

FEMSRE-0301-0047R1.R1.

Exploiting the potential of Insects for *in vivo* pathogenicity testing of Microbial pathogens.

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Keywords: Humoral immunity; Innate immune response; Insect; *in vivo* pathogenicity testing.

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1.0 ABSTRACT

Conventional assays for quantifying the virulence of microbial pathogens and mutants have traditionally relied upon the use of a range of mammalian species. A number of workers have demonstrated that insects can be used for evaluating microbial pathogenicity and provide results comparable to those that can be obtained with mammals since one component of the vertebrate immune system, the innate immune response, remains similar to that found in insects. Larvae of the Greater Wax Moth *Galleria mellonella* have been used to evaluate the virulence of a range of bacterial and fungal pathogens and a correlation with the virulence of these microbes in mice has been established. This review highlights the similarities of the vertebrate and insect innate immune responses to infection and identifies the potential use of insects for the *in vivo* evaluation of the microbial pathogenicity.

2.0 INTRODUCTION

Insects are one of the most successful and geographically widespread groups of animals on Earth. They are found in almost every habitat - the main exception being the oceans, and have succeeded in colonising niches inaccessible to, or unusable by, other animal life forms. Conservative estimates suggest that there are 750,000 species of insect but in reality this figure may be closer to 1,000,000 - making them the most diverse animal life form [1]. They are also the most abundant with as many as 10^{18} individuals at any one time [2].

From an evolutionary perspective insects and vertebrates diverged approximately 500 million years ago however many aspects of their physiology remain similar [3]. The innate immune system, unlike the adaptive immune system [4, 5] of insects and mammals, share a high degree of structural and functional homology [1, 6]. In particular, a number of features of the innate immune response are common to mammals and insects [7, 8] and analysis of insect responses to pathogens can provide an indication of the vertebrate response to infection. [7, 9]. Since the innate immune response is the main line of defence in vertebrates against many microbial pathogens [10], much effort has been focused on examining the mammalian and insect responses to microbial infection and a strong correlation between both systems has been demonstrated [6].

The study of insect immunology has been described as the 'poor relation' of immunology since it was assumed that the vast numbers of insects and their rapid rates of reproduction obviated the need for a sophisticated and finely tuned immune system. However, we are now beginning to appreciate the efficacy of the insect response to

infection [6]. Closer study of the insect immune response demonstrates a highly adapted and effective system that is capable of dealing with a wide range of bacterial, fungal and protozoan pathogens and with microbial loads that would prove fatal to vertebrates [1, 3]. In recent years there has been a recognition of the homology between the insect and mammalian innate immune systems and that a knowledge of the insect response to infection could provide valuable information into the functioning of the mammalian innate system [6]. There may also be an ulterior motive in learning more about the insect immune response since this could be used to design more effective or novel insecticides that function by inhibiting the insect immune response to microbial pathogens [11].

3.0 THE INSECT IMMUNE SYSTEM

3.1 The cuticle

The first line of defence in insects against the majority of pathogens is the cuticle, which serves a function analogous to the skin in mammals. The cuticle is a structurally and chemically complex barrier designed to prevent or retard the entry of pathogens into the haemoceol (the body cavity) [12]. The outer layer of the cuticle (the epicuticle) is covered in a waxy layer containing lipids, fatty acids and sterols, which may display anti-microbial properties [13]. The cuticle itself consists of chitin fibrils embedded in a

protein matrix. The intact cuticle prevents entry of microbial pathogens but once it is ruptured by injury or degradation there is an increased chance of infection [14]. The lesion may be plugged and subsequently repaired to restore the structural and functional integrity of the cuticle. Injury to the cuticle activates the humoral immune response, which leads to the production of cecropins and attacins, which display anti-bacterial activity.

3.2 The Haemolymph

The insect body cavity or haemocoel contains haemolymph, which serves a function analogous to blood in mammals in that it transports nutrients, waste products and signal molecules [15] although it plays no role in respiration. In addition, haemolymph contains cells and anti-microbial peptides capable of immobilising and killing invading microorganisms [2, 16]. The volume of haemolymph within an insect varies between species and even within a species depending upon the developmental stage of the individual insect [1]. The insect immune response to microorganisms has been shown to involve a change in the circulating haemocyte population and synthesis of new haemolymph proteins [17]. The haemolymph is the main site of the immune response to microorganisms. The innate immune response consists of cellular and humoral mechanisms that are tightly interconnected.

