction of outer membrane proteins, their size and their homology with those of *E. coli* are extensively discussed (Forst *et al.*, 1995; Forst and Nealson, 1996; Forst *et al.*, 1997; Forst and Tabatabai, 1997; Kim and Forst, 2005; Leisman *et al.*, 1995; Park and Forst, 2006; Tabatabai and Forst, 1995).

X. nematophila lipopolysaccharide (LPS), found on the outer membrane of the organism, is released into growth media by secretion of outer membrane blebs (Forst *et al.*, 1997; Khandelwal and Banerjee-Bhatnagar, 2003). After entering the host hemolymph, *X. nematophila* releases LPS, a major virulence factor of the bacterium, preventing phenoloxidase activation and provoking hemocyte lysis (Dunphy and Webster, 1988a,b). The toxic moiety of *X. nematophila* LPS for lepidopteran hemocytes is lipid A which can be detoxified with polymyxin B (Dunphy and Webster, 1998a). Both the live and dead, phase I form of *X. nematophila* limit also phenoloxidase activation and hemocyte activities by releasing lipopolysaccharide (Dunphy and Webster, 1988a) which chelate divalent cations; Ca^{+2} in *Agrotis segetum* (Yokoo *et al.*, 1992; Yokoo *et al.*, 1995) and Fe^{+2} in *G. mellonella* (Dunphy *et al.*, 2002). Lipid A, binding to N-acetyl-D-glucosamine hemocyte receptors, damages *G. mellonella* hemocytes releasing entrapped bacteria (Dunphy and Webster, 1988a; Dunphy and Webster, 1988b) and iron-binding proteins; the latter, in concert with iron-chelation by the endotoxin, potentially limits bacterial growth (Dunphy *et al.*, 2002). Fimbriae of *X. nematophila* bacteria damage hemocytes; the fimbrial shaft protein, MrxA, makes pores in hemocytes triggering dose dependent lysis of *Helicoverpa armigera* hemocytes (Banerjee *et al.*, 2006; Forst and Nealson, 1996; Moureaux *et al.*, 1995).

The cellular and humoral immune responses of the lepidopteran species are also suppressed by other mechanisms (Park *et al.*, 2006b). Live X. *nematophila* inhibits eisocanoid biosynthesis by inhibiting phospholipase A_2 (Park *et al.*, 2003; Park and Stanley, 2006) within 1h of infection and subsequently blocks nodulation and phenoloxidase activation (Kim *et al.*, 2005; Park and Kim, 2000; Park *et al.*, 2005a; Park and Stanley, 2006; Stanley, 2006).

X. nematophila protease II, which is capable of digesting cecropin antibacterial proteins in *G. mellonella* and *Pseudaletia unipuncta*, does not harm hemocytes (Caldas *et al.*, 2002), although the bacteria induce septicemia in *B. mori* through hemocytic apoptosis (Cho and Kim, 2004). Homoserine lactone has been implicated in aspects of the later stages of pathogenesis (Dunphy *et al.*, 1997). Bacterial tolerance of host defences is part of the early stages of pathogenesis (Dunphy *et al.*, 1998).

Thoughout this Thesis, dead *X. nematophila* were used. This was necessary to preclude aspects of metabolism (Alavo and Dunphy, 2004; Park and Kim, 2000) influencing results allowing the direct observation of the interaction of the insect hemocytes with bacterial surfaces and their antigens.

Bacillus subtilis

Bacillus subtilis (Bacillaceae) is a rod-shaped, aerobic, Gram-positive, endospore-forming bacterium found in soil (Morohashi *et al.*, 2007; Sonenshein *et al.*, 2002). Under unfavorable growth conditions, *B. subtilis* switches on survival modification mechanisms including chemotaxis, motility, DNA exchange (Sonenshein *et al.*, 2002) and finally sporulation (Wipat and Hardwood, 1999).

In addition to its use in biotechnology and industry (Olempska-Beer *et al.*, 2006), *B. subtilis* has been used in a variety of immunological studies. Such studies have involved for example, inoculation of human leukaemia cell lines with the organism for the induction of reactive oxygen

species (Timm *et al.*, 2006), inoculation of commercial fish feed inducing cellular innate responses (Salina *et al.*, 2005) and challenge of hemolymph *in vivo* or/and *in vitro* of major insect orders, including Lepidoptera, Hymenoptera (Dani *et al.*, 2003), Orthoptera (Da Silva, 2002) and Coleoptera (Sagisaka *et al.*, 2001) for antimicrobial responses (Marin *et al.*, 2005).

Although *B. subtilis* is generally not considered an insect pathogenic bacterium (Dunphy *et al.*, 2002), cyclic lipopeptides produced by *B. subtilis* exhibit mosquitocidal activity due to their surfactant nature (Das and Mukherjee, 2006). *B. subtilis* LTA comprises 1-2% by weight of the vegetative cell wall and demonstrates a negative charge (Foster and Popham, 2002; Kunst *et al.*, 1997; May *et al.*, 2005). *In vivo, B. subtilis* LTA in *G. mellonella* hemolymph activates innate immune responses such as nodulation, depletion of plasmatocyte levels, discharge of granular cells and activation of phenoloxidase cascade activation (Halwani *et al.*, 2000).

Thoughout this Thesis, dead *B. subtilis* was used for the same reasons as cited for *X. nematophila*.

Insect hemolymph

Insect blood (hemolymph) 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 hemolymph initiates wound repairs and limits the entry of microorganisms into the hemocoel (Haine *et al.*, 2007). The insect hemolymph 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 hemolymph has been reported for *Manduca sexta* and *Bombyx mori* (Furusawa *et al.*, 2008; Li *et al.*, 2006b). Herein

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.

Insect hemolymph 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 hemolymph 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 van der 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.

Larval hemocytes in lepidopteran species

Lepidoptera larval hemocytes are classified based on their function, morphology (Price and Ratcliffe, 1974; Lea and Gilbert, 1966), 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. Their role in immunity, depending on insect species and stage, is to recognize the presence of foreign objects within the hemolymph, 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 hemolymph begins when adhesion molecules such as integrins, cover the outer membrane of hemocytes (Nardi *et al.*, 2003). Here, emphasis will be placed on lepidopteran hemocytes with reference to those of other insect orders whenever relevant.

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 hemolymph lepidopteran prohemocytes continue to produce prohemocytes through cell division (Yamashita and Iwabuchi, 2001).

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; Davies and Preston, 1985). 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). The antigenic properties of plasmatocytes and their ploidy levels can be used as criteria to distinguish this cell type from granular cells (Nardi *et al.*, 2003). 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 hemolymph; their contribution to the total hemocyte count is low (~1%) (Dean *et al.*, 2004a,b).

Granular cells

Granular cells comprise approximately 30-50% of the total hemocyte counts (Falleiros *et al.*, 2003). Cell shape is spherical (diameter 7-9μm) 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 Iwabichi, 2001; Ling *et al.*, 2005a). Granular cells are involved in nodulation (see section: Nodulation, page 42) and encapsulation (see section: Encapsulation, page 40) of foreign objects within the hemolymph, 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).

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; Scott, 1972). Spherulocytes transport cuticular material (Lavine and Strand, 2002; Sass *et al.*, 1994). *In vivo*, population levels of spherulocytes are affected by the presence of parasitoids, the hemocyte types differing in numbers depending on the lepidopteran host species and the parasitoid species (Stettler *et al.*, 1998). 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).

Oenocytoids

Oenocytoids are spherical cells (diameter 20-35μm) and represent 1-10% of the total hemocyte population (Butt and Shields, 1996; Jones, 1962; Neuwirth 1973). Mitochondria, polyribosomes and an eccentric nucleus are the main parts of the subcellular anatomy (Hu *et al.*, 2003). These cells can also be identified with non-plasmatocyte-crossreacting, 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).

Insect hemocytes in non lepidopteran species

Comparative elements between lepidopteran and non lepidopteran hemocytes are always useful to define evolutionary patterns of immune responses among different insect orders. Hemocyte types have been studied extensively in Diptera with emphasis on *Drosophila melanogaster* (Williams, 2007) and numerous mosquito species (Araujo *et al.*, 2008; Brayner, *et al.*, 2005). In *Drosophila*, total hemocyte counts are extremely low compared to Lepidopteran species, the larvae containing the major cell types, the prohemocytes, plasmatocytes, crystal cells and lamellocytes (Rizki and Rizki, 1980). Plasmatocytes and lamellocytes are capable of engulfing foreign objects; crystal cells release melanizing factors into the hemolymph in response to the presence of microbial factors (Williams, 2007). In this, crystal cells are similar to the oenocytoids of lepidopteran species. Prohemocytes transform into the major dipteran cell types (Lanot *et al.*, 2001); however at high hemopoetic activity or in the presence of foreign material, the plasmatocytes differentiate into lamellocytes (Qiu *et al.*, 1998; Rizki and Rizki, 1992). The genetics of *D. melanogaster* hemocyte development, unlike those for lepidopterans, have been described (Wood and Jacinto, 2007). Lepidopteran and dipteran hemocytes show substantial morphological and structural differences; nevertheless, their innate (Zheng *et al.*, 2005) and adaptive (Crayton *et al.*, 2006; Dong *et al.*, 2006) immunological functions seem to be similar (Willot *et al.*, 1995). Mosquitoes differ slightly in composition of their hemocyte types compared with other diptera. Apart of prohemocytes, plasmatocytes, granular cells, spherulocytes and oenocytoids, which are similar to the lepidopteran hemocytes, they possess adipohemocytes

(hemocytes with large lipid vesicles; seen, e.g. in *Culex* and *Aedes* species) (Brayner *et al.*, 2005) and thrombocytoids (*Aedes* species only) (Araujo *et al.*, 2008). Adipohemocytes and thrombocytoids do not adhere to glass or contain phenoloxidase; their function needs to be explored futher (Hillyer *et al.*, 2003).

In the order Blattodea the cockroach, *Periplaneta americana*, the hemocytes are classified as motile (plasmatocytes) and non-motile cells (granular cells) (Chain *et al.*, 1992; Tackle and Lackie, 1986). In Coleoptera, most hemocyte types are similar to those in Lepidoptera, with the exception of the fragile hemocyte type, the coagulocyte (Giulianini *et al.*, 2003). Coagulocytes, with their numerous pinocytic vesicles in the outer membrane, contribute to micropinocytosis of small foreign objects (Brehelin *et al.*, 1978; Giulianini *et al.*, 2003).

Insect immunity

Insect immunity against microbial invaders consists of a broad spectrum of insect-host defenses that range from innate immunity (Gillespie *et al.*, 1997) to induced immunity (Gillespie *et al.*, 1997; Kaya and Tanada, 1993; Kurtz and Armitrage 2006). Immune reactions occur against microorganisms (Beckage, 2008), parasites (Infanger *et al.*, 2004), parasitoids (Wertheim *et al.*, 2005), particles/surfaces such as Sephadex beads and glass (Lavine and Strand, 2001) and during the wound healing process (Haine *et al.*, 2007).

Innate immunity refers to the short term nonspecific responses of the hemolymph tissue to invaders (Hoffmann, 1995). The response is the culmination 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 immunologlobulin 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).

Innate immunity within insect hemolymph

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 (Boguś *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 (Lackie, 1986; Lavine and Strand, 2001). Hemocytes of *Schistocerca gregaria* do not adhere to negatively charged modified agarose beads whereas those of *Periplaneta americana* do so avidly, possibly reflecting the repulsion of high negatively charged *S. gregaria* hemocytes and lesser repulsion of the less negatively charged cockroach hemocytes (Lackie 1983, 1986). However the hemocytic removal of the trypanosome, *Trypanosoma rangeli,* from these insect species argues that charged host hemocyte surface interaction with the parasite cannot account totally for host reaction (Tackle and Lackie, 1987). Similarly, for the hyphomycete, *Nomurea rileyi,* negative charge of hydrophobic germ tubes, conidia or mycelial cells did not affect the binding of these fungal stages to *Spodoptera exigua* hemocytes (Pendland and Boucias, 1991). The complexity of surface charge and antigens is further shown by Wang and Leger (2006) who described the *Mcl1* gene product, a collagenous protein, on *M. anisopliae* hyphal bodies that mask antigenic cell wall components, which, in conjunction with the negative charge of hyphal bodies limits *M. sexta* hemocyte involvement. However, negatively charged repulsion affects hemocytes of *Choristoneura fumiferana* (Dunphy and Nolan, 1982) and *Lambdina fiscellaria fiscellaria (*Dunphy and Nolan, 1980) reacting with the entomopathogenic fungus *Entomophaga aulicae*.

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 wettability of polystyrene plates and beads increase adhesion by *P. americana* hemocytes than did *S. gregaria* (Lackie, 1983; 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;

Wittwer and Wiesner, 1996), selectins (Okazaki *et al.*, 2006), collagen (Yasothornsrikul *et al.*, 1997) and immunoglobulin-like containing molecules (Watson *et al.*, 2005).

In the present work, negatively charged polystyrene (Kennedy O' Gara, 2004) and *B. subtilis* were studied as antigens reacting with *M. disstria* hemocyte cell lines.

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 molcules include lectins in *Blaberus discoidalis* (Wilson *et al.*, 1999). Lectins are carbohydrate binding recognition molecules in the hemolymph (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 α2-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 (Aye *et al.*, 2008; 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; Nardi *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). Physicochemical analysis of integrins (Hynes, 2002) and fibronectins show a positive electrostatic charge on various human cell types (Sharma *et al.,* 1999), mollusc hemocytes (Jones *et al.*, 1976; Pierres *et al.,* 2002) and muscle cells of insects (Devenport *et al.*, 2007). Real time PCR transcriptional analysis of α and β subunits of integrins of *Pseudoplusia includens* plasmatocytes and granulocytes show an increase both α- and β- mRNA transcripts during encapsulation (Pech and Strand, 1996; Lavine and Strand, 2003). Moreover, the transmembrane protein Eater with epidermal growth factor repeats, mediates phagocytosis of *E. coli* and *S. aureus* without altering Toll and Imd functions in *Drosophila* hemocytes (Kocks *et al.*, 2005). Other plasmatocyte proteins affecting bacterial phagocytosis in *D. melanogaster* include the single-pass transmembrane protein with EGF-like repeats Nimrod C1, which functions much like Eater (Kurucz *et al.*, 2007). Indirect recognition receptors include Toll and Toll-like (TLR) trasmembrane receptors (Ao *et al.,* 2008a) which are activated by soluble and cellular peptidoglycan binding proteins once the proteins have reacted with bacteria peptidoglycan. Serine proteases activated by pattern recognition receptors binding to antigens elicit proteolytic activation of the cytokine-like transducer Spätzle species which activates Toll in Diptera and Lepidoptera (Wang *et al.*, 2007). 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 hemolymph, are able to activate both Toll and Imd pathways (Dziarski and Gupta, 2006). Ions such as Ca^{+2} (Tojo *et al.*, 2000) and, Fe^{+2} increase the removal of *X. nematophila* and *B. subtilis* in *G. mellonella* (Dunphy *et al.,* 2002) and Zn^{+2} enhance *M. sexta* plasmatocytes spreading (Willot and Tran, 2002). These cations possibly play a significant role in phagocytic non-self responses. Part of foreign surface engulfment process may include the following signalling mechanisms: activation of mitogen-activated protein (MAP) kinases such as c-jun N-terminal (JNK) kinase and p38 mitogen-activated protein which activate phenoloxidase in *C. capitata* against *E. coli* (Lamprou *et al.*, 2005) and, recognition by the soluble form of Down Syndrome Cell Adhesion Molecule with subsquent increase in engulfment of foreign objects (Dong *et al.* 2006). Rho-GTPase promotes actin stabilization beneath the cell membrane and subsequent engulfment of foreign objects by dipteran, and lepidopteran hemocytes (Castillo *et al.*, 2006; Richards and Edwards, 2002; Williams *et al.*, 2006). In contrast, lower enzyme activity of protein kinases A and C result in effective phagocytic activity (Brooks and Dunphy, 2005; Zakarian *et al.*, 2003).

Morphological analysis of phagocytosis of *S. aureus* and *E. coli* by *D. melanogaster* hemocytes revealed similarities to mammalian macrophages (Pearson *et al.*, 2003). Zippering type of extension of pseudopodia of *D. melanogaster* hemocytes and macropinocytic engulfments have been observed around both bacteria (Pearson *et al.*, 2003).

Encapsulation

Encapsulation is divided into two main categories: a) humoral encapsulation which results in a capsule of melanin around foreign objects in the hemolymph 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; Peters and Ehlers, 1997), fungi in *G. mellonella* (Dunphy *et al.*, 2003), and chromatography beads in various lepidopteran host species (Hernadez-Martinez *et al.*, 2002; Lavine and Strand, 2002), 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; Pech *et al.*, 1994). 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,* 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). Superoxide production in lepidopteran hemocytes exposes the microbial invader to reactive oxygen species stress (Bergin *et al.*, 2005) while the phenoloxidase pathway deposits melanin around the microbe (Gillespie *et al.*, 1997). The size of the cellular capsule around the foreign object in the hemolymph is regulated by apoptosis of granular cells at the periphery of the capsule induced by plasmatocytes (Pech and Strand, 2000).

Anti-integrin monoclonal MS13 and MS34 antibodies binds to *M. sexta* hemocytes preventing encapsulation of DEAE–Sephadex beads thus integrins are involved in the adhesion process to the foreign object (Levin *et al.*, 2005). Lepidopteran hemocytes contribute also to encapsulation of foreign surfaces through the RGD binding motifs on integrins (Lavine and Strand, 2002; Zhuang *et al.*, 2008). Moreover, tetraspanins, common to mammalian cell membranes and implicated in cell adhesion, were detected in *M. sexta*

hemocytes interacting with integrins during encapsulation (Zhuang *et al.*, 2007).

Lepidopteran hemocyte responses are mediated by the cytoskeleton (Li *et al.*, 2009). Because integrins affect cytoskeletal Rac small GTPases by signalling (Bokel and Brown, 2002) and Rac1 and 2 participate in insect hemocyte activities (Willams *et al.*, 2005, 2006), integrins besides physically binding to extracellular matrix proteins (Bokel and Brown, 2002), may regulate lepidopteran hemocytic encapsulation.

Nodulation

Nodulation is a cellular response of the hemolymph 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 hemolymph (Howard et al., 1997; 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; Howard *et al.*, 1997). Similarly, host prostaglandins, epoxyeicosatrienoic acids, and lipoxygenase metabolites enhance nodule formation in lepidopteran and non lepidopteran species (Durmus *et al.*, 2008; Stanley, 2006). Phospholipase A_2 and cyclooxygenase inhibitors decrease the ability of hemocytes to spread and nodulate foreign particles (Kwon *et al.*, 2007; Mandato *et al.*, 1997). Rescuing these inhibited hemocytes with arachidonic acid, restores nodulation and prophenoloxidase activity (Mandato, 1998). Secretory phospholipase A2 from *M. sexta* hemocytes triggers nodulation events (Park *et al.*, 2005a). The entomopathogenic bacterium *X. nematophila* in contrast to *B. subtilis,* inhibits eicosanoid biosynthesis by an unknown mechanism (Park *et al.*, 2004).

Humoral immunity

Humoral immunity in lepidopteran insect hemolymph 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; Lamprou *et al.*, 2005). The following sections pertain to humoral proteins examined within this Thesis.

Apolipophorin-III

The existence of apolipophorin-III (ALP) within the hemolymph was reported for at least 19 different insect species (Weers and Ryan 2006). Outside of hemolymph, ALP is to be found also in the antennae of male fire ants *Solenopsis invicta* (Guntur *et al.*, 2004) and in the genital tract (ovaries and testes) of fall webworm *Hyphantria cunea* (Yun and Kim, 1996).

ALP is an exchangable plasma apolipoprotein in the hemolymph of insects (Halwani, 1999) that is both taken up from and released into the plasma (Ferkovich *et al.*, 1995) and constitutively produced by hemocytes with partial storage in hemocyte granules and fat body (Halwani 1999; Kim *et al.*, 2004). 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). The stereochemical structure of the protein between the two forms (e.g.

lipid free and lipid carrying) significantly changes and is reversible (Van der Horst and Ryan, 2005). The structural model of the interaction between apolipophorin–III and lipid involves conformational transitions which are mediated by availability of suitable lipophilic surfaces (Brieter *et al.*, 1991). The concentration of free ALP molecules within insect hemolymph is affected by numerous parameters such as host age, levels of adipokinetic hormone and contamination by LPS and other lipophilic compounds (Mullen and Goldsworthy, 2003). Separate from the protein's function in lipid trasnport and energy supply (Soulages *et al.*, 1996), 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 (Dunphy and Halwani, 1997; 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 β-glucans (Brown and Gordon, 2005; Dunphy *et al.*, 2003; Whitten *et al.*, 2004) providing host protection against a large spectrum of pathogens.

ALP, after binding to both *G. mellonella* hemocytes and bacteria, exhibits temporal dissociation affecting granular cells functions and hence the protein acts as a counter adhesion molecule (Schmidt and Schreiber, 2006; Zakarian *et al.*, 2002). Contributing in cellular responses, ALP enhances attachment and phagocytosis of microbial invaders in the hemolymph (Weisner *et al.,* 1997; Zakarian *et al.*, 2003). Hydrophobic ALP potentiates also lysozyme activity and induced-cecropin activity against bacteria (Halwani and Dunphy, 1999; Park *et al.*, 2005b; Wiesner *et al.*, 1997). Although regarded as a constitutive plasma protein, vaccination of *H. virescens* larvae and pupae (Chung and Ourth, 2002), and larvae of *H. cunea* (Kang *et al.*, 2004) and *G.*

mellonella (Andrejko *et al.*, 2005) elevates ALP, activating or enhancing cell-free immunity by lysozyme and cecropin production (Kim *et al.*, 2004). Despite the increase in concentration in hemolymph with bacterial antigens (Freitak *et al.*, 2007), ALP can be a target of microbial enzymes being degraded by *Pseudomonas aeruginosa* serine proteases during infection of *G. mellonella* (Andrejko *et al.*, 2005).

Phenoloxidase System

Phenoloxidases are a group of copper containing enzymes (Lu and Jang, 2007) responsible for the production of antimicrobial melanin and free radicals (Shelby and Popham, 2006) 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). 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 hemolymph, 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 (Ling *et al.*, 2005b; 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 (Gupta *et al.*, 2005; Ratcliffe, 1993; 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, β-1,3-glucan, peptidoglycan and

lipoteichoic acids in lepidopteran hemolymph increase phenoloxidase activity (Dunphy and Webster, 1988; Jiang *et al.*, 2004; Kanost *et al.*, 2004). Phenoloxidase increases hemocyte attachment to foreign objects, enhancing encapsulation and/or nodulation (Eleftherianos *et al.*, 2008). In contrast, suppresion of phenoloxidase activity within the hemolymph 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 hemolymph of *G. mellonella* increase phenoloxidase activity against *B. subtilis* (Dunphy *et al.*, 2007). Cases of lepidopteran larvae with increased phenoloxidase in hemolymph samples, in absence of microbial antigen or parasitoid, have been attributed to enviromental 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 downregulation which occurs in *C. fumiferana* infected with polydnaviruses (Doucet *et al*., 2008).

Lysozyme

Lysozyme and lysozyme like proteins are major antibacterial proteins. Descriptions of their properties, functions and host members are given in Table 1.1.

Table 1.1: Major antibacterial proteins in lepidopteran hemolymph

Type	Description	References
Attacins	***Properties*:** Attacins (20kDa) in the hemolymph, are peptides secreted from the fat body. ***Function:*** Proteolytic digestion of attacin E can produces attacin F. Gene analysis of attacins in *Hyalophora cecropia* shows that the protein carries an NF-kB 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.	Andra *et al.*, 2001; Boman *et al.*, 1985; Bulet and Stocklin, 2005; Cociancich *et al.*, 1994; Ekengren and

Cecropins (continued)	***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 from *Bombyx mori, Agrius convolvuli; Hinnavin II* from *Artogeia rapae.*	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 report using Gram-positive microbes in *Mamestra brassicae* cell lines. ***Lepidopteran sources:*** Spodoptericin from *Spodoptera frugiperda;* Gallerimycin from *G.mellonella* and *S. frugiperda*; Heliomycin from *Heliothis virescens.*	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; Volkoff *et al.*, 2003; Yamauchi, 2001

Gloverin	***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. ***Lepidopteran sources:*** Gloverin from *H. gloveri*, *Helicoverpa armigera* and *T. ni and* gloverins 1-4 from *B.mori.*	Axén *et al.*, 1997; Boman *et al.*, 1991; Lundström *et al.*, 2002; Mackintosh *et al.*, 1998; Mrinal and Nagaraju, 2008
Lebocin	***Properties and Function:*** Proline-rich peptides with 32 amino acid residues express antibacterial activity against non pathogenic *E. coli* in *B. mori* and *B. thuringiensis* upregulates lebocin synthesis in Mexican strains of *Trichoplusia ni.* Rel protein regulates the expression of lebocin in *B. mori.* Its main source of synthesis is the fat body. ***Lepidopteran sources:*** Lebocins 1 – 5 from *B. mori,* lebocin from *T.ni.*	Eleftherianos *et al.*, 2006; Hara and Yamakawa, 1995; Tamez-Guerra *et al.*, 2008; Tanaka *et al.*, 2005

Lysozymes and lysozyme like proteins	***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 *M. sexta* did not reverse responses to *E. coli*. There are lysozyme-like proteins which lack muramidase activity but are active against bacteria and upregulated during infection in *B. mori*. ***Lepidopteran sources:*** Various species**.**	Bae and Kim, 2003; Bogus *et al.*, 2007; Chang and Deisenhofer, 2007; Dziarski, 2003; Eleftherianos *et al.*, 2006; Gandhe *et al.*, 2007a; Gliński and Jarosz, 1996; Li and Paskewitz, 2006; Li *et al.*, 2005; Royet and Dziarski, 2007; Yu *et al.*, 2002

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

Signal transduction enzymes

Enzymes implicated in the transfer of the non-self message from the outer hemocyte cell membrane towards the nucleus and/or the initiation and regulation of cellular non-self responses are described as signal transduction enzymes (Krauss, 2003; Beckage, 2008). Cell signalling is propagated by hemocytic plasma enzymes resulting in subcellular functional modifications. [For example, BAEEase an enzyme which is able to hydrolyze the synthetic substrate N-benzoyl-L-arginine ethyl ester, isolated from silkworm *B. mori* hemolymph activates *Spatzle*/Toll mechanism (Jang *et al.*, 2006)]. Catalytic activity of phospholipase A_2 increases the synthesis of eicosanoids and enhances phagocytic ability of *S. exigua* larval hemocytes (Shrestha and Kim, 2007). Because the Thesis deals extensively with hemocyte non-self adhesion responses, emphasis in this section will be on signalling factors affecting hemocyte adhesion.

Signal enzymes involved in hemocyte adhesion reactions (e.g. internalization of foreign objects) consist of several kinase superfamilies members of which (e.g. protein kinase A and C), through phosphorylation, transfer the message from the inner cell surfaces (Brooks and Dunphy, 2005). Mitogen activated kinases (MAP Kinases) and extracellular signal-regulated kinases (ER kinases) are required for phagocytosis of bacteria by *C. capitata* and *M. sexta* hemocytes (Lamprou *et al.*, 2005; de Winter *et al.*, 2007). Lamprou *et al.*, (2005) state that c-Jun N-terminal kinase is activated while *C. capitata* hemocytes attempt to engulf latex beads and bacteria. Surface lipopolysaccharide from *E. coli* re-shape medfly hemocytes during LPS endocytosis via Rho family GTPases (Soldatos *et al.*, 2003).

Herein, due to the significance on hemocyte phagocytic responses (Lamprou *et al.*, 2005) and cellular adhesion, protein kinase A and C will be examined for their function in *M. disstria* larval hemocytes from fresh hemolymph samples while adhering to glass surfaces.

Protein Kinase C

The protein kinase C (PKC) family is comprised of numerous isotypes which regulate signal transduction mechanisms within insect cells via phosphorylation of target proteins (Mellor and Parker, 1998). Structurally, PKC (~80kDa) has two major domains, the catalytic (~ 45 kDa) and regulatory (~35kDa) domains (Shieh *et al.*, 2002; Dries, 2007). The catalytic domain consists of an ATP binding region and the substrate binding region (Corbalan-Garcia and Gomez-Fernandez, 2006). The regulatory domain is divided into three major parts: the pseudosubstrate region (it keeps the enzyme inactive) (Hofmann, 1997), the diacylglycerol (DAG) binding region and the Ca^{+2} binding region. Activation occurs with DAG binding and, an increase intracellular Ca^{+2} or phosphatidylinositol trisphosphate (PIP_3) which will subsequently increase cytosol calcium by release from membrane systems (Sando *et al.*, 1998). Based on the primary structure, PKCs are classified into three groups a) *classical or conventional PKC isoforms* activated by DAG and intracellular Ca^{+2}, b) novel PKC isoforms where only DAG activates the enzyme and c) *atypical PKC* isoforms in which PIP_3 plays the role of activator (Corbalan-Garcia and Gomez-Fernandez, 2006).

In insects, PKC isotypes are involved in cell proliferation and tissue differentiation (Hutterer *et al.*, 2004; Cox *et al.*, 2001), apoptosis (Malagoli *et al.*, 2002), secretion (Tobe *et al.*, 2005) and immunity (Avila et al., 2002). In terms of insect immunity, PKC regulates the NF-κB transcriptional factor (Silverman and Maniatis, 2001), involved in antibacterial protein induction (Lanz-Mendoza *et al.*, 1996), and affects also immediate innate hemocyte adhesion (Zakarian et al 2003).

Protein kinase A

Protein kinase A (PKA) consists from two catalytic and two regulatory subunits (Muller, 1999). Phosphorylation of the catalytic domains

turns the PKA complex into the active state (Kim *et al.*, 2006). Cyclic adenosine monophosphate (cAMP) within the insect cells activates the enzyme (Yoshihara *et al.*, 2000). PKA influences insect neurological functions (Armstrong *et al.,* 2006, Diegelmann *et al.*, 2006, Folkers *et al.*, 2006), fat metabolism (Patel *et al.*, 2006), freeze tolerance (Pfister and Storey, 2006) and vittelogenin uptake by the ovaries (Wang and Tefler, 2000). In terms of cellular immunity, inhibition of PKA in lepidopteran hemocytes induces non-self responses *in vivo* and *in vitro* (Brooks and Dunphy, 2005). LPS incubated with isolated hemocytes of *B. mori* increases PKA activity; the latter indirectly regulates cecropin B induction (Choi *et al.*, 1995; Shimabukuko *et al.*, 1996). PKA inhibition *in vitro* of *G. mellonela* hemocytes resulted in filopodia formation on the plasmatocytes in comparison to control (Cytryńska *et al.*, 2007). Those hemocytes remaining in the hemolymph after bacterial nodulation contain elevated PKA (Cytryńska *et al.*, 2007). Four intracellular proteins were phosphorylated by PKA in these hemocytes (Cytryńska *et al.*, 2007).

Insect cell lines

From the beginning of the 20th century, tissue culture was developed as a method for the studying behavior of animal cells free of systemic variations (Freshney, 2005). Insect cell lines were used as tools for: a) observing intracellular activity (Monget, 1975), b) defining signal transduction processes (Ghosh *et al.*, 2004) c) genetics (Deng et al 2006) d) aspects of biotechnology (Verkerk *et al.*, 2007), e) a substitute model for breast cancer cells (Ivanova *et al.*, 2007; Ma *et al.*, 2007), f) enhanced larval protein production for capsule production in drug delivery to human hepatocytes (Takuya *et al.*, 2006) and e) indirectly for pest control with the production of viral insecticides (Wood and Granados, 1991). Insect cell lines are obtained from adult ovaries (Zakarian, 2002), embryos (Lynn, 1996), fat body (Mitsuhashi, 2002), midgut (Goodman *et al.*, 2004), hemocytes (Krywienczyk

and Sohi, 1973; Keddie *et al.*, 1995) and crushed neonate larvae (Charpentier *et al.*, 1995).

In comparison to mammalian cell lines, insect cell lines are capable of prolonged, stable indefinite cell divisions and usually do not exhibit contact inhibition (Vago, 1971; 1972). Most of the insect cell lines grow between 25-30^{o}C (Grace, 1971); however, in polyploid members of *Spodoptera frugiperda* the ovarian cell lines Sf9 and Sf21 growth temperatures are higher e.g. 37^{o}C (Gerbal *et al.*, 2000). In most cases, optimum osmolarity levels are ~ 320mOsm/Kg of solvent (range from 230-450mOsm/Kg of solvent) (Agathos *et al.*, 1990) and the most frequently used pH of the medium ranges between 6.2-6.5 depending on the insect species and the physiological properties of the primary tissue that gives rise to the cell lines (Mitsuhashi, 1989; Zakarian *et al.*, 2002).

The primary insect hemocyte cell lines are collected from hemolymph samples in a Petri dish with medium containing glutathione or cysteine which effectively limits melanization (Lynn, 2001; Mutsihashi, 2002).

Hemocyte cell lines are studied for their non-self responses to selected antigens. Lepidopteran (*M. disstria*) and Dipteran (*D. melanogaster*) hemocyte cell lines exhibit adhesion to polystyrene and phagocytosis of apoptotic cells after ecdysteroid treatments (Manaka *et al.*, 2004; Palli *et al.*, 1995). The effects of isolated hemolymph proteins have been considered as in *D. melanogaster* hemocyte cell lines, e.g. the Ca^{+2} binding plasma protein, calreticulin, regulating phagocytic activities against apoptotic cells (Kuraishi *et al.*, 2007). Isolated hemocyte cell line (mbn-2) from *D. melanogaster* upregulates cationic antimicrobial peptide (diptericin) upon bacterial challenge (Johansson *et al.*, 2006). Through microarray analysis, interaction of *mbn-2* hemocytes with bacterial lipopolysaccharide and intact *E. coli* produce identical and different transcripts. In addition cell morphology and adhesion varied with the antigens used (Johansson *et al.*, 2005). Insect growth blocking peptide is vital for growth regulation of the hemocytes of the cell line SES-MaBr-93 of *Mamestra brassicae* (Narita *et al.*, 2002). Nitric oxides with

antimicrobial properties in the supernatant of the hemocyte cell line of the salt march caterpillar, *Estigmene acreae,* BTI-EA-1174-A are activated when incubation of cells with diverse antigens such as *E. coli* LPS and silica beads (Weiske and Wiesner, 1999; Wittwer *et al.,* 1997). In response to *E. coli* LPS, the BTI-EA-1174-A cell line released a broad spectrum of active proteases (Wittwer and Wiesner, 1998) implying that receptors on lepidopteran hemocytes may sense microbes by microbial surface antigens. We examine the interaction of non adherent cell line with polystyrene and bacteria is adressed in this Thesis. Furthermore, we optimized growth conditions and physiologically and biochemically standarized the cell lines thereafter.

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CHAPTER 2

HEMOCYTES OF LARVAL *Malacosoma disstria* (LEPIDOPTERA: LASIOCAMPIDAE) AND FACTORS AFFECTING THEIR ADHESION TO GLASS SLIDES

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ABSTRACT

Although hemocytes of the forest pest lepidopteran, *Malacosoma disstria* (L.) have been studied, the physico-chemical factors and signalling components affecting their non-self activities have not been examined. Both the ameboid and stellate forms of plasmatocytes and the granular cells from fifth-instar larvae adhere best to glass slides with phosphate-buffered saline (PBS), with maximum granular cell binding within a pH range of 6.0–7.0 and plasmatocyte binding at pH 6.0. The divalent cations, calcium and magnesium, do not affect granular cell attachment. However, calcium in *Galleria*-anticoagulant and PBS and, to a lesser extent, magnesium in the anticoagulant, increase plasmatocyte-glass contact. Based upon the use of selective type I protein kinase A inhibitor (Rp-8-Br-cAMPS) and activator (Sp-8-Br-cAMPS), active protein kinase A inhibits the adhesion of both hemocyte types. Similarly, protein kinase C inhibited by Gö 6976 enhances hemocyte adhesion whereas the enzyme activator, phorbol-myristate-acetate, impairs attachment.

Keywords: Adhesion, granular cells, hemocytes, *Malacosoma*, plasmatocytes, protein kinase A, protein kinase C

INTRODUCTION

The forest tent caterpillar, *Malacosoma disstria*, is a gregarious native pest of North American deciduous trees (Furniss and Carolin, 1977). There are numerous reports of potential biological control agents for use against the insect, including *Bacillus thuringiensis* (van Frankenhuysen *et al*., 1991), parasitoids (Roland and Taylor, 1977) and entomopathogenic fungi (Hajek *et al*., 1996), of which the latter two groups of organisms encounter the insect's innate immune systems in the hemolymph.

In general, the innate hemocytic non-self response of many lepidopteran larvae consists of phagocytosis, nodulation and encapsulation. These reactions are the product of interactive cellular and humoral factors (Gillespie *et al*., 1997). The hemocyte types (i.e. the plasmatocytes and granular cells) are the main cells that, depending on the insect species and antigen type (Howard *et al*., 1998), participate in phagocytosis (Costa *et al*., 2005 Tojo *et al*., 2000). Nodulation is a biphasic response to large numbers of particulate antigens, in which the antigens adhere to proteins discharged about the granular cells forming on the antigen–coagulum complex that is walled off by the plasmatocytes (Ratcliffe *et al*., 1985). Encapsulation is essentially nodulation of foreign particles too large to be phagocytosed (Ratcliffe *et al*., 1985). The described cellular non-self responses share the common feature of adhering to the antigens. Physiochemical properties of insect hemocytes and their environment (Lackie, 1986) and the antigens (Dunphy, 1993; Lavine and Strand, 2001) affect hemocyte adhesion to foreign material. Plasmatocytes and granular cells of most insect species adhere to glass, which some regarded as the initiation of encapsulation (Zakarian *et al*., 2003). Signal transduction components, including eicosanoids (Stanley, 2000) and inactive protein kinase (PK) A and PKC (Zakarian *et al*., 2003), are required for the adhesion of lepidopteran hemocytes to glass. There are few systematic studies on the immediate non-self responses of *M. disstria* larval hemocytes to antigens. Air-dried smears of *M. disstria* hemolymph offer a

preliminary hemogram (Arnold and Sohi, 1976) without describing immunological functions. Stoltz and Guzo (1986) describe behavioural changes to the insect's hemocytes induced by ichneumonid polydnaviruses towards parasitoid eggs, yeast and Sephadex beads with associated diminished melanization. The biogenic amine, octopamine, which contributes to bacterial–hemocyte adhesion in lepidopterous larvae (Dunphy and Downer, 1994), elevates the secondary messenger, cyclic AMP, in *M. disstria* hemocytes (Gole *et al*., 1982). Thus, cyclic AMP-dependent PKA may affect the insect innate hemocyte responses.

The present study describes factors affecting the adhesion of *M. disstria* hemocytes to glass slides in terms of buffer type and pH, divalent cations (calcium, magnesium) and the signal transduction enzymes, PKA and PKC.

MATERIALS AND METHODS

Insects

Laboratory-reared *M. disstria* (supplied by the Canadian Forestry Service, Sault Ste Marie, Ontario) were maintained on a casein dextrose diet at 25 °C (Addy, 1969). Fifth-instar larvae, 3 days into the third, fifth and sixth instars, were used, unless otherwise stated.

Hemocyte monolayers

Hemocyte monolayers were made by adding hemolymph (20 μL) to a 1-cm^2 area on glass slides (previously rendered endotoxin-free by heating at 250 °C for 4 h) containing a selected buffer (100 μL). Slides were incubated at 25 °C at 85% RH for 40 min, after which they were rinsed with 5 ml of the corresponding buffer. Adhering hemocytes were subsequently fixed in glutaraldehyde vapour for 30 min. The total and types of adhering hemocytes were determined by phase contrast microscopy. Hemocyte types in the monolayers and in whole fresh hemolymph were identified according to Price and Ratcliffe (1974) and expressed as cells per mm^2.

The buffers tested included modified *Manduca*-buffered saline [88 mM NaCl, 40 M KCl, 1.7 mM PIPES (Sigma, St Louis, Missouri), 3 mM $CaCl_2$; Kanost *et al*., 1988] in which sucrose was replaced by an osmotic equivalent of NaCl, physiological saline (150 mM NaCl, 5 mM KCl; Yokoo *et al*., 1995), *Galleria* anticoagulant [186 mM NaCl, 13 mM KCl, 17 mM Na_4 EDTA, 10 mM HEPES (Sigma), 1 mM $NaHCO_3$; Mandato *et al*., 1997] with and without 5–20 mM $CaCl_2$, and phosphate-buffered saline (PBS) with and without 5 and 10 mM $CaCl_2$. $MgCl_2$ (10 mM) was also used in the latter two buffers. Buffer pH values ranged from 5.5 to 7.5 except for Ca^{+2} and Mg^{+2} supplemented buffers in which pH values were less than or equal to 7.0 to avoid divalent cation precipitation.

Detection of phenoloxidase activity in *M. disstria* was based on staining the hemocyte monolayers with phenoloxidase substrate, L-

dihydroxyphenylalanine (1 mg/ml in PBS with and without 10 mM calcium supplement). After incubation at 25 °C for 30 min the monolayers were washed, PBS adherent cells were examined for melanization (an indicator of phenoloxidase activity) using a phase contrast microscope (× 2000 magnification).

Signal transduction factors

PKA and PKC affect lepidopteran hemocyte responses to antigens (Lanz-Mendoza *et al*., 1996; Taniai *et al*., 1996; Zakarian *et al*., 2003) and may influence *M. disstria* hemocyte adhesion to glass. Hemocytes were exposed to increasing amounts of the membrane permeable, water soluble PKA activator, Sp-8-Br-cAMPS (Calbiochem, La Jolla, California) and inhibitor Rp-8-Br-cAMPS (Calbiochem), as well as the PKC activator, phorbol-3-myristate (Sigma) or the PKC inhibitor, Gö 6976 (Calbiochem) in 100 µL of PBS on slides. Slides were incubated for 30 min, nonadhering hemocytes were rinsed away with 5 ml of PBS and the monolayers fixed in glutaraldehyde vapor. The total and types of adhering hemocytes were determined as described previously.

Biochemical assays

The link between hemocyte adhesion and PKA and PKC activity was established by measuring the activity of these enzymes in plasma-free hemocytes. Hemocytes were obtained from chilled (5 min at 4 °C) larvae that had been injected with 60 µL of *Galleria* anticoagulant (Mandato *et al*., 1997). Larvae were bled into 1 ml of chilled anticoagulant in 1.5-ml microcentrifuge tubes. Two hundred-µL aliquots were added to 250 µL chilled microcentrifuge tubes. The hemocytes were pelleted by centrifugation (200 ***g***, 2 min at 4 °C) and resuspended in 100 µL of PBS. Hemocytes were rendered

free of plasma and anticoagulant by repeating this centrifuge-washing protocol three times. Trypan blue assays on suspensed hemocytes revealed hemocyte viability ranging from 90–95%. The hemocytes were resuspended in PBS containing PKA or PKC activators or inhibitors, and increasing amounts of enzyme activator with a fixed inhibitor level. After incubation at 25 °C for 30 min, they were centrifuged in 20 μL of calcium-supplemented PBS and lysed by vigorous pipetting. The lysate was quantitatively analysed for PKC and PKA activity using the respective Pre Tag nonradioactivity assay kits (Promega, Madison, Wisconsin). Both assays were based on the enzymes phosphorylating specific chromogenic proteins altering the electrophoretic mobility of the protein in agarose electrophoresis.

