The ontogeny of immunity in the honey bee, *Apis mellifera* L.

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"....you never can tell with bees"

A.A. Milne
1926

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Summary

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This thesis examines the ontogenetic development of the honey bee immune system in an ecological immunity context. I have optimised established immunological techniques to examine how aspects of the constitutive and induced innate immune response in the honey bee, *Apis mellifera* L., are affected by life-history and parasite infection. I have used three optimised immune assays to gain a measure of the timing and magnitude of specific immune effector systems in the honey bee, and explored the consequences of variation in immune efficacy throughout development and aging. The major findings of this thesis are:

1. Neither workers or drones employ phenoloxidase for immune defence in the pre-pupal stages
2. Sexual selection does not eliminate the need for an immune response in adult drones
3. In both sexes, there is an ontogenetic up-regulation in immune function with adult age
4. In the case of workers, this increase in immune response is not linked to age polyethism, but rather a continuous increase in investment beginning immediately after adult eclosion
5. Immune challenge results in a decrease in phenoloxidase activity, and an increase in antimicrobial peptide production in adults
6. The antimicrobial peptide immune response shows signs of senescence in immune challenged adult workers and drones
7. Colony investment in immune responses acts on a temporal scale and responds positively to an increased threat of parasitism
8. Parasitism produces variation in colony defence strategies
9. Rainfall is an important factor in the successful establishment of an infestation by the varroa mite, *Varroa destructor*, in honey bees.

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Chapter 1

Introduction

The risk of infection by, and interaction with, pathogens has resulted in the evolution of an immune system in insects. Despite not possessing the acquired immune system of vertebrates, insects have a well-developed innate immune system, which can be used as a model to investigate the generic function of innate immunity without the conceptual and practical difficulties of a tightly linked and complex acquired system.

Understanding the insect immune system is important in its own right due to their role in economic (the ecological services provided by insects are valued at $60 billion p.a. in the USA, Losey & Vaughan, 2006) and world health issues (as vectors of disease; each year 500 million people are affected by malaria, WHO). In addition, insects are easy to rear and have much shorter generation times than vertebrates, and so allow the investigation of evolutionary and ecological issues within relatively short timescales. Studying the immune profiles of different sexes throughout their lifetime addresses theory on developmental trade-offs (Nylin & Gotthard, 1998), sexual selection (Andersson & Iwasa, 1996) and senescence (Kirkwood & Rose, 1991).

The honey bee, *Apis mellifera*, has great economic value. There are approximately 300 commercial crops grown worldwide, 84% of which are reliant on insect pollination, with honey bees commonly the main pollinator (Allsopp *et al.*, 2008). In the USA alone, the economic value of the honey bee has been estimated at $14.6 billion p.a., yet honey bees are currently under threat from Colony Collapse Disorder (Stokstad, 2007). This thesis establishes a basis from which to approach the issues concerning bee health and presents the honey bee as an excellent study system with which to investigate immunity theory in a social insect. Moreover, it is one of the first longitudinal studies of the ontogeny of immunity in an economically important insect that also explores the consequences of variation in immune efficacy throughout development and aging.

1.1 A background to immunity

1.1.1 Parasite-mediated sexual selection

Parasite-mediated sexual selection theory was proposed by Hamilton and Zuk (1982) based on their findings in North American passerines that females chose mates with the brightest plumage, and that these males also had a higher resistance to parasite infection. Following this, the hypothesis assumes that

1. Females choose mates based on secondary sexual characteristics
2. Expression of these traits is limited by parasitic infection
3. By preferentially choosing a male with the most elaborate trait, females select for parasite resistance in their offspring
4. Heritable variation in immunity is maintained by host-parasite coevolution (Hamilton & Zuk, 1982).

Two predictions arise from this hypothesis. The intra-specific prediction assumes that females should select mates with the most elaborate traits, and that these males have lower levels of parasite infection than their less showy competitors. Females benefit from their choice by gaining good genes for parasite resistance for their offspring. The inter-specific prediction states that there should be a correlation between the level of trait elaboration and parasite load across species. Both of these predictions have been tested and provide mixed empirical support for the hypothesis (e.g. Zuk *et al.*, 1990; Saino & Moller, 1996; Hamilton & Poulin, 1997; Møller *et al.*, 1999). However, Møller *et al.* (1999) found a strong correlation between secondary sexual traits and host immune function (as opposed to parasite load). They proposed that as hosts are subject to a range of parasites throughout their life, it is unlikely that the selection for investment in secondary sexual characteristics relies on the prevalence of a single parasite species, and that measuring host immunity would in fact provide a clearer understanding of parasite-mediated sexual selection (Møller *et al.*, 1999).

Subsequent to Hamilton and Zuk's theory, Folstad & Karter (1992) proposed the immunocompetence handicap hypothesis (ICHH) to explain the mechanistic link between epigamic sexual selection and immunity. Immunocompetence is an all-encompassing term referring to the ability of an individual's immune system to respond to parasites, and is known to be costly (Folstad & Karter, 1992; Siva-Jothy *et al.*, 1998; Barnes *et al.*, 2000; Moret & Schmid-Hempel, 2000; Armitage *et al.*, 2003). The theory is based on the

proposal that testosterone has a dual role in sexual selection, stimulating the development of secondary sexual traits, whilst simultaneously suppressing immune function (Folstad & Karter, 1992). However there is mixed empirical evidence to support this hypothesis (Roberts *et al.*, 2004). Although testosterone is a hormone restricted to vertebrates, research on insects has shown some support for the predictions of the ICHH (Ryder & Siva-Jothy, 2000; Siva-Jothy, 2000), although alternative hypothesis offer equally valid explanations of this relationship (Siva-Jothy, 2000; Rolff, 2002).

1.1.2 The cost of an immune response

Central to the relationship of immunity and parasite-mediated sexual selection is the assumption that parasites have a fitness cost (Sheldon & Verhulst, 1996). Evolutionary ecology assumes that all organisms have a finite level of resources that must be allocated across various life history traits in order to maximise fitness. Maintaining an immune response is one such cost (Schmid-Hempel, 2003). Nutrient resources, reproduction, and levels of metabolic activity have all been found to affect the level of investment in an insect's immune response (Jacot *et al.*, 2005; Boots & Begon, 1993; Adamo *et al.*, 2001; Hosken, 2001; McVean *et al.*, 2002; König & Schmid-Hempel, 1995; Doums & Schmid-Hempel, 2000). Whilst maintaining and displaying immune resistance to infection can be detrimental to other characteristics, similarly strong selection for other fitness-determining traits can be at the cost of an immune response (McKean *et al.*, 2008).

1.1.3 Gaining a measure of immunocompetence

Typically a measure of the immunocompetence of an individual is gained through assaying one or two constituent parts of the immune response, but problems arise due to the lack of a clear definition of 'immunocompetence' and consequently there is no standardised method for measuring it. Assays should rely on general cellular or humoral immune responses to a standardised and novel immune challenge (Siva-Jothy, 1995). These measures are considered to be an indication of the overall immune ability, and a reflection of host fitness, but must be carefully considered in their interpretation (Ryder, 2003; Adamo, 2004). Debate over whether phenoloxidase is a good measure of the immune response can be found in Chapter 6, Section 6.3.6.

1.1.4 Ecological Immunity

The development of theoretical and empirical work into the costs of immunity, and how to measure it, has lead to the emergence of ecological immunity. The field seeks to examine immunity in a broader context, and understand the associated costs in relation to the constraints imposed by ecology and life history (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002; Rolff & Siva-Jothy, 2003). These costs can be split into two types: 1) the evolutionary cost of developing immune defences, and 2) the physiological cost of maintaining them (Rolff & Siva-Jothy, 2003). The life history strategies involved in ecological immunity have been studied both theoretically and empirically, and provide a window into immunity in the context of 'real-world' (cf. laboratory) situations (e.g. Norris & Evans, 2000; Lochmiller & Deerenberg, 2000; Zuk & Stoehr, 2002; Rolff & Siva-Jothy, 2003).

1.1.5 Ontogeny of immunity

This thesis aims to investigate the ontogeny of immunity in insects, by which I mean the changes that occur in immune investment throughout an insect's life, incorporating development, adult selection pressures and aging. Mounting an immune response to infection during development results in lower growth rates and reduced viability (Boots & Begon, 1993; Freitak *et al.*, 2007; Eleftherianos *et al.*, 2008). As insects develop into adults, the selection pressures acting on immunity change (Zuk & Stoehr, 2002, see Sections 3.1, 4.1 & 5.1), and the different life history strategies adopted by the sexes impact on immune investment. Bateman's principle states that males are selected to have high mating success whereas females gain fitness through longevity, which carries an associated risk of increased pathogen exposure (Bateman, 1948; Trivers, 1972). Following this, males should be selected for lower immunity, which seems to be the case in insects (Rolff, 2002; Adamo *et al.*, 2001; Hosken *et al.*, 2001). However, whilst securing a successful mating by investing in sperm production or mating flights is vitally important for male fitness, surviving long enough to mate is also essential, and should be factored into hypothesis about immune investment.

1.2 Insect Immunity

Insects are subject to a wide range of micro- and macro-parasites with a variety of infection modes (Ratcliffe & Rowley, 1987). Insects have responded by evolving an arsenal of defensive mechanisms, from behaviour to the development of innate immune responses.

1.2.1 The innate immune system

The mechanisms for detecting pathogens are largely unknown, but it is thought that haemocytes recognise invading pathogens either by direct interaction with surface receptors, or indirectly by recognition of receptors that bind to, and opsonize, the pathogen (Lavine & Strand, 2002). These receptors stimulate cascade responses and signalling pathways that produce an immune response. Figure 1.1 shows a schematic of the typical responses involved in the innate immune system. The innate immune system can be split into two response categories based on their temporal profiles: constitutive and induced (Schmid-Hempel, 2005). Constitutive responses are mostly associated with the prophenoloxidase activating system and haemocyte (cellular) responses (see Sections 1.2.3 & 1.2.4.1), and whilst not maintained at a constant maximum, are present in the background without any prior challenge (Gillespie & Kanost, 1997; Schimid-Hempel, 2005). They are unspecific but immediate in effect. On the other hand, induced responses only occur once the invading pathogen has been recognised. They are specific, more costly and take longer to produce (hours or days), but are longer lasting (Boman & Hultmark, 1987).

The immune system in insects has 3 levels of response: the physicochemical barriers provided by the cuticle and gut, constitutive cellular responses that occur as soon as pathogens breach the physicochemical barriers, and the induced responses such as the production of antimicrobial peptides and lysozymes, primarily in the fat body, which provide a more specific, long-lasting response (Gillespie & Kanost, 1997; Schmid-Hempel, 2005).

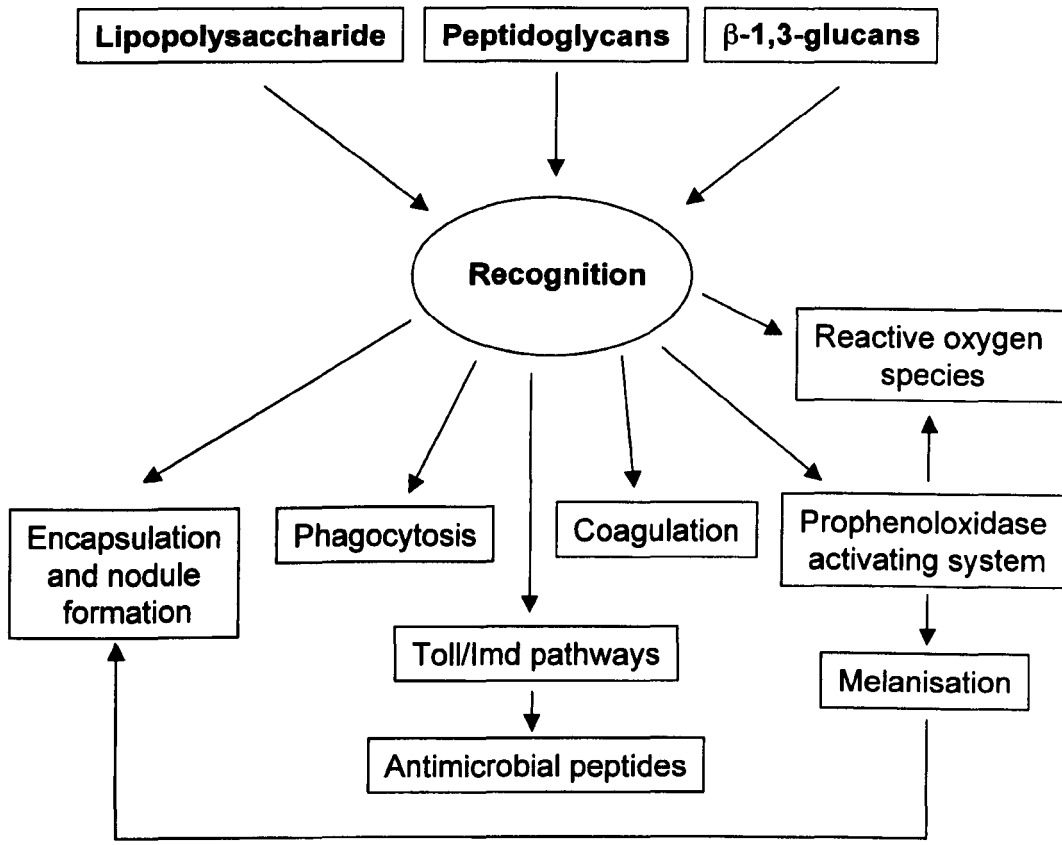


Figure 1.1. Schematic of typical responses of the insect innate immune system following activation from different antigens (lipopolysaccharides, peptidoglycans and β-1,3-glucans). Figure adapted from Tzou *et al.*, 2002.

1.2.2 Physicochemical barriers

The external cuticle is an effective protective barrier against direct attack. The rigid exoskeleton is covered in a secretion of wax and sclerotised with melanin (produced from the phenoloxidase activating system, see Section 1.2.4.1). To avoid having to penetrate the cuticle, many pathogens are transmitted by oral infection. In response to this, the fore- and hindgut and trachea are lined with tough chitinous membranes. The midgut, which lacks this defence, is instead lined with a peritrophic membrane, and mid-gut cells can be sloughed off if infected with a pathogen (Brooks *et al.*, 2002). In addition, the gut represents a hostile environment as the proteolytic enzymes secreted to aid digestion can also cause pathogen breakdown (Schmid-Hempel, 2005).

1.2.3 Cellular responses

Haemocytes are utilised in a number of the innate immune responses including coagulation, phagocytosis, nodule formation and encapsulation, which are briefly described below. Haemocytes first develop during embryogenesis in the mesoderm, and are subsequently produced by division of stem cells or haemocytes already in circulation (Lavine & Strand, 2002). Several different types of haemocytes have been identified in insects, and up to eight different classes are distinguishable based on morphological, histochemical and functional characteristics (Lackie, 1988; Lavine & Strand, 2002). However, complications arise as not all types are found in all insects, and the function of the haemocyte types can change from species to species (Pech & Strand, 1996). The immune function of some types remains unknown, but two classes, the plasmatocytes, and granular cells (granulocytes) are commonly involved in wound healing, phagocytosis, encapsulation and nodule formation (Lackie, 1988; Lavine & Strand, 2002). Products from the prophenoloxidase activating system (ProPO-AS), which is a humoral response (see Section 1.2.4.1), have important roles in several of the cellular responses. This interaction and coordination between immune responses renders the division of the innate immune system into cellular and humoral responses somewhat arbitrary, but it will be organised as such here for clarity.

1.2.3.1 Coagulation

Clotting is initiated as soon as the cuticle is broken and, in addition to preventing mass fluid loss from the insect, limits the spread of pathogens (Siva-Jothy *et al.*, 2005; Haine *et al.*, 2007). Recognition of the wound initiates a proteolytic cascade to activate the clotting response. Two types of haemocyte are involved (granular cells and plasmatocytes), which disintegrate and form extracellular aggregates at the wound site. Activation of phenoloxidase produces melanin (see Section 1.2.4.1), which is laid down over the initial clot, causing it to harden (Theopold *et al.*, 2004).

1.2.3.2 Phagocytosis

Phagocytosis is a multiple step process by which small particles can be removed from circulation by a specialised form of endocytosis (Gillespie & Kanost, 1997). Insect lectins (simple recognition molecules) mark the pathogen to be targeted for removal by the haemocytes (Bayne, 1990; Schmid-Hempel, 2005).

1.2.3.3 Nodule formation

Multicellular aggregations of haemocytes that trap small pathogens such as bacteria, thereby neutralising them, are known as nodules (Lackie, 1988). The nodule is usually removed from circulation by adhering to tissue walls, or may be encapsulated.

1.2.3.4 Encapsulation

Encapsulation is initiated when invading pathogens are too large for phagocytosis or nodule formation, and can be used against a wide range of parasites such as nematodes, cestodes and insects, or against implanted tissues such as parasitoid eggs (Dunn, 1986; Lackie, 1988). There are two types of encapsulation reaction: cellular and melanotic (Gillespie & Kanost, 1997). Melanotic encapsulation is associated with the prophenoloxidase activating system (Section 1.2.4.1) and occurs when melanin cross-links proteins to assemble a capsule around the pathogen (Kumar *et al.*, 2003). Cellular encapsulation is the formation of a capsule by the overlapping of several layers of haemocytes, and is formed by a three-step process: granular cells adhere to the pathogen, which in turn causes layers of plasmatocyes to be

laid down. A final layer of granular cells indicates the capsule formation has finished (Pech & Strand, 1996). This response is the more complicated than the previous cellular responses due to the coordination needed in recruiting the different types of haemocytes (Gillespie & Kanost, 1997).

1.2.4 Humoral responses

Humoral responses refer to those that occur extracellularly. The activation of phenoloxidase, which occurs in the plasma, subsequently results in the production of melanin, a component used in many of the cellular immune responses. Other humoral responses involve complex pathways to produce antimicrobial peptides and agglutination factors, which are described below.

1.2.4.1 Phenoloxidase

The enzyme phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is a major component of the fast acting constitutive part of the innate immune system, used in the defence against, and resistance to, parasite infections (see Ashida & Brey, 1998; Cerenius *et al.*, 2008 for review). PO is widely distributed, being found in plants and fungi as well as animals (Mason, 1955). In insects, PO has several important physiological roles. It is responsible for initiating the biosynthesis of the black pigment melanin, which is involved in cuticle sclerotisation, wound healing and defence responses such as nodule formation and encapsulation, and is associated with the stimulation of phagocytosis (Mason, 1955; Ratcliffe *et al.*, 1984; Cerenius *et al.*, 2008).

The prophenoloxidase-activating system (ProPO-AS) is a series of complex interactions between enzymes and their zymogens, the activation of which occurs in the plasma (Ashida & Brey, 1998). PO is highly active, catalysing the production of toxic byproducts, and is therefore generally stored as its inactive precursor, prophenoloxidase (ProPO), contained in haemocytes and/or plasma (Saul *et al.*, 1987; Brehélin *et al.*, 1989; Brookman *et al.*, 1989). The activation of ProPO into PO through proteolysis occurs as part of a cascade triggered by miniscule amounts of microbial or fungal cell wall components (lipopolysaccharides (LPS), peptidoglycans or β-1,3-D-glucans) (Ratcliffe *et al.*, 1984; Söderhäll & Cerenius, 1998). Recognition molecules in the haemolymph respond to foreign particles as non-self and induce the activation of the ProPO-AS (see Figure 1.2).

The ProPO-AS can be split into 2 parts: 1) recognition reaction occurs between pathogen and pattern recognition proteins, 2) a signal triggered by pathogen invasion relays downstream to ProPO activating factors (Iwanaga & Lee, 2005). Once recognition of the immune challenge has occurred, a serine proteinase cascade mediates the activation of the prophenoloxidase activating enzyme (PPAE), which is itself stored in zymogen form. PPAE then lyses ProPO into PO. PO catalyses the oxidation of phenols into quinines, which subsequently polymerise to produce melanin (Cerenius & Söderhäll, 2004).

During the production of melanin many by-products and intermediates are formed, such as quinones, semiquinones, quinone methides and trihydroxyindoles (Riley, 1988; Hoffmann, 1995; Gillespie & Kanost, 1997). These can generate highly reactive oxygen species such as superoxide anions and hydroxyl radicals, whose cytotoxic effects may contribute to killing pathogens (Riley, 1988; Nappi *et al.*, 1995). These cytotoxic substances do not discriminate between self and non-self, and due to the nature of the open haemocoel in insects, could potentially attack the host (autoimmunity) (Nappi *et al.*, 1995; Nappi *et al.*, 2004). Recent research has focused on the regulation of the ProPO system through various mechanisms, including inhibitory serpins and quinine isomerases (Park *et al.*, 2000; De Gregorio *et al.*, 2002; Park *et al.*, 2006; Sugumaran, 2002), and the pattern recognition proteins that initiate ProPO activation: these have been partially elucidated in several insects (Yoshida *et al.*, 1996; Ochiai & Ashida, 1999; Ma & Kanost, 2000; Cerenius *et al.*, 2008 (review)). It is also thought that the melanin may act as a 'sink', preventing the cytotoxins from damaging the host tissues (Nappi & Vass, 1993; Carton & Nappi, 1997; Riley, 1997).

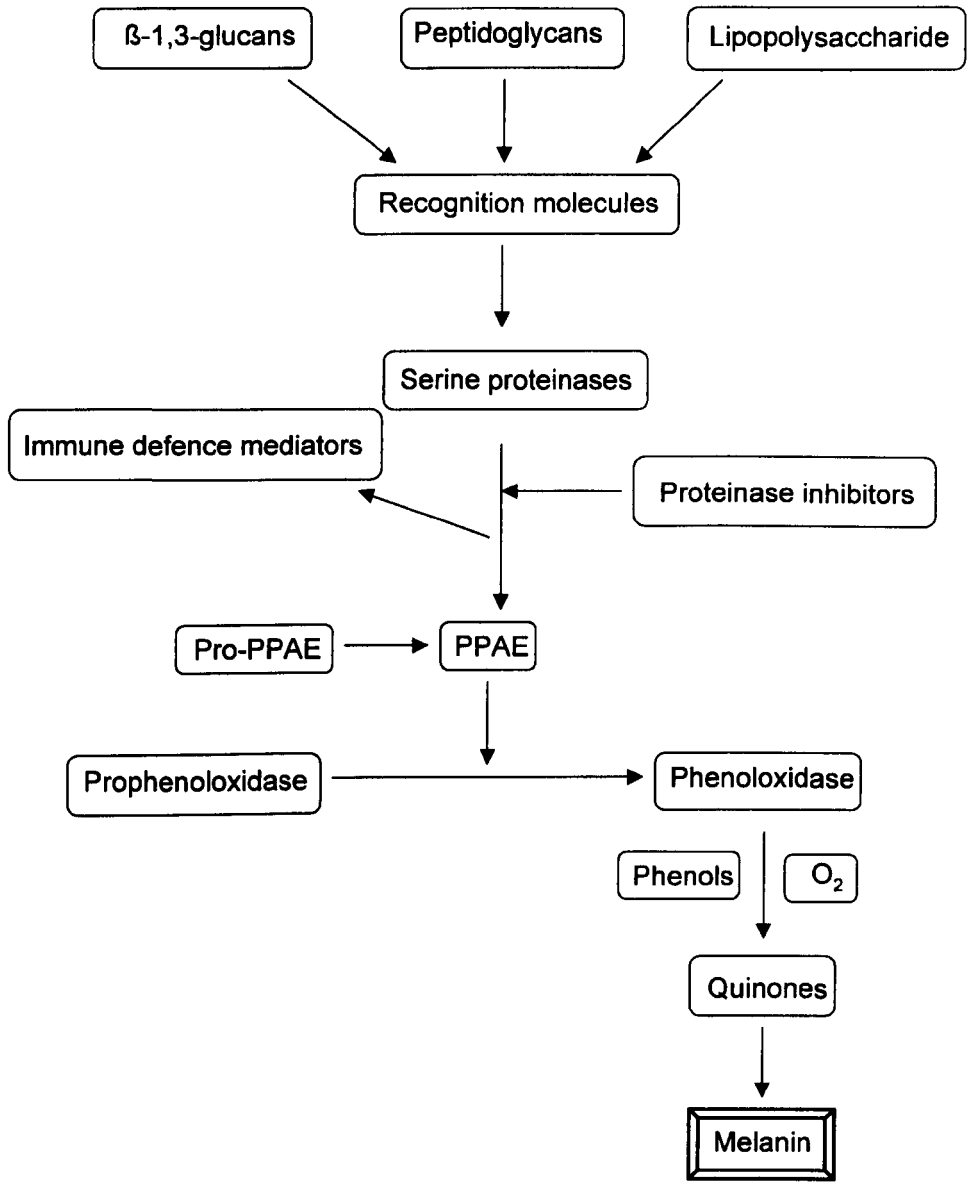


Figure 1.2. Schematic of the prophenolodixase-activating system (ProPO-AS) in arthropods, leading to the production of melanin (adapted from Cerenius & Söderhäll, 2004). Miniscule amounts of microbial or fungal cell wall components can trigger the cascade. The activation of ProPO to PO is catalysed by the ProPO activating enzyme (PPAE), which is stored in zymogen form (Pro-PPAE). Reactive phenols and oxide free radicals are produced during the process of melanin formation.

1.2.4.2 Antimicrobial peptides

Antimicrobial peptides/polypeptides (AMPs) are synthesised in the fat body, a functional homologue of the mammalian liver, although some peptides such as lysozyme and defensin can also be produced in the haemocytes, gut cells, salivary gland cells and reproductive tract (Trenczek & Faye, 1988; Hoffmann *et al.*, 1996; Gillespie & Kanost, 1997). AMPs work by disrupting the permeability of the pathogen membranes, causing lysis and death (Cociancich *et al.*, 1993). Nearly 60 different AMPs have been identified, and are grouped into families based on structure and their specificity to different types of bacteria and fungi (Vilmos & Kurucz, 1998).

Regulation of AMP gene expression in the fat body is through two signal transduction pathways: 1) the Toll pathway, which is activated by fungi and Gram-positive bacteria, and 2) the immune deficiency (Imd) pathway, mainly activated by Gram-negative bacteria (Hoffman *et al.*, 1996; Gillespie & Kanost, 1997). Expression of the genes usually occurs within half an hour of an immune challenge. Transcription intensity peaks at 12 to 48 hours, after which time it decreases, although the effect of antibacterial peptides has been shown to last up to 14 days post challenge in bumble bees (Hoffman, 1995; Korner & Schmid-Hempel, 2004).

1.2.4.3 Other humoral responses

The lectin-agglutination system: Lectins (also known as agglutinins) are defence molecules found in both haemocytes and plasma (Iwanaga & Lee, 2005). In invertebrates, lectins are thought to have a role in non-self recognition, phagocytosis, and in the opsonization of invading pathogens by binding to their surfaces (Gillespie & Kanost, 1997; Wilson *et al.*, 1999). Many lectins have multiple binding sites, which allows for the agglutination activity of target cells (Gillespie & Kanost, 1997).

Haemolin: Haemolin is an inducible haemolymph protein produced following an immune challenge with bacteria (Faye & Kanost, 1998). It is thought to have a role in immune recognition and modulation of defensive responses (Sun *et al.*, 1990; Gillespie & Kanost, 1997). Haemolin can inhibit the aggregation of haemocytes, implying a function in the regulation of the adhesive properties of haemocytes during immune responses (Faye & Kanost, 1998).

1.3 Social insects and immunity

Eusocial behaviour, as shown by the social insects (the social Hymenoptera plus termites) and some aphid, beetle and thrip species (Hughes *et al.*, 2008) presents an opportunity to investigate the role of parasitic selection on immunity in the context of group living. Sociality has many benefits compared to a solitary existence. Cooperative division of labour increases the efficiency of foraging, brood-care and anti-predator defence, allowing these species to become highly productive (Cremer *et al.*, 2007). However, group living comes at a cost. High host densities combined with the warm, resource-rich environment of the colony nest provides the perfect conditions for rapid pathogen proliferation (Schmid-Hempel, 1998; Christe *et al.*, 2003). Social insects are particularly susceptible due to their high-density group living and constant social contact. Consequently, social insects have evolved mechanisms to limit the cost of parasitism. Through cooperation, colonies have developed a 'social immune system': altruistic behaviours of individuals or groups that benefit the colony as a whole (see Cremer *et al.*, 2007 for review). Some examples of this in bees include allo-grooming to remove mites, inducing social fever to heat-kill infecting bacteria, and the hygienic behaviour of workers to remove diseased brood (Starks *et al.*, 2000; Spivak & Boecking, 2001).

Variation in physiological immune defences associated with increasing sociality is also possible. Density-dependent prophylactic immune responses, such as elevated levels of cuticular melanisation, are seen in several insect taxa, but to date has not been shown in a social insect (Barnes & Siva-Jothy, 2000; Wilson *et al.*, 2001; Wilson *et al.*, 2003; Pie *et al.*, 2005). However, antimicrobial cuticular excretions have a prophylactic role in colonies, and have been shown to increase in strength with increasing sociality levels (Stow *et al.*, 2007).

Immune responses may be constrained by the different life histories of the colony members (Boomsma *et al.* 2005; Bocher *et al,* 2007). Within a social colony, individuals are divided into reproductive castes, each with different roles. Queens (only one or a few per colony) and males are responsible for the reproductive output of the colony, whilst large numbers of workers perform all other tasks needed for group survival (Robinson, 1992; Schmid-Hempel, 1998). The reproductive potential, and consequently fitness, of the colony is dependent on the survival of one or a few reproductives. We therefore expect that these

highly valuable colony members should invest in elevated levels of immune protection. Similarly, the tasks performed by workers, which can change throughout life (age polyethism) carry varying levels of associated pathogen risk, and subsequently selection may act on immune investment based on age and role.

The reproductive division of labour in social insects involves all individuals within a colony being the offspring of a single queen, and thus potentially being susceptible to the same pathogens (Baer & Schmid-Hempel, 2003; Palmer & Oldroyd, 2003; Hughes & Boomsma, 2004a). Increased genetic diversity within a colony, either through the presence of multiple queens, or the multiple mating of the single queen, reduces the rate of within-group pathogen transmission (Schmid-Hempel, 1994). Queen polyandry has been shown to reduce colony variation in resistance (Tarpy, 2003), and increase average resistance (Baer & Schmid-Hempel, 1999, 2001; Hughes & Boomsma, 2004a, 2006; Tarpy & Seeley, 2006; Seeley & Tarpy, 2007; Reber *et al.*, 2008).

1.4 The Honey Bee, *Apis mellifera*

1.4.1 The British honey bee

The native British honey bee, *Apis mellifera mellifera* L. (1758, Hymenoptera: Apidae), is also known as the European dark bee, or German black bee due to its dark body colour. *A. m. mellifera* is no longer used as a significant subspecies in the UK due to its aggressive behaviour, and since 1859 beekeepers have imported other subspecies, leading to hybridisation of areas originally occupied by *A. m. mellifera*. The most popular of these are *A. m. ligustica* (Italian bee) and *A. m. carnica* (Carniolan bee), originally imported from Slovenia (Ruttner, 1988). Whilst these subspecies have desirable characteristics such as fast brood build-up, good disease resistance and easy handling nature, they are less resistant to British winters than *A. m. mellifera*, and overwinter in small colonies, which have a low survival rate (Ruttner, 1988). Bees used throughout this thesis were a hybrid known as British Hybrid bees.

The honey bee has a three-tiered caste system based on the reproductive division of labour: a single reproductive queen, up to 50,000 conditionally sterile female workers, and, in the summer, male drones (Hooper, 1997). Figure 1.3 shows the morphological variation between the castes. Queens and drones are approximately twice the size of workers. Drones lack a

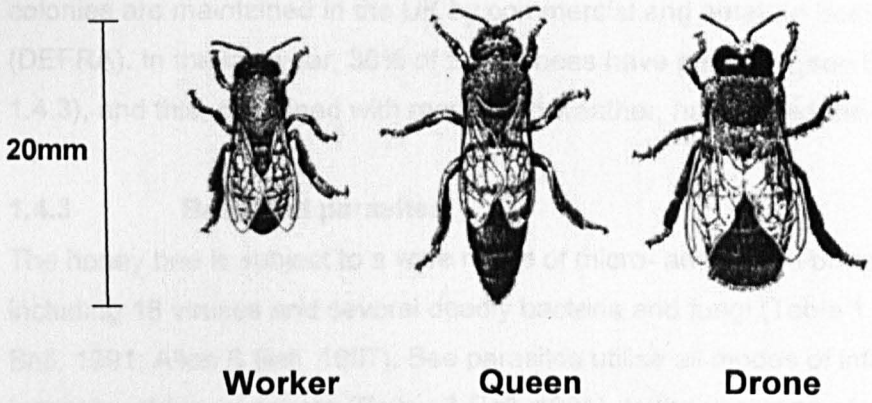


Figure 1.3. Diagram showing morphological differences between the castes of the honey bee, *Apis mellifera*.

sting and have evolved large bulbous eyes used for searching for a mate (Hooper, 1997). Within this thesis I study workers and drones, but do not consider queens. Explanation for this can be found in Chapter 6, Section 6.4.1.

1.4.2 The valuable bee

Honey bees are valued not only for the honey and wax that they produce, but as pollinators of many of our crops and wild flowers. In the UK 39 crops depend on insect pollination (Carreck & Williams, 1998). Honey and bumble bees make up the majority of these pollinators, with honey bees contributing £165 million annually to the agricultural output of the UK (BBKA). Approximately 274,000 colonies are maintained in the UK by commercial and amateur beekeepers (DEFRA). In the last year, 30% of British bees have perished (see Section 1.4.3), and this, combined with recent bad weather, has dented the industry.

1.4.3 Bees and parasites

The honey bee is subject to a wide range of micro- and macro-parasites, including 18 viruses and several deadly bacteria and fungi (Table 1.1, Bailey & Ball, 1991; Allen & Ball, 1997). Bee parasites utilise all modes of infection, from ingestion of fungal spores (Bailey & Ball, 1991), to the vectoring of viruses by ectoparasitic mites (e.g. Bowen-Walker *et al.*, 1999; Brodsgaard *et al.*, 2000; Chen *et al.*, 2004c; Yang & Cox-Foster, 2005), and the immune responses raised against these pathogens have been shown to impact on other life-history traits (Bailey & Ball, 1991; Evans & Pettis, 2005; see Section 5.1). The cost of immunity and how it may be partitioned in worker bees in particular has come under recent scrutiny due to a phenomenon termed Colony Collapse Disorder (CCD). First described in the USA in 2007, CCD refers to unusually low numbers of adults found within a hive with no obvious grounds for collapse. The cause is currently thought to be a combination of factors including multiple parasite infection, nutritional stress and insecticides (Oldroyd, 2007, Cox-Foster *et al.*, 2007). Colony loses in the UK have risen by 200% in the last two years (BBKA survey, 2008). Bad weather conditions during this time lead to bees being confined within their hives, where the stress provided opportunities for bacteria and viruses to proliferate. Consequently, the use of the honey bee as a model system with which to examine the immune profiles of social insect castes, and the responses to parasite infection, now has an additional

Parasite type	Name	Affected life stage	Symptoms
Virus	Deformed wing	Larval	Deformed wings and abdomen in adults
	Sacbrood	Larval	Failure to pupate, and death shortly after cell capping
	Black queen cell	Larval	Dark-walled cells, containing tough, yellow pupae
	Acute bee paralysis	All stages	Death in brood and adults
	Chronic bee paralysis	All stages	Paralysis in adults
	Kashmir	All stages	Death in larvae and adults
Bacteria	American foulbrood (*Bacillus larvae*)	Larval	Discolouration and putrification of propupae
	European foulbrood (*Melissococcus pluton*)	Larval	Larvae displaced in cells; dead larvae discoloured
Fungi	Chalk-brood (*Ascophaera apis*)	Larval	Capped larvae die with white fluffy appearance
	Stone-brood (*Aspergillus flavus*)	Larval	Capped larvae die with white fluffy appearance
Microsporidia	*Nosema apis*	Adult	Reduced lifespan, underdeveloped hypopharyngeal gland
Protozoa	*Malpighamoeba mellificae*	Adult	Cysts on Malpighian tubules
	Crithidia spp.	Adult	Cysts in midgut
	Gregarines, e.g. *Leidyana*	Adult	Crusts in midgut
Mites	Tracheal mite, *Acarapis woodi*	Adult	Reduced survival in severely infested colonies
	Varroa destructor	All stages	Mites visible, reduced colony survival with high infestation
Insects	Greater wax moth, *Galleria mellonella*	All stages	Moth larvae tunnel through wax; may overwhelm weak colonies
	Small hive beetle, *Aethina tumida*	All stages	Beetle larvae tunnel through wax; may overwhelm weak colonies

Table 1.1. Some examples of the range of parasites infecting the honey bee. Parasites comprehensively cover all life stages and modes of infection of the bee, and whilst some have few symptoms, others cause death and can lead to the collapse of a colony (Bailey & Ball, 1991).

importance in its own right in an effort to understand what is causing the current collapse in global bee populations.

1.5 Outline of the thesis

The work presented in this thesis investigates development (ontogeny) of the immune system in honey bees. The questions of how immune responses change during development and aging are addressed for different castes, and finally, the impact of parasites on these immune responses is investigated.

Chapter 2 details the development of assays to quantify phenoloxidase, prophenoloxidase and antimicrobial peptides in the honey bee.

Chapter 3 investigates the changes in immune response for the worker bees throughout their lifecycle, including the development to adulthood, and the effect of age polyethism on immune responses.

Chapter 4 investigates the effect of development and age on the drone immune responses.

Chapter 5 investigates the impact of the ectoparasite, *Varroa destructor*, on the temporal immune responses of worker bees, and the effect that vectored viruses have on immune defences.

Chapter 6 draws together the main findings from the thesis and highlights areas of future research.

Appendix 1 provides further detail on the enzyme kinetic theory dealt with briefly in Chapter 2. Additional results from issues arising from immune assay development are also presented.

Appendix 2 provides figures of the complete data sets from the thesis (for simplicity, two of the control treatment groups are not presented in the figures in the main chapters).

Chapter 2

General Methods

2.1 Introduction

2.1.1 Chapter Aims

This chapter introduces the honey bee, *Apis mellifera*, as a model organism and provides details of the three assays used for measuring the immune response throughout the thesis. The aims of this chapter are:

1. To describe the bee-husbandry methods and protocols used to maintain and challenge bees in the lab (Section 2.2)
2. To develop and optimise a specific assay for measuring phenoloxidase (Section 2.3). This will include:
    a. Ensuring that honey bee phenoloxidase enzyme kinetics conform with the Michaelis-Menton equation
    b. Obtaining a value for the Michaelis constant, Km
    c. Optimising the concentration of L-DOPA used in the assays
3. To develop and optimise a specific assay for measuring prophenoloxidase (Section 2.4)
4. To develop and optimise an assay for antimicrobial activity (Section 2.5)
5. Estimating repeatabilities for all assays with respect to within-individual variation.

2.2 The honey bee as a model organism

2.2.1 Study organism

The honey bees used throughout this thesis were from colonies of hybrid bees kept in Langstroth hives. In 2006 three new nucleus colonies of British Hybrid bees were purchased from Thorne's Bee Supplies. These colonies were allowed to build up from a standard 6-frame nucleus to fill a 10-frame Langstroth hive. A total of 10 different colonies were used in this thesis. With the exception of hives used in Chapter 5, all colonies used in this thesis were free from infestation with the ectoparasitic mite, *Varroa destructor*.

2.2.2 Hive sites

Hives were situated at three different sites within a 1 mile radius of each other, to the west of Sheffield City Centre (Tapton, Fulwood Road and Ranmoor, Figure 2.1). Sites were situated in residential areas with extensive gardens and open parkland, and all were within flying distance (bees can fly over 5 miles from the hive, Beekman & Ratnieks, 2000) of the Peak District National Park, providing heather moorland for foraging.

At the beginning and end of each season (April and October) hives were supplemented with a 50% sugar syrup (50% sugar dissolved in water). At the beginning of the season this helps with the build up of the hive and encourages the start of foraging, while in the autumn the additional food is used for winter stores. At the start of each season hives were checked for surviving colonies. In Sheffield, field sites experience a 70% loss of hive stocks over each winter due to cold temperatures (M. Couvillon, pers. comm.). Surviving hives were checked each week to monitor the build up of brood frames. Colonies were used in experiments when the hive contained at least two frames of capped brood that could be removed for lab use. During the season additional supers (boxes of additional frames placed on top of the original hive) were added to each hive to provide space for food storage and egg laying as the colonies grew.

2.2.3 Lab conditions

Bees used in lab-based experiments were brought into the lab 24 hours before use as a frame of final stage brood. Brood frames were kept in a wooden frame box (i.e. 24 hour dark photoperiod) inside an incubator set at $36^0C \pm 1^0C$. Humidity was maintained at 40% ± 2% using trays of water placed on the incubator floor.

Adult bees were kept in purpose-built bee cages: a box of wooden sides (20cmx15cmx10cm) with a clear Perspex floor to allow easy bee viewing and a sliding metal mesh lid. Adults were also kept in an incubator at $36^0C \pm 1^0C$ with a 12:12h L:D photoperiod. Adults were fed 50% sugar syrup from liquid drop feeders adapted from rabbit water bottles, to which the ball-bearing stopper had been replaced with a glass tube melted to provide a drip dispenser. A piece of filter paper was placed in the floor of the cage to soak up excess drips from the feeder. Each box could comfortably contain several hundred bees.

Figure 2.1. Aerial photograph of Sheffield showing field sites. The three field sites Tapton, Fulwood Road and Ranmoor are pinpointed. The University Animal and Plant Science (APS) Department and Sheffield City Centre are also highlighted. Field sites lie within a 1 mile radius of each other. The Peak District National Park is approximately 3 miles west of the Ranmoor site (aerial photograph taken from Google Earth).

2.2.4 Administering an immune challenge

The procedure for administering an immune challenge was the same for all adult bees used in this thesis. Bees were challenged via haemocoelic injection with a glass microcapillary (1 x 900mm) pulled and ground into a fine needle (tip diameter approximately 130µm) using a Narishige microelectrode puller (model PC-10, and microelectric grinder, model EG-44). Needles were sterilised with ethanol between injecting bees within a treatment group, and fresh needles were used for each treatment group.

The immune challenge was elicited by an injection of lipopolysaccharide (LPS: 0.5mgml^{-1}) in insect Ringer (128mM NaCl, 18mM $CaCl_2$, 1.3mM KCl, 2.3mM $NaHCO_3$, 1l dH_2O, pH 7.2). LPS (derived from *E.coli*) is a component of the outer membrane of Gram-negative bacteria. It is an endotoxin and is widely used as an elicitor of the immune response in insects (Moret & Schmid-Hempel, 2000). Before injection, bees were cold anaesthetised on ice. Injections were made through the pleural membrane between the second and third sternites of the abdomen. Along with a full control (no challenge), additional treatment groups in each experiment provided controls for needle wounding via a sham injection (needle) and the effect of the buffer vehicle (injection with insect Ringer only). Unless otherwise stated, haemolymph was collected for assay 24 hours post challenge (Moret & Schmid-Hempel, 2000).

2.2.5 Statistical analysis

The majority of the statistical analysis in this thesis was performed using the statistical package R (version 2.6.2) on the Mac OS X platform. All data were analysed using parametric tests. Data were tested for normality using a normal Q-Q plot and a plot of residuals against fitted values. Distributions that were significantly non-normal were transformed (as indicated in results). Analysis of assay repeatabilities was performed using the statistical package StatView. In all cases, results presented are for the minimum adequate model, fitted with thorax width as a co-factor to control for body size. Survival data were analysed using a GLM with binary or Poisson distribution families.

2.3 Phenoloxidase

2.3.1 Introduction

Phenoloxidase (PO) is an important constituent of the cellular innate immune system. It is produced as part of a complex cascade, involving the activation of prophenoloxidase (ProPO) which then turns into PO, which subsequently catalyses the reaction for the formation of melanin (Cerenius & Söderhäll, 2004). More detail on the complexities of the cascade and its components can be found in Chapter 1, Section 1.2.4.1. Of concern in this chapter is the development of quantitative assays to measure PO in its different forms. This first section deals with the measurement of PO (the measurement of ProPO is discussed in Section 2.4).

2.3.2 Assaying PO

Two different measurements of PO are commonly taken when assaying immunity. Some researchers measure PO as the amount of activated enzyme present in the haemolymph. Others measure it as the total PO concentration present after the chemical activation of the precursor ProPO. For this thesis, two measurements of PO will be taken: free-standing activated PO (PO), and total potential PO activity, recorded after the artificial chemical activation of ProPO *in vitro* (ProPO – see Section 2.4).

Several assays of PO have been developed over the years, but since 1952 (Horowitz & Shen, 1952) assays typically use the addition of L-dopa (3,4-dihydroxy-L-phenylalanine) to quantify PO levels in a haemolymph sample (e.g. Barnes & Siva-Jothy, 2000; Thompson, 2002). PO catalyses the conversion of L-dopa to dopachrome. The maximum linear rate of colour change (the Vmax) as the L-dopa (colourless) is converted to dopachrome (red-brown colour) is recorded using a spectrophotometer. To assay total PO, samples were incubated with a suitable chemical activator prior to the addition of L-dopa (see section 2.4). Although PO has been shown to follow the Michaelis-Menton equation (see Section 2.3.4) for enzyme kinetics in the mealworm beetle, *Tenebrio molitor*, and all aspects of the assay thoroughly explored (Thompson, 2002), there is some evidence that both the type and concentration of chemicals used in assaying PO and its precursor ProPO can have varying affects when used for different study organisms (Nayar & Bradley, 1994; Thompson, 2002). As no standardised method exists for studying PO and

ProPO in the honey bee, established protocols were further developed to ensure the assays were optimal for measuring honey bee immunity.

2.3.3 Assaying PO: Materials and Methods

The assay used was adapted from Barnes and Siva-Jothy (2000). Haemolymph for assaying was collected using a perfusion bleed method. Adult bees were cold-anaesthetised on ice for approximately 10 minutes. A small cut was made at the bottom of the abdomen using fine surgical scissors. A blade of the scissors was inserted inside the groove in which the sting sits (in the case of drones a sharp blade was inserted to make an initial incision), and a small cut made upwards in the abdominal wall. This cut has two uses: a) perfused haemolymph can drain and be collected from this opening and b) it allows the intestine to prolapse out of the body during the bleed, as the pressure of fluid used in the perfusion can cause the intestine to rupture if it is kept within the body cavity. Contamination of the sample with gut contents can give a false PO reading, even if the contaminating particles are filtered out (pers. obs.). A hypodermic needle (25G) was inserted into the dorsal prothorax, slightly off centre to avoid rupturing the digestive tract. 0.5ml of ice-cold sodium cacodylate buffer (0.001M sodium cacodylate, 0.005M calcium chloride, pH 6.5) was steadily perfused through the body cavity. 0.5ml buffer was used for the perfusion bleed as it provided enough haemolymph extract to be split into three aliquots for use in different assays.

The haemolymph was collected into a 1.5ml centrifuge tube. The sample was vortexed (HATI Rotamixer) for a few seconds to ensure thorough mixing before being divided into three aliquots by splitting the sample into two further 0.5ml centrifuge tube. The samples were immediately frozen at –90°C in a Revco Ultima II chest freezer for a minimum of 24 hours. Freezing the sample at –90°C is necessary to disrupt the haemocytes and allow stable storage of the enzyme, and does not affect the enzyme activity (Pye, 1978).

After freezing, samples were defrosted on ice, vortexed for a few seconds and then centrifuged (4°C, 80,000G, 15 minutes) to remove cell debris. The supernatants were then used in the reaction mixture. For a standard PO assay, 140µl dH_20, 20µl phosphate buffered saline (PBS, 8.74g NaCl, 1.78g $Na_2HPO_4.2H_20$, 1l dH_20, pH 6.5) and 20µl sample are added to a well of a pre-chilled 96-well plate. 20µl L-dopa (concentration to be confirmed, see Section

2.3.6) were added and the plate loaded into a plate reader (VersaMax tunable microplate reader, Molecular Devices) preheated to 30 °C. The reaction was allowed to proceed for 1 hour, with automatic mixing of the sample before the first reading, and between subsequent readings (automixing). The standard PO assay quantifies the rate of conversion of substrate (L-dopa) into product (dopachome) when measured spectrophotometrically as the change in absorbance at a wavelength of 490nm. The rate of conversion is proportional to the concentration of PO present in the sample. The absorbency at 490nm is recorded every 15 seconds (241 readings in an hour) and the results plotted in an enzyme curve using the software Softmax Pro v5.0.1.

2.3.4 Assaying PO: Finding Vmax and Km

To determine the concentration of L-dopa needed for maximum conversion in bee haemolymph, the Km and Vmax of the reaction must first be determined. The assay must show conformity to the Michaelis-Menton equation for Km and Vmax to be calculated. For further information on enzyme kinetics see Appendix 1, Section A1.1.

Thompson (2002) analyses the reaction curve of the PO assay for the mealworm beetle, *Tenebrio molitor* in detail to determine which area of the standard PO curve is the rate of reaction, and what, if any, factors cause departure from this linearity. It was found that the initial lag phase of the curve is due to differences between the temperature of the initial reaction solution and the required reaction temperature. The end lag phase is due to inhibition by reaction products such as melanin. Neither of these factors effects the linear rate phase of the slope, and lag phases are not included in the final Vmax calculation. As with *T. molitor*, the haemolymph of the honey bee was shown to increase linearly with increasing L-dopa concentrations, thereby conforming to the Michaelis-Menton equation (pers. obs.).

2.3.5 Assaying PO: Finding Vmax and Km - Methods

Km and Vmax need to be calculated for the PO assay of honey bee haemolymph as the concentration of substrate (L-dopa) needed for the assay can differ between study systems (Thompson, 2002; Haine *et al.*, 2007; K. Hammerschmidt, pers.comm.). By finding Km we can be sure that the Vmax (2

x Km) of the reaction is achieved, and that the substrate does not limit the rate of reaction.