4.0 CELLULAR ELEMENTS IN HAEMOLYMPH.

The insect haemolymph contains haemocytes, which function in a similar manner to phagocytes of mammals. The majority of haemocytes circulate freely within the

haemolymph but a significant number (up to 30% in some insect species) can be found associated with internal organs such as the fat body, trachea or digestive system [1]. The haemocyte density varies upon infection and in the case of *Galleria mellonella* infected with *Stinerema* nematodes there is a decrease in haemocyte numbers by 80% in the first 4 hours followed by a gradual increase over the next 12 hours to 160% of the control. Subsequently, numbers decline to less than 5% of the control haemocyte density [15]. The initial decline in haemocyte numbers has been attributed to the formation of clumps consisting of haemocytes and invading microbe [18]. The subsequent rise in their numbers is thought to be due to the release of haemocytes bound to internal organs.

At least six types of haemocytes have been identified in lepidopterous (e.g. *Galleria mellonella*) although more types may exist in other species [3] (Figure 1). Price and Radcliffe [19] classified haemocytes as prohaemocytes, plasmatocytes, granulocytes (granular cells), coagulocytes, spherulocytes and oenocytoids. Although a later study by Brehelin [20] provided an alternative classification containing nine groups. Prohemocytes (6-13 μ M in diameter) are small rounded cells with large nuclei, which divide and may differentiate into other cell types (Figure 1). Plasmatocytes (40-50 μ M) and granulocytes (45 μ M) are the predominant phagocytic cells. Plasmatocytes contain lysosomal enzymes and are the most abundant cell type. Granulocytes possess a relatively small nucleus and granule rich cytoplasm. Spherulocytes are oval or round cells (25 μ M) with varying numbers of small spherical inclusions. Oenocytoids are large, binucleate, nonphagocytic cells which may contain prophenoloxidase. Coagulocytes have also been termed hyaline hemocytes and are involved in the clotting process. Adipohemocytes are characterised by the presence of fat droplets (Figure 1).

The plasmatocytes and granulocytes participate in phagocytosis, nodule formation and encapsulation [21] which are important elements of the insect's cellular defence against bacteria and unicellular fungi [22]. Within *Drosophila melanogaster*, plasmatocytes participate, to some extent, in the synthesis of antimicrobial peptides during the humoral response [23, 24] and assume the function of phagocytosis of microorganisms [25] while lamellocytes and crystal cells play respective roles in encapsulation and melanization of larger intruders [26].

4.1 Phagocytosis

In mammalian cells phagocytosis requires recognition with subsequent engulfment of particles such as pathogens that accumulate during infection and inflammation [27] and is mainly performed by migrating professional phagocytes derived from the myeloid cell line. They are the neutrophils and monocytes that circulate in the blood, and tissue residing macrophages [28]. Phagocytosis requires sequential signal transduction events, which lead to the recruitment of the phagocyte to the site of infection followed by recognition of the particle as foreign [29] and the subsequent ingestion within a phagosome.

While the process of phagocytosis in insects is not fully understood receptors on the surface of plasmatocytes and granulocytes are similar to receptors on mammalian phagocytes [2]. The insect proteins malvolio and dSR-C1 show a high degree of homology to mouse natural resistance associated macrophage protein-1 (NRAMP-1). *Drosophila melanogaster* proteins peroxodasin and Croquemort [30, 31], follow the classical distribution of macrophages as described by Tepass and colleagues [32] and

may mediate the breakdown of apoptotic cells, similarly to murine macrophage proteins [2].

The process of phagocytosis in insects and mammals appears to be very similar. In both cases there is the binding of opsonic ligands to the surface of the particle which is then followed by recognition by specific receptors. An intracellular cascade results in the internalisation of the foreign body. It was originally thought that only plasmatocytes were involved in phagocytosis of foreign material in *G. mellonella* however recent work has demonstrated that granular cells are also involved [21]. Activation of the prophenoloxidase (PPO) cascade is required for granular cells to bind to non-self matter and conduct phagocytosis while calcium is required for the adherence of plasmatocytes. Phagocytosis is a lectin-mediated process and lectins are found in the insect haemolymph along with lysozyme - an anti-microbial peptide usually associated with the humoral response. Lysozyme has been found within haemocytes and the intra-haemolymph levels of lysozyme and lectin increase upon infection indicating that these act synergistically upon the process of phagocytosis [33]. Upon invasion with gram-negative bacteria N-acetylglucosamine (GlcNAc) specific lectins (BDL-2 lectins) recognise and bind to peptidoglycans on the bacterial cell surface. These bind to plasmatocytes and facilitate phagocytosis. At the same time lysozyme degrades the peptidoglycan layer releasing sugars and exposing teichoic acid and lipomannans which are recognised by BDL-1 lectins. This process gives the insect the ability to recognise and engulf a range of bacteria despite the changing nature of the exposed bacterial surface [33]. This also indicates how the cellular and humoral arms of the innate immune systems co-operate in combating infection.