Statistical analysis

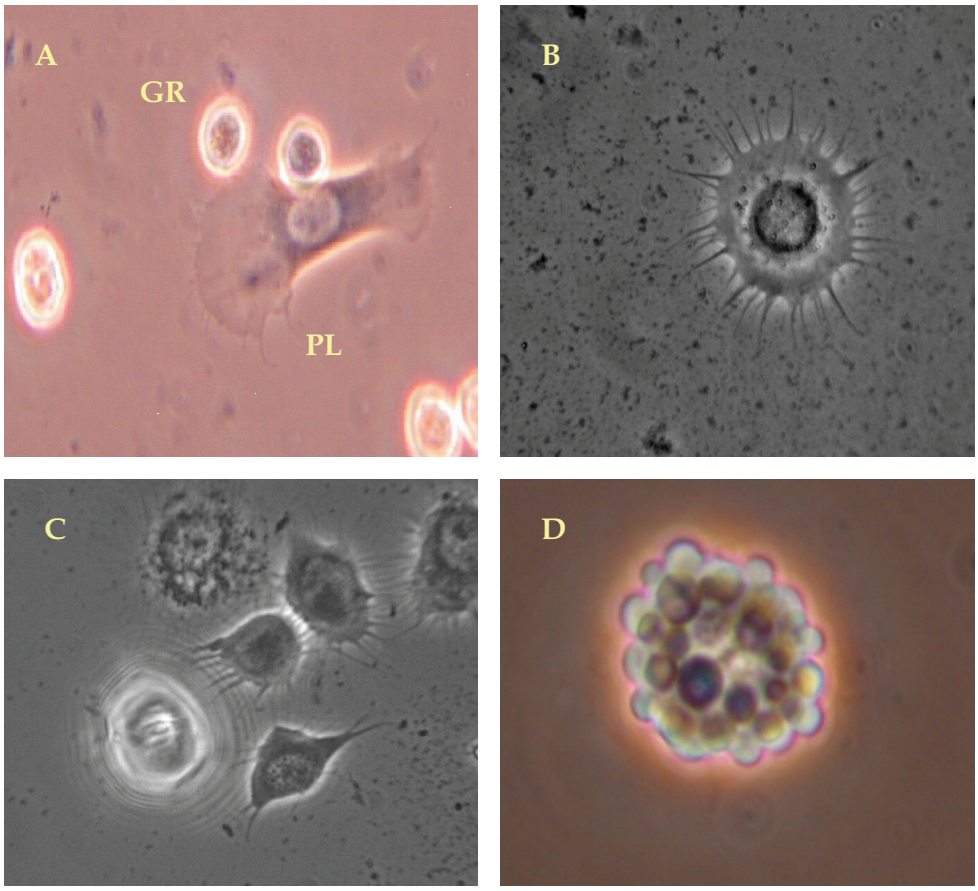
Hemocyte counts were analysed using the 95% confidence limits overlap protocol (Sokal and Rohlf, 1969). Data are presented as the mean ± SEM. An α level of 0.05 was chosen. Each insect was itself a replicate receiving individually prepared treatments. Groups of 5 insects were used to produce a mean ± SEM and analyzed as described with other groups within the treatment series. This protocol was repeated at least three times on different days or different times on a given day.

RESULTS

Hemocyte types

The major hemocyte types in the third, fifth and sixth instars included the granular cells (52%, range 67–84%) and the plasmatocytes (27%, range 20–32%). Granular cells were spherical (9.3 × 9.2 μm, $n > 100$) and phase bright (Figure 2.1A). Two types of large, nucleated plasmatocytes were seen; ameboid forms (Figure 2.1A) with single fans (42 × 10 μm minimum width, $n > 100$) were frequently found in smaller (320 mg) larvae whereas previously unreported stellate plasmatocytes (25.5 × 25.4 μm, $n > 100$) were more common (comprising 85% of the plasmatocytes) in large (720 mg) fifth- and sixth-instar insects (Figure 2.1B). Intermediate forms of plasmatocytes were frequently detected (10%) varying in characteristics between the amoeboid and stellate forms; they were often in clusters (Figure 2.1C). The spherulocytes (23%, range 15–20%) were oval (15.7 × 10.1 μm, $n > 100$) with the central nucleus often being occluded by the spherules (Figure 2.1D). Oenocytoids (29%, range 0–89%) with eccentric nuclei and rod-shaped crystals lysed rapidly upon bleeding. Staining the hemocytes with L-dihydroxyphenylalanine revealed melanization and thus phenoloxidase activity in the granular cells and oenocytoids, with the intensity of the reaction being greatest in calcium-supplemented buffer. The granular cells and both forms of plasmatocytes adhered to glass slides.

Figure 2.1: Hemocytes of fifth instar larval *Malacosoma disstria* in phosphate-buffered saline, pH 6.2 (× 2000 magnification, phase contrast microscopy). (A) Granular cells and ameboid plasmatocyte. (B) Stellate plasmatocyte found in later instars. (C) Clusters of plasmatocytes varing in form between the ameboid and stellate forms. (D) Spherulocyte with phase dark nucleus.


A
GR
PL
B
C
D

Physicochemical factors affecting hemocyte binding to glass

Because both buffer type and pH similarly affected hemocyte attachment for the three instars, data for the fifth instar are presented. PBS (Figure 2.2) and modified *Manduca* saline (Figure 2.3) were the most conducive to hemocyte attachment to slides. Granular cells adhesion was essentially constant in PBS at pH 6.0–7.5, whereas granular cell attached in *Manduca* saline peaked at pH 6.5. Maximum plasmatocyte-glass binding occurred at pH 6.0 in both buffers. Hemocyte clumping was common in *Manduca* saline, especially at pH values in excess of 6.5. Although hemocyte binding to slides with physiological saline at pH 6.0 (Figure 2.4) was slightly in excess of those in PBS at pH 6.0, the cells were stressed based on morphology, and granular cells were shrunken at pH ≤ 6.0 or degranulated at pH ≥ 6.5. Anticoagulant (Figure 2.5) without calcium limited total hemocyte and plasmatocyte adhesion compared with PBS, with maximum plasmatocyte and granular cell adhesion occurring at pH 5.5 and 6.5, respectively. Calcium at 20 mM (but not at lower concentrations) in the anticoagulant enhanced both total and plasmatocyte adhesion (Figure 2.5) but the increase adhesion was less than in PBS with similar Ca^{+2} levels. Plasmatocyte adhesion in the anticoagulant revealed a pH-calcium effect, with the adhesion levels increasing to a maximum at pH 7.0. Calcium did not affect granular cell adhesion in either the PBS or anticoagulant buffer. At 10 but not 5 mM levels, calcium marginally increased plasmatocyte adhesion in PBS; however, no evidence of a calcium–pH interaction was seen. Maximum total hemocyte adhesion and cellular differentiation occurred within 30 min of incubation of hemocytes in PBS (with 10 mM calcium) at pH 6.15 (Figure 2.6); the same incubation time was used in subsequent experiments. The ratio of adhering granular cells: plasmatocytes was approximately 5 : 2, and the ratio was approximately 2 : 1 in the differential hemocyte count in whole hemolymph, which was not significantly different ($P > 0.05$). Magnesium (10 mM) marginally increased plasmatocyte binding in *Galleria* anticoagulant

(anticoagulant, pH 6.5, no magnesium: 12.2 ± 0.7 plasmatocytes/mm^2; anticoagulant, pH 6.5 ± 10 mM $MgCl_2$: 18.7 ± 1.3 plasmatocytes/mm^2, $P < 0.05$) but not in PBS (PBS, pH 6.5, no magnesium: 26.2 ± 1.3 plasmatocytes/mm^2; PBS, pH 6.5 ± 10 mM $MgCl_2$: 29.9 ± 0.3 plasmatocytes/mm^2, $P > 0.05$).

Figure 2.2: Adhesion and differentiation of *Malacosoma disstria* hemocytes on glass slides in phosphate-buffered saline (PBS) without and with 20 mM calcium at different pH values.

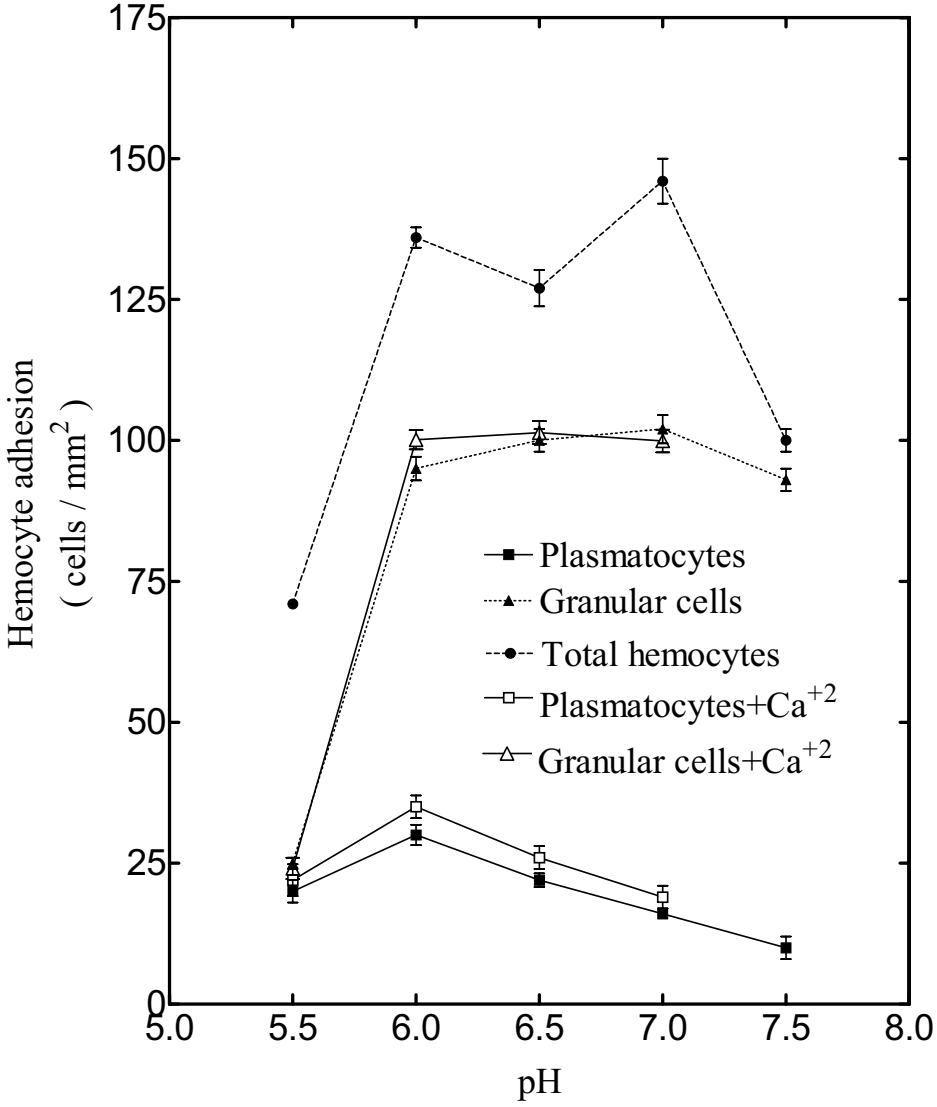

Hemocyte adhesion
(cells / mm^2)
175
150
125
100
75
50
25
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
Plasmatocytes
Granular cells
Total hemocytes
Plasmatocytes+Ca^{+2}
Granular cells+Ca^{+2}

Figure 2.3: Adhesion and differentiation of *Malacosoma disstria* hemocytes in modified *Manduca*-saline at different pH values on glass slides.

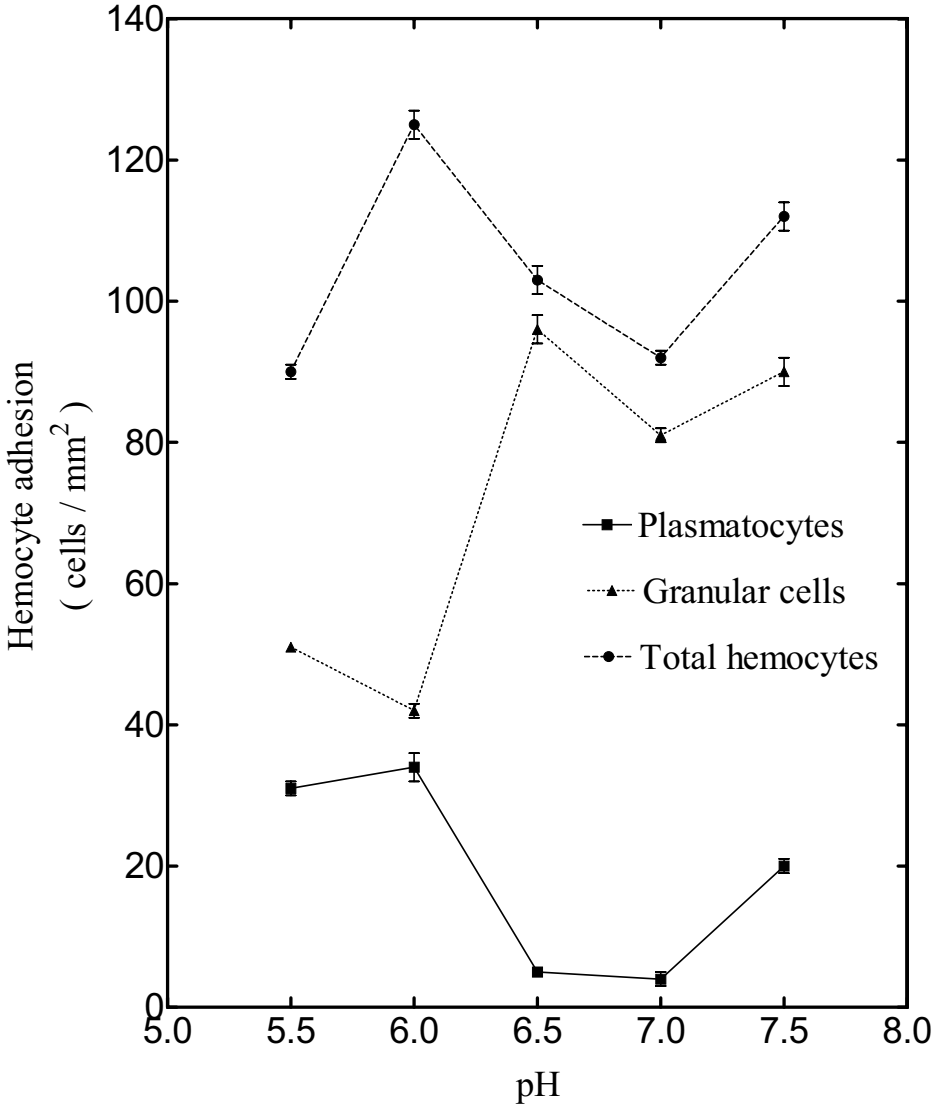

Hemocyte adhesion
(cells / mm2)
140
120
100
80
60
40
20
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
Plasmatocytes
Granular cells
Total hemocytes

Figure 2.4: Adhesion and differentiation of *Malacosoma disstria* hemocytes in insect physiological saline at different pH values on glass slides.

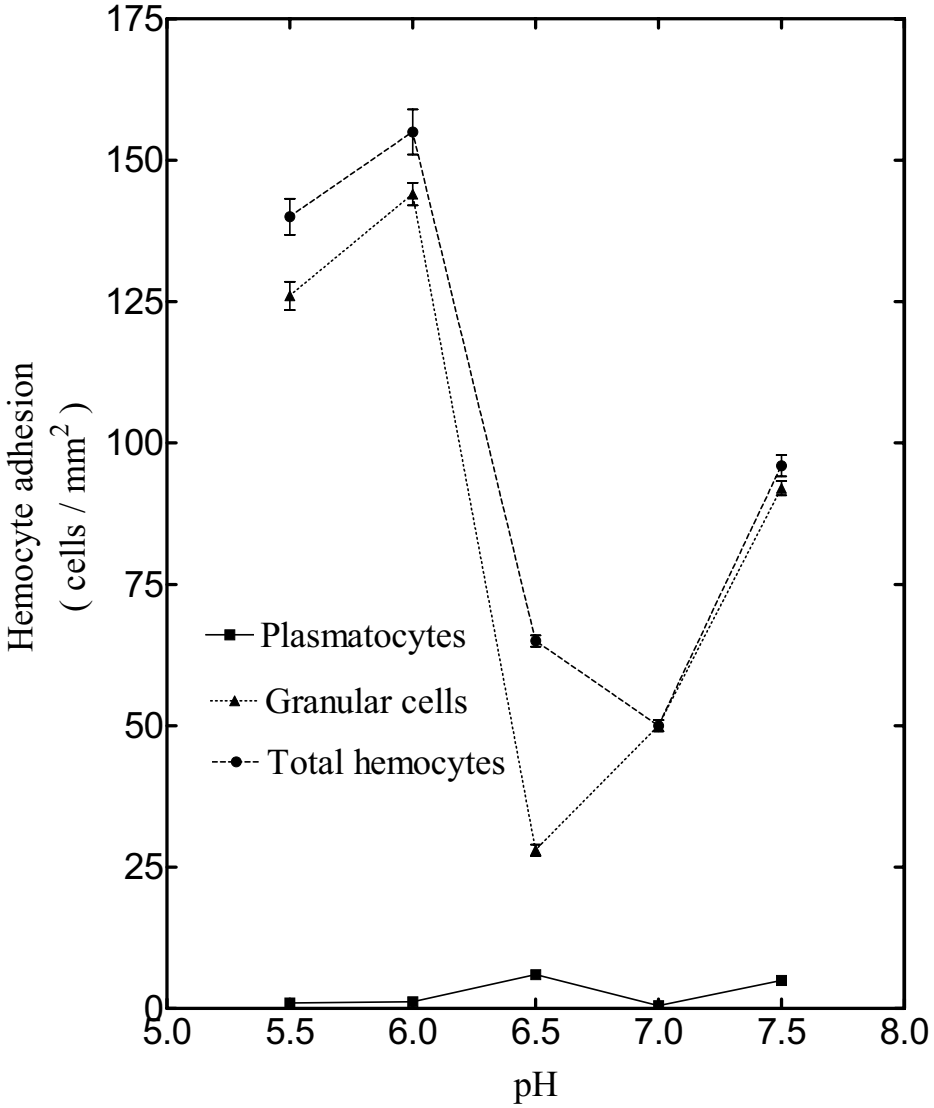

175
150
125
100
75
50
25
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
Hemocyte adhesion
(cells / mm2)
Plasmatocytes
Granular cells
Total hemocytes

Figure 2.5: Adhesion and differentiation of *Malacosoma disstria* hemocytes on glass slides in *Galleria* anticoagulant without and with 20 mM calcium at different pH values.

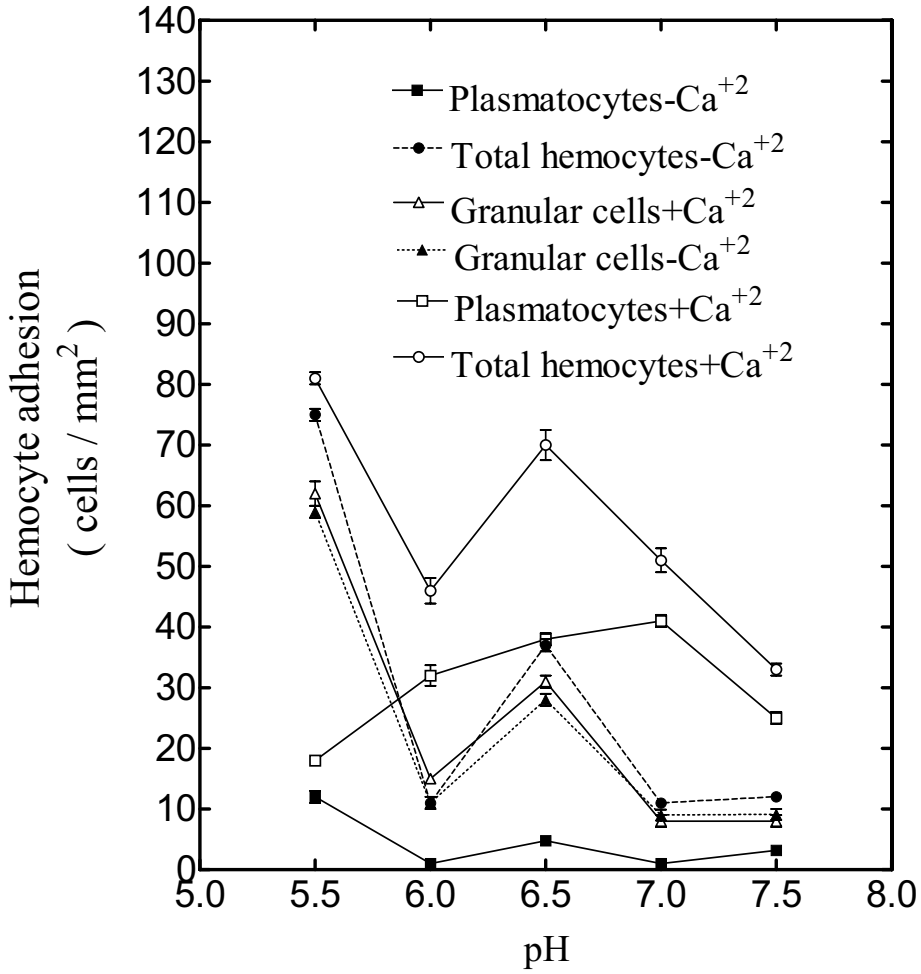
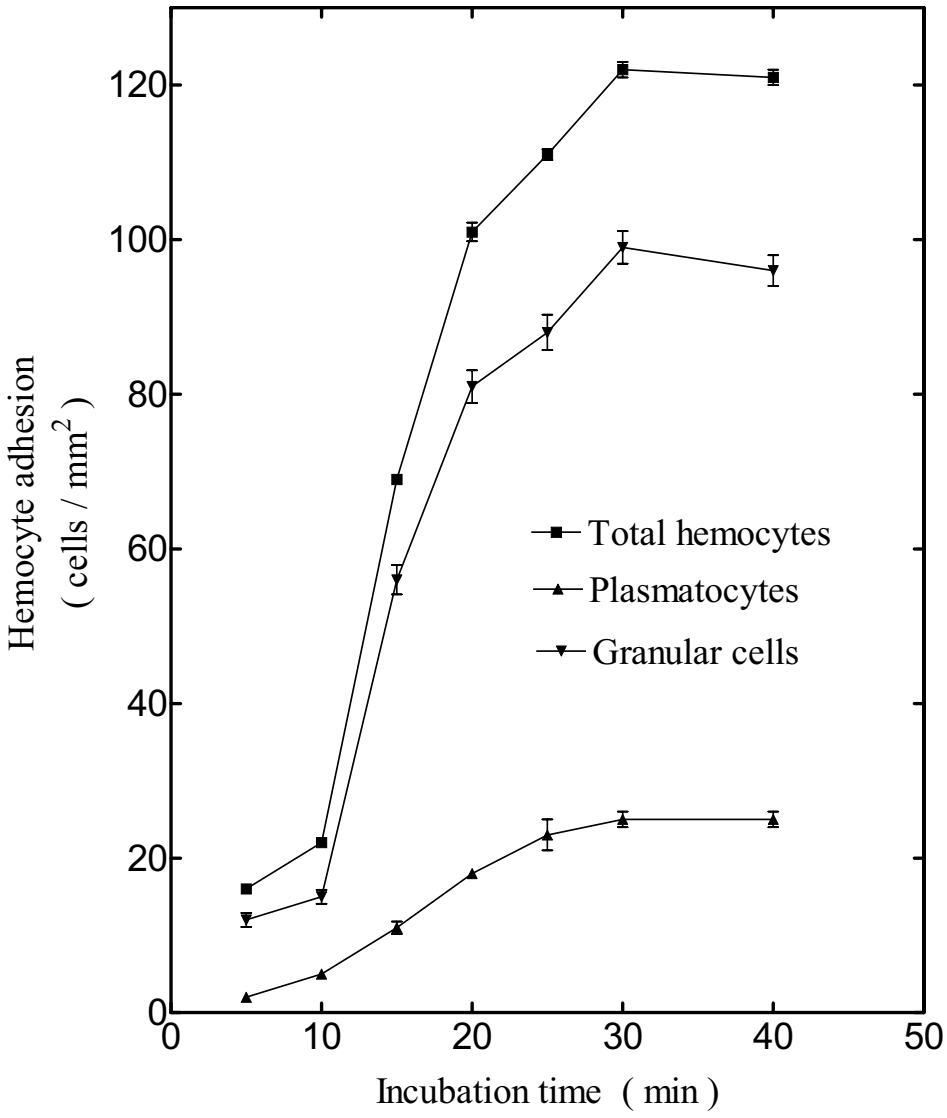

Hemocyte adhesion
(cells / mm^2)
pH
Plasmatocytes-Ca^{+2}
Total hemocytes-Ca^{+2}
Granular cells+Ca^{+2}
Granular cells-Ca^{+2}
Plasmatocytes+Ca^{+2}
Total hemocytes+Ca^{+2}
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
5.0
5.5
6.0
6.5
7.0
7.5
8.0

Figure 2.6: Adhesion and differentiation of *Malacosoma distria* hemocytes on glass slides in phosphate-buffered saline (PBS, pH 6.5) at different times.


Hemocyte adhesion
(cells / mm2)
120
100
80
60
40
20
0
0
10
20
30
40
50
Incubation time (min)
Total hemocytes
Plasmatocytes
Granular cells

Signal transduction factors

The PKA activator, Sp-8-Br-cAMPS significantly lowered the adhesion of granular cells and plasmatocytes compared with the control group (Table 2.1). Rp-8-Br-cAMPS, a type 1 PKA inhibitor, substantially increased the adhesion of both hemocyte types. Similarly, increasing concentrations of the PKA inhibitor with a fixed level of Sp-8-Br-cAMPS increasingly offset the PKA activator effect. Activating PKC with phorbol-myristate acetate concentration-dependently diminished granular cell and plasmatocyte adhesion to slides, whereas the PKC inhibitor, Gö 6976, increased cell adhesion (Table 2.2). The increased hemocyte adhesion induced by the PKC inhibitor, Gö 6976, diminished with increasing concentrations of the PKC activator, phorbol-myristate-acetate. The enzyme modulators affected PKC and PKA activity in parallel with the adhesion data. The PKA antagonist, Rp-8-Br-cAMP, and the agonist, Sp-8-Br-cAMP, inhibited and enhanced PKA activity, respectively (Table 2.3). The agonist also offset some of the PKA inhibition caused by Rp-8-Br-cAMP. Similarly, the PKC inhibitor, Gö 6976, and activator, phorbol-myristate-acetate, lowered and enhanced PKC activity in the hemocytes, respectively, and, together, the agonist offset PKC inhibition by Gö 6976 (Table 2.4).

Table 2.1: Effect of protein kinase A (PKA) modulators on the adhesion of fifth-instar larval *Malacosoma disstria* hemocytes to slides.

Enzyme	Modulator	Concentration (nM)	Hemocytes per mm²*		
			Total	Granular cells	Plasmatocytes
PKA	Rp-8-Br-	0	25.2 ± 0.3[a]	15.7 ± 0.2[a]	10.1 ± 0.2[a]
	cAMPs	25	47.3 ± 1.1[b]	26.1 ± 0.1[b]	19.3 ± 1.1[b]
	(antagonist)	50	86.5 ± 0.9[c]	49.8 ± 0.7[c]	37.4 ± 2.1[c]
	Sp-8-Br-	0	27.3 ± 1.1[a]	16.2 ± 1.2[a]	9.8 ± 0.3[a]
	cAMPS	25	16.1 ± 0.7[b]	9.4 ± 0.7[b]	3.5 ± 0.7[b]
	(agonist)	50	6.2 ± 0.3[c]	6.8 ± 0.3[b]	1.1 ± 0.3[b]
	Antagonist	0	52.2 ± 2.3[a]	28.7 ± 1.1[a]	22.2 ± 0.7[a]
	(25 mM)	25	41.0 ± 1.0[b]	23.7 ± 1.2[a]	18.2 ± 0.2[b]
	+ agonist	50	22.1 ± 0.7[c]	9.8 ± 0.7[b]	12.1 ± 0.3[c]

*Mean ± SEM (n = 5 replicates with 10 samples per replicate). Values within a column for a given treatment with the same superscript letter are not significantly different (P> 0.05).

Table 2.2: Effect of protein kinase C (PKC) modulators on the adhesion of fifth-instar larval *Malacosoma disstria* hemocytes to slides.

Enzyme	Modulator	Concentration (nM)	Hemocytes per mm²*		
			Total	Granular cells	Plasmatocytes
PKC	Gö 6976 (antagonist)	0	31.7 ± 2.1[a]	18.2 ± 0.3[a]	13.9 ± 0.1[a]
		25	72.3 ± 3.6[b]	36.1 ± 1.7[b]	36.8 ± 0.8[b]
		50	97.1 ± 2.8[c]	52.2 ± 1.9[c]	48.0 ± 2.1[c]
	phorbol-myristolate acetate (agonist)	0	29.7 ± 1.1[a]	17.8 ± 1.3[a]	10.1 ± 1.1[a]
		25	14.6 ± 0.7[b]	8.8 ± 0.9[b]	5.2 ± 0.7[b]
		50	6.1 ± 0.8[c]	1.0 ± 1.7[c]	2.1 ± 0.9[c]
	Antagonist (25 nM) + agonist	0	27.3 ± 0.7[a]	18.9 ± 1.1[a]	9.2 ± 0.3[a]
		25	15.7 ± 0.3[b]	8.0 ± 2.0[b]	7.9 ± 1.1[b]
		50	8.9 ± 0.2[c]	3.8 ± 1.1[c]	5.1 ± 0.8[b]

*Mean ± SEM (n = 5 replicates with 10 samples per replicate). Values within a column for a given treatment with the same superscript letter are not significantly different (P> 0.05).

Table 2.3: Effect of protein kinase A modulators on PKA activity from hemocytes of fifth instar larval *Malacosoma disstria.*

		Concentration	Enzyme activity
Enzyme	**Modulator**	**(nM)**	**(pmol per 10^3 cells)***
PKA	Rp-8-Br-cAMPS	0	62 ± 1^a
	(antagonist)	25	15 ± 1^b
	Sp-8-Br-cAMPS	0	65 ± 1^a
	(agonist)	25	121 ± 9^c
	Antagonist (25 nM)	0	98 ± 8^a
	+ agonist	25	62 ± 3^d

*Mean ± SEM (n = 3 samples from autonomous hemocyte collections). Values with the same superscript letter are not significantly different.

Table 2.4: Effect of protein kinase C modulators on PKC activity from hemocytes of fifth instar larval *Malacosoma disstria*.

Enzyme	Modulator	Concentration (nM)	Enzyme activity (pmol per 10^3 cells)*
PKC	Gö 6976	0	65 ± 3^a
	(antagonist)	25	27 ± 7^e
	Phorbol-myristate-	0	76 ± 7^a
	acetate (agonist)	25	133 ± 4^c
	Antagonist (25 nM)	0	67 ± 10^a
	+ agonist	25	121 ± 8^c

*Mean ± SEM (n = 3 samples from autonomous hemocyte collections). Values with the same superscript letter are not significantly different.

DISCUSSION

The hemocyte types, the granular cells, spherulocytes and elliptical plasmatocytes of *M. disstria*, are known from cell air-dried hemolymph smears (Arnold and Sohi, 1976; Kurtti and Brooks, 1970). The stellate form of the plasmatocyte reported in the present study is a first for this insect species and is similar to those in the post fourth-instar larval stage of the lepidopteran noctuid, the clear dart, *Euxoa declarata* (Arnold and Hinks, 1974). Both plasmatocyte forms and the granular cells comprise the majority of immunoreactive hemocytes of *M. disstria* larvae adhering to glass slides. Hyperphagocytic plasmatocytes as shown in tobacco hornworm, *M. sexta* based on cells being challenged with microbial antigens (Dean *et al.*, 2004) were not observed in fresh hemocytes samples or on adherent *M. disstria* hemocyte populations on glass slides. Oenocytoids have not been reported previously, possibly reflecting the failure of these cells to survive air-drying. Both the oenocytoids and granular cells stain for phenoloxidase, with melanization occurring more rapidly in hemocytes in calcium-supplemented buffer. This implies a possible non-self role for oenocytoids and further supports the antiforeign role of granular cells. Zymogenic, prophenoloxidase is synthesized in oenocytoids of *M. sexta* where lacking of signal peptides, it's released to the plasma occurs by oenocytoid lysis, the zymogen enzyme subsequently binding to other hemocyte types including granular cells with possible mediation by immunolectin-2 (Yu and Kanost, 2004; Ling and Yu, 2005). Although in *M.disstria* phenoloxidase may be on the granular cell surfaces as it is for *M. sexta* (Ling and Yu, 2005). It may also be in the cytoplasm and surfaces as it is for medfly, *Ceratitis capitata* (Mavrouli *et al.*, 2005; Charalambidis *et al.*, 1996). Both the activation and activity of phenoloxidase in most insect species are calcium-dependent (Brookman *et al.*, 1988; Dunphy, 1991). Phenoloxidase of *Lymantria dispar* hemocytes is also converted from a zymogenic form to an active form by cell wall and envelope

components of microorganisms initiating serine protease activity (Dunphy, 1991).

Buffer type and pH affect the adhesion of granular cells and plasmatocytes to slides. pH affects hemocytes from numerous orders in terms of hemocyte aggregation and integrity (Da Silva et al., 2000; Miranpuri *et al*., 1991), phagocytosis (Brookman *et al*., 1988) and adhesion to slides (Da Silva *et al*., 2000). Extracellular calcium favours the adhesion of *M. disstria* plasmatocytes but not granular cells. Similar results are known for *G. mellonella* (Anggraeni and Ratcliffe, 1991; Tojo *et al*., 2000) and *Agrotis segetum* (Yokoo *et al*., 1995). By contrast, the spreading but not initial adhesion of a subpopulation of *M. sexta* plasmatocytes on glass slides requires calcium (Willott *et al*., 2002). Calcium requirements for adhesion are not limited to extracellular calcium; intracellular calcium increases *G. mellonella* plasmatocyte adhesion by decreasing the activity of the signal transduction enzyme, PKC (Zakarian *et al*., 2003).

The effects of magnesium on hemocyte activity are rarely considered. The divalent cation induces limited *M. sexta* plasmatocyte spreading on slides in the absence of calcium (Willott *et al*., 2002). The contribution of calcium and magnesium to the phagocytosis of three bacterial species and chicken erythrocytes by hemocytes of the cockroach, *Gromphadorhina portentosa*, and the horseshoe crab, *Limulus polyphemus*, is inconclusive (Gupta and Campenot, 1996). In the present study, the Mg^{+2} effect varies with the buffer type increasing plasmatocyte adhesion in *Galleria* anticoagulant but not in PBS. The granular cells are Mg^{+2}-insensitive in both solutions. Magnesium enhances the activities of PKA in the bacterium *Escherichia coli* (Zhou and Adams, 1997) and PKC in chloroplasts (Siegenthaler and Bovet, 1993). Active PKA and PKC of *M. disstria* hemocytes is associated with a decline in hemocyte adhesion to glass slides. On this basis, less hemocyte adhesion is expected but not seen in magnesium supplemented solutions. Thus, Mg^{+2} may not be affecting PKA or PKC.

The initial binding of lepidopterous hemocytes to foreign matter is mediated by the discharge of adhesive proteins (Lavine and Strand, 2002; Pech *et al*., 1995). Such discharge may be triggered by antigens adhering to two types of receptor, nonpattern recognition receptors and pattern-recognition receptors (Dettloff *et al*., 2001) with or without mediation by plasma (Ma *et al*., 2006; Ohta *et al*., 2006). RGD receptors participate in binding hemocyte-antigen of granular cells of *M. sexta* via integrins (Zhuang *et al*., 2008). The receptors transfer the information into the hemocytes by signal transduction to continue the non-self responses (Bedick *et al*., 2000). Plasma proteins such as hemolin, C-type of lectins and lipophorin particles possibly reacting with hemocyte receptors trigger signalling that eventually, elicits hemocyte adhesion (Schmidt and Schreiber, 2006).

Signal transduction by the PKA pathway leads to the *de novo* synthesis of the antimicrobial cecropins protein is known for lepidopteran hemocytes (Choi *et al*., 1995; Tania *et al*., 1996). However, little is known about signalling with respect to the immediate adhesion of lepidopteran hemocytes to foreign materials. However, for dipteran hemocytes the signal factors contribute to antigen-adhesion responses include, p38 mitogen-activated protein kinases (Lamprou *et al*., 2005) and Ras/mitogen-activated protein kinase (Foukas *et al*., 1998; Mavrouli *et al*., 2005). *Galleria mellonella* requires active phospholipase A2 and cyclooxygenase for prophenoloxidase activation, phagocytosis and hemocyte spreading (Mandato *et al*., 1997). Active PKC isoforms are required for hemolin-enhanced phagocytosis of yeast by *Hyalophora cecropia* hemocytes (Lanz-Mendoza *et al*., 1996). However, for *G. mellonella*, active, calcium- and lipid-dependent PKC isoforms limit plasmatocyte and granular non-self responses, such as cell adhesion to glass slides (Zakarian *et al*., 2003). The latter is similar to *M. disstria* in which PKC inhibited by Gö 6976 increases hemocyte adhesion whereas the activator decreases adhesion. That Gö 6976, an inhibitor of both conventional and novel PKC isotypes (Gschwendt *et al*., 1996), offset the agonist implies specificity in

PKC inhibition. Both hemocyte types are more sensitive to phorbol-myristate-acetate than Gö 6976, especially the granular cells.

Active PKA is attributed to diminished *G. mellonella* non-self responses when the enzyme is inhibited by H-89 (Zakarian *et al*., 2003), an inhibitor of numerous PKA subtypes and cGMP-dependent kinase (Wang, 2000). Using a specific PKA type I antagonist and an agonist, the present study demonstrates that active PKA limits *M. disstria* hemocyte adhesion. Active PKA in the hemocytes of the bivalve, *Crassostrea gigas*, caused by stress-induced elevation of noradrenalin, inhibits bacterial phagocytosis (LaCoste *et al*., 2001). The active enzyme also lowers *G. mellonella* hemocyte binding to slides and bacterial nodulation *in vivo* (Brooks and Dunphy, 2005). Because of changes in adhesion levels for both *G. mellonella* and *M. disstria*, the granular cells are more sensitive to the PKA inhibitor than the plasmatocytes, whereas the plasmatocytes are more sensitive to Sp-8-Br-cAMPs. Collectively, the data for the two hemocyte types imply heterogeneity in the contribution of the two enzymes to non-self responses. Subpopulations of both hemocyte types that differ in their antimicrobial and adhesion responses (Tojo *et al*., 2000; Zakarian *et al*., 2003) may represent this heterogeneity.

How PKA affects intracellular hemocyte activity remains unknown. PKA types phosphorylate three forms of the inositol-1,4,5-triphosphate receptors in rat neurones, increasing calcium-influx from the endoplasmic reticulum (Wang, 2000) and increasing cytosol Ca^{+2} in *Drosophila melanogaster* neuromuscular junctions (Yoshihara *et al*., 2000). In view of the similarity of the response of *M. disstria* and *G. mellonella* to PKC and PKA modulators, PKA activity possibly ensures calcium-sensitive PKC activity and hence diminishes hemocyte-adhesion. Cytryńska *et al*., (2007) reported similar morphological changes such as filopodia formation in *G. mellonella* plasmatocytes when there were incubated with Rp-8-Br-cAMPS *in vitro*, in comparison to control. These data were correlated with low total hemocyte counts and low PKA *in vivo* that would occur after nodulation (Cytryńska *et*

al., 2007). Thus, those hemocytes remaining in the hemolymph represents cells with elevated PKA (relative to control cells) reflecting the complex effects of hemogram changes induced by different bacterial species and insect homeostasis; the latter being an attempt to restore hemocyte profiles to the native state (Cytryńska *et al*., 2007). In some hemocyte subtypes, PKC-independent pathways may participate also because PKA inhibits T-cell activation by both PKC-dependent and -independent signalling routes (Tamir *et al*., 1996).

In summary, *M. disstria* hemocytes demonstrate optimum adhesion rates in PBS at pH value 6.5. Signal transduction enzymes PKA and PKC affect the adhesion process *in vitro*.

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CONNECTING STATEMENT – I

In the 2nd chapter, factors affecting the adhesion of *M. disstria* larval hemocytes to glass slides were determined. The information was used to develop an *in vitro* assay for *M. disstria* hemocytes in the 3rd chapter. Chapter 3 examines the interaction between intact *X. nematophila* and *B. subtilis* and host hemocytes.

CHAPTER 3

INTERACTION OF THE BACTERIA *Xenorhabdus nematophila* (Enterobacteriaceae) AND *Bacillus subtilis* (Bacillaceae) WITH THE HEMOCYTES OF LARVAL *Malacosoma disstria* (LEPIDOPTERA: LASIOCAMPIDAE)

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ABSTRACT

Malacosoma disstria larvae are a pest of deciduous trees. Little is known on the interaction of bacteria with the immediate innate hemocytic antimicrobial responses of these insects. Incubating dead *Xenorhabdus nematophila* and *Bacillus subtilis* with a mixture of serum-free granular cells and plasmatocytes *in vitro* revealed differential bacterial-hemocyte adhesion and differential discharge of lysozyme and phenoloxidase but not total protein. Although active phenoloxidase adhered equally to both bacterial species, *X. nematophila* limited enzyme activation whereas *B. subtilis* enhanced activation. Serum with active phenoloxidase (as opposed to tropolone-inhibited phenoloxidase) and purified insect lysozyme increased bacterial-hemocyte adhesion of both bacterial species. Apolipophorin-III protein, when incubated with hemocytes, limited their responses to glass slides and bacterial adhesion. However, initial binding of the protein to both bacterial species increased granular cell levels with bacteria while lowering the plasmatocyte levels with adhering procaryotes. Although *B. subtilis in vivo* elicited a nodulation-based decline in total hemocyte counts and did not affect hemocyte viability, dead *X. nematophila* elevated hemocyte counts and damaged the hemocytes as lipopolysaccharide levels increased and *X. nematophila* emerged into the hemolymph. Apolipophorin-III protein once bound to the bacteria slowed their removal from the hemolymph.

Keywords: *Malacosoma*; *Xenorhabdus*; hemocytes; lysozyme; phenoloxidase; plasma protein

INTRODUCTION

There are few well-defined models of the hemocytic antimicrobial systems of the Lepidoptera, the salient examples being the economic pest insects, the tobacco hornworm, *Manduca sexta,* and the greater wax moth, *Galleria mellonella*, and the beneficial silkworm, *Bombyx mori* (Gillespie *et al.*, 1997), all of which occupy different niches and have nuance differences in their antibacterial systems (Gillespie *et al.*, 1997). *Malacosoma disstria,* the tent caterpillar, was chosen as a model because, unlike the previously considered insects, it is a major gregarious native pest of North American deciduous trees (Furniss and Carolin, 1997). Additionally, *M. disstria* hemolymph supports the growth and development of numerous biological control agents including parasitoids and microorganisms (Stoltz and Guzo, 1986) as well as the nematode, *Steinernema carpocapsae*, and its mutualistic bacterium, *Xenorhabdus nematophila* (personal observation).