To find Km, the PO assay was run using 5 different concentrations of L-dopa. Haemolymph was collected from perfusion bleeds of 23 newly emerged worker bees taken from the same colony. Bees were perfuse bled with 0.5ml ice-cold sodium cacodylate buffer, and the samples pooled and frozen at –90°C. 24 hours later the sample was defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes). Based on the findings for *T. molitor* (Vmax required an L-dopa concentration of 3mM, Thompson (2002)), L-dopa was assayed in the following concentrations: 0.0mM, 0.5mM, 1.0mM, 2.0mM, 3.0mM and 4.0mM. These concentrations were calculated for the total sample volume (150µl). Each sample was replicated 3 times. Following Thompson (2002), samples were added to the wells of a pre-chilled 96-well plate in a ratio of 1:2. 50µl of sample were mixed with 100µl L-dopa. The plate was loaded into the plate reader and the reaction was allowed to proceed as standard (30 °C, 490nm, 1hour, automixing). The results were plotted in a direct linear plot (Figure 2.2), considered to be both easier and more accurate to calculate Km from than the more traditional Lineweaver-Burk double reciprocal plot (Cornish-Bowden, 1995). The mean Vmax for each L-dopa concentration was plotted on the y-axis, and the negative of the L-dopa concentration plotted on the x-axis. Corresponding points were joined with a straight line and extended beyond the y-axis. The lines for different substrate concentrations should all cross each other. Points where lines cross give the values for Vmax (on the y-axis) and Km (on the positive x-axis). If the lines do not all cross in the same place, the crossing points are ranked left to right and the medium point is used to give the Km and Vmax (Cornish-Bowden, 1995).

2.3.6 Assaying PO: Finding Vmax and Km - Results

The average Vmax for each L-dopa concentration was plotted using a direct linear plot (Figure 2.2). The crossing points of the lines were not in the same place, so the medium point was used to determine Km and Vmax. This gave a Vmax value of 14.75 and a Km value of 5.75mM. The substrate (L-dopa) concentration needed to achieve Vmax for this assay was therefore 2 x Km = 11.5mM.

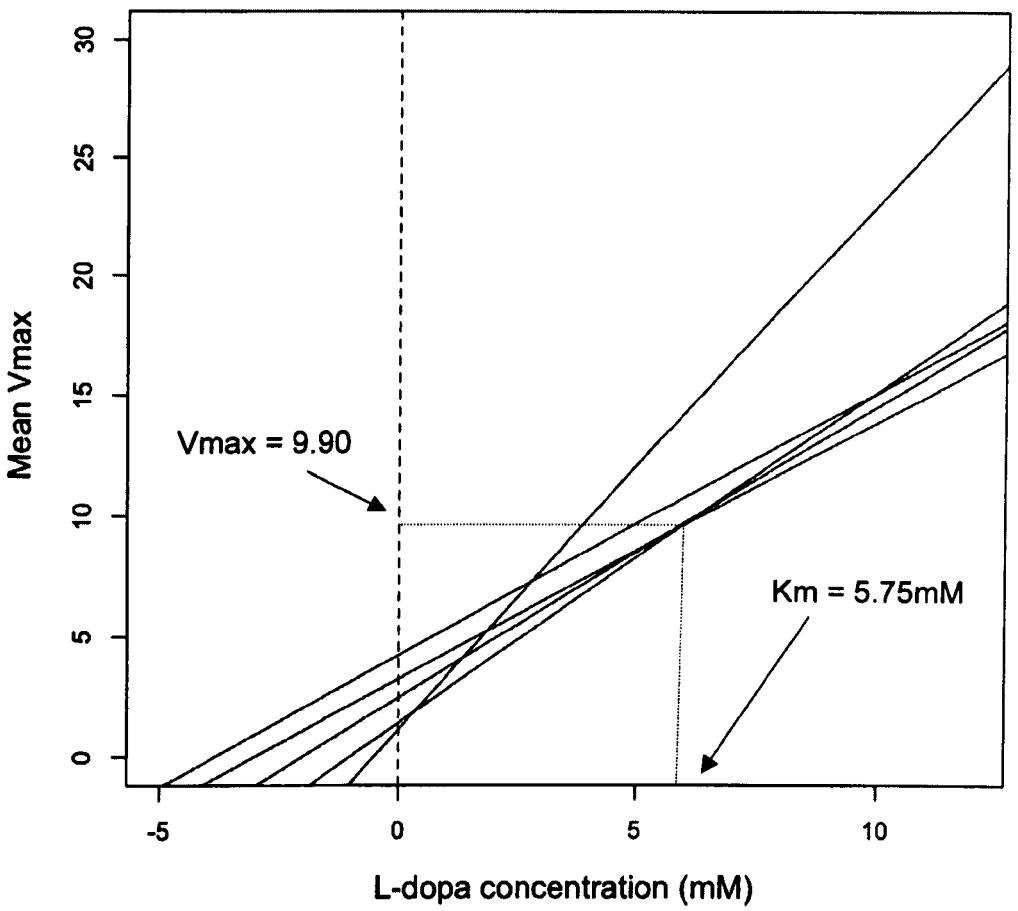


Figure 2.2. Direct linear plot to determine Km and Vmax for honey bee phenoloxidase. The negative L-dopa concentration was plotted against the corresponding mean Vmax. The two points were joined and extended well into the positive x-axis. This was repeated for all L-dopa concentrations. The lines did not intersect at a single point, so crossing points were ranked from left to right. The Km and Vmax values were taken from the medium intersect (Cornish-Bowden, 1995). This gives a Km value of 5.75mM for the honey bee.

2.3.7 Assaying PO: Evaluating Km

Results from the determination of Km and Vmax (previous section) showed that the concentration of L-dopa needed for the PO assay to proceed at Vmax for honey bee haemolymph was 11.5mM, or 22.68 $mgml^{-1}$ (to achieve this final concentration in the total sample volume). This concentration is several times greater than that found in *T. molitor*, but is similar to values subsequently found for the American cockroach *Periplaneta americana*, and the field cricket, *Gryllus bimaculatus* (Thompson, 2002; Haine *et al.*, 2007; K. Hammerschmidt, pers. com.). L-dopa has a low solubility in water: the Merck Index reports a solubility of L-dopa in water in the range of 66mg in 40ml, or $1.65mgml^{-1}$ (Merck Index, 2006). A concentration of 10 mM of L-dopa in water has been reported (Sinsabaugh *et al.*, 1991), but L-dopa is described as "sparingly soluble" (Bredberg *et al.*, 1993). This makes it impossible to produce a solution of L-dopa in the concentration derived from the Km calculations. An experiment was carried out to investigate what concentration of soluble L-dopa could be used in experiments with bee haemolymph that would not be rate-limiting, and what affect this would have on PO and ProPO concentrations. Initially, L-dopa solutions were produced using the following concentrations: half Km (2.88mM), Km (5.75mM) and 2Km (11.50mM). The concentrations were made up in dH_2O and vortexed for 15 minutes to ensure thorough mixing. As expected, the 2Km solution failed to fully dissolve, and despite previous reports, the Km solution also reached saturation. Therefore, the experiment was carried out using just two L-dopa concentrations: half Km (2.88mM) and the saturated solution (made up using 5.75mM L-dopa, but with a final concentration = half Km < saturated < Km). The saturated solution was filtered (0.2µm polypropylene filter, 25mm diameter, Whatman) to remove any un-dissolved particles that might have distorted the light absorbency in the spectrophotometer before use.

Haemolymph was collected from perfusion bleeds of 10 newly emerged worker bees with 0.5ml sodium cacodylate buffer. All bleeds were pooled and the sample vortexed and frozen at –90°C. 24 hours later the sample was defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes). To ensure that the concentration of PO in the haemolymph sample was not a limiting factor in the assay, the sample was tested with both L-dopa concentrations at the following dilution factors: 1, 1/2, 1/4, 1/8, 1/16. It was predicted that as dilution levels increased, we would see the PO concentration starting to become a limiting factor. Any difference between the L-dopa

concentrations would therefore be cancelled out at the higher dilution levels. 20µl of the sample was loaded into a pre-chilled 96-well plate, with 140µl dH_2O and 20µl PBS buffer. A control containing 20µl sodium cacodylate buffer (no haemolymph) was also added to the plate. 20µl of the L-dopa solutions were added to the samples. Each dilution level:L-dopa combination was repeated 7 times.

2.3.8 Assaying PO: Evaluating Km - Results

Vmax decreased significantly with increasing dilution (Figure 2.3, regression, $F_{1,48} = 186$, $p<0.0001$, $r^2 = 0.795$, data are log10 transformed). There was no significant effect of L-dopa concentration on PO Vmax. At the highest sample concentration, the enzyme curves become asymptotic for both L-dopa concentrations (Figure 2.3), implying that the concentration of PO in the samples was the limiting factor, and not the L-dopa concentration. This result, combined with those for ProPO (Section 2.4.3) confirmed that the concentration of L-dopa was conservative: consequently a saturated L-dopa concentration (made up using 5.75mM ($11.34 mgml^{-1}$) with a final concentration = half Km < saturated < Km) was used for all subsequent experiments.

2.3.9 Assaying PO: Repeatability

The perfusion method for sampling haemolymph in bees is destructive, so multiple samples cannot be taken from the same individual. To test the repeatability of the PO assay, repeatability, r, was calculated using the formula described in Lessels & Boag (1987).

To test the repeatability of the PO assay, haemolymph samples were collected from 8 newly emerged workers (24 hours old). Samples were collected from a perfusion bleed with 0.5ml sodium cacodylate buffer. Each sample was split into 3 aliquots, briefly vortexed and frozen immediately for 24 hours (–90°C). Samples were defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes). 20µl of each aliquot for each sample was added to a pre-chilled 96-well plate, along with 240µl dH_2O and 20µl PBS. 20µl L-dopa (saturated) was added and the plate run as standard.

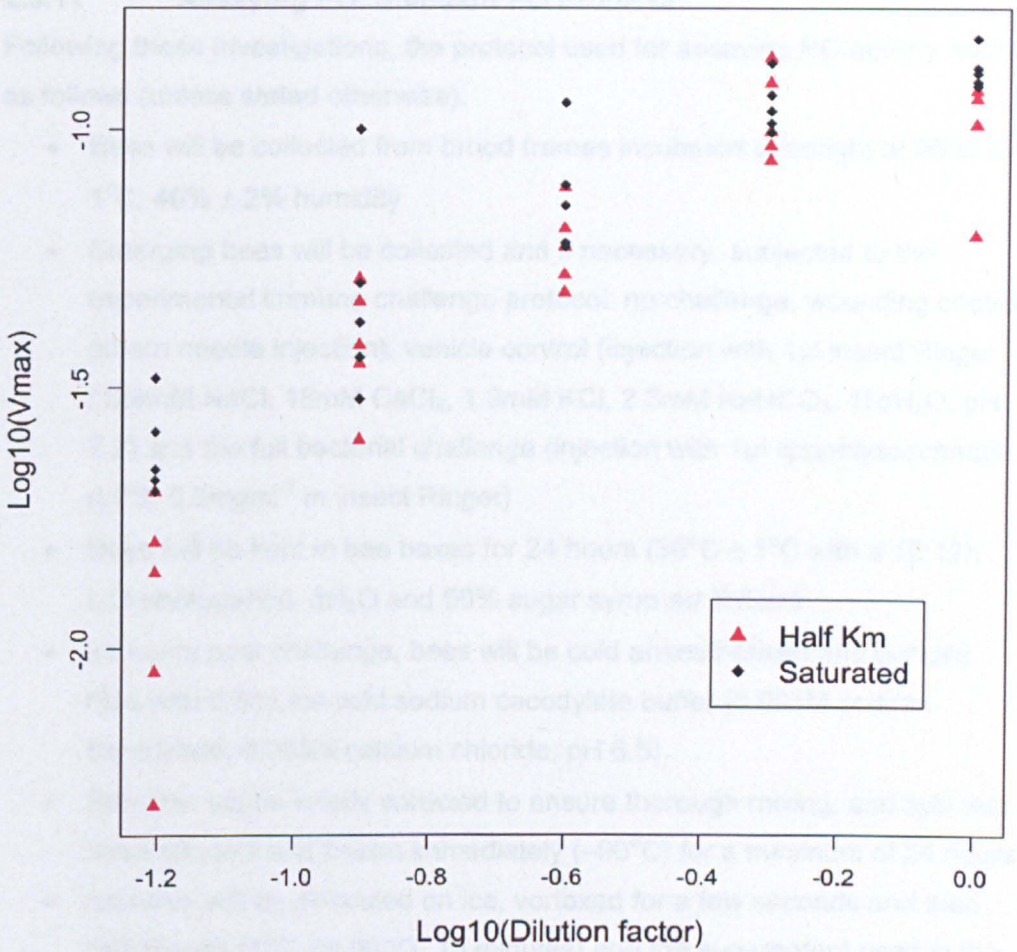


Figure 2.3. PO assay changes in Vmax with different L-dopa concentrations. There was a significant decrease in Vmax with dilution factor, with the enzyme curve starting to level off at the highest concentrations. There was no significant effect of the L-dopa concentration on PO production. The L-dopa curve became asymptotic at saturation.

2.3.10 Assaying PO: Repeatability - Results

The PO assay was highly repeatable with 90.3% of the total variation made up of variation among individuals (ANOVA, $F_{7,16}$= 28.7, p< 0.0001, r = 0.903).

2.3.11 Assaying PO: Standard PO Protocol

Following these investigations, the protocol used for assaying PO activity will be as follows (unless stated otherwise):

- Bees will be collected from brood frames incubated overnight at 36°C ± 1°C, 40% ± 2% humidity
- Emerging bees will be collected and if necessary, subjected to the experimental immune challenge protocol: no challenge, wounding control (sham needle injection), vehicle control (injection with 1µl insect Ringer (128mM NaCl, 18mM $CaCl_2$, 1.3mM KCl, 2.3mM $NaHCO_3$, 1l dH_2O, pH 7.2) and the full bacterial challenge (injection with 1µl lipopolysaccharide (LPS: 0.5mgml^{-1} in insect Ringer)
- Bees will be kept in bee boxes for 24 hours (36°C ± 1°C with a 12:12h L:D photoperiod, dH_2O and 50% sugar syrup *ad libitum*)
- 24 hours post challenge, bees will be cold anaesthetised and perfuse bled with 0.5ml ice-cold sodium cacodylate buffer (0.001M sodium cacodylate, 0.005M calcium chloride, pH 6.5)
- Samples will be briefly vortexed to ensure thorough mixing, and split into three aliquots and frozen immediately (–90°C) for a minimum of 24 hours
- Samples will be defrosted on ice, vortexed for a few seconds and then centrifuged (4°C, 80,000G, 15 minutes) and the supernatent used in the reaction mixture
- 20µl sample, 140µl dH_20 and 20µl phosphate buffered saline (PBS, 8.74g NaCl, 1.78g $Na_2HPO_4.2H_20$, 1l dH_20, pH 6.5) will be added to a well of a pre-chilled 96-well plate
- 20µl L-dopa (saturated solution, made using 5.75mM, 11.34mgml^{-1} in dH_2O, solution will be vortexed for 15 minutes and filtered, with a final concentration = half Km < saturated < Km) will be added to the well
- The plate will be run in a spectrophotometer at 490nm for 1 hour at 30°C, with readings taken every 15 seconds, and automatic mixing of the sample occurring before the first reading, and between subsequent readings

- The rate of change of absorbency will be plotted in an enzyme curve and the maximum linear slope of the line calculated to obtain a Vmax value for the sample.

2.4 Prophenoloxidase

2.4.1 Introduction

Prophenoloxidase (ProPO) is the inactive precursor to PO. Its activation into PO allows us to measure the total PO within an individual, and consequently gain a measure of their potential immune response. Further information on the complexities of the ProPO activating system can be found in Chapter 1, Section 1.2.4.1.

2.4.2 Assaying ProPO

This assay involves the activation of stored zymogen ProPO into PO, which is then able to react with L-dopa as in the standard PO assay. The assay used was adapted from Thompson, 2002. 20µl of the sample are added to 135µl dH_2O and 20µl PBS in the well of a 96-well plate. To activate the ProPO, 5µl of ProPO activator is added (see Section 2.4.5), and the plate taken off ice and incubated at room temperature (19 °C ± 2 °C) for 5 minutes. 20µl of L-dopa solution is then added, and the plate is loaded into the plate reader. The reaction is allowed to proceed as normal for 1 hour (see Section 2.4.14).

2.4.3 Assaying ProPO: Evaluating Km

As discussed in Section 2.3.7, the value of Km calculated from the direct linear plot corresponded to a much higher concentration of L-dopa than was achievable. The experiment in Section 2.3.7 was repeated with a second set of samples, but this time ProPO was activated to gain a measure of the effect of L-dopa concentration on total PO. 5µl of dH_2O was replaced with 5µl α-chymotrypsin (a ProPO activator – see Section 2.4.5) in dH_2O ($5mgml^{-1}$). Samples were incubated at room temperature (19 °C ± 2 °C) with the dH_2O, PBS and α-chymotrypsin for 5 minutes before the L-dopa was added and the samples run on the plate reader as standard (Section 2.4.14).

2.4.4 Assaying ProPO: Evaluating Km - Results

There was a significant interaction with the L-dopa concentration and the dilution factor (Figure 2.4, ANCOVA, $F_{1,46}$= 10.2, p=0.002549, data log10 transformed): as the dilution factor increased, the difference between the L-dopa concentrations was reduced. This was due to ProPO becoming the

limiting factor as the haemolymph samples become more dilute. As the dilution factor got closer to 1, a higher level of ProPO activity was recorded with the saturated L-dopa solution than the half Km. Although the L-dopa curve did not become asymptotic at saturation as with the PO result (Figure 2.3), conducting subsequent assays using the saturated solution (5.75mM (11.34mgml^{-1}) with a final concentration = half Km < saturated < Km) was thought to be conservative compared to concentration of L-dopa used in previous studies (Thompson, 2002).

2.4.5 Assaying ProPO: Activator Suitability

ProPO is naturally activated by bacterial and fungal cell wall components lipopolysaccharides (LPS), peptidoglycans and β-1,3-D-glucans (Ratcliffe *et al.*, 1984; Söderhäll & Cerenius, 1998). Several other substances have been found to activate ProPO, including detergents (e.g. Sugumaran & Nellaiappan, 1990), alcohols (Asada, 1998) and nitrocellulose membrane (Brey *et al.*, 1991). Commonly laminarin, LPS and α-chymotrypsin are used as ProPO activators, but have been found to have different success levels in different insects (Brookman *et al.*, 1989; Thompson, 2002; Korner & Schmid-Hempel, 2004). Laminarin (a storage polysaccharide of brown algae, made up of 1,3-glucans) induces ProPO activation in *T.molitor*, and activation and nodule formation in the locust, *Locusta migratoria migratorioides* (Thompson, 2002; Mullen & Goldsworthy, 2006). LPS has been shown to induce nodule formation when injected into the desert locust, *Schistocerca gregaria*, and increased phagocytosis in wax moth, *Galleria melonella*, (Gunnarsson & Lackie, 1985; Ratcliffe *et al.*, 1984). Chymotrypsin is a protein-lysing enzyme that fills the role of serine proteinases in the ProPO-AS, hydrolysing the peptide bonds in ProPO to break it down into active PO, and has been shown to activate ProPO in many insects (e.g. Saul & Sugumaran, 1988; Kopacek *et al.*, 1995). Laboratory grade chymotrypsin (α-chymotrypsin, Type II, SIGMA C-4129) is synthesised from bovine pancreas.

2.4.6 Assaying ProPO: Activator Suitability – Materials and Methods

To test the suitability of the activators in the honey bee, each was made up in the concentrations that activated the maximum PO for *T. molitor* (Thompson,

2002). The concentration of laminarin used was 1mgml^{-1} and the concentration of LPS was 0.5mgml^{-1}. In the case of α-chymotrypsin where no concentration was specified, the concentration of 5 mgml^{-1} was used (R. Naylor, pers. comm.). All solutions were made up in dH_2O.

Twelve newly emerged workers (24 hours old) were perfuse bled with 0.5ml sodium cacodylate buffer. All samples were vortexed and frozen individually at –90°C. 24 hours later the samples were defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes). Four 20μl aliquots of each sample were added to a pre-chilled 96-well plate. Each aliquot received 5μl of either laminarin, LPS, α-chymotrypsin or a control of dH_2O. 20μl of PBS and 135μl dH_2O were also added. Additional controls replacing perfused haemolymph with sodium cacodylate buffer were run for each activator. The plate was taken off ice and incubated at room temperature for 5 minutes. The plate was gently shaken at the start of the incubation period to ensure thorough mixing. After 5 minutes, 20μl L-dopa (saturated) was added and the plate loaded in to the plate reader and run as standard (Section 2.4.14).

2.4.7 Assaying ProPO: Activator Suitability - Results

There was a significant difference between ProPO activators (Figure 2.5, ANOVA, $F_{3,40} = 46.5$, $p<0.001$, data log10 transformed), with much more ProPO activated by α-chymotrypsin than any of the other activators.

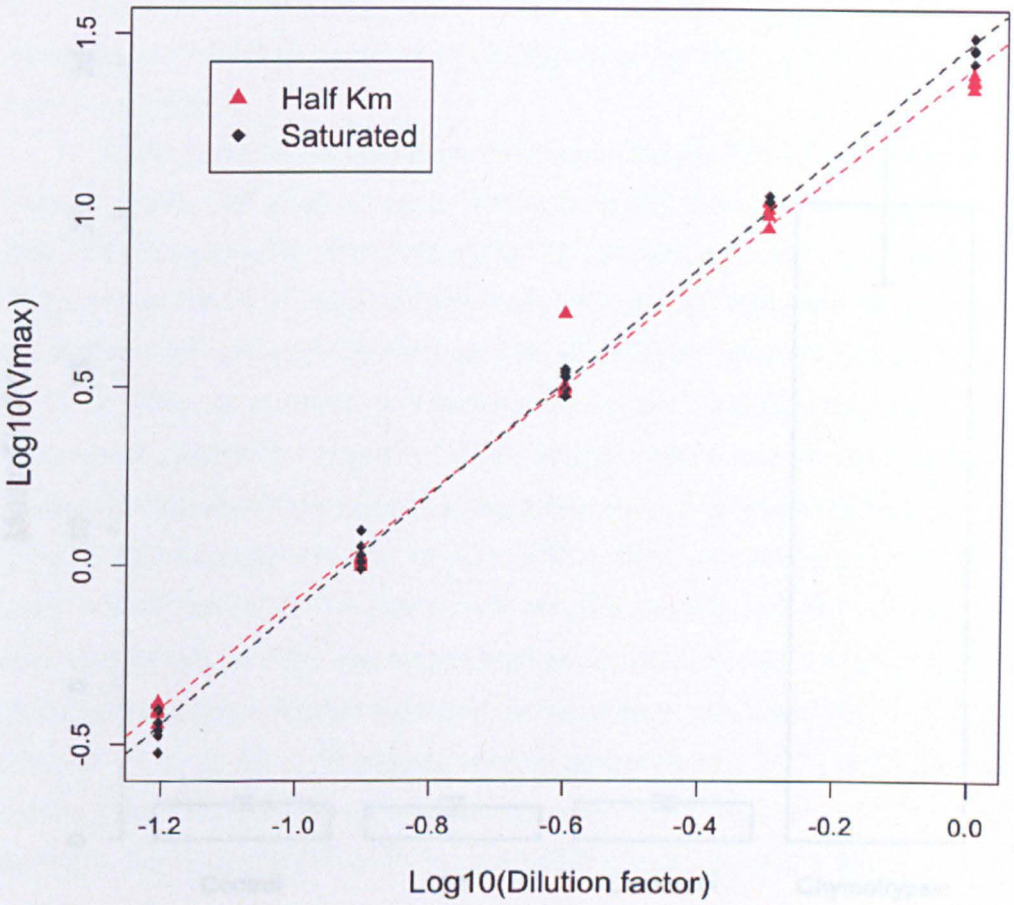


Figure 2.4. ProPO assay: changes in Vmax with different L-dopa concentrations. There was a significant interaction between L-dopa concentrations and the dilution factor; at higher concentrations, the ProPO activity was higher in the saturated L-dopa solution than the half Km concentration.

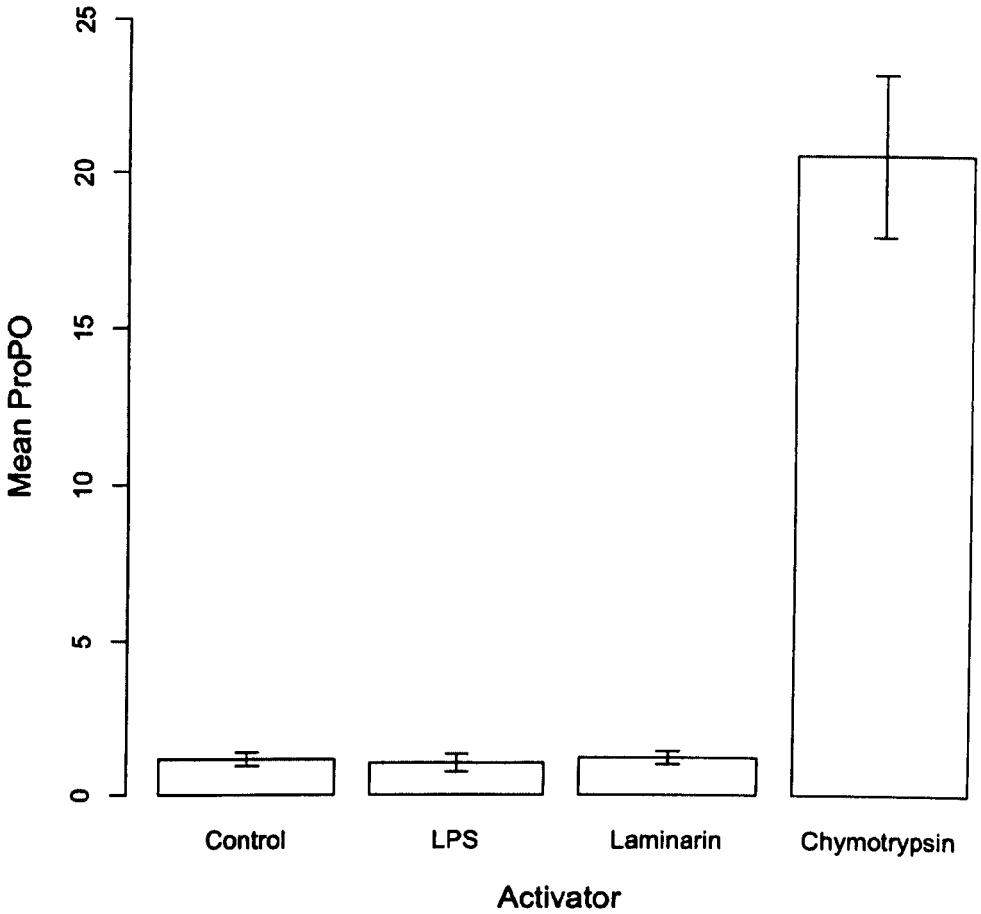


Figure 2.5. Effect of different ProPO activators. There was a significant difference between the activators, with significantly more ProPO activated by α-chymotrypsin than by any of the other activators.

2.4.8 Assaying ProPO: Activator timecourse

In his work on ProPO activators, Thompson incubated his samples at 30 °C for 30 minutes before adding L-dopa (Thompson 2002). Preliminary lab studies showed that samples left on ice for several hours gave very different ProPO measurements, depending on the length of time spent out of the freezer (pers. obs.). The potential degrading effects of a long incubation period on the activation of ProPO, and subsequent measurement of PO, have not previously been investigated.

To test the effect of incubation time with different ProPO activators on final PO activity, 48 newly emerged worker bees (24 hours old) were perfuse bled with 0.5ml sodium cacodylate buffer. All samples were split into 4 aliquots, vortexed and frozen at –90°C. 60 randomly selected aliquots were defrosted on ice and 20µl of each were pooled together. The pooled sample was centrifuged (4 °C, 50,000G, 15 minutes), and then kept on ice for the duration of the experiment. Laminarin (1mgml^{-1}), LPS (0.5mgml^{-1}) and α-chymotrypsin (5mgml^{-1}) were used as ProPO activators, along with a control of dH_2O. Additional controls replacing haemolymph samples with sodium cacodylate buffer were used for each activator. The plates were set up as before, with 5µl of activator, 20µl sample, 20µl of PBS and 135µl dH_2O added to each well. To test the effect of incubation time on ProPO activation, samples were incubated with each activator for 5, 10, 20 or 30 minutes at room temperature (19 °C ± 2 °C) before L-dopa (saturated) was added and the plates run in the plate reader as standard. Each time period:activator combination was repeated 5 times.

2.4.9 Assaying ProPO: Activator timecourse - Results

There was a significant effect of activator and incubation time on the activation of ProPO (Figure 2.6). Across all activator groups the concentration of ProPO assayed significantly decreased with increasing incubation time. There was a significant effect of activator on ProPO activation (ANOVA, $F_{3,64} = 4730$, $p<0.0001$), with α-chymotrypsin activating vastly more ProPO than the other 3 activators. Therefore, to gain a measure of the maximum level of ProPO activity, an incubation time of 5 minutes with the α-chymotrypsin activator will be used in future assays.

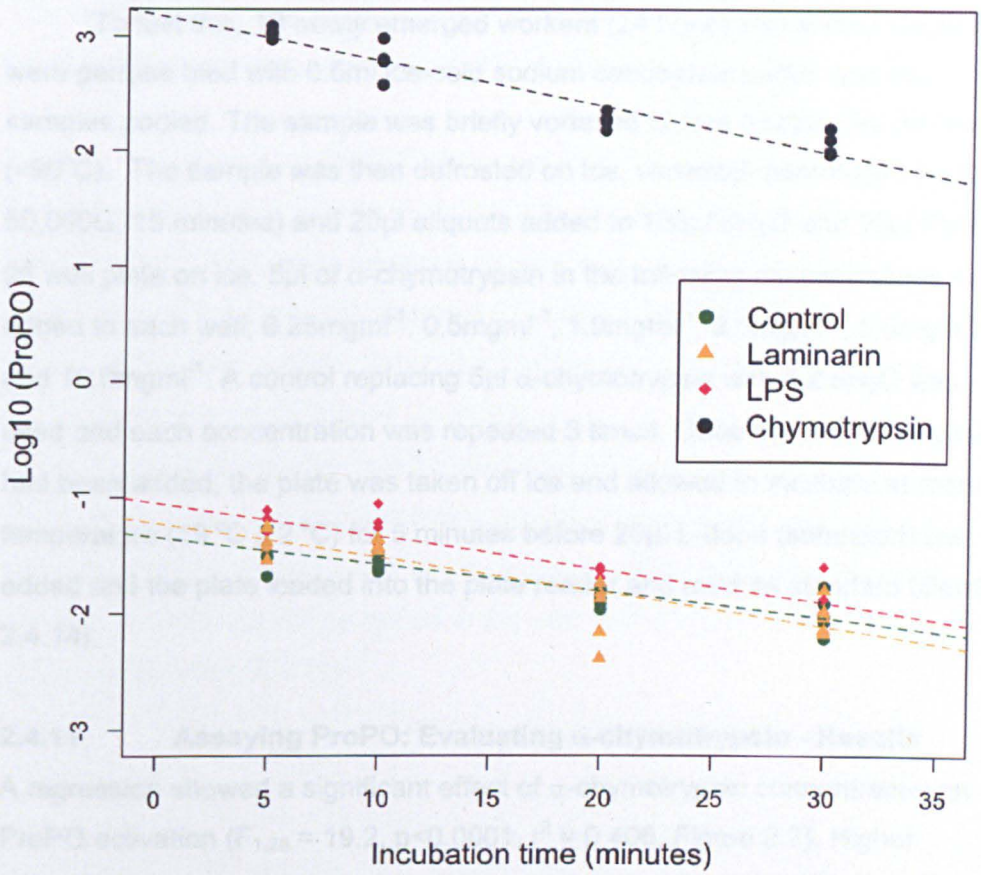


Figure 2.6. ProPO activator timecourse. Three activators (laminarin, LPS and α-chymotrypsin) and a control were incubated for 5, 10, 20 and 30 minutes with the haemolymph sample prior to the addition of L-dopa and subsequent plate reading. There was a significant interaction between activator and time; longer incubation periods resulted in lower levels of ProPO activity for all activators, although incubation time had a greater effect on the ProPO activated by α-chymotrypsin.

2.4.10 Assaying ProPO: Evaluating α-chymotrypsin

When used as an *in vitro* activator of ProPO, α-chymotrypsin has no regulatory mechanism attached to it. This means the hydrolysis of ProPO will potentially continue unchecked. To minimise any "damage" caused by the α-chymotrypsin, it is important to use as low a concentration as possible.

To test this, 10 newly emerged workers (24 hours old) from a single hive were perfuse bled with 0.5ml ice-cold sodium cacodylate buffer, and the samples pooled. The sample was briefly vortexed before freezing for 24 hours (–90°C). The sample was then defrosted on ice, vortexed, centrifuged (4 °C, 50,000G, 15 minutes) and 20µl aliquots added to 135µl dH_2O and 20µl PBS in a 96 well plate on ice. 5µl of α-chymotrypsin in the following concentrations was added to each well: 0.25mgml^{-1}, 0.5mgml^{-1}, 1.0mgml^{-1}, 2.0mgml^{-1}, 5.0mgml^{-1} and 10.0mgml^{-1}. A control replacing 5µl α-chymotrypsin with 5µl dH_2O was used and each concentration was repeated 5 times. Once the α-chymotrypsin had been added, the plate was taken off ice and allowed to incubate at room temperature (19 °C ± 2 °C) for 5 minutes before 20µl L-dopa (saturated) was added and the plate loaded into the plate reader and read as standard (Section 2.4.14).

2.4.11 Assaying ProPO: Evaluating α-chymotrypsin - Results

A regression showed a significant effect of α-chymotrypsin concentration on ProPO activation ($F_{1,28} = 19.2$, $p<0.0001$, $r^2 = 0.406$, Figure 2.7). Higher concentrations of α-chymotrypsin produced lower levels of ProPO activation. As α-chymotrypsin is potentially destructive, it is preferable to use the lowest concentration possible whilst still yielding maximum results. Consequently the lowest concentration was taken from the three concentrations that showed a higher level of ProPO activation (points above the regression line). All future experiments will therefore use an α-chymotrypsin concentration of 0.50 mgml^{-1}.

2.4.12 Assaying ProPO: Repeatability

As with the PO assay, the method of sampling haemolymph is destructive, so the same bee cannot be measured twice. To test the repeatability of the ProPO assay, 8 newly eclosed workers (24 hours old) were perfuse bled with 0.5ml sodium cacodylate buffer. Each sample was vortexed and split into three

aliquots and frozen for 24 hours (–90°C). All samples were defrosted on ice and run as a standard ProPO assay (Section 2.4.14). Repeatability was calculated using the formula described in Lessels & Boag (1987).

2.4.13 Assaying ProPO: Repeatability - Results

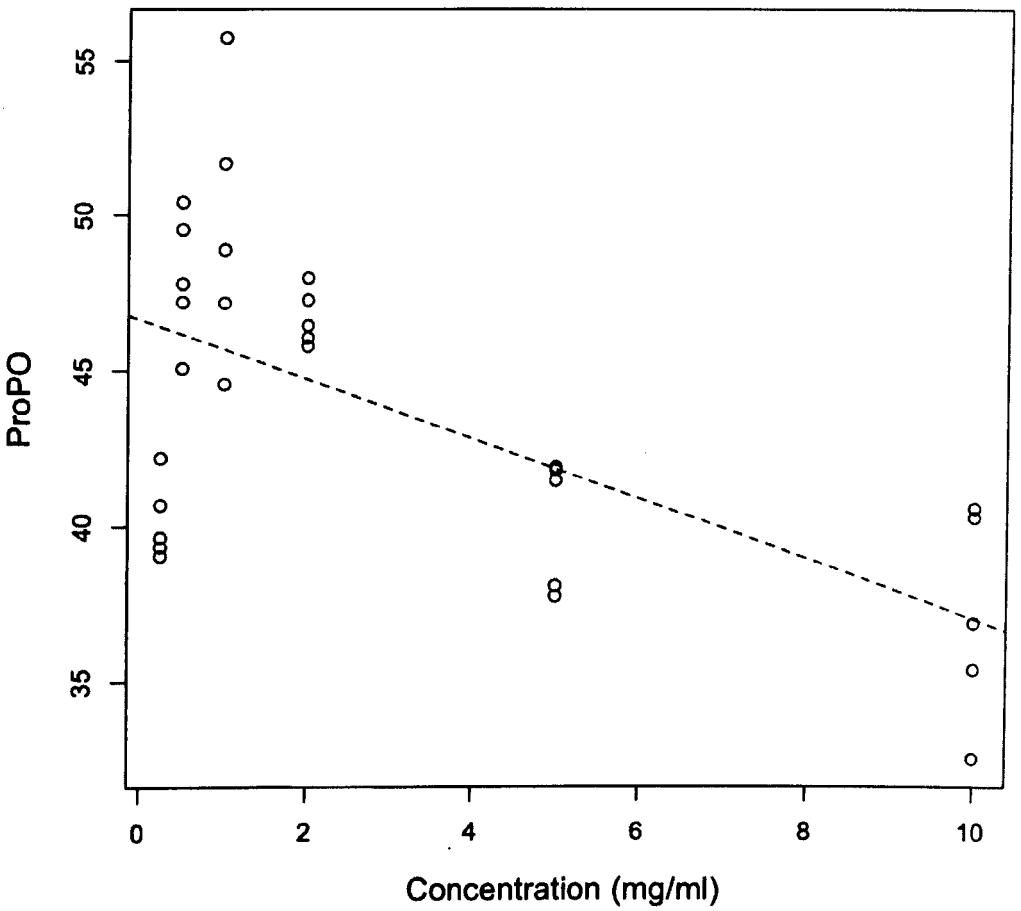
The ProPO assay was highly repeatable with 99.6% of the total variation made up of variation among individuals (ANOVA, $F_{7,16}$= 730, $p< 0.0001$, $r = 0.996$).

2.4.14 Assaying ProPO: Standard ProPO Protocol

Following the results of these investigations, the standard procedure for assaying ProPO will be as follows (unless otherwise stated):

- Method will be as the standard PO assay (Section 2.3.11)
- Within each sample, 5µl of dH_2O will be replaced with 5µl α-chymotrypsin ($0.5mgml^{-1}$ in dH_2O)
- The reaction mixture will be incubated for 5 minutes at room temperature

20µl L-dopa (saturated solution made using 5.75mM ($11.34mgml^{-1}$) in dH_2O with a final concentration = half Km < saturated < Km, solution vortexed for 15 minutes and filtered) will be added to the well, and the plate will be run as in the PO assay (Section 2.3.11).



- **Figure 2.7**. Effect of α-chymptrypsin concentration ($mgml^{-1}$) on ProPO activity. There was a significant effect of α-chymptrypsin concentration on ProPO activation, with higher concentrations of α-chymptrypsin producing lower levels of ProPO activity.

2.5 Determining sampling time in new bees

2.5.1 Determining sampling time: Introduction

Cuticle sclerotisation occurs in the first few hours post-eclosion, but in some cases may take several days (Andersen, 1974). During this period, PO and ProPO have a dual role, being utilised both in the immune response, and in sclerotisation, which involves the oxidative incorporation of phenolic compounds into the cuticle (Andersen *et al.*, 1996). Consequently, assays carried out during this period may provide a false record of the use of PO and ProPO as an immune response. In honey bees, the process of cuticular sclerotisation takes approximately 1-2 days, but this must be balanced with the length of time it is practical to keep bees in the lab prior to testing (Hooper, 1997). To determine at what time post-eclosion PO and ProPO can successfully be measured as having a role in immune function, the following experiment was carried out.

2.5.2 Determining sampling time: Materials and methods

To investigate the affect that the post-eclosion period has on PO and ProPO levels and their role in the immune response, a timecourse was run over the first 72 hours post-eclosion, with bee samples from 2 different hives (Hive F and Hive G). To see the response of PO and ProPO in bees to an immune challenge, bees within the two hives were spilt into 4 treatment groups (No Challenge, Needle, Ringer injection (0.5µl) and LPS injection (0.5µl)). Haemolymph samples were collected from perfusion bleeds (0.5ml sodium cacodylate) at 0, 5hr, 12hr, 24hr, 36hr, 48hr, 60hr and 72hr post challenge. Samples were collected and assayed for PO and ProPO as standard (Sections 2.3.11 & 2.4.14). Thorax widths were measured using QCapture Pro 51 to control for body size.

2.5.3 Determining sampling time: Results

2.5.3.1 Hives Combined

PO: Results were analysed first by combining the two hives as one large data set and fitting Hive as a cofactor. The results for PO (Figure 2.8) show that PO activity significantly increased with age across all of the treatment groups. There was a significant interaction of time with treatment, with the No Challenge group having a higher rate of PO production over time and the LPS challenged group exhibiting lower levels of PO activity (ANCOVA, $F_{3,476} = 8.65$, $p<0.0001$). There

was no effect of thorax width on PO concentration (ANCOVA, $F_{1,476} = 1.08$, $p=0.2993$), but there was a significant effect of Hive (ANCOVA, $F_{1,476} = 190$, $p<0.0001$). The continued increase in PO activity throughout the first 72 hours post-eclosion was subsequently seen to continue for at least the first 14 days of adult life (Section 3.3.4), and may be due to bees responding to cues in the hive, such as the presence of pathogens, or pheromone contact with incoming foragers, or the PO/ProPO immune response may simply increase because of the physiological processes of aging.

ProPO: The results for ProPO (Figure 2.9) showed a significant interaction of time with treatment, with the No Challenge group having a higher rate of ProPO production over time (ANCOVA, $F_{3,476} = 8.65$, $p<0.0001$). Challenge with LPS produced lower ProPO concentrations. There was a significant effect of Hive (ANCOVA, $F_{1,476} = 294$, $p<0.0001$) and thorax width (ANCOVA, $F_{1,476} = 6.68$, $p=0.01007$) on ProPO activity.

Thorax width was further investigated for each of the hives. Bees in Hive F were significantly larger (mean thorax width = 3.31mm) than in Hive G (mean thorax width = 3.23mm; $t = 6.59$, $p< 0.0001$ (two tailed), d.f. = 452), but there was no significant difference in the variation in thorax widths between the two hives (HiveF = 0.0204, Hive G = 0.0172, F-test, $p=0.1817$). We expect some variation in the average body size between hives, as although similar hives were chosen for experiments based on estimated colony size, environmental factors that may influence body size could not be controlled. By adding thorax width as a co-factor in all future analysis models, we can control for the effect of differences in body size between hives.

As the results for PO and ProPO show a significant effect of Hive on enzyme activity, the data were re-analysed by Hive to compare the differences between the two.

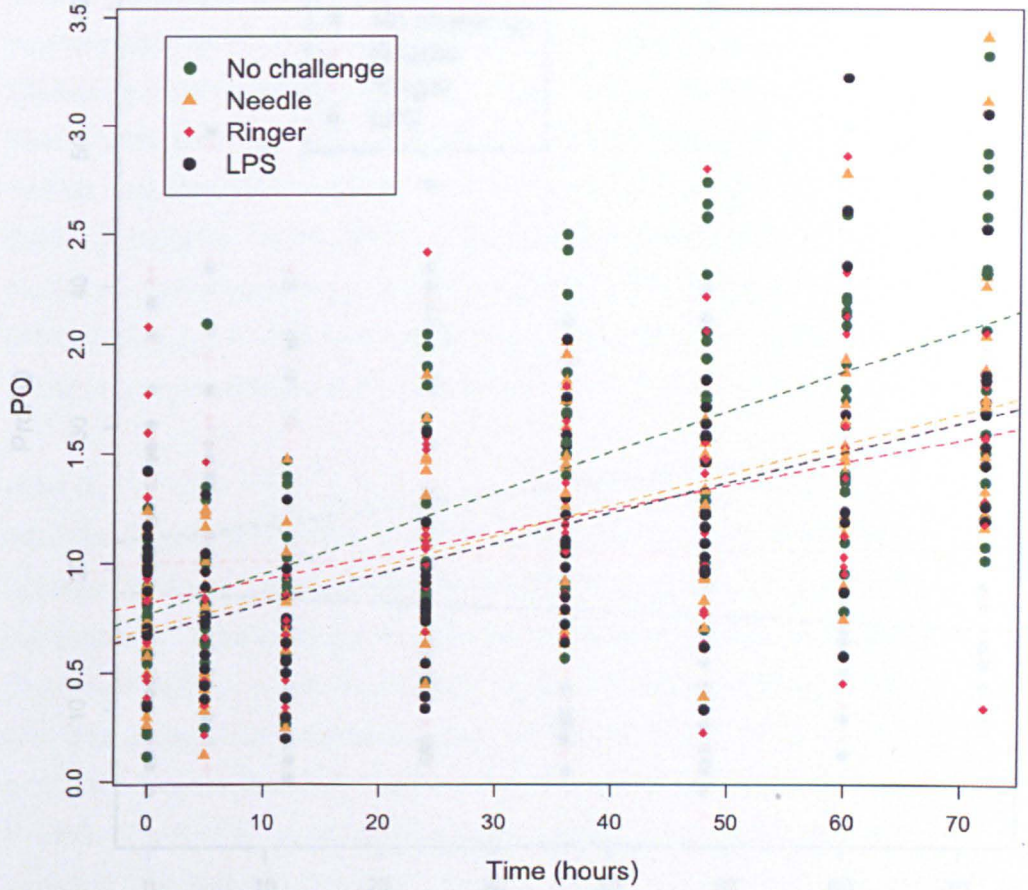


Figure 2.8. Change in PO concentration over the first 72 hours post-eclosion. Samples were split into 4 treatment groups: No Challenge, Needle, Ringer and LPS challenged groups. Results shown are combined for 2 hives. All treatment groups showed an increase in PO concentration over time. There was a significant interaction of time with treatment, with the No Challenge group having a higher rate of PO production over time.

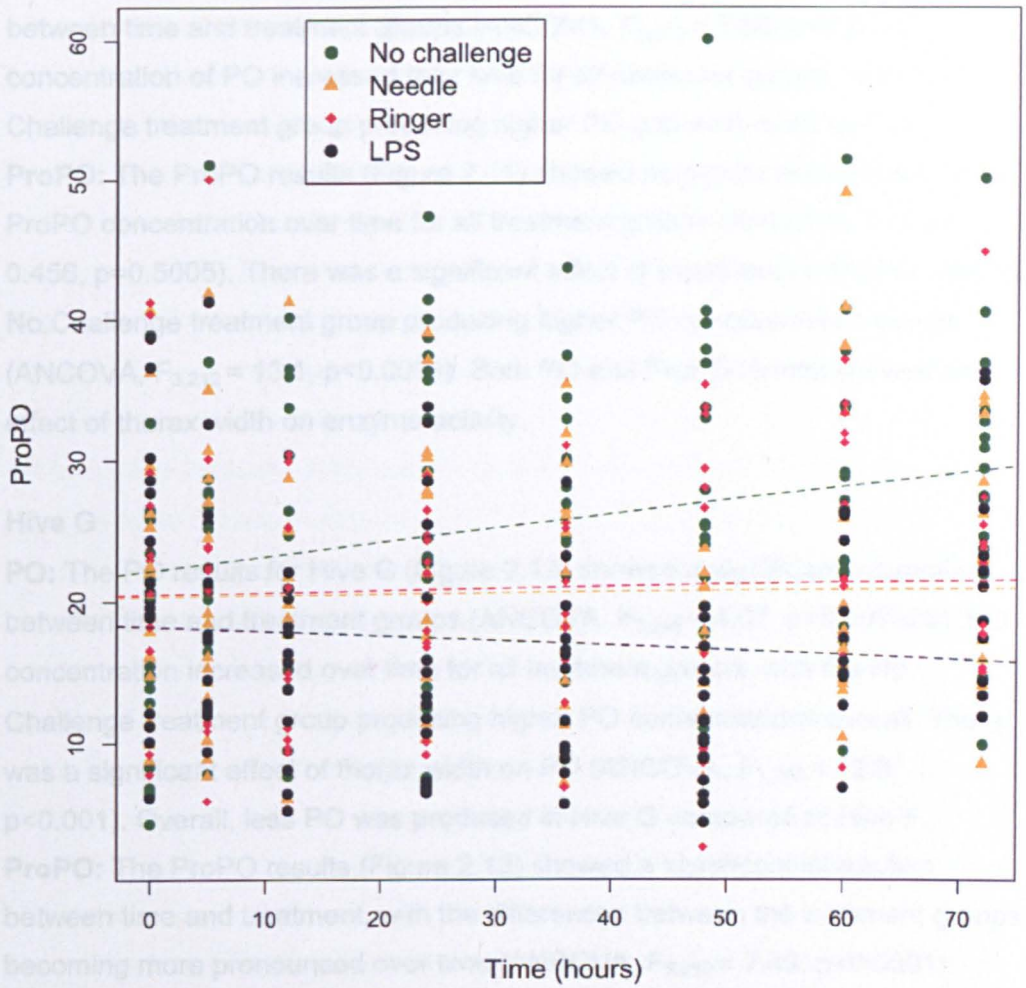


Figure 2.9. Change in ProPO concentration over the first 72 hours post-eclosion. Samples were split into 4 treatment groups: No Challenge, Needle, Ringer and LPS challenged groups. Results shown are combined for 2 hives. There was a significant interaction between time and treatment, with the No Challenge group increasing in ProPO production over time whilst the challenged groups all fell.

2.5.3.2 Hives Separated

Data were split into the two Hive groups: Hive F and Hive G.