The burst in oxidative metabolism associated with phagocytosis has been interpreted as indicating that the oxygen becomes converted into anti-microbial products and indeed phagocytes manufacture a battery of reactive oxygen intermediates (ROI) (Figure 2), that *in vitro* have been shown to successfully kill microorganisms [34, 35] (Table 1; Figure 2). The production of ROI has also been detected in the haemolymph and haemocytes of many insects. The *in vitro* generation of ROI was studied by the method of nitroblue tetrazolium reduction [36] and was observed in haemocytes of *Galleria mellonella*, *Aporia crataegi*, *Dendrolimus sibiricus* and *Gryllus bimaculatus*. With the use of a fluorescent probe, evidence of both superoxide and its dismutation product hydrogen peroxide was found in plasmatocytes of *D. melanogaster* larvae [37] and similarly using electron spin resonance (ESR) spectroscopy in *G. mellonella* [38].

It has been abundantly documented in mammalian phagocyte studies that O_2^- is not strongly bactericidal by itself, but may participate in the innate defence systems by interaction with nitric oxide ($NO\cdot$) to generate peroxynitrite ($ONOO^-$) [39, 40] (Figure 2). $ONOO^-$ then undergoes a secondary reaction to produce an agent that is able to nitrate tyrosine. These include $ONOOH$ that breaks down to NO_2^- [40], $O_2N-O-CO_2^-$ produced by reaction of $ONOO^-$ with carbon dioxide [41] and NO_2Cl , formed when $HOCl$ reacts with nitrite [42] a degradation product of $ONOO^-$.

In vitro studies of peroxynitrite, show it to be a strong oxidant which possesses highly cytotoxic properties against *E. coli* [43], parasites such as *Trypanosoma cruzi* [41] or fungi such as *Candida albicans* [44]. In addition nitric oxide synthase knock-out mice have demonstrated the enzyme's essential contribution to host defence against a restricted set of pathogens, including *Mycobacterium tuberculosis* [45] and *Leishmania major* [46].

Suggestions of conservation between the mammalian and insect innate immunity recently led to the discovery of a role for nitric oxide in the insect immune responses. Nitric oxide synthase (NOS) was induced by infection, and inhibition of NOS activity increased susceptibility of *Drosophila* larvae to gram-negative bacterial infection [47].

4.2 Nodulisation

Nodules are formed in response to a number of invading microorganisms [48], consist of viable and degraded haemocytes, non-self materials and melanised debris and may be attached to tissue or surrounded by haemocytes. Nodule formation in insects is not fully characterised although it is known that it is lectin-mediated [2]. A lectin, Scolexin, is thought to be involved in nodule formation in *Manduca sexta* - the Tobacco hornworm, where it is produced by epidermal and mid-gut cells upon injury to the insect or following invasion by bacteria.

4.3 Encapsulisation.

Large structures such as protozoa, nematodes and eggs and larvae of parasitic insects are encapsulated by being surrounded by layers of haemocytes, which form a capsule of overlapping layers of cells [3]. Capsule formation begins within 30 minutes of the entry of a pathogen and initially involves the attachment of granular cells to the target followed by binding of plasmatocytes which are attracted to the site by plasmatocyte spreading peptides (PSP) which are released by granulocytes and induce aggregation [2]. A study in *G. mellonella* showed the enrichment of a 47kDa protein at an early stage of encapsulation, the amino acid sequence of which revealed this protein to share strong homology with both human and *Drosophila* calreticulin [49]. Calreticulin was recently

reported to partially localise on the surface of neutrophils, and anti-microbial bound calreticulin transmitted a signal into cells via G-protein to activate neutrophils to generate superoxide anions [50]. Humoral factors may also be involved in encapsulation specifically in the recognition and binding of non-self matter. Lavine and Strand [51] have demonstrated that humoral recognition receptors bind to foreign material and stimulate aggregation of plasmatocytes to the target. In mammals pathogen recognition receptors bind to pathogen-associated molecular pattern molecules or PAMPs bind to molecules produced by microorganisms. Examples of such molecules include mannan of the yeast cell wall and lipopolysaccharides and lipotechoic acids of bacterial wall.

5.0 HUMORAL IMMUNITY

The humoral immune response of insects consists of the processes of melanisation, haemolymph clotting and wound healing in response to injury [2]. In addition, the humoral response also involves the synthesis of a range of anti-microbial peptides and heat shock proteins [6]. Although insects do not produce antibodies they are capable of generating a series of proteins which confer a degree of non-specific immunity to a range of microorganisms.