In general, the innate antimicrobial systems in lepidopteran hemolymph consist of interactive humoral and cellular factors (Gillespie *et al.*, 1997). Salient humoral elements include the opsonic, melanizing phenoloxidase system (Lockey and Ourth, 1996; Ling and Yu, 2005), lysozyme (a cationic, constitutive plasma protein) (Lockey and Ourth, 1996; Wilson and Ratcliffe, 2000), C-type lectins, β-1,3-glucan-binding proteins, hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002) and apolipophorin-III (ALP), (Halwani *et al.*, 2000), many of which facilitate the binding of microorganisms to hemocytes. ALP is a multifunctional plasma protein in *G. mellonella* that binds to lipoteichoic acid from avirulent *Bacillus subtilis* impairing phenoloxidase activation (Halwani *et al.*, 2000), neutralizes toxic lipopolysaccharide (endotoxin) of virulent *X. nematophila* (Dunphy and Halwani, 1997), adheres to bacterial surfaces affecting hemocyte activity (Zakarian *et al.*, 2002, 2003), activates the prophenoloxidase cascade in *G. mellonella* (Park *et al.*, 2005b), potentiates lysozyme activity against bacterial

surfaces (Halwani and Dunphy, 1999) and is a β-1,3-glucan recognition protein (Dunphy *et al.*, 2003; Whitten *et al.*, 2004) enhancing encapsulation of yeast (Whitten *et al.*, 2004). ALP, by interacting with lipophorins, forms cages around toxic antigens limiting antigen damage (Ma *et al.*, 2006). The cellular antibacterial components include the hemocyte types, the plasmatocytes and granular cells, which, depending on the insect species and bacterial species and concentration (Howard *et al.*, 1998), participate in phagocytosis (Tojo *et al.*, 2000) and nodule formation (Ratcliffe *et al.*, 1985). Nodulation is a biphasic response in which microorganisms adhere to granular cells producing a coagulum that is walled off by the plasmatocytes (Ratcliffe *et al.*, 1985).

Little is known about the immediate innate hemocyte responses of *M. disstria* to foreign materials. Air-dried hemolymph smears of *M. disstria* provide a foundation hemogram (Arnold and Sohi, 1976). Polydnaviruses suppress phenoloxidase activity and phagocytosis of yeast (Stoltz and Guzo, 1986). As part of a non-self response, the biogenic amine, octopamine, elevates the secondary cellular messenger, cyclic AMP in a *M. disstria* hemocyte tissue culture (Gole *et al.*, 1982). Protein kinase C and cyclic AMP-dependent protein kinase A influence the adhesion of fifth instar *M. disstria* granular cells and two plasmatocyte morphotypes to glass (Giannoulis *et al.*, 2005). Herein, emphasis is placed on the immediate interaction of the insect hemocytes with the surfaces of dead *B. subtilis* and *X. nematophila*. Dead bacteria were used to define their surface factors participating in the antimicrobial hemocyte response without influence from bacterial metabolism. Consideration is given to the involvement of phenoloxidase, lysozyme and ALP, molecules known to affect lepidopteran hemocyte responses (Gillespie *et al.*, 1997; Halwani *et al.*, 2000).

Bacillus subtilis binds avidly to hemocytes of *G. mellonella* (Zakarian *et al.*, 2002). Lipoteichoic acids of these bacteria elicit nodulation, deplete the plasmatocyte concentration in the hemocoel and irreversibly damage granular cells while activating phenoloxidase of larval *G. mellonella* (Halwani *et al.*,

2000). Vectored into insect hemolymph by the entomopathogenic nematode, *S. carpocapsae* (Akhurst, 1980), *X. nematophila* limits phenoloxidase activation (Dunphy and Webster, 1988; Dunphy and Webster, 1991) possibly by chelating Ca^{+2} ions (Yokoo *et al.*, 1992). This microorganism impairs also hemocyte function by releasing iron-chelators from hemocytes damaged by bacterial lipopolysaccharide (Dunphy *et al.*, 2002), while tolerating lysozyme-induced modification of the bacterial envelope (Dunphy and Webster, 1991). In *Spodoptera exigua*, live *X. nematophila* impairs eicosanoid biosynthesis (Park and Kim, 2000). Eicosanoids are part of the signal transduction system of insect hemocytes (Morishima, 1998) affecting hemocyte phagocytosis and spreading (Mandato *et al.*, 1997).

Here, we determine that the extent of binding of *X. nematophila* and *B. subtilis* to *M. disstria* hemocytes varies with the bacterial species and hemocyte type with mediation by lysozyme and phenoloxidase and possibly phenoloxidase metabolites. ALP binds to the bacteria limiting the adhesion of both bacterial species to plasmatocytes and enhances the number of granular cells with either bacterial species. *B. subtilis* is removed from the hemolymph by nodulation more rapidly than *X. nematophila*, the removal of both bacterial species being slowed by ALP. Unlike the former bacterial species, *X. nematophila* subsequently emerges into the hemolymph as endotoxin levels increase and hemocyte viability declines independently of ALP bound to the bacteria.

MATERIALS AND METHODS

Bacteria

Stock cultures of the phase one form of *X. nematophila* ATCC19061 [the form released from the infective stage of the nematodes upon entering the insect hemolymph (Akhurst, 1980)] were grown on Luria agar supplemented with triphenyltetrazolium chloride (30 mg/L) and bromthymol blue (25 mg/L). *B. subtilis* (Boreal Biological Co.) was grown on Luria agar. Both bacterial species were incubated at 25 °C in darkness and subcultured biweekly.

For experimental purposes, bacteria were grown to the mid-exponential phase of the growth cycle (turbidity at 660 nm = 0.75) in 5 ml of Luria broth in 20 ml scintillation vials at 28 °C on a horizontal gyratory shaker (250 rpm). Bacteria were washed three times by centrifugation (12,000*g*, 2 min, 25 °C) and resuspension in 5 ml of phosphate-buffered saline (138 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2.8 mM KH_2PO_4, adjusted to pH 6.5 with 5M HCl; PBS). The bacteria were killed by UV-irradiation for 3 h and were then stored overnight at 5 °C. Bacterial death was confirmed by (i) the absence of a change in turbidity of Luria broth inoculated with UV-irradiated bacteria and incubated for 96 h and (ii) no discernible colony formation when the bacteria were plated on Luria agar and incubated for 96 h at 28 °C. Cultures were centrifuge-washed in PBS prior to use. Dead bacteria precluded the effects of formyl peptides (Alavo and Dunphy, 2004) and other aspects of metabolism (Park and Kim, 2000) influencing results allowing the direct observation of the interaction of the insect hemocytes with bacterial surfaces.

Insects

Laboratory reared *M. disstria* (supplied by the Canadian Forestry Service, Sault Ste. Marie, Ontario) were maintained on a casein dextrose diet at 25 °C (Addy, 1969). Fourth instar larvae 3 days into the stadium were selected, unless otherwise stated.

Isolation of Apolipophorin-III

Apolipophorin-III in *M. disstria* hemolymph was isolated by diluting the hemolymph 1:1 (v/v) in PBS, removing the hemocytes by centrifugation (12,000*g*, 4 min, 20 °C) and heating (100 °C, 30 min) the supernatant. Following centrifugation (15,000*g*, 20 min, 20 °C) the non-precipitated serum proteins in the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and rendered visible with copper chloride (Lee *et al.*, 1987). An 18.7 kDa band was excised from the gel, destained in 250 mM ethylenediaminetetraacetic acid in 250 mM Tris (pH 9.0 with HCl), and dialyzed overnight against 2 L of 25 mM Tris–192 mM glycine (pH 8.3). The protein was electroeluted in the Tris–glycine buffer (2 h at 100 mV), dialyzed in PBS (2 L) and lyophilized. ALP purity and identity was determined based on the first 20 amino acid sequence according to Halwani and Dunphy (1999). The protein was tentatively identified as ALP by immunoblotting of the protein onto nitrocellulose membrane (Millipore). This was followed by blocking non-specific binding sites with a 5% (w/v) solution of skim milk. The membrane probed using anti-*G. mellonella* ALP antibodies. Binding of this primary antibody was detected using a 1:10,000 (v/v) solution of γ-chain specific alkaline phosphatase-conjugated monoclonal anti-rabbit antibody (Sigma, St. Louis, MO) as the secondary antibody (Halwani *et al.*, 2001).

Hemocyte monolayers

To examine bacterial adhesion to hemocytes *in vitro* hemocyte monolayers were prepared by adding 20 µl of hemolymph [collected from a prothoracic leg lesion of chilled (5 °C, 10 min) larvae] to 80 µl of 10 mM $CaCl_2$ supplemented PBS (pH 6.5) on a 1 cm^2 area on glass slides. The level of the cation used is equal to that in hemolymph of fourth instar larvae (Howard *et*

al., 1998) and potentiates non-self responses of *M. disstria* cultured hemocytes (Jahagirdar *et al.*, 1987), to more closely approximate the ionic environment of the hemocytes during the initial adhesion of the hemocytes, 10 mM $CaCl_2$ was used. The slides were rinsed three times with 5 ml PBS to remove non-adhering hemocytes and larval serum, and the resulting monolayers were used in the following experiments.

Interactions of *X. nematophila* and *B. subtilis* with hemocytes were based on incubating 100 µl of bacterial suspension (1×10^7 bacteria/ml PBS) with the hemocyte monolayers (producing a bacteria:hemocyte ratio of 100:1) for 30 min at room temperature and 95% RH. To determine the effects of selected larval serum components on bacteria–hemocyte interactions, hemocytes adhering to the slides were rinsed with PBS to remove larval serum and then were covered with 100 µl of PBS prior to the addition of 100 µl of bacterial suspension in PBS. Bacterial suspensions contained PBS alone, lysozyme (isolated from *G. mellonella* larvae by Dr. J. Chadwick, Queen's University, Canada; 1 mg/ml PBS), larval serum with active or tropolone (1.5 mM)-inhibited phenoloxidase [tropolone specifically inhibits phenoloxidase activity (Li *et al.*, 1996)] or PBS containing 10 ng of ALP. ALP was used at the lowest concentration that produced a discernible effect, and which was below that found in other lepidopteran larvae (Halwani *et al.*, 2000). Similarly, bacteria incubated with ALP and centrifuge-washed to remove non-bound protein were exposed to hemocyte monolayers. Non-attached bacteria were removed from the slides by rinsing with PBS and hemocyte-bacterial complexes were fixed in glutaraldehyde vapour (30 min). The percentages of each hemocyte type with bacteria were determined using phase contrast microscopy at 400× magnification. Hemocytes were identified according to Arnold and Hinks(1974) and Price and Ratcliffe, (1974).

Hemocyte discharge

Hemocytic discharge of lysozyme and phenoloxidase (enzymes known to affect bacteria–hemocyte adhesion) (Dunphy *et al.*, 2007) was induced by adding bacteria (1.6×10^6 cells) of either species in 100 µl of PBS to serum-free monolayers. Hemocyte controls for spontaneous discharge consisted of hemocyte monolayers with 100 µl of PBS alone. Bacterial controls involved either bacterial species in 100 µl of PBS without hemocytes. After incubating at 25 °C and 95% RH for selected times, 50 µl aliquots of buffer were removed for analysis. These aliquots were centrifuged (12,000*g*, 3 min, 25 °C) to remove bacteria and hemocyte debris and the supernatants were assayed for total protein (Bradford, 1976), lysozyme and phenoloxidase. Lysozyme activity was based on the clearing zone produced by the lysis of a suspension of *Micrococcus lysodeikticus* (1%, w/v) in 1.5% (w/v) agar containing PBS (Morishima, 1998). Phenoloxidase activity was assayed by measuring dopachrome formation (Leonard *et al.*, 1985). Briefly, supernatant (10 µl) was incubated with 50 µl of phenoloxidase activator (laminarin, 1 mg/ml PBS) for 60 min at 25 °C. A 25 µl volume of the resulting solution was added to a 500 µl freshly prepared solution of the phenoloxidase substrate, L-dihydroxyphenylalanine (1.5 mg/ml) and the formation of dopachrome was spectrophotometrically monitored at 495 nm. To ensure dopachrome formation was not due to residual hemocyte peroxidase, phenoloxidase was inhibited with 1.5 mM of the phenoloxidase-specific inhibitor tropolone and dopachrome formation determined. To preclude auto-oxidation of dihydroxyphenylalanine influencing results, only freshly made solution was used in the assay. Specific activity was calculated according to Leonard *et al.* (1985).

Two protocols were used to detect the binding of phenoloxidase to the bacteria. In protocol one increasing concentrations of either bacterial

species in 10 µl of PBS were added to 50 µl of hemocyte lysate containing previously laminarin-activated phenoloxidase. The bacterial suspensions were incubated for 30 min, centrifuged (15,000*g*, 5 min, 25 °C) and the supernatant analyzed for residual phenoloxidase activity. In protocol two *X. nematophila* and *B. subtilis* (1×10^4 cells) in 10 µl PBS were incubated with hemocyte discharge (20 µl) for 30 min. Negative controls consisted of bacteria incubated with hemocyte discharge containing tropolone-inhibited phenoloxidase and bacteria incubated with PBS. The bacteria were washed three times by centrifugation (15,000*g*, 5 min, 25 °C) from and resuspension in 30 µl PBS. The suspensions were added to dihydroxyphenylalanine solutions and dopachrome formation determined spectrophotometrically. Lysozyme binding to the bacteria was analyzed initially by incubating 50 µl hemocyte lysate with 10 µl of bacterial suspension (1×10^{10} bacteria) at 25 °C for 30 min. Bacteria and debris were removed by centrifugation (15,000*g*, 5 min, 25 °C) and the supernatant analyzed for lysozyme as described previously. Additionally, bacteria were incubated in 50 µl of purified lysozyme (1 mg/ml) in place of hemocyte lysate. Lysozyme activity was measured after 30 min incubation.

Innate antibacterial responses *in vivo*

To identify the hemocytic responses of the larvae to intact dead bacteria *in vivo*, insects were injected with 1×10^9 cells of either bacterial species in 10 µl PBS. Changes in total hemocyte counts and, where appropriate changes in concentrations of bacteria not attached to hemocytes were determined over time using a hemocytometer and phase contrast microscopy. Bacteria coated with ALP were similarly injected and their concentration determined over 6 h post-injection. Total phenoloxidase in hemolymph with lysed hemocytes from these larvae was determined as previously described. Hemocyte viability in the collected hemolymph was

based upon the exclusion of the vital stain, trypan blue (0.1%, w/v, PBS) by hemocyte suspensions. Limulus amebocyte chromogenic assay kit (Cedar Lane, ON) was used to detect endotoxin in the hemolymph at designated times post-injection.

Induced immunity

Larvae were injected with UV-irradiated *X. nematophila* (1×10^5 bacteria) in 10 µl PBS. Control larvae were injected with PBS. Insects were bled after incubating at 25 °C for 4, 6, 12 and 24 h and larval serum isolated by centrifugation (15,000*g*, 5 min, 25 °C).

Induced antibacterial activity was based on the lysis of *E. coli* D31 by larval serum (Chalk and Suliamin, 1998). Briefly, melted Luria agar containing 10 ml of filter-sterilized hen lysozyme (Sigma, 100 mg/1 ml, to enhance bacterial sensitivity to antibacterial proteins) and 1 ml of streptomycin (10 mg/ml) solution was inoculated with *E. coli* culture ($OD_{660\ nm} = 0.4$). Ten milliliters was poured into 9 cm diameter Petri dishes. Larval serum (10 µl) was added to (2 mm diam) holes punched in the agar. After 24 h of incubation at 25 °C, lytic zones were measured and compared against a gentamycin standard curve. Lysozyme assays were done as previously described except that larval serum (10 µl) was used.

Insects injected with the bacteria were bled, and the hemolymph centrifuged to collect bacteria. The bacteria were centrifuge-washed and added to 5 ml of Luria broth in 20 ml scintillation vials and to Luria agar in Petri dishes. Following incubation (96 h) cultures were examined for growth either visually by colony formation on plate cultures or turbidimetrically ($OD_{660\ nm}$) for liquid culture.

Statistics

Bacterial and hemocyte counts were analyzed using the 95% confidence limits overlap protocol (Sokal and Rohlf, 1969). Percentage data were analyzed using overlap of confidence limits on arcsin $\sqrt{p}$ -transformed data. Graphic data are presented as means ± standard error of the mean. An *a* level of 0.05 was chosen. A minimum of five replicates containing 10 samples were used for each value.

RESULTS

Hemocyte-bacterial interaction *in vitro*

Xenorhabdus nematophila in PBS adhered to plasmatocytes and granular cells in hemocyte monolayers to similar extents whereas *B. subtilis* adhered more extensively to granular cells; both bacterial species bound to plasmatocytes to equal extents (Table 3.1). Two enzymes with the potential to influence bacterial adhesion to hemocyte are lysozyme and phenoloxidase. The binding patterns of the hemocytes with the two bacterial species may reflect differences in the release rates of the two enzymes from the hemocytes. The two bacterial species equally promoted protein discharge from serum-free hemocyte monolayers (Figure 3.1) precluding a link between total protein release rates and the level of hemocyte types with bacteria. Each bacterial species (which did not release detectable lysozyme) elevated lysozyme activity from the hemocytes in a biphasic pattern which was above the values of the control hemocytes. *B. subtilis* was more effective than *X. nematophila* during the initial 50 min of incubation (Figure 3.2). Thereafter, lysozyme activity was comparable for both bacterial treatments ($P > 0.05$) and similar to the activity of hemocyte lysates (19.4 ± 0.6 U/mg total protein, $n = 10$, $P > 0.05$). Lysozyme activity at 50 min was correlated with increased levels of granular cells ($r = 0.892$, $P < 0.05$) and plasmatocytes ($r = 0.962$, $P < 0.05$) with *B. subtilis* and granular cells ($r = 0.778$, $P < 0.05$) but not plasmatocytes ($r = 0.115$, $P > 0.05$) with *X. nematophila*. Incubating *X. nematophila* and *B. subtilis* with purified lysozyme in PBS produced comparable results ($P > 0.05$; Table 3.1). Phenoloxidase activity released from monolayers with *B. subtilis* continuously increased above the control hemocyte values (Figure 3.3) achieving the maximum level equal to the total phenoloxidase activity of lysed hemocytes (16.8 ± 0.4 U/mg protein, $n = 10$, $P > 0.05$). However, with *X. nematophila* phenoloxidase activity was less than the control levels. Although

low enzyme activity may represent less zymogen discharge, it is possible that *X. nematophila* also limited phenoloxidase activation and activity. Limited enzyme activation was proven by the significantly less phenoloxidase activity in total hemocyte lysate with *X. nematophila* (0.7 ± 0.3 U/mg protein, $n = 20$) compared with enzyme activity in lysate without bacteria (17.4 ± 1.1 U/mg protein, $n = 10$, $P < 0.05$). Adding *X. nematophila* to serum with fully activated phenoloxidase did not alter enzyme activity (14.9 ± 0.8 U/mg protein, $n = 10$, $P > 0.05$). Phenoloxidase activity at 50 min in the bacteria–hemocyte monolayer was correlated with the levels of both hemocyte types with *B. subtilis* (plasmatocytes: $r = 0.782$, $P < 0.05$; granular cells: $r = 0.882$, $P < 0.05$). However, for *X. nematophila* there was a correlation of plasmatocytes ($r = 0.758$, $P < 0.05$) but not granular cells ($r = 0.095$, $P > 0.05$) with bacteria. There was no discernible phenoloxidase released from the dead bacteria (0.0 ± 0.2 U/10^4 bacteria, $n = 10$, $P > 0.05$).

Table 3.1: Adhesion of bacteria to hemocytes of *Malacosoma disstria* larvae *in vitro.*

Bacteria	Treatment	Percentage of hemocytes with bacteria[a]	
		Plasmatocytes	Granular cells
X. nematophila	PBS[b]	11.6 (22.8–17.4)^{g,g}	14.7 (25.5–20.7)^{g,g}
	Serum with active phenoloxidase[c]	20.5 (27.9–25.8)^{h,h}	22.9 (30.3–26.2)^{g,h}
	Serum with tropolone-inhibited phenoloxidase[d]	18.7 (28.1–19.7)^{g,i}	19.5 (26.9–24.7)^{g,i}
	Lysozyme[e]	18.6 (28.9–18.8)^{g,i}	22.7 (30.2–26.9)^{g,j}
	Apolipophorin-III[f]	7.8 (15.1–13.4)^{i,k}	12.4 (18.3–16.5)^{g,k}
B. subtilis	PBS	13.0 (25.1–18.5)^{g,k}	25.5 (31.6–28.3)^{g,l}
	Serum with active phenoloxidase	38.0 (40.9–35.1)^{i,m}	41.3 (41.6–38.1)^{k,m}
	Serum with tropolone-inhibited phenoloxidase	17.3 (30.3–22.3)^{g,n}	29.1 (35.6–32.3)^{k,n}
	Lysozyme	29.4 (33.4–31.3)^{i,o}	29.3 (36.1–32.7)^{k,o}
	Apolipophorin-III	5.7 (14.8–12.3)^{i,k}	9.4 (17.9–11.7)^{i,k}

[a] Decoded mean (with 95% confidence limits of arcsin √p transformed data, $n \geq 10$); values within a column with the same left, primary superscript letter and values between rows with the same right superscript letter were not significantly different compared with the PBS control group, $P > 0.05$.

[b] Phosphate-buffered saline, pH 6.5.

[c] 100% insect plasma with laminarin activated phenoloxidase (specific activity 12.7 ± 0.4 U/mg protein).

[d] 100% insect plasma with 1 mM tropolone-inhibited active phenoloxidase (specific activity 0.3 ± 0.4 U/mg protein).

[e] Lysozyme (1 mg/ml) in PBS. Enzyme isolated from *Galleria mellonella* by Dr. J. Chadwick, Queen's University, Canada.

[f] Apolipophorin-III, 10 µg in 100 µl PBS.

Figure 3.1: Total protein discharge from larval *Malacosoma disstria* hemocytes induced by the bacteria *Xenorhabdus nematophila* or *Bacillus subtilis* in the absence of larval serum in phosphate-buffered saline (PBS).

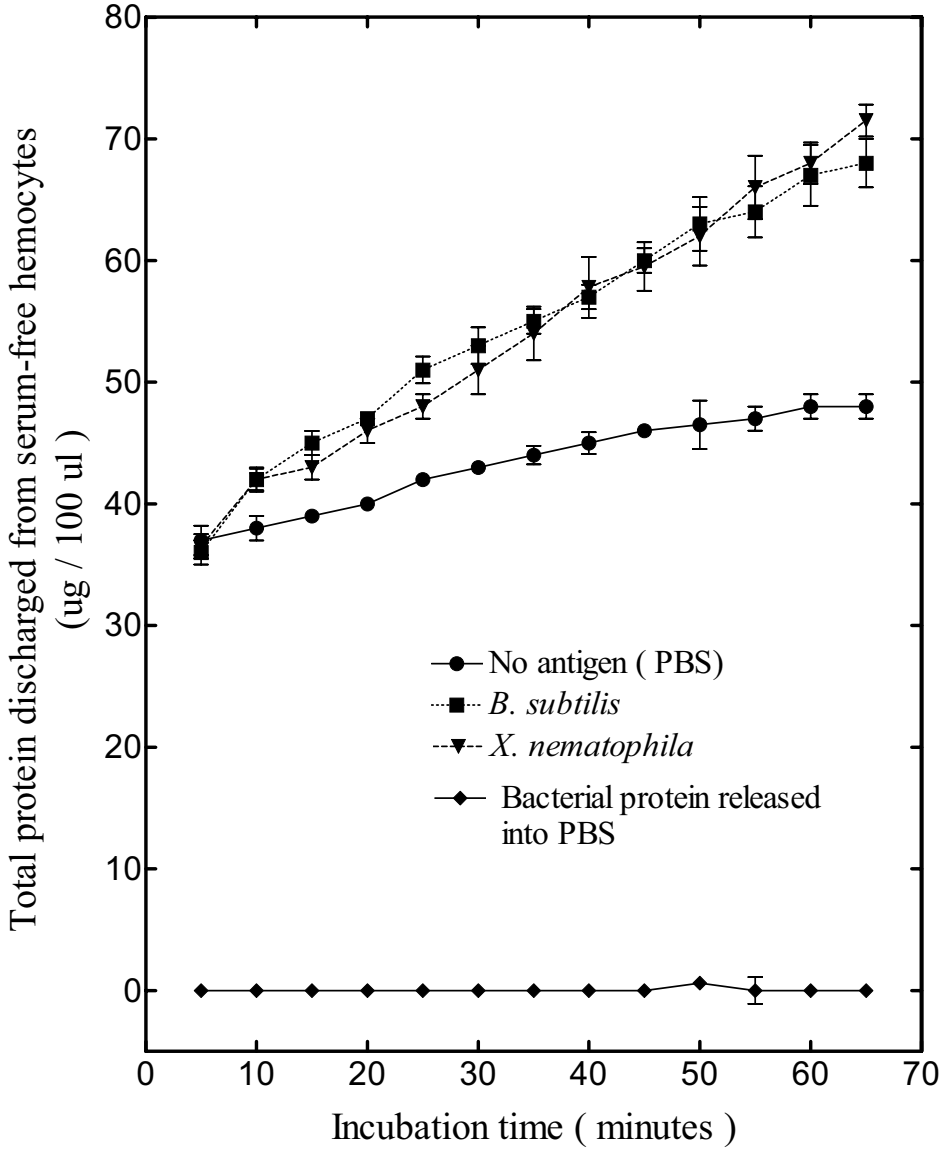

Total protein discharged from serum-free hemocytes
(ug / 100 ul)
Incubation time (minutes)
No antigen (PBS)
B. subtilis
X. nematophila
Bacterial protein released
into PBS
0
10
20
30
40
50
60
70
80

Figure 3.2: Discharge of lysozyme from *Malacosoma disstria* larval hemocytes induced by the bacteria *Xenorhabdus nematophila* or *Bacillus subtilis* in the absence of larval serum in phosphate-buffered saline (PBS).

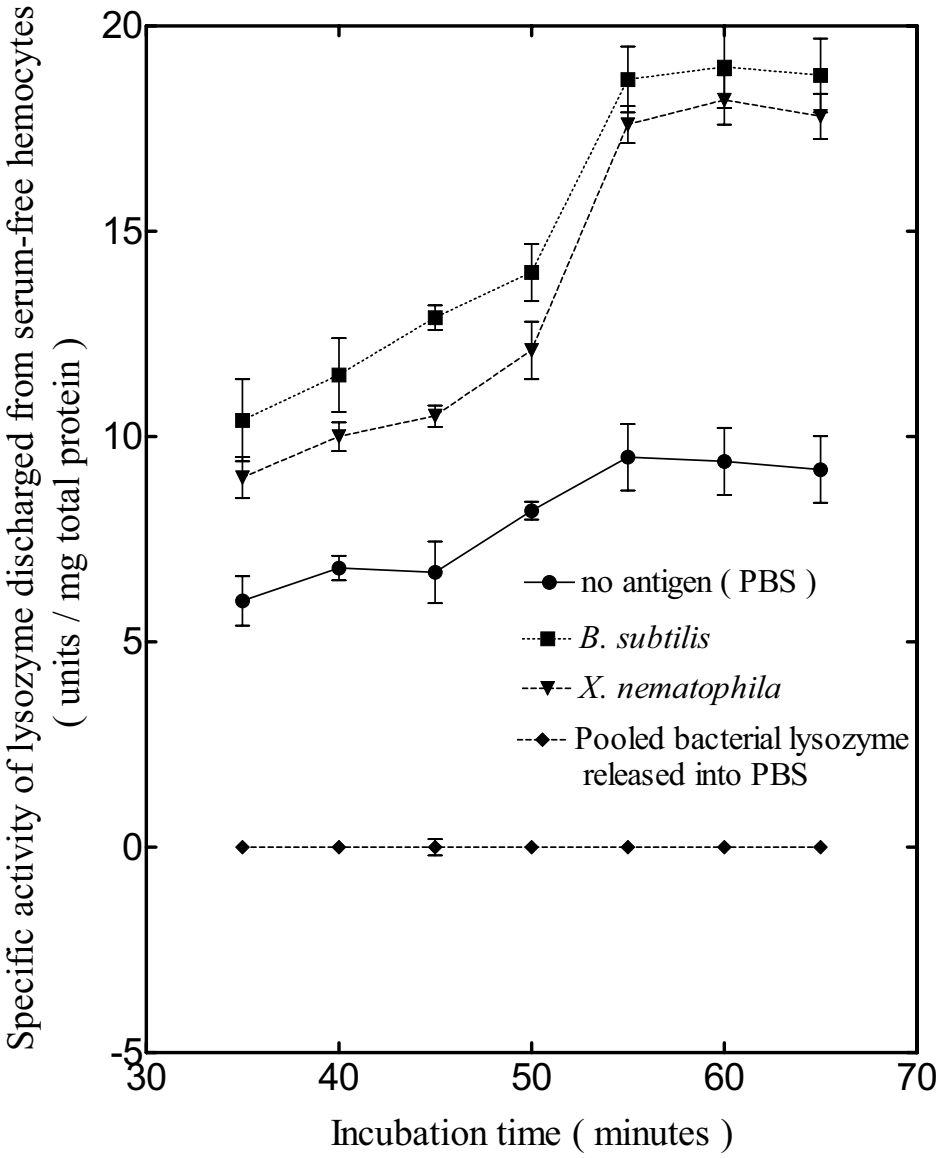
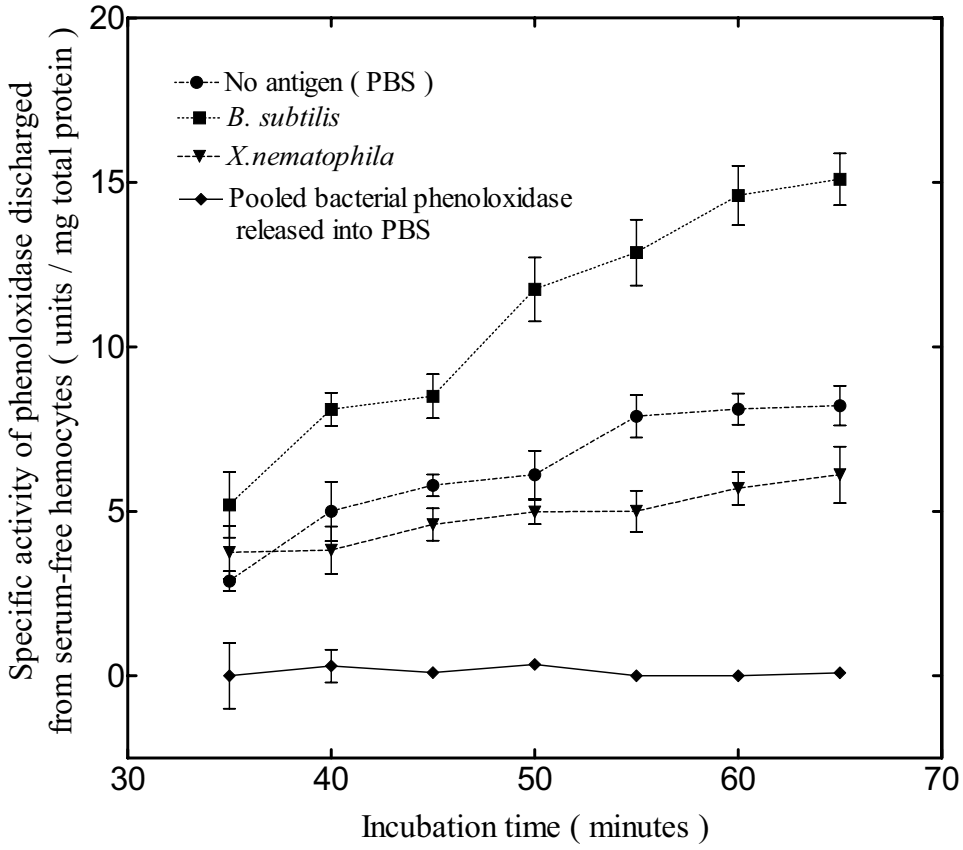

Specific activity of lysozyme discharged from serum-free hemocytes
(units / mg total protein)
20
15
10
5
0
-5
30
40
50
60
70
Incubation time (minutes)
no antigen (PBS)
B. subtilis
X. nematophila
Pooled bacterial lysozyme
released into PBS

Figure 3.3: Phenoloxidase activity in PBS containing *Malacosoma disstria* larval hemocytes incubated with *Xenorhabdus nematophila* or *Bacillus subtilis* bacteria in the absence of larval serum.


Specific activity of phenoloxidase discharged from serum-free hemocytes (units / mg total protein)
Incubation time (minutes)
No antigen (PBS)
B. subtilis
X.nematophila
Pooled bacterial phenoloxidase released into PBS
0
5
10
15
20
30
40
50
60
70

The different levels of hemocyte types with bound bacteria may reflect different amounts of the enzymes binding to the bacteria thus altering their recognition as non-self antigens by the hemocytes. Comparing changes in enzyme activity of serum indirectly adsorbed by bacteria revealed that prolonged incubation of insect serum with the highest concentrations of *X. nematophila* and *B. subtilis* did not affect serum lysozyme activity (*X. nematophila*: 0–60 min = 8.2 ± 0.3 mm – 8.4 ± 0.2 mm; *B. subtilis*: 0–60 min = 8.0 ± 0.4 mm – 7.9 ± 0.3 mm, $n \geq 10$, $P > 0.05$). Also, the activity of purified lysozyme incubated with both bacterial species was not altered (*X. nematophila*: 0–60 min = 9.1 ± 0.2–9.3 ± 0.7 mm, *B. subtilis*: 0–60 min = 8.7 ± 0.2–9.0 ± 0.3 mm, $n \geq 10$, $P > 0.05$). Both bacterial species equally ($P > 0.05$) lowered phenoloxidase levels in a concentration dependent manner (Table 2) implying phenoloxidase may adhere equally to both bacterial species. Bacteria that were previously reacted with the supernatants of the hemocyte monolayers and subsequently washed, produced significant ($P < 0.05$) and comparable ($P > 0.05$) dopachrome formation (*X. nematophila*: 76.2 ± 1.2 U/10^4 bacteria; *B. subtilis* = 85.2 ± 2.2 U/10^4 bacteria; $n = 10$) when incubated in dihydroxyphenylalanine solution, implying similar amounts of phenoloxidase (and possibly phenoloxidase cascade components) bound to the bacteria. Neither control bacterial species exhibited phenoloxidase activity.

Table 3.2: Phenoloxidase activity in *Malacosoma disstria* larval hemocyte lysate after adsorption with bacteria.

Bacterial concentration (cells/ml)	**Phenoloxidase activity (U/mg protein)**[a]	
	B. subtilis	***X. nematophila***
0	$122.7 \pm 15.1^{a,b}$	$134.3 \pm 12.4^{b,b}$
1×10^4	$98.2 \pm 9.5^{b,c}$	$89.3 \pm 11.7^{b,c}$
1×10^6	$61.1 \pm 10.2^{c,d}$	$72.7 \pm 8.5^{c,d}$
1×10^8	$26.5 \pm 7.4^{d,e}$	$35.8 \pm 6.2^{d,e}$
1×10^{10}	$8.3 \pm 2.7^{f,g}$	$6.8 \pm 1.8^{f,g}$

[a] Phenoloxidase activity 30 min post incubation. Mean ± standard error of the mean, $n \geq 10$. Values within a column with the same primary superscript letter were not significantly different, $P > 0.05$. Values between rows with the same secondary superscript were not significantly different, $P > 0.05$.

Incubating *X. nematophila* in serum with active phenoloxidase as opposed to bacteria in PBS, increased the levels of granular cells and plasmatocytes with the bacteria, whereas in serum with tropolone-inhibited phenoloxidase the number of both hemocyte types with *X. nematophila* was comparable to those in PBS ($P > 0.05$; Table 3.1). Active phenoloxidase increased the responding levels of both hemocyte types with *B. subtilis*. Tropolone-inhibited phenoloxidase, although lowering the level of granular cells with *B. subtilis*, did not lower the levels to those in the PBS control groups. Inhibited phenoloxidase lowered the plasmatocyte levels with *B. subtilis* to the PBS control level (Table 3.1). There were no signs of agglutination of either bacterial species in tests with active or inhibited phenoloxidase precluding changes in the hemocyte levels with bacteria representing agglutination artifacts.

Apolipophorin-III when incubated with floating hemocytes before forming hemocyte monolayers, impaired hemocyte adhesion to slides (PBS: granular cells, 154.6 ± 17.1 cells/mm^2; ALP, granular cells, 22.5 ± 11.2 cells/mm^2, $n \geq 10$, $P < 0.05$, PBS: plasmatocytes, 67.2 ± 5.4; ALP, plasmatocytes, 32.5 ± 6.1 cells/mm^2, $n \geq 10$, $P < 0.05$). There was no evidence of hemocyte lysis. ALP equally limited the number of granular cells and plasmatocytes with either bacterial species (Table 3.1). Although this may represent the effect of ALP directly on the hemocytes, binding of the protein to the bacteria may also be a factor. Within 30 min of incubation, ALP bound equally to both bacterial species [based upon the decline in protein with increasing bacterial concentration (Table 3.3)]. Prolonged (2 h) incubation of the protein with the highest concentration of either bacterial species (1×10^{10} bacteria/ml) did not lower the protein concentration (*X. nematophila*: 6.9 ± 0.3 µg/ml, *B. subtilis*: 5.3 ± 0.2 µg/ml) compared with the 30 min results (Table 3.3) ($P > 0.05$) precluding possible bacterial proteolytic enzymes lowering the protein concentration. Bacteria incubated in PBS with and

without ALP, washed in PBS and exposed to hemocytes adhered to different levels of hemocytes. Fewer plasmatocytes had ALP-coated bacteria than non-coated bacteria [*X. nematophila*: PBS, 12.5% (17.5–23.0); ALP 2.1% (6.8–14.6), $n \geq 10$, $P < 0.05$, *B. subtilis*: PBS, 14.2% (22.4–26.1); ALP 5.1% (13.6–19.8), $n \geq 10$, $P < 0.05$]. However, more granular cells had ALP-coated bacteria than did control hemocytes [*X. nematophila*: PBS, 15.2% (21.7–25.2); ALP, 29.1% (27.1–32.6), $P < 0.05$, $n \geq 10$; *B. subtilis*: PBS, 21.3% (22.5–26.4), ALP, 52.1% (46.2–56.5), $P < 0.05$, $n \geq 10$]. There was no indication of bacterial agglutination.

Table 3.3: Binding of a *Malacosoma disstria* heat-stable, 18.7 kDa protein to dead bacteria within 30 min incubation *in vitro.*

Bacterial species	**Bacterial concentration (cells/ml)**	**Non-adsorbed protein remaining in solution (µg/ml)[a,b]**
X. nematophila	0	12.5 ± 0.3[a]
	1×10^4	9.7 ± 0.2[b]
	1×10^6	7.2 ± 0.5[c]
	1×10^8	5.3 ± 9,2[d]
	1×10^{10}	4.1 ± 0.2[e]
B. subtilis	0	12.5 ± 0.3[a]
	1×10^4	10.8 ± 0.2[b]
	1×10^6	9.3 ± 0.1[b]
	1×10^8	6.5 ± 0.4[c]
	1×10^{10}	5.2 ± 0.1[d]

[a] Mean ± standard error of the mean, $n \geq 5$. For a given bacterial species means with the same letters are not significantly different ($P > 0.05$).

[b] Protein from 1×10^{10} bacteria/ml of dead *X. nematophila* and *B. subtilis* in phosphate-buffered saline, pH 6.5, was not detected (0.0 ± 0.5 µg/ml).

Bacteria in the hemolymph *in vivo*

X. nematophila was not removed from the hemolymph during the initial 5 min to 0.5 h post-injection but was removed linearly from 0.5 to 2.5 h post-injection (Figure 3.4). There was no indication of bacterial agglutination. By 4.5 h post-injection the bacterial concentration increased to a plateau level. Larvae receiving *X. nematophila* had a constant hemocyte level similar to the control insects ($P > 0.05$) until 2.5 h post-injection after which a three fold increase in hemocyte counts occurred followed by a decline in hemocyte counts by 4.5 h. Hemocyte viability in the presence of *X. nematophila* decreased rapidly to nearly zero by 3 h post-injection (Figure 2B). Lipopolysaccharide were detected by 30 min post-injection, the amounts rapidly increasing thereafter (Table 3.4). The increase in endotoxin concentration was negatively correlated with hemocyte viability ($r = 0.79$, $P < 0.05$). Hemocyte viability in control larvae was not altered by PBS injections (Figure 3.4) nor were endotoxins detected.

Figure 3.4: The interaction of *Malacosoma disstria* larval hemocytes with injected dead bacteria. The concentration of *Xenorhabdus nematophila* with changes in total hemocyte levels.

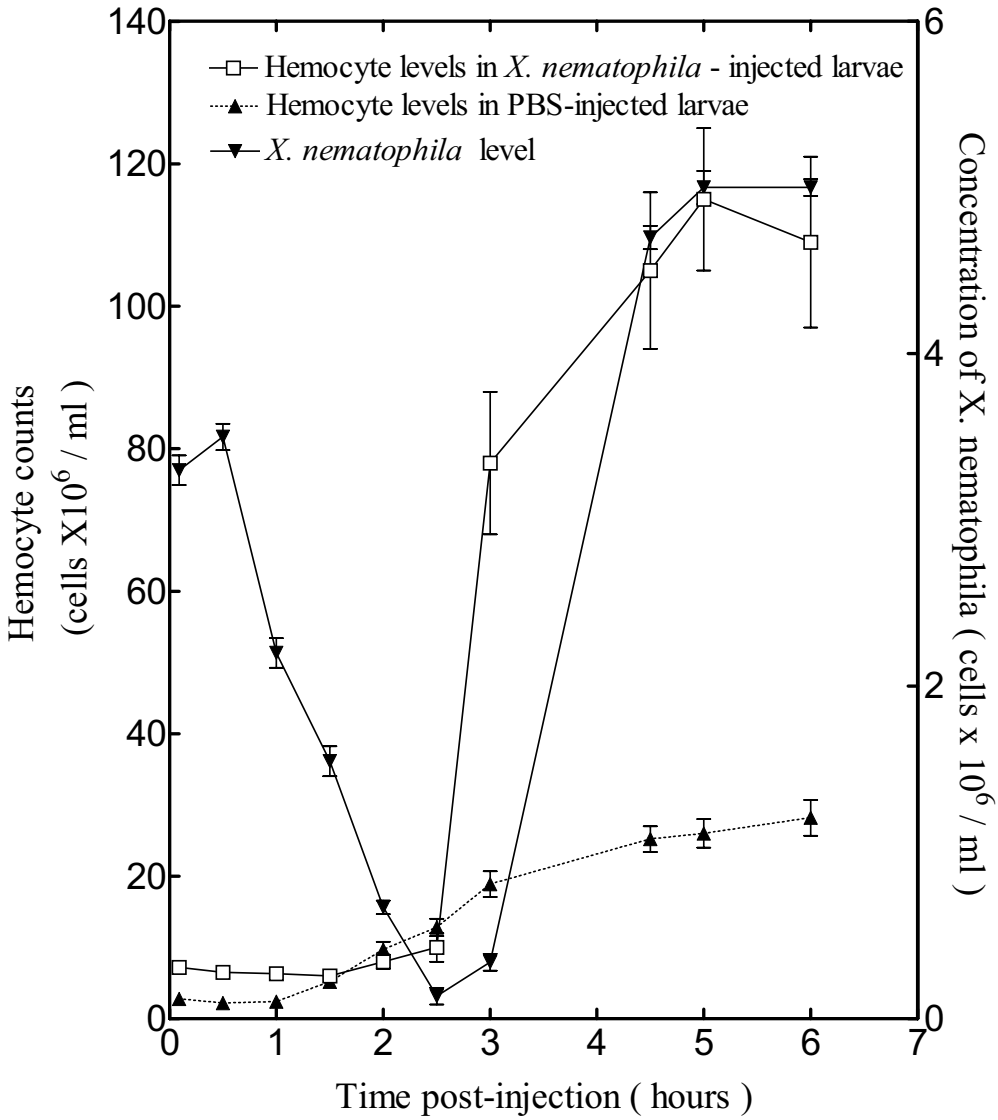

Hemocyte levels in X. nematophila - injected larvae
Hemocyte levels in PBS-injected larvae
X. nematophila level
Hemocyte counts (cells X10^6 / ml)
Concentration of X. nematophila (cells x 10^6 / ml)
Time post-injection (hours)
0
20
40
60
80
100
120
140
0
2
4
6
0
1
2
3
4
5
6
7

Figure 3.5: Effect of injections of *Bacillus subtilis* or *Xenorhabdus nematophila* on hemocyte viability of *M. disstria* larvae.