Hive F

PO: The PO results for Hive F (Figure 2.10) showed a significant interaction between time and treatment groups (ANCOVA, $F_{3,212} = 3.66$, $p=0.01333$). The concentration of PO increased over time for all treatment groups, with the No Challenge treatment group producing higher PO concentrations overall.

ProPO: The ProPO results (Figure 2.11) showed no significant increase in ProPO concentration over time for all treatment groups (ANCOVA, $F_{1,212} = 0.456$, $p=0.5005$). There was a significant effect of treatment on ProPO, with the No Challenge treatment group producing higher PO concentrations overall (ANCOVA, $F_{3,212} = 13.1$, $p<0.0001$). Both PO and ProPO results showed no effect of thorax width on enzyme activity.

Hive G

PO: The PO results for Hive G (Figure 2.12) showed a significant interaction between time and treatment groups (ANCOVA, $F_{3,256} = 4.07$, $p=0.007529$). PO concentration increased over time for all treatment groups, with the No Challenge treatment group producing higher PO concentrations overall. There was a significant effect of thorax width on PO (ANCOVA, $F_{1,256} = 12.9$, $p<0.001$). Overall, less PO was produced in Hive G compared to Hive F.

ProPO: The ProPO results (Figure 2.13) showed a significant interaction between time and treatment, with the differences between the treatment groups becoming more pronounced over time (ANCOVA, $F_{3,256} = 7.49$, $p<0.0001$). ProPO activity increased over time for all treatment groups, with the No Challenge treatment group producing higher PO concentrations overall. There was a significant effect of thorax width on ProPO (ANCOVA, $F_{1,256} = 6.47$, $p=0.01158$).

Comparing the two hives

Splitting the two hives showed that PO values followed the same pattern for both hives. Quantitatively, Hive G had a lower PO levels than Hive F (ANCOVA, $F_{1,476} = 190$, $p<0.0001$), but the main effects and interactions were qualitatively the same, with the No Challenge treatment group having a higher concentration of PO than any of the other treatment groups.

The ProPO assay for the two hives also produced the same qualitative result for both hives, with the No Challenge treatment group having the highest concentration of ProPO. Hive F showed no effect of time on ProPO, whilst Hive G had a significant interaction of time with treatment groups. Again, Hive G overall had lower ProPO concentrations than Hive F (ANCOVA, $F_{1,476} = 293$, $p<0.0001$). The lower levels of ProPO stored in Hive G bees may predispose them to an increased sensitivity to immune challenges.

The pattern of responses seen in the PO and ProPO assays, with PO activity increasing with age, whilst ProPO activity increases at a lower rate or stays constant, is seen in subsequent assays with workers and drones (Sections 3.3.4 & 4.3.2). In the PO assay, the level of response of PO activity following an immune challenge is similar in all challenged treatment groups, including the control challenges. This suggests that PO is used up immediately following any immune challenge. PO activity increases with age, which may be a response to pathogen exposure, or the physiological process of aging. The maximum capacity of haemolymph for active PO unknown, but the fact that it continues to rise with worker age suggests that the levels stored in "resting" bees never reaches its maximum limit. The pattern of activity in ProPO shows a significant staggered response in the reaction to different treatment groups, which is maintained across time. It may be that ProPO activity is maintained at a maximum for a given set of ecological constraints (e.g. immune challenge), or that ProPO is being used up at a constant rate relative to the level of immune challenge.

Most importantly, at 24 hours post challenge, Hive F and Hive G show the same pattern of results for both the PO and ProPO assay, and there is a clear difference in response between the four treatment groups. This indicates that PO and ProPO both have a role in the immune response at 24 hours post-eclosion, and consequently this sampling time will be used when collecting haemolymph from new bees in all future assays.

Analysis of Hive G showed a significant effect of thorax width, with larger bees having higher levels of PO and ProPO. We expect larger bees to contain a greater volume of haemolymph, and given the method of collection (i.e. a perfusion bleed), this will be seen as an increase in haemolymph concentration per unit bleed. By controlling for body size we take this into account.

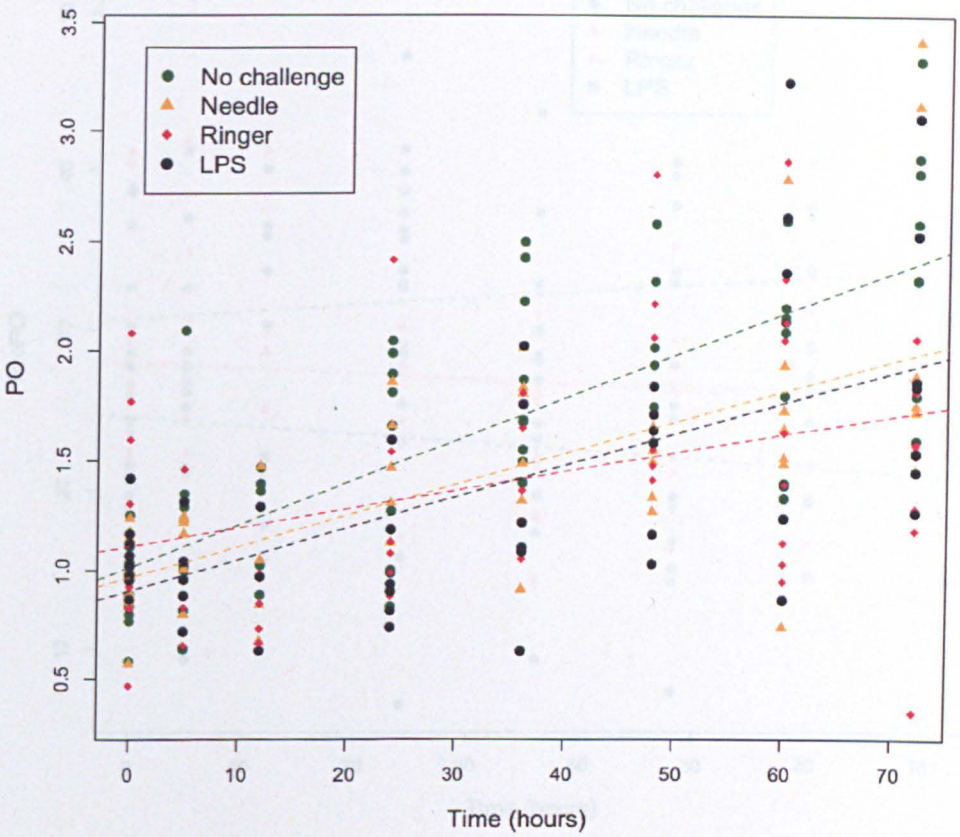


Figure 2.10. Change in PO over first 72 hours post eclosion for Hive F. All treatment groups showed an increase in PO concentration over time. There was a significant interaction between time and treatment.

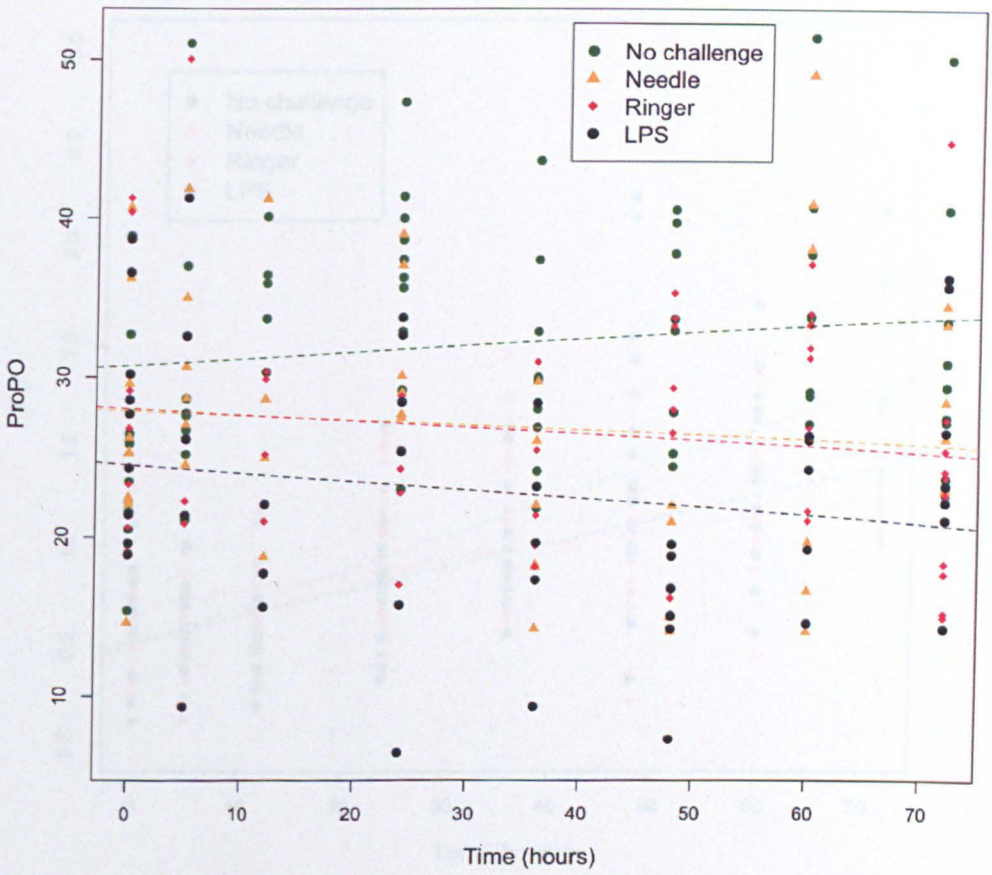


Figure 2.11. Change in ProPO over first 72 hours post eclosion for Hive F. There was no significant increase in ProPO concentration over time for any of the treatment groups, but there was a significant effect of treatment on ProPO, with the No Challenge treatment group producing higher PO concentrations overall.

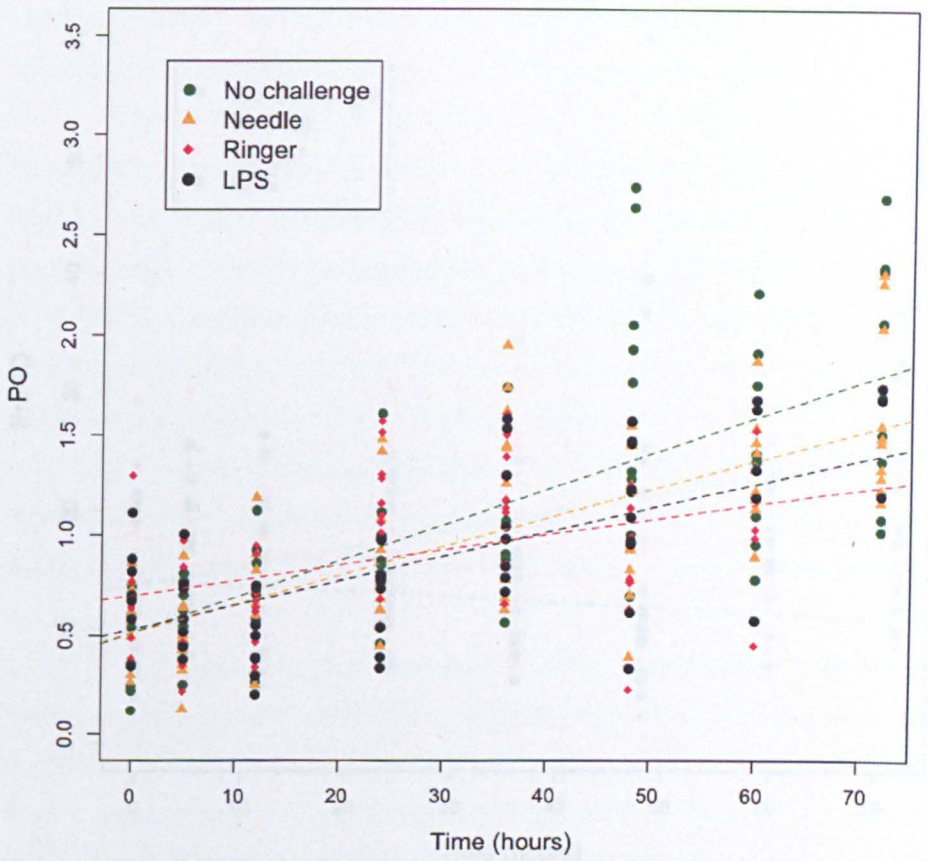


Figure 2.12. Change in PO over first 72 hours post eclosion for Hive G. All treatment groups showed an increase in PO concentration over time. There was a significant interaction between time and treatment groups.

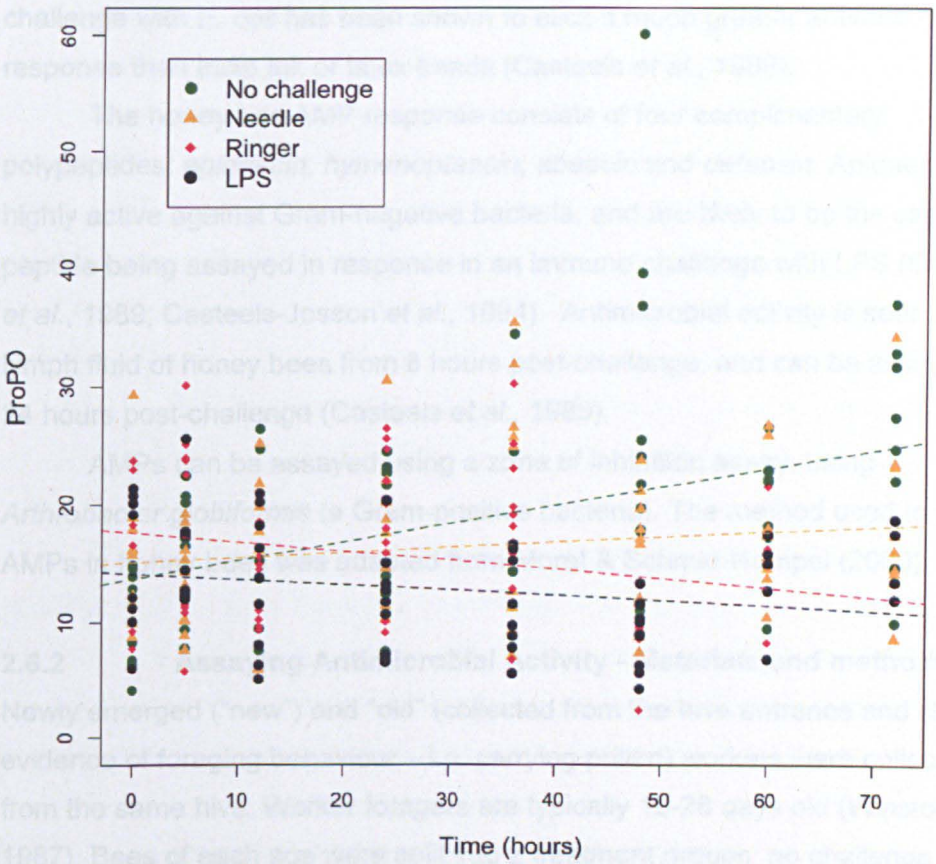


Figure 2.13. Change in ProPO over first 72 hours post eclosion for Hive G. There was a significant interaction of time and treatment on ProPO concentration.

2.6 Assaying antimicrobial activity

2.6.1 Assaying Antimicrobial Activity - Introduction

In honey bees, the up-regulation of antimicrobial peptides (AMPs) is seen in response to wound infections and injection of bacterial cells into the haemocoel, with increasingly severe immune challenges inducing higher levels of up-regulation of antimicrobial production (Casteels *et al.*, 1989). An immune challenge with *E. coli* has been shown to elicit a much greater antimicrobial response than India ink or latex beads (Casteels *et al.*, 1989).

The honey bee AMP response consists of four complimentary polypeptides: *apidaecin*, *hymenoptaecin*, *abaecin* and *defensin*. Apidaecins are highly active against Gram-negative bacteria, and are likely to be the candidate peptide being assayed in response in an immune challenge with LPS (Casteels *et al.*, 1989; Casteels-Josson *et al.*, 1994). Antimicrobial activity is seen in the lymph fluid of honey bees from 8 hours post-challenge, and can be assayed at 24 hours post-challenge (Casteels *et al.*, 1989).

AMPs can be assayed using a zone of inhibition assay, using *Arthrobacter globiformis* (a Gram-positive bacteria). The method used to assay AMPs in honey bees was adapted from Moret & Schmid-Hempel (2000).

2.6.2 Assaying Antimicrobial Activity - Materials and methods

Newly emerged ("new") and "old" (collected from the hive entrance and showing evidence of foraging behaviour – i.e. carrying pollen) workers were collected from the same hive. Worker foragers are typically 18-28 days old (Winston, 1987). Bees of each age were split into 2 treatment groups: no challenge, or immune challenged with 0.5µl LPS in Ringer (0.5 $mgml^{-1}$). Bees were incubated overnight as standard. Haemolymph was collected 24 hours post challenge from the bees, which were anaesthetised on ice. The original assay, using the bumble bee, *Bombus terrestris*, collected haemolymph by homogenising the thorax with Ringer solution. Honey bees have a much lower haemolymph volume than bumble bees, and it was not known if a perfusion bleed (i.e. a diluted sample) would produce measurable levels of AMPs. Therefore, for each age and treatment grouping, half of the bees had haemolymph collected via a perfusion bleed with 0.5ml sodium cacodylate, and half was collected as a neat haemolymph sample. Neat haemolymph was collected from the stump of a pair of legs, removed using fine scissors. A pre-chilled glass capillary tube that was

pulled and ground into a point fine enough to enter the wound was used to collect the haemolymph. A maximum volume of 3µl can be obtained this way. In all cases, haemolymph samples were immediately frozen for 24 hours (–90°C). A total of 12 new and 12 old bees were used to test this assay; each age:treatment:bleed combination contained three bees.

The method for assaying antimicrobial activity in bees was adapted from the original assay by Moret & Schmid-Hempel (2000, Figure 2.14). A single *A. globiformis* colony was selected from a plate of colonies and grown in 10ml LB (Luria-Bertani) broth overnight (30 °C, automatic shaker). 50ml of 1% agar solution was autoclaved and placed in a water bath at 44 °C. When the solution had cooled to 44 °C, 100µl of *A.globiformis* broth was added, and the solution swirled to mix. 5ml of the resulting solution was pipetted into Petri dishes within a laminar flow cabinet. The solution was left to solidify for 30 minutes. Petri dishes can be stored in a fridge (4 °C) until needed. Just before use, wells were punched into the set agar using a sterile glass Pasteur pipette (area = $2mm^2$). A paper template placed underneath the Petri dish allowed well position and contents to be recorded.

1µl samples of the haemolymph collected from the bees was carefully pipetted into the wells, ensuring no overspill. Petri dishes were incubated for 48 hours at 30°C. After incubation, a uniform lawn of bacterial growth could be seen, with clear zones plainly visible where inhibition of the bacteria by the haemolymph had occurred. The area was photographed and measured.

2.6.3 Assaying Antimicrobial Activity - Results

There was a significant 3-way interaction between age, bleed type and immune challenge on antimicrobial peptide production (ANOVA, $F_{1,16} = 41.4$, $p=<0.001$, Figure 2.15, Table 2.1). When using a neat bleed method, antimicrobial activity was recorded in old bees both with and without an immune challenge, but antimicrobial peptides were only measurable in new bees following an immune challenge. The perfuse bleed method only produced recordable results for the immune challenged old bees.

2.6.4 Assaying Antimicrobial Activity: Repeatability

As collecting haemolymph samples was destructive, whether collected as neat haemolymph or by a perfusion bleed, individuals could not be multiply sampled

to test repeatability. Repeatability of the method used to measure the area of bacterial inhibition was tested using the samples from the previous experiment. The diameter of each clear zone was measured 3 times to get a mean diameter, from which the area of the inhibition zone can be calculated. This was repeated three times for each zone. Repeatability of the measurement method was calculated using the formula described by Lessels & Boag (1987).

2.6.5 Assaying Antimicrobial Activity: Repeatability - Results

The method of measuring the antimicrobial assay was highly repeatable with 99.9% of the total variation between repeated measures was made up of the variation among individuals (ANOVA, $F_{10,22} = 1167$, $p<0.001$, $r=0.999$).

2.6.6 Assaying Antimicrobial Activity: Standard AMP Protocol

Following these results, the standard protocol for assaying AMP used throughout this thesis will be as follows:

- When relevant, bees will be subjected to the experimental immune challenge protocol: no challenge, wounding control (sham needle injection), vehicle control (injection with 1µl insect Ringer (128mM NaCl, 18mM $CaCl_2$, 1.3mM KCl, 2.3mM $NaHCO_3$, 1l dH_2O, pH 7.2) and the full bacterial challenge (injection with 1µl lipopolysaccharide (LPS: 0.5mgml^{-1} in insect Ringer)
- 24 hours post challenge, bees will be cold anaesthetised and neat haemolymph collected from a leg stump
- Haemolymph samples will be immediately frozen for 24 hours (–90°C)
- A single *Arthrobacter globiformis* colony will be selected and grown in 10ml LB (Luria-Bertani) broth overnight (30 °C, automatic shaker)
- 50ml of 1% agar solution will be autoclaved and placed in a water bath at 44 °C. When the solution has cooled to 44 °C, 100µl of *A.globiformis* broth will be added, and the solution swirled to mix
- 5ml of the resulting solution will be pipetted into Petri dishes within a laminar flow cabinet and left to solidify for 30 minutes
- Just before use, wells will be punched into the set agar using a sterile glass Pasteur pipette (area = 2mm^2)
- Haemolymph samples will be defrosted on ice and 1µl samples pipetted into the wells

- Petri dishes will be incubated for 48 hours at 30°C
- Clear zones of inhibition will be photographed and measured for area (QCapture Pro 51)

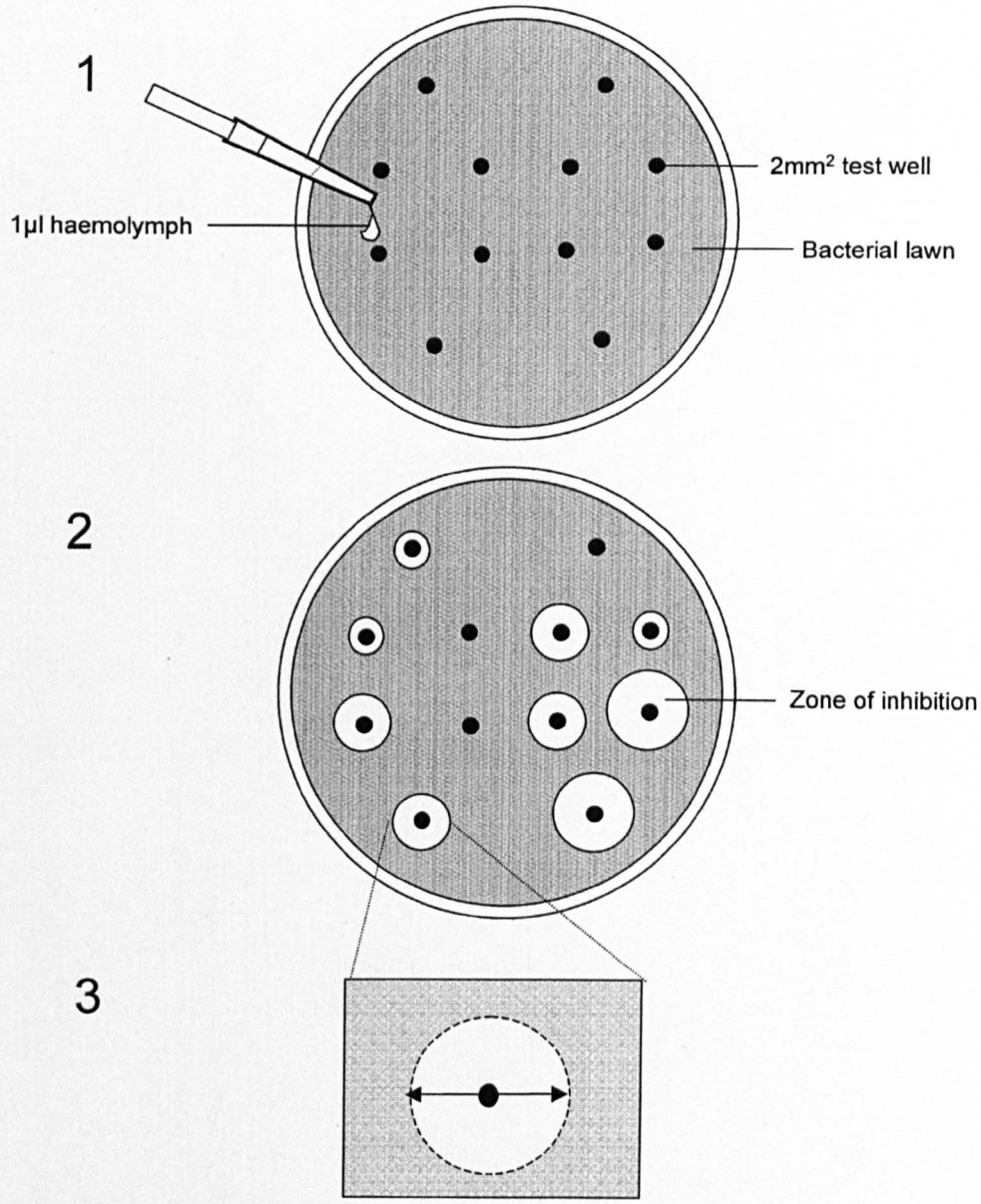


Figure 2.14. Method for antimicrobial assay. A petri dish containing agar colonised with a lawn of *Arthrobacter globiformis* has 12 x 2mm^2 wells punched in it. 1µl of haemolymph sample is added to each well (1). Plates are incubated for 48 hours, after which clear zones can be seen caused by the inhibitory effect of antimicrobial activity on the *A. globiformis* (2). Clear zones are photographed and the area calculated (3).

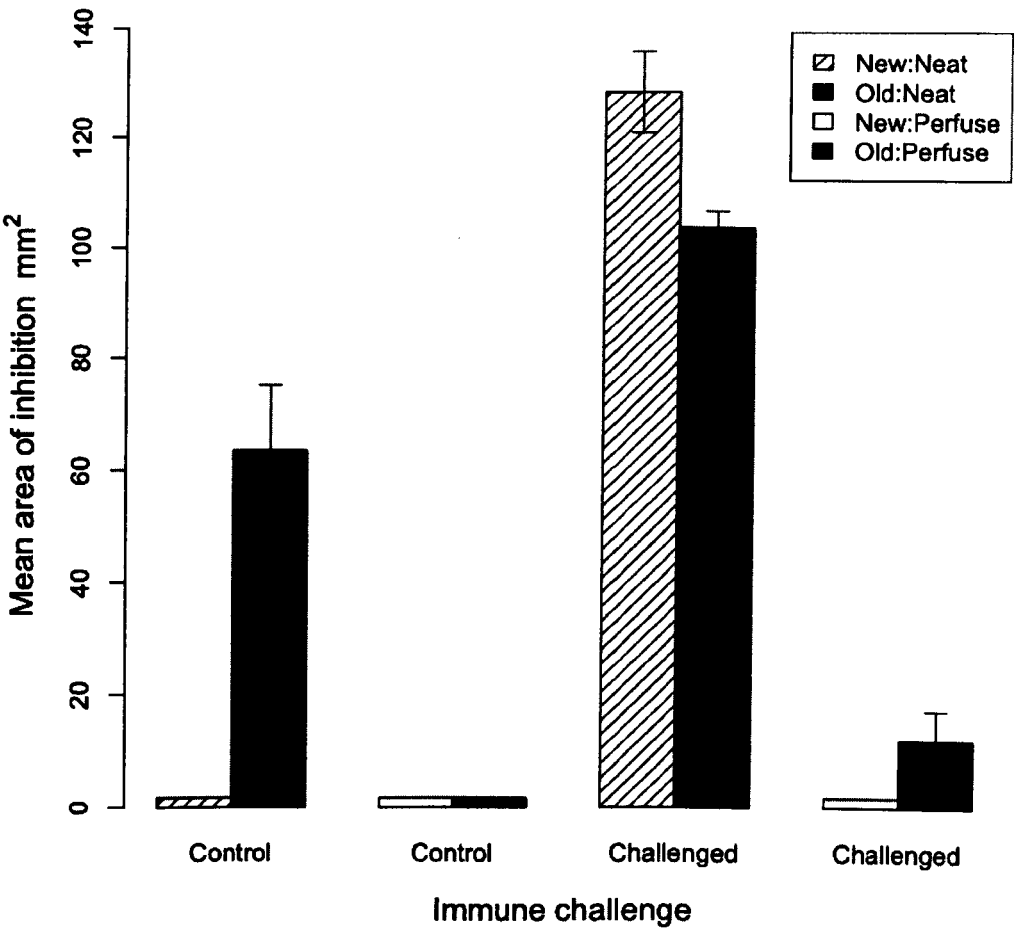


Figure 2.15. Antimicrobial Assay Test. Figure shows the results for control (no challenge) and challenged (LPS injected) bees, split by bleed type (neat or perfuse) and age (new and old). There was a significant 3-way interaction between age, bleed type and challenge on antimicrobial peptide production. When using a neat bleed method, antimicrobial activity was recorded in foragers both with and without an immune challenge. Antimicrobial peptides were only measurable in new bees following an immune challenge. The perfuse bleed method only produced recordable results for the immune challenged old bees.

Source	d.f.	Sum of squares	F-value	P
Age	1	834	9.80	0.006445
Treatment	1	11900	139	<0.0001
Bleeding method	1	29600	346	<0.0001
Age x treatment	1	2190	25.5	<0.0001
Age x bleed	1	264	3.08	0.09862
Treatment x bleed	1	9290	108	<0.0001
Age x treatment x bleed	1	3540	41.4	<0.0001
Residuals	16	1370		

Table 2.1. ANOVA results for the effect of age, immune challenge and haemolymph collection method on antimicrobial peptide assay.

2.7 Discussion

This chapter has focussed on three main areas: firstly the beekeeping techniques and methods used in the lab, second the optimising of the PO and ProPO assays for use with the honey bee and thirdly, optimising the use of an antimicrobial assay.

2.7.1 Bee Specificity

The optimisation of the PO and ProPO assay to be bee-specific highlighted some species-specific responses. The use of α-chymotrypsin as the most successful activator of ProPO in bees contrasts with the results of Thompson (2002) in *T. molitor* where LPS was a stronger activator. α-chymotrypsin is a general protease, and as such is capable of causing damage if not controlled. Higher levels of α-chymotrypsin were shown to have a negative effect on ProPO activation, and highlighted the sensitivity of the assay to changes in α-chymotrypsin concentration. High concentrations of LPS and α-chymotrypsin, when used as activators, have also been shown to have a negative effect on ProPO activation in *T. molitor* (Thompson, 2002).

Further research into the effect of the activators has shown that long incubation times with the activator, as used by many previous researchers (e.g. Schmid *et al.*, 2008), can dramatically reduce the concentration of ProPO available for assay. Consequently an incubation time of 5 minutes was chosen for all subsequent ProPO assays in this thesis. Longer incubation times allow more of the PO (activated from the ProPO) to be degraded by the PPAE, or removed by combining into larger protein aggregates, both regulatory mechanisms to avoid the deleterious autoimmune effects of high concentrations of active PO (Nappi *et al.*, 1995; Nappi *et al.*, 2004; K. Söderhäll, pers. comm.). Although samples assayed with long incubation times within an experiment will be comparable with each other, it is difficult to say exactly what the assay is measuring, as it is neither the maximum immune investment (i.e. all ProPO and all PO), nor just the PO or ProPO responses.

2.7.2 Determining sampling time in new bees

Results for the timecourse showed an increase in PO concentration with age, with a significant difference in the PO responses of the different treatment groups. This difference became more pronounced with time: up to 12 hours there was little difference between the responses of the treatment groups. This suggests that during the first 12 hours post-eclosion, PO is being utilised in the sclerotisation of the cuticle and is therefore not available for immune response.

Overall, ProPO concentrations within each treatment group remained constant throughout the timecourse, although there was a significant difference in the responses between the treatment groups. The contrast between the PO and ProPO results, with PO activity increasing over time whilst ProPO activity remained relatively constant, were seen in subsequent experiments (Sections 3.3.4 and 4.3.2, see Section 2.5.3.2 for discussion). Results from the timecourse showed a decrease in PO and ProPO activity levels following an immune challenge with LPS. Previous studies have shown the opposite response, with active PO levels increasing as ProPO is activated during the immune response (Ashida and Yamazaki, 1990). However, the response seen with the honey bees has recently been replicated in the African armyworm, *Spodoptera exempta*, and damselfly larvae, *Ischnura elegans* (S. Povey, S. Slos, pers. comm.). The lower levels of PO and ProPO activity seen following an immune challenge may be due to the rapid utilisation of PO in the immune response, which in turn leads to the activation of ProPO to replace the active PO. The reduction in levels may therefore be due to the inability of the haemocytes to replenish ProPO at the same rate as it is being used.

The experiment highlighted a difference in the immune responses of different hives. Whilst Hive F recorded higher concentrations of PO, and Hive G showed a change in ProPO with time, the overall immune responses of the four treatment groups were qualitatively similar. Previous research on honey bee immunity differs in the number of colonies included within an experiment, although there are obvious differences in levels of parasite infection and immunity between colonies (e.g. Yang & Cox-Foster, 2007; Wilson-Rich *et al.*, 2008; Chen *et al.*, 2004a; Evans & Pettis, 2005; Decanini *et al.*, 2007). Future experiments in this thesis will compare the immune responses in two hives as a check that immune responses measured are consistent and repeated in more than one hive.

2.7.3 AMP Assay in Bees

Antimicrobial peptide (zone of inhibition) assay can be successfully used to measure antimicrobial peptide production in honey bees. Haemolymph samples collected using a perfusion bleed showed little antimicrobial response, and this is undoubtedly due to the dilution of the active compounds and hence the subsequent lack of sensitivity of the assay. Future assays will therefore be conducted using neat haemolymph samples. Both an immune challenge with LPS and increasing age produced an increase in antimicrobial peptide production, and this will be fully explored in later chapters. These results, combined with the low error in measuring the zones of inhibition suggest that this assay can successfully be added to the arsenal of techniques for measuring immune responses in the honey bee.

2.8 Summary

In this chapter, I have:

1. Described the honey bee husbandry methods and protocols used to maintain and challenge bees in this thesis
2. Developed and optimised an assay for measuring phenoloxidase in the honey bee and have:
    a. Shown that honey bee phenoloxidase enzyme kinetics conform to the Michaelis-Menton equation
    b. Obtained a value for the Michaelis constant, Km
    c. Calculated the optimal workable concentration of L-DOPA used in the assays
3. Developed and optimised an assay for measuring prophenoloxidase in the honey bee
4. Developed and optimised an antimicrobial peptide assay in the honey bee
5. Shown all assays to be highly repeatable

Chapter 3

Ontogeny of worker immunity

3.1 Introduction

3.1.1 Developmental Immunity

Relatively few studies have followed the changes that occur in invertebrate immune systems throughout development and metamorphosis (Schmid *et al.*, 2008; Wilson-Rich *et al.*, 2008). The expression of immunity-related genes is known to differ both between and within developmental stages, but little is known about the impact of this variation on immune responses (Evans, 2004; Chan *et al.*, 2006). Cellular and antimicrobial responses have been shown to vary with developmental stage in several different insects (Natori *et al.*, 1999; Chung & Ourth, 2000; Evans & Lopez, 2004; Meylaers *et al.*, 2007; Freitak *et al.*, 2007; Dubovskiy *et al.*, 2008; Elefthianos *et al.*, 2008; Wilson-Rich *et al.*, 2008), with small changes in age resulting in significant changes in immune response, which are variable across different study species (e.g. Eleftherianos *et al.*, 2008; Wilson-Rich *et al.*, 2008).

Honey bees are holometabolous with a developmental period of 21 days for workers (see Figure 3.1, Hooper, 1997). Larvae are fed by nurse bees, which produce brood food from their hypopharyngeal and mandibular glands and feed the larvae until the cell is sealed with a wax cap (Winston, 1987). Brood food contains honey, which has been shown to have antibiotic properties, and may offer some bacterial resistance to the larvae (Winston, 1987; Lusby *et al.*, 2005; Maeda *et al.*, 2008). Brood is kept at the centre of the hive, and this structural organisation provides a prophylactic defence barrier, minimising forager's (potentially carrying pathogens) contact with brood (Schmid-Hempel, 1998; Cremer *et al.*, 2007).

In addition, the hygienic behaviour of adult bees towards the larvae, and physiological processes that occur during development, make it hard for a pathogen to become established (Bailey & Ball, 1991; Schmid-Hempel, 1998; Spivak & Boecking, 2001; Cremer *et al.*, 2007). Once the cell is capped, larvae form a propupa and stop feeding. The capped cell represents a protective

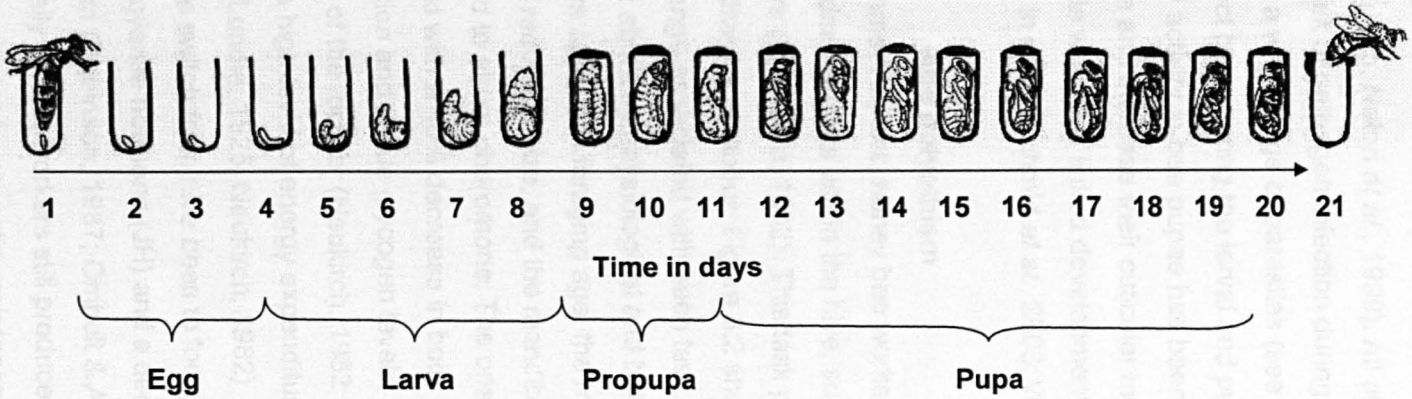


Figure 3.1. Developmental stages of the worker honey bee (taken from Winston, 1987). Development from egg to imago takes approximately 21 days. Pupal stages can be distinguished by their eye colour: day 12-14 = white-eyed pupae, day 15-20 = red eyed pupae (red stage may be further split into pink-eyed (days 15-17) and purple-eyed (days 18-20)). Older red-eyed stages are accompanied by an increase in cuticular melanisation (Hooper, 1997; Michelette & Soares, 1993).

environment in which to undergo metamorphosis (see Chapter 5 for exceptions), during which time the high levels of proteases needed for the degradation of larval tissue present a hostile environment for invading pathogens (e.g. Natori *et al.*, 1999). All of these factors contribute to the reduced risk of pathogen infection during development. Despite this, bees are subject to a wide range of parasites (see Section 1.4.3, Table 1.1), many of which infect bees during the larval and pupal stages of development.

PO activity in bee pupae has been shown to increase successively as pupae age and increase their cuticular melanisation (Zufelato *et al.*, 2004), but to date little work has linked developmental stages and response to immune challenge in bees (Schmid *et al.*, 2008; Wilson-Rich *et al.*, 2008).

3.1.2 Age polyethism

In the summer months, honey bee workers exhibit age polyethism, with younger bees performing tasks within the hive, such as nursing, and older bees working as foragers (Robinson, 1992). This task partitioning represents a temporary, dynamic division of labour. Figure 3.2 shows the different tasks performed, and the age range associated with each task. The transition from hive bee to forager is brought about by physiological and behavioural changes (Robinson, 1992). As workers approach foraging age, the mandibular, hypopharyngeal and wax glands all reduce in size, and the mandibular glands switch production from brood food to alarm pheromone. The onset of foraging activity is also associated with a 40% decrease in body mass and an increase in oxygen consumption and tissue glycogen levels, all of which help to increase the efficiency of the forager (Neukirch, 1982; Winston, 1987). Foraging activity involves a high level of energy expenditure, and 98% of all bees die whilst foraging (Lundie, 1925; Neukirch, 1982).

The switch from hive bees to foragers is associated with an increase in levels of juvenile hormone (JH) and a decrease in the yolk precursor vitellogenin (Robinson, 1987; Omholt & Amdam, 2004). Despite being conditionally sterile, workers still produce vitellogenin, which has an additional role as a regulator for somatic maintenance in foragers (Omholt & Amdam, 2004). Vitellogenin is the main carrier protein for zinc in the haemolymph, a lack of which causes pycnosis (cell death) of the haemocytes (Falchuk, 1998; Amdam *et al.*, 2004, Amdam *et al.*, 2005). This decrease in functioning

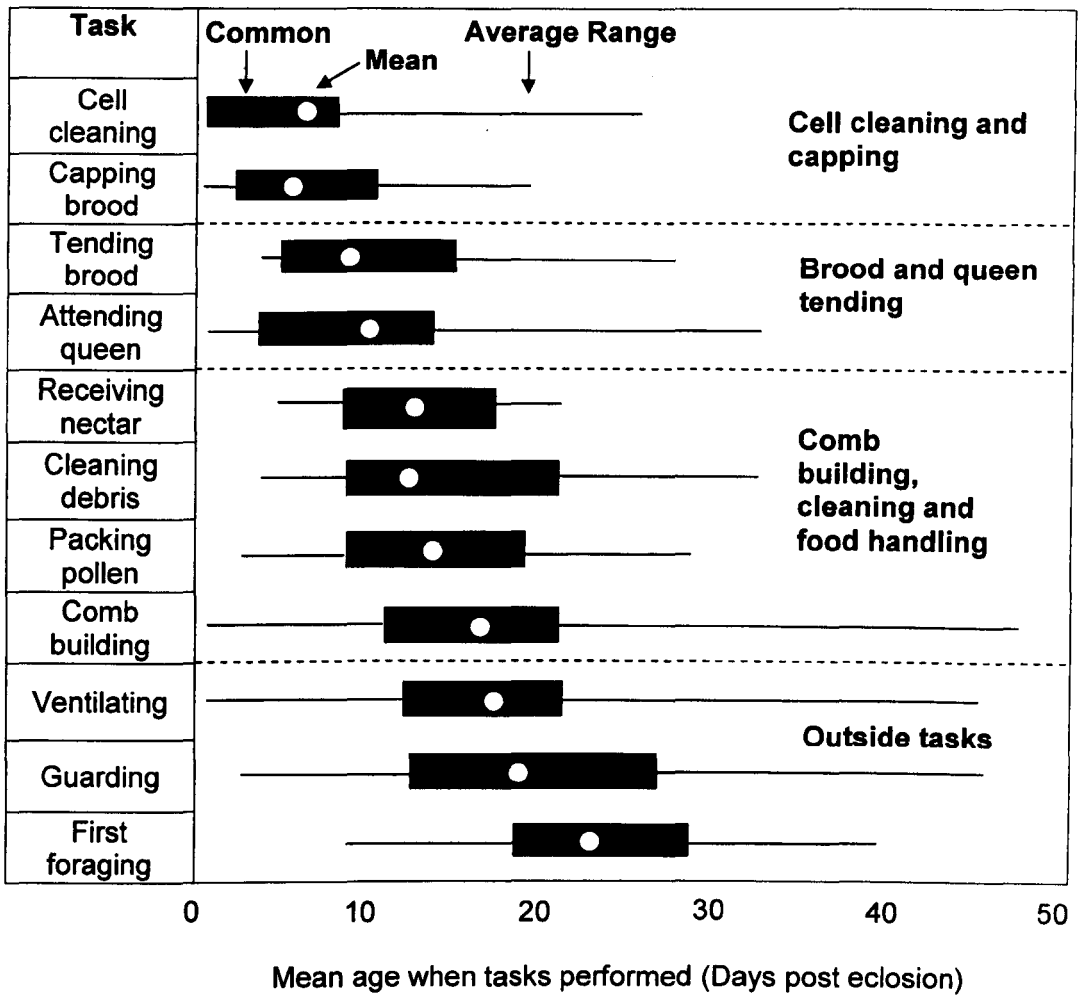


Figure 3.2. Overlap of age-related tasks performed by workers. Table taken from Winston, 1987. There is considerable variation in the ages at which these tasks are performed, and a worker may carry out several different tasks at the same time.

haemocyte number should reduce the bee's capacity for cellular-based immune responses such as PO, encapsulation and phagocytosis (Bedick *et al.*, 2001; Amdam *et al.*, 2004; Robinson, 1992; Omholt & Amdam, 2004; Amdam *et al.*, 2005). Support for this is seen by the loss of a nodulation response (a cellular immune response) in foragers compared to newly emerged bees (Bedick *et al.*, 2001), but other immune responses have yet to be investigated.

Commonly, aging in both vertebrates and invertebrates is associated with increased activation of the innate immune system (DeVeale *et al.*, 2004; Zerofsky *et al.*, 2005), but the maintenance of an immune system throughout life is costly (e.g. Kaaya & Darji, 1988; Zerofsky *et al.*, 2005; König & Schmid-Hempel, 1995; Moret & Schmid-Hempel, 2000). Foraging bees leaving the hive experience a higher risk of pathogen infection, and may therefore favour an increased level of immune investment (Bocher *et al.*, 2007). Conversely, the process of age polyethism selects nutrient depleted foragers to undertake the most hazardous tasks towards the end of their life, and therefore the need and ability to invest in an immune response may be removed. It is therefore unclear whether newly emerged bees or foragers should have the higher immune system.

3.1.3 Chapter Predictions

Based on this information, we can make predictions about the expected outcome of the investigation into ontogeny and the immune response. I predict that:

1. Larvae, which are nursed in a protective environment will exhibit little or no investment in the immune response
2. During the physiological changes that occur throughout the transition into the pupal stage, and subsequent melanisation of the cuticle, PO levels will increase, but not as a function of the immune response
3. There will be an effect of age polyethism on immune investment but the direction of this change is unclear.

3.1.4 Chapter Aims

This chapter aims to investigate the impact of different aspects of aging on immunity in worker bees. Using assays developed in Chapter 2, I examine:

1. The impact of immune challenge on bee survival (Section 3.2.1)

2. Developmental changes in a) PO and b) ProPO responses (Section 3.2.2)
3. The effects of age polyethism on measures of a) PO, b) ProPO and c) antimicrobial peptides (Sections 3.2.3, 3.2.4 & 3.2.5).

3.2 Materials and Methods

3.2.1 Bee longevity

Bee longevity in the lab was examined to ensure that lab conditions did not significantly reduce mortality. When kept in hives, the life expectancy of a worker bee is approximately 36 days in the summer months (Hooper, 1997). This extends to 6 months for overwintering individuals, although this figure is subject to wide variation both within and between subspecies of bee (Hooper, 1997). Although none of the lab experiments designed during this research required bees to be kept alive in the lab for more than 3 days, it was important to ensure that, as far as possible, the lab-based bee boxes provided a suitable habitat for the bees over this time-frame. In addition, bees were divided into treatment groups to monitor the effect of immune challenge on longevity.

A brood frame was collected from a single hive and incubated overnight to allow adults to emerge. Bees were split into 4 treatment groups: no challenge (n=42), sham injected (n=56), injected with 1μl insect Ringer (n=88), injected with 1μl LPS (0.5mgml^{-1}) in insect Ringer (n=99). Each treatment group was housed in a bee box (36^{0}C ± 1^{0}C with a 12:12h L:D photoperiod) and provided with a 50% sugar solution and dH_2O *ad libitum*. Bees were checked daily for deaths, and dead bees were removed and recorded.

3.2.2 Developmental Immunity

Brood frames were collected from two hives (Hives A & B). Frames contained brood at different stages of development. Bees were assayed at five different developmental stages: 4th instar larvae (uncapped), 5th instar larvae (capped), white-eyed pupae (white-eyed), red-eye pupae (red-eyed) and newly emerged adults (adult). Preliminary experiments found that no PO or ProPO could be measured in early instar larvae (pers.obs.). Bees were assessed on their developmental stage based on size, positioning within the cell and, in the case of pupae, eye and thorax colouring (Hooper, 1997; Michelette & Soares, 1993).

Bees in each developmental stage were removed from the brood cells. Care was taken not to damage the fragile larval and pupal stages. Individuals that sustained damage (seen as a dark melanin response) were discarded. Individuals were housed in 25-well sectioned boxes. They were laid on moist cotton wool to maintain a high humidity and, in the case of larvae, provided with brood food obtained from brood cells. Adults were incubated in bee boxes as standard. Body mass (wet weight) was recorded as a measure of body size.

Each of the 5 developmental stages was split into 4 treatment groups to measure the effect of an immune challenge on immune response (No Challenge, Needle, 1µl Ringer, 1µl LPS in Ringer, n = 11-79 per treatment:age group). Larvae are essentially a fluid-filled sac, so extra fine needles (110µl diameter) were used to puncture the cuticle without causing loss of haemolymph. Injections were made approximately one third of the way down the right-hand side of the larval body. Bees were incubated for 24 hours (45% humidity ± 5% in incubator, 24 hour dark photoperiod). 24 hours post challenge, bees were assessed for survival. In pupae, this was recorded as a continued development, confirmed by changes in eye and body colour. In uncapped larvae, survival was recorded as any voluntary movement. In capped larvae, individuals that lacked body shape and definition, showed no initiation of head development or had turned an opaque white colour throughout were judged to be dead, and discarded.