Humoral factors involved in insect immunity to infection include lysozyme, lectins and the prophenoloxidase cascade [52] and serine proteases and carbohydrases [53].

5.1 Clotting mechanisms

There are two clotting mechanisms characteristic of insects. The first involves the polymerisation of clottable proteins and is catalysed by a calcium dependent transglutaminase [54]. The clottable proteins are lipophorins and the vitellogenin-like proteins, which contain a cysteine-rich region homologous to the "d" domain of the mammalian clottable proteins of the Von Willebrand's factor [2], which is involved in the blood coagulation process. The second clotting mechanism is the haemocyte-derived clotting cascade where clottable proteins are released from the cytoplasmic L-granules of the haemocytes into the haemolymph in response to activation by cell wall components of invading microbes. Gram negative bacteria activate factor C while fungi activate factor G which results in a conformational change [54]. This leads to cleavage of factor B by a serine protease, which in turn leads to the cleavage of pro-clotting enzyme (PCE). This catalyses the cleavage of a soluble protein coagulogen, into coagulin which is an insoluble aggregate which forms a gel-like clot that traps pathogens [55].

5.2 Melanisation

The process of melanisation is key to the defence against a wide range of pathogens and results in the deposition of melanin on the microbe within the haemolymph [1]. The formation of melanin is catalysed by phenoloxidase-monophenyl L-dopa: oxygen oxidoreductase (EC 1.14.18.1) [56]. PO is found in insects in its inactive form pro-phenoloxidase (ProPO) located in the haemocytes as a zymogen. It is released from haemocytes by rupture and is either actively transported to the cuticle or deposited around wounds or encapsulated parasite. PO catalyses the o-hydroxylation of monophenols and oxidation of phenols to quinines which then polymerise non-

enzymatically to form melanin [56]. Insect ProPO has a sequence similar to the thiol-ester region of the vertebrate complement proteins C3 and C4. In vertebrates following cleavage by an activating protease the thiol-ester region becomes active and can react with hydroxyl or amino groups on biological surfaces leading to immobilisation of the molecule on the foreign material.

The process of melanisation is initiated by soluble pattern recognition receptors that bind target surfaces thus initiating the serine protease cascade leading to cleavage of ProPO to PO and ultimately the cross-linking and melanisation of proteins. This process is frequently referred to as the Prophenoloxidase activating system (ProPO-AS). Pattern recognition receptors (PRRs) activate the complement system in vertebrates and the PrpPO-AS system in insects. A number of PRRs are present in insects (Table 2) and include: the C type lectins which bind bacterial LPS, the peptidoglycan recognition protein which is expressed in the fat body, haemocytes and epithelial cells and the β -1,3 glucan binding protein which recognises fungal glucan and activates the ProPO-AS pathway.

The serine protease cascade controlling the cleavage of PPO or PO is highly controlled and counter-balanced by protease inhibitors since the reaction must be maintained near the site of invasion because active PO can produce highly reactive and detrimental oxygen intermediates [56].

Mammalian immune cells express several Toll-like receptors (TLR) that are considered cellular PPRs, because they directly recognise LPS and other microbial products. Within the *Drosophila* genome, Toll and several additional *Toll*-related genes (*Toll-3-8*, *18 wheeler*) and the gene *immune deficiency (imd)* [57] have been identified. In

1997 Medzhitov *et al.*, cloned the human homologue of Toll (TLR4) [58] and to date ten members of the TLR family have been identified in humans [59].

Drosophila Toll is a transmembrane protein with an extracellular leucine-rich domain and a cytoplasmic domain [58] (Figure 3) that resembles the cytoplasmic domain of the interleukin (IL)-1 receptor. Toll was originally identified as a critical factor for the dorsoventral polarity of the *Drosophila* embryo [60]. Toll induces the activation of Dorsal, a member of the Rel family of rapid inducible transcription factors through the degradation of the protein cactus (Figure 3). The latter causes retention of Dorsal in the cytoplasm and the complex Toll/Tube/Pelle mediates its phosphorylation and degradation. Spaetzle, the extracellular Toll ligand controls the generation of microbicidal peptides and involves the gene cassette spaetzle/Toll/cactus but instead of Dorsal, the Dorsal-related immunity factor (Dif) is involved. As illustrated in Figure 3, there are remarkable structural and functional similarities between the systems mediating *Drosophila* Toll and mammalian IL-1 receptor mediated signalling [61]. Nuclear factor NF- κ B, inhibitory I- κ B and the serine threonine kinase IRAK are mammalian homologues of the *Drosophila* Dorsal, cactus and Pelle respectively.