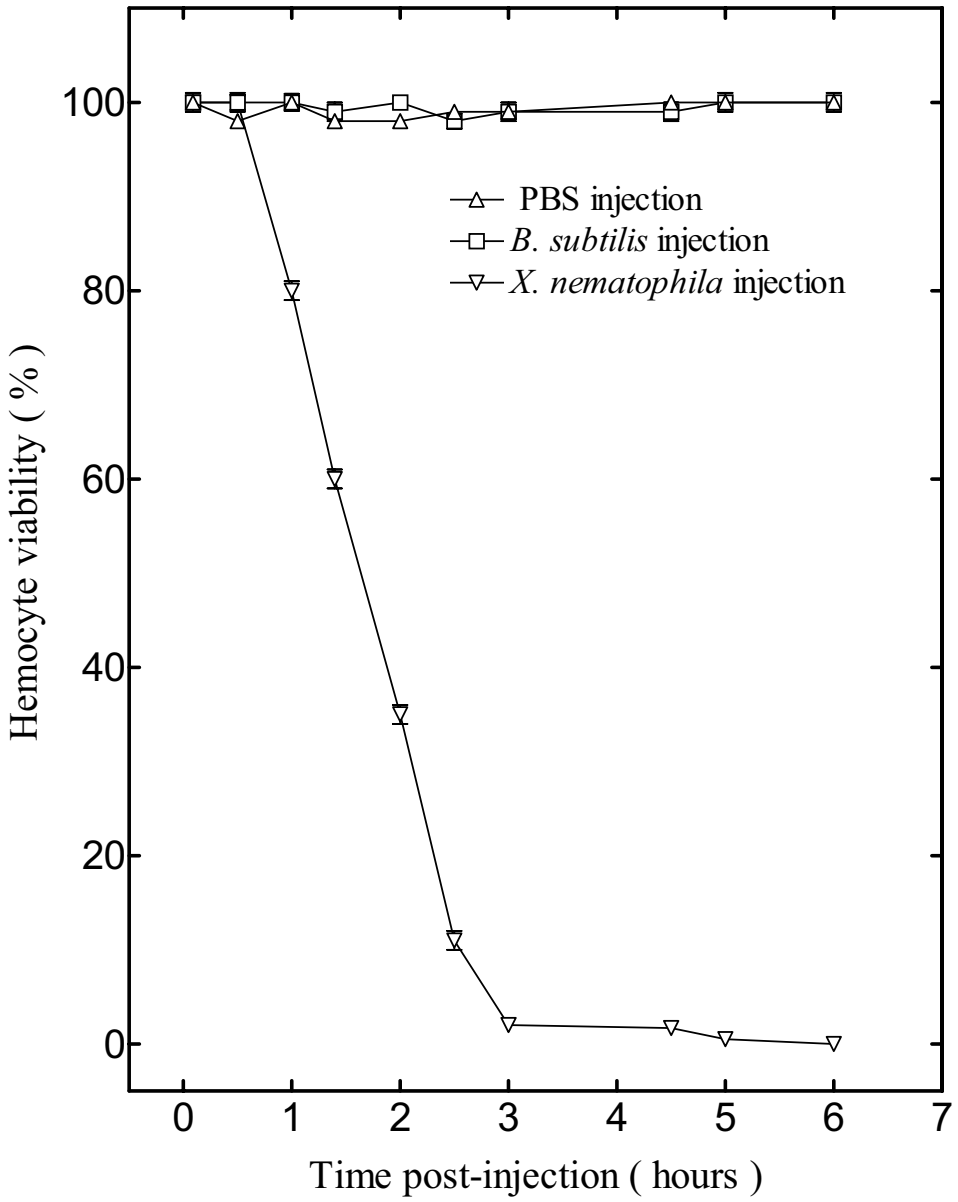

100
80
60
40
20
0
Hemocyte viability (%)
0
1
2
3
4
5
6
7
Time post-injection (hours)
PBS injection
B. subtilis injection
X. nematophila injection

Figure 3.6: Changes in the levels of *Malacosoma disstria* larval hemocytes levels and the concentration of *Bacillus subtilis* following bacterial injection.

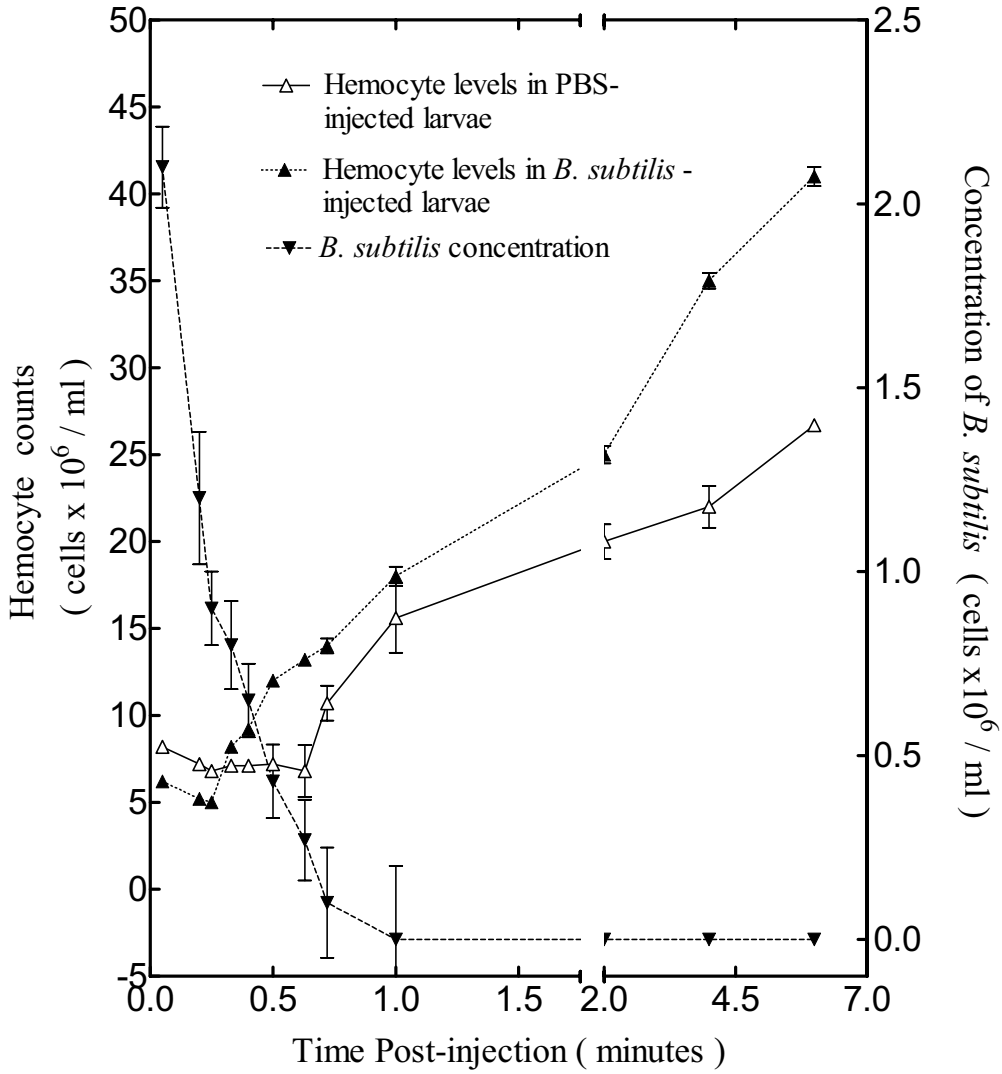

Hemocyte levels in PBS-injected larvae
Hemocyte levels in B. subtilis - injected larvae
B. subtilis concentration
Hemocyte counts (cells x 10^6 / ml)
Concentration of B. subtilis (cells x10^6 / ml)
Time Post-injection (minutes)
50
45
40
35
30
25
20
15
10
5
0
-5
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
4.5
7.0

Table 3.4: Lipopolysaccharide released from dead *Xenorhabdus nematophila in vivo.*

Time post-injection (min)	**LPS level[f] (ng/10 µl)**
0	0.0 ± 0.1[a]
15	2.7 ± 1.9[a]
30	28.2 ± 10.1[b]
60	456.1 ± 10.2
120	972.3 ± 62.1[d]
180	1191.7 ± 108.2[e]

[f] Mean ± standard error of the mean, n = 10. Values within a column with the same primary letter superscript were not significantly different, $P > 0.05$.

Larvae injected with *X. nematophila* did not show induced antibacterial activity between 0 and 6 h post-injection ($n = 15$, $P > 0.05$), but did by 12 h post-injection (105.2 ± 0.9 µg gentamycin equivalents/ml, $n = 10$, $P < 0.05$) with maximum activity (116.7 ± 1.2 µg gentamycin equivalents/ml, $n = 10$, $P < 0.05$) occurring by 24 h. An increase in lysozyme activity was detected by 6 h post-injection (0 h = 8.5 ± 0.3 mm, 4.5 h = 9.2 ± 1.1 mm, 6.0 h = 19.8 ± 0.7 mm, $P > 0.05$; 24 h = 25.2 ± 1.7 mm, $n \geq 10$, $P < 0.05$). Adding suspensions of washed *X. nematophila* previously removed from larvae 4.5 h post-injection to Luria broth did not reveal a change in suspension turbidity ($OD_{600\ nm}$ 0 h = 0.01 ± 0.5; 96 h = 0.00 ± 0.05, $P > 0.05$, $n = 10$) indicating no bacterial growth; no colonies formed from bacterial suspensions plated on Luria agar. Thus, the increase in *X. nematophila* following the initial decline in bacteria did not represent a few live bacteria (injected with dead bacteria) multiplying early in the infection and subsequently being held in check by induced antibacterial proteins.

Dead *X. nematophila* decreased the darkening of hemolymph throughout the incubation time in concert with a decline in phenoloxidase activity (Table 3.5). By 3 h post-injection, hemolymph darkening did not occur even after incubation with the phenoloxidase activator, laminarin, for 24 h at 25 °C. There was a significant negative correlation between lipopolysaccharide levels (Table 3.4) and phenoloxidase activity ($r = 0.86$, $P < 0.05$). Control hemolymph (without *X. nematophila*) by 1 min post-injection with the phenoloxidase activator exhibited dophachrome formation, the effect increasing with time (phenoloxidase activity: 1 min, 12.6 ± 0.3 U/mg total protein, 3 h, 67.1 ± 1.7 U/mg total protein, $n \geq 15$, $P < 0.05$).

Table 3.5: Effect of *Xenorhabdus nematophila* injected into larval *Malacosoma disstria* on hemolymph phenoloxidase activity

Time post-injection (min)	**Phenoloxidase activity (U/mg protein)**[a]	
	Bacteria[b]	**PBS**[b]
0	16.2 ± 0.3[a]	14.7 ± 0.8[a]
15	14.7 ± 0.2[a]	21.1 ± 1.1[b]
30	10.1 ± 0.4[b]	32.1 ± 2.1[c]
60	1.3 ± 0.3[c]	45.3 ± 2.7[d]
120	0.4 ± 0.3[c]	52.1 ± 1.5[d]
180	0.0 ± 0.2[d]	73.6 ± 2.7[e]

[a] Mean ± standard error of the mean, $n > 10$. Values within a column with the same superscript are not significantly different, $P > 0.05$.

[b] Bacteria injected into the larvae 1×10^9 bacteria in 10 µl of PBS. PBS injection served as the negative control.

Dead *B. subtilis* were also removed from the hemolymph (Figure 3.6), bacterial concentration declining rapidly shortly after injection until 2 h post-injection when they were no longer visual. There was no evidence of bacteria emerging into the hemolymph by 6 h post-injection. In larvae with *B. subtilis*, the total hemocyte counts declined until 25 min post-injection and increased thereafter to values greater than larvae injected with PBS alone (Figure 3.6). Total hemocyte counts did not change in larvae injected with PBS until 0.75 h. There was no discernible change in hemocyte viability (Figure 3.5). Phenoloxidase activity was detected in hemolymph samples with *B. subtilis* by 5 min post-injection (phenoloxidase activity: 0 min, 0.5 ± 0.2 U/mg protein; 5 min, 7.1 ± 0.8 U/mg protein, $n = 10$, $P < 0.05$) without the use of laminarin. Enzyme activity increased to toxic levels by 3 h post-injection (85.2 ± 0.9 U/mg protein, $n = 10$); the larvae darkened substantially and exhibited sluggishness. Melanized nodules were commonly detected around the injection site and on the visceral fat body.

Both bacterial species, coated with ALP, were removed more slowly from the hemolymph by 1 h post-injection compared with non-coated bacteria (*X. nematophila* removal rate: non-coated, $1.0 \pm 0.2 \times 10^6$ bacteria/ml/min; ALP coated bacteria, $0.3 \pm 0.2 \times 10^6$ bacteria/ml/min; *B. subtilis* removal rate: non-coated, $4.1 \pm 0.3 \times 10^6$ bacteria/ml/min; ALP coated bacteria, $2.7 \pm 0.2 \times 10^6$ bacteria/ml/min, $n = 8$, $P < 0.05$).

DISCUSSION

Responses of *M. disstria* hemocytes towards *X. nematophila* and *B. subtilis* differed in terms of the levels of granular cells and plasmatocytes with adhering bacteria *in vitro* and the rate and pattern of bacterial removal from the hemolymph *in vivo*. Differences in antibacterial responses are attributed to bacterial species and virulence levels in *M. sexta* (Howard *et al.*, 1998; Horohov and Dunn, 1982) and in *G. mellonella*, to bacterial species with different surface antigens (Dunphy *et al.*, 1986; Dunphy and Webster, 1984; Dettloff *et al.*, 2001) that may react with different types of hemocyte receptors (Dettloff *et al.*, 2001). Specific hemocyte membrane receptors directly reacting with the bacteria could include peptidoglycan receptor proteins, long chain (PGRP-LC), which bind to peptidoglycan (Dziarski and Gupta, 2006). Pattern recognition receptors that react with lipoteichoic acid, lipopolysaccharide and/or β-1,3 glucans as occur with *B. mori* hemocytes (Ohta *et al.*, 2006), may occur on *M. disstria* hemocytes because the microorganisms bound to the hemocytes in the absence of larval plasma.

Antigen stimulation of *M. disstria* hemocytes by the slides resulted in the discharge of proteins, including lysozyme and phenoloxidase, from the hemocytes. Similar results are known for hemocytes of other species of insects (Gillespie *et al.*, 1997) and crustaceans (Söderhall and Cerenius, 1998; Sugumaran and Nellarappan, 1991). Phenoloxidase and lysozyme enhance bacterial nodulation and phagocytosis, respectively (Dunphy and Webster, 1991; Söderhall and Cerenius, 1998). Although the two bacterial species in the present study induced similar levels of discharged total protein, the bacteria adhered to different levels of hemocytes which may indicate the selective discharge of adhesive proteins including the enzymes phenoloxidase and/or lysozyme by subpopulations of hemocytes.

Compared with control hemocytes phenoloxidase activity increased more rapidly during hemocyte stimulation by *B. subtilis* than by *X. nematophila*. The increase with *B. subtilis* may represent increased activation of prophenoloxidase in the serum and/or increased discharge and activation of zymogenic phenoloxidase from the hemocytes. The latter two possibilities were supported by increased enzyme activity in monolayers without insect serum. Low phenoloxidase activity from hemocytes with adhering *X. nematophila* may reflect either less zymogen discharge and/or impaired activation of phenoloxidase. Blocking enzyme activation is known to occur in *G. mellonella* (Dunphy and Webster, 1991) and the turnip moth, *Agrotis segetum* (Yokoo *et al.*, 1992). Herein, as for *G. mellonella* (Dunphy and Webster, 1991), active phenoloxidase is not inhibited by *X. nematophila*. However, prolonged incubation of *X. nematophila* with fresh serum inhibited phenoloxidase activation. Thus, low phenoloxidase activity in *M. disstria* serum reflects impaired activation which may contribute to low levels of hemocytes with the bacteria. Discharged phenoloxidase caused by both bacterial species may be greater than actually recorded by enzyme activity because the enzyme bound to both species and *X. nematophila* inhibited zymogenic activation.

The tentative contribution of discharged phenoloxidase and/or its byproducts to bacterial-hemocyte attachment is implied by correlational analysis in which enzyme activity is linked to bacterial-hemocyte adhesion as opposed to differential binding of the enzyme to the bacterial species. Larval serum contributes to the activation of phenoloxidase on *M. sexta* hemocytes (Ling and Yu, 2005). However, if this occurred during the formation of *M. disstria* hemocytes, prior to washing the serum from the hemocytes, it is unlikely hemocytic surface phenoloxidase contributed to the different bacteria-hemocyte binding patterns because the enzyme binds equally to both *B. subtilis* and *X. nematophila*. The relationship of bacteria-hemocyte binding and discharged enzyme is further established using tropolone, a

phenoloxidase-specific inhibitor, that substantially lowers the percentage of both hemocyte types with adhering *X. nematophila* and plasmatocytes with *B. subtilis*. For *G. mellonella*, granular cell adhesion to glass and phagocytosis of silica beads is linked to phenoloxidase and/or its activation cascade (Tojo *et al.*, 2000). The levels of *M. disstria* granular cells with *B. subtilis*, although lowered by tropolone, were still greater than the control groups implying other humoral factors such as soluble C-type lectins (Kanost *et al.*, 2004), peptidoglycan recognition proteins (Dziarski and Gupta, 2006) and/or lysozyme may participate in hemocyte reactions to the bacteria. Lysozyme increases *Bacillus cereus* adhesion to hemocytes of the cockroach, *Blaberus discoidalis* (Wilson and Ratcliffe, 2000). Purified lysozyme and lysozyme interacting synergistically with insect plasma carbohydrases increase *X. nematophila* hemocyte interaction in *G. mellonella* (Dunphy and Webster, 1991). Herein, both bacterial species elevate the discharge of hemocyte lysozyme, *B. subtilis* being more effective than *X. nematophila*. Because neither bacterial species incubated with a fixed amount of lysozyme in serum or purified lysozyme altered enzyme activity, lysozyme may have differentially increased bacteria–hemocyte binding by altering to different extents the structure of the outer membrane of *X. nematophila* and the peptidoglycan of *B. subtilis* as opposed to different amounts of enzyme irreversibly binding to the bacteria. The increased bacteria–granular cell reactions would then represent exposing recognized antigens and/or removing immunosuppressive antigens. For *M. disstria*, the effect of lysozyme on bacterial adhesion to hemocytes may represent both the different rates of lysozyme release from the hemocytes and/or different extents of digestion of the bacterial surfaces.

Apolipophorin-III affected bacteria binding to hemocytes, the extent varying with the nature of antigen presentation. Exposure of hemocytes without bacteria to ALP impaired hemocyte adhesion to slides. If this represents cellular shut-down of the non-self response, it explains the limited binding of bacteria to hemocytes. When ALP bound to the bacteria the

extent of hemocytes with bound bacteria differed from the previous experiment and may represent a change in ALP conformation altering its impact on the hemocytes, the mechanism(s) of adhesion varying with the hemocyte type. The protein increased granular cell attachment to bacteria in *G. mellonella* while impairing bacteria attachment to the plasmatocytes (Zakarian *et al.*, 2002). Diminished *M. disstria* plasmatocyte levels with bacteria may reflect impaired plasmatocyte activity but not reduced availability of bacteria due to bacterial reaction with increased numbers of granular cells because the total number of bacteria was in excess of those bound to the latter hemocytes.

In the presence of ALP-free bacteria, phenoloxidase may not have participated in the reactions of ALP-treated hemocytes because lipoteichoic acid of *B. subtilis* reacting with the laccase in the presence of ALP in *G. mellonella* impair phenoloxidase activation (Halwani *et al.*, 2000). Lysozyme modifies the cell walls of bacteria including *B. subtilis* (Selsted and Martinez, 1978) and *X. nematophila* (Dunphy and Webster, 1991) increasing bacteria–hemocyte contact (Dunphy and Webster, 1991). Although ALP of *G. mellonella* potentiates the effect of lysozyme on bacterial cell walls (Halwani and Dunphy, 1999), the effect of lysozyme with ALP on procaryotic reactions to *M. disstria* granular cells was not determined. ALP reactions with live *X. nematophila* and *B. subtilis* compared with their dead counterparts may vary, since other live bacterial species such as *Pseudomonas aeruginosa* enzymatically degrade ALP via protease IV in *G. mellonella* hemolymph (Andrejko *et al.*, 2008).

Xenorhabdus nematophila was removed from *M. disstria* hemolymph *in vivo* even as the total number of damaged hemocytes increased and hemocyte viability decreased. This removal was due not to bacterial agglutination but to bacterial adhesion to hemocytes and some nodulation. Hemocytic removal of and damage by live *X. nematophila* in *S. exigua* (Park and Kim, 2000) and live and dead *X. nematophila* in *G. mellonella* (Dunphy and

Webster, 1988) are similar to the results with dead bacteria in *M. disstria*. Dead *X. nematophila* damaged *M. disstria* hemocytes implying a bacterial surface component was the hemocytotoxin. That (i) endotoxins were detected in *M. disstria* hemolymph with *X. nematophila* in concert with an increase in damaged hemocytes and (ii) *X. nematophila* lipopolysaccharide damage lepidopteran insect hemocytes (Dunphy and Webster, 1988) implies the hemocytotoxin may be lipoplysaccharides as opposed to possible peptidoglycan fragments [which initiate nodulation in lepidopterans (Yu *et al*., 2002) and orthopterans (Brookman *et al*., 1989)]. Research has shown that injections of *X. nematophila* endotoxin into *M. disstria* elevates damaged hemocyte counts and the early emergence of the bacteria into the hemolymph support this contention (see chapter 4). Herein, *B. subtilis* was rapidly and irreversibly removed from the hemolymph by nodulation. However, inexplicably, ALP slowed the removal of both bacterial species even as it enhanced bacteria–granular cell contact.

In summary, the differential interaction of *X. nematophila* and *B. subtilis* with the granular cells and plasmatocytes of larval *M. disstria in vitro* was linked to (1) different extents of lysozyme discharge and possible bacterial sensitivity to the enzyme and (2) phenoloxidase activation and/or the product(s) of the enzyme. ALP, when incubated with hemocytes inhibited hemocyte adhesion to slides but when bound to bacteria, the protein impaired bacterial adhesion to the plasmatocytes and increased bacterial reaction with the granular cells. Dead bacterial species without bound ALP were differentially removed from the hemolymph by nodulation. ALP, when bound to the bacteria, slowed the bacterial removal rates but *B. subtilis* was still more rapidly removed than *X. nematophila*. Whereas *B. subtilis* was rapidly and effectively nodulated, *X. nematophila* was more slowly nodulated and emerged from nodules and hemocytes as the levels of damaged hemocytes increased. A hemocytotoxin (maybe lipopolysaccharide) of *X. nematophila* was released into the hemolymph from the outer membranes of

the dead bacteria.

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CONNECTING STATEMENT – II

In the previous chapter, the reactions of *M. disstria* larval hemocytes with intact dead bacterial cells were determined using *in vitro* and *in vivo* assays. Different responses occurred with the Gram-negative entomopathogenic *X. nematophila* and Gram-positive non pathogenic *B. subtilis*. Recognizing these responses represented interactions of the hemocytes with bacterial surface antigens, the role of major *X. nematophila* and *B. subtilis* surface antigens on non-self hemocyte activities was investigated. In the following chapter *X. nematophila* lipopolysacharide, *X. nematophila* lipid A, and *B. subtilis* lipotechoic acid were used to determine if these antigens are responsible for the reactions observed by *M. disstria* hemocytes under *in vitro* and *in vivo* conditions in the presence of the intact bacteria.

CHAPTER 4

SURFACE ANTIGENS OF *Xenorhabdus nematophila* (F. Enterobacteriaceae) AND *Bacillus subtilis* (F. Bacillaceae) REACT WITH ANTIBACTERIAL FACTORS OF *Malacosoma disstria* (Lepidoptera: Lasiocampidae) HEMOLYMPH

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ABSTRACT

Previous research established different interactions of the insect pathogen, *Xenorhabdus nematophila* and nonpathogen, *Bacillus subtilis*, with the innate antimicrobial hemocytes and humoral factors of larval *Malacosoma disstria* (Giannoulis *et al*., 2007); the antimicrobial systems were inhibited by *X. nematophila* and stimulated by *B. subtilis*. The bacterial surface antigens participating in these reactions were unknown. Thus, herein the effects of lipopolysaccharide (endotoxin) from *X. nematophila* and lipoteichoic acid from *B. subtilis* on the larval *M. disstria* immune factors, the hemocytes and phenoloxidase, were determined. Endotoxin elevated the level of damaged hemocytes limiting the removal of *X. nematophila* from the hemolymph and enhancing the rapid release of bacteria trapped by nodulation. Similar effects were observed with the lipid A moiety of the endotoxin. The effects of lipopolysaccharide and lipid A on the hemocyte activities were abrogated by polymyxin B (an antibiotic that binds to lipid A) confirming lipopolysacharide as the hemocytotoxin by virtue of the lipid A moiety. Lipoteichoic acid elicited nodulation and enhanced phenoloxidase activation and/or activity. Although lipoidal endotoxin and lipid A inhibited phenoloxidase activation they enhanced the activity of the previously activated enzyme. Apolipophorin-III precluded the effects of lipopolysaccharide, lipid A and lipoteichoic acids on the hemocytes and prophenoloxidase until the antigens exceeded a critical threshold.

Key words: *Xenorhabdus nematophila, Bacillus subtilis, Malacosoma disstria*, lipoteichoic acid, lipopolysaccharide, hemocytes

INTRODUCTION

The interaction of lepidopteran antimicrobial systems with microorganisms has been extensively studied in *Manduca sexta*, *Bombyx mori* and *Galleria mellonella* (Gillespie *et al*., 1997). The salient lepidopteran humoral element examined most frequently is phenoloxidase, a melanizing enzyme (Leonard *et al*., 1985), which alone or in conjunction with its reaction products, may be bactericidal and opsonic (Yu *et al*., 2002). Other humoral factors include lysozyme (a cationic, constitutive plasma protein) with pattern-recognition (Wilson and Ratcliffe, 2000; Lee, 2004) and antibacterial activity (Lockey and Ourth, 1996), C-type lectins including immulectins-2,-3 (Yu and Ma, 2006) and -4 (Yu *et al*., 2006), β-1,3-glucan binding proteins (see Yu and Kanost, 2002), hemolin (Bao *et al*., 2007; Eleftherianos *et al*., 2007), apolipophorin-III (Halwani *et al*., 2000) and interactions of these components with lipophorin particles (Rahman *et al*., 2006). Many of these factors bind to molecular antigens as part of cell-free immunity (Rahman *et al*., 2006; Ma *et al*., 2006) and participate in hemocyte adhesion reactions (Schmidt and Schreiber, 2006).

The main lepidopteran hemocyte types responding to foreign materials are the plasmatocytes and granular cells, which, depending on the insect species and bacterial species and concentration (Howard *et al*., 1998) participate in phagocytosis (Tojo *et al*., 2000) and nodulation (Ratcliffe *et al*., 1985). Nodulation is a biphasic activity in which direct bacterial contact with granular cells and/or their receptors (Ohta *et al*., 2006) or indirect contact with humoral factors and hemocytes elicit the discharge of sticky extracellular matrix proteins (Beckage, 2008). The proteins increase microbial and granular cell entrapment and by binding to RGD-integrin receptors on plasmatocytes, activate plasmatocyte adhesion to the granular cell-bacterial mass producing nodules (Levin *et al*., 2005; Nardi *et al*., 2005). Antigen adhesion to lepidopteran hemocytes may involve the signal transduction enzymes protein

kinase A (Brooks and Dunphy, 2005) and C (Zakarian *et al*., 2003) and actin cytoskeleton activity (Glatz *et al*., 2004). Antigen-hemocyte adhesion and subsequent antimicrobial activities may occur also by signal-dependent, leverage-mediated mechanisms (Schmidt and Schreiber, 2006) or by signal-independent scavenger receptors like those on hemocytes of the dipteran, *Drosophila melanogaster* (Franc *et al*., 1999).

Apolipophorin-III, a multifunctional, non-glycosylated, protein that is highly conserved within the Lepidoptera (Kim *et al*., 2004), participates in both cell-free and hemocyte-mediated antimicrobial responses (Yang *et al.,* 2005). At the humoral level, this β-1,3-glucan binding protein (Dunphy *et al*., 2003; Whitten *et al*., 2004) impairs phenoloxidase activation by binding to soluble and bacterial bound lipoteichoic acids (Halwani *et al*., 2000). Lipoteichoic acids are polyglycerol phosphate compounds with 1,3-phosphodiester linked glycerol units, isolated from the cell membranes of Gram-positive bacteria (Hammond *et al*., 1984). Apolipophorin-III blocks also the inhibition of phenoloxidase activation by endotoxin (lipopolysaccharide) release from entomopathogenic *X. nematophila* (Dunphy and Webster, 1988 a,b). Lipopolysaccharide, from the outer membrane of Gram-negative bacteria, is composed of an outer core of polymerized heptoses linked to polysaccharides of varying chain lengths (O-side chains); the outer core is bound to an inner core of two or three 3-deoxy-D-manno-octulosonic acid residues, which, in turn binds to the outer membrane by lipid A, a glucosaminyl-glucosamine dissaccharide containing esterified and amidated fatty acids (Hammond *et al*., 1984). Hydrophobic apolipophorin-III potentiates also lysozyme activity (Halwani and Dunphy, 1999) and induced-cecropin (Weisner *et al*., 1997) activity (Park *et al*., 2005b) against bacteria. *Heliothis virescens* larvae and pupae (Chung and Ourth, 2002), and larvae of *Hyphantria cunea* (Kang and Seo, 2004) and *G. mellonella* (Andrejko *et al*., 2005) elevate apolipophorin-III upon vaccination, activating cell-free immunity by lysozyme and cecropin production (Kim *et al*., 2004).

Apolipophorin-III is both taken up from and released into the plasma (Ferkowich *et al*., 1995) and constitutively produced by hemocytes and stored in hemocyte granules (Kim *et al*., 2004). It enhances yeast phagocytosis in *G. mellonella* (Wiesner *et al*., 1997) and when loaded with lipid inhibits hemocyte attachment and spreading in the short-term and in prolonged association with plasmatocytes enhances nodulation (Whitten *et al*., 2004). Apolipophorin-III both slows bacterial removal from *G. mellonella* hemolymph *in vivo* and enhances bacterial adhesion to a subpopulation of granular cells *in vivo*, the protein binding to both hemocytes and bacteria. In the case of the latter, binding exhibits temporal dissociation (Zakarian *et al*., 2002), the protein acting as a counter adhesion molecule (Schmidt and Schreiber, 2006). Many of the immulectins and hemolin in conjunction with lipophorins of Lepidoptera neutralize endotoxins (Ma *et al*., 2006). In *G. mellonella* apolipophorin-III neutralizes the toxicity of *X. nematophila* endotoxin on hemocytes by binding to the lipid A moiety (Dunphy and Halwani, 1997) and 2-keto-deoxyoctonate (Limura *et al*., 1998). Like other immunological factors, apolipophorin-III is limited in effects at critical levels, *X. nematophila* endotoxin can cause hemocyte damage indicating apolipophorin-III exhaustion (Halwani *et al*., 2002). Additionally, limited effectiveness is evident during development of *Pseudomonas aeruginosa* septicemia in *G. mellonella*, the protein in hemocytes declining (possibly secreted) during infection while the plasma apolipophorin-III concentration increases out by 24 h post-infection antigenic fragments comparable to those produced by *P. aeruginosa* protease IV digestion of apolipophorin-III *in vitro* (Andrejko *et al*., 2005) are detected *in vivo* (Andrejko *et al*., 2007).

Live *X. nematophila* and related species inhibit phenoloxidase and hemocyte activity in lepidopterans within one hour of infection by inhibiting phospholipase A_2 and hence eicosanoid biosynthesis (Park and Kim, 2000; Park *et al*., 2005a; Kim *et al*., 2005; Park and Stanley, 2006). Production of protease II by *X. nematophila*, which is capable of digesting cecropin

antibacterial proteins in *G. mellonella* and *Pseudaletia unipuncta*, does not harm hemocytes (Caldas *et al*., 2002), although the bacteria release metabolites inducing hemocytic apoptosis in *B. mori* (Cho and Kim, 2004). Homoserine lactone has been implicated in aspects of the later stages of pathogenesis (Dunphy *et al*., 1997). Bacterial tolerance of host defences is part of the early stages of bacterial pathogenesis (Maxwell *et al*., 1995).

Live or dead, the phase one form of *X. nematophila* limits also phenoloxidase activation and hemocyte activities by releasing lipopolysaccharide (Dunphy and Webster, 1988a) which chelates divalent cations; Ca^{+2} in *Agrotis segetum* (Yokoo *et al*., 1992) and Fe^{+2} in *G. mellonella* (Dunphy *et al*., 2002). Lipid A, binding to N-acetyl-D-glucosamine hemocyte receptors, damages *G. mellonella* hemocytes releasing entrapped bacteria (Dunphy and Webster, 1988 a,b) and iron-binding proteins, the latter, in concert with iron-chelation by the endotoxin, potentially limits bacterial growth (Dunphy *et al*., 2002). In contrast, lipoteichoic acid from *B. subtilis* produces nodules, depletes plasmatocyte levels and damages granular cells while activating phenoloxidase in *G. mellonella* larvae (Halwani *et al*., 2000).

Little is known about the interaction of larval *M. disstria* hemocytes with microorganisms including polydnaviruses (Stoltz and Guzo, 1986), and the bacteria *X. nematophila* and *B. subtilis* (Giannoulis *et al*., 2007) or with inert foreign matter (Giannoulis *et al*., 2005). Adhesion of *M. disstria* hemocytes to glass is enhanced by extracellular calcium and the inactivation of the signal transduction enzymes, protein kinase C and cyclic AMP-dependent protein kinase A (Giannoulis *et al*., 2005). Germain to the adhesion of established cell lines of *M. disstria* hemocytes is the production of extracellular matrix proteins, avid adhesion possibly reflecting the ability of the cells to inhibit matrix degrading proteases (Keddie *et al*., 1995).

Artificially activated phenoloxidase, adhering equally to dead *X. nematophila* and *B. subtilis*, and interacting synergistically with lysozyme, increases the adhesion of both bacterial species to *M. disstria* granular cells *in*

vitro (Giannoulis *et al*., 2007). However, in the normal situation in *M. disstria*, *X. nematophila* inhibits phenoloxidase activation (Giannoulis *et al*., 2007), whereas *B. subtilis* activates phenoloxidase (Giannoulis *et al*., 2007). This results in enhanced nodulation and removal of *B. subtilis* from the hemolymph compared with the diminished responses to *X. nematophila*. In part, hemocyte antigenic responses may reflect increased levels of the biogenic amine, octopamine, which increases intracellular cyclic AMP in the *M. disstria* hemocyte cell line, Md66 (Gole *et al*., 1982). Octopamine acts as a bifunctional molecule binding to bacteria and lepidopteran hemocytes (Dunphy and Downer, 1994). *Malacosoma disstria* apolipophorin-III increases the number of granular cells with bacteria *in vitro* and slows bacteria removal from the hemolymph *in vivo* (Giannoulis *et al*., 2007). Unlike *B. subtilis*, which induces a nodulation-mediated decline in both bacterial and hemocyte counts, *X. nematophila*, after limited nodulation and phagocytosis, emerges into the hemolymph in parallel with an increase in damaged hemocyte levels and lipopolysaccharide concentration (Giannoulis *et al*., 2007). The types of antigen(s) responsible for the hemocytic and phenoloxidase responses to both bacterial species, until now, were not known for *M. disstria* larvae.

Using antigens from *B. subtilis* and the phase one form of *X. nematophila* [the form released from *S. carpocapsae* into the insect hemocoel (Akhurst, 1980)], bacterial surface molecules eliciting the hemocyte-bacteria interactions previously described (Giannoulis *et al*., 2007) are reported. Endotoxin impaired phenoloxidase activation and hemocyte activity whereas lipoteichoic acid enhanced both factors once the antigens exceeded a threshold. The threshold represented neutralization of the bacterial surface antigens by an 18.7 kDa plasma protein identified as apolipophorin-III.

MATERIALS AND METHODS

Bacteria, lipopolysaccharide, lipid A and lipoteichoic acid

The phase one form of *X. nematophila* 19061 was used. Phase one was confirmed by the blue colony coloration produced by the bacteria on Luria agar supplemented with triphenyl-tetrazolium chloride (30 μg/ml) and bromthymol blue (25 μg/ml), the presence of proteolytic activity and antibiotic production (Akhurst, 1980). *B. subtilis* (Boreal Biological Co., Ont., Canada) was cultured on Luria agar. Both species were incubated at 25 ^{o}C in darkness and subcultured biweekly.

For lipopolysaccharide extraction, *X. nematophila* was grown in 50 ml Luria broth in 250 ml Erlenmeyer flasks on a horizontal gyratory shaker (250 rpm) at 28 ^{o}C until the bacteria reached the mid-exponential phase of the growth cycle (optical density at 660 nm ≈ 0.75). Bacteria were washed three times by centrifugation (12,000 x g, 2 min, 25 ^{o}C) from and resuspension into 50 ml phosphate-buffered saline (PBS, 138 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2.8 mM KH_2PO_4, adjusted to pH 6.5 with 5M HCl) and once with endotoxin-free distilled water. Bacterial suspensions were frozen at -80 ^{o}C and lyophilized. Lipopolysaccharide was obtained from isolated outer membranes. Outer membranes, prepared from lyophilized bacteria by means of Sarkosyl extraction and centrifugation (100,000 x g, 1 h, 4 ^{o}C), were subsequently lyophilized. Lipopolysaccharide, which were extracted with phenol-water (1:1, v/v, Hancock and Poxton, 1988), contains little peptidoglycan (<1%) (Deslauriers *et al*., 1990). Lipopolysaccharide (1 mg) was partially purified by incubation with 20 μg each of DNase I and RNase A in PBS (5 ml). The lipopolysaccharide was re-extracted with phenol-water, inactivating the nucleases, and pelleted by centrifugation (100,000 x g, 3.5 h, 4 ^{o}C). Endotoxin was suspended in lipopolysaccharide-free distilled water and stored at -80 ^{o}C until required. Lipid A was cleaved from lipopolysaccharide

suspension by acid hydrolysis (1% acetic acid, v/v, 100 °C, 1 h) and isolated by centrifugation (20,000 x g, 1 h, 4 °C). Subsequently the pellet was extracted with chloroform followed by aqueous washing of the chloroform extract (Dunphy and Webster, 1988a).

B. subtilis lipoteichoic acid was purchased (Sigma-Aldrich Chemical Co., Ont., Canada).

Insect rearing

Laboratory-reared *M. disstria* (supplied by the Canadian Forestry Service, Sault Ste. Marie, Ont., Canada) was maintained on a casein dextrose diet at 25 °C (Addy, 1969). Fourth instar larvae, 3 days into the stadium, were selected, unless otherwise stated.

Endotoxin, lipid A and lipoteichoic acid reacting with hemocytes and phenoloxidase *in vitro*

Endotoxin [within the range detected in the hemolymph during early exposure to *X. nematophila in vivo* (1-2 μg/60 μl hemolymph; Giannoulis *et al*., 2007)], lipid A (60 μg) and lipoteichoic acid (0-5 μg) in 10 μl PBS were incubated with hemolymph (25 μl) in PBS (25 μl) in wells of 96-well polystyrene tissue culture plates (Costar). The total number of hemocytes and level of vacuolated hemocytes were determined using a hemocytometer and phase contrast microscopy.

The effects of the antigens on the conversion of prophenoloxidase to phenoloxidase was established by adding lipopolysaccharide (1 μg), lipid A (60 μg) or lipoteichoic acid (1 or 5 μg) in PBS (10 μl) to hemocyte lysate. Briefly, hemocyte lysate (10 μl) was produced from hemocyte suspensions (i.e. hemolymph diluted 50% (v/v) with PBS) by vigorously pipetting to damage the cells and release zymogenic phenoloxidase. The resulting mixture

was clarified by centrifugation (12,000 x g, 2 min, 25 °C) and the supernatant containing prophenoloxidase inoculated with the bacterial antigens. After incubating the supernatant with 50 µl of the phenoloxidase activator, laminarin (1 mg/ml PBS), for 60 min at 25 °C, an aliquot (25 µl) was added to 500 µl of the phenoloxidase substrate, L-dihydroxyphenylalanine (1.5 mg/ml PBS) and dopachrome formation determined spectrophotometrically at 495 nm. To ensure dopachrome formation was not due to hemocyte peroxidase, phenoloxidase was inhibited with 1.5 mM tropolone, a phenoloxidase-specific inhibitor (Li *et al*., 1996), and dopachrome formation determined. To limit autooxidation of enzyme substrate, only fresh dihydroxyphenylalanine solution was used.

Endotoxin contaminants in isolated peptidoglycan and commercial sources of lipoteichoic acid activate the antibacterial immunodeficiency pathway of *Drosophila melanogaster* (Kaneko *et al*., 2004) and mouse macrophages *in vitro* (Gao *et al*., 2001), respectively. To determine if, in this study, endotoxin alone or as a contaminant influenced *M. disstria* hemocyte and phenoloxidase responses, antigen types were saturated with the antibiotic, polymyxin B and used in the aforementioned assays unless stated otherwise. Polymyxin B is a nonapeptide that electrostatically binds to the lipid A moiety of lipopolysaccharide (Coyne and Fenwick, 1993) including that of *X. nematophila* (Dunphy and Webster, 1988a), and does not bind to lipoteichoic acid (Lin *et al*., 2001).

Antigens were saturated by incubation with excess polymyxin B in PBS for 24 h, unbound antibiotic being removed by exhaustive dialysis against 10l of diluted PBS (1:10 v/v) using cellulose nitrate dialysis tubing (5,000 Da molecular weight cut off). Following lyophilization, antigens were rehydrated in endotoxin-free distilled water producing initial antigen levels in regular PBS (Dunphy and Webster, 1988a).