Haemolymph samples from surviving bees were collected through perfusion bleeds with 0.25ml sodium cacodylate buffer. A volume of 0.25ml of buffer was used for all bees in order to obtain recordable PO and ProPO levels in the earlier developmental stages. Samples were assayed for PO and ProPO by the standard method (Chapter 2, Sections 2.3.11 & 2.4.14). Larval bees contain a lot of fat, which was unavoidably collected in the haemolymph samples, and the effect of the lipid on the PO and ProPO assays was investigated (Appendix 1, A1.3). Lipids were shown to breakdown during the assay, which resulted in negative enzyme curves. Samples that recorded this breakdown contained no recordable PO or ProPO, and the presence of lipids did not affect this immune result.

After defrosting, all samples were centrifuged twice (4°C, 80,000G, 15 minutes), with as much lipid as possible removed by pipette between cycles. The clear supernatant was then used in the reaction mixture as standard.

3.2.3 Age Polyethism and Phenoloxidase

To investigate the difference in immune responses of hive (new) and forager (old) bees, workers were collected from two hives (Hives A and B) at two distinctly different life-stages. New bees were collected as newly emerged workers from a brood frame incubated overnight (36°C ± 1°C, 40% ± 2% humidity). Old bees were collected as they returned to the hive following a foraging trip. Although not controlled for age, old bees had to exhibit a sign of foraging activity (pollen collected on legs) in order to distinguish them from younger workers making test flights.

To examine how the different ages responded to an immune challenge, each age group was split into 4 treatment groups (No Challenge, Needle, 1µl Ringer and 1µl LPS in Ringer, n=15-44 for each age:treatment group per hive). Treatment groups were housed in separate bee boxes and haemolymph samples collected 24 hours post-challenge. Samples were assayed for PO and ProPO as standard (Chapter 2, Sections 2.3.11 & 2.4.14). Thorax width was recorded for all bees to obtain a control for body size.

3.2.4 Age Polyethism: Timecourse

Bees venture out of the hive on test flights at approximately 5 days old (Hooper, 1997). Therefore, they are likely to be exposed to pathogens outside of the hive before they become foragers. If foragers increase their immune investment in response to the increase in pathogens encountered outside the hive, this up-regulation in immunity should be initiated as soon as they start to leave the hive, regardless of polyethism structure. If an up-regulation in immune investment occurs in response to cumulative pathogen encounters, we would expect to see immunity increased continually with age. If however, the up-regulation of the immune system in foragers occurs as part of the suite of physiological changes associated with the shift in polyethism, then we would expect investment in immune response to remain low in younger hive bees. To establish whether changes in immune response with age are a reaction to increased interaction with pathogens as bees start to venture outside, worker brood frame was collected from two hives (Hives G and H), and incubated overnight (36°C ± 1°C, 40% ± 2% humidity). Newly emerged workers were marked with a dot of acrylic paint on the thorax and released back into their

original hive. Marked bees were recaptured at 1, 2, 3, 4, 5, 6, 7,and 14 days old. None of the bees should have started foraging at this age.

To examine how different ages responded to an immune challenge, each age group was split into 4 treatment groups (No Challenge, Needle, 1µl Ringer and 1µl LPS in Ringer, n = 4 – 20 per treatment group, per hive (low sample size in some groups is due to incubator overheating causing death)). Treatment groups were housed in separate bee boxes with 50% sugar syrup and dH_2O *ad libitum*. Haemolymph samples were collected 24 hours post challenge and assayed for PO and ProPO as standard (Chapter 2, Sections 2.3.11 & 2.4.14). Thorax width was recorded for all bees to obtain a control for body size.

3.2.5 Age Polyethism and Antimicrobial Peptides (AMPs)

Preliminary studies had found an immune challenge and increasing age lead to an increase in AMP production (Section 2.6.3). Bees were sampled from two different hives (Hives F and G). The presence of AMPs in the haemolymph was investigated using the zone of inhibition assay (Chapter 2, Section 2.6.2). Workers were sampled at two different ages, corresponding to the extremes of age polyethism that are observed with honey bees. New bees were sampled at 24 hours old, and old bees were collected as they returned to the hive following a foraging trip. Although not controlled for age, foragers had to exhibit a sign of foraging activity (pollen collected on legs) in order to distinguish them from younger workers making test flights.

To obtain the new bees, frames of worker brood were collected from two hives and incubated over night ($36^0C \pm 1^0C$, 40% ± 2% humidity). Newly emerged workers were collected the following day, as were the old bees, which were collected directly from the hives. To investigate the effect of an immune challenge on the two age groups, the bees in each age group were further split into 4 treatment groups (No Challenge, Needle, 1µl Ringer and 1µl LPS in Ringer, treatment sample sizes ranged from 13-28, average n = 20). Treatment groups were housed in separate bee boxes and neat haemolymph was collected 24 hours post challenge. Samples were assayed for AMP activity as standard (Chapter 2, Section 2.6.6). The thorax width of each bee was measured to control for body size, and the mortality rate for each treatment group was recorded.

3.2.6 Presentation of Results

In all experiments, the No Challenge and LPS treatment groups showed the extremes of the range of immune responses seen. For clarity, graphical data are only presented for these two treatment groups. The Needle and Ringer treatment groups provided wounding and vehicle controls, and showed a staggered response between the two extremes. Graphs with all four treatment groups can be found in Appendix 2, Section A2.1.

3.3 Results

3.3.1 Bee Longevity

Bee longevity was recorded using a binomial score for time to death, and an analysis of deviance run using a GLM with a Gamma family error structure (as the variance is proportional to the square of the means).

There was a significant effect of treatment on bee longevity (Figure 3.3). Survival analysis showed that there is a significant effect of treatment on survival (GLM, $p<0.001$, d.f. =3,281, gamma errors), with the challenge of LPS and Ringer leading to lower survival. This suggests that there is a cost to mounting the immune response.

3.3.2 Developmental Immunity

Both hives produced qualitatively similar results, but were analysed separately due to quantitative differences in the immune responses.

Within both hives, the earlier developmental stages, particularly the larvae, commonly contained no recordable PO (samples recorded negative enzyme curves – see Appendix 1, Section A1.3 for explanation). This resulted in a positive skew in the distribution, and subsequently the dataset could not be transformed to conform to the assumptions of normality. Data were scored categorically as having a positive or zero PO response, and the proportion of each was analysed using a binomial distribution. Figures 3.4 and 3.5 show the full results for the 5 different developmental stages samples for Hives A and B. The proportion of samples recording zero PO concentration was then compared for each age:treatment group combination for Hive A (Figure 3.6) and Hive B (Figure 3.7). Samples containing no recordable PO were then removed from the data sets, and remaining samples were analysed against each other for each hive.

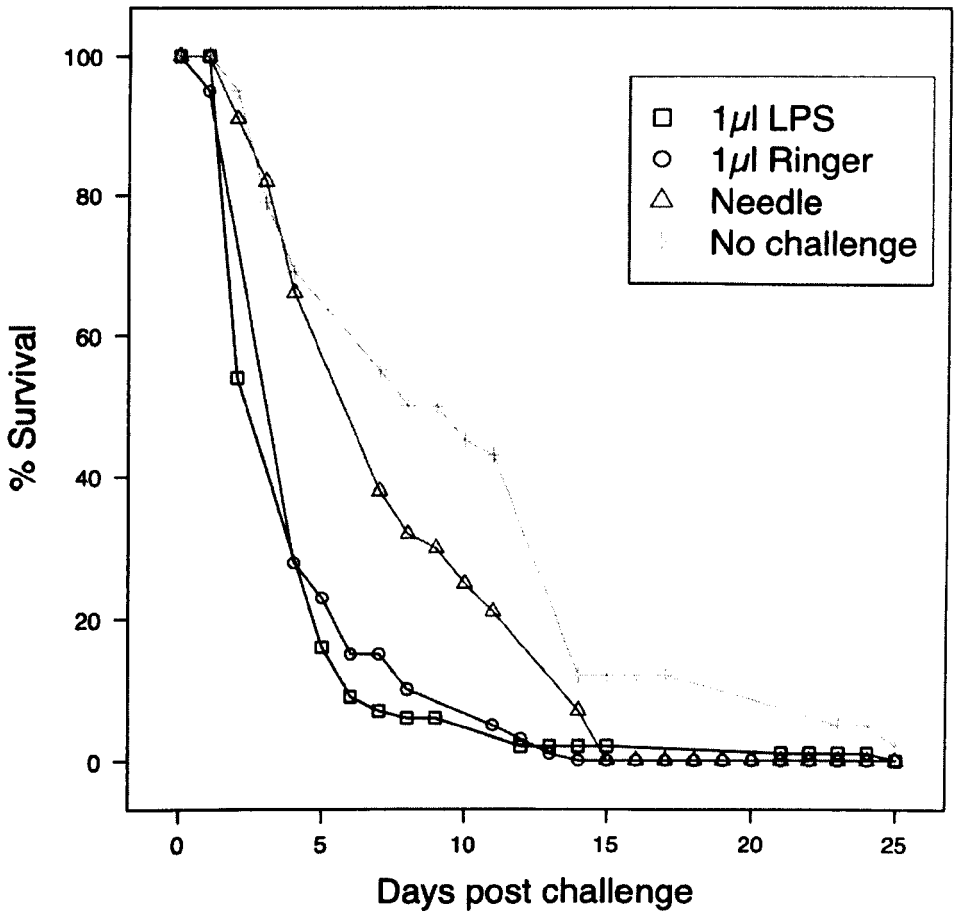


Figure 3.3. Bee longevity showing the percentage survival of worker bees over time post challenge. Four treatment groups were used to measure the effect of an immune challenge on survival (No Challenge, Needle (sham injection), 1μl Ringer and 1μl LPS in Ringer). Survival analysis using a GLM with gamma errors showed that there was a significant effect of treatment on survival, with the challenge of LPS and Ringer leading to lower survival.

Hive A

PO: Figure 3.6 shows the proportion of samples that contained no recordable PO concentrations. There was a significant effect of developmental age on the proportion of samples containing no recordable PO (Figure 3.6, $F_{4,522} = 60.5$, $p<0.0001$, binomial errors), with early developmental stages commonly containing no recordable PO, whilst adults always had a measurable level of PO activity.

Figure 3.8 shows the PO results with those samples that recorded a PO concentration of zero removed. There was a significant effect of body size, age and immune challenge on PO activity (Table 3.1). Challenge with LPS produced lower levels of PO activity, and, where present, larval and pupal stages had higher levels of PO activity than adults.

ProPO: There was a significant effect of body size, age and immune challenge on ProPO activity (Figure 3.10, Table 3.2), with adults containing higher concentrations of ProPO than the larval and pupal stages.

Hive B

PO: Figure 3.7 shows the proportion of samples that contained no recordable PO activity. There was a significant interaction between age and treatment groups (Figure 3.6, $F_{12,625}= 2.08$, $p=0.01519$, binomial errors), with all developmental stages except adults commonly containing at least 60% samples with no recordable PO levels.

Figure 3.9 shows the PO results with all samples recording a PO concentration of zero removed. There was a significant effect of age and body size on PO activity, with adults having lower PO levels compared to the other developmental stages that produced an immune response (Table 3.3, ANOVA, $F_{4,224} = 10.9$, $p<0.0001$, data log10 transformed). There was no effect of treatment group on PO activity.

ProPO: There was a significant effect of body size, age and treatment on ProPO concentration (Figure 3.11, Table 3.4). ProPO activity was maintained at a constant level throughout development, and increased in adults following eclosion. ProPO activity was lower overall in LPS challenged bees.

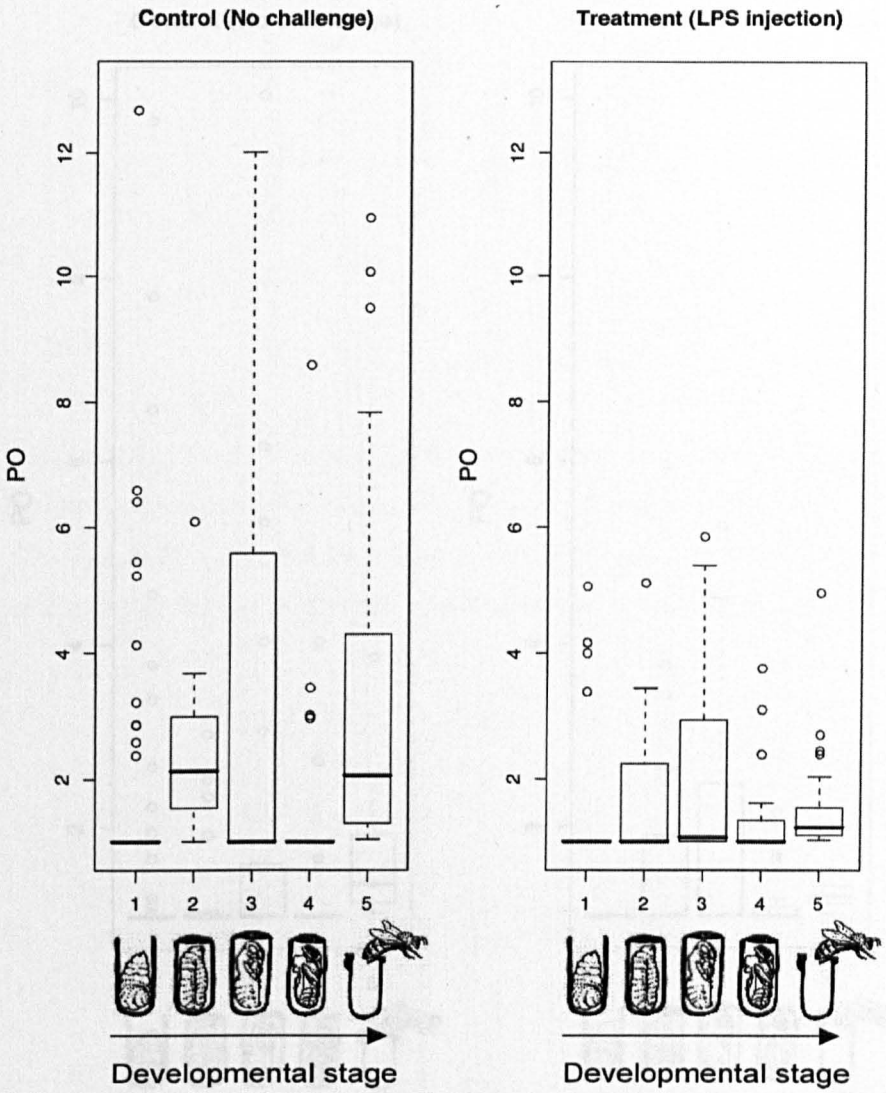


Figure 3.4. Hive A: PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS immune challenge treatment groups. The larval and pupal stages contained a high proportion of individuals with no recordable PO concentration.

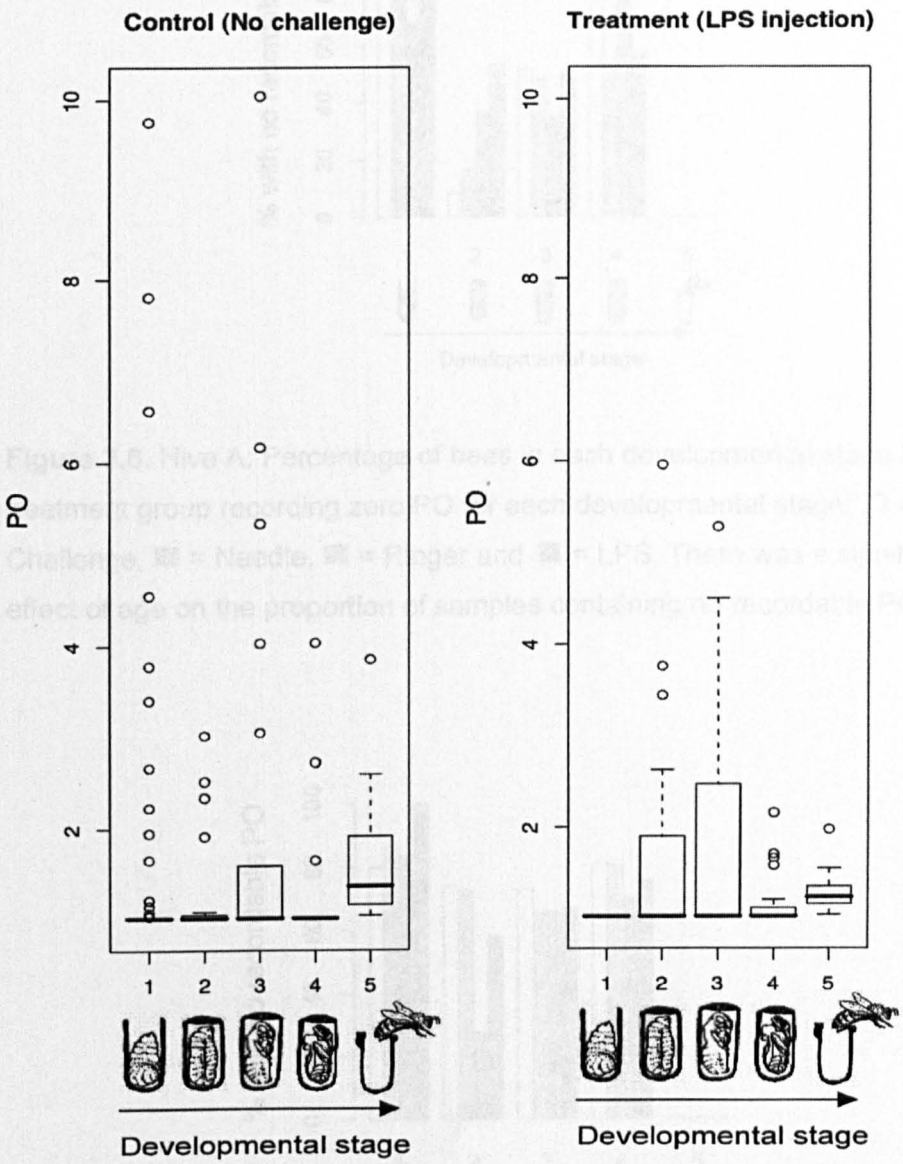


Figure 3.5. Hive B PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS immune challenge treatment groups. The larval and pupal stages contained a high proportion of individuals with no recordable PO concentration.

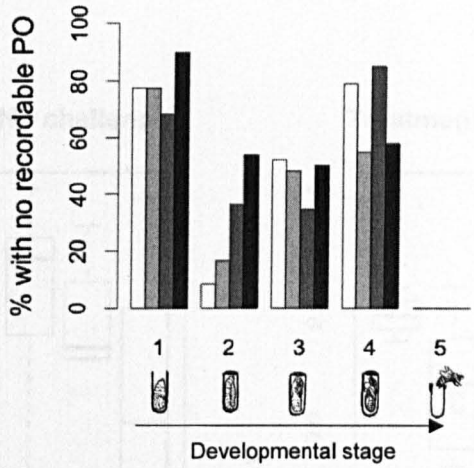


Figure 3.6. Hive A: Percentage of bees in each developmental stage by treatment group recording zero PO for each developmental stage: □ = No Challenge, ▨ = Needle, ■ = Ringer and ■ = LPS. There was a significant effect of age on the proportion of samples containing no recordable PO.

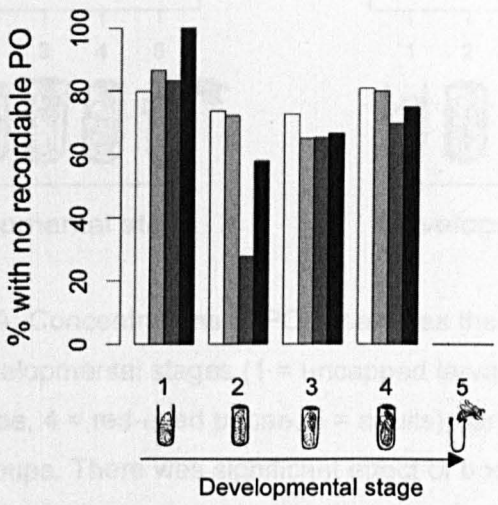


Figure 3.7. Hive B: Percentage of bees in each developmental stage by treatment group recording zero PO for each developmental stage: □ = No Challenge, ▨ = Needle, ■ = Ringer and ■ = LPS. There was a significant interaction between age and treatment group.

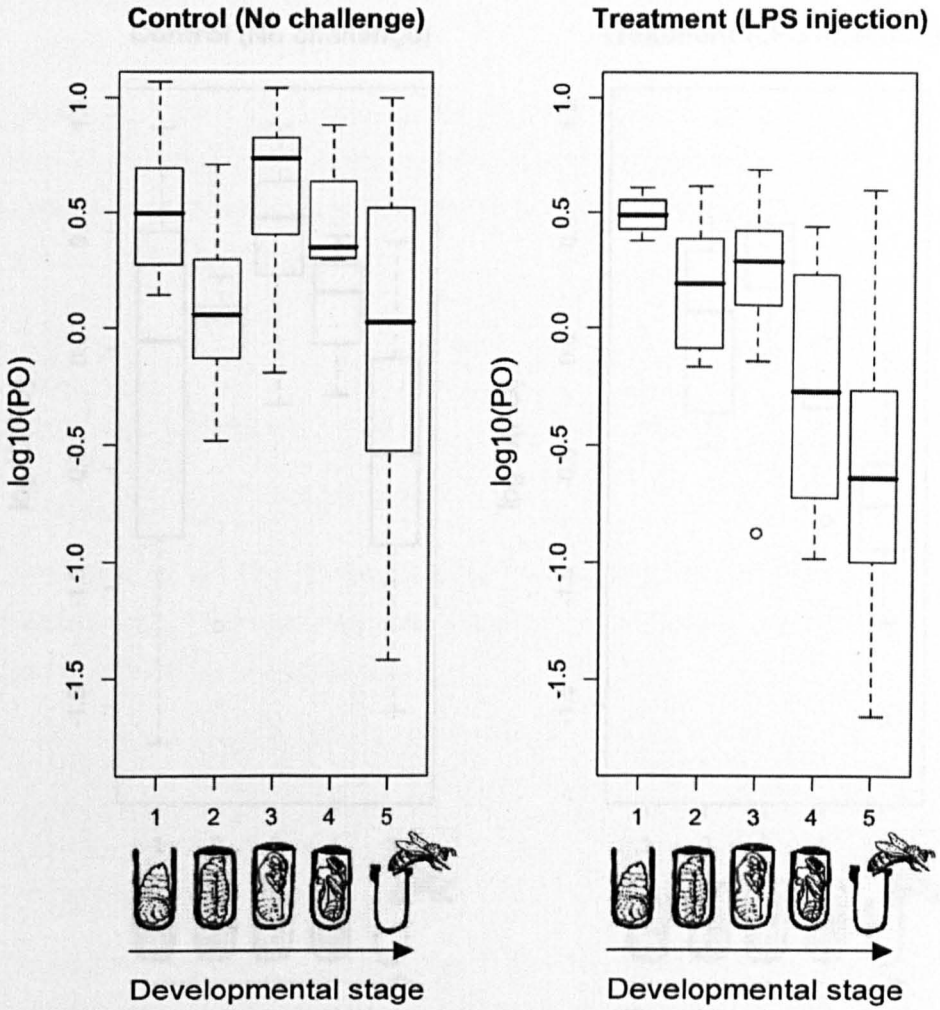


Figure 3.8. Hive A: Concentrations of PO in samples that returned a recordable value for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was significant effect of body size, age and immune challenge on PO concentration (Table 3.1, data are log10 transformed).

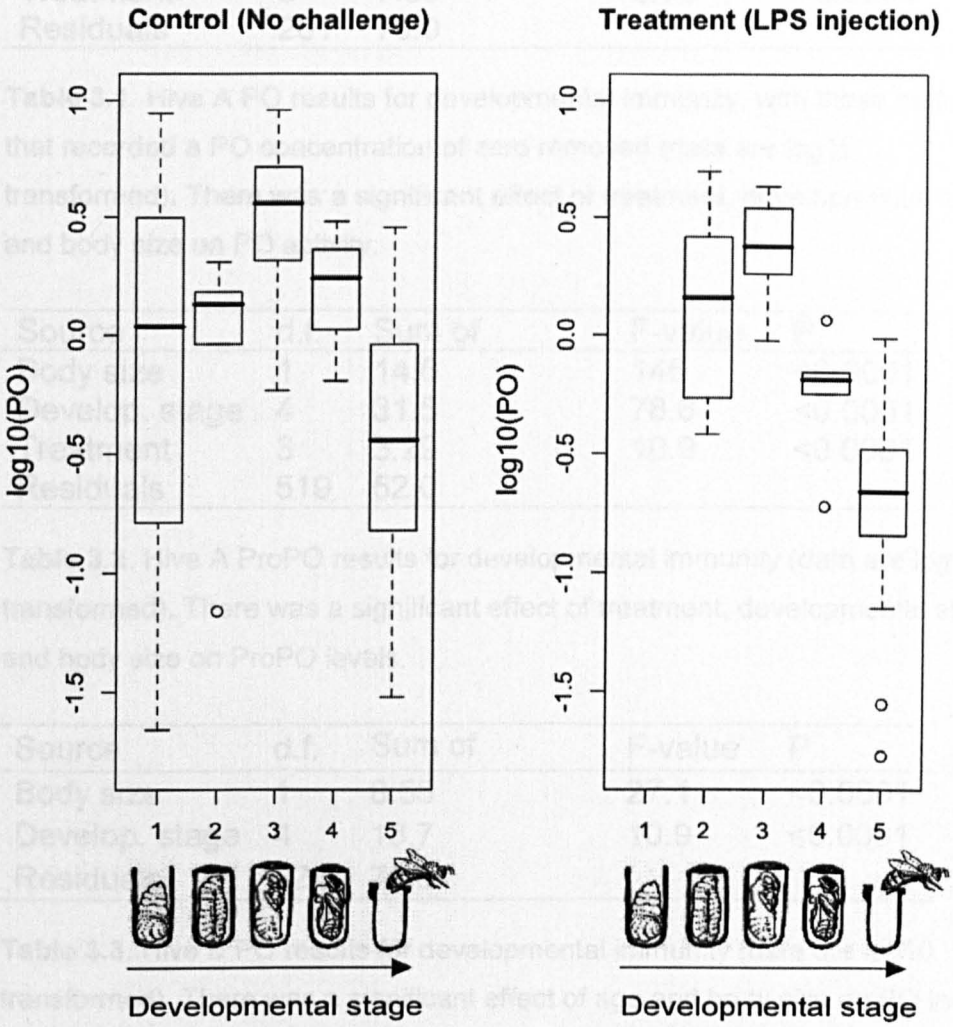


Figure 3.9. Hive B: Concentrations of PO in samples that returned a recordable value for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size and age on PO concentration (Table 3.3, data are log10 transformed).

Source	d.f.	Sum of	F-value	P
Body size	1	10.1	33.9	<0.0001
Develop. stage	4	9.51	7.96	<0.0001
Treatment	3	7.85	8.75	<0.0001
Residuals	261	78.0		

Table 3.1. Hive A PO results for developmental immunity, with those samples that recorded a PO concentration of zero removed (data are log10 transformed). There was a significant effect of treatment, developmental stage and body size on PO activity.

Source	d.f.	Sum of	F-value	P
Body size	1	14.6	146	<0.0001
Develop. stage	4	31.5	78.6	<0.0001
Treatment	3	3.29	10.9	<0.0001
Residuals	519	52.0		

Table 3.2. Hive A ProPO results for developmental immunity (data are log10 transformed). There was a significant effect of treatment, developmental stage and body size on ProPO levels.

Source	d.f.	Sum of	F-value	P
Body size	1	8.55	27.1	<0.0001
Develop. stage	4	13.7	10.9	<0.0001
Residuals	224	70.8		

Table 3.3. Hive B PO results for developmental immunity (data are log10 transformed). There was a significant effect of age and body size on PO levels.

Source	d.f.	Sum of	F-value	P
Body size	1	29.0	363	<0.0001
Develop. stage	4	16.3	51.1	<0.0001
Treatment	3	3.84	16.0	<0.0001
Residuals	603	48.2		

Table 3.4. Hive B ProPO results for developmetal immunity (data are log10 transformed). There was a significant effect of treatment, developmental stage and body size on ProPO activity.

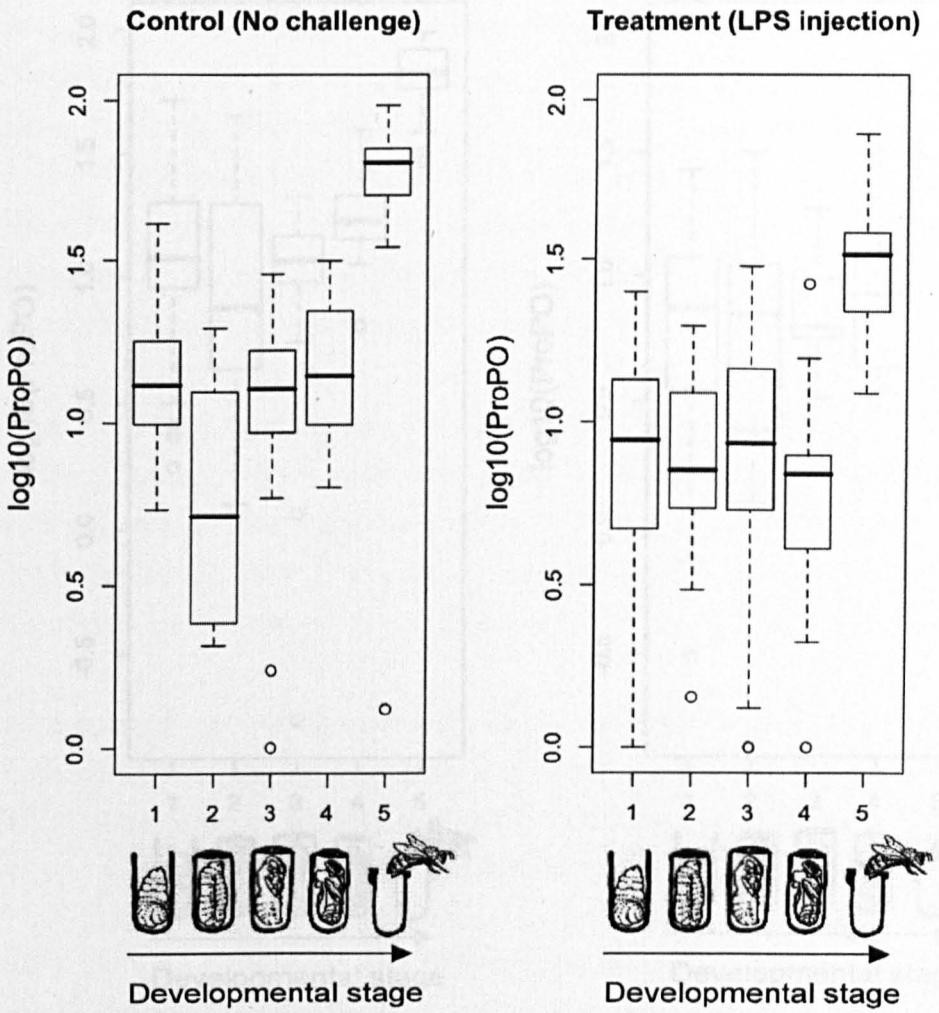


Figure 3.10. Hive A: Concentrations of ProPO in samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size, age and immune challenge on ProPO concentration (Table 3.2, data are log10 transformed)

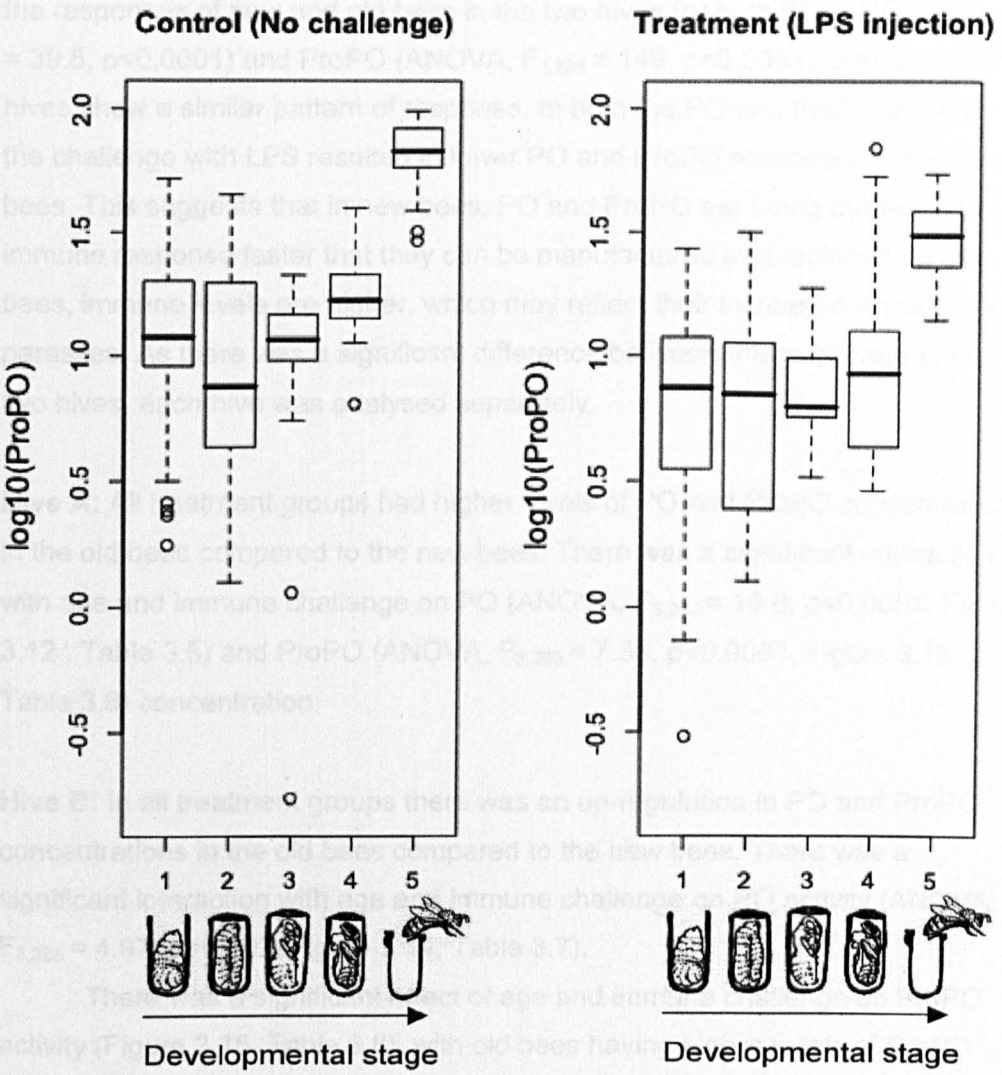


Figure 3.11. Hive B: Concentrations of ProPO in samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size, age and treatment on ProPO concentration (Table 3.4, data are log10 transformed).

3.3.3 Age Polyethism and Phenoloxidase

In all cases, old bees had higher PO and ProPO concentrations than new bees (Figures 3.12, 3.13, 3.14 & 3.15). There was a significant difference between the responses of new and old bees in the two hives for both PO (ANOVA, $F_{1,524} = 39.5$, $p<0.0001$) and ProPO (ANOVA, $F_{1,524} = 149$, $p<0.0001$), although both hives show a similar pattern of response. In both the PO and ProPO assays, the challenge with LPS resulted in lower PO and ProPO responses in the new bees. This suggests that in new bees, PO and ProPO are being utilised in the immune response faster that they can be manufactured and replaced. In old bees, immune levels are higher, which may reflect their increased exposure to parasites. As there was a significant difference between the responses of the two hives, each hive was analysed separately.

Hive A: All treatment groups had higher levels of PO and ProPO concentrations in the old bees compared to the new bees. There was a significant interaction with age and immune challenge on PO (ANOVA, $F_{3,209} = 10.8$, $p<0.0001$, Figure 3.12 , Table 3.5) and ProPO (ANOVA, $F_{3,209} = 7.56$, $p<0.0001$, Figure 3.13, Table 3.6) concentration.

Hive B: In all treatment groups there was an up-regulation in PO and ProPO concentrations in the old bees compared to the new bees. There was a significant interaction with age and immune challenge on PO activity (ANOVA, $F_{3,305} = 4.97$, $p=0.002$, Figure 3.14, Table 3.7).

There was a significant effect of age and immune challenge on ProPO activity (Figure 3.15, Table 3.8), with old bees having higher levels of ProPO activity. Treatment with LPS produced lower ProPO levels in both age groups.

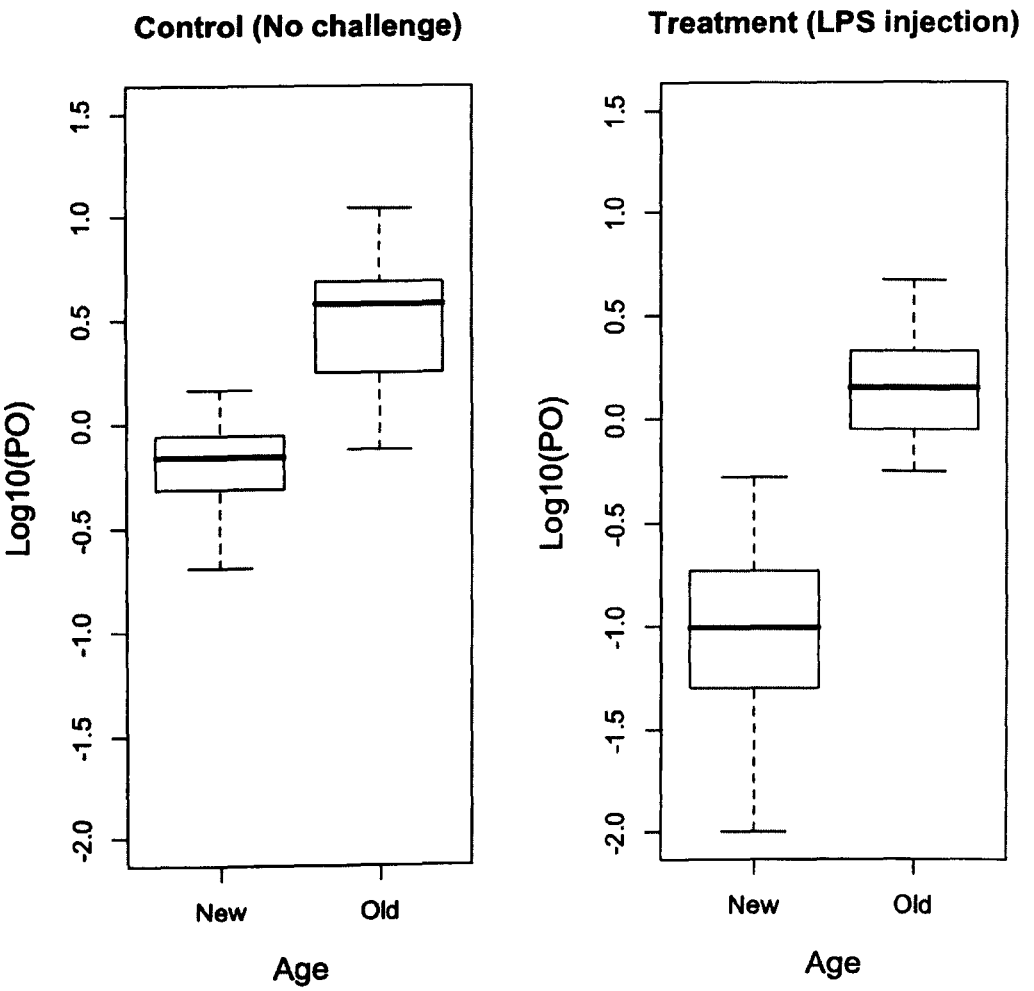


Figure 3.12. PO response of the two age groups, new and old, in Hive A to the No Challenge and LPS treatment group. There was a significant interaction with age and immune challenge, with LPS challenged bees producing lower PO concentrations than the controls (results are log10 transformed).

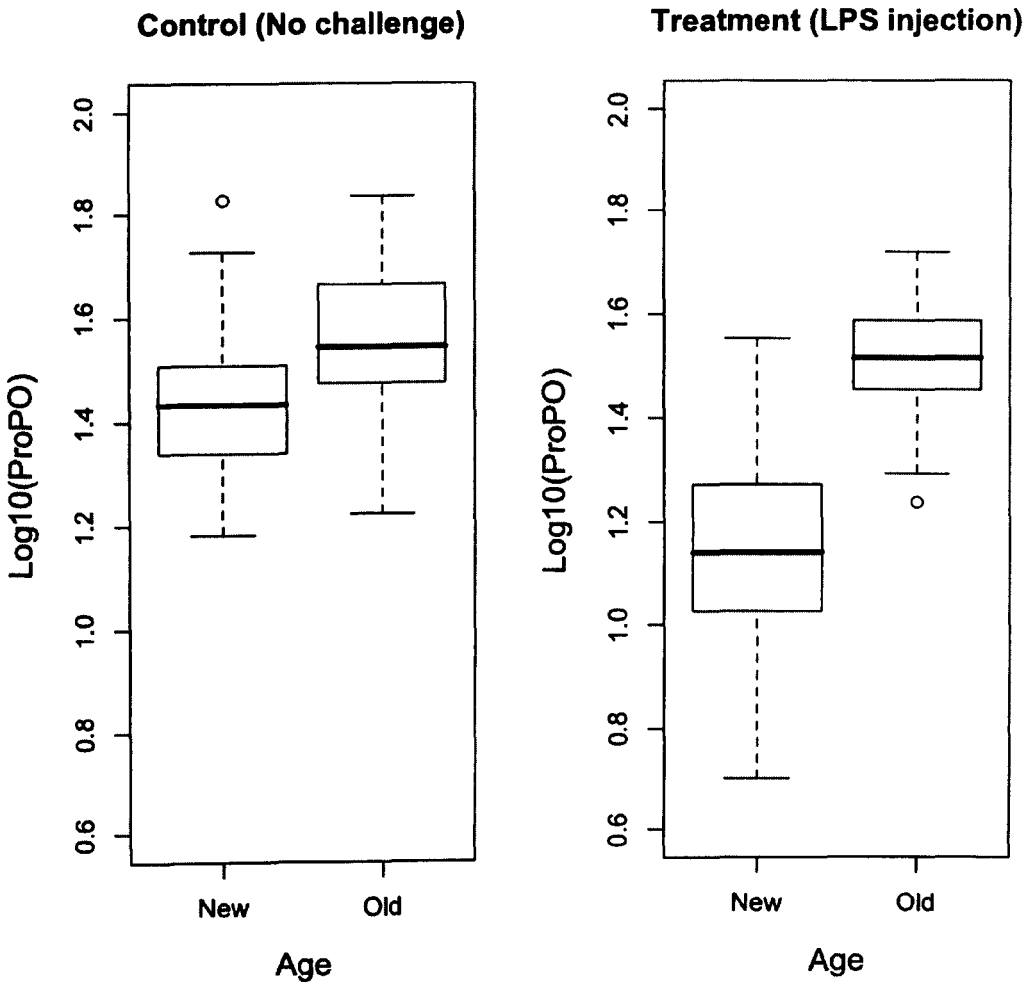


Figure 3.13. ProPO response of the two age groups, new and old, in Hive A to the No Challenge and LPS treatment groups. There was a significant interaction of age with immune challenge (results are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.077	0.772	0.3808
Age	1	31.3	312	<0.0001
Treatment	3	19.0	63.3	<0.0001
Age x treatment	3	3.25	10.8	<0.0001
Residuals	209	20.9		

Table 3.5. PO results for age polyethism in Hive A (results are log10 transformed). There was a significant interaction of age with treatment, with all treatment groups showing an increase in PO activity with age. Treatment with LPS resulted in lower PO levels overall.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.104	4.64	0.03233
Age	1	1.73	76.8	<0.0001
Treatment	3	1.60	23.7	<0.0001
Age x treatment	3	0.510	7.56	<0.0001
Residuals	209	4.70		

Table 3.6. ProPO results for age polyethism in Hive A (results are log10 transformed). There was a significant interaction with age and treatment, with old bees having increased levels of ProPO activity. Treatment with LPS resulted in lower concentrations of ProPO, which was more pronounced in new bees.

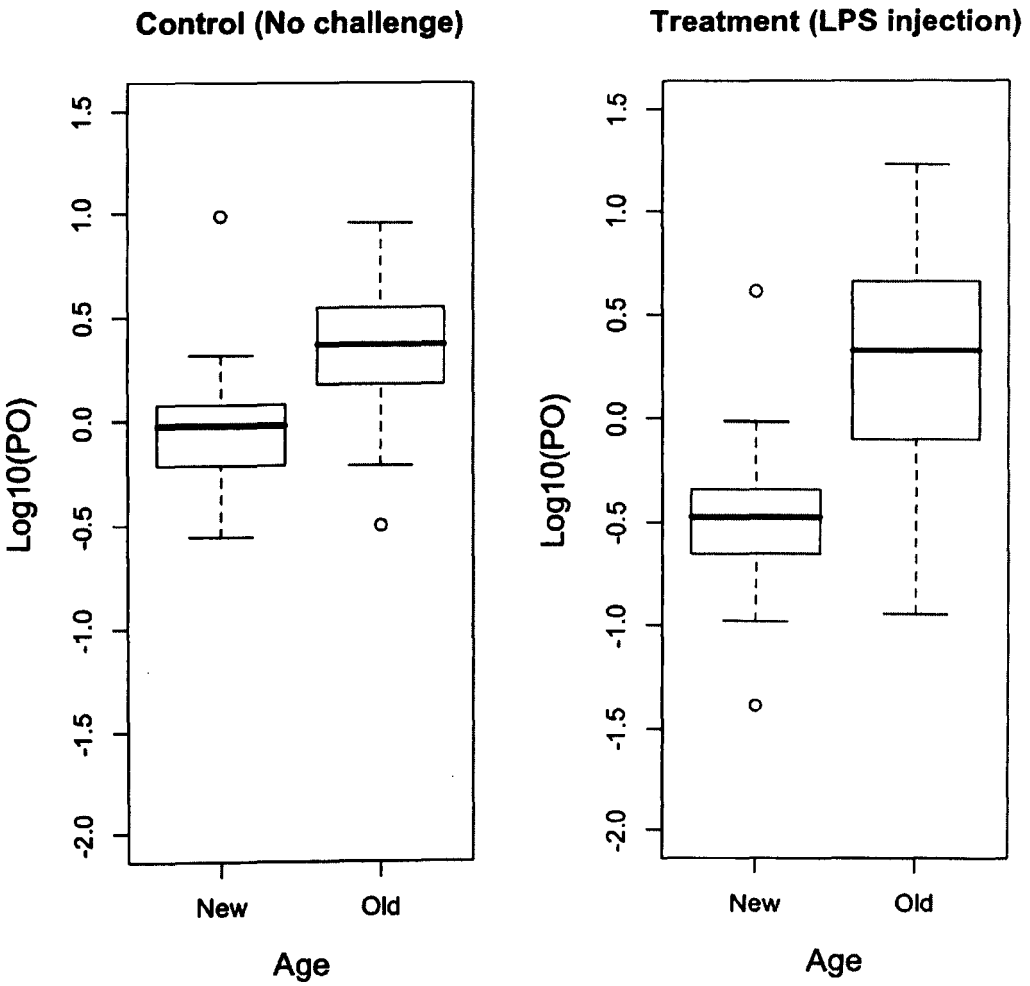


Figure 3.14. PO response of the two age groups, new and old, in Hive B to the No Challenge and LPS treatment groups. There was a significant interaction with age and immune challenge on PO activity (Table 3.7, results are log10 transformed).

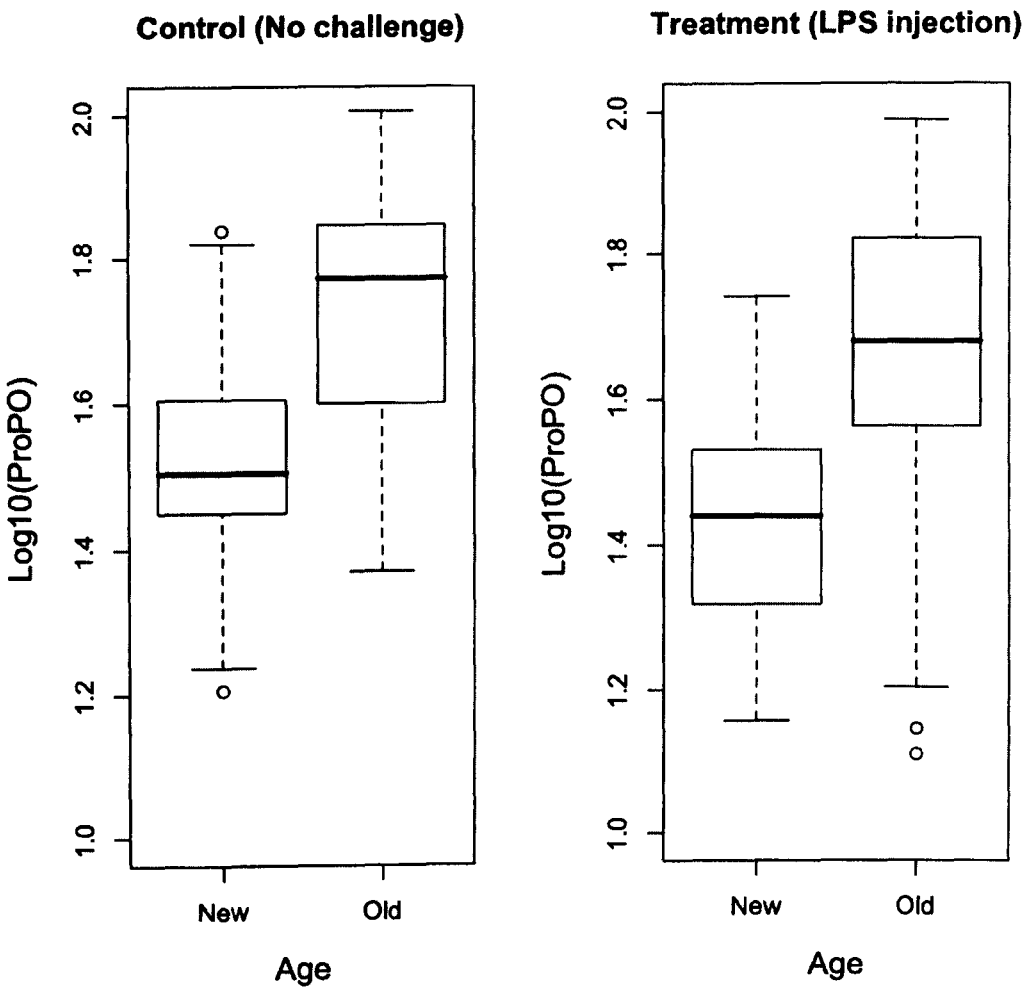


Figure 3.15. ProPO response of the two age groups, new and old, in Hive B to the No Challenge and LPS treatment groups. There was a significant effect of treatment and immune challenge on ProPO activity (Table 3.8, results are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.002	0.0145	0.9042
Age	1	20.7	175	<0.0001
Treatment	3	2.94	8.33	<0.0001
Age x treatment	3	1.75	4.97	0.002221
Residuals	305	35.9		

Table 3.7. PO results for age polyethism in Hive B (results are log10 transformed). There was a significant interaction of age with treatment, with old bees exhibiting higher levels of PO activity. Challenge with LPS resulted in lower concentrations of PO in new bees.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.0675	2.70	0.1013
Age	1	3.54	142	<0.0001
Treatment	3	0.280	3.73	0.01162
Residuals	308	7.69		

Table 3.8. ProPO results for age polyethism Hive B (results are log10 transformed). There was a significant effect of age and treatment on levels of ProPO activity, with old bees having higher levels of ProPO activity. Challenge with LPS resulted in reduced levels of ProPO activity.