Signalling processes leading to production of antimicrobial peptides have been studied by subtractive hybridization and RACE PCR with the positive identification of immunorelevant genes and LPS-induced defensin-like antimicrobial molecules in *G. mellonella* [62]. *In vivo* knock-out studies revealed adult flies lacking Toll, had compromising levels of drosomycin resulting in reduced resistance to fungal infection [63]. In addition mutants of the other Toll family member, *18-wheeler* are more susceptible to bacterial infection [64].

5.3 Anti-microbial peptides.

Although the cellular and humoral responses so far described are effective in combating microbial invasion they are unable to totally clear the haemocoel if a large number of microorganisms enter. The last line of defence is the synthesis of a range of anti-microbial peptides, which are released into the haemolymph where they attack elements of the bacterial or fungal cell wall [1]. These peptides play a crucial role in combating infection and similar classes of proteins are found in vertebrates, invertebrates and plants [2, 6]. (Table 2).

The main sites of synthesis of anti-microbial peptides in the insect are the fat body, haemocytes, the digestive tract, salivary glands and the reproductive tract. The fat body functions as a biosynthetic organ, is analogous to the liver in mammals and is also a site of binding for many haemocytes [65]. A number of peptides are produced and all are amphipatic basic molecules that act in a detergent-like manner on cell membranes causing the death of the microorganism by lysis. Anti-microbial peptides are synthesised as pre-proteins at a rate up to 100 times faster than IgM in mammals [65]. Their small size allows diffusion through the haemolymph to counteract invading pathogens.

In humans the antimicrobial neutrophil proteins are located within intracellular granules which are released into newly formed phagocytic vacuoles. The proteins and peptides stored in the granules are of two kinds: 1) those with cytotoxic properties, including bactericidal/permeability-increasing protein, azurocidin and defensins, and 2) a range of enzymes, capable of contributing to the destruction of killed bacteria by digesting their macromolecules [66, 67]. Among these are lysozyme, proteinases, some

with independent antimicrobial activity (elastase and cathepsin G), nucleases, and saccharidases (Table 2). Enzymes degrading bacterial phospholipids [68] and lipopolysaccharides (LPS) [69] are also known to be granule associated.

Attacins display a relatively narrow spectrum of anti-bacterial and anti-fungal activity and are believed to act on the outer membranes of microbial cells [17]. It appears that the primary function of attacins may be to facilitate the action of lysozyme and cecropins thereby allowing the three immune proteins to work in consort.

Proline-rich peptides, Glycine-rich peptides and Dipterocins: Proline rich peptides are small, 15-34 residues and between 2 - 4 kDa [7]. These were first isolated in larvae of *Phormia terranova* [70]. Other examples of such peptides include abaecin and the apidaecins from honey bees and other hymenoptera and drosocin from *Drosophila*. These peptides appear to function by increasing membrane permeability of bacteria and lyse gram-negative bacteria. Glycine-rich peptides are 9-30 kDa and are active against gram-negative bacteria. Dipterocins are only found in dipterin species and are induced by and active against gram-negative bacteria.

Lysozyme is a 14.4kDa (pI>10) cationic protein with the ability to kill a wide range of Gram-positive bacteria, by virtue of its ability to hydrolyze cell wall components. It is present in both azurophilic and specific granules of human neutrophils and is also found in the granules of monocytes and macrophages, in blood plasma, tears, saliva and airway secretions. Lysozyme is extremely active against such bacteria as *Bacillus subtilis*, *B. megaterium* [71, 72] and *Micrococcus lysodeikticus*, indeed, the

susceptibility of this latter organism to lysozyme forms the basis of a laboratory assay for this enzyme [73]. Bacterial cell walls consist in general of linear polysaccharide chains containing repeating units of N-acetylglucosamine and N-acetylmuramic acid residues in β -1-4 linkage. Lysozyme hydrolyses β -(1,4) glycosidic bonds in peptidoglycan of bacterial cell wall, is proteinaceous in nature with insect lysozyme possessing a high degree of similarity with mammalian lysozymes [2]. Lysozymes in insects are 14kDa proteins [2], may be found in haemostatic cells [1] and were the first anti-bacterial factor purified from insect haemolymph [74]. Lysozyme has been located in the gut of several insects, in haemocytes of *Spodoptera eridania* and *Locusta* and in haemocyte cell lines [3]. While lysozyme displays anti-bacterial activity it appears to work in combination with cecropins and attacins [17].