Endotoxin, lipid A and lipoteichoic acid reacting with hemocytes and phenoloxidase *in vivo*

To further establish the effects of the bacterial antigens on the hemocytes and phenoloxidase, larvae were injected with increasing amounts of lipopolysaccharide or lipoteichoic acid in 10 µl of PBS. Thirty min post-injection (p.i.), insects were bled and the total hemocyte counts and, where applicable, the types of hemocytes present, were determined on a hemocytometer and as hemocyte monolayers on slides, respectively. Hemocyte viability was determined using the trypan blue viability assay on hemocyte suspensions (Giannoulis *et al*., 2005).

Two approaches were used to further investigate the effects of the antigens on hemocyte counts. In one method larvae were injected with a fixed amount of endotoxin (1 µg) or lipid A (60 ng) and increasing amounts of polymyxin B. In protocol two, larvae were injected with endotoxin, its lipid A or lipoteichoic acid either alone or saturated with polymyxin B. For both methods total hemocyte counts were determined 30 min p.i. as previously described above.

The physiological effect of lipopolysaccharide on antibacterial cellular responses was determined using two protocols. Protocol one involved co-injecting one set of larvae with 10 µl PBS containing 1 µg lipopolysaccharide (determined in the present study to increase hemocyte counts) and *X. nematophila* (1×10^{10} bacteria/ml PBS) and a second set (the control group) with *X. nematophila* only. Bacterial concentrations at selected times p.i. were determined using a hemocytometer. In a second protocol, larvae were injected with *X. nematophila*, incubated for 1 h to facilitate both bacteria – hemocyte binding and nodulation, after which one subgroup was injected with 10 µl PBS (control group) and the other with 1 µg lipopolysaccharide in 10 µl PBS. Concentration of bacteria in the hemolymph

was determined 30 min p.i. using a hemocytometer and phase contrast microscopy.

Apolipophorin-III reactions with bacterial antigens, hemocytes and phenoloxidase *in vivo*

Herein it was necessary to increase endotoxin and lipoteichoic acid concentrations above a critical level before the antigens affected hemocyte counts implying a plasma protein may limit antigen reactions with hemocytes. Apolipophorin-III from *G. mellonella* and *M. disstria* binds to *X. nematophila* and *B. subtilis* (Zakarian *et al*., 2002; Giannoulis *et al*., 2007) and also impairs endotoxin activity against *G. mellonella* hemocytes (Dunphy and Halwani, 1997) and phenoloxidase activation (Halwani *et al*., 2000). Isolation of a similar protein from *M. disstria* hemolymph was achieved using the protocol of Halwani *et al*. (2000). This required diluting the hemolymph 1:1 (v/v) in PBS, removing the hemocytes by centrifugation (12,000 x g, 4 min, 20^{o}C) and heating the supernatant (100^{o}C, 30 min). Following centrifugation (15,000 x g, 20 min, 20^{o}C), the nonprecipitated serum proteins in the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and rendered visible with copper chloride (Lee *et al*., 1987). An acrylamide segment containing a 18.7 kDa polypeptide was excised, destained in 250 mM ethylenediaminetetraacetic acid in 250 mM Tris (pH 9.0 with HCl), and dialyzed overnight against 2 L of 25 mM Tris-192 mM glycine (pH 8.3). The polypeptide was electroeluted (2 h, 100 mV) in the latter buffer (Halwani and Dunphy, 1999; Halwani *et al*., 2000), dialyzed against PBS (2 L), lyophilized and purity and protein identity were determined according to Halwani *et al*. (1999) based on aminoacid primary sequences. Antigenicity of the protein was compared to *G. mellonella* apolipophorin-III by immunoblotting using anti-*G. mellonella* apolipophorin-III antibodies, produced in New Zealand white male rabbits, as the primary antibody and

alkaline phosphatase-conjugated anti-rabbit antibody (Sigma-Aldrich Chem. Co., Ont., Canada) as the secondary antibody (Halwani *et al*., 2000).

Herein, increasing amounts of apolipophorin-III were co-injected with fixed levels of endotoxin, lipid A and lipoteichoic acid in PBS or in PBS lacking bacterial antigens and compared with the control lacking the protein. Changes in hemocyte counts and phenoloxidase activity were determined. When lipoteichoic acid was injected, the phenoloxidase assay required the release of the zymogen into the plasma by the co- injection of laminarin (1 mg/ml PBS); otherwise, the antigen induced nodulation with little prophenoloxidase discharge.

Statistics

Hemocyte counts were analyzed using the 95% confidence limits overlap protocol and graphic and tabular data are presented as means ± standard error of the mean (Sokal and Rholf, 1969). An α level of 0.05 was chosen. A minimum of 5 replicates containing 10 samples were used for each value.

RESULTS

Reactions of antigens with hemocytes *in vitro*

Lipopolysaccharide lowered hemocyte counts in tissue culture plates when the concentration of endotoxin exceeded 100 ng/60 µl of diluted hemolymph, the effect being more pronounced with incubation time (Table 4.1, $P < 0.05$). There was no evidence of hemagglutination or hemocytes adhering to reaction vessel surfaces; thus, the hemocyte decline represented lysis. Both lysis and hemocyte vacuolation increased with increasing lipopolysaccharide levels. Saturating lipopolysaccharide (1 µg) with polymyxin B abrogated the hemocyte effects when compared with the PBS control hemolymph which lacked endotoxin (Table 4.1). Purified lipid A lowered also hemocyte counts by lysis (Table 4.1, $P < 0.05$) suggesting lipid A may be the hemocytotoxin. Lipid A (60 ng) saturated with the antibiotic produced hemocyte counts that were higher than those obtained with lipid A alone and were comparable to the control groups (Table 4.1). Thus, the hemocyte toxic activity of the endotoxin is due to the lipid A moiety.

Lipoteichoic acid decreased the counts of individually floating hemocytes (Table 4.1, $P < 0.05$) due to agglutination. Vacuolation was more prevalent in aggregated hemocytes than in non-attached cells. The effect of lipoteichoic acid on hemocyte numbers increased also with incubation time. Polymyxin B did not alter the effects of 1 µg lipoteichoic acid on hemocyte counts; hence, any possible endotoxin contamination of lipoteichoic acid did not contribute to the results.

Table 4.1: Changes in *Malacosoma disstria* hemocyte counts *in vitro* by *Xenorhabdus nematophila* lipopolysaccharide.

Antigen	Antigen concentration (60 ng/60 µl diluted hemolymph)[a]	Vacuolation (%) at 30 min[b]	Hemocyte counts at selected times (×10^6 cells/ml of diluted hemolymph)[c]	
			30 min	60 min
LPS	0	5^{q} (9.9–17.6)	4.1 ± 0.3^{e,e}	3.0 ± 0.2^{e,f}
	100	27^{f} (27.8–38.1)	3.2 ± 0.1^{e,g}	2.3 ± 0.2^{f,h}
	1000	58^{g} (42.3–54.2)	2.1 ± 0.2^{f,h}	1.0 ± 0.1^{h,i}
	5000	91^{h} (67.6–83.0)	1.6 ± 0.7^{f,i}	0.4 ± 0.1^{i,j}
LPS (1 µg) saturated with polymyxin B	—	ND	3.9 ± 0.4^{e}	ND
Lipid A	60	89^{h} (61.2–79.4)	2.3 ± 0.1^{f,f}	1.2 ± 0.2^{h,j}
Lipid A (60 ng) saturated with polymyxin B	—	ND	4.0 ± 0.3^{e}	ND
Lipoteichoic acid	0	6^{e} (5.7–19.1)	4.8 ± 0.2^{e,e}	2.7 ± 0.1^{f,f}
	100	10^{e} (13.6–23.1)	3.6 ± 0.3^{f,g}	1.9 ± 0.1^{g,g}

	1000	26[f] (26.9–33.8)	1.5 ± 0.1[g,h]	0.7 ± 0.3[h,h]
	5000	41[g] (36.6–41.8)[g]	0.9 ± 0.1[g,i]	0.3 ± 0.2[1,j]
Lipoteichoic acid (1 µg) saturated with polymyxin B		ND	1.4 ± 0.1[g]	ND

[a] Hemocyte suspension consisted of 25 µl PBS (pH 6.5), 25 µl of hemolymph, and 10 µl of antigen in PBS. ND, not determined.

[b] Decoded mean (with 95% confidence limits of arcsin √p transformed). Values with the same superscript for a given antigen are not significantly different, $n \geq 10$, $P > 0.05$.

[c] Means ± standard error of the mean, $n \geq 10$. Values with the same left superscript within a column are not significantly different, $P > 0.05$. Values with the same right superscript between the time parameters column for a given antigen are not significantly different, $P > 0.05$.

Reaction of antigens with phenoloxidase *in vitro*

Lipopolysaccharide, in a concentration-dependent fashion, and lipid A (60 ng), impaired the activation of phenoloxidase by laminarin (Table 4.2). Both of these antigens, when saturated with polymyxin B, did not alter phenoloxidase activation compared with the control samples containing laminarin-activated enzyme. Polymyxin B alone increased phenoloxidase activity above that caused by laminarin in a dose-dependent manner (Table 4.2). Lipopolysaccharide impaired the increase in phenoloxidase activity caused by a fixed amount of polymyxin B confirming polymyxin B binding to, and neutralization, of the endotoxin.

Lipoteichoic acid at increasing concentrations enhanced laminarin activation of phenoloxidase and/or enzyme activity. Alone, lipoteichoic acid enhanced enzyme previously spontaneously-activated (i.e. without laminarin) in serum (Table 4.2, $P < 0.05$). Rather than compromising the effects of lipoteichoic acid on laminarin-based activation of phenoloxidase, saturation of lipoteichoic acid with polymyxin B did not enhance phenoloxidase activity for a given amount of lipoteichoic acid (Table 4.2) again precluding possible endotoxin contamination influencing an immunological response.

Table 4.2: Changes in *Malacosoma disstria* phenoloxidase *in vitro* caused by selected antigens.

Antigen	Laminarin activated[a]	Concentration (ng/60 µl diluted hemolymph)	Phenoloxidase activity (U/mg protein)[b]
LPS	+	0	14.2 ± 1.7[c]
		100	9.7 ± 1.4[d]
		1000	1.3 ± 0.7[d]
		5000	0.3 ± 0.2[e]
LPS (1 µg) saturated with polymyxin B	+	—	15.7 ± 1.9[c]
Lipid A	+	60	1.0 ± 0.3[d]
Lipid A (60 ng) saturated with polymyxin B	+		16.1 ± 3.2[c]
Polymyxin B	+	0	16.2 ± 2.8[c]
		10	25.2 ± 3.7[f]
		50	37.9 ± 1.8[g]
		100	64.3 ± 2.2[h]
Polymyxin B (10 ng) plus LPS	+	0	26.2 ± 3.3[f]
		10	21.1 ± 2.1[f]
		50	14.1 ± 1.5[c]
Lipoteichoic acid	+	0	14.7 ± 2.2[c]
		1000	30.7 ± 3.2[g]
		5000	60.3 ± 3.2[h]

Lipoteichoic acid saturated with polymyxin B	+	1000	48.7 ± 3.5[g]
		5000	69.1 ± 4.8[h]
Lipoteichoic acid	−	0	5.7 ± 1.1[d]
		1000	9.5 ± 0.8[d]
		5000	15.1 ± 0.2[c]

[a] + Phenoloxidase activated with laminarin, − spontaneous activation of phenoloxidase (no laminarin).

[b] Means ± standard error of the mean, 30 min p.i. $n \geq 10$. Values with the same superscript are not significantly different, $P > 0.05$.

Reactions of antigens without and with polymyxin B with hemocytes *in vivo*

Lipopolysaccharide, at concentrations below 300 ng/larva did not affect hemocyte counts and marginally influenced cell viability; however, at levels ranging from 300 ng/larva to 10 μg/larva, endotoxin increased damaged vacuolated hemocyte counts and decreased hemocyte viability (Figure 4.1). The hemocytes from the latter group of insects did not adhere to slides precluding their reliable identification. Injections of lipopolysaccharide in excess of 10 μg/larva caused a concentration-dependent decline in total hemocyte counts without eliciting discernible nodulation. Co-injecting insects with PBS containing 1 μg of lipopolysaccharide with increasing amounts of polymyxin B lowered hemocyte counts to the level of those in PBS-injected larvae (Figure 4.2). Polymyxin B alone increased hemocyte counts. Implication of lipid A as the toxic moiety of the endotoxin, as inferred from the *in vitro* experiments, was reinforced in the *in vivo* experiments, the administration of lipid A (60 ng) increasing damaged hemocyte levels to values comparable to those obtained with 1 μg of lipopolysacharide per larva by 30 min p.i. (Table 4.3, $P > 0.05$); also, the percentage of vacuolated hemocytes in hemolymph from lipid A-treated larvae was comparable to that in larvae injected with lipopolysaccharide (Table 4.3, $P > 0.05$). Injections of increasing concentrations of polymyxin B abrogated the lipid A effect lowering hemocyte counts and vacuolation (Table 4.3) until the values of these parameters were similar to those obtained with the control larvae ($P > 0.05$).

Elevation of vacuolated, non adhesive hemocyte counts by 1 μg of lipopolysaccharide would be expected to affect the removal of *X. nematophila* from the hemolymph. Compared with control larvae receiving *X. nematophila* (in PBS) alone, co-injecting *X. nematophila* with lipopolysaccharide (1

μg/larva) compromised the removal of the bacteria from the hemolymph [bacterial level (bacteria X 10^7/ml hemolymph) in control larvae; 10 min p.i., 1.7 ± 0.4; 30 min p.i., 0.8 ± 0.0 and 60 min p.i., 2.1 ± 0.3; bacteria co-injected with lipopolysaccharide, 10 min p.i., 2.1 ± 0.3; 30 min p.i., 2.4 ± 0.1; 60 min p.i., 2.5 ± 0.3; $n \geq 10$, $P < 0.05$]. By 60 min p.i. endotoxin elevated bacterial counts implying release of the bacteria from the few nodules formed and freeing bacteria from the floating hemocytes. This was confirmed by bacteria previously extensively nodulated being released more rapidly from nodules 30 min after injecting 1 μg of lipopolysaccharide (17.1 ± 0.2 X 10^6 bacteria/ml hemolymph) compared with control larvae (2.9 ± 0.4 X 10^6 bacteria/ml of hemolymph; $n \geq 10$, $P < 0.05$).

Lipoteichoic acid injections at 500 ng/larva raised hemocyte counts, the hemocytes appearing normal and lacking vacuoles; lipoteichoic acid in excess of this concentration lowered hemocyte counts, the responses being accentuated with incubation time (Figure 4.3). Both 1 μg and 5 μg but not 500 ng of lipoteichoic acid per larva produced melanotic nodules on the fat body and at the injection portal within 24 h of injection. Five hundred ng of lipoteichoic acid per larva elevated the number of plasmatocytes and granular cells in the hemolymph, whereas higher concentrations lowered these hemocyte types (Table 4.4) due to nodulation. Prior treatment of lipoteichoic acid with polymyxin-B had little, if any, effect on the lipoteichoic acid-mediated reduction in hemocyte counts (Figure 4.3) precluding, once again, the involvement of endotoxin contamination.

Reactions of antigens without and with polymyxin B with phenoloxidase *in vivo*

Lipopolysaccharide (1 µg/larva) and lipid A (60 ng/larva) impaired plasma phenoloxidase activation to equal degrees compared with the PBS-injected control insects (Table 4.3, $P < 0.05$). The inhibition by lipid A,

of phenoloxidase activation by injected laminarin, was blocked by polymyxin B in a concentration-dependent manner ($P < 0.05$). Rather than inhibiting previously activated phenoloxidase, lipopolysaccharide and lipid A unexpectedly enhanced activity (phenoloxidase activity: preactivated control phenoloxidase, 12.4 ± 1.9 units/mg protein; 500 ng lipopolysaccharide/larva, 16.9 ± 3.1 units/mg protein, 60 ng lipid A/larva, 24.6 ± 2.7 units/mg protein, $n = 10$, $P < 0.05$).

Figure 4.1: Effects of increasing concentrations of *Xenorhadus nematophila* lipopolysaccharide on hemocyte viability and total hemocyte counts in *Malacosoma disstria* larvae with increasing lipopolysaccharide concentrations at 30 mins post injection. All data represent the mean ± the standard error of the mean, n ≥ 10

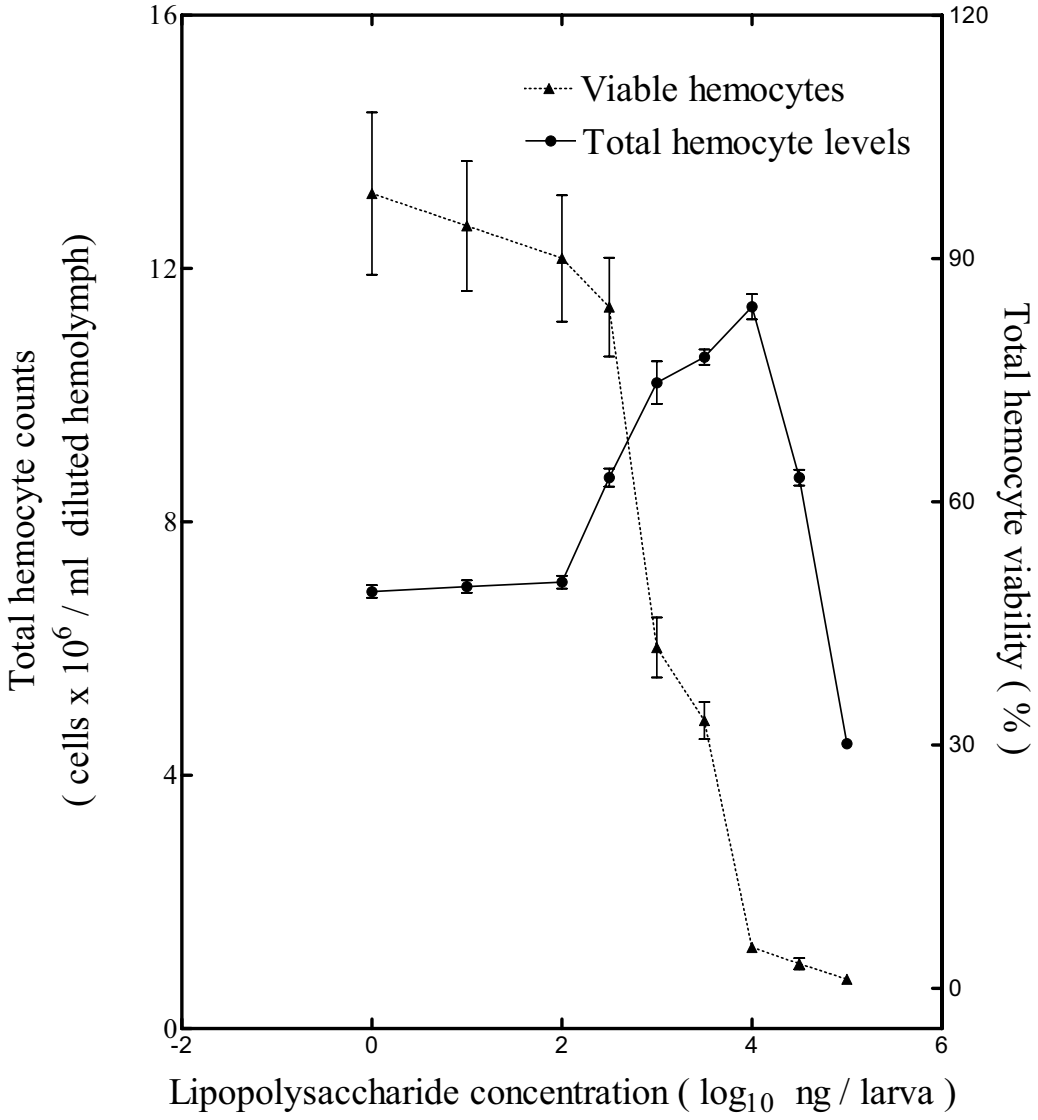
Viable hemocytes
Total hemocyte levels
Total hemocyte counts
(cells x 10^6 / ml diluted hemolymph)
Total hemocyte viability (%)
Lipopolysaccharide concentration (log10 ng / larva)
16
12
8
4
0
120
90
60
30
0
-2
0
2
4
6

Figure 4.2: Effects of increasing amounts of polymyxin B without and with a fixed amount of *Xenorhadus nematophila* lipopolysaccharide (1 µg) on the total hemocyte counts of *Malacosoma disstria* larvae, 30 mins post injection. All data represent the mean ± the standard error of the mean, n ≥ 10.

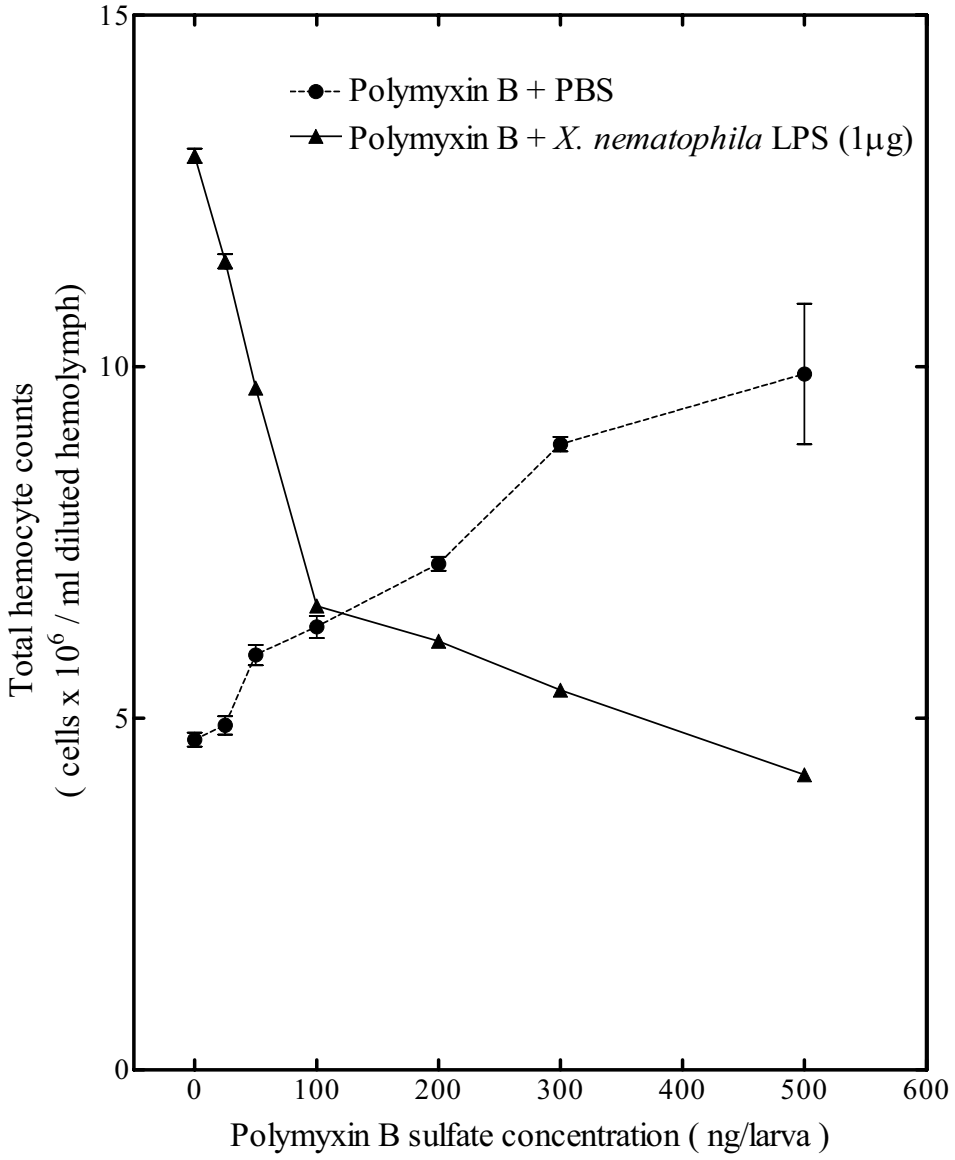
Polymyxin B + PBS
Polymyxin B + X. nematophila LPS (1μg)
Total hemocyte counts
(cells x 10^6 / ml diluted hemolymph)
Polymyxin B sulfate concentration (ng/larva)
0
5
10
15
0
100
200
300
400
500
600

Figure 4.3: Effects of different amounts of *Bacillus subtilis* lipoteichoic acid, and lipoteichoic acid saturated with polymyxin B on total hemocyte counts of larval *Malacosoma disstria*. All data represent the mean ± the standard error of the mean, n ≥ 10.

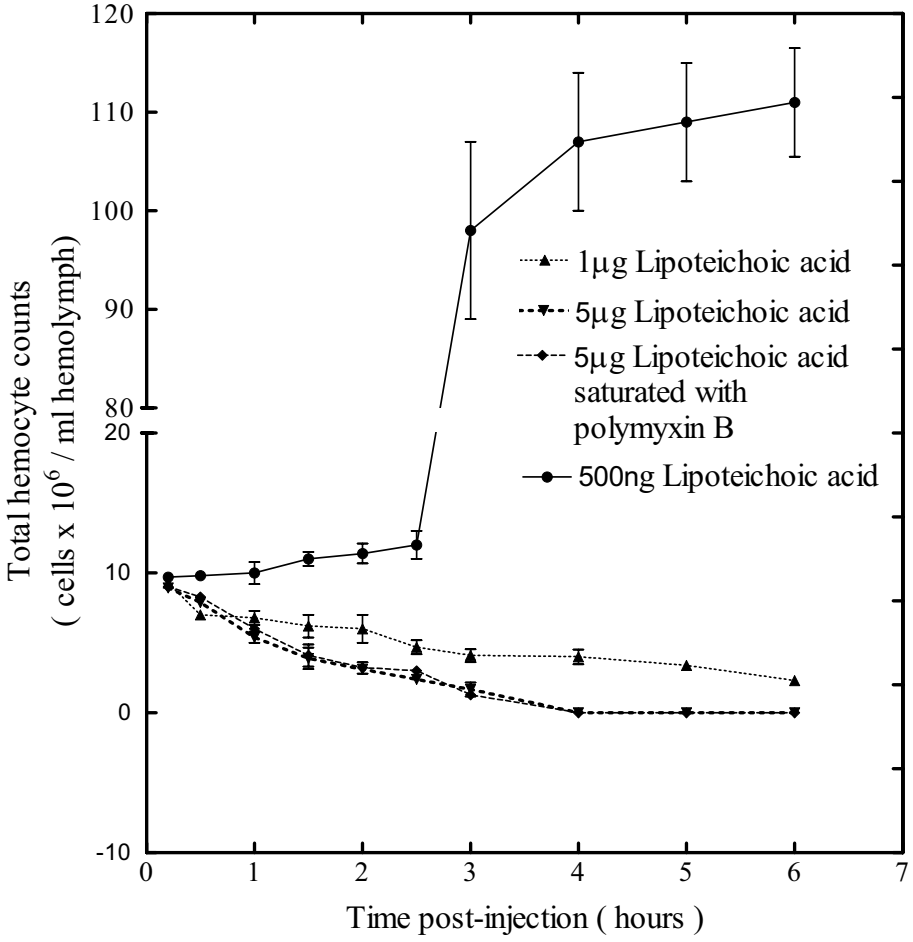
Total hemocyte counts
(cells x 10^6 / ml hemolymph)
Time post-injection (hours)
1µg Lipoteichoic acid
5µg Lipoteichoic acid
5µg Lipoteichoic acid saturated with polymyxin B
500ng Lipoteichoic acid
120
110
100
90
80
20
10
0
-10
0
1
2
3
4
5
6
7

Table 4.3: Effects of injected *Xenorhabdus nematophila* lipopolysaccharide and lipid A on larval *Malacosoma disstria* hemocytes and phenoloxidase activity 30 min post-injection

Antigen	**Concentration (ng/larva)**	**Hemocyte vacuolation (%)**[a]	**Hemocyte counts (cells × 10⁶/ml hemolymph)**[b]	**Phenoloxidase activity (U/mg protein)**[b]
LPS	1000	81[c] (56.8–72.5)	15.9 ± 0.3[c]	1.3 ± 0.7[c]
Lipid A	60	75[c] (53.5–63.2)	15.7 ± 0.1[c]	2.1 ± 0.6[c]
Lipid A (60 ng) plus polymyxin B	0	78[c] (55.1–56.2)	16.9 ± 1.4[c]	1.8 ± 0.7[c]
	100	53[d] (39.6–54.3)	12.1 ± 0.4[d]	7.9 ± 0.6[d]
	300	0[e] (5.2–5.7)	6.9 ± 0.4[e]	12.8 ± 1.1[e]
Phosphate-buffered saline	—	3[e] (5.2–12.9)	7.2 ± 0.3[e]	14.9 ± 0.6[e]

[a] Decoded means ± standard error of the mean (with 95% confidence limits of arcsin √p transformed data), $n \geq 10$. Values with the same superscript are not significantly different, $P > 0.05$.

[b] Means ± standard error of the mean, $n \geq 10$. Values with the same superscript are not significantly different, $P > 0.05$.

Table 4.4: Levels of plasmatocytes and granular cells in *Malacosoma disstria* larvae 120 min post injection of *Bacillus subtilis* lipoteichoic acid.

Lipoteichoic acid concentration (µg/larva)[a]	**Hemocytes/mm²[b]**	
	Plasmatocytes	**Granular cells**
0	29.2 ± 3.2[c]	116.7 ± 8.1[c]
0.5	46.1 ± 7.3[d]	138.9 ± 3.5[d]
1.0	13.8 ± 1.4[e]	12.9 ± 1.1[e]
5.0	5.1 ± 2.2[f]	1.2 ± 0.8[f]

[a] Lipoteichoic acid dissolved in PBS, pH 6.5.

[b] Means ± standard error of the mean, $n \geq 10$. Values with the same superscript are not significantly different, $P > 0.05$.

Effects of apolipophorin-III on hemocytes and phenoloxidase *in vivo*

A critical concentration of *X. nematophila* endotoxin (Figure 4.1) and *B. subtilis* lipoteichoic acid (Figure 4.3) was required to elevate damaged hemocyte counts and lower hemocyte levels, respectively, *in vivo*, implying the existence of a hemolymph neutralizing factor which would limit hemocyte reaction with bacterial antigens during the initial stages of infection. *M. disstria* apolipophorin-III, when co-injected with lipopolysaccharide or lipid A into the insects, limited the increase in hemocyte counts in a concentration-dependent fashion (Table 4.5). The protein alone did not alter hemocyte counts. Apolipophorin-III also countered, in a concentration-dependent manner, the inhibition of phenoloxidase by 60 ng of lipid A injected into the larvae (Table 4.6, $P < 0.05$). Enhancement of phenoloxidase activity by 5 µg of lipoteichoic acid/larva was inhibited by apolipophorin-III (Table 4.6, $P < 0.05$). Although apolipophorin-III alone at 10 µg/larva impaired phenoloxidase activation by laminarin (Table 4.6, $P < 0.05$), it did not alter the activity of phenoloxidase once activated (phenoloxidase activity: laminarin-treated larvae, 21.0 ± 2.9 units/mg protein; laminarin + 10 ng or 100 ng apolipophorin-III/larva, 20.3 ± 4.1 or 19.6 ± 1.7 units/mg protein, respectively; $n = 10$, $P > 0.05$). In both experiments, the absence of an apolipophorin-III effect may be due to binding of this β-1,3-glucan-binding protein to laminarin (a β-1,3-glucan) precluding interaction between phenoloxidase and the protein. Apolipophorin-III injections without laminarin activation enhanced spontaneous phenoloxidase activity in a concentration-dependent manner (Table 4.6, $P < 0.05$).

Table 4.5: Total hemocyte counts 30 min post-injection of *Malacosoma disstria* of fixed amounts of *Xenorhabdus nematophila* lipopolysaccharide or lipid A with increasing amounts of an apolipophorin-III.

Protein concentration (ng/larva)[a]	**Total hemocyte counts × 10^6/ml hemolymph[b,c]**	
	Lipopolysaccharide (1 µg/larva)	**Lipid A (60 ng/larva)**
0	$12.7 \pm 0.2^{ed,d}$	$14.2 \pm 0.7^{ed,d}$
500	$11.5 \pm 0.7^{ed,e}$	$10.1 \pm 0.3^{e,e}$
1000	$8.2 \pm 0.3^{fe,f}$	$6.8 \pm 0.2^{f,f}$
2000	$4.7 \pm 0.2^{f,g}$	$3.2 \pm 0.1^{g,g}$
5000	$4.9 \pm 0.3^{f,h}$	$3.3 \pm 0.2^{h,i}$

[a] Injected in 10 µl of PBS, pH 6.5, per larva.

[b] Means ± standard error of the mean, $n \geq 10$. Values within a column with the same right superscript are not significantly different, $P > 0.05$. Values within a row with the same left superscript are not significantly different, $P > 0.05$.

[c] There were no differences in total hemocyte counts between larvae injected with PBS alone (total hemocyte counts = $4.6 \pm 0.3 \times 10^6$ hemocytes/ml hemolymph, $n = 10$) and those injected with the different concentrations of apolipophorin-III (pooled hemocyte counts for protein-injected larvae = $4.9 \pm 0.6 \times 10^6$ hemocytes/ml hemolymph, $n = 40$, $P > 0.05$).

Table 4.6: Effect of apolipophorin-III in the presence and absence of bacterial antigens on phenoloxidase activity in the hemolymph of larval *Malacosoma disstria*

Antigen	**Laminarin**	**Apolipophorin-III concentration (ng/larva)**	**Phenoloxidase activity (U/mg protein)[a]**
Lipid A (60 ng) plus apolipophorin-III	+	0	3.5 ± 0.7[b]
		10	9.7 ± 1.5[c]
		100	21.1 ± 1.3[d]
Lipopolysaccharide (1 µg) plus apolipophorin-III	+	0	2.9 ± 1.1[b]
		10	11.1 ± 0.7[c]
		100	19.7 ± 1.0[d]
Lipoteichoic acid (5 µg) plus apolipophorin-III	+	0	71.1 ± 8.2[e]
		10	52.5 ± 7.2[f]
		100	13.4 ± 2.7[d]
Apolipophorin-III	+	0	19.4 ± 3.2[d]
		10	12.1 ± 2.6[c]
		100	3.2 ± 0.7[b]
Apolipophorin-III	−	0	4.1 ± 1.3[b]
		10	15.2 ± 1.1[d]
		100	42.1 ± 0.7[e]
Phosphate-buffered saline	+	—	17.2 ± 1.4[d]

[a] Means ± standard error of the mean, $n \geq 10$. Values with the same superscripts are not significantly different, $P > 0.05$.

DISCUSSION

Dead *X. nematophila* but not *B. subtilis* damage larval *M. disstria* hemocytes (Giannoulis *et al*., 2007) implying an outer membrane component rather than peptidoglycan and/or products of ongoing bacterial metabolism, contributed to the hemocyte toxicity of the former bacterium. The most likely toxic component is lipopolysaccharide because (1) it increases as the level of damaged hemocytes increases *in vivo* (Giannoulis *et al*., 2007), (2) herein purified endotoxin elevated the level of vaculoated, nonadhesive hemocytes, (3) polymyxin B, which binds to the lipid A of endotoxin (Coyne and Fenwick, 1993; Dunphy and Webster, 1988a), prevented hemocyte damage by endotoxin and (4) it both limited the removal of *X. nematophila* from the hemolymph by nodulation and enhanced the release of bacteria from previously formed nodules. *X. nematophila* lipopolysaccharide has multifaceted properties, chelating Ca^{+2} in *Agrotis segetum* (Yokoo *et al*., 1992) and Fe^{+2} from the hemolymph of *G. mellonella* (Dunphy *et al*., 2002), which limit both phenoloxidase activation and hemocyte function. Inhibition of phenoloxidase activation by endotoxins has been reported for *X. nematophila* and the closely related bacterium, *Photorhabdus luminescens*, the fatty acids of lipid A effectively inhibiting the enzyme (Dunphy and Webster, 1988b; Dunphy, 1995). However, other lipopolysaccharide species are known to activate phenoloxidase (Goldsworthy *et al*., 2003; Altincicek *et al*., 2007). Enzyme inhibition may occur by more than one method, metabolites from several *Xenorhabdus* species and *Photorhabdus temperata* subsp. *temperata* inhibiting phenoloxidase by inhibiting phospholipase A_2, the product of which, arachidonic acid, is known to activate phenoloxidase (Kim *et al*., 2005). For hemocytes of both *G. mellonella* (Dunphy and Webster, 1988a) and *M. disstria* (this study), *X. nematophila* endotoxin limits non-self responses to bacteria and slides. Based on hemocyte damage caused by purified lipid A and the prevention of damage with polymyxin B, lipid A is regarded as the

toxic moiety of *X. nematophila* endotoxin, the fatty acids of which, in *G. mellonella*, like those of *P. luminescens* endotoxin (Dunphy and Webster, 1988b) induce hemolysis (Dunphy and Webster, 1988a). Inhibition of phospholipase A_2 is also associated with the blocking of hemocyte adhesion by living *Xenorhabdus* spp. (Kim *et al*., 2005).

Antigens from Gram-positive bacterial cell walls affect also cellular and humoral systems of insects (Gillespie *et al*., 1997). Nodulation is induced by peptidoglycan fragments in *M. sexta* larvae (Dunn, 1986) and by numerous lipoteichoic acid species in *G. mellonella* (Halwani *et al*., 2000) and *B. subtilis* lipoteichoic acid in *M. disstria* (this study). Herein, *B. subtilis* lipoteichoic acid, depending on antigen concentration, caused nodulation and while a poor activator of the prophenoloxidase system when present alone, with laminarin, it enhanced enzyme activity dramatically. When added to previously maximally activated phenoloxidase, lipoteichoic acid increased enzyme activity farther. Although *X. nematophila* lipopolysaccharide and lipid A inhibited phenoloxidase activation, like lipoteichoic acid, the hydrophobic antigens enhanced the activity of previously activated enzyme. The amphiphilic nature of these three antigens may be responsible for the enhancement of enzyme activity, amphiphilic molecules increasing phenoloxidase activity in the cockroach, *Periplaneta americana* (Sugumaran and Nellaiappan, 1991) and the armyworms, *Spodoptera littoralis* (Lee and Anstee, 1995) and *Spodoptera frugiperda* (Yong *et al*., 1999). Polymyxin B did not alter the cellular or humoral response to lipoteichoic acid, confirming that what little endotoxin contamination may be associated with commercial lipoteichoic acid, it did not elicit discernible effects.

A critical concentration of lipopolysaccharide and lipoteichoic acid was required to alter hemocyte counts impling the existence of (a) neutralizing factor(s) in *M. disstria* hemolymph. Hemolymph serum proteins and hemocyte proteins binding species of lipopolysaccharide (DeLucca *et al*., 1995; Fabrick *et al*., 2003; Jamori and Natori, 1991; Kawabata and Iwanaga,

1999; Sun *et al*., 1990; Taniai *et al*., 1996; Xu *et al*., 1995; Yu and Kanost, 2002; Yu *et al*., 2006) and lipoteichoic acid (Yu *et al*., 2006) in arthropod hemolymph, limiting antigen toxicity and/or initiating immune induction, are common. Two heat-stable plasma proteins in *G. mellonella* larvae inhibit the hemocyte toxicity of lipopolysaccharide of *X. nematophila*; particularly effective is apolipophorin-III (Dunphy and Halwani, 1997), a β-1, 3-glucan-binding protein (Dunphy *et al*., 2003; Whitten *et al*., 2004) which inhibits hemocytopenia by *B. subtilis* lipoteichoic acid (Halwani *et al*., 2000), potentiates lysozyme digestion of bacterial cell walls (Halwani and Dunphy, 1999), binds to bacterial surfaces (Zakarian *et al*., 2002) and non-specifically binds to non-toxic amounts of *Escherichia coli* 055:B5 lipopolysaccharide (Pratt and Weers, 2006). Apolipophorin-III, in lepidopterans including *G. mellonella*, exceeds concentrations for participating in the conversion of high density lipophorin particles to low density particles favoring other roles including immunological activity (Pratt and Weers, 2004). This does not preclude other mechanisms limiting toxin activity including endotoxin inactivation by lipophorin particles and associated endotoxin binding proteins (Ma *et al*., 2006).

Herein, *M. disstria* apolipophorin-III exhibited many physiological similarities with *G. mellonella* apolipophorin-III neutralizing endotoxemia by lipid A (Dunphy and Halwani, 1997), lipoteichoic acid-induced nodulation (Halwani *et al.* 2000), binding to both *X. nematophila* and *B. subtilis* (Zakarian *et al.,* 2002) and reacting with *β*-1,3 glucans (Dunphy *et al*., 2003; Whitten *et al*., 2004). The protein also abrogated the inhibition of phenoloxidase activation by endotoxin and lipid A and activation by lipoteichoic acid in *M. disstria* (this study) and *G. mellonella* (Halwani *et al*., 2000). Recognition of structurally different hydrophobic antigens by apolipophorin-III is similar to hemolin (Yu *et al*., 2002) and immulectins-2, -3 and -4 (Yu and Ma, 2006). *M. disstria* reaction with laminarin is comparable to the β-1, 3-glucan binding proteins of the pyralid, *Plodia interpunctella* (Fabrick *et al*., 2003) and *M. sexta* immulectins

(Yu *et al.,* 2006). However, apolipophorin-III, unlike hemolin, limits the binding of bacteria to hemocytes *in vivo* and unlike other β-1, 3-glucan-binding proteins including the immulectins, does not agglutinate bacteria (Giannoulis *et al*., 2007). Additional differences are also seen among other invertebrate groups possessing physiologically similar molecules. *M. disstria* apolipophorin-III is smaller than the endotoxin-β-1, 3-glucan binding proteins of the crayfish, *Pacifastacus leniusculus* (Lee *et al*., 2000) and earthworm, *Eisenia foetida* (Beschin *et al*., 1998), but like these proteins it activates phenoloxidase. However, the antigen-protein complex did not activate the enzyme in the present study whereas the *E. foetida* coelomic fluid protein effectively activates phenoloxidase when bound to yeast and wall components of Gram-negative bacteria (Lee *et al*., 2000) as do recognition protein counterparts in many crustaceans (Söderhall and Cerenius, 1998). *M. disstria* apolipophorin-III complexed with antigens did not induce discernible coagulation whereas in the horseshoe crab, *Tachypleus tridentatus*, endotoxin and β-1, 3-glucan binding proteins, once reacted with antigen produce pathogen-limiting coagulation (Muta *et al*., 1991).

In view of the toxicity of prophenoloxidase products such as quinones (Pardini, 1995) and radicals (Kamarov *et al*., 2005) and the ability of apolipophorin-III of *M. disstria* to activate prophenoloxidase (*al beit* to a limited extent), the apolipoprotein may not exist in the free form unattached to other molecules to ensure insect survival. In *G. mellonella,* apolipophorin-III is associated with apolipophorin-II (Halwani *et al*., 2000) and in other species, with lipophorin particles or other recognition receptors which collectively form a cage around foreign molecules limiting antigen damage (Ma *et al*., 2006; Rahman *et al*., 2006).

In summary the lipid A moiety of *X. nematophila* endotoxin damages *M. disstria* hemocytes and inhibits the activation of prophenoloxidase whereas *B. subtilis* lipoteichoic acid enhances hemocytic nodulation and activates phenoloxidase. The activities of both surface

antigens are initially prevented by the plasma protein, apolipophorin-III, a pattern recognition protein binding to laminarin and hydrophobic molecules.