3.3.4 Age Polyethism: Timecourse

None of the recaptured bees showed signs of foraging activity (pollen on legs). As foraging occurs at around 18+ days old (Seeley, 1982) it was assumed that none of the bees used in this experiment were working as foragers. There was a significant effect of Hive on both PO (ANCOVA, $F_{1,617} = 121$, $p<0.0001$) and ProPO (ANCOVA, $F_{1,612} = 229$, $p<0.0001$) results, so hives were analysed separately.

Hive G

PO: There was a significant increase in PO activity with age (ANCOVA, $F_{1,373} = 15.8$, $p<0.0001$, Figure 3.16, Table 3.9). There was also a significant effect of treatment group on PO, with the LPS challenged group having lower PO levels than the control (ANCOVA, $F_{3,373} = 3.68$, $p=0.01229$).

ProPO: ProPO activity increased significantly over time (ANCOVA, $F_{1,373} = 15.6$, $p<0.0001$, Figure 3.17, Table 3.10), with LPS challenged bees having lower levels of ProPO than the controls throughout the timecourse (ANCOVA, $F_{3,373} = 4.36$, $p=0.00495$).

Hive H

PO: There was a significant increase in PO activity with worker age (Regression, $F_{1,238} = 9.26$, $p=0.002599$, Figure 3.18, Table 3.11), but no effect of treatment group on immune response.

ProPO: LPS challenged bees had significantly lower levels of ProPO activity than control bees (ANOVA, $F_{1,236} = 6.29$, $p=0.0004$, Figure 3.19, Table 3.12), but there was no significant increase in activity with age ($p=0.05155$). However, as age is nearly statistically significant, it does support the trend for increasing PO and ProPO levels with increasing age.

If we look at these results compared to those with old workers that have started foraging activities (Section 3.3.3), it can bee seen that foraging old bees had slightly higher levels of PO and ProPO activity than the 14-day old bees seen here. However, they are comparable with a continued increase in immunity at the rate seen during the first 14 days of adult life. In both hives, the start of test flights does not correspond to a sudden increase in immune investment; rather the levels of PO and ProPO activity seem to be continuously increasing from the moment of eclosion.

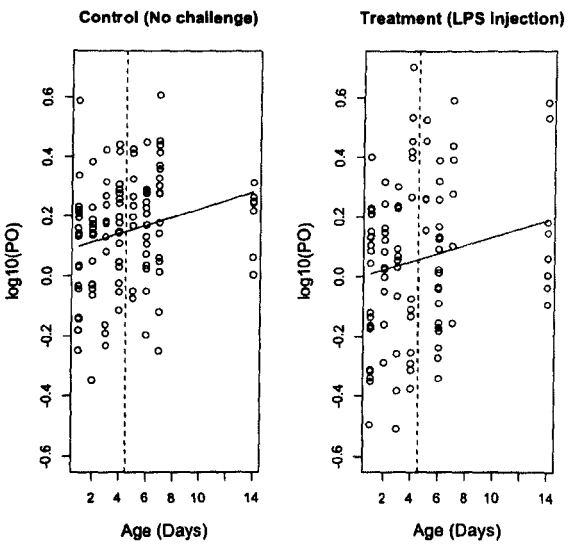


Figure 3.16. Hive G: Change in PO with age, for the first 14 days of adult worker life, for the No Challenge and LPS treatment groups. Dashed line represents the age at which test flights start. There was a significant effect of age and treatment on PO activity (Table 3.9, data are log10 transformed).

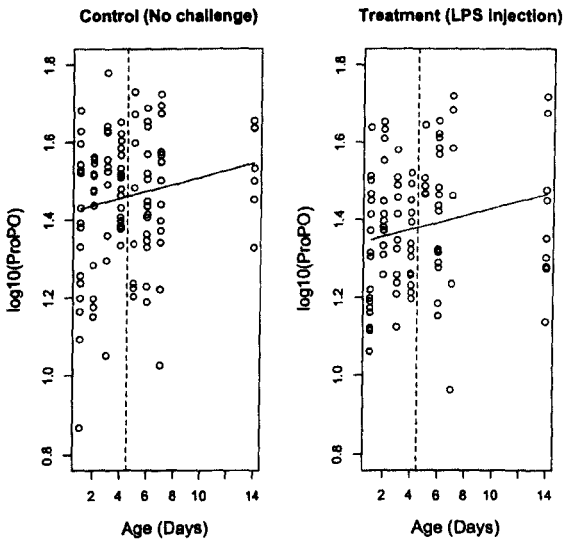


Figure 3.17. Hive G: Change in ProPO with age, for the first 14 days of adult worker life, for the No Challenge and LPS treatment groups. Dashed line represents the age at which test flights start. There was a significant effect of age and treatment on ProPO activity (Table 3.10, data are log10 transformed).

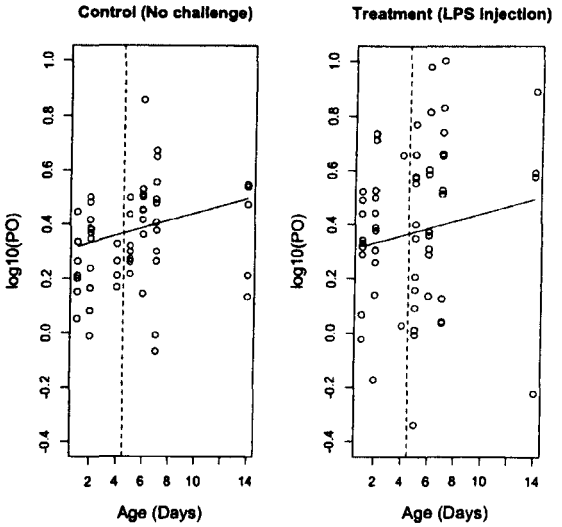


Figure 3.18. Hive H: Change in PO with age, for the first 14 days of adult worker life, for the No Challenge and LPS treatment groups. Dashed line represents the age at which test flights start. There was a significant effect of age on PO activity (Table 3.11, data are log10 transformed).

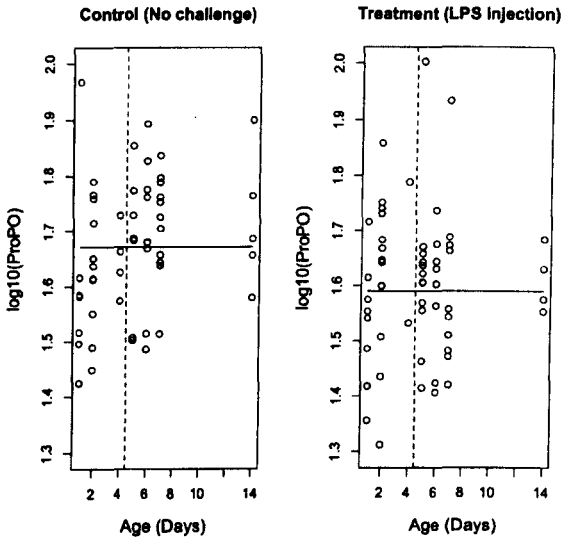


Figure 3.19. Hive H: Change in ProPO with age, for the first 14 days of adult worker life, for the No Challenge and LPS treatment groups. Dashed line represents the age at which test flights start. There was a significant effect of treatment on ProPO activity (Table 3.12, data are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.0078	0.161	0.6883
Age	1	0.767	15.8	<0.0001
Treatment	3	0.535	3.68	0.01229
Residuals	373	18.1		

Table 3.9. PO results for polyethism timecourse in Hive G (results are log10 transformed). There was a significant increase in PO activity with age. LPS challenged individuals had lower levels of PO activity overall.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.000	0.0152	0.9019
Age	1	0.448	15.6	<0.0001
Treatment	3	0.375	4.36	0.00495
Residuals	373	10.7		

Table 3.10. ProPO results for polyethism timecourse in Hive G (results are log10 transformed). There was a significant increase in ProPO activity with age. LPS challenged individuals had lower levels of ProPO activity overall.

Source	d.f.	Sum of	F-value	P
Thorax width	1	0.544	10.3	0.001533
Age	1	0.490	9.26	0.002599
Residuals	238	12.6		

Table 3.11. PO results for polyethism timecourse in Hive H (results are log10 transformed). There was a significant increase in PO activity with age.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.113	7.48	0.006726
Treatment	3	0.284	6.29	<0.001
Residuals	236	3.55		

Table 3.12. ProPO results for polyethism timecourse in Hive H (results are log10 transformed). LPS challenged individuals had significantly lower levels of ProPO activity.

3.3.5 Age Polyethism and Antimicrobial Peptides

Mortality rate: There was no effect of immune challenge on mortality, but there was a significantly higher mortality rate in the old bees compared to the new bees ($F_{1,577} = 24.5$, $p<0.0001$, binomial errors, data from both hives combined). The overall percentage mortality for new bees = 29.6%, and old bees = 50%.

Antimicrobial peptides: Overall, there was an up-regulation of AMP production following an immune challenge with LPS. Figure 3.20 shows the zones of inhibition plates for new and old bee samples. In both plates, the effect of an immune challenge can be seen as larger zones of inhibition around the wells. Old bees showed much higher levels of melanin (seen as brown pigment) in their haemolymph samples, which is expected given the elevated levels of PO and ProPO found in the previous experiment. The presence of the melanin has been shown to have no effect on the inhibition assay (E. Haine, pers. comm.), so will not have biased the results.

Hive F: There was a significant interaction between age and immune challenge (Figure 3.21, Table 3.13, ANOVA, $F_{3,130} = 9.94$, $p<0.0001$, results are square root transformed). Across both age groups, AMP production increased following an immune challenge. In the No Challenge group, AMP production was seen to increase in old bees. The LPS challenged group showed the opposite pattern, with a decrease in AMP production with increasing age.

Hive G: There was a significant interaction between age and immune challenge (Figure 3.22, Table 3.14, ANOVA, $F_{3,163} = 8.75$, $p<0.0001$, results are square root transformed). Hive G showed a similar pattern of responses as Hive F, with both age groups exhibiting an increase in AMP production following an immune challenge. Again, AMP production increased with increasing age in all but the LPS treatment group.

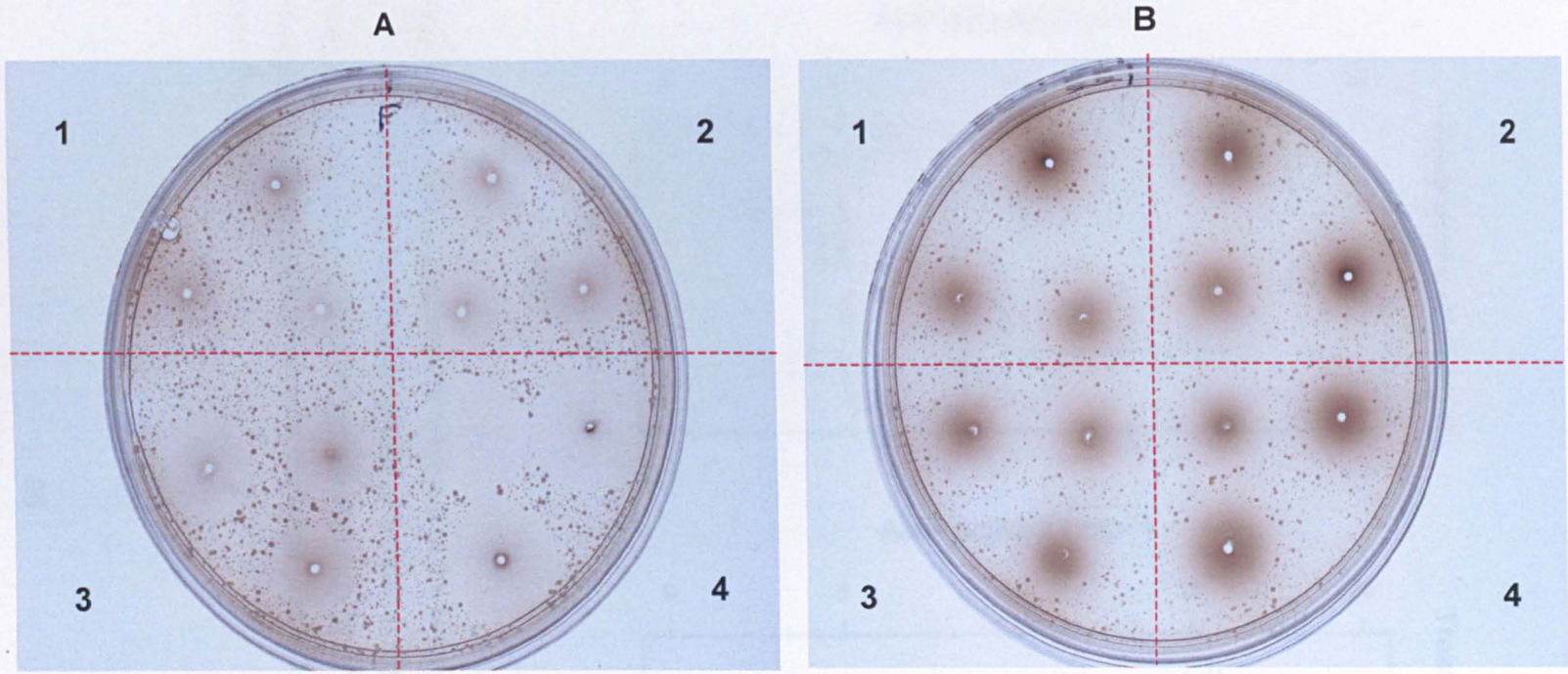


Figure 3.20. Zones of inhibition within a lawn of *A. globiformis*, caused by antimicrobial peptides from haemolymph samples of A) new bees, and B) old bees. The plate was split between the 4 different treatment groups: 1 = No challenge, 2 = Needle, 3 = Ringer, 4 = LPS. In old bees there was an obvious increase in melanin, seen as the dark areas around the wells. The presence of the melanin has been shown to have no effect on the inhibition assay (E. Haine, pers. comm.), and is likely to be a result of the elevated PO and ProPO concentrations found in old bees.

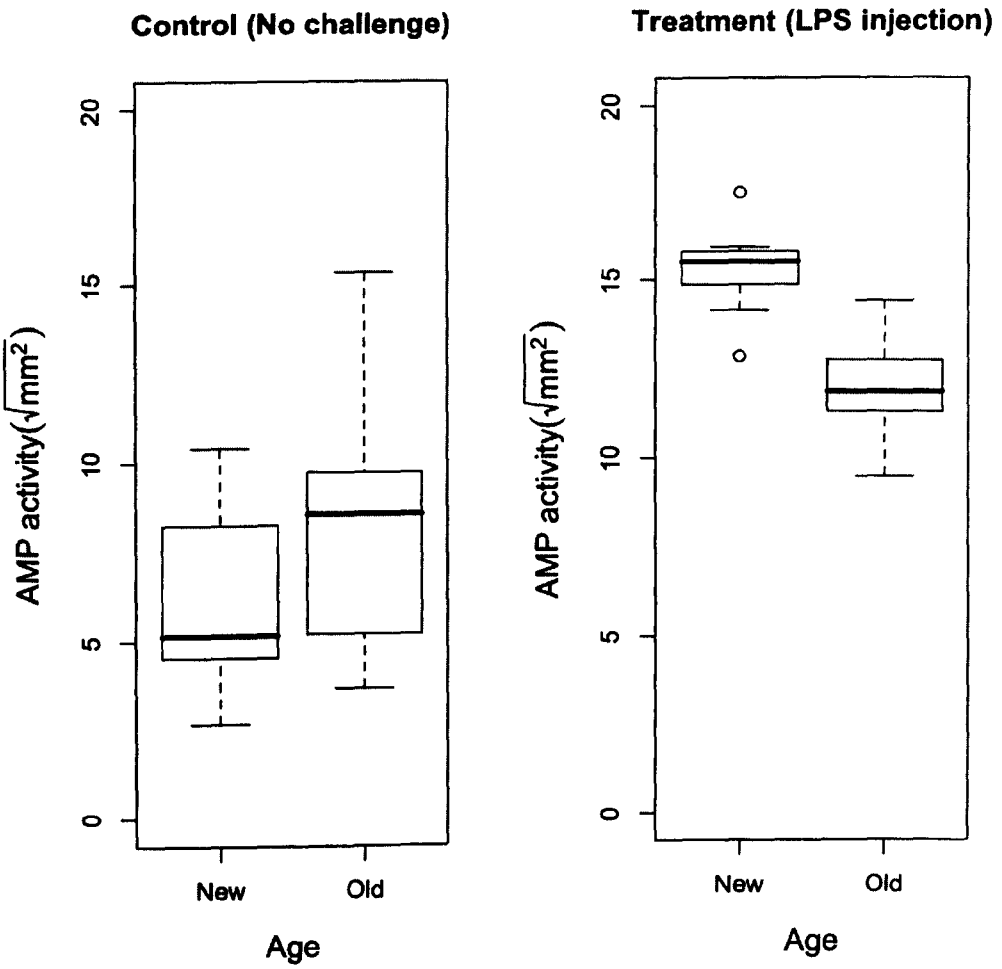


Figure 3.21. Antimicrobial peptide response of the two age groups, new and old bees, in Hive F in the No Challenge and LPS treatment groups. AMP activity was measured as the area of bacterial growth inhibition (mm^2). There was a significant interaction between age and immune challenge (Table 3.13, results are square root transformed).

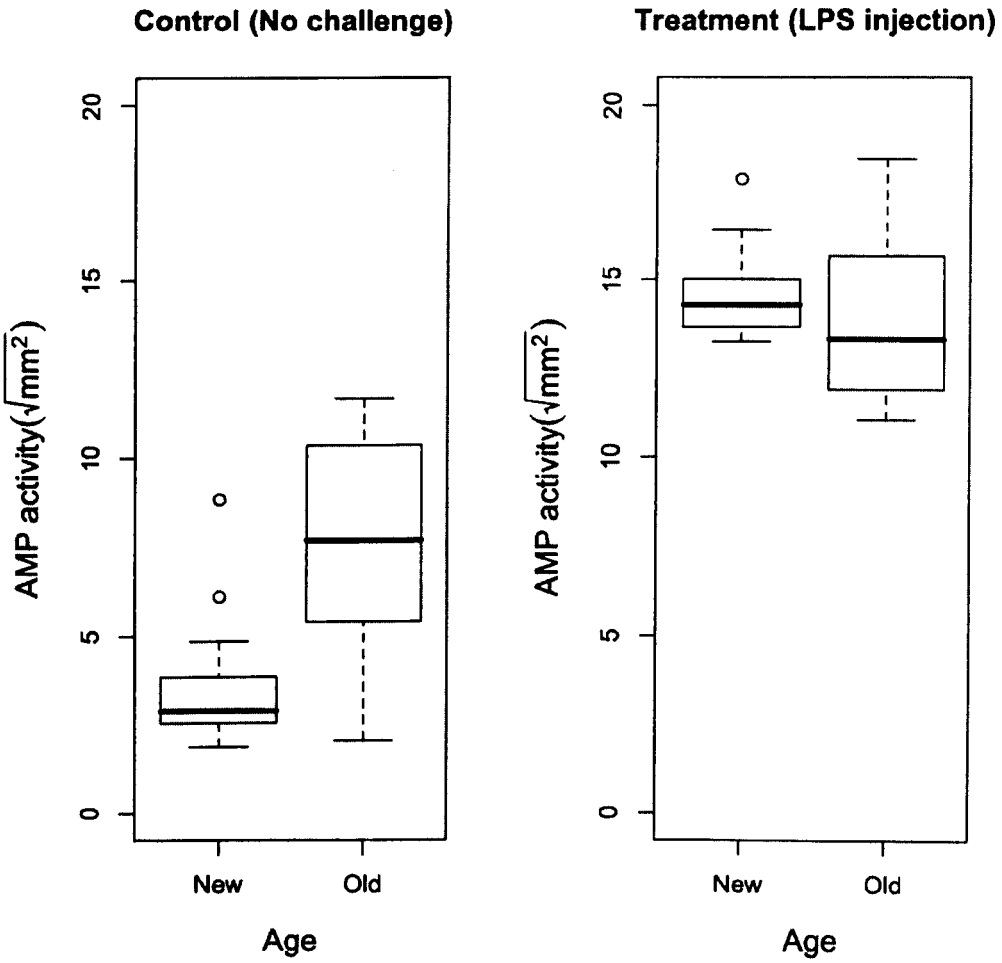


Figure 3.22. Antimicrobial peptide response of the two age groups, new and old bees, in Hive G in the No Challenge and LPS treatment groups. AMP activity was measured as the area of bacterial growth inhibition (mm^2). There was a significant interaction between age and immune challenge (Table 3.14, results are square root transformed).

Source	d.f.	Sum of squares	F value	P
Thorax width	1	4.74	0.984	0.3230
Age	1	6.71	1.40	0.2398
Treatment	3	786	54.4	<0.0001
Age x treatment	3	143	9.94	<0.0001
Residuals	130	626		

Table 3.13. Antimicrobial results for Hive F (results are square root transformed). There was a significant interaction of age and treatment group, with AMP production increased following an immune challenge with LPS. There was some indication of senescence, with the old bees unable to produce the same high levels of AMPs seen in new bees following an immune challenge.

Source	d.f.	Sum of squares	F value	P
Thorax width	1	19.9	3.70	0.05606
Age	1	98.5	18.4	<0.0001
Treatment	3	1590	98.8	<0.0001
Age x treatment	3	141	8.75	<0.0001
Residuals	163	874		

Table 3.14. Antimicrobial results for Hive G (results are square root transformed). There was a significant interaction of age and treatment group, with LPS challenged bees having higher concentrations of AMPs. The different response of the age groups suggests a senescence of the immune response.

3.4 Discussion

This chapter has demonstrated that there are clear differences in the immune activity at different stages in honey bee development. Larvae and pupae have little immune activity, which may be because they are unlikely to be challenged with parasites: the brood is effectively sterile. However, adults are challenged with parasites and therefore invest in immune activity. Immune responses increased with age, which may be a reflection of the increased risk of pathogen encounters. There is no evidence that the increase in activity is associated with the switch to foraging activities, and may instead be due to an increase in cumulative pathogen exposure with age.

3.4.1 Developmental Immunity

As predicted, a large proportion of the larval and pupal samples did not contain a recordable level of PO activity (an average of 56% in Hive A and 72% in Hive B). This result is unlikely to be due to experimental error as all factors of this assay have been thoroughly investigated (Chapter 2, Section 2.3 and Appendix 1, Section A1.3). These developmental stages may not need to maintain a costly active constitutive immune response as threat of pathogen infection is reduced due to the hygienic behaviour of nurse bees, or the protective housing of the capped brood cell. Active PO may be present but constantly utilised for functions outside of immunity such as cuticle sclerotisation and melanin production, and so be undetectable with this assay. Across all developmental stages, where present, active PO was maintained at very low concentrations (compared to the levels stored as ProPO), which is likely to be due to the cytotoxic effects of storing excess active PO in the haemolymph.

Pre-adult developmental stages that did contain a recordable concentration of PO had levels of activity higher than those found in adults. Developing individuals with a high level of PO activity may be responding to previous immune challenge, such as feeding by *Varroa* mites, which were subsequently removed by the grooming practices of nurse bees (see Chapter 5). Alternatively, the high PO concentrations seen may be a consequence of the natural variation in immunity. As the analysis was carried out on developing stages from which a large percentage

of the population had been removed due to the lack of PO response, what remains may not be comparable to the adult population results as a whole.

However, the capped larval and white-eyed pupal stages in both hives contained a higher proportion of individuals with recordable levels of PO activity than the other pre-adult stages (57% and 43% respectively), and this level of activity was, on average, higher than that in adults (Figures 3.8 & 3.9), which suggests that there may be a physiological reason for up-regulation in PO activity during this developmental period.

Where recordable, PO levels were lower in individuals from the pupal and adult stages following an immune challenge with LPS. This response has been seen in several other insect species, and we believe is an indication of the use of PO in the immune response (S. Povey, S. Slos, pers. comm.) Larval stages with recordable PO levels showed little change in PO activity following a challenge with LPS, which suggests that either PO is not used for immunity at this life stage, or that PO levels recorded in these individuals are already a response to an undetermined immune challenge.

Developmental results show that larval and pupal stages had a lower ProPO response compared to adult bees, and activity levels were qualitatively similar throughout development in both hives. In contrast to PO however there was a measurable level of ProPO in all larvae and pupae samples. In the control groups there was a stage-specific decrease in ProPO activity in the capped larval stage (see full graph in Appendix 2, Figure A2.1.5 & A2.1.6). This response was lost in the LPS challenged group, when ProPO activity increased to the same level as the other developmental stages. Processes occurring in the capped larval stage, such as the spinning of the cocoon may utilise ProPO, but resource allocation may be diverted towards the immune response following a challenge. ProPO activity was maintained at a steady level in all other larval and pupal stages, and may represent a maximum capacity for ProPO storage.

Body mass was included in the analysis as a control for body size. As the uncapped larval stage stops feeding and undergoes metamorphosis, body mass decreases as lipid stores are utilised. Therefore, the adult bees in this experiment had the lowest body mass, yet overall the highest levels of PO and ProPO activity.

This resulted in a significant effect of body size on both the PO and ProPO assays in both hives.

3.4.2 Age Polyethism

Results showed a universal up-regulation in PO, ProPO and AMP responses with increasing age in unchallenged bees. Previous research suggests that the opposite should be true, as the increase in haemocyte apoptosis with transition from hive bee to forager should reduce the level of cellular immune responses available (Robinson, 1992; Bedick *et al.*, 2001; Amdam *et al.*, 2004; Omholt & Amdam, 2004; Amdam *et al.*, 2005). Foragers have reduced numbers of functioning haemocytes (Amdam *et al.*, 2005), which suggests that remaining cells may increase their levels of PO and ProPO production in order to produce the results obtained. Alternatively, as types of haemocytes have different functions in different insects, selection may be acting towards the survival of haemocytes with immune activity whilst causing pycnosis in those cell types with no link to immunity (Lavine & Strand, 2002). Recent work has shown both a decrease in haemocyte counts and a juxtaposed increase in PO activity with age in the honey bee (Wilson-Rich *et al.*, 2008).

Following an immune challenge with LPS, PO and ProPO levels decreased in new bees. This response was reduced or lost altogether in the old bees. Old bees may maintain a constant maximum level of PO and ProPO production. If they are under continuous attack from pathogens, the mechanisms for manufacturing and replacing PO and ProPO as it is used up in the immune response may already be operational.

Results from the timecourse experiment showed that the difference in PO and ProPO levels between new and old bees was the product of a continuous increase in activity levels, beginning immediately after eclosion (see Section 2.5). It was predicted that an up-regulation of the immune response in old bees could be a prophylactic measure as the risk of pathogen encounter increases, or may be due to a greater cumulative exposure to pathogens. As the increase in PO and ProPO activity began even before new bees had left the hive for their first test flights, this cannot be the case. The increase may be due to bees responding to cues in the hive, such as the presence of pathogens, or pheromone contact with incoming

foragers, or the PO/ProPO immune response may simply increase as part of the physiological processes of aging.

Workers up-regulated AMP production following an immune challenge with LPS. This response is seen in several other insects (Boulanger *et al.*, 2001; Boulanger *et al.*, 2002). As with PO and ProPO, there was also an increase in activity with age in unchallenged bees. The level at which AMP production was maintained in non-challenged bees was much higher than that seen in drones (Section 4.3.3), suggesting that workers have the resources to invest in a costly immune response even when not under threat.

The AMP response in LPS-challenged bees decreased with age in Hive F (although not in Hive G), which may be a sign of immune senescence (Doums *et al.*, 2002; Hillyer *et al.*, 2005). Recent research has found a decrease in the fat body mass in old bees compared to new bees (Wilson-Rich *et al.*, 2008). Although the reduced fat body still produced AMPs in old bees, age-imposed limits on functioning ability would support the decrease in AMP production seen in the immune challenged old bees.

The transition to foragers in bumble bees has been shown to correlate to a decrease in 'immunocompetence' (König & Schmid-Hempel, 1995; Doums & Schmid-Hempel, 2000), but evidence for this in the honey bee is mixed and so far such conclusions cannot be drawn (Amdam *et al.*, 2005; Schmid *et al.*, 2008; Wilson-Rich *et al.*, 2008).

Summary

This chapter investigated the impact of different aspects of aging on immunity in worker bees. Using assays developed in Chapter 2, I have shown that:

1. Immune challenge significantly increased mortality (Section 3.3.1)

2a. PO responses varied with developmental age (Section 3.3.2)

2b. The ProPO immune response was maintained at a constant level throughout development, and increased following eclosion to an adult (Section 3.3.2)

3a. PO and ProPO responses were higher in old bees compared to new bees, but this seemingly large up-regulation was the result of a

continuous increase that began immediately post-adult eclosion (3.3.4)

3b. AMPs were up-regulated in old bees compared to new bees, and their production was greatly increased following an immune challenge (Section 3.3.5)

4. Overall, immune challenge produced a decrease in PO and ProPO activity, and an increase in AMP production.

Chapter 4

Ontogeny of drone immunity

4.1 Introduction

This chapter examines the development, and changes with age, of the immune responses of drones. Workers and drones have very different life histories, and drones provide an opportunity to investigate the immune investment of sexuals in the colony. Although in some circumstances the suppression of reproduction in workers of a colony may be reversed, this is rare and difficult to predict or reproduce for experimental purposes. The reproductive queens can be artificially reared to large numbers, but as only one can reside in a hive at a time, it is impractical to work on them under natural conditions. Therefore, by investigating immune investment in drones throughout development and adult life, this chapter aims to highlight the differences that exist between two of the honey bee castes, and attempt to link them to life-history.

4.1.1 The Drone

Unfertilised eggs laid by the queen develop into male drones. Drones have a slightly longer developmental time (24 days) compared to workers (21 days) (Winston, 1987), and their life history is very different. A colony produces only 200-1,000 drones (compared to 50,000 workers) during the most productive summer months, and their sole function is to mate with a queen (Hooper, 1997). Drones are twice as big as workers, although they are reared on similar food (Hooper, 1997). They reach sexual maturity at 13 days post eclosion, and live approximately 22 days (Winston, 1987). They have no age polyethism and do not work for the colony, and all leave the hive for test-flights soon after emergence.

During their lives, drones are potentially exposed to a similar range of parasites that infect workers. Workers and drones start their lives in the same brood environment, where they are subject to infection with a variety of parasites (see Table 1.1), and in the case of the *Varroa* mite, *Varroa destructor*, drone brood is favoured over workers for infestation (Hooper, 1997; Otten and Fuchs, 1988; Fuchs, 1992). Although as adults they have different behavioural patterns (for example, drones do not land on flowers), both castes return to the

hive at night, where the mixing of drones and foraging workers provides opportunities for the transfer of novel pathogens (Hooper, 1997; Boomsma *et al.*, 2005; Cremer *et al.*, 2007).

Immune responses in the bumblebee, *Bombus terrestris*, have found that drones have a less intense encapsulation response than workers (Gerloff *et al.*, 2003; Baer & Schmid-Hempel, 2006), although results show no difference between male and female susceptibility to transmissible parasites (Ruiz-González & Brown, 2006). Male ants also have a lower immune response than both workers (Baer *et al.*, 2005) and queens (Vainio *et al.*, 2004). As honey bee drones are relatively short-lived compared to workers, we may also expect them to invest even less in an immune response, and instead channel their resources into sperm production and mating flights.

4.1.2 Chapter Predictions

From their life-history, we can formulate some predictions about the immune responses we might expect to see in drones throughout their development and adult life. I predict that:

1. There will be little or no immune response in the larval stages, as they are tended by nurse bees and raised in a relatively sterile environment
2. There will be an increase in PO and ProPO activity as it is needed for cuticle formation and melanisation in pupal drones
3. Adult drones will have little or no immune responses, due to their short lifespan and selection to invest all resources into obtaining a successful mating.

4.1.3 Chapter Aims

This chapter will investigate the impact of different aspects of aging on immunity in the drone. Using assays developed in Chapter 2, I examine:

1. Developmental changes in a) PO and b) ProPO responses (Section 4.2.1)
2. The effects of age on measures of a) PO, b) ProPO, c) mortality and d) antimicrobial peptides (Sections 4.2.2 and 4.2.3).

4.2 Materials and Methods

4.2.1 Developmental Immunity

The protocol of this experiment is identical to that used for investigating workers (Section 3.2.2). The same five developmental stages (uncapped larvae, capped larvae, white-eyed and red-eyed pupae and newly eclosed adults) were collected from two similar hives (D & E). Individuals were housed in 25-well sectioned boxes. They were laid on moist cotton wool to maintain a high humidity and, in the case of larvae, provided with brood food obtained from brood cells. Adult bees were incubated in bee boxes as standard. Body mass (wet weight) was recorded as a measure of body size.

Each developmental stage was split into four treatment groups to measure the effect of an immune challenge on the immune response (No Challenge, Needle, 1µl Ringer, 1µl LPS, n=5-41, average n=19). Post challenge, bees were incubated for 24 hours (36°C, 45% humidity ± 5% in incubator, 24 hour dark photoperiod). Haemolymph samples from surviving bees were collected through perfusion bleeds with 0.25ml sodium cacodylate buffer. Samples were assayed for PO and ProPO as standard (Chapter 2, Sections 2.3.11 & 2.4.14). To reduce the lipid contamination of haemolymph samples (see Appendix 1, A1.3), after defrosting, all samples were centrifuged twice (4°C, 80,000G, 15 minutes), with as much lipid as possible removed by pipette in between cycles. The clear supernatant was then used in the reaction mixture as standard.

4.2.2 Age and Phenoloxidase

My work on worker bees found an increase in PO and ProPO activity with age, and a decrease in activity following an immune response (Section 3.3.3). To investigate if PO and ProPO immune responses were the same in new and old drones, drones were collected at two different ages.

A frame of drone brood was brought into the lab and allowed in hatch overnight (36°C ± 1°C, 40% ± 2% humidity). Approximately 180 newly emerged drones were marked with a spot of acrylic paint on the thorax, and released back into the hive. These drones were recaptured for assaying 2 weeks later, when they had reached sexual maturity ('old' drones). A second set of drones from the same brood frame was assayed at 24 hours old as 'new' drones. Drones (both new and old) were split into four treatment groups to test the response to an immune challenge (No Challenge, Needle, 1µl Ringer, 1µl LPS,

n=11-25). Insects were challenged and samples collected and assayed for PO and ProPO as in Section 3.2.3 and Chapter 2, Sections 2.3.11 & 2.4.14. Thorax width was measured to control for body size. Percentage mortality was also recorded for each age:treatment group combination.

4.2.3 Age and Antimicrobial Peptides (AMPs)

My work on worker bees found an increase in AMP production with age and immune challenge (Section 3.3.5). To test whether this increase also occurs in drones, drone frame was collected from a single hive. Brood was incubated overnight (36°C ± 1°C, 40% ± 2% humidity) and emerged drones were collected. Samples were split into two groups: approximately 50 drones were marked with a spot of acrylic paint and released back into the hive. These were recaptured two weeks later for assaying ('old' drones, 22 recaptured, of which 17 survived). The second group ('new') drones were assayed at 24 hours old. Before assaying, bees from both groups were split into four treatment groups to test the response to an immune challenge (No Challenge, Needle, 1µl Ringer, 1µl LPS, n=5-32). Post challenge, treatment groups were housed in separate bee boxes and neat haemolymph was collected 24 hours post challenge. Samples were assayed for AMP activity as standard (Chapter 2, Section 2.6.6). The thorax width of each bee was measured to control for body size.

4.2.4 Presentation of Results

In all experiments, the No Challenge and LPS treatment groups showed the extremes of the range of immune responses seen. For simplicity, graphical data are only presented for these two treatment groups. The Needle and Ringer treatment groups provided wounding and vehicular controls, and showed a staggered response between the two extremes. Figures for all four treatment groups can be found in Appendix 2, Section A2.2.

4.3 Results

4.3.1 Developmental Immunity

Hives produced qualitatively similar results, but were analysed separately due to quantitative differences in the immune response (ANCOVA, $F_{1,742} = 20.7$, $p<0.0001$). As predicted, within both hives, earlier drone developmental stages commonly contained no recordable concentrations of PO (see Appendix 1,

Section A1.3 for explanation). This resulted in a positively skewed distribution, and subsequently the dataset could not be transformed to conform to the assumptions of normality. The complete data sets for Hives D (Figure 4.1) and E (Figure 4.2) show the large number of samples in the larval and pupal stages that contained no measurable levels of PO. The proportion of samples recording a zero PO concentration was compared for each age:treatment group combination. Samples containing no recordable PO were then removed from the data sets, and remaining samples were analysed against each other for each hive.

The complete data sets for ProPO in Hives D (Figure 4.7) and E (Figure 4.8) also showed a number of samples in some developmental stages that contained no recordable levels of ProPO. As with the PO assay, the proportion of samples containing no recordable ProPO was analysed for each hive. Samples containing no recordable ProPO were then removed from the data sets, and remaining samples were analysed against each other for each hive.

Hive D

PO: There was a significant effect of developmental stage on the proportion of samples containing no recordable PO (Figure 4.3, $F_{4,379} = 36.9$, $p<0.0001$, binomial errors). There was a significant effect of both body size and developmental stage on PO concentration for those samples that contained a recordable concentration of PO (Figure 4.5, Table 4.1). Where PO was recordable, larval and pupal stages produced higher concentrations than the adult drones (see Section 4.4.1 for discussion).

ProPO: There was a significant decrease in the proportion of samples with no recordable ProPO with increasing developmental age (Figure 4.9, $F_{4,379} = 32.4$, $p<0.0001$, binomial errors). There was a significant effect of body size and developmental stage on ProPO concentration for those samples that contained a recordable concentration of ProPO (Figure 4.11, Table 4.3). ProPO concentrations increased sequentially with developmental stage.

Hive E

PO: There was a significant effect of developmental stage on the proportion of samples containing no recordable PO (Figure 4.4, $F_{4,359} = 26.2$, $p<0.0001$, binomial errors). There was a significant effect of body size, developmental

stage and treatment on PO concentration for those samples that contained a recordable concentration of PO (Figure 4.6, Table 4.2). Where PO was recordable, larval and pupal stages produced higher concentrations than the adult drones. Treatment with LPS resulted in lower PO concentrations.

ProPO: There was a significant effect of developmental stages on the proportion of samples containing no recordable ProPO, with later developmental stages containing more samples with recordable ProPO (Figure 4.10, $F_{4,359} = 53.1$, $p<0.0001$, binomial errors). There was a significant effect of body size, developmental stage and treatment on ProPO activity for those samples that contained a recordable concentration of ProPO (Figure 4.12, Table 4.4). ProPO activity increased with age, with the LPS treated drones recording lower levels of ProPO activity over all.

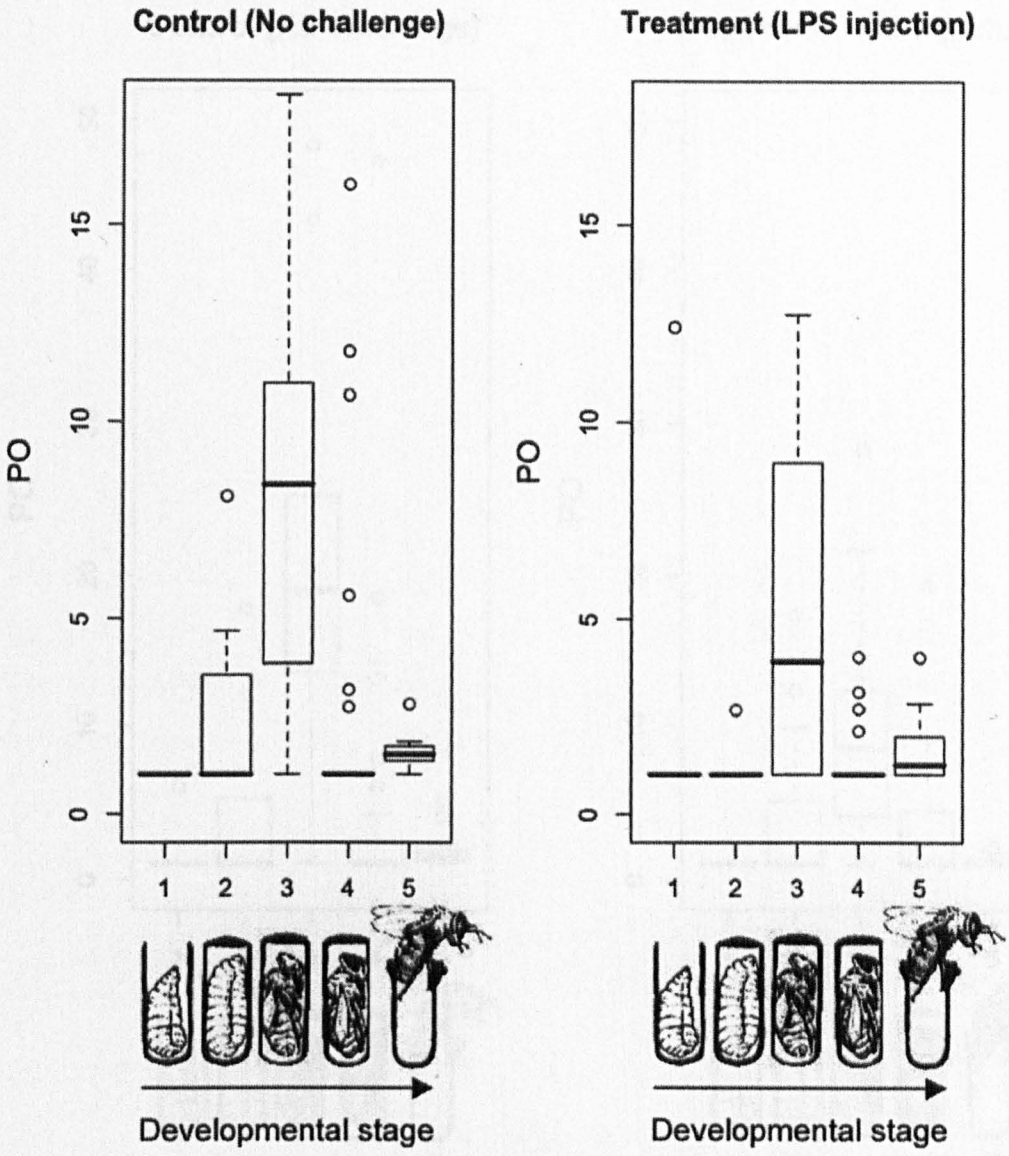


Figure 4.1. Hive D: PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. The larval and pupal stages contained a high proportion of individuals with no recordable PO concentration.

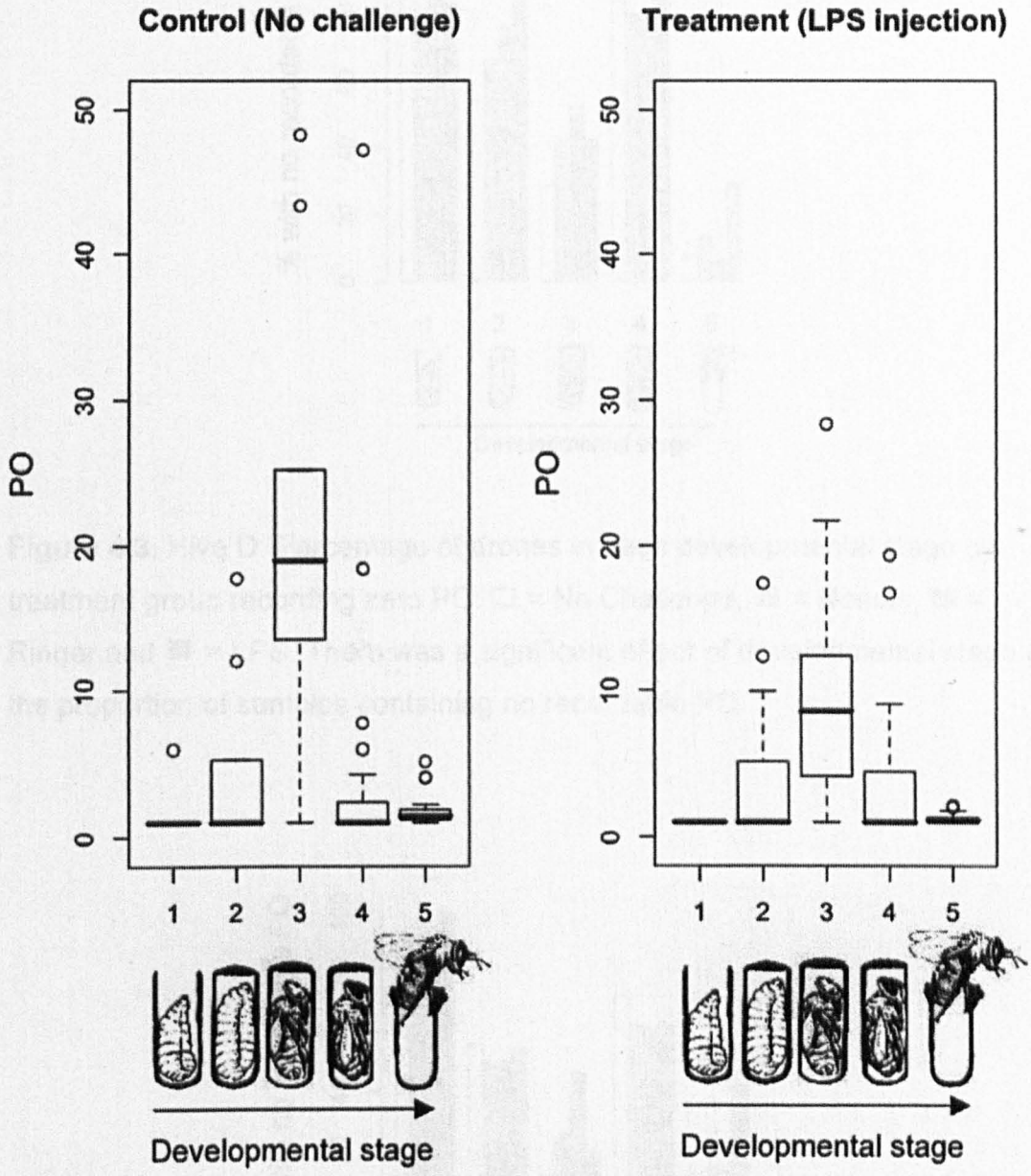


Figure 4.2. Hive E: PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. The larval and pupal stages contained a high proportion of individuals with no recordable PO concentration.

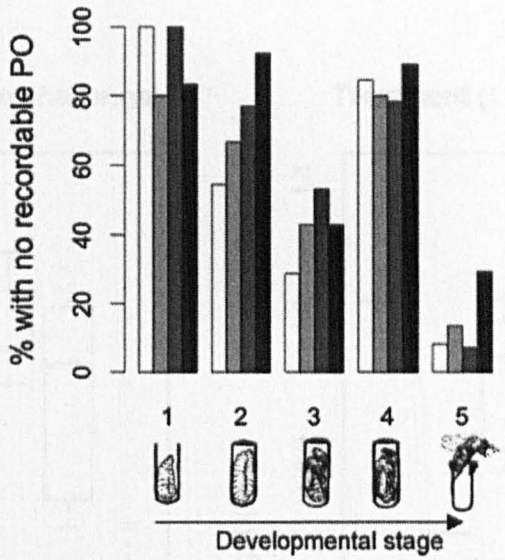


Figure 4.3. Hive D: Percentage of drones in each developmental stage by treatment group recording zero PO: □ = No Challenge, ■ = Needle, ■ = Ringer and ■ = LPS. There was a significant effect of developmental stage on the proportion of samples containing no recordable PO.