LPS-binding proteins. In insects, bacterial LPS-binding protein facilitates the clearance of bacteria by promoting nodule formation. Smooth strains of *E. coli* are cleared slowly from larvae of *Bombyx mori* since they possess O-specific polysaccharides which protect the lipid A binding site. In contrast rough strains are cleared within 30 minutes by nodule formation and have no polysaccharides protecting the relevant binding site [75].

Transferrin: In insects transferrin has an iron binding domain in the N-terminal region and may function by sequestering iron from pathogens thus inhibiting their growth [65]. Human lactoferrin is a member of the transferrin family and displays antimicrobial properties against Gram-positive and Gram-negative bacteria [76] by limiting the

availability of environmental iron [77]. However, since iron-saturated lactoferrin is also able to kill certain bacteria, mechanisms other than iron depletion are involved [78].

Defensins: Another important group of antimicrobial peptides is the group of beta-sheet defensins that comprise four members in humans: HNPI-HNP4 [79]. Defensins in insects are anti-microbial cationic peptides of 4 kDa and 40 residues long which play an important role in innate and adaptive immunity [80]. In insects, defensins show activity against gram-positive bacteria and some gram-negative species [2]. Defensins are cysteine rich cationic peptides containing three or four disulphide bridges and represent an early defence against invading microorganisms. Defensins may be produced within 3 hours of infection and their level declines 12-36 hours post-infection suggesting a correlation between expression and presence of bacteria. Defensins act on the cytoplasmic membrane of bacteria and lyse cells by forming voltage-dependent ion channels, which lead to leakage of potassium and other ions [7]. The widespread occurrence of defensins in higher animals and more distant defensin relatives in plants [81] and insects [82] is consistent with an early evolutionary origin [79, 83]. *In vitro* studies reveal the microbicidal activity of defensins against a variety of bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, many fungi, and some viruses [84 -86].

Cecropins are active against gram positive and negative bacteria [87] and are approximately 4kDa with 35 - 39 residues. Cecropins are amphipathic molecules that penetrate bacterial cell walls resulting in pore formation and subsequent ion leakage [65].

6.0 THE USE OF *Galleria mellonella* AS A MODEL FOR MICROBIAL VIRULENCE TESTING

The use of mammals for evaluating the virulence of microbial pathogens has provided much information on the functioning of the immune system but such tests can be time consuming, labour intensive and expensive in terms of purchasing animals and the need to provide feed and housing facilities. In addition, there is a legal requirement to obtain permission to work with animals and to perform such procedures. Over the last two decades there has been a growing international awareness of the need to reduce the use of mammals for routine product and microbial virulence testing [88]. Alternative systems that could provide comparable data without the need to use mammals for *in vivo* testing would be useful and desirable for evaluating microbial pathogenicity. The use of cell, tissue and organ cultures has been adopted in many instances but the number of animals used for such tests remains high. An alternative system that would be more cost effective than mammalian testing, ethically acceptable and provides comparable data would be of great benefit.

The study of the response of insects to infection was pioneered by Pasteur in the nineteenth century who used insects to demonstrate the microbial origin of disease in Silk moths. More recently the response of insects to entomopathogenic nematodes and their endosymbiotic bacteria has been studied in an effort to optimise the use of nematodes for the control of insect pests in the field [87]. The larvae of *G. mellonella* have also been used to evaluate the pathogenicity of *Proteus mirabilis* [16], *Escherichia coli* and

Bacillus cereus [22]. *D. melanogaster* and *Spodoptera littoralis* have been used to determine the toxicity of secondary metabolites of *Penicillium* species [89].

Given the role of the innate immune response in protecting mammals from microbial infection [10, 90] and the high degree of similarity that exists between the mammalian and insect innate immune responses [2, 6], studying the insect response to infection may provide comparable data to those which may be obtained using mammals. The Greater Wax Moth, *G. mellonella*, is being used increasingly as a model for assessing the virulence of a range of microorganisms. *G. mellonella* belongs to the order Lepidoptera and the family Pyralidae. *G. mellonella* is a pest of beehives feeding upon pollen and destroying the combs of weak or diseased hives. The larvae are dull white in colour, about 3 cm in length, weigh approximately 0.3 - 0.5gr and undergo a metamorphosis to give a grey moth. Larvae of *G. mellonella* can be purchased from a variety of commercial sources and can be maintained for a fraction of the cost associated with mammals used in conventional *in vivo* pathogenicity testing.