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CONNECTING STATEMENT – III

In the previous research chapters, the responses of fresh larval hemolymph samples and hemocytes to microbial (LPS, Lipid A, LTA) and non microbial (glass) origin antigens were determined. Established hemocyte cell lines of *M. disstria* larvae were used in the following chapter to observe hemocyte non-self responses without mediation by insect plasma factors.

CHAPTER 5

Development of hemocyte cell lines of *Malacosoma disstria* (C. Insecta: O. Lepidoptera) and their interactions with biotic and abiotic antigens

A version of this chapter will be submitted in the
In Vitro Cellular and Developmental Biology– Animal

ABSTRACT

Studies pertaining to the responses of *Malacosoma disstria* hemocytes to antigens are often limited by hemocyte availability. Maintainable *M. disstria* hemocyte cell lines would alleviate this situation and would have an added benefit in that they could be used to study the responses of hemocytes to foreign antigens in the absence of plasma. Herein, selected *M. disstria* cell lines were investigated with respect to optimum temperature for growth and characterized with respect to morphogenesis, blebbing and extracellular enzyme profiles. Subsequently, the interactions of the cells with polystyrene and the surface of the bacterium, *Bacillus subtilis,* with the hemocytes were assessed in terms of antigen modification of hemocytic phenoloxidase activity and released total protein.

The optimum temperature for growth of the adhesive hemocyte cell lines UA-Md221, and the Md108 was 28 ^{o}C, whereas for the cell line UA-Md203, the optimum was 21 ^{o}C. Compared with the other adhering cultures, Md108 exhibited a tolerance for a wider temperature growth range. Both Md108 and UA-Md211 adhered to polysterene surfaces within the first 24 h of subculturing; however, their morphology underwent unique changes in modified Grace's medium up to 48 and 72 h, respectively, post subculturing due to the effect of proteases used in subculturing of the cells. Md66 grew best at 21-28 ^{o}C.

The release of enzymes, which based on substrate activity, were regarded as like- alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C 14), leucine–aminopeptidase, valine–aminopeptidase, cystine–aminopeptidase, serine proteases trypsin and chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase and β-glucosidase, N-acetyl-β-glucosamidinase, α-mannosidase and α-fucosidase from the cell lines at 50% confluency in modified Grace's medium was determined to establish a biochemical baseline to ensure quality. The fetal bovine serum component contained high levels of

esterase-lipase (C8) –like and phosphoamidase-like enzymes when it was diluted in Grace's tissue culture medium and phosphate-buffered saline (PBS). Dead *B. subtilis* in PBS altered quantitatively and qualitatively the enzyme secretion profile as well as total protein release from Md66 and Md108 cultures. Neither Md66 nor Md108 in PBS with or without *B. subtilis* discharged detectable amounts of lysozyme and phenoloxidase.

Keywords: *Malacosoma disstria*, insect, hemocytes, cell adhesion, abiotic, biotic and responses

INTRODUCTION

Salient lepidopteran hemocytes that differentiated from the prohemocyte progenitor include plasmatocytes, granular cells, oenocytoids and spherulocytes (Strand, 2008). The major non-self adhering hemocyte types in Lepidoptera, including *M. disstria*, are the granular cells and plasmatocytes (Giannoulis *et al.*, 2005).

The main hemocytic non - self responses towards particulate antigens consist of phagocytosis, nodulation and encapsulation (Lavine and Strand, 2002). These responses are triggered by the adhesion of foreign microbial antigens (Foukas *et al.,* 1998), carboxy- modified latex, and agarose and dextran beads (Lamprou *et al.,* 2007; Lavine and Strand, 2001) to the immune responsive hemocytes. Phagocytosis is the mechanism by which cells internalize foreign particulates (Garcia-Garcia, 2005; Ribeiro and Brehelin, 2006). The process involves antigen recognition and attachment leading to signal transduction, and consequently, increased pseudopod formation and ingestion of the antigens (Gillespie *et al.*, 1997). Ingestion involves the cytoskeletal regulatory proteins D-SCAR, D-WASp and profilin (Pearson *et al.*, 2003).

The major response of insect hemocytes to large numbers of bacteria and non-hyphal fungal structures is called nodulation (Gandhe *et al.*, 2007). Nodulation consists of antigen stimulated hemocyte aggregations around the antigens which are entrapped by matrix proteins from granular cells (Beckage, 2008). The aggregates are subsequentially surrounded by plasmatocytes (Ratcliffe, 1993). Nodulation promotes clearance of microorganisms from the hemolymph (Da Silva *et al.*, 2000; Howard *et al.*, 1998). Phagocytosis and nodulation, prompted by cell signalling in response to the surface binding of foreign materials (Lamprou *et al.*, 2005), culminates also in the release of extracellular matrix proteins discharged from the granular cells and spherulocytes (Kaya and Tanada, 1993; Nardi *et al.,* 2005; Ratcliffe 1975; Ratciffe and Gagen, 1976). Extracellular matrix proteins of

insect blood cells include lacunin protein (Nardi *et al.*, 2001), noduler protein (Gandhe *et al.*, 2007) and type IV collagen (Lavine and Strand, 2002). These proteins enhance adhesion of the hemocytes to invading microorganisms. Calreticulin, 60-kDa endoplasmic reticulum protein, is associated with the adhesive and phagocytic activities of hemocytes of *Pieris rapae* larvae towards yeast cells (Asgari and Schmidt, 2003). Polydnavirus infection of the larval blood cells of the lepidopteran fall armyworm, *Spodoptera frugiperda,* reduces transcription levels of calreticulin and subsequently prophenoloxidase activity enzyme, immulectin-2 and hemocyte scavenger receptors limiting the insect's immune responses (Barat-Houari *et al.,* 2006). The encapsulation process exhibited by lepidopteran hemocytes is similar to nodulation; however, the size of the foreign object is larger than the hemocytes (Strand, 2008). Nodulation and encapsulation interfere with the acquisition of nutrients and oxygen by the trapped microorganisms and as a result of phenoloxidase-mediated melanin deposition, the activities of other enzymes released from hemocytes and enzyme - generated radical production, there is no proliferation of the microbes (Strand, 2008).

Adhesion of plasmatocytes to foreign surfaces can lead to apoptosis of granular cells evidenced by chromatin condensation, intranucleosomal DNA fragmentation and cell blebbing (Pech and Strand, 2000). Blebbing is a common response of insect hemocytes to bacterial toxins produced by entomopathogenic bacteria such as *Photorhabdus luminescens* subsp. *akhurstii* strain W14 (Daborn *et al.,* 2002) and polydnaviruses released during oviposition by the hymenopteran parasitoid *Cotesia congragata* wasp (Amaya *et al.,* 2005) and it is regarded as major general stress response (Brooks and Dunphy, 2005).

Foreign recognition is defined by lepidopteran humoral pattern recognition proteins binding to antigenic receptors (Beckage, 2008) and by hemocyte receptors including RGD-integrin receptors on plasmatocytes (Levin *et al.,* 2005; Nardi *et al.*, 2005). RGD-integrin receptors bind to collagen

type IV or their fragments generated by metalloproteinases released by invading bacteria (Altincicek *et al.,* 2007), and to discharged granular cell extracellular matrix proteins (Lavine and Strand, 2002). Indirect recognition receptors are represented by Toll and Toll like (TLR) transmembrane receptors (Ao *et al.,* 2008a). Serine proteases activated by pattern recognition receptors binding to antigens elicits proteolytic activation of the cytokine-like transducer Spätzle in Diptera and Lepidoptera species which activates Toll (Wang *et al.*, 2007). Indepedent of Toll protein family function, the Imd pathway also senses the presence Gram negative bacteria in insect hemocytes (Wang and Ligoxygakis, 2006). Although the Imd pathways found in lepidopteran and dipteran fat body cells are homologous (Gandhe *et al.*, 2006), the similarities of Imd pathways in hemocytes of the two insect orders is not known (Ao *et al.*, 2008b). Peptidoglycan recognition proteins (PGRP), which are found on hemocytes and in soluble form in the hemolymph, are able to activate Toll and Imd pathways depending on the PGRP subtype (Dziarski and Gupta, 2006). Signalling in hemocytes in response to adhesion and engulfment of foreign particles may involve numerous molecules, for example, activation of mitogen-activated protein (MAP) kinases such as c-jun N-terminal (JNK) kinase and p38 mitogen-activated protein (Lamprou *et al.*, 2005), recognition by the soluble form of Down Syndrome Cell Adhesion Molecule (Dong *et al.* 2006) and intracellular cascades linked to Toll transmembrane receptors (Imler and Hoffmann, 2000). Additional factors associated with hemocytic signalling include: GTPase function (Williams *et al.*, 2006), PKA and PKC (Brooks and Dunphy, 2005; Giannoulis *et al.*, 2005) and re-modelling of actin (Castillo *et al.*, 2006; Richards and Edwards, 2002).

Non-self responses by hemocyte cell lines have been described for lepidopterans in the presence of microbial antigens. Antimicrobial nitric oxide derivates are detected in the supernatant of the hemocyte cell line of the salt marsh caterpillar, *Estigmene acreae* BTI-EA-1174-A when the cells are incubated with *E. coli* LPS or silica beads (Wittwer *et al.,* 1997; Weiske and

Wiesner, 1999). The addition of *E. coli* LPS to the BTI-EA-1174-A cell line induces also the extensive release of active proteases (Wittwer and Wiesner, 1998) suggesting that lepidopteran hemocytes outside the hemocoel may sense microbes via microbial surface antigens.

Malacosoma disstria is a major pest of North America's forests (Fitzgerald, 1995; Furniss and Carolin, 1977). Like other lepidopteran hemocyte cell lines (Aoki *et al*., 2004; Tanaka *et al*., 2003), *M.disstria* hemocyte lines have been developed for research convience, and have been used, for example, to study gene expression, ecdysteroid action (Palli *et al.,* 1995; Shuler *et al*., 1995), polyploidy (Ennis and Sohi, 1976) , and virus activity (Erlandson *et al*., 2006; Keddie *et al*., 1995). Unlike the innate cellular immunity which is expressed in presence of insect plasma in fresh hemolymph (Giannoulis *et al*., 2005; Giannoulis *et al*., 2007), the *M. disstria* cell lines allow examination of such immunity without plasma and hence facilitate direct studies of hemocyte-antigen interaction. Most *M. disstria* cell lines vary in their adhesiveness to foreign surfaces (Keddie *et al*., 1995) and thus possibly in their immune responses. The floating *M. disstria* hemocyte cell line, Md66, adheres to polystyrene flasks after 6 days post-treatment with the moulting hormone 20-hydroxyecdysterone, by producing cytoplasmic projections (Palli *et al*., 1995). Fragments of Md66 cell line hormone receptor 2 (MdHR2) and 3 (MdHR3) cDNA show homology with the ecdysone-inducible E75 gene of tobacco hornworm, *M. sexta*, *G. mellonella*, *D. melanogaster* and hormone receptor 3 of *M. sexta. G. mellonella, D. melanogaster,* respectively (Palli *et al*., 1995). In mosquitoes, the E75 gene product affects indirectly insect immunity enhancing vitellogenin upregulation and subsequently the synthesis of antimicrobial defensins by the fat body and release to the hemolymph (Raikhel *et al*., 2002).

Adherent *M. disstria* hemocyte cultures demonstrate extensive variation with respect to time for dissociation from polystyrene surfaces (Keddie *et al*., 1995; Sohi, 1973). Enzymatic treatment of Md108, UA-Md203

and UA-Md221 cell lines with proteases (trypsin, with or without collagenase), causes the release of adhering *M. disstria* hemocytes from the surfaces of tissue culture flasks (Keddie *et al.*, 1995; Sohi, 1973). Initial adhesion of insect hemocytes to foreign surfaces is physico-chemically influenced by hydrophobic (Wang and St Leger, 2006) and electrostatic interactions between those surfaces and the hemocyte membrane (Tackle and Lackie, 1985). In most studies of attachment by insect hemocytes emphasis is placed on hemocyte receptors and extracellular matrix proteins. Protein families of integrins (Levin *et al.*, 2005; Wittwer and Wiesner, 1996) and selectins (Okazaki *et al.*, 2006), collagen formation (Yasothornsrikul *et al.*, 1997) and immunoglobulin containing molecules (Watson *et al.*, 2005) have been implicated in adhesion process. Physicochemical analyses of integrins (see review Hynes, 2002) and fibronectins indicate that they present positive electrostatic charge on the surfaces of various human cell types (Sharma *et al.,* 1999), mollusc hemocytes (Jones *et al.*, 1976; Pierres *et al.,* 2002) and insect muscle cells (Devenport *et al.*, 2007); this is in contrast to the negatively-charged surface of polystyrene (Kennedy and O'Gara, 2004) and thus, cellular charge may influence the capacities of hemocytes to attach to the latter. Adhesion to negative surfaces varies among insect species. For example, desert locust, *Schistocerca gregaria,* larval hemocytes, in the absence of plasma do not engulf negatively-charged carboxyl methyl Sepharose beads (Lackie, 1983; Lackie, 1986); in contrast, hemocytes of the American cockroach, *Periplaneta americana,* adhere to and accumulate on the surfaces of such beads (Lackie, 1983).

Polystyrene is an aromatic hydrocarbon styrene polymer (Albright 1974; Li *et al.,* 2007). Polystyrene in standard tissue culture flasks is treated with ionized gas in an electric field, increases surface wettability, facilitating contact of biological material to the substrate (Chinn *et al.,* 1994). Hydroxyl groups on polystyrene favor the cell adhesion of human leucocytes through interations with complement factor C3b to the surface(Curtis *et al.*, 1983;

Sperling *et al.*, 2007); however, the mode of action has yet to be delineated (Sperling *et al.*, 2007). Since little is known of the ways in which the hemocytes interact with polystyrene, studies pertaining to the cellular responses of hemocytes to polystyrene were conducted as part of the research for this Thesis.

Bacillus subtilis is a rod-shaped, aerobic, Gram-positive, endospore-forming bacterium found in soil (Sonenshein *et al.*, 2002) and it has been used in many immunological studies (Timm *et al.*, 2006; Salina *et al.*, 2005; Marin *et al.*, 2005; Dani *et al.*, 2003; Da Silva *et al.*, 2000; Sagisaka *et al.*, 2001). Herein, the negatively charged bacterium was used to compare the responses of selected hemocyte lines with the response to polystyrene.

Reactions of *M. disstria* hemocytes *in vivo* (Giannoulis *et al.*, 2007) and *in vitro* (as primary cultures) with bacteria and their surface antigens (Giannoulis *et al.* 2005; Giannoulis *et al.*, 2008) are known. However, no research has been done on the non-self responses of the hemocyte cultures of *M. disstria***.**

Herein, I a) determined the optimum temperature for growth *in situ* of sticking and floating cell lines of *M. disstria,* b) characterized the extracellular enzyme profiles of these cells (deemed necessary to ensure the cultures did not degenerate) c) investigated the morphogenesis of selected cell lines, d) determined cell type frequencies and e) evaluated the occurrence of cell blebbing as a stress marker within the tissue culture flask environment to select for cell lines that most closely approximate freshly obtained hemocytes isolated from the larvae. Such cell lines were then used to examine non–self hemocyte responses to polystyrene and *B. subtilis*. Non-self responses were investigated at the optimum growth temperature in phosphate-buffered saline in terms of the types of enzymes, including lysozyme and phenoloxidase, and levels of total protein released from the cell lines in response to contact with polystyrene and *B. subtilis.*

MATERIALS AND METHODS

Hemocyte cell lines

Four hemocyte cell lines of larval *M. disstria* hemocytes (UA-Md203, UA-Md221, Md108, Md66) were used initially. UA-Md203 was derived from hemocytes of *M. disstria* larvae hatched from eggs collected from *Malus sp* in Edmonton (Alberta) and UA-Md221 from eggs on *Populus tremuloides* in the Peace River district of Alberta (Keddie *et al.*, 1995). Md108 (provided by Forestry Canada, Sault Ste Marie, Ontario) was derived from *M. disstria* larvae hatched from eggs found in the Sault Ste Marie area and Md66 was derived from Md108 (Ennis and Sohi, 1976; Sohi, 1971; Sohi, 1973). The culture medium was based on Grace's insect tissue culture medium enriched with Bacto tryptose broth (0.25 g/100 ml of medium) (Sohi, 1973), adjusted to pH 6.2 with 1 N KOH and filter sterilized (0.22 µm pore size). This medium was subsequently supplemented with sterile heat-inactivated (56 ^{o}C for 30 min) fetal bovine serum (final concentration 8% v/v) and is referred to as culture medium throughout this study.

The cell lines Md108, UA-Md203 and UA-Md221, which grow attached to the polystyrene substrate, were subcultured by dissociating the hemocytes after they achieved 80-90% confluency of growth. Dissociation involved the removal of the culture medium and the rinsing of the adherent cells with 5ml Rinaldini's balanced salt solution (Rinaldini 1959, 0.8 g NaCl, 0.02 g KCl, 0.1 g Na citrate, 0.005 g NaH_2PO_4, 0.1 g $NaHCO_3$, 0.1 g glucose in 100 ml deionized water). The cell lines UA-Md203 and Md108 were then incubated in 0.05% (w/v) trypsin in Rinaldini's balanced salt solution for 1h (Keddie *et al.*, 1995) and 5 min (Sohi, 1973), respectively. The cells were subsequently rinsed with and finally suspended in culture medium (5 ml). For cell line UA-Md221, dissociation involved incubation for 1 h at 21 ^{o}C with both trypsin (as above) and collagenase (0.05%, Keddie *et al.*, 1995). This cell line was rinsed free of proteases using culture medium and suspended by

gentle pipetting. Stock cultures were maintained by inoculating 25 cm^2 flasks (Corning) with 2 x 10^4 cells per 5 ml of culture medium and incubating at 21 ^oC in the dark. A 1ml aliquot of the floating cell line, Md66, at 80% confluency was added to 5ml of fresh culture medium. For experimental purposes, cells from the four types of culture were used when they reached 50% confluency in the tissue culture flasks, unless stated otherwise. Fifty per cent confluency was chosen because the hemocyte density in the tissue culture flasks per mm^2 was similar to the total hemocyte counts obtained with adherent cells in the hemocyte monolayer assays in which whole hemolymph of *M. disstria* was used (Chapter 2).

Bacteria

Stock cultures of *B. subtilis* (Boreal Biological Company) were subcultured every 2 weeks on tryptic soy agar and incubated at 5 ^oC. For experimental tests, the bacteria were incubated at 25 ^oC in tryptic soy broth (10 ml) in scintillation vials (20 ml) on a gyrotary shaker (250 rpm) until they achieved an optical density at 660 nm of 0.75. Dead bacteria were used to avoid ongoing bacterial metabolism from influencing results (Alavo and Dunphy, 2004).The bacteria were killed by irradiation for 3 h with ultraviolet light (203 nm, 20 ml, 25 ^oC). After centrifugation (20 ml, 12,000 x g, 2 min, 25°C) and suspension of the cells in PBS (1 ml) three times (henceforth referred to as centrifuge-washing) viability was determined by spreading 100µl of aliquots of bacterial suspension on tryptic soy agar and following 96h incubation at 25 ^oC and 30 ^oC, colony counting; the absence of colonies established the bacteria as dead.

Optimum temperature for hemocyte growth

Flasks containing 10 ml of culture medium were inoculated with 4x10^4 cells prior to incubation at selected temperatures. Because the ambient

temperature experienced in the field by *M. disstria* last instar larvae ranges from 15 °C to 31 °C (Fitzgerald, 1995; Blais *et al.*, 1955; Kraus, 2006), the cell lines were incubated at 15, 21, 28 and 31 °C. Cell growth was assessed *in situ*, in terms of cells per mm^2 using a stereo-dissecting microscope (magnification 50X). The effect of selected temperatures on the growth of cell lines was monitored for 132 h. Thereafter cell counting was complicated because the cultures formed three dimensional cell masses in which cell adhesion occurred partially to polystyrene and partially to other hemocytes limiting accurate counting. Trypsin did not dissociate the aggregated hemocytes. Unless stated otherwise, the temperature found to be optimum for the growth of each cell line was used in subsequent experiments.

To determine if the effects of growth at suboptimal temperatures were reversible, two of the most temperature-sensitive cell lines, UA-Md221 and UA-Md203, which exhibited little or no growth at 15 °C, were shifted from 15 °C to 21 °C, a temperature that does permit their growth, and the effects of this shift on growth parameters were determined. The recovery test was done using these temperatures since this would simulate environmental conditions experienced by fifth and sixth instar *M. disstria* larvae in the collection areas (Peace River and Edmonton) (average maximum temperature in Edmonton and the Peace River area for the period May 15 to June 15 during years 1997-2007 were 18.3 ± 5.5 °C and 17.8 ± 4.9 °C, respectively).

Discharge of enzymes from hemocyte cell lines in culture medium

Culture medium (10 ml) from flasks with adhering cell cultures at 50% confluency, grown at the optimum temperature was collected and centrifuged (325 x g, 4 min, 21 °C) to remove hemocyte debris. The supernatant was examined for its enzyme content.

For the continuously floating cell line Md66, enzyme content was assessed at two different times, at the time of flask inoculation and when the cell culture reached 50% confluency. At each time points, samples of the cell

culture were centrifuged (325 x g, 4 min, 21 °C) and the supernatant was collected and examined for its enzyme content. Relative differences of enzyme activities between inoculation time and 50% confluency of the Md66 cell culture were used to assess the enzyme discharge in the culture medium.

The release of enzymes which based on specific substrate hydrolysis, only, were regarded as enzymes with activity like alkaline phosphatase and acid phosphatase, esterase (C4), esterase-lipase (C8), lipase (C14), leucine-aminopeptidase, valine-aminopeptidase, cystine-aminopeptidase, trypsin, chymotrypsin, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase by hemocyte cell lines was determined qualitatively and semiquatitatively using the API ZYM test strips (bioMerieux Inc., Montreal, Canada). Henceforth, all API ZYM detected enzymes will be written without prepositional adjective "like". Test strips capsules were incubated with 65 µl volumes of culture medium and the strips were incubated (4 h, 21 °C) and developed as directed by the manufacturer. Identification of enzymes and semiquantitive estimation of enzyme activities were based on visual estimations of reaction-associated color development. Heat–inactivated fetal bovine serum was also checked for enzyme content. Fetal bovine serum (8% (v/v) in PBS and Grace's medium) was tested similarly for the presence of enzymes.

Hemocyte morphological characterization during growth

Cell morphogenesis, cell type frequencies, and blebbing were examined with phase contrast microscopy *in situ* for the adhering hemocyte cultures. Examination of Md66, the floating hemocytes, involved the spreading of 80 µl aliquots of tissue culture fluid on 1 cm^2 glass slide surface. Morphometrics of the cell types in the different cultures involved determination of cell shapes, the maximum length or diameter of the cell and where applicable, additional information on appendage formation.

Photographs of hemocytes were taken with Nikon camera Coolpix 990 on Olympus BH2 phase contrast microscope at 40 X magnification.

Blebbing, an indicator of stress and apoptosis in hemocytes of lepidopteran larvae (Pech and Strand, 2000; Richards and Parkison, 2000; Daborn *et al.*, 2002) and associated with the release of cytoplasmic contents from stressed mammalian cells (Charras, 2008), was quantified during cell growth.

Discharge of enzymes and total protein from hemocytes reacting to foreign matter in phosphate - buffered saline (PBS)

The responses of hemocytes to antigens, including enzyme discharge, may be affected by the nature of the culture medium. Fetal calf serum contains growth factors that affect eukaryotic cell differentiation and spreading (Zheng *et al.,* 2006) and could affect lepidopteran hemocyte activity; for example, insulin-like molecules in fetal bovine serum act as a hemocyte population growth factor in plasma of *B. mori* (Nakahara *et. al*., 2006) and promotes mitotic division in granular cells of the same insect species (Saito and Iwabuchi, 2003). Additionally, integrin β1 in fetal bovine serum (Zheng *et al*., 2006) could have an effect on hemocyte adhesion, such protein receptors being known to participate in plasmatocyte encapsulation of Sephadex beads (Levin *et al*., 2005). Sugars in the medium are known to decrease lectin mediated hemocyte coagulation (Minnick *et al*., 1986) and opsonic phenoloxidase activity (Dunphy and Chadwick, 1989). Thus, to elucidate the response of the hemocytes to polystyrene and dead *B. subtilis* without possible modulation by the culture medium, the hemocytes were washed free of culture medium using PBS. Md66, derived from Md108, and Md108 were used to preclude any possible heterogeneity in non-self responses which could be attributed to geographic locations. This particulary significant since, based on the mitochondria cytochrome oxidase I gene, *M. disstria* may represent a morphologically similar species complex (Herbeck, 2002). Md66

was washed by centrifugation (325 x g, 4 min, 21 ^{o}C) and the pellet resuspended in PBS (5 ml). Md108 was rinsed with PBS (5 ml). Hemocyte viability, based on the exclusion of the vital stain, trypan blue (0.1% w/v in PBS), was greater than 85-90%. Both Md66 and Md108 cell lines at 50% confluency (Md66: 1.4 x 10^6 cells/ml PBS; Md108: 150 cells/mm^2) were incubated in culture flasks without and with *B. subtilis* (1.6 x 10^8 bacteria/ml PBS) in PBS (5 ml) for selected periods of time (0 – 40 min) at 21 ^{o}C. Bacterial controls for possible protein and enzyme release consisted of PBS with *B. subtilis*. Samples were centrifuged (325 x g, 4 min, 21 ^{o}C) to remove the cell debris and the supernatants were then analyzed for enzymes (using the APIZYM system previously described) and for total protein (Bradford, 1976).

Lysozyme and phenoloxidase released from Md66 and Md108 in PBS without and with *B. subtilis* after 0-40 min at the optimum temperatures of two cell lines. PBS with *B. subtilis* only served as the negative control. Supernatants produced by centrifugation were used in the following assays.

Lysozyme activity in 10µl of supernatant was based on the lytic zone diameter of *Micrococcus lysodeikticus*(2 mg/ml) PBS solidified with agar (1.5% w/v). Control plates were inoculated with 10 µl chicken lysozyme (Sigma) in PBS (0.5 – 25 mg lysozyme/ml). Total protein released from the hemocytes over 40 min of incubation was determined with Bradford assay (Bradford, 1976).

Phenoloxidase activity was assayed by measuring dopachrome formation (Leonard *et al.*, 1985). Briefly, 100 µl of L-dihydroxyphenylalanine (2 mg/ml), a phenoloxidase substrate, was added to 100 µl of supernatant. Following 5 min of incubation at room temperature, the absorbance of the solution was measured at 490 nm to indicate the amount of dopachrome formation reflecting the amount of activated phenoloxidase (Leonard *et al.*, 1985). This protocol measures bacteria - activated enzyme which may not be the total phenoloxidase in the system. Therefore, five microliters of laminarin

(1 mg/ml), an activator of zymogenic prophenoloxidase (Goldworthy *et al.*, 2002; Giannoulis *et al.*, 2007), was added to the supernatants (100 µl) which were incubated for 60 min at room temperature after which enzyme subtrate (100µl) was added. Absorbance was determined at 490 nm.

Statistics

Data were analyzed using the 95% confidence limits overlap protocol and graphic and tabular data are presented as means ± standard error of the mean (Sokal and Rohlf, 1969). Ten replicates were used unless stated otherwise.

RESULTS

Optimum temperature for cell growth

The optimum temperature for growth was 28 °C for all isolates except UA-Md203 for which 21°C was optimal and Md66 which grew best at 21-28 °C (Figure 5.1 – Figure 5.4). However, this does not mean that the cell lines exhibited similar growth profiles at their optimum or suboptimum temperatures. Although UA-Md221 cells grew marginally during the first 24 h at 15 °C, returning these cells to 21°C at 96h post-inoculation did not restore growth during the remaining 72 h of observation indicating temperature-induced hemocyte damage as opposed to cytostatic effects. Cell multiplication was slow but marginal at 31 °C. UA-Md203, unlike the other cell lines, grew more rapidly at 21 °C than 28 °C, slowly at 15°C and not at 31 °C (Figure 5.2). UA-Md203 cell line grew more quickly upon shifting the culture from 15°C to 21 °C.

Md108, which exhibited comparable growth profiles and rates at 21 °C and 28 °C, grew to a significantly lesser extent and slower rate ($P < 0.05$) at 31 °C and lesser still at 15 °C, respectively (Figure 5.3). Growth patterns similar to Md108 were seen for Md66 ($P > 0.05$).

The projected level of inoculum adhesion to the flasks by 24h was anticipated to be 8 cells/mm^2. However, the actual number of cells at 24 h post inoculation varied with hemocyte cell line; for Md108 the adhesion numbers were 2-7 cells/mm^2 (in all tested temperatures), for UA-Md221 2-4 cells/mm^2 (in all tested temperatures) and for UA-Md203 1-4 cells/mm^2 (in all tested temperatures). The differences in cell adhesion are due possibly to combination of protease effect, possible cell lysis, conditioned medium adaptation and/or temperature effects. In the floating cell line there is also variation in actual cell counts for possibly the same reasons.

Figure 5.1: Growth of *Malacosoma disstria* hemocyte cell line UA-Md221 on polystyrene surfaces at selected temperatures. (n=3 flasks, 25 random fields examined in each flask)

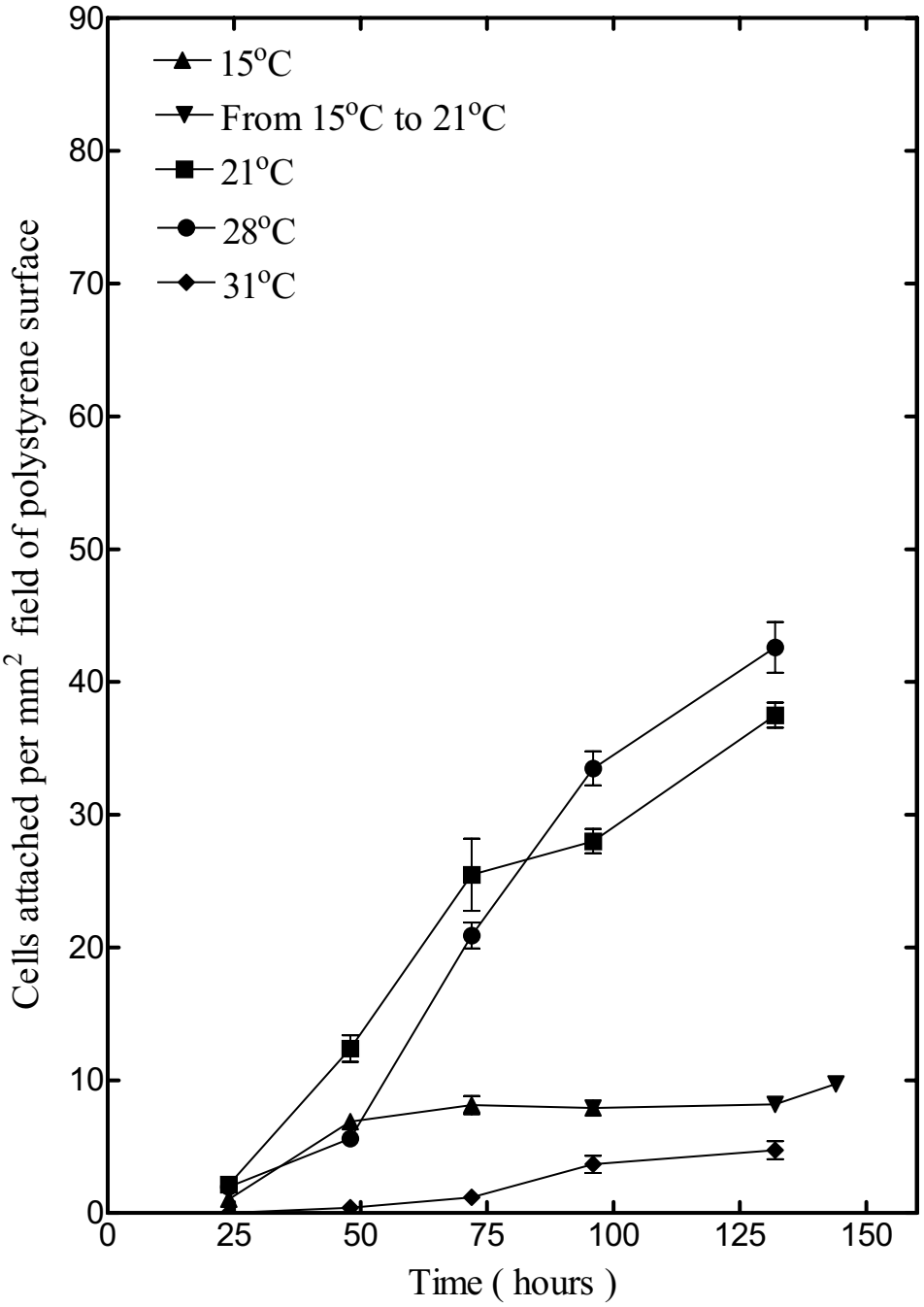

15°C
From 15°C to 21°C
21°C
28°C
31°C
Cells attached per mm2 field of polystyrene surface
Time (hours)
0
10
20
30
40
50
60
70
80
90
0
25
50
75
100
125
150

Figure 5.2: Growth of *Malacosoma disstria* hemocyte cell line UA-Md203 on polystyrene surfaces at selected temperatures. (n=3 flasks, 25 random fields examined in each flask)

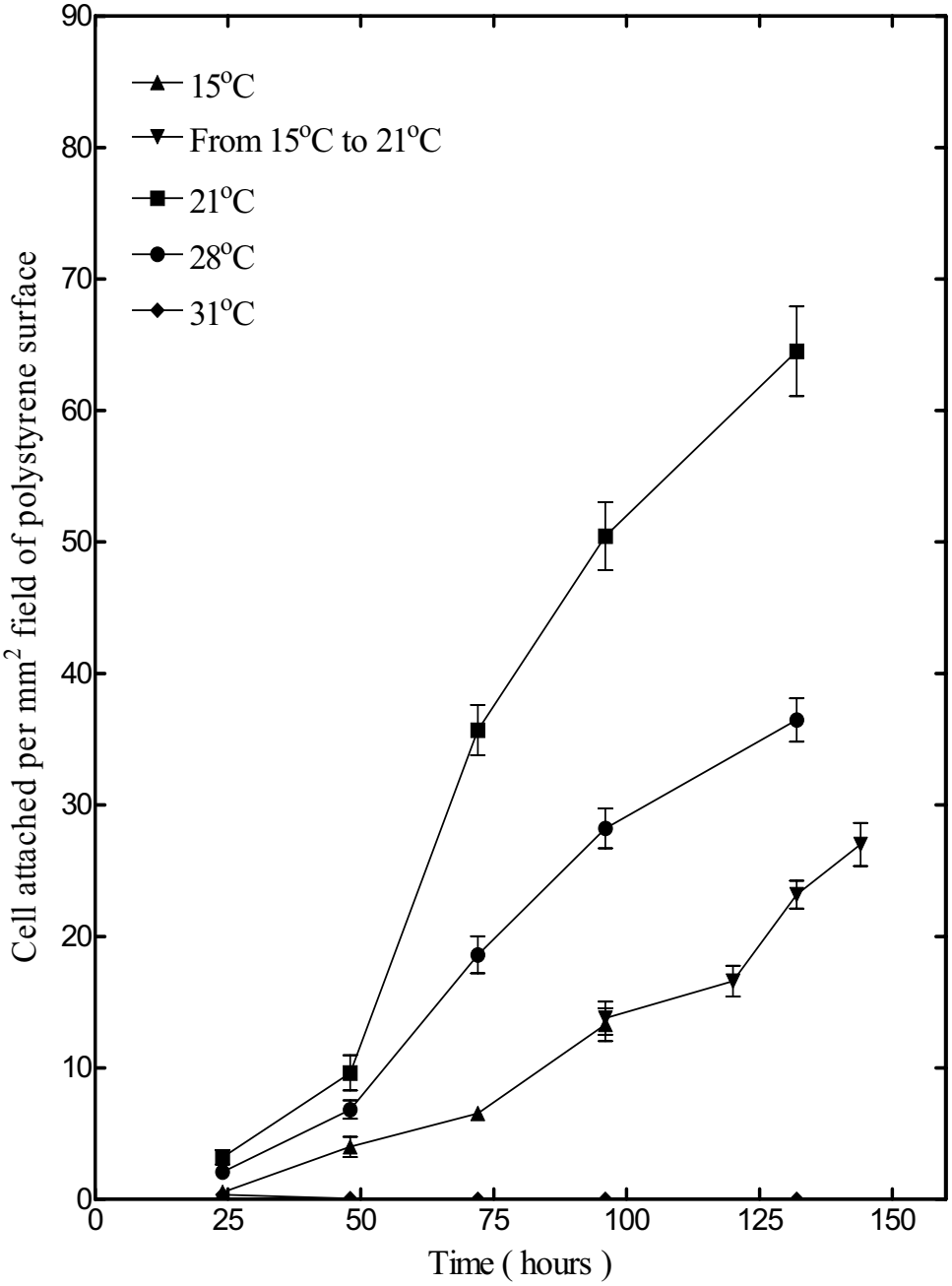

15°C
From 15°C to 21°C
21°C
28°C
31°C
Cell attached per mm2 field of polystyrene surface
Time (hours)
0
10
20
30
40
50
60
70
80
90
0
25
50
75
100
125
150

Figure 5.3: Growth of *Malacosoma disstria* hemocyte cell line Md108 on polystyrene surfaces at selected temperatures. (n=3 flasks, 25 random fields examined in each flask)

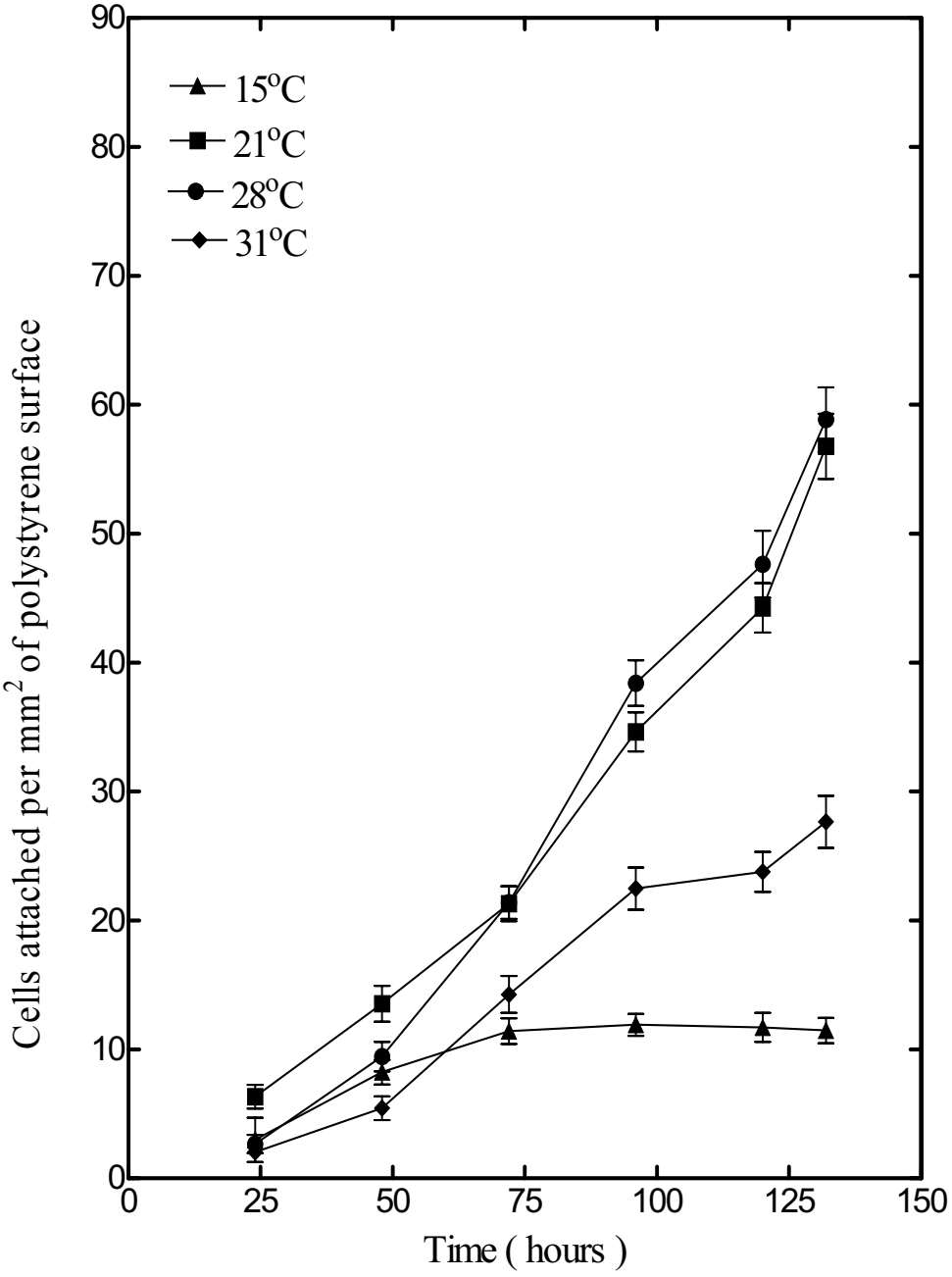
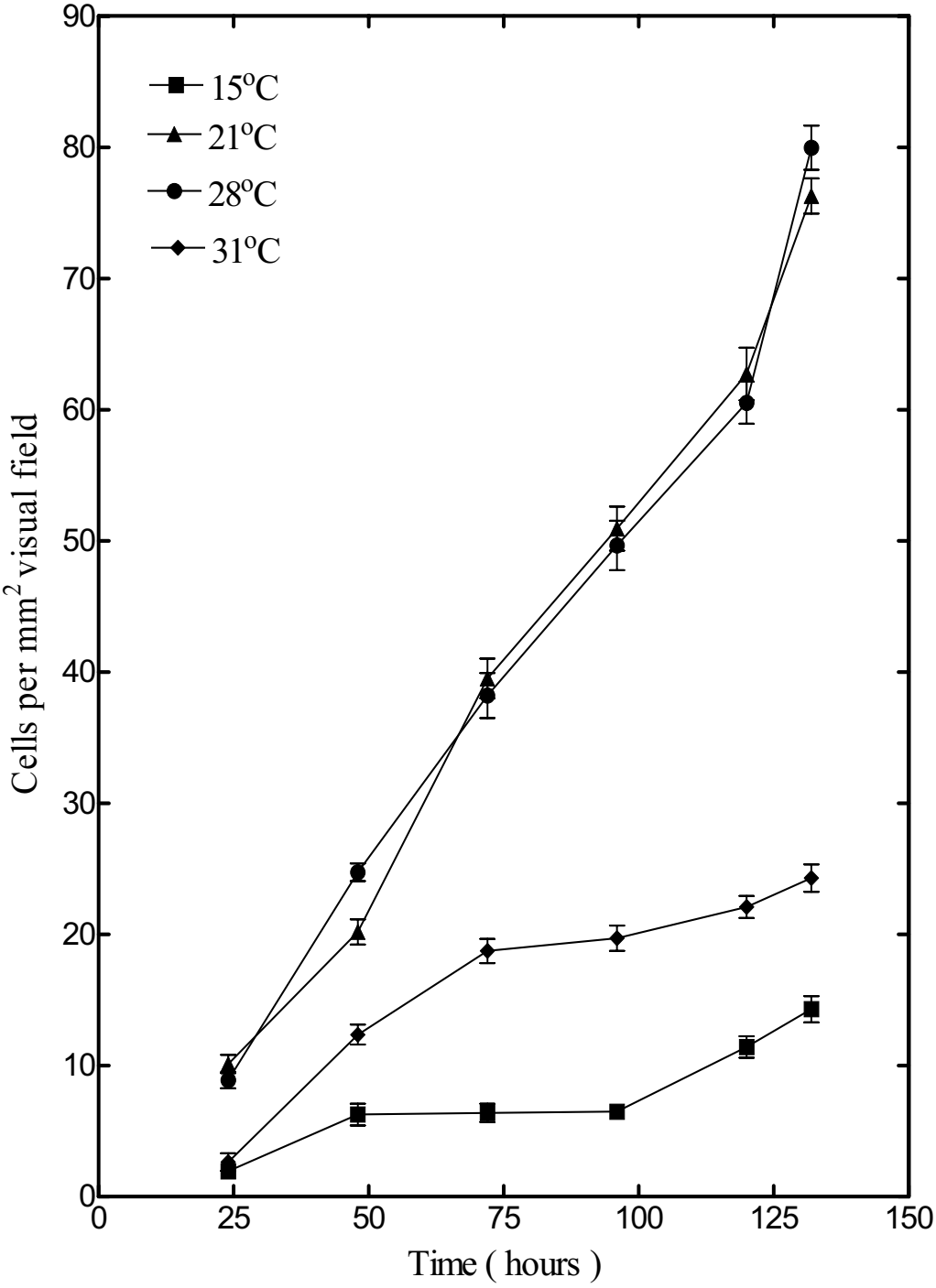

15°C
21°C
28°C
31°C
Cells attached per mm2 of polystyrene surface
Time (hours)
0
10
20
30
40
50
60
70
80
90
0
25
50
75
100
125
150

Figure 5.4: Growth of *Malacosoma disstria* hemocyte cell line Md66 at selected temperatures. (n=3 flasks, 25 random fields examined in each flask)


15°C
21°C
28°C
31°C
Cells per mm2 visual field
Time (hours)
0
10
20
30
40
50
60
70
80
90
0
25
50
75
100
125
150

Characterization of cell lines in terms of blebbing

Cell stress in culture medium, as exemplified by blebbing (Figure 5.5), was marginally discernible (less than 10% of the total number of hemocytes) during the first 10 days of cell growth when all cell lines were less than 50% confluency (50% confluency occurred within 10-15 days post inoculation, the time varing with the cell line). Blebbing increased with increasing cell confluency levels, especially after 90% confluency (30 post inoculation days) (Figure 5.6). Blebbing was least in UA-Md203 at all confluency levels compared with the other cell lines which exhibited a similar extent of blebbing between day 10 and 35 post-inoculation time. Cell aggregation for all cell lines was evident during late culture (cell levels at more than 90% confluency). Blebbing was not detected in any cell line during their short exposure to PBS even though the hemocytes were at the 50% confluency level; hence it is unlikely that blebbing contributed to the observed release of enzymes and total protein from the cells in PBS.