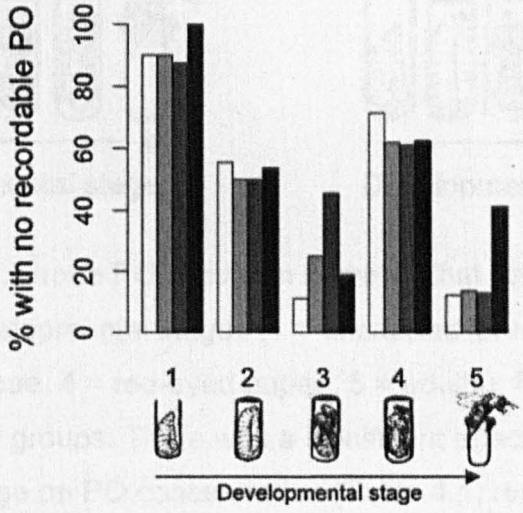


Figure 4.4. Hive E: Percentage of drones in each developmental stage by treatment group recording zero PO: □ = No Challenge, ■ = Needle, ■ = Ringer and ■ = LPS. There was a significant effect of developmental stage on the proportion of samples containing no recordable PO.

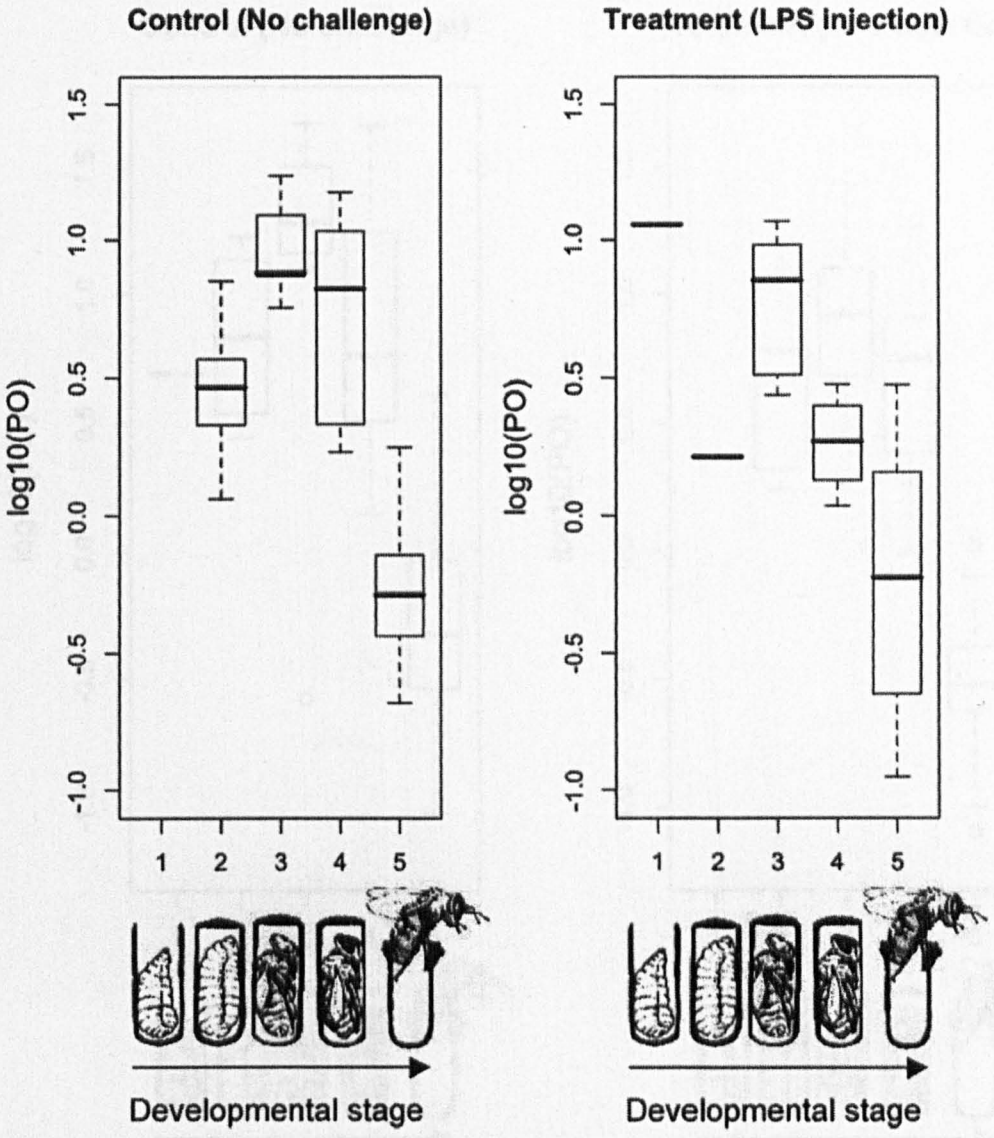


Figure 4.5. Hive D: Drone PO activity in samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size and developmental stage on PO concentration (Table 4.1, results are log10 transformed).

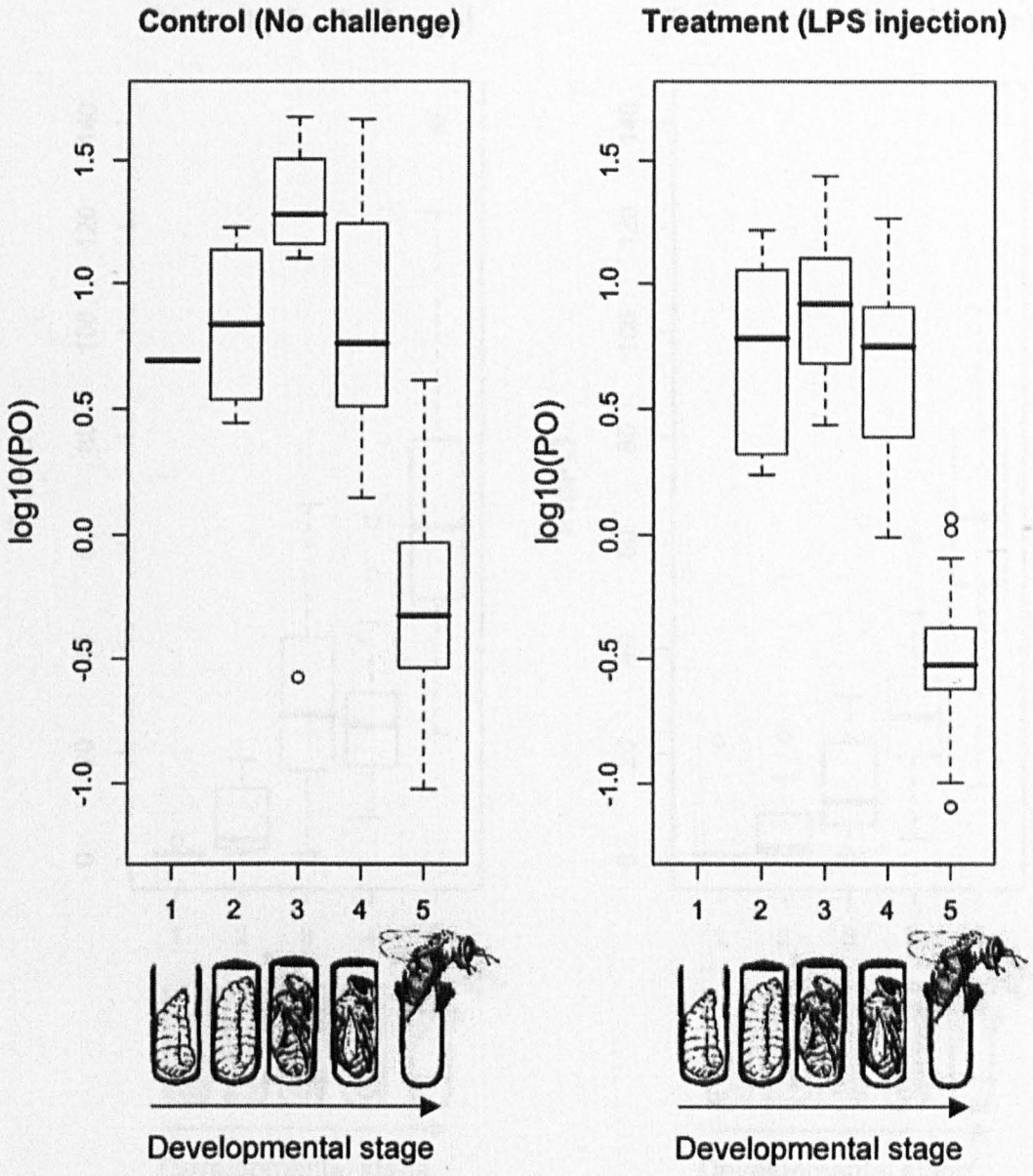


Figure 4.6. Hive E: Drone PO activity for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size, developmental stage and treatment on PO concentration (Table 4.2, results are log10 transformed,).

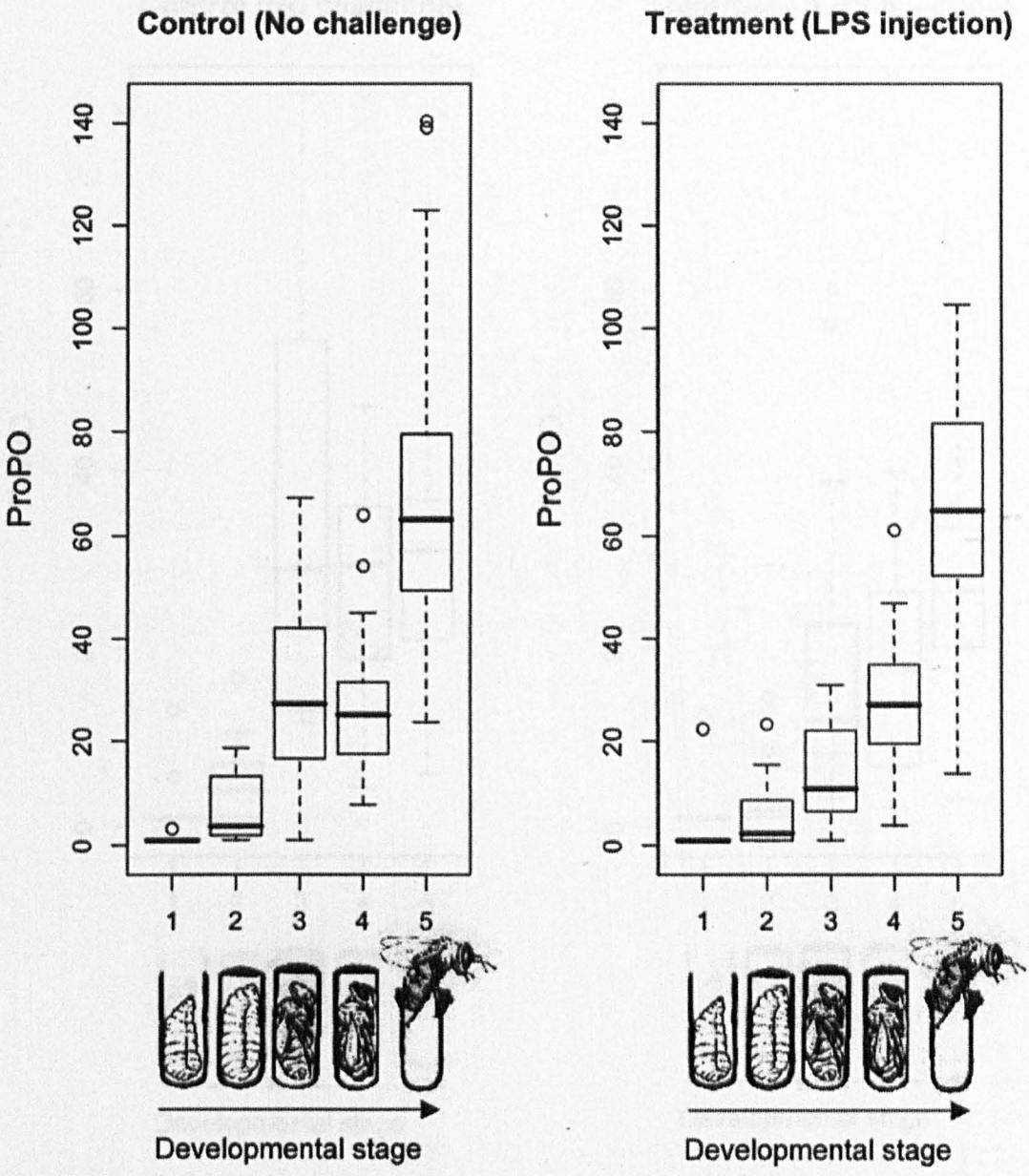


Figure 4.7. Hive D: ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. The larval stages commonly contained no recordable level of ProPO.

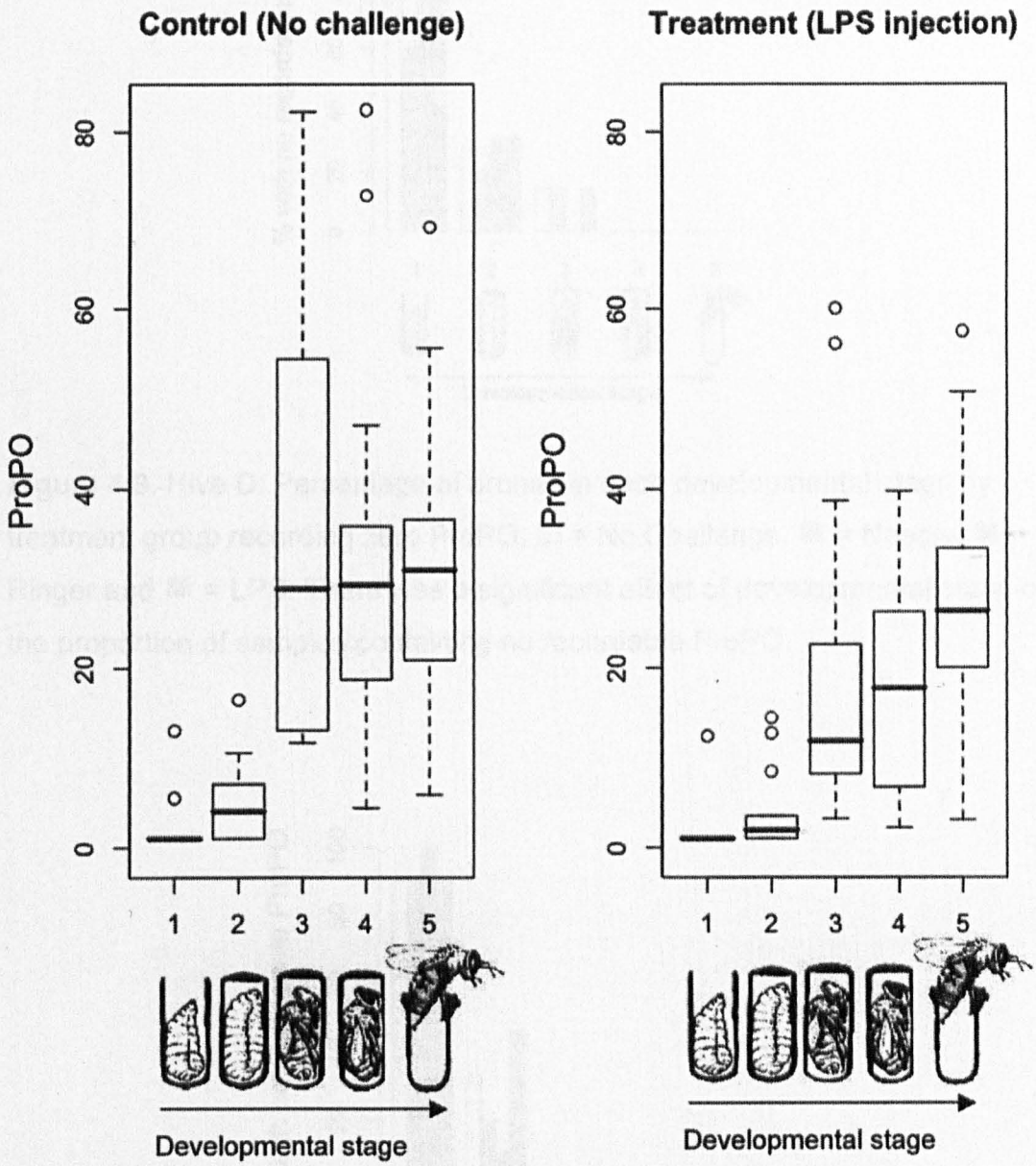


Figure 4.8. Hive E: ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. The larval stages contained a high proportion of individuals with no recordable ProPO activity.

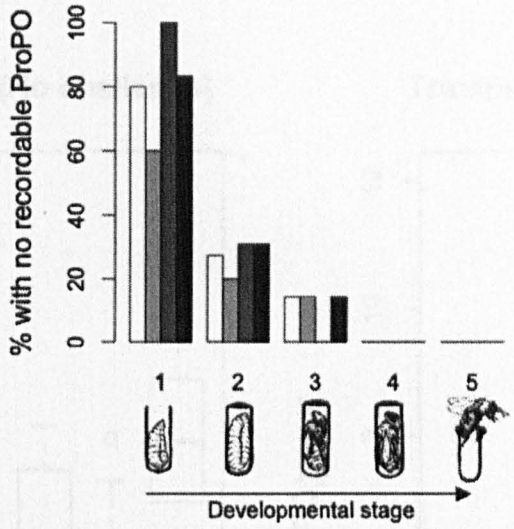


Figure 4.9. Hive D: Percentage of drones in each developmental stage by treatment group recording zero ProPO: □ = No Challenge, ▩ = Needle, ■ = Ringer and ■ = LPS. There was a significant effect of developmental stage on the proportion of samples containing no recordable ProPO.

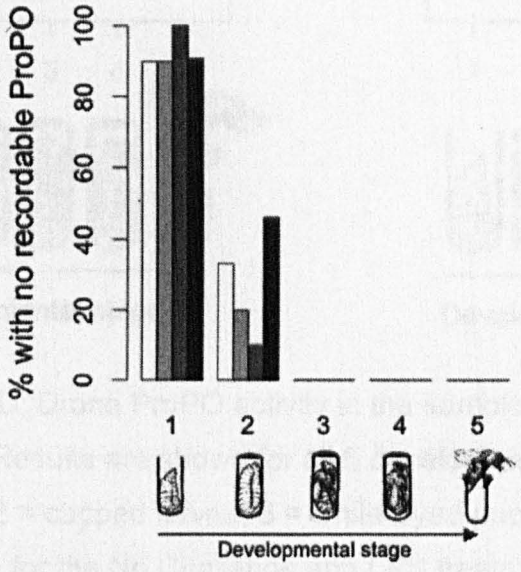


Figure 4.10. Hive E: Percentage of drones in each developmental stage by treatment group recording zero ProPO: □ = No Challenge, ▩ = Needle, ■ = Ringer and ■ = LPS. There was a significant effect of developmental stage on the proportion of samples containing no recordable ProPO.

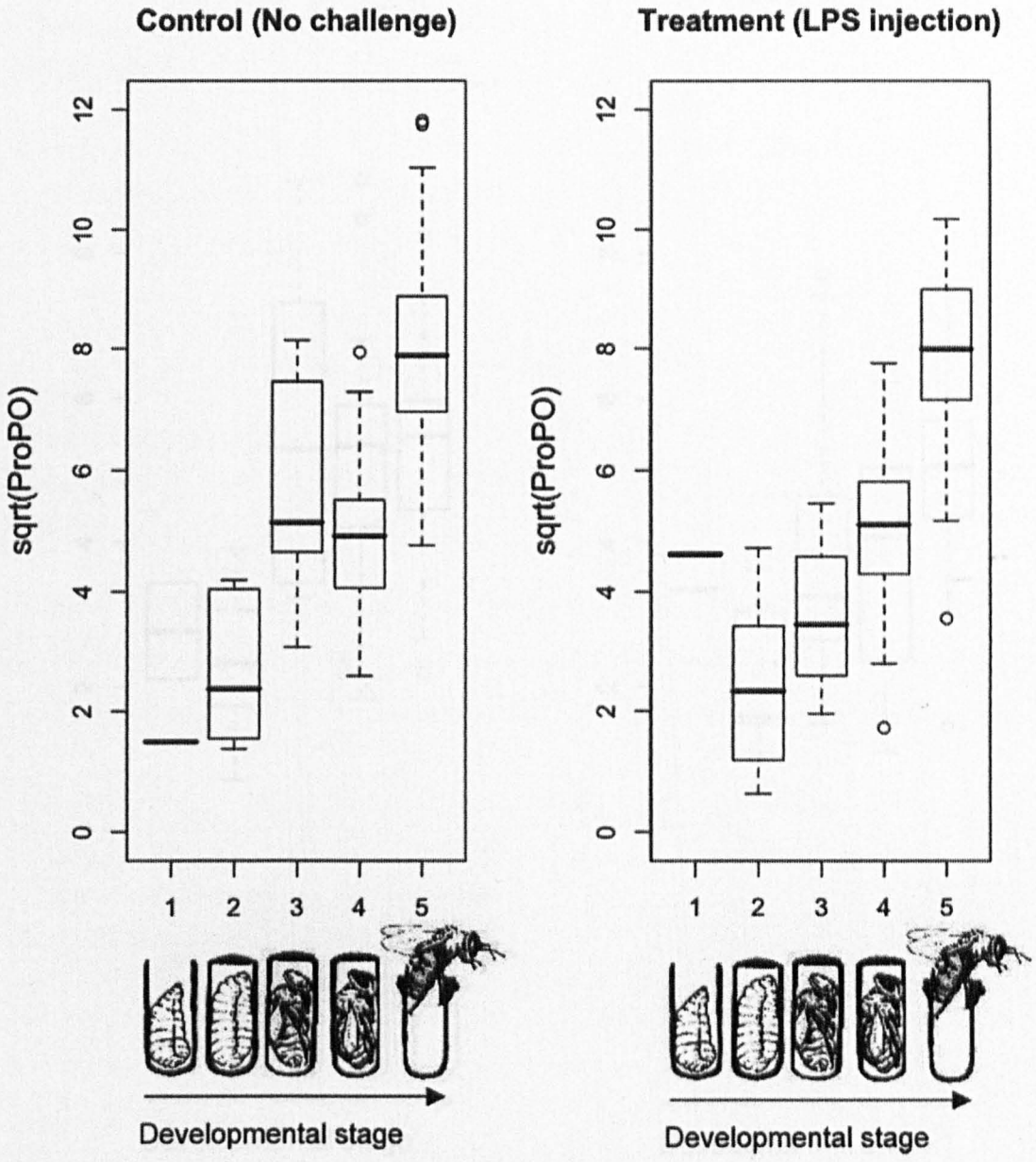


Figure 4.11. Hive D: Drone ProPO activity in the samples that returned a recordable value. Results are shown for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size and developmental stage on ProPO concentration (Table 4.3, results are square root transformed).

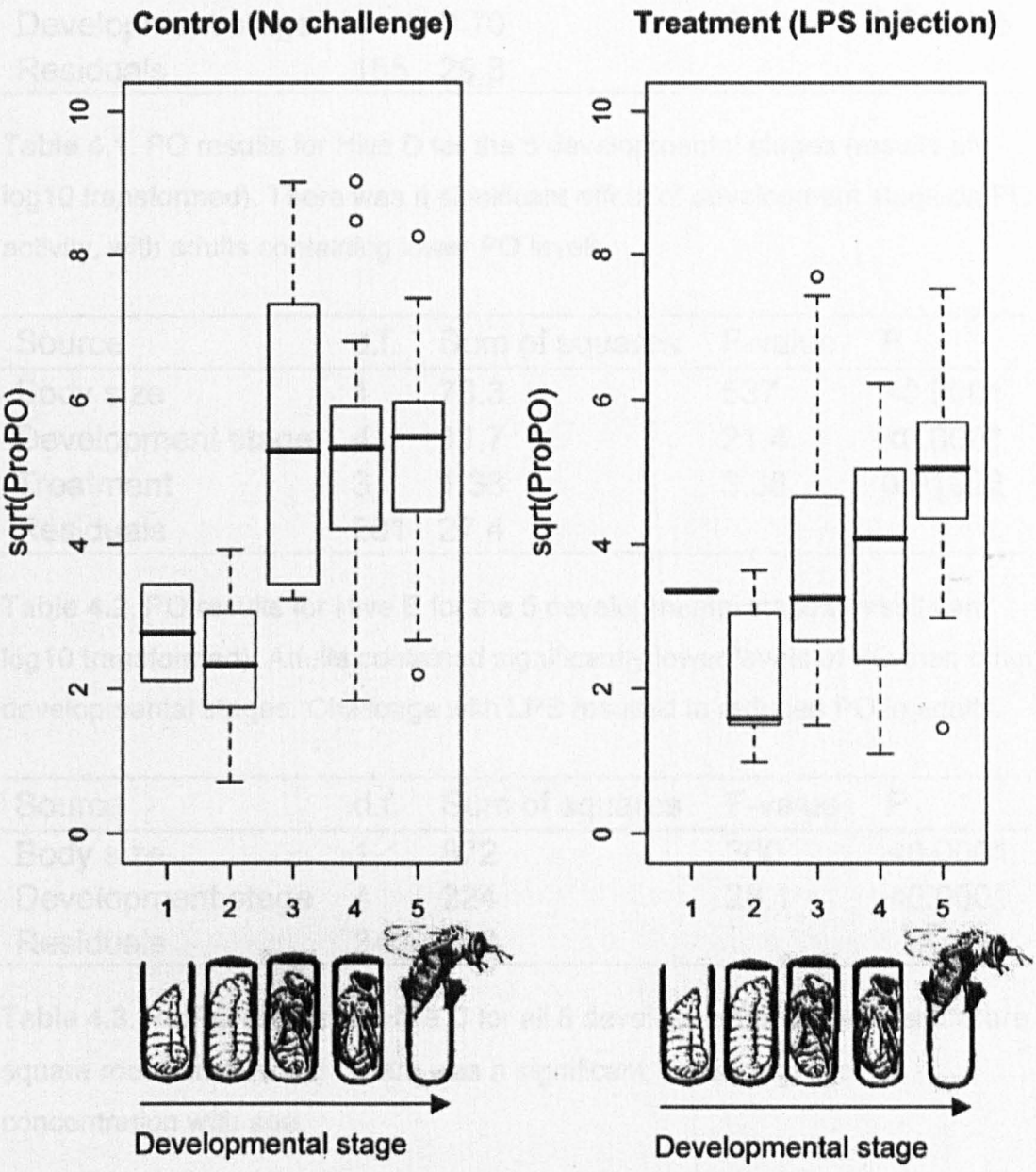


Figure 4.12. Hive E: Drone ProPO activity in samples that returned a recordable value. Results are shown for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for the No Challenge and LPS treatment groups. There was a significant effect of body size, developmental stage and treatment on ProPO activity (Table 4.4, results are square root transformed).

Source	d.f.	Sum of squares	F-value	P
Body size	1	30.6	159	<0.0001
Development stage	4	3.70	4.81	0.001095
Residuals	155	29.8		

Table 4.1. PO results for Hive D for the 5 developmental stages (results are log10 transformed). There was a significant effect of development stage on PO activity, with adults containing lower PO levels.

Source	d.f.	Sum of squares	F-value	P
Body size	1	73.3	537	<0.0001
Development stage	4	11.7	21.4	<0.0001
Treatment	3	1.38	3.38	0.01922
Residuals	201	27.4		

Table 4.2. PO results for Hive E for the 5 developmental stages (results are log10 transformed). Adults contained significantly lower levels of PO than other developmental stages. Challenge with LPS resulted in reduced PO in adults.

Source	d.f.	Sum of squares	F-value	P
Body size	1	872	360	<0.0001
Development stage	4	224	28.1	<0.0001
Residuals	340	822		

Table 4.3. ProPO results for Hive D for all 5 developmental stages (results are square root transformed). There was a significant increase in ProPO concentration with age.

Source	d.f.	Sum of squares	F-value	P
Body size	1	73.6	32.8	<0.0001
Development stage	4	112	12.5	<0.0001
Treatment	3	33.3	4.94	0.002296
Residuals	308	691		

Table 4.4. ProPO results for Hive E for the 5 developmental stages (results are square root transformed). There was a significant effect of age and treatment on ProPO activity. There was a general increase in ProPO concentration with age.

4.3.2 Age and Phenoloxidase

Mortality: 91 old drones (~50%) were recaptured from the original released sample. Of those that survived to be used in the experiment, there was a significant difference in the percentage mortality between the new and old drones, with new drones having an average 4% mortality rate, compared to 29% in the old drones ($F_{1,214} = 26.8$, $p<0.0001$).

PO: There was a significant effect of age and treatment on PO activity (Figure 4.13, Table 4.5). Old drones had higher concentrations of PO in the haemolymph. An immune challenge with LPS produced lower levels of PO in new drones, but there was no effect of immune challenge on PO activity in the old drones, where PO activity remained constantly high.

ProPO: There was a significant effect of treatment on ProPO concentration, with the LPS challenged groups having lower levels of ProPO activity than the controls (Figure 4.14, Table 4.6, $F_{3,148} = 2.97$, $p=0.03397$). This provides support for the hypothesis that ProPO is being activated into PO and utilised in an immune response faster than it can be manufactured and replaced. There was no effect of age on ProPO concentration.

4.3.3 Age and Antimicrobial Peptides (AMPs)

There was a significant effect of treatment on AMP response, with challenged individuals containing higher concentrations of AMPs. Figure 4.15 shows all of the results for the No Challenge and LPS treatment groups. A significant number of the No Challenge treatment samples did not produce a recordable AMP response (58% of young and 71% of old drones), which produced a positively skewed distribution ($F_{3,114} = 18.8$, $p<0.0001$, binomial errors). Samples that did not contain a recordable level of AMPs were removed from the data set before analysis, so that data conformed to normality.

Figure 4.16 shows the AMP results for No Challenge and LPS treatment groups, after the samples containing no recordable AMP were removed. There was a significant interaction of age and treatment on AMP concentration (Table 4.7). AMP activity increased with immune challenge, but production was lower in old, immune challenged drones. This decrease may be a sign of immune senescence.

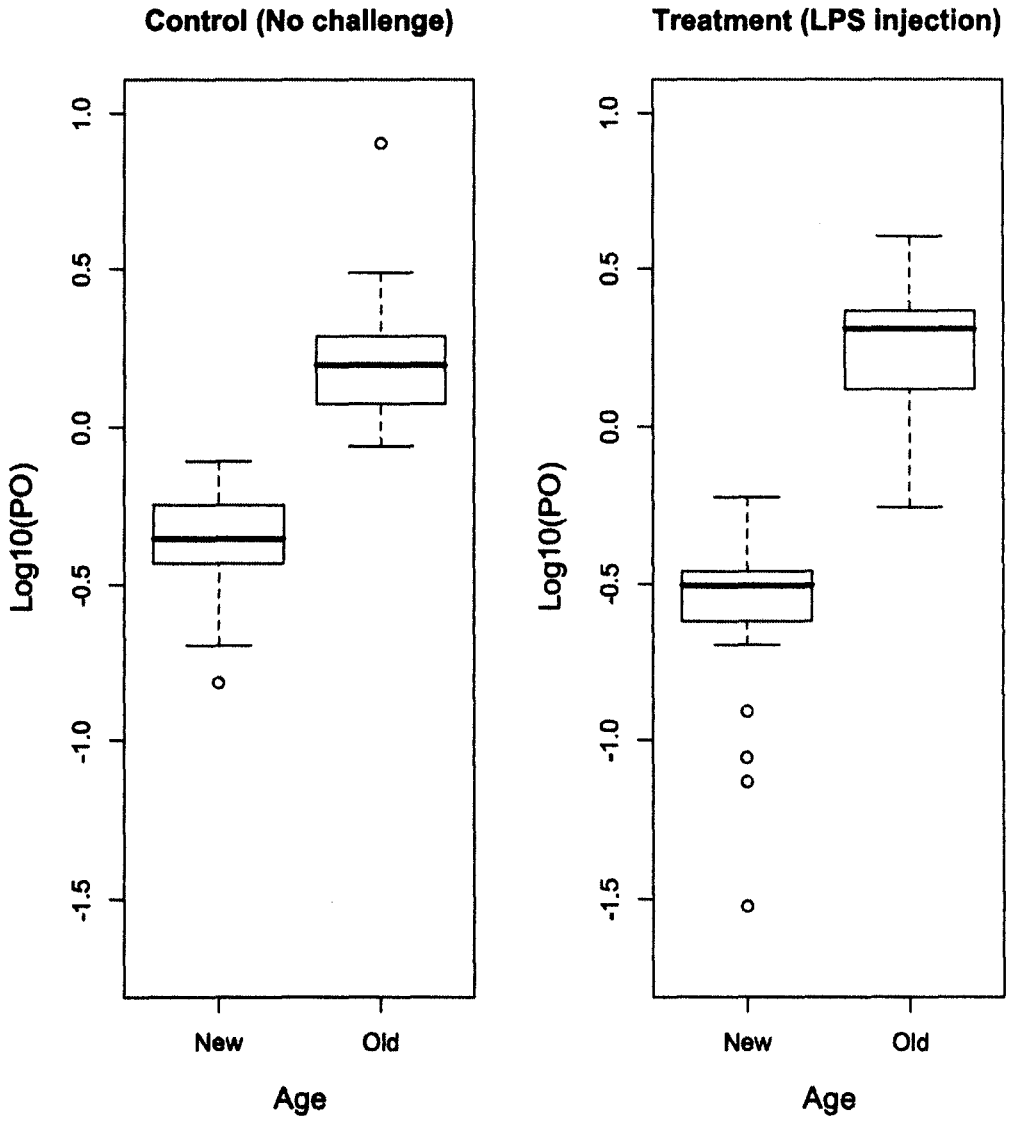


Figure 4.13. PO response of the two age groups, new and old drones, to the No Challenge and LPS treatment groups. There was a significant interaction with age and immune challenge, with new drones challenged with LPS producing lower PO concentrations than the controls (data are log10 transformed, see Table 4.5).

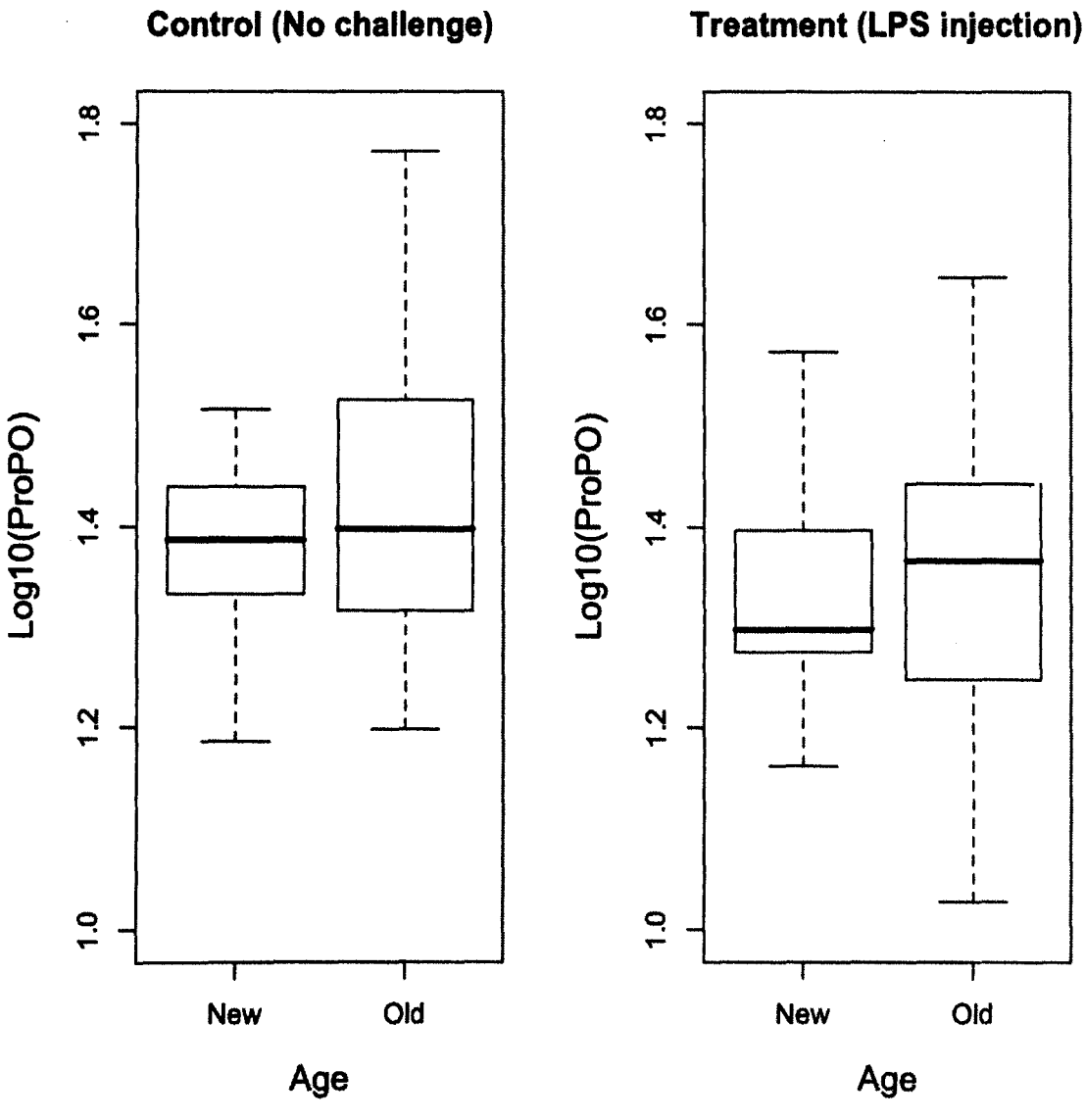


Figure 4.14. ProPO response of the two age groups, new and old drones, to the No Challenge and LPS treatment groups. There was a significant effect of immune challenge on ProPO concentration, with LPS challenged drones producing lower ProPO concentrations than the controls (data log10 transformed, see Table 4.6).

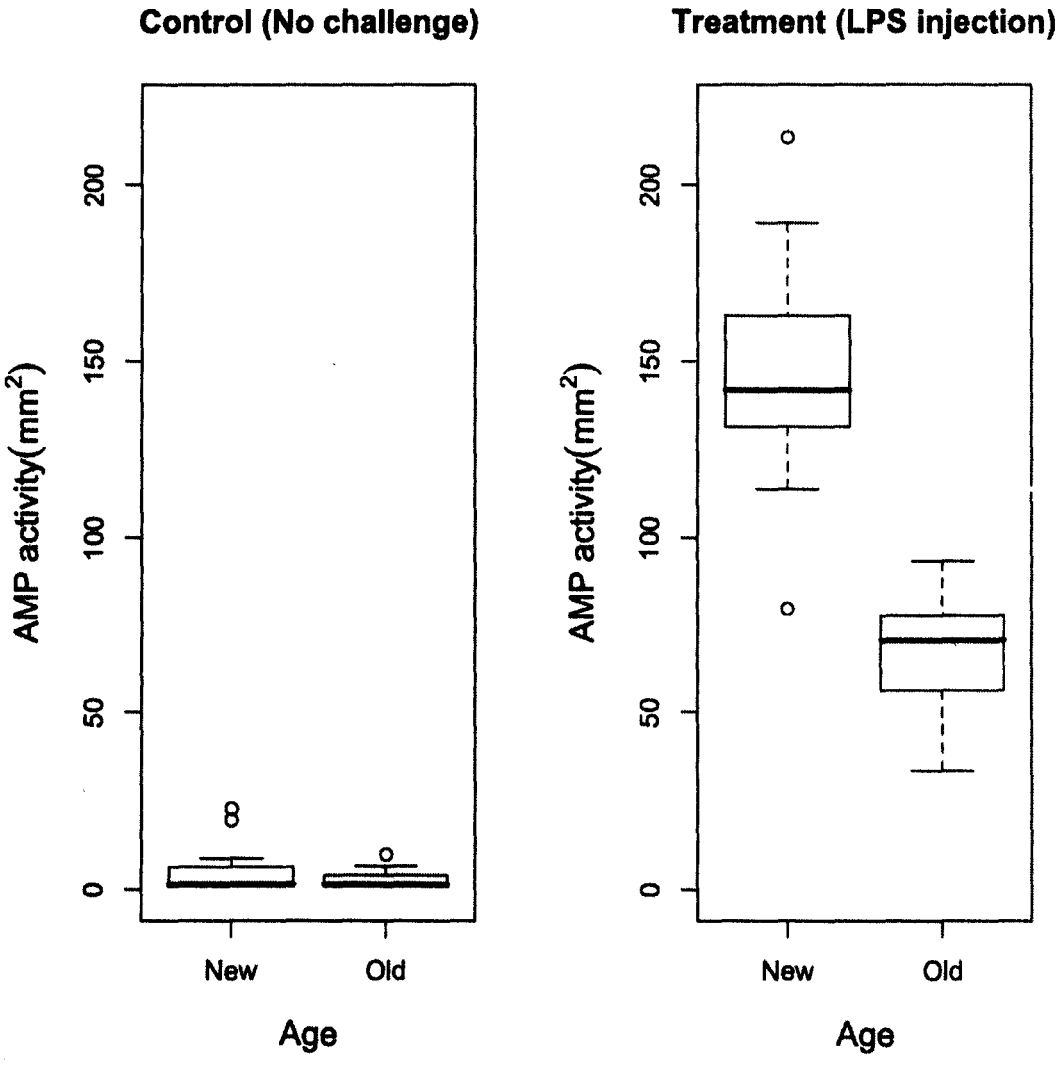


Figure 4.15. Antimicrobial peptide (AMP) response for new and old drones to the No Challenge and LPS treatment groups. The figure shows the results for all samples. AMP activity was measured as the area of bacterial growth inhibition (mm^2). A significant number of the No Challenge samples did not contain any recordable AMPs, which produced a positively skewed distribution. These results were removed before analysis (Figure 4.16, Table 4.7).

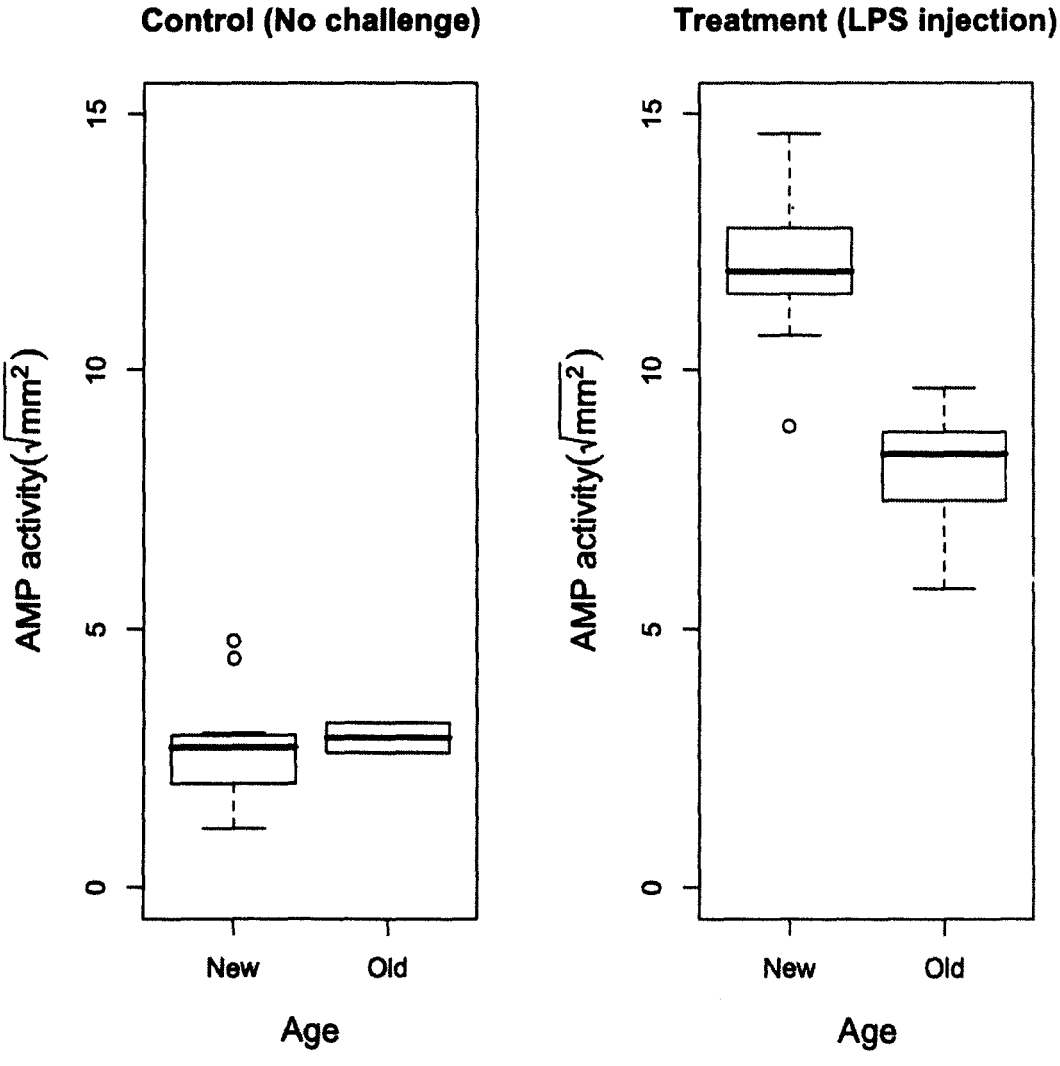


Figure 4.16. Antimicrobial peptide (AMP) response for new and old drones to the No Challenge and LPS treatment groups, for those samples that contained a recordable level of AMPs. AMP activity was measured as the area of bacterial growth inhibition (mm^2). There was a significant interaction with age and treatment (Table 4.7, data are square root transformed). AMP production increased with immune challenge, but this increase became less with age.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.0510	1.22	0.2710
Age	1	16.8	402	<0.0001
Treatment	3	0.670	5.35	0.001589
Residuals	147	6.14		

Table 4.5. PO results for new and old drones. PO response increased with age, but levels were lower following an immune challenge (data are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.00505	0.306	0.5810
Treatment	3	0.147	2.97	0.03397
Residuals	148	2.44		

Table 4.6. ProPO results for new and old drones. ProPO responses were lower in new drones following an immune challenge, but in general levels were similar across both age groups (data are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	49.4	19.6	<0.0001
Age	1	18.8	7.46	0.007688
Treatment	3	1130	150	<0.0001
Age * treatment	2	31.7	6.30	0.002849
Residuals	83	209		

Table 4.7. Results for AMP production in new and old dones. There was a significant interaction of age and treatment (data are square root transformed).

4.4 Discussion

This chapter has demonstrated that PO, ProPO and AMP are all present in drones, and that the allocation of resources towards these responses varies throughout development and adult life. Larvae and pupae have little immune activity, which may be a response to the low risk of parasite infection in brood. However, adult drones do invest in immune responses, although age has a significant impact on the level of response.

4.4.1 Developmental Immunity

The presence of large numbers of developmental samples containing no recordable levels of PO activity are unlikely to be caused by experimental error due to the extensive investigation of the PO assay carried out in Chapter 2 and Appendix 1 Section A1.3. The proportion of samples containing no recordable levels of PO during development may be interpreted in two ways. Firstly, if the data for red-eyed pupae are removed from Figures 4.3 and 4.4, the proportion of samples with a recordable level of PO activity increases linearly with developmental age. The high proportion of red-eyed pupae containing no recordable PO levels may be due to a stage-specific physiological process. Spermatogenesis and spermiogenesis occur at this stage, and PO may be required for these processes (Page & Peng, 2001).

The second interpretation of the proportion data is that developmental stages up to adult have low levels of active PO in the haemolymph, but that there is an increase in recordable PO levels during the white-eyed pupal stage, and to some extent the capped larval stage, similar to that seen in workers (Section 3.3.2). On average, 77% of the white-eyed pupae samples contained a recordable concentration of PO, compared to an average of 24% for all the other developmental stages (excluding adults), which suggests that its presence is unlikely to be due to experimental error. This increase in PO activity may be connected to physiological processes associated with the formation of the pupal stage (Chumurzynska & Wojtczak, 1963; Hopkins & Kramer, 1992; Russell & Dunn, 1996). Both of these scenarios suggest that it is unlikely that PO is being used for immunity during development, which questions whether drones utilise PO for an immune response at all. However, if we look at the proportion of newly emerged adult drones with recordable levels of PO we see a different pattern. An average of 11% of drones from the control groups in both hives did

not contain a recordable concentration of PO. This proportion rose to 35% for the LPS challenged adults, supporting my theory that PO levels decrease as it is used in response to an immune challenge (S. Povey, S. Slos, pers. comm.), and that drones may, in adult life, employ it as an immune defence.

The presence of ProPO increased with developmental age, and the older developmental stages showed a significant effect of immune challenge on ProPO activity. In contrast to the workers, ProPO was much lower in the early larval stages. In line with the first interpretation of the PO results, this suggests that drones may not use ProPO as an immune response, and only employ it in the latter developmental stages when it is required for cuticle formation and melanisation (Chumurzynska & Wojtczak, 1963; Hopkins & Kramer, 1992).

ProPO results for workers showed little response to immune challenge during development, although ProPO activity was maintained at a high concentration throughout development (Section 3.3.2). PO levels in developing workers did not show the stage-wise increase as suggested in drones, although workers were seen to use PO in response to an immune challenge in the pupal and adult stages. These results suggest that neither workers nor drones employ PO and ProPO for an immune defence in the pre-pupal developmental stages, which may be due to lack of resources and/or a lack of need. The findings also highlight the differences in levels of investment in PO and ProPO between the two castes at different life stages, the causes of which may only be speculated.

There was a significant negative relationship of body size with PO and ProPO for both hives. In the developmental stages measured, body size (measured as wet weight) decreases with age, as larvae stop feeding and undergo metamorphosis. Therefore, adults had the lowest body weight, but the highest immune response. Incorporating body size into the analysis controls for this effect.