The ability of *G. mellonella* larvae to detect differences in the pathogenicity of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* has been demonstrated [52], and a good correlation exists between the virulence of *P. aeruginosa* in *Galleria* larvae and in mice [91]. Larvae of *G. mellonella* have been used to assess the virulence of *Bacillus thuringiensis* and *Bacillus cereus* and strong agreement has been established between the results obtained in insects and mice [92]. In an examination of the responses of plants, nematodes, insects and mice to a *P. aeruginosa* PA14 *rpoN* mutant [93] it was determined that virulence in nematodes was reduced but, interestingly, that virulence in plants, insects and mice was unimpaired suggesting that *rpoN* does not regulate virulence

genes required for infection in a range of hosts. The insect in this case was *G. mellonella* and the result suggests that similar virulence genes may be required for infection in insects and mice. Larvae of *G. mellonella* have recently been employed to determine the relative virulence of *C. albicans* isolates and to differentiate between pathogenic and non-pathogenic yeast species [94]. A positive correlation between the virulence of *C. albicans* mutants when tested in *G. mellonella* and in BalbC mice has been established [95]. The above examples demonstrate the potential for using *G. mellonella* as a model for evaluating microbial pathogenicity and show how comparable data may be obtained using species as diverse as *G. mellonella* and mice.

6.1 Inoculation of *G. mellonella* larvae.

Larvae of *G. mellonella* are easy to inoculate *via* injection into the haemocoel through the last left pro-leg [94]. The base of the pro-leg can be opened by applying gentle pressure to the sides of the leg and this aperture will re-seal after removal of the syringe needle without leaving a scar. Inoculation of larvae with test microorganisms must be accompanied by inoculation of larvae with the buffer used to re-suspend the test microorganisms to ensure that this has no effect on larval viability. A number of workers also suggest the 'mock-inoculation' of a number of larvae per experiment to ensure that the handling and inoculation procedures are not deleterious to the health of the larvae [87, 94]. Larvae can be stored at 15°C prior to use and, once inoculated, may be maintained at temperatures up to 37°C as long as appropriate controls are implemented to quantify the

effect of temperature on survival. Larvae should be handled with care as rough handling affects survival and also leads to the expression of stress proteins.

End points that may be used with *G. mellonella* larvae include the percentage survival at different time points following inoculation [94], the fluctuations in the haemocyte density [15, 18] and the expression of anti-microbial proteins in response to infection [2]. Larval death is the easiest parameter to measure whereas the other end-points are more labour intensive but may give insights into the immune response(s) of the insect to challenge with sub-lethal doses of micro-organisms or to inoculation with pathogens of low or attenuated virulence.

7.0 CONCLUSION.

The innate immune response in insects shares many common features with that in mammals [6] and as a result an increasing number of workers are evaluating microbial virulence in insects as well as in mammals. While the insect immune response is not as complex as the mammalian response and lacks the acquired responses of the mammalian system, the fact that large elements of the innate immune response are similar in both mammals and insects [2, 6, 10] opens the possibility of using insects to model the innate immune response to pathogens in order to fine-tune subsequent experimentation in mammals. The use of insects may allow the comparison of the effect of specific

pathogens in insects and mice but it does not facilitate the study of many of the disease processes that may be specific to mammalian tissue. Consequently, the use of insects may be limited to quantifying the alterations in virulence of microbial cells or mutants rather than elucidating the processes involved in disease progression and dissemination in mammals. While it is extremely unlikely that the use of insects will replace the need for mammals for *in vivo* pathogenicity testing it does offer the possibility of refining mammalian experimentation so that the least number of mammals are used with additional data being supplied by studying the innate immune responses of insects.

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LEGENDS TO FIGURES

Figure 1. Haemocyte types involved in cellular immune response.

Linear maturation process in *Lepidoptera* (closed arrow) or differentiation of prohemocytes into different cell types (open arrows).

Figure 2. Diagrammatic representation of activation of the respiratory burst and possible oxidant generation during phagocytosis by neutrophils.

Microorganisms are phagocytosed and encapsulated within a phagocytic vacuole. NADPH oxidase is activated and accepts electrons from NADPH to reduce O_2 to O_2^- (superoxide). The cytoplasmic granules migrate to the phagocytic vacuole and discharge their contents. Myeloperoxidase (MPO) enters the vacuole and utilises H_2O_2 in the presence of chloride, to generate hypochlorous acid (HOCl). Hydroxyl radical ($\cdot OH$) formation within the vacuole may occur by a number of mechanisms. O_2^- interacts with nitric oxide ($NO\cdot$) to generate peroxynitrite ($ONOO\cdot$). The other granule enzymes (e.g. defensins, proteases) can attack the phagocytosed microorganism.