Cell types

The cell types during and after subculturing varied between the cell lines UA-Md221, Md108 and Md66 as shown in Figures 5.7 - 5.9. Descriptions of the characteristics of each cell type category are given in Table 5.1. For UA-Md221 and Md108 cells lines, recovery from stress induced by the dissociation solution, as seen with the appearance of morphological forms not seen during growth and only detected after protease treatment, occurred from 48-76 h and 24-48 h, respectively.

Cell frequencies

The cell type that appeared most frequently in the Md66 culture was type 1 and it remained at relatively constant levels throughout the 288h

incubation period (Figure 5.10). Cell types 3, 4 and 6 also remained at relatively constant levels during most of the incubation period. However the levels of cell type 2 declined at 216 h and returned to the usual level at 288 h whereas cell type 3 declined at 288 h. Cell type 5 increased bimodally to a plateau level from 72 h-216 h and then again by 288 h (Figure 5.10).

For cell line Md108, trypsin treatment altered the incidence of the hemocyte types. Md108 cell types 3-5 were not evident until 48 h post trypsin exposure by which time cell types 1 and 2 had ceased to exist. Thus, Md108 cell types 1 and 2 may represent the stress response of the cell types to trypsin. The levels of Md108 cell types 3-5 although differing between each other, were constant from 48 h onwards.

Extensive morphological diversity of hemocytes of Md221 cell line did not permit cell type frequency analysis at their optimum growth temperature.

Figure 5.5: Example of blebbing in Md108 cell type 1 (see Figure 5.8) from a 40 day old culture, grown at 31 ^{o}C.

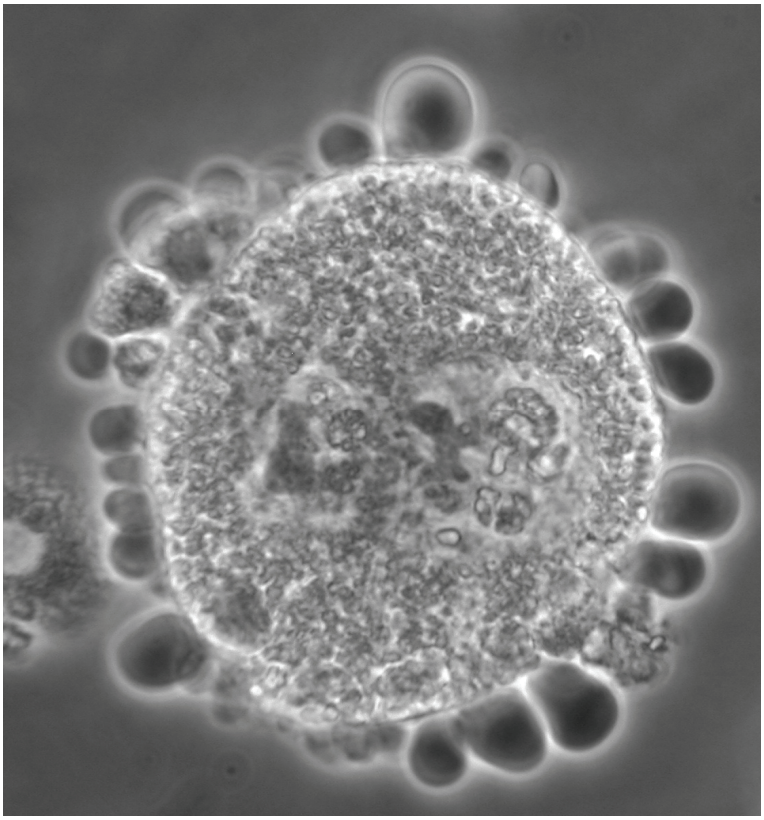


Figure 5.6: Levels of cell blebbing in Md66, UA-Md203, Md108 and UA-Md221 *Malacosoma disstria* cell lines during their growth at their respective optimum temperatures (n=10).

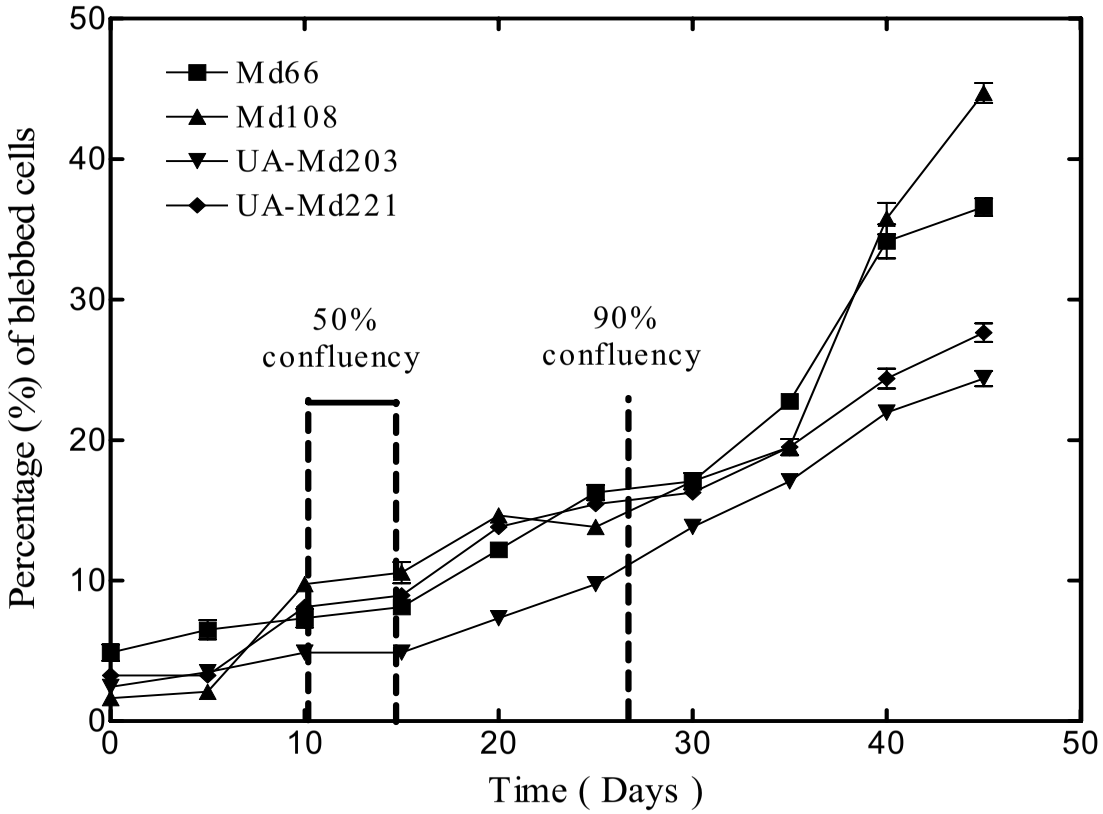
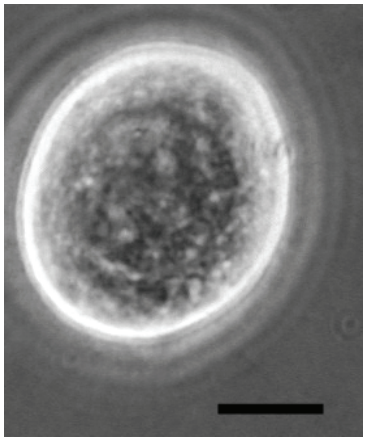
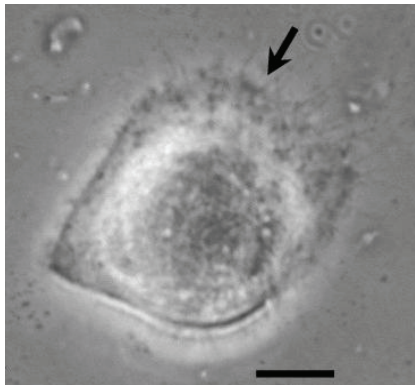

Md66
Md108
UA-Md203
UA-Md221
50% confluency
90% confluency
Percentage (%) of blebbed cells
Time (Days)
0
10
20
30
40
50

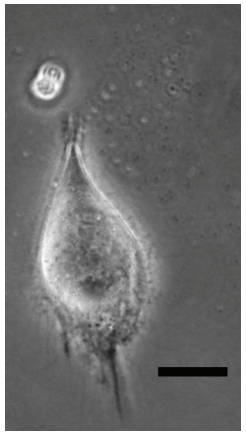
Figure 5.7: Morphogenesis of *Malacosoma disstria* Md66 cell line hemocytes. (Bars = 5µm, cell morphology is decribed in Table 5.1. Arrows are also defined in same table)



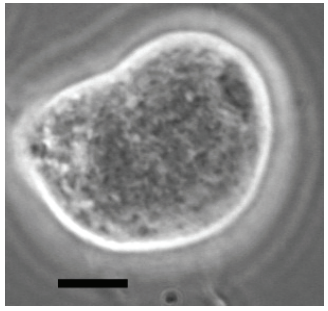
Md66 cell type 1



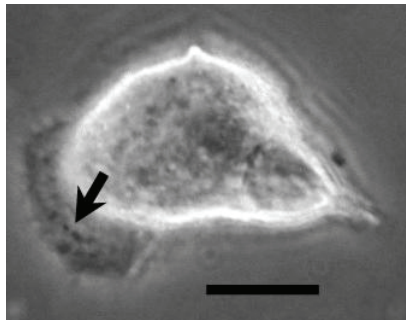
Md66 cell type 2



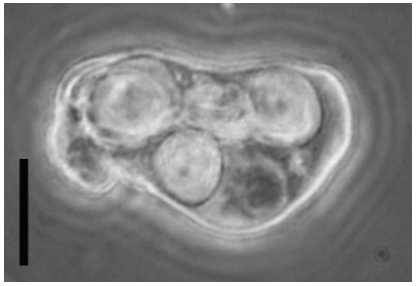
Md66 cell type 3



Md66 cell type 4

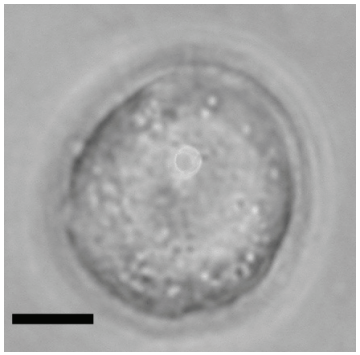


Md66 cell type 5

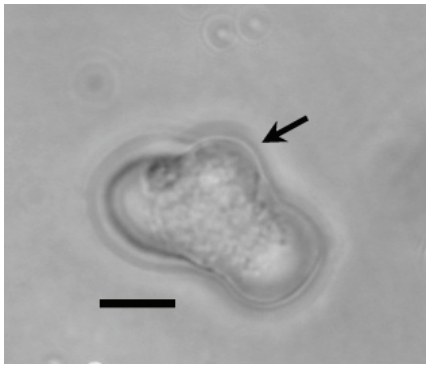


Md66 cell type 6

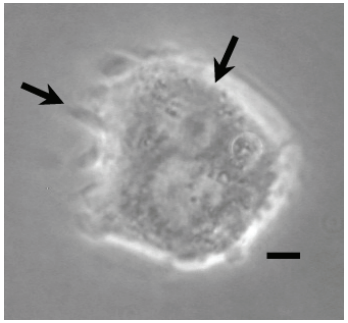
Figure 5.8: Morphogenesis of *Malacosoma disstria* Md108 cell line hemocytes. (Bars = 5µm, cell morphology is decribed in Table 5.1. Arrows are also defined in same table)



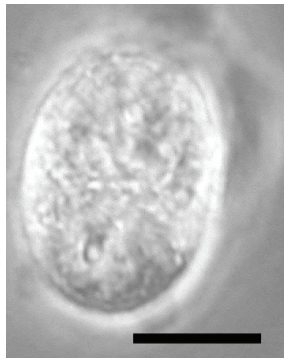
Md108 cell type 1



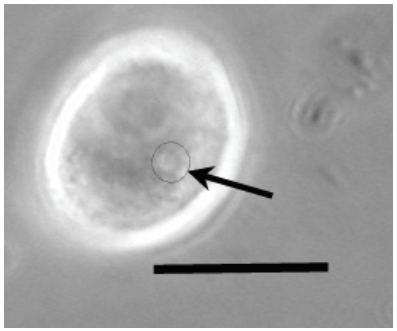
Md108 cell type 2



Md108 cell type 3

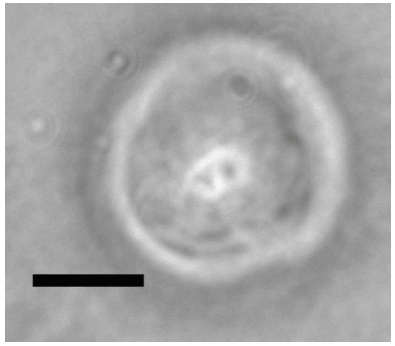


Md108 cell type 4

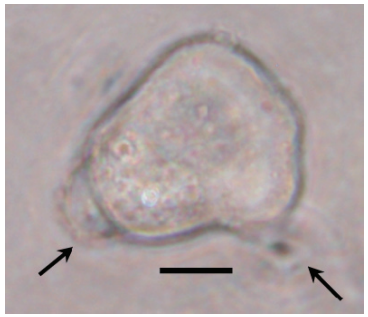


Md108 cell type 5

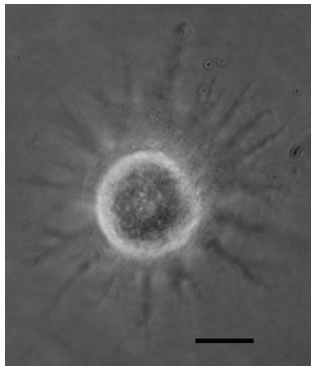
Figure 5.9: Morphogenesis of *Malacosoma disstria* UA-Md221 cell line hemocytes. (Bars = 5µm, cell morphology is decribed in Table 5.1. Arrows are also defined in same table)



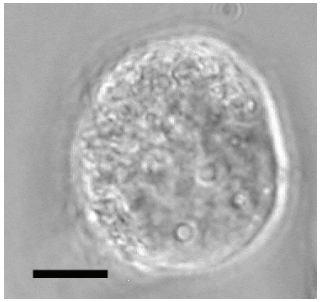
UA-Md221 cell type 1



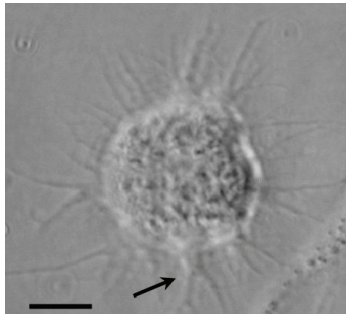
UA-Md221 cell type 2



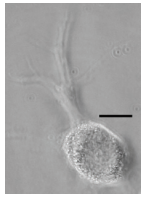
UA-Md221 cell type 3



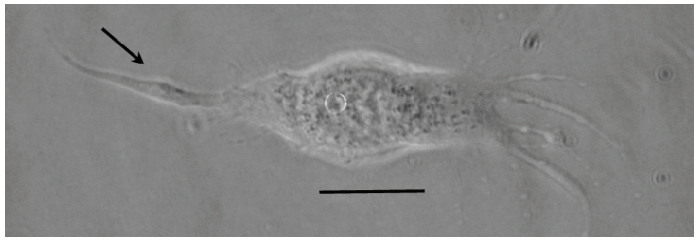
UA-Md221 cell type 4



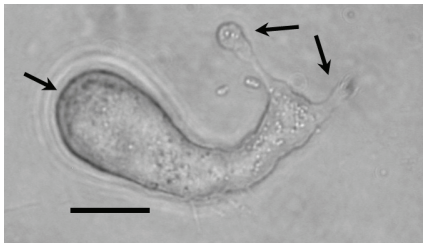
UA-Md221 cell type 5



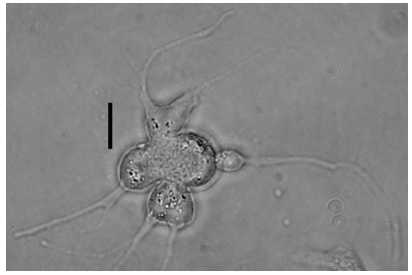
UA-Md221 cell type 6



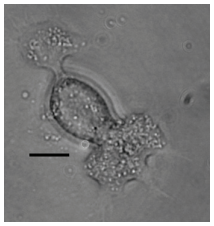
UA-Md221 cell type 7



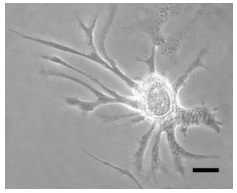
UA-Md221 cell type 8



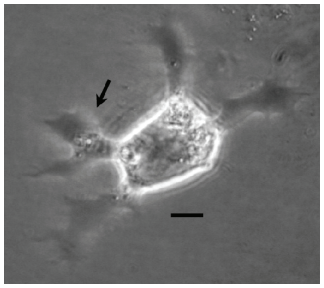
UA-Md221 cell type 9



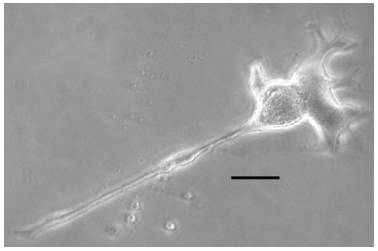
UA-Md221 cell type 10



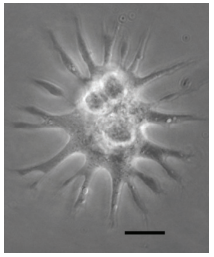
UA-Md221 cell type 11



UA-Md221 cell type 12



UA- Md221 cell type 13



UA-Md221 cell type 14

Table 5.1: Description of cell forms detected in different *Malacosoma disstria* hemocyte cell lines in culture medium at their optimum temperatures.

Hemocyte cell line	Hemocyte type	Description	Cell Size[a] (µm)
Md66	1	Round cells, smooth edged with large central nucleus (~ 8µm diameter).	7-24
	2	Oval cells with large central nucleus (~ 8µm diameter), chromatin not visible, filopodia with some membrane extension beyond the main cell mass and between the filopodia (arrow).	8-13
	3	Elongated cells with one or more thick projections at one or both ends without or with an apical membrane marginalized with numerous filopodial structures; central nucleus with or without discernible chromatin.	9-17
	4	Oval cell with nucleus lacking discernible chromatin.	13-20
	5	Oval-trapezoid plasmatocyte-like cell with or without thick projections and with an occasional crenated membrane opposite the projections (arrow)	8-10
	6	Elongated cell containing 5-6 spherical inclusions (~5-4µm diameter)	9-13

Md108	1	Spherical cell with a large central nucleus (~8µm diameter) and phase bright granules in the cytoplasm	4-12
	2	Oval-ablong hemocytes with more than one projection (arrow), no discernible nucleus; phase dark with few granules	7-13
	3	Oval cell with filopodia extending beyond the plasmacytoid membrane, ≥2 nuclei (5-8µm diameter)	9-12
	4	Oval granulated hemocyte, no detectable nucleus	5-7
	5	Spherical cels with small eccentric nucleus (1µm diameter)(arrow)	4
UA-Md221	1	Spherical cell with a large central nucleus.	7-12
	2	Pear-shaped cells with more than one lobopodial projection (arrow), nucleus, cytoplasm phase dark with few granules	10-14
	3	Stellate plasmatocyte with clearly visible nucleus in the center.	17-22
	4	Oval, granulated cell, no detectable nucleus. Similar, but smaller ($P < 0.05$) cell type was observed in Md108, (cell type 4; Figure 5.8).	13-20

UA-Md221 continued	5	Granular cell with filopodial projections (arrow)	7-16
	6	Round cell with monopolar projection, that ends with dendritic-type tips.	17-22
	7	Elongated, polarized cell with one (or more) stylet-type projections (arrow) on one side and numerous projections on the other. Large nucleus (3-4µm) is visible in the center.	(24-31) x (5-8)
	8	Polymorphic elongated cell with one side oval shaped and the other ending in globular projections (arrow)	14-18
	9	Multifilopodial cell with round central body and peripheral globular condensations	16-24
	10	Oval cell with flat projected cytoplasmic extensions at both tips	19-26
	11	Multi-branched cell with asymmetrical radiations	22-34
	12	Polygonal cell shaped main cell with enlarged lobopodia	15-17 body 7-8 projection

UA-Md221 continued	13	Dendritic hemocyte, with a linear axis	8-12 main body axis 15-17
	14	“Triploid” stellate cell, bifurcated, with linear filopodia	24-29

[a]Maximum dimensions (μm)

Figure 5.10: Cell type frequencies in *Malacosoma distria* hemocyte cell line Md66 (n=10). Bar values with the same letter on the top are not significantly different (P > 0.05).

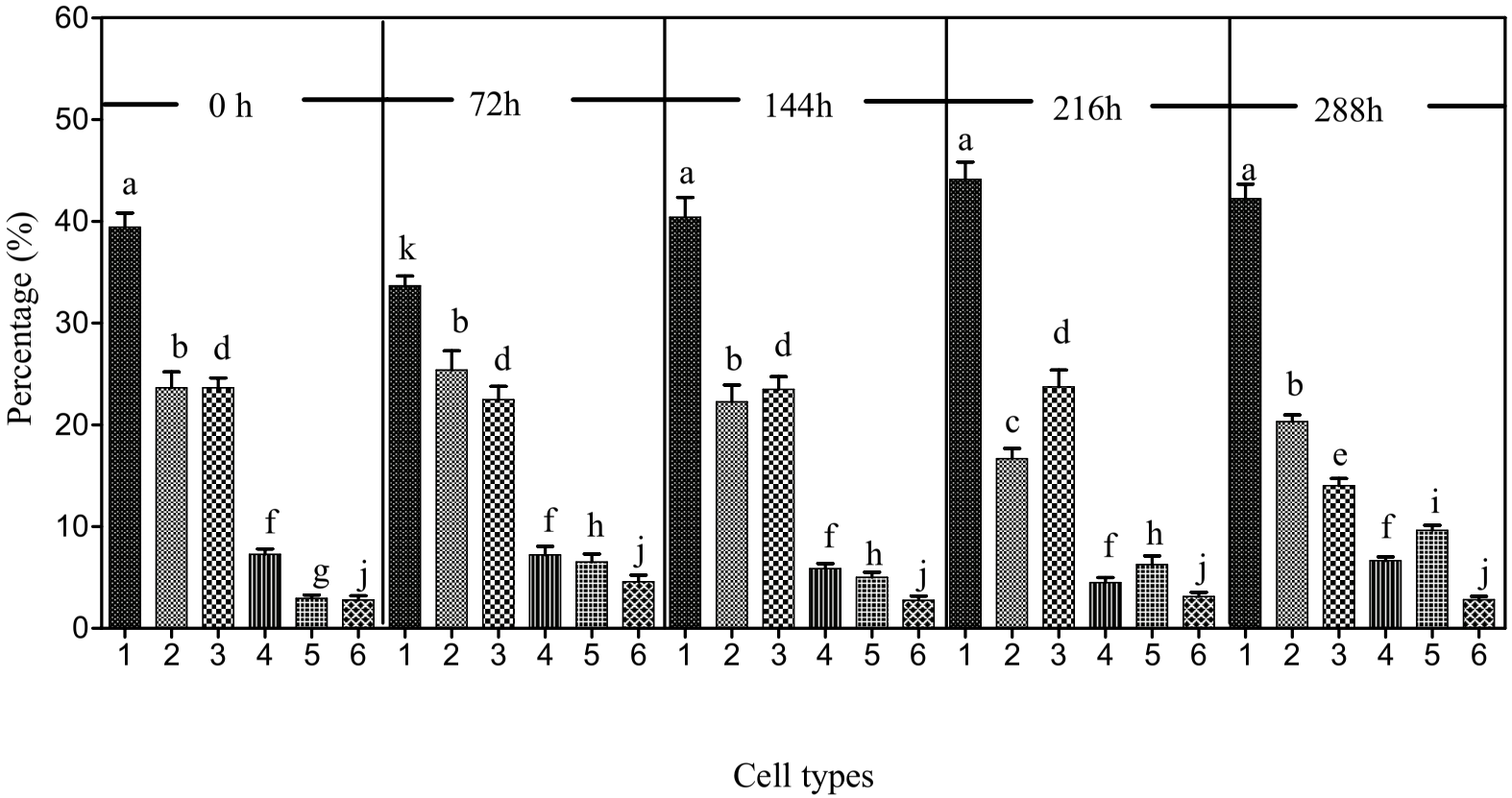
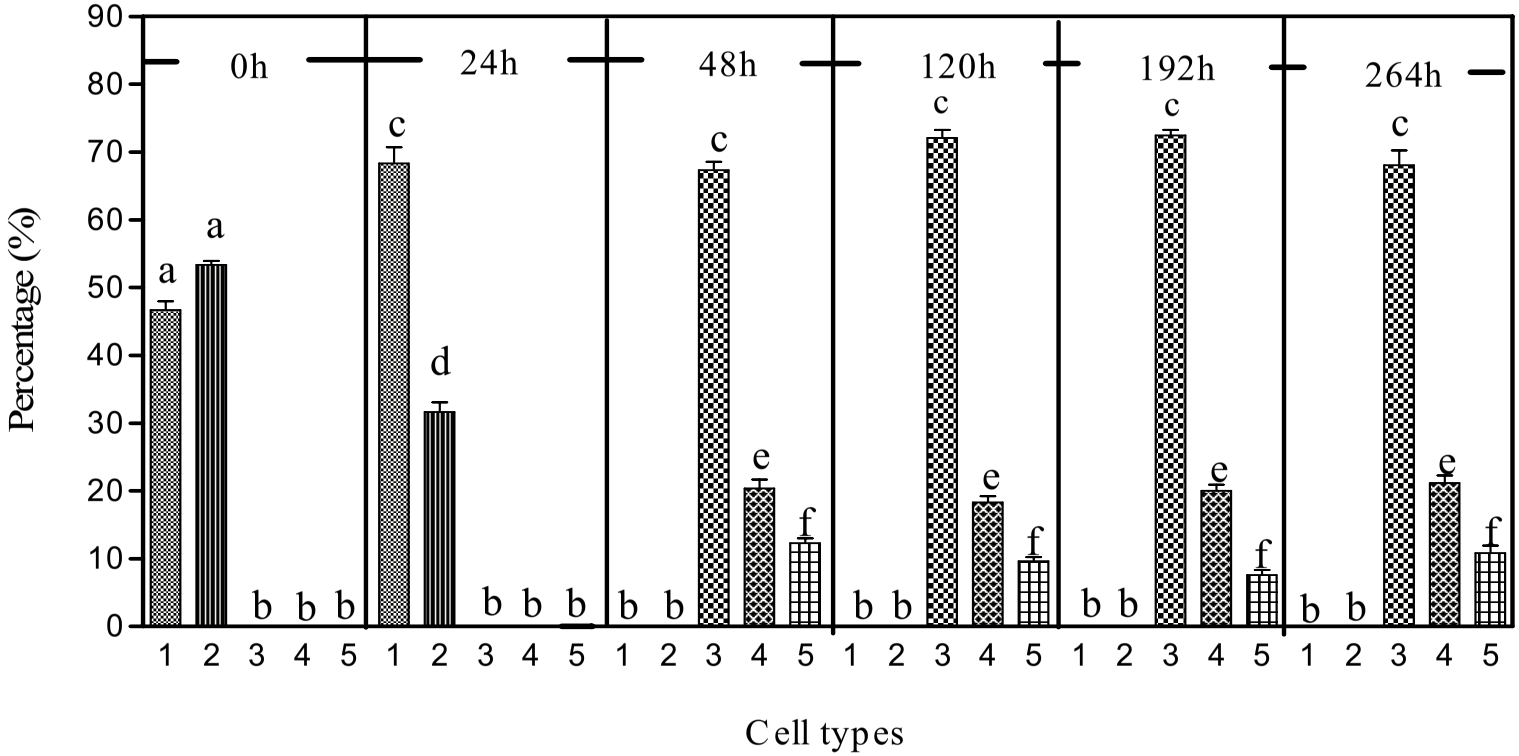

0 h
72h
144h
216h
288h
Percentage (%)
Cell types
0
10
20
30
40
50
60
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
a b d f g j k b d f h j a b d f h j a c d f h j a b e f i j

Figure 5.11: Cell type frequencies of *Malacosoma distria* hemocyte cell line Md108 (n=10). Bar values with the same letter on the top are not significantly different (P > 0.05).


Percentage (%)
0h
24h
48h
120h
192h
264h
Cell types

Discharge of enzymes from *Malacosoma disstria* cell lines in culture medium

Centrifuged spent media from cultures of UA-Md221 and UA-Md203 contained comparable levels of the following salient enzymes, esterase-lipase (C8), phosphoamidase and N-acetyl-β-glucosaminidase, whereas the supernatant of culture UA-Md221 contained more acid phosphatase and cells of UA-Md203 released more valine–aminopeptidase, cystine-aminopeptidase and α-galactosidase and β-glucuronidase (Table 5.2). Both adhering lines released the serine proteases trypsin and chymotrypsin, UA-Md203 spent medium containing more activity of both enzymes than did UA-Md221 ($P < 0.05$).

Spent supernatants from adhering Md108 cells contained significantly less ($P < 0.05$) esterase (C4) activity than did spent media from cultures UA-Md221 and UA-Md203. Esterase-lipase (C8) levels in supernatants of UA-Md221 were higher than those of the other cell lines, the enzyme activity being the same in other two adhering cell lines. The moderately adhering cell lines Md108 and UA-Md203 released higher levels of more types of enzymes than did UA-Md221; Md108 released more esterase, leucine-aminopeptidase and N-acetyl-β-glucosaminidase than did UA-Md221 and UA-Md203 whereas supernatant of the UA-Md203 medium contained more valine-aminopeptidase, cystine-aminopeptidase and trypsin and chymotrypsin than did supernatants from the other adhering lines. There was no discernible relationship between trypsin and chymotrypsin activity with hemocyte adhesion avidity between the three adhering cell lines.

α-Mannosidase and acid and alkaline phosphatases were released into culture medium for all hemocyte cell line types. High α-mannosidase was observed in media with UA-Md221 and UAMd-203 cell lines whereas Md108, which released the lowest amount of α-mannosidase, released the highest levels of the two types of phosphatases.

Md66 cell line discharged fewer types of enzymes into culture medium than the other cell lines tested (Table 5.2). There was no evidence of trypsin or chymotrypsin release. Due to the nature of subculturing of Md66 (the inoculum used in the fresh medium included enzymes from previous culture), no comparisons can be made for the enzyme content of the medium with adherent cell lines.

Fetal bovine serum diluted with PBS or culture medium revealed detectable amounts of esterase-lipase (C8) and phosphoamidase (Table 5.2). Incubating the cell lines in the fetal bovine serum supplemented medium produced a decrease in esterase-lipase. The medium containing adhering cell lines had more phosphoamidase levels than did Md66-spent medium.

Discharge of enzymes and total protein from hemocytes in response to foreign matter

The protein levels in PBS with Md66 without bacteria were constant throughout the study (Figure 5.12) whereas, in the supernatants of Md66 hemocytes incubated with *B. subtilis,* the levels increased from 10 min to 40 min post inoculation (Figure 5.12). However, the protein levels at 10 and 20 min in the hemocyte cultures with bacteria were significantly less than the protein concentrations in supernatants of PBS containing hemocytes only (Figure 5.12) ($P > 0.05$). Protein release from Md108 increased over the incubation time and to the same concentration regardless of the presence or absence of the microbial antigen (Figure 5.13). Dead *B. subtilis* did not release detectable amount of proteins (0.02 ± 0.03 µg/ml PBS).

Enzyme type and activity released from the hemocytes in PBS varied with the hemocyte cell line, incubation time and antigenic stimulus. Md66 in PBS without bacteria continously increased esterase, acid phosphatase and phosphoamidase levels, and elevated esterase lipase from 10 min to 40 min post-inoculation. Alkaline phosphatase increased from 20 min post incubation time, the other enzymes were either absent or exhibited spurious activities

(Table 5.3). The number of enzymes detected at 0 min post incubation increased from 4 in bacterial-free hemocyte cultures in PBS to 17 enzymes in Md66 cultures with *B. subtilis*, eliciting consistently high activities for acid and alkaline phosphatase, phosphoamidase, esterase and esterase-lipase (Table 5.3). Comparable activity values for these enzymes occurred at 20 or 40 min post inoculation for Md66 cells loosely contacting the polystyrene surface of the tissue culture flasks. Valine and cystine aminopeptidases, although absent in PBS containing hemocytes without bacteria during 0-40 min, were detected at statistically equal activity levels (which were higher than the control cultures) during 0-20 min incubation with *B. subtilis*, the former aminopeptidase substantially increasing thereafter by 40 mins post inoculation. Leucine aminopeptidase consistently exhibited higher activity in the presence of bacteria than in the absence of bacteria during 0 and 10 min pi; the activity levels from 10-40 min were comparable. Trypsin and chymotrypsin were present in cell lines with *B. subtilis* but not in the bacteria-free hemocyte cultures. Spurious low levels of the carbohydrases occurred from 0-40 min post inoculation of the hemocytes with bacteria, their profiles differing from bacteria-free control hemocyte samples. *B. subtilis* did not release detectable levels of enzymes into PBS.

Supernatants from adhering Md108 with and without *B. subtilis* contained essentially the same enzyme activities throughout the study. The exception was for the bacterial-free hemocytes in that phosphoamidase increased by 40 mins post incubation (Table 5.4). In the presence of *B. subtilis* Md108, unlike the Md66 cultures, marginally altered the enzyme profiles by elevating phosphoamidase by 20 min post inoculation.

Whether with or without bacteria, no lysozyme (0.0 ± 0.0 mm of clearing zone of *M. lysodeikticus* plates) or phenoloxidase (0.0 ± 0.0 U/mg protein) activity at 60 mins and 24h was observed in supernatants of Md108 and Md66 cell lines in PBS. The bacteria alone did not release any detectable form of the assayed enzymes (0.0 nmoles of hydrolyzed subtrate).

Figure 5.12: Protein discharged from Md66 hemocyte cell line in the presence and absence of *Bacillus subtilis.*

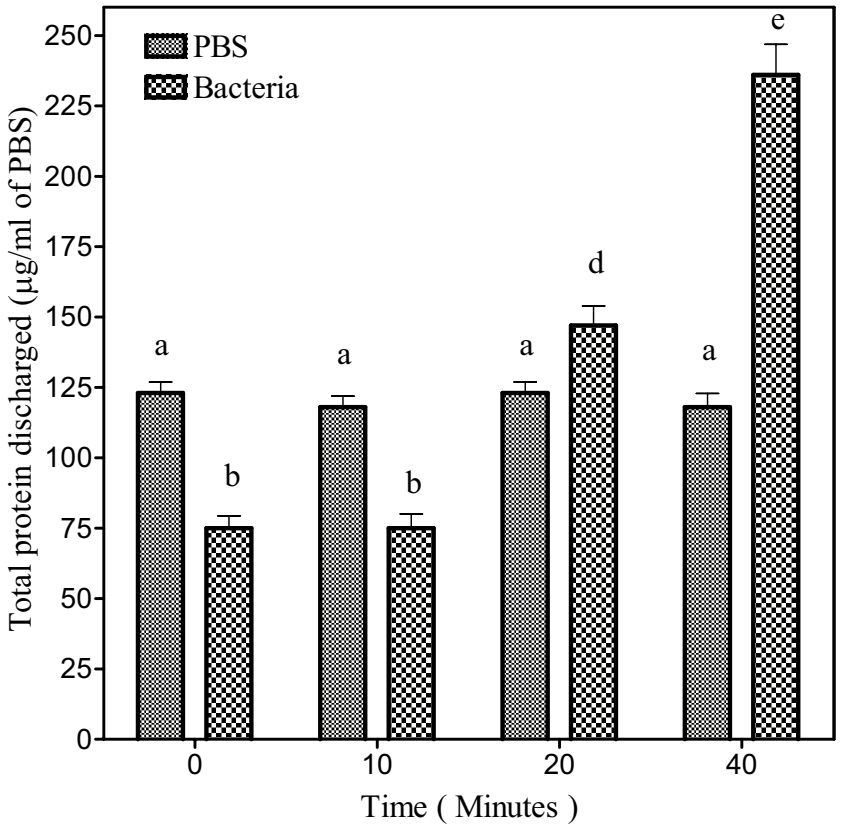

PBS
Bacteria
Total protein discharged (µg/ml of PBS)
250
225
200
175
150
125
100
75
50
25
0
a
b
a
b
a
d
a
e
0
10
20
40
Time (Minutes)

Figure 5.13: Protein discharged from Md108 hemocyte cell line in the presence and absence of *Bacillus subtilis.*

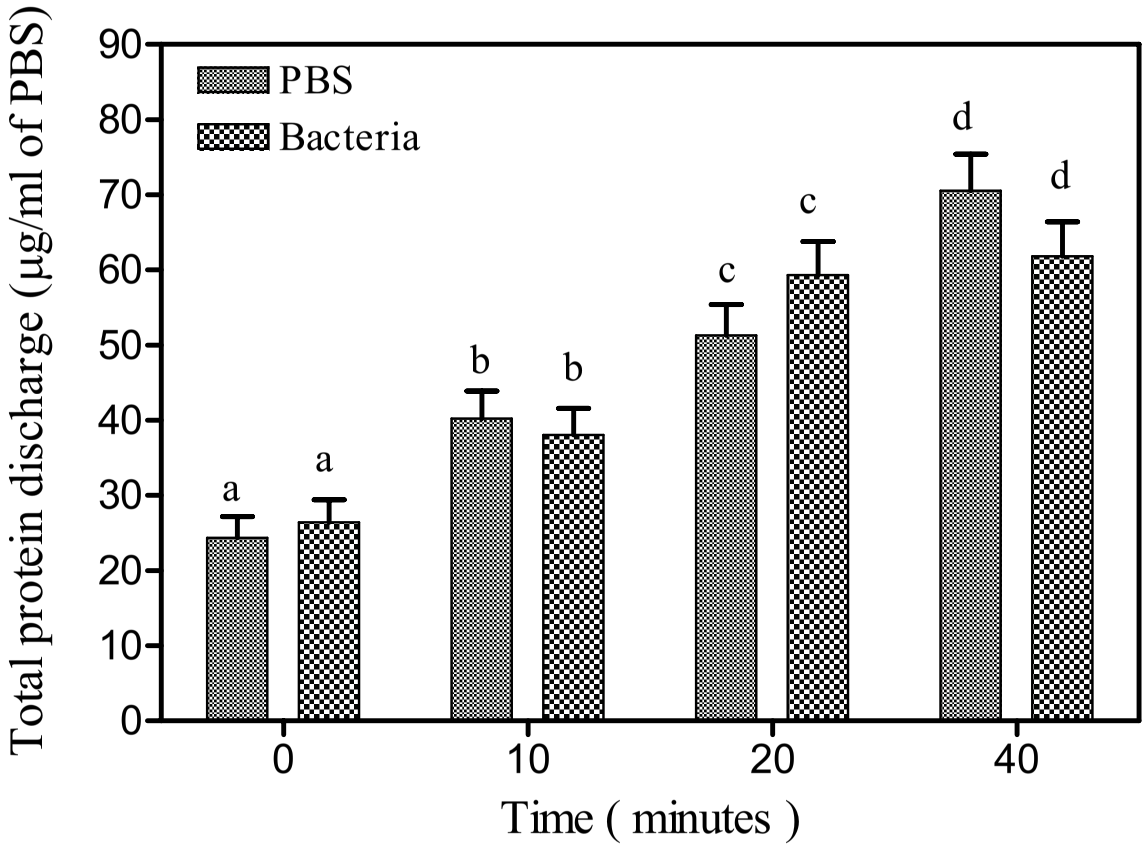

Total protein discharge (µg/ml of PBS)
90
80
70
60
50
40
30
20
10
0
PBS
Bacteria
a
a
b
b
c
c
d
d
0
10
20
40
Time (minutes)

Table 5.2: Basal levels of enzymes released from cell lines of *Malacosoma disstria* hemocytes grown at their optimum temperature at 50% confluency in culture medium.