4.4.2 Age and Immunity

Results of the changes in immune response with age showed an increase in PO activity with age, at a similar level to that found in workers (Section 3.3.3). This suggests that adults in both sexes maintain a higher level when outside the hive, possibly in response to increased exposure to pathogens. There was no effect of immune challenge on PO in old drones (an effect also seen in workers): the continual maintenance of such elevated levels of PO activity may

mean that any drops caused by response to a challenge are not seen. Alternatively, the increase of PO with age in drones may be associated with the onset of sexual maturity, and not a role in immunity. There is some evidence that PO is involved in production of gametes in females (Li *et al.*, 1996; Bai *et al.*, 1997), and this may be the case in drones. However, as with the previous experiment, new drones responded to a challenge with LPS with a decrease in PO concentration, which suggests an immune function.

ProPO levels were maintained at a constant level throughout adult life, a level that is lower than that found in corresponding workers (Section 3.3.3). LPS challenged drones had a lower ProPO response than the controls, which suggests that, as in workers, ProPO does have a role in immunity in adult life. It should be remembered when making comparisons with the immune response in workers that drones are twice the size, so although immune responses may be of a similar magnitude, this cost represents a lower absolute level of investment.

Unlike workers, AMP production was only induced following an immune challenge. The amount of AMPs produced in drones when challenged was lower than that found in the corresponding worker age groups, suggesting that drones have fewer resources to invest in this immune response. If PO and ProPO have limited use as immune defences in the drone, the importance of induced responses, such as AMPs, to the immune arsenal is amplified. As seen in workers, levels of AMP production decreased with age, even when challenged, which may be an indicator of immune senescence (Doums *et al.*, 2002; Hillyer *et al.*, 2005). Recent research has found a decrease in the fat body mass with age in workers (Wilson-Rich *et al.*, 2008). If such a decrease also exists in drones, limits on functioning ability of the fat body would support the decrease in AMP production seen. Alternatively, as sperm viability reaches its peak in 2-week old drones, resources from the fat body may be needed for sperm production in the older, sexually mature males (Page & Peng, 2001).

4.5 Summary

This chapter has investigated the impact of different aspects of aging on immunity in drone bees. Using assays developed in Chapter 2, I have shown:

1a. Variation in the PO activity in developing drones, with all developmental stages containing samples with no recordable PO.

White-eyed pupae have the highest level of PO activity (Section 4.3.1)

1b. An increase in ProPO activity with developmental stage, with the uncapped larval stage commonly having no recordable levels of ProPO (Section 4.3.1)

2a. PO activity increased with adult drone age (Section 4.3.2)

2b. ProPO activity remained constant throughout adult drone life (Section 4.3.2)

2c. AMPs were produced in response to an immune challenge, but the level of response was significantly reduced with age (Section 4.3.3).

Chapter 5

Varroa destructor and the honey bee immune response

5.1 Introduction

A. mellifera is subject to a range of internal and external parasites (Bailey & Ball, 1991). The ectoparasitic mite *Varroa destructor* is one of the most destructive parasites of managed honey bee colonies (De Jong *et al.*, 1982; Sammataro *et al.*, 2000) and is thought to be the cause of parasitic mite syndrome, or *Varroa*sis, a disease which, if left untreated, may destroy colonies worldwide (Ball & Allen, 1988; Martin *et al.*, 1998; Martin, 2001a).

5.1.1 *Varroa destructor*

First discovered in 1904, the *Varroa* mite shifted from its natural host, the Asian honey bee, *A. cerana*, to *A. mellifera* in 1957 when *A. mellifera* was introduced into areas inhabited by *A. cerana* (Oldroyd, 1999; Gregory *et al.*, 2005). From this initial infection it rapidly spread to *A. mellifera* colonies worldwide, although it was not found in the UK until 1992 (Bailey & Ball, 1991; Oldroyd, 1999). Adult female mites (Figure 5.1, A and B) are reddish-brown in colour, 1mm long, dorso-ventrally flattened, with a concave dorsal shield and weigh approx. 0.14mg (De Jong *et al.*, 1982; Rath, 1999). Their shape allows them to slide between sternal plates avoiding detection and removal during grooming (Rath, 1999). The males are smaller and lighter in colour (Sammataro *et al.*, 2000). The adult female mites live either attached to an adult honey bee (phoretic phase, Figure 5.1C), or within a sealed drone or worker cells where they undergo the reproductive phase of their lifecycle (Martin, 2001b). Mites move between adult hosts, preferentially moving onto nurse bees over foragers, and preferring drone brood to worker brood for the reproductive phase (Otten & Fuchs, 1988; Boot *et al.*, 1992; Fuchs, 1992; Kuenen & Calderone, 1997).

Mites can only survive off the host for 18 – 70 hours, depending on the substrate (de Guzeman *et al.*, 1993). They reproduce on a 10-day cycle within capped brood cells (Figure 5.2). Female mites attached to nurse bees drop off into larval brood cells. A female enters a brood cell approximately 24 hours prior to capping for worker cells, and 48 hours prior to capping for drone cells. The

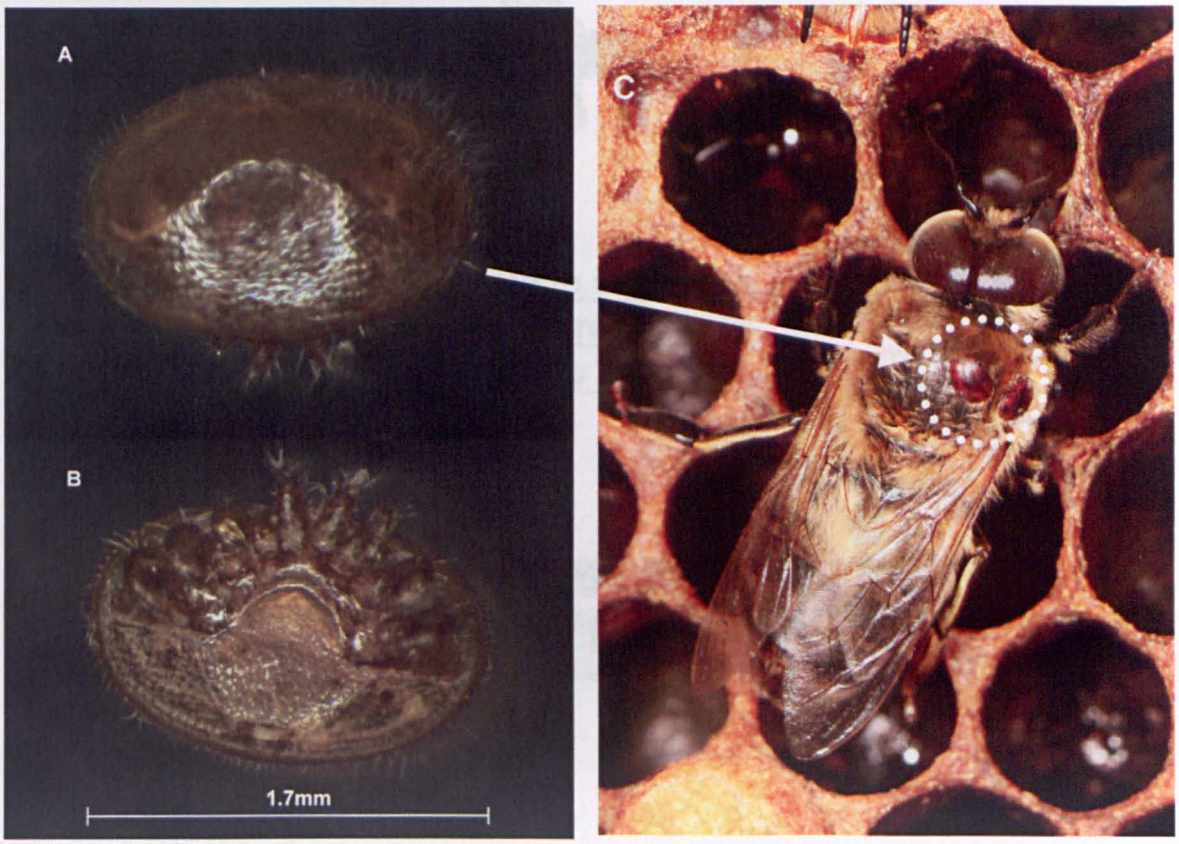


Figure 5.1. Dorsal (A) and ventral (B) side of adult female *Varroa*. Female offspring emerge on bees as they eclose and can be seen clinging to the thorax (C, two mites on thorax of a newly eclosed drone), or more often slide in between the sternal plates (photos by R. Naylor).

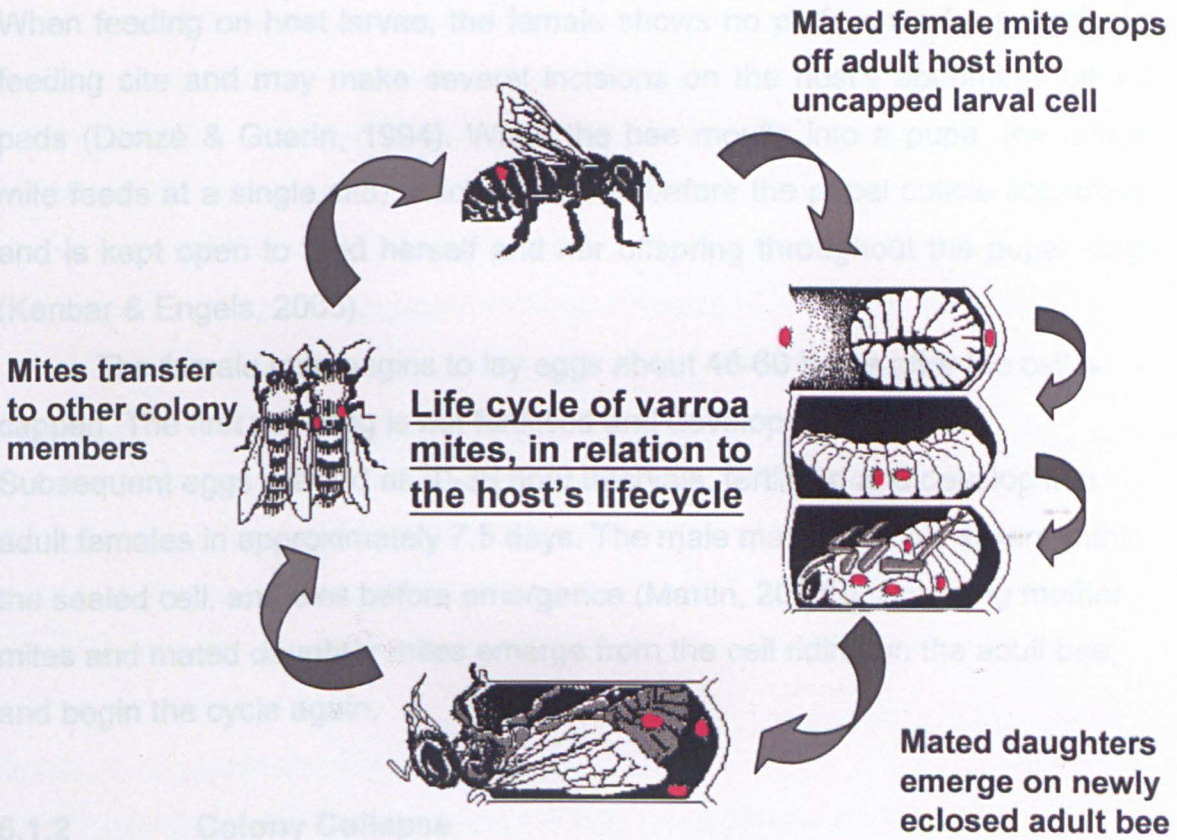


Figure 5.2. Life cycle of the *Varroa* mite. Female mites, depicted in red, drop from the host into uncapped larval cells. The reproductive cycle, which lasts about 10 days, occurs within capped brood cells. New adult bees emerge from their cells carrying the mated female offspring, which are then free to transfer to other bees during close contact, and seek another cell in which to repeat the cycle.

female mite submerges herself in the larval food (provided by the nurse bees). After the cell has been capped the larva eats all remaining food, so freeing the mite, which begins to feed on the larva. The mite feeds on the host's haemolymph, which is obtained by piercing the inter-segmental membrane of adults, or the cuticle of pupae with their chelicerae (Sammataro *et al.* 2000). When feeding on host larvae, the female shows no preference for a particular feeding site and may make several incisions on the host's abdominal pleural pads (Donzé & Guerin, 1994). When the bee moults into a pupa, the female mite feeds at a single site, which is chosen before the pupal cuticle sclerotises and is kept open to feed herself and her offspring throughout the pupal stage (Kanbar & Engels, 2003).

The female mite begins to lay eggs about 48-60 hours after the cell is capped. The first mite egg is not fertilised and develops into a male. Subsequent eggs are laid at 30-36 hour intervals, fertilised and develop into adult females in approximately 7.5 days. The male mates with his sisters within the sealed cell, and dies before emergence (Martin, 2001b). Surviving mother mites and mated daughter mites emerge from the cell riding on the adult bee, and begin the cycle again.

5.1.2 Colony Collapse

The mechanism by which *Varroa* can cause the collapse of a bee colony is not properly understood. "Parasitic mite syndrome" is a non-specific term that refers to colonies simultaneously infested with mites and infected with viruses, with a high mortality rate (Shimanuki *et al.*, 1994). Symptoms of parasitic mite syndrome include morphological deformities in adult bees, reduced flying and learning ability, increased mortality of brood and general colony weakening, with colony collapse occurring within four years after infestation (Shimanuki *et al.*, 1994; De Jong & De Jong, 1983; Akratanakul & Burgett, 1975; Hung *et al.*, 1995; Martin *et al.*, 1998; Sammataro *et al.*, 2000; Kralj *et al.*, 2007). The highest death rate in colonies occurs during the winter, as mite populations are allowed to build up in a relatively small, and resource limited population of bees (De Jong *et al.*, 1982). In addition, the cessation of drone production towards the end of the summer causes mites to switch to reproducing solely in worker cells. This increased pressure on workers may contribute towards colony collapse during the winter months.

The feeding of *Varroa* on bees has been shown to cause a reduction in the protein content of the haemolymph (Glinski & Jarosz, 1984), weight loss of up to 25% in the newly emerged bee (De Jong *et al.*, 1982), reduced longevity and wing and body deformity (De Jong & De Jong, 1983; Akratanakul & Burgett, 1975). Wing deformity may be due to reduced hydrostatic pressure brought about through loss of haemolymph (Daly *et al.*, 1988). Higher numbers of mites feeding on each bee leads to a greater proportion of emerging bees exhibiting deformities (De Jong *et al.*, 1982). However, bees with a single or no mites can develop deformities, and bees with high numbers of mites can show no deformity at all (Bowen-Walker *et al.*, 1999). There is no direct evidence of a link between mite populations and colony collapse, and similar sized mite populations have been found in both collapsing and surviving colonies (Martin *et al.*, 1998; Martin, 2001a). Consequently doubts are raised over whether mites acting alone cause bee deformity and colony death. It is suggested that these symptoms are in fact due to the vectoring of viruses or other pathogenic agents during mite feeding (Ball, 1985; Hung *et al.*, 1995; Hung *et al.*, 1996; Martin *et al.*, 1998).

5.1.3 Deformed Wing Virus

There are 18 viruses known to infect honey bees (Bailey & Ball, 1991, Allen & Ball, 1997). Of these, deformed wing virus (DWV) is a positive, single-stranded, picorna-like RNA virus, belonging to the genus *Iflavirus* (Christian *et al.*, 2002), with isometric particles approximately 20-30nm in diameter (Allen & Ball, 1996; Chen *et. al.*, 2004a). *Varroa* did not arrive in the UK until 1992, but like many other honey bee viruses, DWV can persist in a population as a covert infection (Dall, 1985; Bailey, 1967; Tentcheva *et al.*, 2004; Yang & Cox-Foster, 2005), and can be detected in adult workers of *Varroa*-free colonies in the UK (Martin, 2001a).

DWV replicates slowly, and infected pupae either die, or emerge with deformed or poorly developed wings, a shortened abdomen and discolouration; symptoms which were previously attributed to the feeding activity of *Varroa* (Ball, 1989; Bailey & Ball, 1991; Bowen-Walker *et al.*, 1999). Infected bees also show a reduction in lifespan (Kovac & Crailsheim, 1988). DWV can cause mortality in naturally infected populations, but is thought to have increased in prevalence due to transmission by *Varroa* (Ball, 1997).

Mites may affect a colony by vectoring DWV, or by the activation of covert infections (Ball & Allen, 1988; Bowen-Walker *et al.*, 1999; Brodsgaard *et al.*, 2000). In the most straightforward scenario, the mites could directly transmit DWV during feeding (Bowen-Walker *et al.*, 1999; Hung *et al.*, 2000; Nordstrom, 2003; Ongus *et al.*, 2004; Tentcheva *et al.*, 2004; Shen *et al.*, 2005a; Shen *et al.*, 2005b). Alternatively, there is some evidence that the presence of more mites may increase the stress exerted on the pupae, either through loss of haemolymph or increased immune suppression, to an extent which triggers the replication of latent infections (Anderson & Gibbs, 1988; Ball & Allen, 1988; Bowen-Walker *et al.*, 1999; Brodsgaard *et al.*, 2000; Chen *et al.*, 2004c; Yang & Cox-Foster, 2005). Significantly higher levels of DWV are found associated with mite infestations (Shen *et al.*, 2005b), and there is a strong body of evidence that mites can effectively transmit DWV in the field (Ball & Allen, 1988; Bowen-Walker *et al.*, 1999; Nordstrom, 2003; Chen *et al.*, 2004b).

5.1.4 *Varroa* and the Immune Response

Recent studies have started to investigate the impact of ectoparasites on the immunity of invertebrate hosts (Rivers *et al.*, 2002), and more specifically, of *Varroa* on the honey bee (Gregory *et al.*, 2005; Yang & Cox-Foster, 2005; Yang & Cox-Foster, 2007).

The population dynamics of *Varroa* closely follows that of the bees but lags a month behind, peaking during August and September, and within two years can exceed thresholds for colony survival to the following summer (Martin, 2001a). As the threat of *Varroa* cycles annually with the bee population, it may be adaptive for bees to up-regulate their immune response as the season progresses, in line with increasing *Varroa* infestation levels.

The act of puncturing the cuticle of the pupae or adult bee during feeding produces a wound, the healing of which is initiated by an accumulation of haemocytes, a cellular immune response (Kanbar & Engels, 2003). The process of scarring and healing increases towards the end of the pupal phase, although large wounds may not heal until just before adult emergence, providing a longer window of opportunity for secondary pathogen infections (Kanbar & Engels, 2003). However, all wounds are healed before the adult bee emerges, preventing secondary infection from pathogens outside the brood cell.

There is some evidence that mite-parasitised bees have a reduced survival, and are less able to mount an immune response to secondary infections (Yang & Cox-Foster, 2005; Yang & Cox-Foster, 2007). Wounds are known to harbour a wide variety of bacteria, including foulbrood agents *Melissococcus pluton* and *Paenobacillus larvae* (Glinski & Jarosz, 1992; Ball, 1997; Kanbar & Engels, 2003). Bacterial infections of wounds do not carry a corresponding mortality rate, suggesting that such secondary infections are not directly fatal to the bees, and the level of infection does not differ between mite infested and non-infested bees (Kanbar & Engels 2003; Gregory *et al.*, 2005). However, bacterial infections have been shown to stimulate the multiplication of DWV, so although secondarily infected bees may survive to eclosion, their survival may be severely compromised during their adult life (Yang & Cox-Foster, 2005).

Little is known about other cellular and humoural immune responses to *Varroa* infestation. The response of antimicrobial peptide transcripts seems to vary between the peptides they encode, although all show some level of suppression with mite infestation (Gregory *et al.*, 2005; Yang & Cox-Foster, 2005). The expression of genes encoding PO was found to be lower in *Varroa* infested bees compared to those without mites, within a heavily infested colony (Yang & Cox-Foster, 2005). As it is not known how these levels relate to unparasitised colonies, it is difficult to gain a measure of the direct cost of the mites on the immune response.

5.1.5 The Control of *Varroa*

The chemical control of *Varroa* is difficult. As *Varroa* must live on the bee hosts, only pesticides that cause little or no harm to the bees can be used. Whilst various acaricides have been produced around the world, no single one is able to completely eliminate *Varroa* from a colony (De Jong *et al.*, 1982). A large part of this problem is due to the fact that once sealed inside brood cells, mites cannot be targeted by the acaricides. On top of this, honey produced by colonies must be fit for human consumption, and so cannot have absorbed the pesticide. Currently the most popular treatment in the UK are the acaricide strips "Apistan", polymer beehive strips containing 824mg of the pyrethriod tau-fluvalinate (10.3% w/w), which has acaricidal properties but is tolerated by bees. The strips are suspended between brood frames for a period of between 6-8 weeks, which is a short enough time to prevent the evolution of resistance.

Apistan is applied in late summer and after the main honey is harvested to prevent contamination of honey, and to reduce mite numbers before bees overwinter. However, even with controlled and rotational use, resistance to pyrethriods has been detected in *Varroa* across Europe since 1991 (Trouiller, 1998).

5.1.6 Chapter Predictions

Given what we know about immune response to pathogen infections in other insects, we could make some predictions about the response of bees to the *Varroa* mite. Throughout this chapter, I will refer to "immune investment" as the level of PO or ProPO measured in an individual i.e. the level of resources maintained in an individual available for an immune response, and "immune response" as the difference in PO and ProPO levels between the control and challenged groups of bees. Following this, I predict that:

1. Colonies will up-regulate their levels of immune investment in response to the increasing threat of a parasite infection. By "stockpiling" resources in the form of PO and ProPO, bees will be better able to respond to a parasitic infection
2. If bees face extra stress on their resources to maintain an elevated immune investment when the threat of *Varroa* infestation is high, any additional immune challenge (LPS) will result in a greater immune response (larger drop in PO and ProPO levels) than that seen in *Varroa*-free colonies
3. Successful removal of *Varroa* with a pesticide will result in lowered levels of immune investment in the colony
4. There will be an effect on the immune response in bees infested with *Varroa*, with and without deformed wings (symptomatic of DWV), but the direction of this change is unpredictable.

5.1.7 Chapter Aims

The impact of mite infestations on honey bee immunity is largely unknown. This chapter aims to examine the effect of mite population dynamics on the phenoloxidase (PO) and prophenoloxidase (ProPO) responses in worker bees. Using the assays developed in Chapter 2, I will investigate:

1. The immune response to *Varroa* in infested colonies, in terms of a) PO and b) ProPO (Section 5.2.1)
2. The effect of eliminating a mite population using an acaricide on the bee's immune response in terms of a) PO and b) ProPO (Section 5.2.2)
3. The immune response in *Varroa*-infested bees exhibiting deformities akin to those caused by DWV, in terms of a) PO and b) ProPO (Section 5.2.3).

5.2 Materials and Methods

5.2.1 Effect of *Varroa* on seasonal immune response

To gain an understanding of how the standing immune response of workers changes temporally in line with *Varroa* populations, the immune responses of two similar hives (each hive had one super (total of 20 frames), and at least 6 frames of brood. Colonies were judged to be strong and of similar size by eye) were measured throughout the season (May-October 2007). Neither hives were treated for *Varroa*, so any infestation present was allowed to build up naturally over the season. Although the hives were similar in terms of size, treatment and environment, as the season progressed, one hive showed high levels of *Varroa* infestation, whilst the other had consistently low levels of infestation. This provided an opportunity to investigate the impact that different levels of *Varroa* infestation have on immune responses within the hive. Of interest in this experiment were the effects of a parasite infestation on the immune responses of uninfested bees, as we predict that bees will maintain an elevated level immune response when the threat of *Varroa* infestation is high, but as immunity is costly, immune responses may be reduced when this threat is low or absent.

Brood frames from each hive were brought into the lab at the same time each month. To ensure that bees used in the experiment had no known exposure to *Varroa*, individuals were collected as they eclosed from the brood cells. Bees carrying mites, or those with evidence of mites left within their cell were discarded. Where sample sizes could not be collected within one day, frames were incubated overnight (36°C ± 1°C, 40% ± 2% humidity). The following day, any bees that had emerged overnight were discarded, as *Varroa* could have transferred between individuals during the night. The process of

sample collection was repeated. All samples were collected over a maximum 2-day period.

The response of the bees to an immune challenge was recorded to investigate how the pressure of a *Varroa* infestation may affect the ability to respond to an additional immune challenge. In order to test the response to an immune challenge, the monthly samples of bees from each hive were split into 4 treatment groups (No Challenge, Needle, 1µl Ringer and 1µl LPS in Ringer, sample size ranged from 14-48 per group, average sample size = 36). Treatment groups were housed in separate bee boxes with 50% sugar syrup and dH_2O *ad libitum*. Haemolymph samples were collected via a perfusion bleed 24 hours post-challenge with 0.5ml ice-cold sodium cacodylate buffer. Haemolymph samples were split into 2 aliquots and frozen for 24 hours (-90°C). Samples were then defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes) before being run on a plate reader for standard PO and ProPO assays (Chapter 2, Sections 2.3.11 & 2.4.14). Thorax width was recorded for all bees to obtain a control for body size.

In addition, mite numbers were regularly recorded for each hive from May to October. Mite numbers were recorded by fitting a *Varroa* floor to each hive. This consists of a 3-sided wooden frame that sits between the brood box and the hive floor that holds a sheet of fine epoxy coated mesh, through which mites can fall as they die or are dislodged. The mesh is too fine for bees to fall through, and prevents bees from removing dead mite bodies. A thin sheet of aluminium was cut to the same dimensions as the *Varroa* floor and the leading edge bent upwards so that it could be slid in and out between the *Varroa* floor and the hive floor like a drawer. In order for *Varroa* to stick to the aluminium to allow counting, the drawer was sprayed with vegetable oil. To record mite numbers, the tray was slid out of the hive and all debris (frass and mites) scraped into a collecting bag. The drawer was re-sprayed with oil and returned to the hive. The content of the drawer was inspected under a dissecting microscope, and mite numbers recorded.

Using the actual mite drop numbers collected, samples were added up over each month, and divided by the number of days to get the mite drop per day, per month. The total proportion of the emergent mite population dropped daily during brood emergence is 30% (Martin, 1998), so the estimated total monthly mite population for each hive can be calculated. This can then be

compared with the expected total mite population, as taken from Martin (2001a). As this calculation averages out data within each month, some of the detail of population growth will be lost, but it will give a good measure of the overall mite populations in our hives to compare with the model.

The weather for the spring/summer season 2007 was poor – it was Britain's wettest May-July since records began in 1776. June was one of the wettest months on record in Britain (more than double the average for the month) and Sheffield experienced severe flooding throughout this period. Although bees can fly and forage in rain, if the weather is severe, few bees leave the hive to forage (Riessberger & Crailsheim, 1997). This was the case at Sheffield field sites, with few individuals observed to be foraging during the heavy rain (pers. obs.). During this time, hives were supplemented with 50% sugar syrup to prevent starvation, but generally hives were very slow to build up during the start of the summer. As the *Varroa* population dynamics are dependant both on the increasing bee population within the hive, and on transmission by foragers to other hives, it was predicted that the weather might have impacted on the population dynamics of the *Varroa*. Therefore, weather data (and in particular, rainfall) was collected from Western Park Weather Station, a station situated next to the Animal and Plant Sciences Department, (supplied by Western Park Museum) to investigate the effect of weather on mite population dynamics.

5.2.2 Effect of acaricide on the immune response

Varroa at the Tapton field site show no sign of resistance to the acaricide Apistan (pers. obs.) In susceptible mites, exposure to Apistan for a 12 hour period typically results in a 90% death rate (Martin *et al.*, 2002). By treating hives with Apistan we can therefore effectively remove the *Varroa* infestation from a colony, and observe how the immune response reacts to the removal of this parasitic threat.

Two similar colonies (Hives A and B) were sampled at the start of September (Hive A = 07/09/06, Hive B = 01-03/09/06), October (Hive A = 01-04/10/06, Hive B = 01-06/10/06) and throughout November 2006 (Hive A = 04-08/11/06 and 20-22/11/06; Hive B = 07-14/11/06 and 20-22/11/06). The late summer that year meant that colonies continued to forage into the autumn months. Due to the late season, hives initially showed no mite infestation, but in

October increasing mite populations were present in both hives. Immediately after bees were collected for the October samples, Apistan acaricide strips were added to each hive to reduce *Varroa* populations within the colonies.

To measure the immune response to Apistan treatment, brood frames from each hive were brought into the lab and emerging workers collected after an overnight incubation period (36°C ± 1°C, 40% ± 2% humidity). In addition to monitoring the effect of Apistan, bees from each sample were tested for their response to an immune challenge. Bees from each hive were split into 4 treatment groups (No Challenge, Needle, 1µl Ringer and 1µl LPS in Ringer, treatment groups sample size ranged from 8-50, mean sample size = 32). If bees down-regulate their immune system following the removal of *Varroa* by Apistan, we would expect to see this reflected in their response to a novel immune challenge.

Treatment groups were housed in separate bee boxes with 50% sugar syrup and dH_2O *ad libitum*. Haemolymph samples were collected via a perfusion bleed 24 hours post-challenge with 0.5ml ice-cold sodium cacodylate buffer. Haemolymph samples were split into 2 aliquots and frozen for 24 hours (-90°C). Samples were then defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes) before being run on a plate reader for standard PO and ProPO assays (Chapter 2, Sections 2.3.11 & 2.4.14). Thorax width was recoded for all bees to obtain a control for body size.

Mite numbers were recoded as the proportion of the sample of new workers emerging with one or more mites attached to them. Workers that were infested were not used in the experiment, and therefore all bees assayed had no known previous exposure to mites. November bees are sampled twice – once just after the addition of Apistan, and once at the end of the month, to see how the response to Apistan changed post treatment. Sampling in Hive B ran over a longer period in November, as brood production slowed down so samples took longer to collect.

5.2.3 Effect of deformed wings on the immune response

To test what the effect of a physical deformity might have on ProPO and PO activity, bees were collected from a single hive infested with *Varroa* mites. A single brood frame was brought into the lab, and workers were collected as they emerged from the brood cells.

Workers were split into 3 groups: those with had no mites and no deformities (Normal – mites, n=26), those with one or more mite on them, but with no visible deformity (Normal + mites, n=19), and those with one or more mites on them and showing visible signs of deformed wings (Deformed wings, n=10). The 3 treatment groups were housed in separate bee boxes for 24 hours (and provided with 50% sugar syrup and dH_2O *ad libitum*. When bees were 24 hours old they were perfuse bled with 0.5ml ice-cold sodium cacodylate buffer. Haemolymph samples were split into 2 aliquots and frozen for 24 hours (-90°C). Samples were then defrosted on ice, vortexed and centrifuged (4 °C, 2800G, 15 minutes) before being run on a plate reader for standard PO and ProPO assays (Chapter 2, Sections 2.3.11 & 2.4.14).

5.2.4 Presentation of Results

In all experiments, the No Challenge and LPS treatment groups showed the extremes of the range of immune responses seen. For simplicity, graphical data are only presented for these two treatment groups. The Needle and Ringer treatment groups provided wounding and vehicle controls, and showed a staggered response between the two extremes. Graphs for all four treatment groups can be found in Appendix 2, Section A2.3.

5.3 Results

5.3.1 Effect of *Varroa* on seasonal immune response

There was no significant difference overall in the PO responses between the low and high level *Varroa*-infested hives (ANCOVA, $F_{1,1724} = 1.94$, $p = 0.1636$), but there was a significant difference in the level of ProPO activity (ANCOVA, $F_{1,1719} = 229$, $p<0.0001$). Consequently, hives were analysed separately.

Low level infected hive

Figures 5.3 and 5.4 show the results for PO and ProPO in the low level *Varroa* infected hive. There was a steady reduction in PO and ProPO over time, following a quadratic, rather than a linear curve. Therefore, time was fitted as both a linear and a quadratic effect. Both PO and ProPO results were log10 transformed to produce normal distributions.

PO: The results for PO (Figure 5.3, Table 5.1) show that there was a significant effect of time, treatment and thorax width on PO. All treatment groups showed a reduction in PO concentration over time.

ProPO: Results for ProPO (Figure 5.4, Table 5.2) showed a significant interaction between treatment and time (ANCOVA, $F_{3,856} = 2.96$, $p=0.03139$). All groups showed a reduction in ProPO over time, with the LPS immune challenged group showing the biggest decrease.

High level infected hive

As can be seen from Figures 5.5 and 5.6, the results for PO and ProPO in the high level infested hive follow a quadratic curve over time for all treatments, dropping mid-season (July) and rising again towards the end of the summer. Time was fitted as both a linear and a quadratic effect. Both PO and ProPO results were log10 transformed to follow a normal distribution.

PO: Results for PO (Figure 5.5, Table 5.3) showed a significant interaction with time and treatment (ANCOVA, $F_{3,858} = 13.4$, $p<0.0001$). All treatment groups showed a drop in PO response down to July, rising sharply again in September and October. The PO activity in the LPS treatment group dipped later in the season, and had a lower response towards the end of the season than the other treatment groups.

ProPO: Results for ProPO (Figure 5.6) follow a quadratic curve for all treatment groups, dropping in July and rising sharply in September and October. There was a significant effect of time, treatment and thorax width on ProPO concentrations (Table 5.4). The challenged groups produced lower levels of ProPO activity throughout the season.

Mite populations and the immune response

Mite numbers for the two hives can be seen in Figure 5.7. Analysis of the mite count data confirmed that there was a significant difference between the mite counts for low and high level infected hives (GLM, $p<0.0001$, d.f. = 1,29, Poisson errors) and that these counts varied significantly over time (GLM, $p<0.0001$, d.f. = 1,29, Poisson errors).

Summary: Although the curves fitted to the two hives appear different, there was in fact no significant difference between the levels of PO activity in the two

hives. The slightly different responses seen towards the end of the season, although not statistically significant, may represent the effect of *Varroa* infestation on the immune response. However, there were highly significant different in the levels of ProPO activity between the hives with low and high infestation. The lower concentrations of ProPO in the hive with low level infestation may be a response to the lack of *Varroa* in this hive, so eliminating the need to maintain a costly level of immune investment. The high level infested hive also showed an initial decrease in ProPO immune response, but this was up-regulated immediately following the increase in *Varroa* numbers in September.

The extreme variation in the ProPO immune response seen between the hives may be attributed to the presence of the *Varroa* mites. However, whilst all other *Varroa*-free colonies used in this thesis have shown qualitatively similar immune responses (a trait which is lacking in this data), their significantly different quantitative results has lead to them being analysed individually. The differences seen between the low and high level *Varroa*-infested colonies may therefore be due to the natural variation in colony response. Surveying a larger sample size (n=8) of colonies at low, medium and high levels of *Varroa* infestation would enable the effect of hive to be fitted as a random factor and so allow focus on the difference in colony responses to *Varroa* infection levels. If the variation in colony response was found to be due to mites, then the presence of *Varroa* should be taken into consideration when choosing colonies to use as a representative sample of honey bees in future experiments.

Thorax width had a significant positive relationship with enzyme activity in all but the PO activity in the high level infested hive. Larger bees are likely to have more haemolymph, and consequently with the sampling method used (perfusion bleed), this will be recorded as a higher haemolymph concentration per unit buffer. Incorporating a measure of body size into the analysis controls for this effect.

Mite populations and the weather

Figure 5.8 shows the mite population for the high level infected hive (calculated from weekly mite drops using Martin, 1998, see Section 5.2.1 for explanation), plotted with the expected mite population (see Martin, 2001a for predictive model). It can be seen that the observed mite population for the high level

infected hive was much lower than that expected. Total monthly rainfall was also plotted on the graph, with the solid black line representing the actual rainfall in Sheffield over summer 2007. The dashed black line shows the average rainfall (data collected over 29 years from 1971-2000), giving an indication of what is expected for these months. Sheffield experienced extreme rainfall in summer 2007, with total monthly rainfall far exceeding that expected. Heavy rainfall prevents foraging activity in bees, and consequently a reduction in the rate of colony reproduction, a factor vital for mite reproduction. These results suggest that the extreme rainfall was correlated with a significant impact on *Varroa* population dynamics, leading to decreased levels of infestation in both hives.

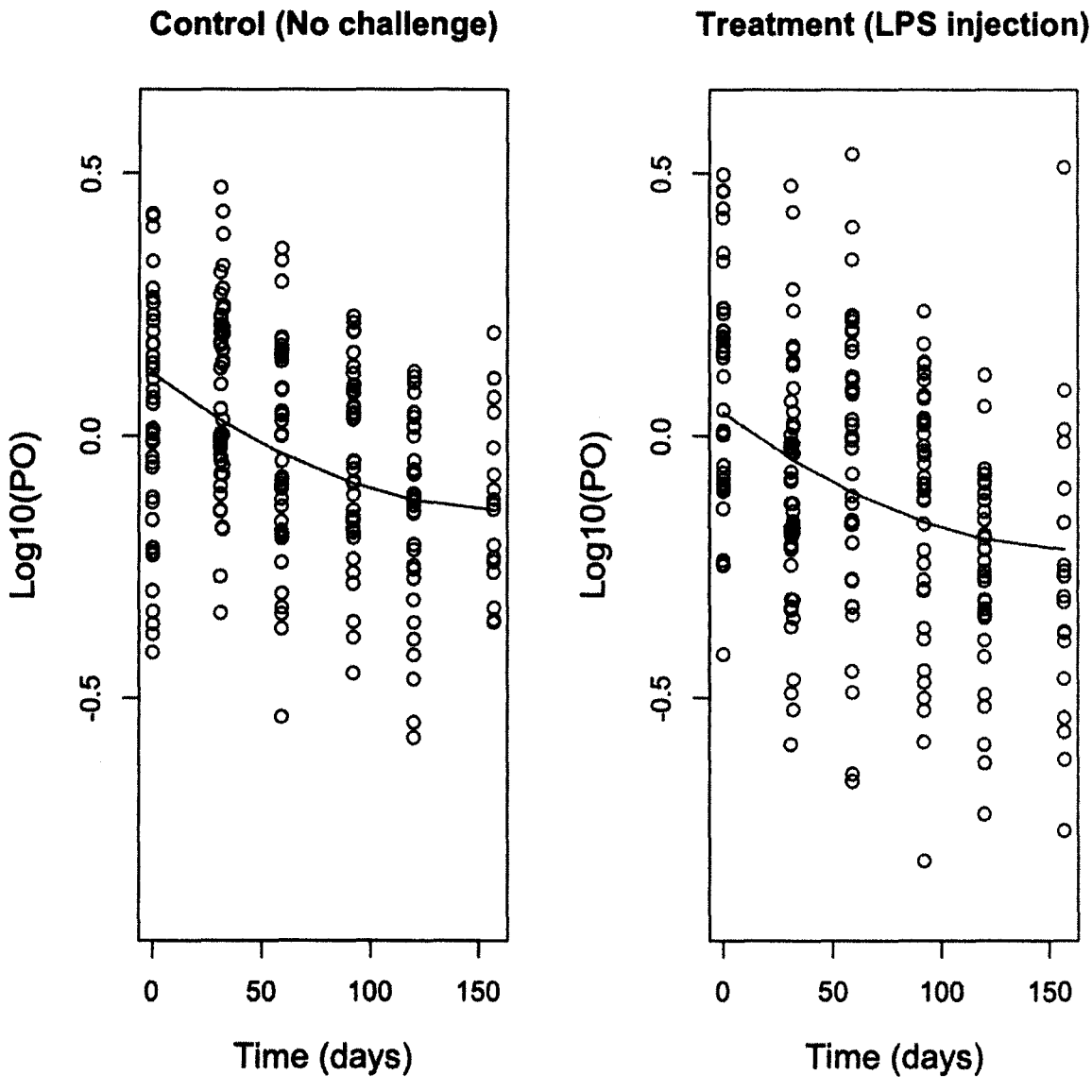


Figure 5.3. PO response of bees in low level infected hive to No Challenge and LPS treatment groups. Samples were taken over a period of 157 days (one set of samples every month) for 6 months, with Day 0 = 30th May 2007. There was a significant effect of time, treatment and thorax width on PO (Table 5.1). All treatment groups showed a reduction in PO concentration over time. Data are log10 transformed.

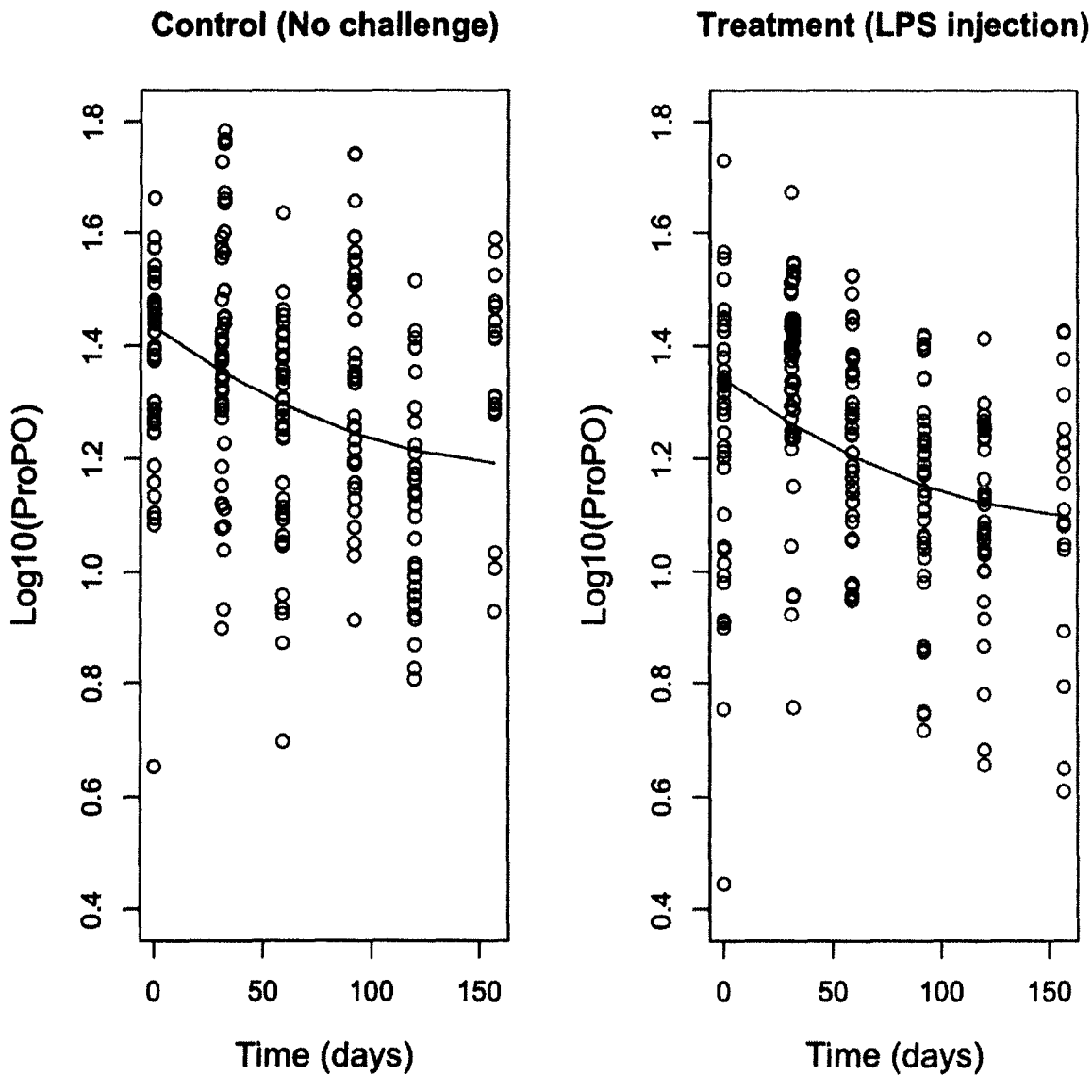


Figure 5.4. ProPO response of bees in the low level infected hive to No Challenge and LPS treatment groups. Samples were taken over a period of 157 days (one set of samples every month) for 6 months, with Day 0 = 30th May 2007. There was a significant interaction between treatment and time (data are log10 transformed).

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.555	11.1	<0.001
Treatment	3	0.650	4.35	0.00475
Time (linear)	1	5.75	115	<0.0001
Time (quadratic)	1	0.373	7.49	0.006348
Residuals	859	42.8		

Table 5.1. PO results for low level *Varroa*-infested hive throughout summer 2007 (results are log10 transformed). There was a significant quadratic effect of time on PO activity. The LPS treatment group produced lower levels of PO than the controls.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	1.01	22.2	<0.0001
Treatment	3	0.977	7.17	<0.0001
Time (linear)	1	4.69	103	<0.0001
Time (quadratic)	1	0.266	5.85	0.01581
Treatment x time (linear)	3	0.404	2.96	0.03139
Residuals	856	38.9		

Table 5.2. ProPO results for low level *Varroa*-infested hive throughout summer 2007 (results are log10 transformed). There was a significant interaction of time on ProPO activity

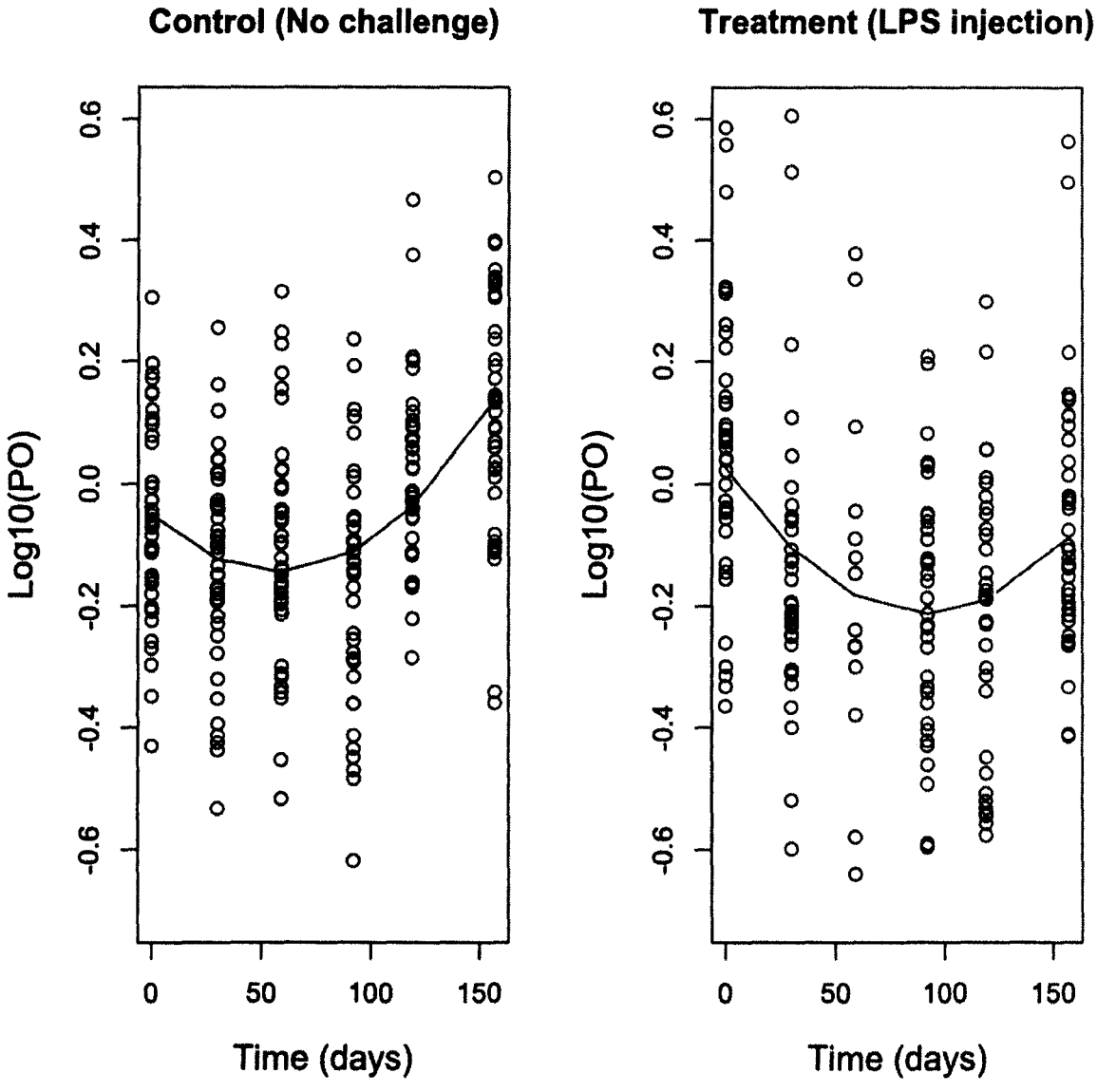


Figure 5.5. PO response of bees in high level infested hive to the No Challenge and LPS treatment groups. Samples were taken over a period of 157 days (one set of samples every month) for 6 months, with Day 0 = 30th May 2007. There was a significant interaction of time with treatment (data are log10 transformed). All treatment groups showed a drop in PO response down to July, rising sharply again in September and October. The LPS treatment group showed a lower drop and a reduced rise at the end of the season compared to the controls.