Figure 3. A diagrammatic representation of the similarities between *Drosophila* and human Toll cascades.

Signalling through Toll and Cactus proteins resulting in activation of Dorsal or Dif, parallels signalling induced by Toll/I- κB and activation of NF- κB . In insects, microbicidal peptide synthesis is controlled by spaetzle, the extracellular Toll ligand, involving the gene cassette spaetzle/Toll/cactus/Dif (illustrated by dashed line). TLR2 and TLR4 are the best studied in humans as the co-receptor for LPS, the other co-receptor being CD14 leading to the expression of pro-inflammatory cytokines. Abbreviations used: LBP; lipopolysaccharide binding protein, I- κB ; inhibitory κB , NF- κB ; nuclear factor κB .

Table 1. Properties of reactive oxygen species.

Proposed ROI and RNI production in mammalian phagocytes (*) and in cells of the insect haemolymph (+).

Table 2. A comparison of humoral and cellular PPRs, and anti-microbial peptides and enzymes in humans and insects.

Antibacterial activity (AB) and antifungal activity (AF)

Table 1. Kavanagh and Reeves.

Superoxide: * +	$O_2 + e^- + H^+ \rightarrow O_2^- + H^+$ Superoxide is both a one-electron reductant and a one-electron oxidant, with limited levels of biological activity. Does not have direct toxic effects on targets, but rather exerts its toxicity by conversion to other ROI.
Hydrogen peroxide: * +	$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ Reacts with heme proteins and peroxidases to initiate radical reactions and lipid peroxidation. Membrane permeable. Reacts with reduced iron and copper salts or superoxide to generate hydroxyl radicals.
Hydroxyl radical: *	$Fe_2^+ + H_2O_2 \rightarrow Fe_3^+ + OH^- + \cdot OH$ Extremely reactive with most biological molecules. Involved in microbicidal and cytotoxic reactions, causes DNA modifications and breaks.
Hypochlorous acid: *	$H_2O_2 + Cl^- + H^+$ (presence of MPO) $\rightarrow H_2O + HOCl$ Hypochlorous acid induced death occurs very rapidly and is 100 to 1000 times more effective than H_2O_2 . Strong nonradical oxidant of a wide range of biological compounds. Preferred substrates thiols and thioesters.
Singlet oxygen: *	$HOCl \rightarrow H^+ + OCl^-$ $OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2$ Electronically excited state of oxygen. Reactivity with a range of biological molecules. Toxicity by inactivation of membrane respiratory chain enzymes has been reported.
Nitric oxide: * +	Reacts very rapidly with superoxide to generate the highly toxic peroxynitrite. Inactivates iron/sulphur centres.
Peroxynitrite: *	$NO \cdot + O_2^- \rightarrow ONOO^-$ Strong lived, unstable strong oxidant with properties similar to hydroxyl radical. May be transformed in an acid milieu to peroxynitrite acid and then to hydroxyl radical.

Table 2. Kavanagh and Reeves.

	Vertebrates	Invertebrates
Humoral PRRs	Macrophage mannose receptor (175kDa). f-Met-Leu Phe receptor (binds to N-formyl peptide). c-type lectins	LPS Binding Protein. Lectins.

	C2-type immunoglobulin domain.	Hemolin.
	Complement/ α 2 macroglobulin. von Willebrand platelet aggregation factor. Scavenger receptor.	β -1,3 glucan binding protein. Gram (-ve) bacterial recognition protein. Peptidoglycan recognition protein. α TEPI. Hemocytin.
Cellular PRRs	Toll like receptors.	<i>Toll</i> <i>Toll 3-8</i> <i>18 wheeler</i> <i>immune deficiency (imd)</i>
	Integrins (CD11b/(CD18) and LFA-1.	Integrins (α , β) heterodimeric proteins
Cationic Proteins	Elastase (29-31kDa) AB, AF Cathepsin G (25-29kDa) AB, AF BPI (55-60kDa) AB Lactoferrin (78kDa) AB Proteinase 3 Azurocidine (29kDa) AB, AF Lysozyme (14.4kDa) AB, AF MPO/H ₂ O ₂ (150kDa) AB, AF	Attractin/ Sarcotoxin (20-28kDa) AB Lysozyme AB, AF
Metalloproteinases	Collagenase Gelatinase	Metalloproteinase (297, 198 & 95kDa)
Peptides	Defensins (4kDa) AB, AF	Defensins AB Cecropins (4kDa) AB,AF Diptericins (9kDa) AB Drosocin AB Metchnikowin AB, AF Proline rich antimicrobial peptides AB Drosomycin AF AFP AF