Enzymes[2,3,5]	Hemocyte cell lines[1]				Grace's Medium	Grace's Medium + 8%(v/v) FBS[4]	PBS + 8%(v/v) FBS[4]
	Md108	Md66	UA-Md221	UA-Md203			
1	0 ± 0.0[a]	0.0 ± 0.0	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
2	10.0 ± 0.0[a]	26.0 ± 0.0	12.0 ± 2.0[a]	4.0 ± 1.0[c]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]
3	20.0 ± 3.7[a]	26.0 ± 2.4	11.0 ± 1.0[b]	8.0 ± 2.0[b]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]
4	3.0 ± 2.0[a]	4.0 ± 3.9	16.0 ± 2.4[b]	5.0 ± 2.2[a]	0.0 ± 0.0[d]	30.0 ± 0.0[e]	30.0 ± 0.0[e]
5	0.0 ± 0.0[a]	9.0 ±1.0	9.5 ± 2.7[b]	4.0 ± 1.0[c]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
6	28.0 ± 2.0[a]	0.0 ± 0.0	15.0 ± 3.2[c]	21.0 ± 4.5[cd]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
7	3.0 ± 2.0[a]	0.0 ± 0.0	9.0 ± 2.9[c]	20.0 ± 0.0[d]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
8	2.0 ± 1.2[a]	0.0 ± 0.0	11.0 ± 3.7[c]	22 ± 2.0[d]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
9	0.0 ± 0.0[a]	0.0 ± 0.0	11.0 ± 3.7[b]	22.0 ± 2.0[c]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
10	0.0 ± 0.0[a]	0.0 ± 0.0	5.0 ± 0.0[b]	20.0 ± 0.0[c]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
11	21.0 ± 3.3[a]	11.5 ± 5.8	16.0 ± 2.4[b]	9.0 ± 1.0[c]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]

12	24.0 ± 2.4[a]	7.0 ± 2.4	18.0 ± 3.7[ce]	20.0 ± 0.0[c]	0.0 ± 0.0[d]	15.0 ± 0.0[e]	15.0 ± 0.0[e]
13	3.0 ± 1.2[a]	7.0 ± 1.2	6.0 ± 2.4[ba]	16.0 ± 2.4[c]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]
14	2.0 ± 1.0[a]	8.0 ± 1.2	12.0 ± 3.7[b]	6.0 ± 2.4[b]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]
15	1.0 ± 1.0[a]	0.0 ± 0.0	8.0 ± 2.0[b]	16.0 ± 2.4[c]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
16	9.0 ± 1.0[a]	8.0 ± 1.2	8.0 ± 2.0[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
17	5.0 ± 1.5[a]	7.0 ± 1.2	16.0 ± 2.4[b]	9.5 ± 2.7[c]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]
18	40.0 ± 0.0[a]	11.0 ± 2.4	20.0 ± 0.0[c]	20.0 ± 0.0[c]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
19	2.0 ± 1.2[a]	4.0 ± 1.0	11.5 ± 3.8[b]	8.0 ± 2.0[b]	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
20	3.0 ± 1.2[a]	6.0 ± 1.0	9.5 ± 2.7[bc]	4.0 ± 2.4[c]	0.0 ± 0.0[d]	0.0 ± 0.0[d]	0.0 ± 0.0[d]

[1] Hemocyte cell lines were incubated at their optimum temperatures.

[2] Enzyme activities are in nmol substrate hydrolyzed /65µl culture supernatant /4h.

[3] Enzyme identities: 1. Control (no subtrate); 2. Alkaline phosphatase; 3. Esterase (C4); 4. Esterase-lipase (C8); 5. Lipase (C 14); 6. Leucine aminopeptidase; 7. Valine aminopeptidase; 8. Cystine aminopeptidase; 9. Trypsin; 10. Chymotrypsin; 11. Acid phosphatase; 12. Phosphoamidase; 13. α-Galactosidase; 14. β-Galactosidase; 15. β-Glucuronidase; 16. α-Glucosidase; 17. β-Glucosidase; 18. N-Acetyl-β-glucosaminidase; 19. α-Mannosidase; 20.α-Fucosidase

[4] FBS, fetal bovine serum heat-inactivated at 56^{o}C for 30 mins; PBS, phosphate-buffered saline, pH=6.5

[5] Mean ± standard error of the mean, (n ≥ 3). Values with the same superscript character set are not significantly different for the specific enzyme, $P > 0.05$.

Table 5.3: Enzymes released from Md66 hemocyte cell line[1] into phosphate-buffered saline (PBS) in the presence and absence of *Bacillussubtilis*.

Enzyme[2,3,4]	0 min		10 min		20 min		40 min	
	PBS	PBS + *B. subtilis*	PBS	PBS + *B. subtilis*	PBS	PBS + *B. subtilis*	PBS	PBS + *B. subtilis*
1	$0.0 \pm 0.0^{a,c}$	$0.0 \pm 0.0^{b,c}$	$0.0 \pm 0.0^{a,d}$	$0.0 \pm 0.0^{b,d}$	$0.0 \pm 0.0^{a,f}$	$0.0 \pm 0.0^{b,f}$	$0.0 \pm 0.0^{a,g}$	$0.0 \pm 0.0^{b,g}$
2	$0.0 \pm 0.0^{a,d}$	$21.7 \pm 1.7^{c,e}$	$0.0 \pm 0.0^{a,f}$	$20.0 \pm 0.0^{c,g}$	$0.0 \pm 0.0^{a,h}$	$26.7 \pm 3.3^{c,i}$	$20.0 \pm 0.0^{b,k}$	$23.3 \pm 3.3^{c,k}$
3	$0.0 \pm 0.0^{a,g}$	$21.7 \pm 1.7^{e,i}$	$5.0 \pm 0.0^{b,k}$	$20.0 \pm 0.0^{e,l}$	$10.0 \pm 0.0^{c,m}$	$23.3 \pm 3.3^{ef,n}$	$26.0 \pm 2.4^{d,o}$	$26.7 \pm 3.3^{f,0}$
4	$0.0 \pm 0.0^{a,g}$	$15.0 \pm 5.0^{d,h}$	$0.0 \pm 0.0^{a,i}$	$23.3 \pm 3.3^{f,k}$	$8.0 \pm 1.2^{b,l}$	$26.7 \pm 3.3^{f,m}$	$20.0 \pm 0.0^{c,n}$	$23.3 \pm 3.3^{f,n}$
5	$0.0 \pm 0.0^{a,e}$	$1.7 \pm 1.7^{c,e}$	$0.0 \pm 0.0^{a,f}$	$6.7 \pm 1.7^{d,g}$	$5.0 \pm 0.0^{b,h}$	$20.0 \pm 5.7^{e,i}$	$0.0 \pm 0.0^{a,k}$	$8.3 \pm 6.0^{d,l}$
6	$0.0 \pm 0.0^{a,f}$	$10.0 \pm 5.7^{d,g}$	$0.0 \pm 0.0^{a,h}$	$8.3 \pm 1.7^{d,i}$	$4.0 \pm 1.0^{b,k}$	$5.0 \pm 2.9^{d,l}$	$8.0 \pm 2.0^{c,m}$	$13.3 \pm 6.7^{e,m}$
7	$0,0 \pm 0,0^{a,e}$	$3.3 \pm 1,7^{b,f}$	$0.0 \pm 0.0^{a,g}$	$8.3 \pm 1.7^{c,i}$	$0.0 \pm 0.0^{a,k}$	$5.0 \pm 2.9^{c,l}$	$0.0 \pm 0.0^{a,m}$	$30.0 \pm 0.0^{d,n}$
8	$0,0 \pm 0,0^{a,d}$	$3.3 \pm 1,7^{b,e}$	$0.0 \pm 0.0^{a,f}$	$10.0 \pm 0.0^{c,g}$	$0.0 \pm 0.0^{a,i}$	$1.7 \pm 1.7^{b,i}$	$0.0 \pm 0.0^{a,k}$	$10.0 \pm 5.0^{c,l}$
9	$0,0 \pm 0,0^{a,f}$	$1.7 \pm 1.7^{b,f}$	$0.0 \pm 0.0^{a,g}$	$6.7 \pm 1.7^{c,h}$	$0.0 \pm 0.0^{a,i}$	$3.3 \pm 1.7^{d,k}$	$0.0 \pm 0.0^{a,l}$	$13.3 \pm 3.3^{e,m}$

10	0.0 ± 0.0^{a,d}	1.7 ± 1.7^{b,d}	0.0 ± 0.0^{a,e}	5.0 ± 0.0^{c,f}	0.0 ± 0.0^{a,g}	5.0 ± 2.9^{c,h}	0.0 ± 0.0^{a,i}	5.0 ± 0.0^{c,k}
11	2.5 ± 1.4^{a,h}	33.3 ± 3.3^{fg,i}	14.0 ± 2.4^{b,k}	30.0 ± 5.7^{f,m}	20.0 ± 0.0^{c,n}	33.3 ± 3.3^{fg,o}	34.0 ± 2.4^{d,p}	36.7 ± 3.3^{g,p}
12	0.8 ± 0.8^{a,f}	36.7 ± 3.3^{e,g}	9.0 ± 1.0^{b,h}	36.7 ± 3.3^{e,i}	20.0 ± 0.0^{c,k}	30.0 ± 5.8^{e,l}	28.0 ± 2.0^{d,m}	36.7± 3.3^{e,n}
13	0.0 ± 0.0^{a,c}	6.0 ± 3.7^{b,d}	0.0 ± 0.0^{a,e}	1.7 ± 1.7^{c,e}	0.0 ± 0.0^{a,f}	5.00 ± 0.0^{b,g}	0.0 ± 0.0^{a,h}	5.0 ± 0.0^{b,i}
14	0.0 ± 0.0^{a,d}	3.3 ± 1.7^{c,e}	0.0 ± 0.0^{a,f}	3.3 ± 1.7^{c,g}	4.0 ± 1.0^{b,h}	5.00 ± 0.0^{c,h}	0.0 ± 0,0^{a,i}	5.0 ± 0.0^{c,k}
15	0.0 ± 0.0^{a,d}	1.7 ± 1.7^{b,d}	0.0 ± 0.0^{a,e}	5.0 ± 0.0^{c,f}	0.0 ± 0.0^{a,g}	1.7 ± 1.7^{b,g}	0.0 ± 0.0^{a,h}	5.0 ± 0.0^{c,i}
16	0.0 ± 0.0^{a,d}	1.7 ± 1.7^{b,d}	0.0 ± 0.0^{a,e}	6.7 ± 1.7^{c,e}	0.0 ± 0.0^{a,f}	3.3 ± 1.7^{bc,g}	0.0 ± 0.0^{a,h}	5.0 ± 0.0^{bc,i}
17	0.0 ± 0.0^{a,d}	1.7 ± 1.7^{b,d}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{b,f}	0.0 ± 0.0^{a,g}	3.3 ± 3.3^{bc,g}	0.0 ± 0.0^{a,h}	5.0 ± 2.9^{c,i}
18	0.0 ± 0.0^{a,f}	1.7 ± 1.7^{c,f}	0.0 ± 0.0^{a,g}	3.3 ± 1.7^{c,h}	0.0 ± 0.0^{a,i}	1.7 ± 1.7^{c,i}	4.0 ± 1.0^{b,i}	6.7 ± 1.7^{d,i}
19	0.8 ± 0.8^{a,d}	0.0 ± 0.0^{c,d}	0.0 ± 0.0^{a,f}	1.7 ± 1.7^{c,f}	4.0 ± 1.0^{b,g}	0.0 ± 0.0^{c,h}	3.0 ± 1.2^{b,i}	0.0 ± 0.0^{c,l}
20	0.8 ± 0.8^{ab,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,g}	1.7 ± 1.7^{c,g}	1.0 ± 0.0^{b,h}	0.0 ± 0.0^{c,h}	0.0 ± 0.0^{a,i}	3.3 ± 1.7^{d,k}

[1] Hemocyte cell line grown to 50%confluency at their optimum temperature prior to washing by centrifugation and resuspension in PBS.

[2] Enzymes are expressed in nmol substrate hydrolyzed /65µl culture supernatant /4h.

[3] Enzyme identities: 1. Control (no subtrate); 2. Alkaline phosphatase; 3. Esterase (C4); 4. Esterase-lipase (C8); 5. Lipase (C 14); 6. Leucine aminopeptidase; 7. Valine aminopeptidase; 8. Cystine aminopeptidase; 9. Trypsin; 10. Chymotrypsin; 11. Acid

phosphatase; 12. Phosphoamidase; 13. α-Galactosidase; 14. β-Galactosidase; 15. β-Glucuronidase; 16. α-Glucosidase; 17. β-Glucosidase; 18. N-Acetyl-β-glucosaminidase; 19. α-Mannosidase; 20. α-Fucosidase

[4] Mean ± standard error of the mean, (n = 3). Values with the same superscript character set are not significantly different for the specific enzyme, $P > 0.05$. First superscript refers to statistical comparison values PBS or PBS with *B. subtilis* among different incubation times for the same enzyme. Second superscript refers to statistical comparison of values for the same incubation time for the same enzyme.

Table 5.4: Enzymes released from Md108 hemocyte cell line[1] into phosphate-buffered saline (PBS) in the presence and absence of *Bacillus subtilis*.

Enzyme[2,3,4]	0 min		10 min		20 min		40 min	
	PBS	PBS + *B.subtilis*	PBS	PBS + *B. subtilis*	PBS	PBS + *B. subtilis*	PBS	PBS + *B. subtilis*
1	0.0 ±0.0^{a,b}	0.0 ± 0.0^{a,b}	0.0 ± 0.0^{a,c}	0.0 ± 0.0^{a,c}	0,0 ± 0,0^{a,d}	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{a,e}
2	1.7 ± 1.7^{a,i}	3.3 ± 1.7^{d,i}	5.0 ± 0.0^{b,k}	5.0 ± 2.9^{f,k}	8.3 ± 1.7^{c,l}	10.0 ± 0.0^{g,m}	6.7± 1.7^{c,n}	13.3 ± 3.3^{h,o}
3	1.7 ± 1.7^{a,f}	3.3 ± 1.7^{c,f}	5.0 ± 0.0^{b,g}	8.3 ± 1,7^{d,h}	8.3 ±1.7^{b,i}	5,0 ± 2.9^{d,i}	6.7± 1.7^{b,k}	11.7 ± 4.4^{e,k}
4	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{c,f}	5.0 ± 2.9^{b,g}	6,7 ± 3.3^{d,g}	8.3± 6.0^{b,h}	6.7 ± 1.7^{d,h}
5	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{b,d}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{b,f}	1.7 ± 1.7^{a,g}	6.7 ± 3.3^{c,h}	0.0± 0.0^{a,i}	8.3 ± 1.7^{c,k}
6	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{c,f}	1.7 ± 1.7^{a,g}	0.0 ± 0.0^{c,g}	5.0± 2.9^{b,h}	5.0 ± 0.0^{d,h}
7	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	1.7 ± 1.7^{c,f}	1.7 ± 1.7^{a,g}	0.0 ± 0.0^{c,g}	6.7± 3.3^{b,h}	3.3 ± 1.7^{d,h}
8	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	5.0 ± 2.9^{b,f}	0.0 ± 0.0^{c,g}	1.7 ±1.7^{a,h}	0.0 ± 0.0^{c,h}	6.7± 1.7^{b,i}	8.3 ± 1.7^{d,i}
9	1.7 ± 1.7^{a,e}	1.7 ± 1.7^{c,e}	5.0 ± 0.0^{b,f}	1.7 ± 1.7^{c,g}	5.0 ± 2.9^{b,h}	5.0 ± 2.9^{d,h}	6.7± 1.7^{b,i}	8.3 ± 1.7^{d,i}

10	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{b,d}	0.0 ± 0.0^{a,e}	1.7 ± 1.7^{b,e}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{b,f}	1.7 ± 1.7^{a,g}	8.3 ± 1.7^{c,h}
11	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{b,e}	0.8 ± 0.8^{a,f}	3.3 ± 1.7^{c,g}	3.3 ± 1.7^{b,h}	5.0 ± 2.9^{cd,h}	0.0 ± 0.0^{a,i}	6.7 ± 1.7^{d,k}
12	6.7 ± 1.7^{a,g}	8.3 ± 1.7^{d,g}	5.0 ± 0.0^{a,h}	15.0 ± 5.0^{e,i}	11.7± 4,1^{b,k}	26.7 ± 3.3^{f,l}	26.7 ± 3.3^{c,m}	20.0 ± 0.0^{e,n}
13	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{b,e}	0.0 ± 0.0^{a,f}	1.7 ± 1.7^{b,f}	1.7 ± 1.7^{a,g}	3.3 ± 1.7^{bc,g}	0.0 ± 0.0^{a,h}	5.0 ± 0.0^{c,i}
14	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	1.7 ± 1.7^{c,f}	0.0 ± 0.0^{a,g}	3.3 ± 1.7^{cd,h}	3.3 ± 1.7^{b,i}	5.0 ± 0.0^{d,i}
15	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{c,f}	0.0 ± 0.0$^{a.g}$	0.0 ± 0.0^{c,g}	3.3 ± 1.7^{b,h}	4.2 ± 0.8^{d,h}
16	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{c,f}	0.0 ± 0.0^{a,g}	0.0 ± 0.0^{c,g}	3.3 ± 1.7^{b,h}	5.0 ± 0.0^{d,h}
17	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{c,e}	1.7 ± 1.7^{ab,f}	0.0 ± 0.0^{c,f}	3.3 ± 1.7^{b,g}	0.0 ± 0.0^{c,h}	1.7 ± 1.7^{ab,i}	3.3 ± 1.7^{d,i}
18	1.7 ± 1.7^{a,f}	0.0 ± 0.0^{c,f}	0.0 ± 0.0^{a,g}	3.3 ± 1.7^{d,h}	5.0 ± 0.0^{b,i}	5.0 ± 2.9^{de,i}	10.0 ± 5.0^{b,k}	6.7 ± 1.7^{e,k}
19	0.0 ± 0.0^{a,f}	0.0 ± 0.0^{c,f}	1.7 ± 1.7^{ab,g}	3.3 ± 1.7^{d,g}	3.3 ± 1.7^{b,h}	5.0 ± 2.9^{d,h}	0.0± 0.0^{a,i}	3.3 ± 1.7^{d,k}
20	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{b,d}	0.0 ± 0.0^{a,e}	0.0 ± 0.0^{b,e}	0.0 ± 0.0^{a,f}	5.0 ± 2.9^{c,g}	0.0± 0.0^{a,h}	0.0 ± 0.0^{b,h}

[1] Hemocyte cell lines grown to 50%confluency at their optimum temperature prior to washing by centrifugation and resuspension in PBS.

[2] Enzymes are expressed nmol substrate hydrolyzed /65µl culture supernatant /4h.

[3] Enzyme identities: 1. Control (no subtrate); 2. Alkaline phosphatase; 3. Esterase (C4); 4. Esterase-lipase (C8); 5. Lipase (C 14); 6. Leucine aminopeptidase; 7. Valine aminopeptidase; 8. Cystine aminopeptidase; 9. Trypsin; 10. Chymotrypsin; 11. Acid

phosphatase; 12. Phosphoamidase; 13. α-Galactosidase; 14. β-Galactosidase; 15. β-Glucuronidase; 16. α-Glucosidase; 17. β-Glucosidase; 18. N-Acetyl-β-glucosamidinase; 19. α-Mannosidase; 20. α-Fucosidase

[4] Mean ± standard error of the mean, (n = 3). Values with the same superscript character set are not significantly different for the specific enzyme, $P > 0.05$. First superscript refers to statistical comparison values PBS or PBS with *B. subtilis* among different incubation times for the same enzyme. Second superscript refers to statistical comparison of values for the same incubation time for the same enzyme.

DISCUSSION

The cell lines grew in a temperature sensitive manner, the optimum (or optimum range) and the effects of sub- and super- optimal temperature varying with the cell lines. The hemocyte lines, although varying in temperature sensitivity, grew poorly at suboptimal temperatures. Herein, adhesion of Md108, UA-Md203, UA-Md221 was temperature – independent. The hemocyte responses of dipteran encapsulation of parasitoids (Nappi and Silvers, 1984) and lepidoptera larval nodulation of bacteria (Mostafa *et al.*, 2005) and hemocytic erythrocyte phagocytosis (Brookman *et al.*, 1988) diminished with decreasing temperature. This may explain, in part, why *M. disstria* larvae, which grow slowly at low temperatures (Levesque *et al.*, 2002), exhibit an increase in mycoses when exposed to the entomopathogenic fungus *Furia gastropachae* (Zygomycetes: Entomophthorales) (Filotas and Hajek, 2004), the hemocytes possibly having diminished activity (present study) even as the temperature enhances the pathogen (Filotas *et al.*, 2006; Filotas and Hajek, 2004).

UA-Md203 demonstated a different optimum temperature for growth (21 ^{o}C) from other cell lines (which exhibited optimum temperatures of 21-28 ^{o}C). Both UA-Md203 and UA-Md221 grew slowly at 15 ^{o}C, the growth profiles varying with the cell lines. UA-Md203, which grew continuously throughout the incubation time at 15 ^{o}C, increased in growth rate when shifted to 21 ^{o}C. Although this suggests temperature had a cytostatic effect, the fact that the hemocytes grew more slowly than hemocytes continuously grown at 21 ^{o}C, implies either chronic damage occurred and/or cultivation at 15 ^{o}C selected for a subtype of UA-Md203 cells that grew best at low temperature. UA-Md221 exhibited growth during the initial incubation (15 ^{o}C) followed by a plateau of no growth that did not change after shifting the cultures to 21 ^{o}C indicating a cytocidal effect. Herein, the growth rates of UA-Md221 and UA-Md203 were faster than those reported by Keddie *et al.*, (1995) which may be attributed to differences in the

amount of fetal bovine serum in culture (6% v/v, Keddie et al., 1995; 8% v/v herein) and/or to variation in fetal bovine serum composition.The limited growth of cells Md66 and Md108 at 15 °C may reflect properties of hemocytes obtained during the initial selection of Md66 from the adhering cell line (Sohi, 1971) and thus would reflect differences due to using larvae from different geographical sources.

The temperature response differences that were observed in hemocytes during these studies may reflect the possibility of *M. disstria* being a species complex (Herbeck, 2002) that is morphologically difficult to separate, a phenomenon that occurs also in other insect species (Clarke *et al.*, 2005). The nucleotide sequences of the mitochondrial cytochrome oxidase I genes from *M. disstria* from different locations support this contention (Herbeck, 2002). It is unlikely that the differences in hemocyte growth profiles represent selection by cultivation temperature because the lines were all grown at similar temperatures of 25-28 °C (Keddie *et al.*, 1995; Sohi, 1973; Wilson and Sohi, 1977). Growth at 31 °C was highly variable among the cell lines with lowest rates in UA-Md203 and highest at Md108. The limited-to-no growth and slow growth at 31 °C may reflect the degeneration of the medium as occurred for a *G. mellonella* ovarian cell line (Zakarian *et al.*, 2002) and a larval fat body cell line (Eguchi and Iwabuchi, 2006).

Hemocyte cell lines exhibited a greater variation in morphological cell types in comparison to those found in freshly isolated *M. disstria* larval hemolymph samples (Chapter 2). Charpentier *et al.* (2002) refer to the polyploidy state of insect hemocyte cell lines of *Leptinotarsa decemlineata* as a possible factor in cellular polymorphism. RNAi studies with embryonic *D. melanogaster* hemocyte cell lines established the existence of 994 cell shape regulators (Kiger *et al.*, 2003) which if applicable to present cell lines may explain the spectrum of hemocyte types. Despite the morphological differences between the cell lines and the fresly isolated hemocytes (Chapter 2), the serological properties of the Md66 cell line showed similarities with the *M. disstria* larvae hemolymph samples which implies that Md66 floating

hemocytes demonstrate similar properties to fresh blood samples (Krywienczyk and Sohi, 1973). Herein, the UA-Md221 cell line exhibited extensive polymorphism. Such polymorphism has not been detected in direct blood sampling from the larval hemolymph (Giannoulis *et al.*, 2005) due possibly to the absence of plasma factors in the cell lines. The contribution of protease treatments for one hour on UA-Md221 cells to their cell shape pattern and stability should be futher investigated since trypsin alters chick fibroblast cell surface glycoproteins and their *in situ* regenerative sythesis, with consequences for the overall cell shape (Vernay *et al.*, 1978).

One of the major components of insect tissue culture medium, fetal bovine serum, contains animal growth factors (Zheng *et al.*, 2006) and enzymes (Spence *et al.*, 1989) which might play a role in cell development and function. TGF-β (transforming growth factor–β) found in fetal bovine serum (Zheng *et al.*, 2006), affects molluscan immunocyte cell shape (Kletsas *et al.*, 1998), induces chemotactic, calcium-dependent migration of immunocytes (Ottaviani *et al.*, 1997b) and upregulates epinephrine release from them (Ottaviani *et al.*, 1997a). The TGF-β growth factor also prevents apoptosis in a fat body cell line from the lepidopteran, *Lymantria dispar* (Ottaviani *et al.*, 2000; Ottaviani *et al.*, 2001). Inoculation of hemolymph samples from the tick, *Ornithodoros moubata,* with fetal bovine serum increases the phagocytic activity of the granulocytes but not that of the plasmatocytes (Inoue *et al.*, 2001). Additionally, serum proteins generally bind rapidly to cells and their physical supports (Gray, 2004), the consequence for animal cells varying with the type of physical support; for example, polystyrene-serum interactions substantially affect rat glioma cell adhesion and cellular functions (Wang *et al.*, 2006). Phosphoamidase and esterase-lipase (C8) are the two enzymes which are consistently present in fetal bovine serum whether diluted with Grace's medium or PBS. Phosphoamidase, while it is frequently described in insect tissue culture (Doty and Schofield, 1972; Mitsuhashi and Shozawa, 1985), is commonly released by insect pathogens (Simoes *et al.*, 2000), and may participate in breakdown of the extracellular matrix proteins (Meyer and

Weinmann, 1955). However, that the cells adhered to the flasks questions the extent of matrix protein hydrolysis by this enzyme because extensive hydrolysis would be expected to limit hemocyte adhesion. Phosphoamidase is present also in the gut of predatory pentatomids (Stamopoulos *et al.*, 1993) and saliva of parasitic arthropods (Kerlin and Hughes, 1992) assisting protein digestion. It is not known if the enzyme digests proteins in the culture medium. Strong phosphoamidase and weak esterase-lipase activities were detected during the growth of NIAS-MaBr-92 and NIAS-MaBr-93 hemocyte cell lines of cabbage army moth, *Mamestra brassicae,* while the cells grew (Mitsuhashi and Shozawa, 1985). Esterase-lipase (C8) was detected in the venom of the wasp *Pimpla hypochondriaca,* hymenopteran parasitoid of bright-line brown-eye moth, *Lecanobia oleracea,* larvae; however, the exact contribution of phosphoamidase to the immunosuppresive venom antihemocytic activity of the parasitoid is not known (Dani *et al.*, 2005). The antimicrobial properties of esterase-lipase have been demonstrated in mollusc hemocytes against bacteria (Cheng, 1983a). There is no discernible link between esterase-lipase and adhesion of *M. disstria* hemocytes to polystyrene flasks since all cell lines, independently of their adhesion properties, produced these enzymes in the tissue culture medium.

Keddie *et al.* (1995) propose that the most avidly adhering hemocyte cell line, UA-Md221, may inactivate external proteases explaining the low levels of trypsin and chymotrypsin in the culture medium compared with lesser adhesive Md203 observed in the Thesis. It is possible also that the higher enzyme activities in Md203 cultures are due to the greater production and/or release of proteases which could neutralize the serine protease inhibitors in fetal bovine serum (Chen *et al.*, 1992).

There was no obvious relationship between hemocyte adhesion avidity to polystyrene and serine protease activity in the culture medium. Both mildly adhering Md108 and the floating Md66 cell line did not produce trypsin and chymotrypsin in the culture medium compared to more strongly adhering UA-Md203 and UA-Md221. The absence of trypsin and

chymotrypsin in Md108 and Md66 – spent medium could be the result of enzyme inhibition by the fetal bovine serum serine protease inhibitors e.g. an inter-α-trypsin inhibitor (Chen *et al.*, 1992; Kanda *et al.*, 2007). However, neither enzymes were detected for either cell lines in PBS without fetal bovine serum in presence of polystyrene which implies that the antigen may elicit the selective release of enzymes in PBS and possibly in Grace's medium. The release of trypsin and chymotrypsin by both cell lines in PBS with bacteria show that the absence of these enzymes in both bacteria-free culture medium and PBS did not represent the absence of the enzymes in the hemocytes.

Although the objective of documenting the activities of serine proteases and other enzymes was to establish a biochemical characterization baseline for hemocytes to ensure culture quality, examination of possible enzyme functions (as alluded to for several enzymes earlier in the Discussion) is also useful because the enzymes may contribute to hemocytic non-self responses. Many of the enzymes showing increased activities in the culture medium are lysosomal enzymes with known antimicrobial activity (Cheng, 1983a,b; Rowley and Ratcliffe, 1979) in molluscs (Cheng, 1983a,b) and insects (Rowley and Ratcliffe, 1979), these enzymes being released from the hemocytes as part of the non-self activities (Foley and Cheng, 1977; Dani *et al.*, 2003). Consideration of these enzymes follows.

Aminopeptidases (Monis, 1965) are enzymes that can degrade bioactive peptides [by removing amino acids sequentially from the unblocked N termini] (Akada *et al.*, 2002) and can limit tumor necrosis factor–alpha (TNF-α)-induced apoptosis in human neutrophils (Cowburn *et al.*, 2006). In terms of non-self responses, molluscan aminopeptidases exhibit statistically higher activity in circulating hemocytes and serum of the gastropod mollusc *B. glabrata* during infections with the parasitic trematode *Echinostoma lindoense* compared with non treated controls (Cheng, 1983a). Leucine-, valine- and cystine- aminopeptidases may not be linked to the inhibition of hemocyte adhesion to polystyrene in the present study, because the enzymes are absent in the floating Md66 cell line and they are present in high amounts in more

adhesive cell lines. Leucine aminopeptidase contributes to immune cellular responses, such as increasing viral uptake by T-cell lymphocyte cell lines (Pulido-Cejudo *et al.*, 1997). Secretion enzyme profile of human blood cells after exposure to type I interferon shows enhanced leucine aminopeptidase activity within the first 12 h (Rani *et al.*, 2007) indicating that this enzyme is connected by cytokine related immunological stimulation. Interferon response elements are found in the promoter region of the peptidoglycan recognition protein (PGRP) gene in Lepidoptera (Ochiai and Ashida, 1999) which may imply that the release of leucine aminopeptidase by hemocytes is linked with insect cellular non self responses against microbial surfaces.

Secretion of N-acetyl-β-glucosaminidase occurs in the hypopharyngeal gland in the eusocial bees, *Scaptotrigona postica,* and newly emerged individuals of the honeybee, *Apis melifera* (Costa and da Cruz-Landim, 2005) which may protect the larvae from infection in the alimentary canal. Bovine neutrophils in culture plates release N-acetyl-β-glucosaminidase when leukocyte Fc receptors are stimulated by aggregated bovine immunoglobulin G; this enzyme is participating in non-self responses (Nagahata *et al.*, 1996). Herein, there is no obvious relationship in between adhesion of *M. disstria* hemocyte cell lines to the tissue culture plates and N-acetyl-β-glucosaminidase levels. Also the low levels of secretion of N-acetyl-β-glucosaminidase by hemocytes in PBS with or without bacteria do not indicate that the enzyme supports antimicrobial activity in the hemocyte cell lines.

Although β-galactosidase was present in growth medium from all hemocyte cultures at 50% confluency, enzyme activity was very low in PBS containing hemocytes with polystyrene or bacteria, implying the enzyme may have a marginal role in immunity. Because fetal bovine serum contains oligosaccharides with terminal β-galactose residues (Saxena *et al.*, 1997), the elevated β-galactosidase levels in hemocyte culture medium may reflect hemocytes obtaining nutrition by hydrolysis of the sugar from oligosaccharides. β-galactosidase does have a protective role in invertebrates.

In molluscs the enzyme increases in the plasma of the European flat oyster, *Ostrea edulis*, during infection by the protozoan *Bonamia ostrea* (Da Silva *et al.*, 2008) and in hemocyte phagosomes of the bivalve, *Mercenaria mercenaria* exposed to dinoflagellate, *Isochrysis galbana in vitro* (Moore and Gelder, 1985). The arthropod, *Homarus americanus,* similarly shows elevated β-galactosidase when challenged with bacteria (see Córdoba-Aguilar *et al.*, 2008) and is enhanced in pathogen resistant damsel fly species (see Córdoba-Aguilar *et al.*, 2008). The β-galactosidase gene is highly induced in presence of ecdysterone in the Kc embryonic cells of *D. melanogaster* (Best-Belpomme *et al.*, 1978). The hormone activates also attachment mechanisms in the floating Md66 cell line (Palli *et al.*, 1995). The relationship between this enzyme and ecdysteroids for Md66 was not addressed but in view of antigen-hemocyte attachment, and hormone-mediation of antimicrobial innate humoral (Flatt *et al.*, 2008) and hemocyte activity (Zou *et al.*, 2005), β-galactosidase may initiate aspects of hemocyte non-self functions.

β-Glucuronidase, an enzyme with a possible antimicrobial role observed in hemocytes of *G. mellonella* (Chain and Anderson, 1983). Herein the enzyme was not linked to antimicrobial responses of Md66 and Md108 cell lines to *B. subtilis* or polystyrene in PBS. The enzyme does have an antimicrobial or general non-self role in invertebrates and vertebrates. β-glucuronidase increases in the plasma of the snail *B. glabrata* implying a role for the enzyme in the host's immunity during infection by *Schistosoma mansoni* (Zelck *et al.*, 1996; Zelck, 1999). In addition antibacterial proctective role of β-glucuronidase occurs in the oviduct of domestic hen *Gallus domesticus* (Skolek *et al.*, 1978).

α-Mannosidase may serve a protective role on the cytoplasmic membranes of *D. melanogaster* spermatozoa (Cattaneo *et al.*, 2002) and α-mannosidase deficiency in humans is associated with humoral and cellular immunodeficiencies (Malm *et al.*, 2000). The amino acid sequence of *Spodoptera frugiperda* Sf9 α-mannosidase exhibits 35-57% homology with the corresponding sequences for α-mannosidases from a variety of sources

(Kawar *et al.*, 1997) but there are differences in the catalytic activities of the enzymes (Kawar *et al.*, 2001) suggesting, perhaps, diverse roles *in vivo*. Serum-free hemocytes of the Eastern oyster, *Crassostrea virginica*, selectively enhance the release of α-mannosidase in response to the presence of *E. coli* but not in response to *Micrococcus roseus* or *Klebsiella oxytoca* (Cheng, 1992). In the present study, a positive correlation was observed between the avidity of adhesion of *M. disstria* hemocyte cell lines to polystyrene and the release of α-mannosidase by the hemocytes to tissue culture medium.

α-Glucosidase was released from the Md66, Md108 and UA-Md221 cell lines in culture medium. Two hemocyte cell lines, NIAS-MaBr-92 and NIAS-MaBr-93, from *M. brassicae* demonstrate α-glucosidase activity in MTCM-1601 culture medium in which sucrose was included (Mutsihashi, 1989) impling that these lepidopteran hemocytes are capable of sucrose digestion (Mutsihashi, 1989). The enzyme was not detectable in UA-Md203 which may indicate this cell line do not use sucrose. A similar absence of the enzyme occurs in *B. glabrata* hemocytes when the activity of the enzyme was measured with photometric assays; however, α-glucosidase was readily apparent using polyacrylamide gels (Zelck *et al.*, 1996). It is possible the APIZYM assay was insufficiently sensitive to detect the enzyme.

The strongly adherent cell lines UA-Md221 and UAMd203 released more β-glucosidase to the culture medium than did mildly and non adherent cell lines. β-Glucosidase released by hemocytes contributes to snail immunity against *S. mansoni* by increasing in activity in the snail plasma altering the parasite integument (Zelck, 1999).

Investigating the interactions of insect hemocytes with microorganisms in a tissue culture system provides a unique type of information; hemocytes, while reacting to polystyrene proliferate at the same time, which is difficult to study *in vivo*. Floating Md66 and adhesive Md108 hemocyte cell lines were tested for their responses to bacteria because they have the same primary culture origin and similar temperature optima for growth and one cell line may be regarded as a control for the other.

Collectively, the API ZYM enzyme discharge data imply that *M. disstria* cell lines respond to polystyrene and *B. subtilis* by releasing possible antimicrobial enzymes. In PBS with *B. subtilis,* as opposed to bacteria-free PBS the activities of numerous enzymes of both cell lines rapidly increased the enzyme possibly interacting with the selected antigens, supporting the contention that the enzymes may be part of effector mechanisms of Md108 and Md66 hemocyte cell lines.

Acid phosphatase, which was active in PBS supernatants of Md66 cells without bacteria, exhibited higher activity in presence of *B. subtilis*. This lysosomal enzyme is found in the immunoreactive granular cells of insects (Hillyer *et al.*, 2003), the granular and hyaline hemocytes of the decapod crustaceans, *Homarus americanus*, *Panulirus interruptus* and *Loxorhynchus grandis* (Hose *et al.*, 1990) and in the hemocytes of the mollusc, *Crassotrea madrasensis* (Gijo *et al.*, 2006). Ultrastructure and cytochemical studies of *G. mellonella* hemocytes demonstrated that lysosomal acid phosphatase is released when blood cells attempt to engulf latex beads (Rowley and Ratcliffe, 1979). Acid phosphatase in hemolymph plasma of flesh fly, *Neobellieria bullata,* larvae represents secretions from two tissues, the fat body and hemolymph (Csikós and Sass, 1997) implying that during an *in vivo* immune challenge of the insect larvae with antigens, acid phosphatase may have several origins. Similar conclusions were made for the origin of enzymes in the hemolymph from the fat body in molluscs (Cheng, 1983b).

Interestingly, no lysozyme or phenoloxidase was discharged during innate responses of *M. disstria* hemocytes to polystyrene surfaces or dead *B. subtilis* in PBS. Initial incubation of a lepidopteran *Estigemene acreae* hemocyte cell line with *E. coli* lipopolysaccharide did not elicit detectable release lysozyme; however, lysozyme was released with prolonged incubation (Wittwer *et al.*, 1997). Hemolymph samples from *M. disstria* larvae demonstrated differential primary hemocytic lysozyme and phenoloxidase discharge during interaction with pathogenic and non-pathogenic bacteria (Giannoulis *et al.,* 2007) and their surface antigens (Giannoulis *et al.*, 2008),

which facilitated hemocyte adhesion (Giannoulis *et al.*, 2007). The absence of lysozyme and phenoloxidase in media with Md66 and Md108 may represent hemocyte degeneration or selection for the absence of release of these enzymes either way this provides a basis to study adhesion of hemocytes to cell surface receptors without the involvement of both of these adhesion-promoting enzymes (Giannoulis *et al.*, 2007).

In conclusion, *M. disstria* hemocyte cell lines exhibits a diverse spectrum of growth rates and optimum growth and variety of extensive cell morphogies not seen in fresh hemolyph samples. PBS serum-free hemocytes expressed non-self responses to polystyrene and bacteria by altering quatitatively and qualitatitavely their secretion enzyme profile. Total protein, secreted from cell lines while interacting with polystyrene and or microorganism in PBS, demonstrate an overall quantitative interaction profile for adhering and floating hemocytes even if lysozyme and phenoloxidase were not detectable.

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CHAPTER 6

GENERAL DISCUSSION AND CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

GENERAL DISCUSSION

The main objective of this Thesis was to investigate innate non-self responses of *M. disstria* larval hemocytes to entomopathogenic and non-pathogenic bacteria with attention being focused primarly on their surface antigens. This was accomplished using both *in vitro* and *in vivo* assays.

In Chapter 2, it was reported that PBS was found to be the best buffer to be used as a reaction medium for the hemocytes. Due to the fact, that calcium is a major inorganic intracellular and extracellular messenger (Zakarian *et al.*, 2003), the contributions of calcium to hemocyte activities were determined. The types of hemocyte found in *M. disstria* hemolymph did not differ from those found in the hemolymph of most Lepidopteran species (Arnold and Sohi, 1976), the only exception being the stellate type of plasmatocyte. Stellate plasmatocytes were not observed by Arnold and Sohi (1976) due possibly to their use of a different method of cell preparation (e.g. smearing of hemolymph samples over the glass slide). Protein kinases A and C, major cell signalling enzymes, were involved also in the non-self responses as they were for other lepidopteran hemocytes, the enzymes participating in adhesion, phagocytosis (Brooks and Dunphy, 2005; Lanz-Mendoza *et al.*, 1996) and nodulation (Marin *et al.*, 2005). Activation and inhibition of protein kinases A and C inhibited and stimulated *M. disstria* hemocyte cell adhesion to glass, respectively.

In Chapter 3, the responses of hemocytes to challenge with the microorganisms, *X. nematophila* and *B. subtilis* were reported. Bacterial challenge resulted in increased levels of phenoloxidase and lysozyme, both of which enhanced bacterial attachment to larval hemocytes. The hemolytic effects of *X. nematophila* were attributed to the surface antigen, LPS.

In Chapter 4, LPS and lipid A *per se* were shown to provoke hemocyte lysis, an effect that could be abrogated by the lipid A-binding antibiotic, polymyxin B.

The plasma protein, apolipophorin-III, was detected in *M. disstria* hemolymph. Apolipophorin-III is a pattern recognition protein that participates in lipid transport (Weers and Ryan, 2006). It effectively limited the toxicity of LPS and LTA protecting the hemocytes and preventing phenoloxidase activation until LPS and LTA concentrations exceeded a critical threshold. Apolipophorin III affects many types of immune factors of insects (Dunphy and Halwani, 1997; Wiesner *et al.*, 1997; Whitten *et al.*, 2004). Despite the protection of *M. disstria* immune status that apolipophorin III provides against intact bacterial antigens and their surface molecule (Chapter 3 and 4), it increased the activity of spontaneously activated phenoloxidase (Chapter 4).

All chapters prior to Chapter 5 dealt with hemocytes from fresh hemolymph samples. In Chapter 5, *M. disstria* hemocyte cell lines were used allow investigations of hemocytes in an insect-plasma-free environment. The cell lines were characterized with respect to extracellular enzyme complements. Cell lines were studied in terms of their extracellular enzymes released while responding to polystyrene surfaces and *B. subtilis*. No production of lysozyme and phenoloxidase were detected.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. This Thesis represents the first detailed report on innate hemocyte immune responses in the hardwood forest lepidopteran, *M. disstria*.

2. A buffer system, optimal for the study of *M.disstria* hemocytes, was developed.

3. Protein kinases A and C are identified as cell signaling factors involved in the adhesion of *M. disstria* hemocytes to glass.

4. The effect of *in vitro* and *in vivo* challenges of *M. disstria* hemocytes with intact bacteria and surface components are presented for the first time.

5. The levels of *M. disstria* lysozyme, phenoloxidase and total protein levels were shown to increase when fresh larval hemocytes were challenged with bacterial antigens.

6. The cellular responses of *M. disstria* hemocytes to an entomopathogenic bacterium (*X. nematophila*) and a non-pathogenic microorganism (*B. subtilis*) is reported for the first time.

7. Surface antigens mediating *M. disstria* hemocyte and phenoloxidase responses to *X. nematophila* and *B. subtilis* are identified.

8. *A*polipophorin III-protein was detected in *M. disstria* hemolymph and its role in host response against bacteria and their surface antigens was established.

9. The effect of selected temperatures on the growth of four *M. disstria* hemocyte cell lines is reported for first time.

10. The cell forms present in *M. disstria* Md66, Md108 and Md221 hemocyte cell lines are described for the first time.

11. Cell type frequencies in *M. disstria* Md66 floating and M108 adhering hemocyte cell lines are described.

12. Enzyme discharge by four *M. disstria* hemocyte cell lines and comparison enzyme between the adherent ones are described for the first time.

13. No lysozyme and phenoloxidase discharge by *M. disstria* hemocytes.

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APPENDIX