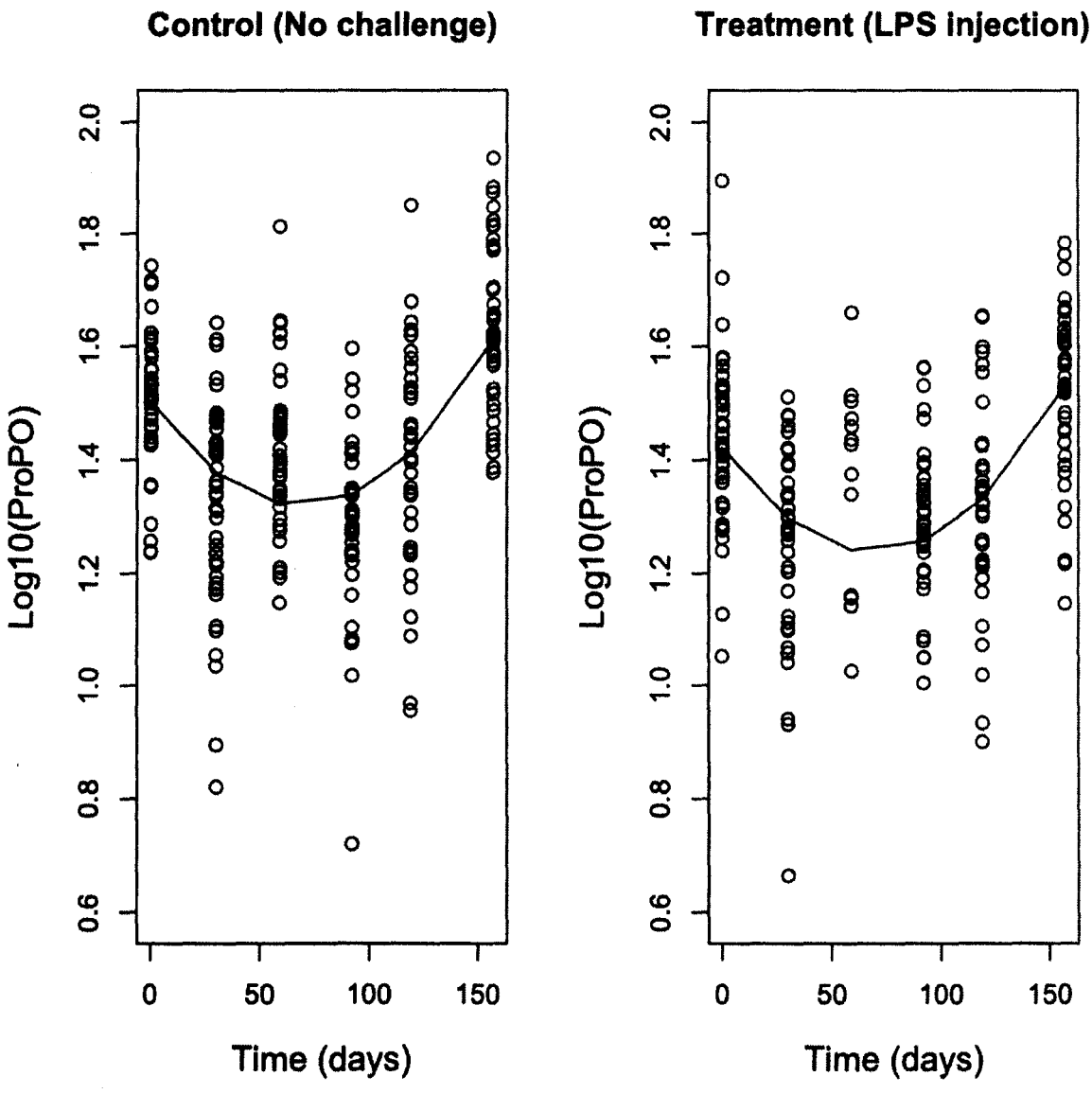


Figure 5.6. ProPO response of bees in high level infested hive to the No Challenge and LPS treatment groups. Samples were taken over a period of 157 days (one set of samples every month) for 6 months, with Day 0 = 30th May 2007. There was a significant effect of time, treatment and thorax width on ProPO concentrations (Table 5.4). The LPS challenge resulted in lower ProPO concentrations throughout the season. Data are log10 transformed.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.083	1.96	0.1621
Treatment	3	0.430	3.36	0.01830
Time (linear)	1	1.17	27.4	<0.0001
Time (quadratic)	1	4.62	108	<0.0001
Treatment x time (linear)	3	1.71	13.4	<0.0001
Residuals	858	36.6		

Table 5.3. PO results for high level *Varroa*-infested hive throughout summer 2007 (results are log10 transformed). There was a significant interaction of time and treatment on PO activity.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.503	17.5	<0.0001
Treatment	3	0.496	5.76	<0.001
Time (linear)	1	1.00	34.9	<0.0001
Time (quadratic)	1	7.90	275.	<0.0001
Residuals	861	24.7		

Table 5.4. ProPO results for high level *Varroa*-infested hive throughout summer 2007 (results are log10 transformed). There was a significant effect of time and treatment on ProPO levels. Activity followed a quadratic curve, with the LPS treatment group having lower levels of ProPO activity than the controls.

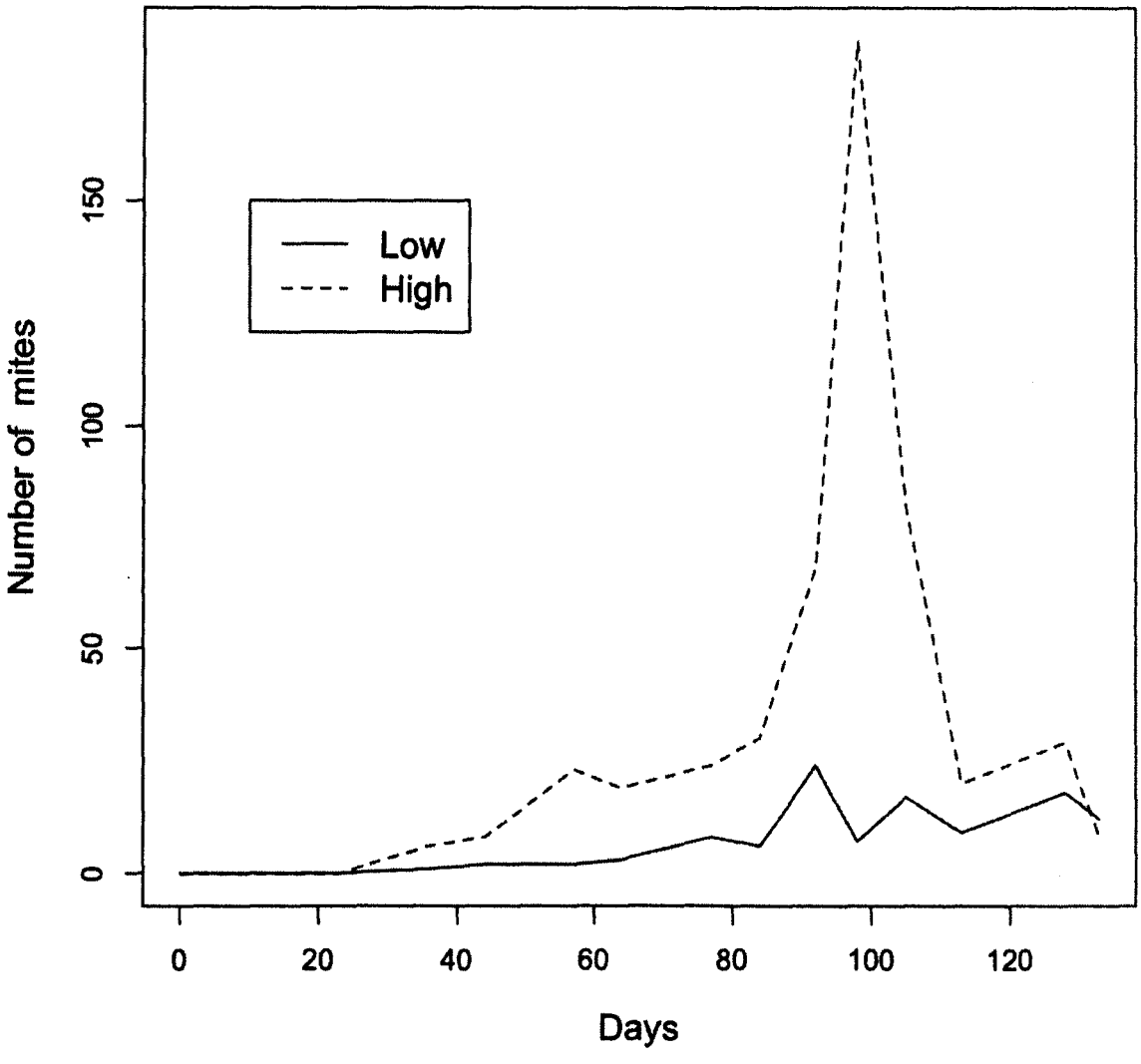


Figure 5.7. Mite counts for low and high level infested hives for the period June-October 2007. There was a significant difference between the mite counts of the two hives, and counts varied significantly over time.

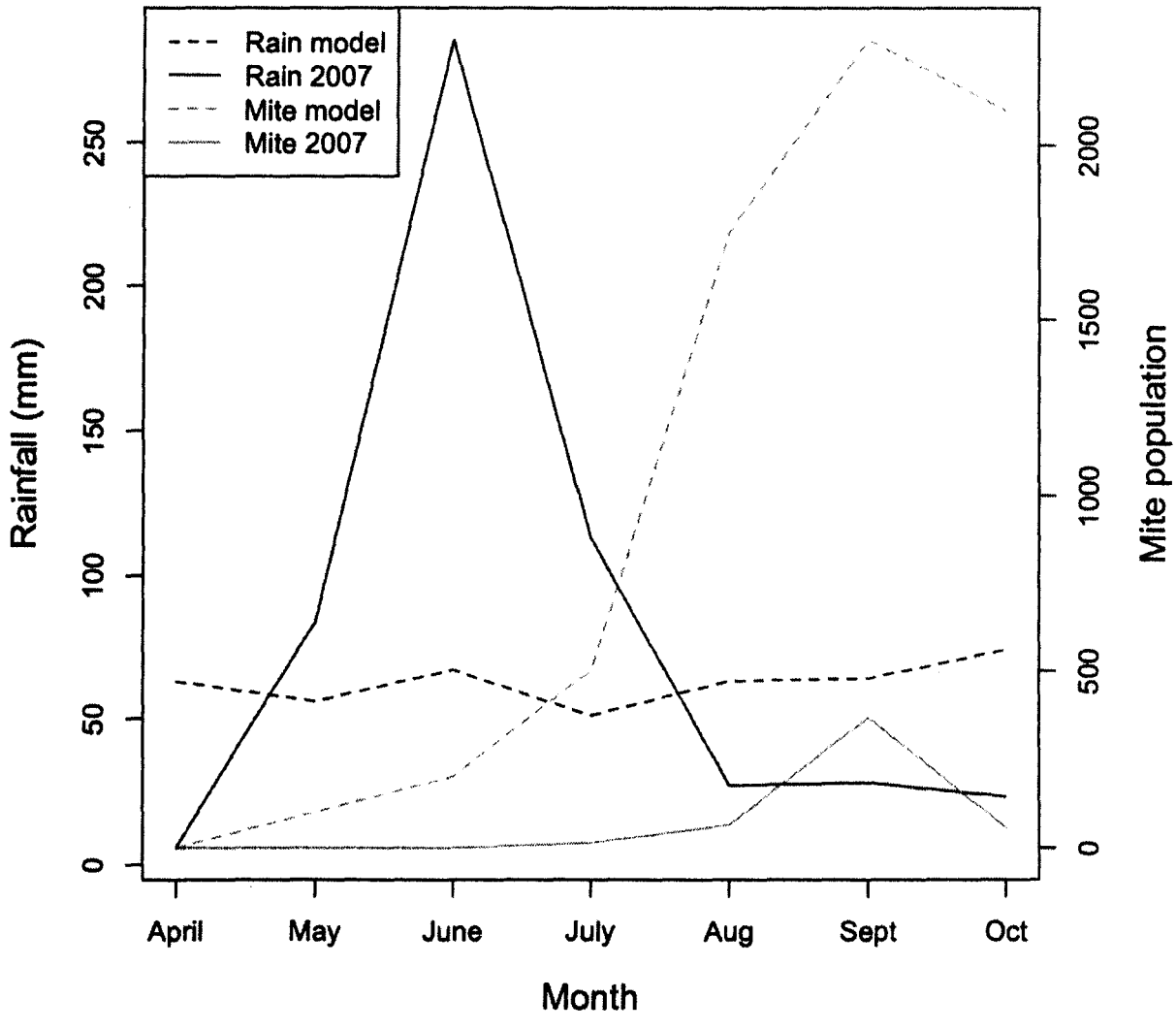


Figure 5.8. Rainfall and mite population dynamics. Mite population (grey lines) for high level infested hive in 2007 (solid line) compared to a model population growth (see Martin, 2001a, dashed line). Rainfall in Sheffield (black lines) was plotted as total rainfall for each month in 2007 (solid line) against the expected rainfall (dashed line), taken as the average monthly rainfall in Sheffield from data collected over 29 years (1971-2000).

5.3.2 Effect of acaricide on immune response

Effect of Apistan on mites:

Figure 5.9 shows the mite numbers (as a % of bees infested with one or more mites) for hives A and B over the period September – November 2006. Sampling dates for the two hives varied slightly, but overall the proportion of mite infestation was very similar for the two hives. Following treatment with the acaricide Apistan, the proportion bees infested with mites was effectively reduced to zero within a month. This strongly suggests that the hives did not contain Apistan-resistant *Varroa*.

Effect of Apistan on bees:

When added into the models, there was a significant effect of hive on both the PO (ANCOVA, $F_{1,997} = 8.59$, $p=0.003450$, data are log10 transformed) and ProPO (ANCOVA, $F_{1,1017} = 5.24$, $p=0.02232$, data are log10 transformed) results. Hives were therefore analysed separately.

Hive A: PO and ProPO showed different patterns of response. The results for both were log10 transformed to follow a normal distribution.

PO: The results showed a significant interaction with time and treatment on PO concentration (Figure 5.10, Table 5.5, data are log10 transformed). The immune response follows a quadratic curve, with all treatment groups up-regulating PO concentration at the end of November. Challenge with LPS resulted in a much lower drop in the curve.

ProPO: The results showed a significant interaction of time and treatment on ProPO concentration (ANCOVA, $F_{3,541} = 3.14$, $p=0.02510$, Figure 5.11, Table 5.6, data are log10 transformed). The immune response followed a quadratic distribution, with all treatment groups increasing ProPO concentration towards the end of the season. Challenge with LPS resulted in lower levels of ProPO acivity.

Hive B:

PO: There was a significant interaction of time and immune challenge on PO concentration (ANCOVA, $F_{3,467} = 14.2$, $p<0.0001$, Figure 5.12, Table 5.7, data are log10 transformed). The No Challenge and LPS groups showed a reduction in PO concentration over time, with LPS producing a much steeper decline.

ProPO: The results showed significant effects of time and immune challenge on ProPO concentration (Figure 5.13, Table 5.8, data are log10 transformed). All treatment groups showed an up-regulation in ProPO concentration at the beginning of November, which decreased again towards the end of the month.

In all cases, across both hives, challenging the immune system with LPS led to a decrease in PO and ProPO activity. Both hives were infested with similar mite numbers (Figure 5.9), yet exhibited huge variation in PO and ProPO responses.

Hive A showed an up-regulation of PO activity in response to the increase in mite numbers, whilst Hive B showed the opposite result. As with the previous experiment (Figure 5.5), this up-regulation was seen in bees assayed in the following month's sample. ProPO activity in both hives showed an increase in activity towards the end of the season.

5.3.3 Effect of deformed wings on the immune response

PO: There was no significant difference between the PO values for bees with/without mites (Figure 5.14, $F_{1,52} = 0.233$, $p=0.6314$) and with/without visible wing deformities ($F_{1,52} = 0.414$, $p=0.5231$).

ProPO: There was no significant difference between the ProPO values for bees with/without mites (Figure 5.15, $F_{1,52}= 0.031$, $p=0.5563$) and with/without visibly deformed wings ($F_{1,52} = 1.49$, $p=0.2278$).

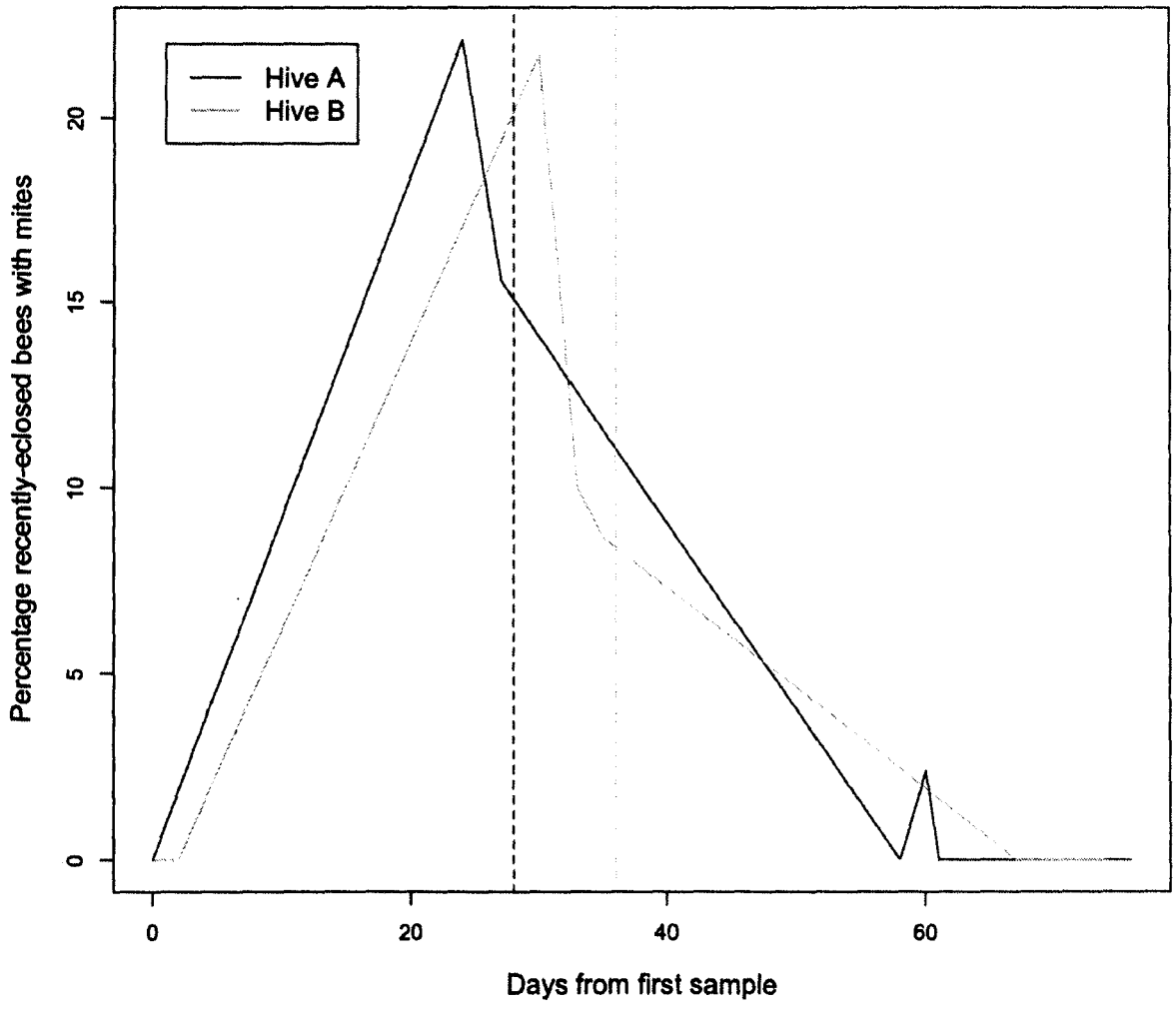


Figure 5.9. Percentage of recently eclosed bees in Hives A and B with at least one *Varroa* mite, over a period of 76 days from the beginning of September to late November 2006 (Days = days from first sample). The acaricide Apistan was added to the hives immediately after October sampling (black and grey dashed lines, respectively), and was successful at removing 100% mites in both hives within a month.

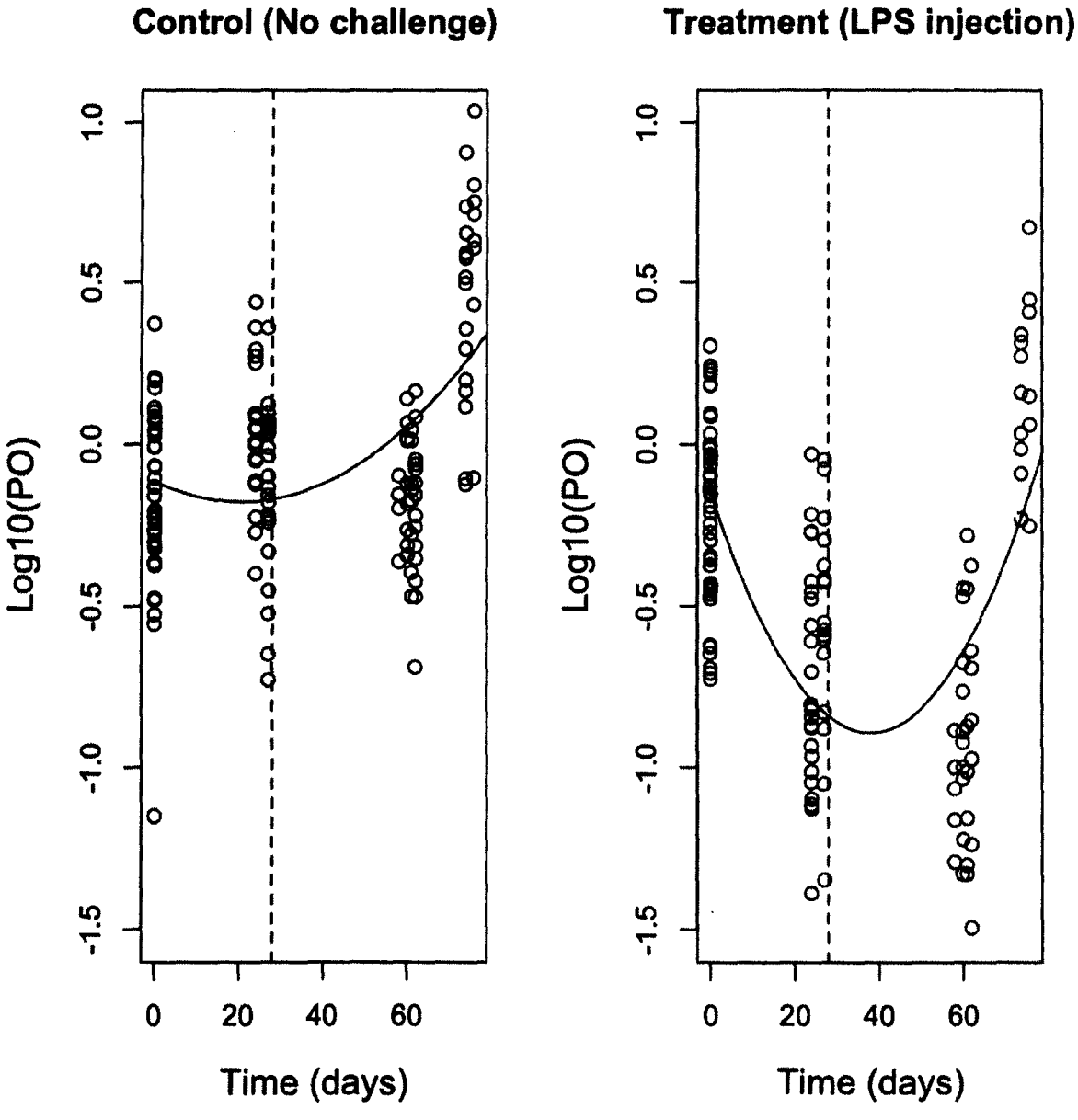


Figure 5.10. PO response of bees in Hive A to No Challenge and LPS treatment groups. Dashed lines indicate the point at which Apistan was added. There was a significant interaction of time with treatment on PO concentration (Table 5.5). All treatment groups showed an increase in PO towards the end of the sampling period. Results are log10 transformed.

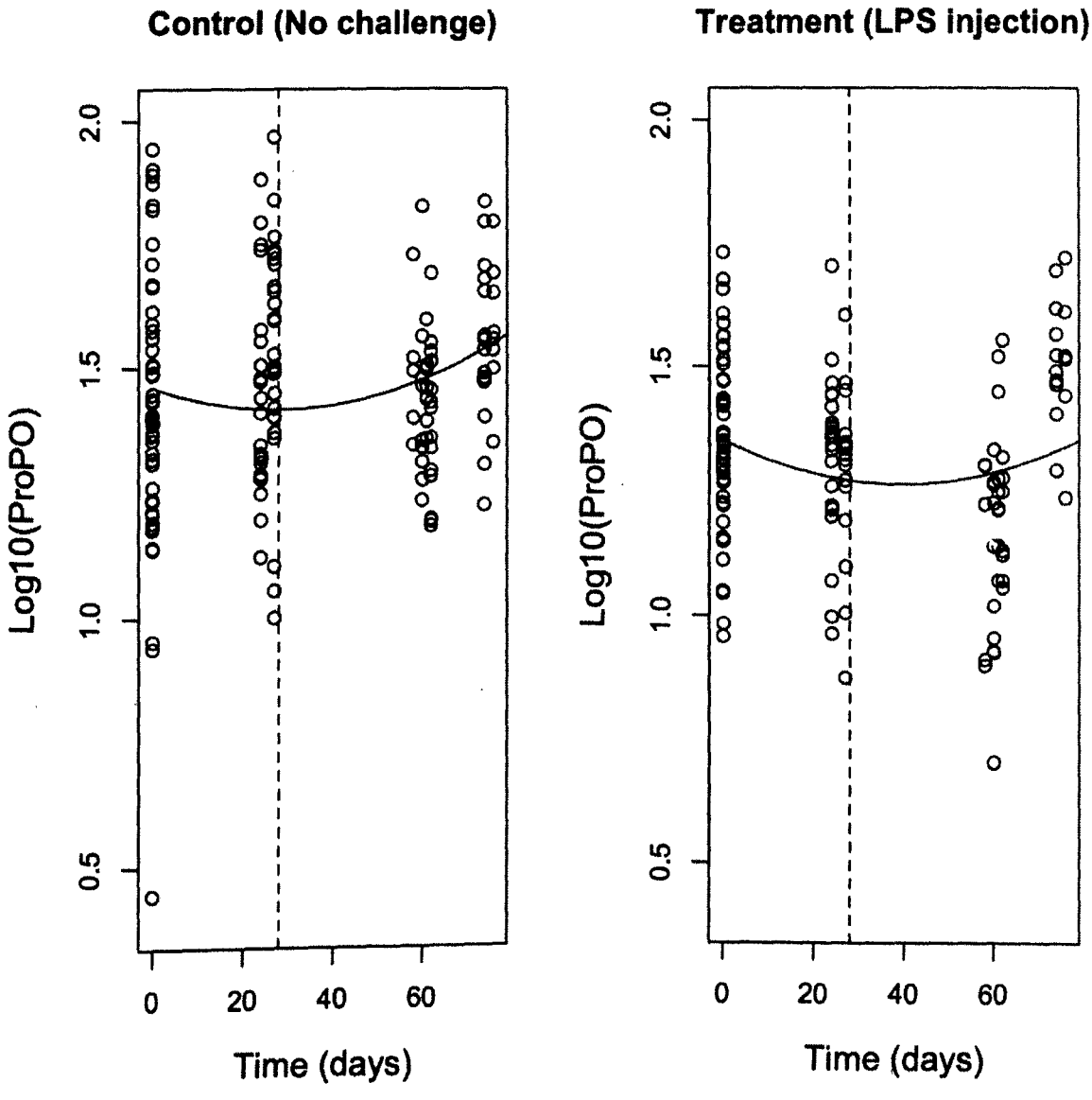


Figure 5.11. ProPO response of bees in Hive A to the No Challenge and LPS treatment groups. Dashed line indicates the point at which Apistan was added. There was a significant interaction of immune challenge and time on ProPO concentration (ANCOVA, $F_{3,541} = 3.137$, $p=0.0251$, see Table 5.6). Results are log10 transformed.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.019	0.175	0.6756
Treatment	3	15.4	46.6	<0.0001
Time (linear)	1	3.49	31.8	<0.0001
Time (quadratic)	1	18.9	172	<0.0001
Treatment x time (linear)	3	4.78	14.5	<0.0001
Treatment x time (quadratic)	3	3.11	9.44	<0.0001
Residuals	538	59.1		

Table 5.5. PO results for Hive A (results are log10 transformed). There was a significant interaction of treatment with time, with the LPS treatment group producing a significantly lower quadratic curve of PO activity over time than the control groups.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.001	0.0350	0.8517
Treatment	3	1.71	15.7	<0.0001
Time (linear)	1	0.426	11.7	<0.001
Time (quadratic)	1	0.527	14.5	<0.001
Treatment x time (linear)	3	0.342	3.14	0.02510
Residuals	541	19.7		

Table 5.6. ProPO results for Hive A (results are log10 transformed). There was a significant interaction of treatment with time, with the LPS treatment group producing lower ProPO activity levels throughout the experiment.

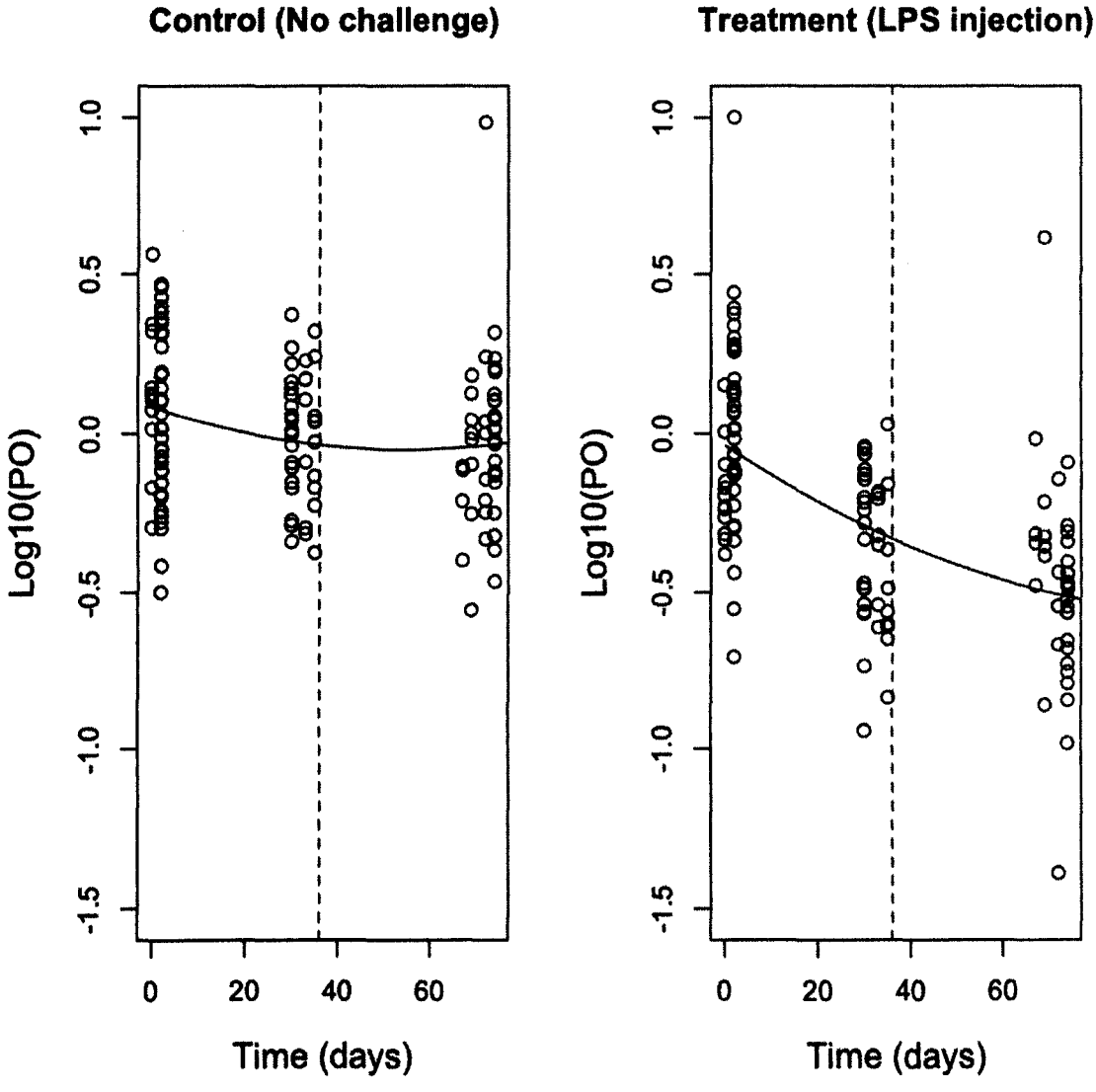


Figure 5.12. PO response of bees in Hive B to the No Challenge and LPS treatment groups. The dashed line indicates the point at which Apistan was added. There was a significant interaction of time with immune challenge on PO concentration (see Table 5.7). Results are log10 transformed.

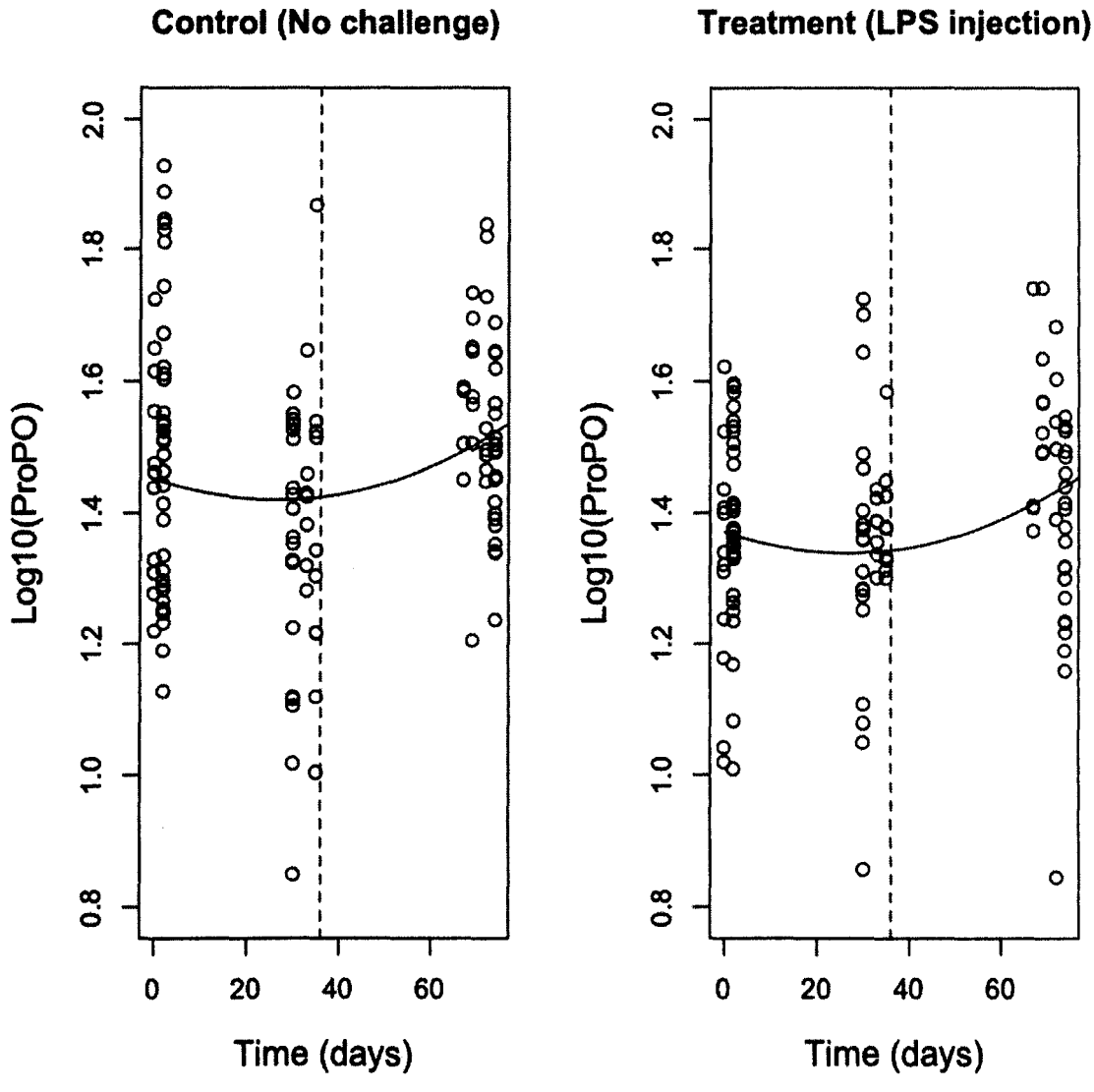


Figure 5.13. ProPO response of bees in Hive B to No Challenge and LPS treatment groups. The dashed line indicates the point at which Apistan was added. There were significant effects time, immune challenge and thorax width on ProPO concentration (see Table 5.8). Results are log10 transformed.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.049	0.695	0.4048
Treatment	3	6.04	28.4	<0.0001
Time (linear)	1	1.63	22.9	<0.0001
Time (quadratic)	1	0.347	4.89	0.02754
Treatment x time (linear)	3	3.02	14.2	<0.0001
Residuals	467	33.2		

Table 5.7. PO results for Hive B (results are log10 transformed). There was a significant interaction of treatment and time, with the LPS treatment group having both a lower and steeper decline in PO activity over time than the controls.

Source	d.f.	Sum of squares	F-value	P
Thorax width	1	0.273	8.371	0.003990
Treatment	3	0.504	5.145	0.001652
Time (linear)	1	0.291	8.925	0.002961
Time (quadratic)	1	0.330	10.121	0.001563
Residuals	470	15.332		

Table 5.7. ProPO results for Hive B (results are log10 transformed). There was a significant effect of treatment and time on ProPO activity. The LPS treatment group produced lower ProPO activity levels than the controls.

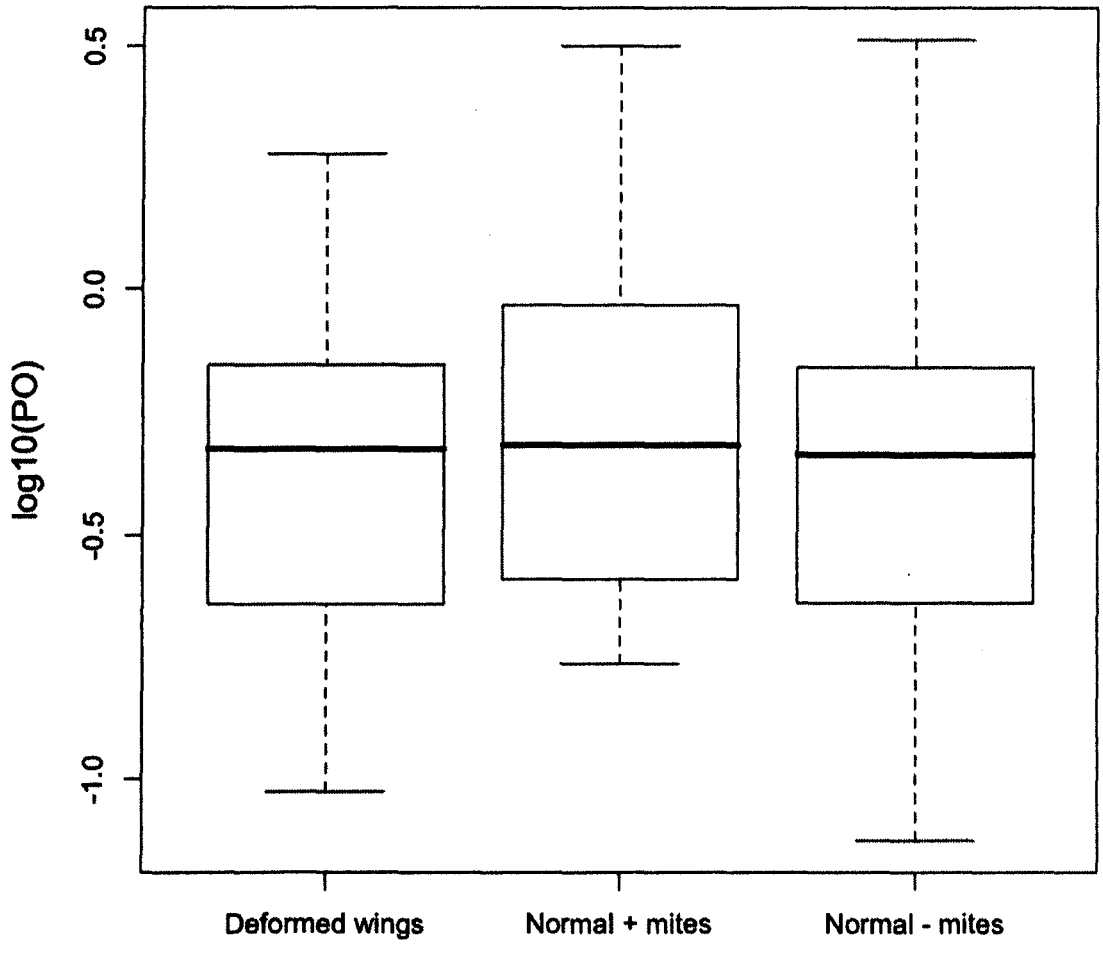


Figure 5.14. PO concentrations for bees with deformed wings and mites (Deformed wings), normal wings and mites (Normal + mites), and normal wings with no mites (Normal – mites). There was no significant difference between the PO values for bees with/without mites and with/without visible deformities. Data are log10 transformed.

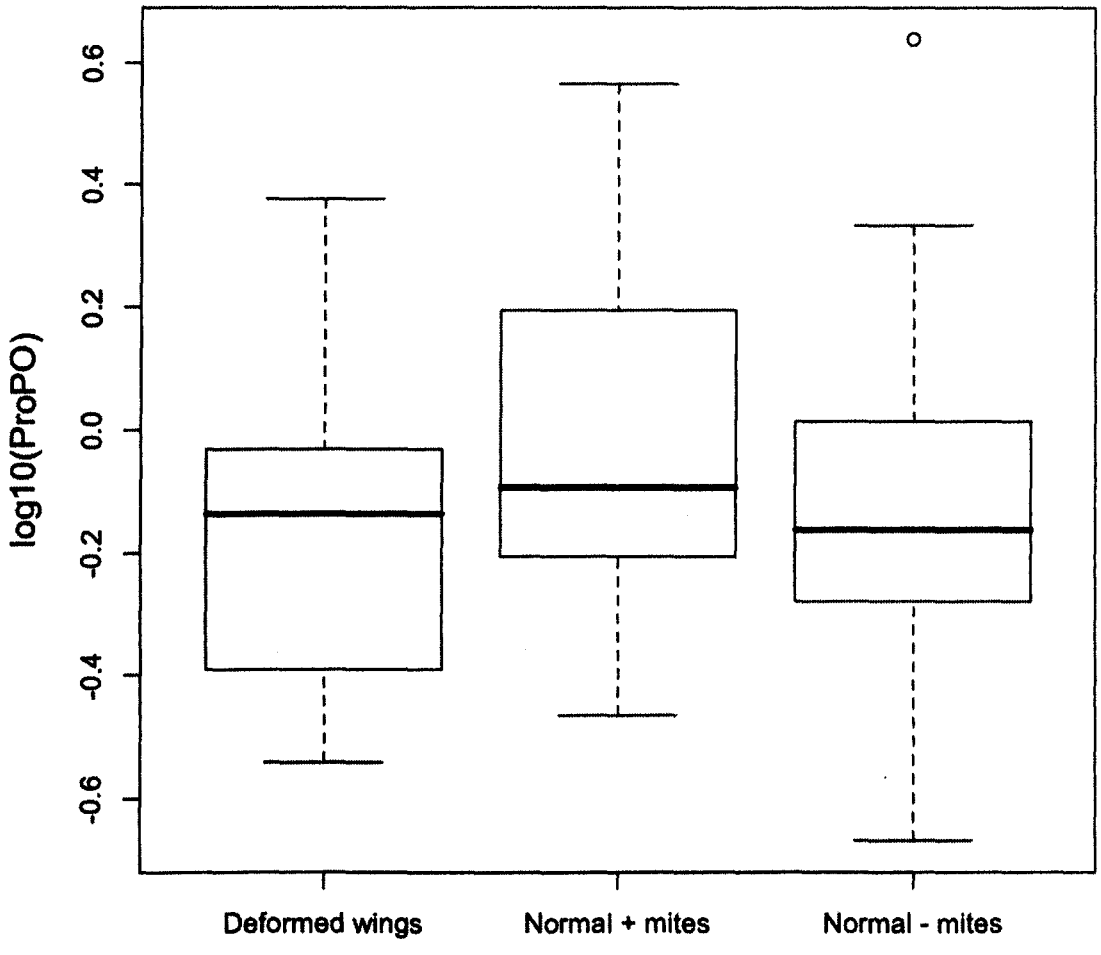


Figure 5.15. ProPO concentrations for bees with deformed wings and mites (Deformed wings), normal wings and mites (Normal + mites), and normal wings with no mites (Normal – mites). There was no significant difference between the ProPO values for bees with/without mites and with/without visible deformities. Data are log10 transformed.

5.4 Discussion

The results suggest that, when infested with *Varroa* mites, bees alter investment in their immune response at a colony level. This change in immune investment varied between colonies, but was generally seen as an up-regulation in PO and ProPO concentrations.

5.4.1 Seasonal immune response

PO and ProPO investment was up-regulated in response to increased mite populations (Figures 5.5 & 5.6). The immediate threat to a bee of infection by *Varroa* is cuticle wounding and subsequent bacterial infection. Recent work has shown that following an infection in the mealworm beetle, *Tenebrio molitor*, 99% of bacteria are cleared from the haemolymph within 1 hour, and that this clearance is due to constitutive immune responses rather than induced antimicrobial peptides (Haine *et al.*, 2008). With limited resources available, bees may preferentially invest in the immediate action of PO and ProPO rather than longer lasting immune responses. Bees in both hives initially exhibited a high immune response at the start of the season. This investment decreased over time as the threat of parasites remained absent, and maintaining a costly immune response proved unnecessary.

Up-regulation of the immune response in the infested colony was initiated approximately one month after the peak in the mite population: new cohorts of bees produced elevated immune responses despite not physically encountering a mite themselves (to my knowledge). Cues for this response may come from pheromones or a change in the composition of food that is fed to larvae, or may be due to trans-generational immune priming (Moret & Schmid-Hempel, 2001).

The up-regulation of the immune response in the high level infested hive undoubtedly increases stress on the bees. In this experiment, both hives survived to the next season. The low level infested hive was effectively mite-free, and although the high level infested hive had a surge in the mite population in September, the numbers were not maintained into the winter, and levels did not breach the threshold for colony survival (Martin, 2001a). More severe levels of infestation may impact on up-regulation; if the majority of bees are infested, mounting an immune response to both the direct effect of *Varroa*,

and any transmitted diseases may prove impossible if they are already compromised through a reduction in haemolymph from *Varroa* feeding.

Although the results found from this seasonal work show quantitative and qualitative differences in the hive responses, this variation may be due to natural colony variation, or other factors associated with temporal change rather than a direct impact of *Varroa* infestation. Experiments from Chapters 2, 3 and 4 also showed quantitative differences in colony responses. Whilst the results obtained are suggestive, as only two hives were surveyed in this experiment, further replication at the colony level is needed to draw any firm conclusions.

The weather has a major effect on *Varroa* populations. As predicted, the prolonged heavy rainfall experienced at the start of the summer led to reduced foraging and delayed brood build up in the hives (Riessberger & Crailsheim, 1997). As mites are dependent on brood cells for their reproduction, this resulted in vastly reduced populations.

5.4.2 Effect of acaricide treatment

The ability of Apistan to eradicate *Varroa* proved successful. It is difficult to ascertain how this loss of *Varroa* may have affected the immune response of the bees. Results from this experiment showed variation between the colonies, but showed a general trend for the up-regulation in PO and ProPO in response to increasing mite numbers. The different responses of PO and ProPO within each hive may be a result of differing sensitivity towards the effects of Apistan. However, again, only two colonies were surveyed in this experiment, with no control (no Apistan added). Therefore, as with the previous experiment, the variation in immune responses seen may be due to natural colony variation over time. Additionally, Apistan may directly impact on the immune response itself. Although Apistan is reported to have no detrimental effects on colonies (Delaplane, 1995), there has been little investigation into its impact on the individual immune response, despite the fact that pyrethroids have been shown to cause sub-lethal effects on the physiology of the honey bee (Desneux *et al.*, 2007). Alterations in physiology and resource partitioning due to the effect of the pesticide may therefore explain the immune responses seen in this experiment.

The methods for measuring mite populations differed between the two experiments, as they were run over different years, and techniques were improved. Mite numbers in the previous experiment (low and high levels of

infection) were measured as drop numbers, from which an estimate of total population can be obtained. Mite numbers in Hives A and B were taken as the proportion of emerging bees infested with one or more mites. As such, it is difficult to know how the numbers in the two experiments correspond to each other. However, I believe that mite infestations in all the hives for the two experiments were much lower than expected due to the bad weather and late summer, with mite numbers unable to sufficiently increase population numbers before brood production decreased (Martin, 2001a).

5.4.3 Effect of deformed wings

Within the *Varroa*-infested colony there was no difference in PO and ProPO responses between normal infested and non-infested bees, and those exhibiting typical wing deformities similar to those caused by DWV. Mite-free bees from infested colonies have been shown to have a higher expression of genes encoding PO than deformed bees (Yang & Cox-Foster, 2005), but these results have not been compared to uninfested colonies. If, as shown by the experiments in this chapter, the response of colonies to *Varroa* infestation is highly variable, both these results may hold true, and pinpointing a specific consistant immune response may prove difficult.

5.4.4 Summary

This chapter examined the effect of mite population dynamics on the phenoloxidase (PO) and prophenoloxidase (ProPO) responses in worker bees. Using the assays developed in Chapter 2, I have shown that:

1a. The PO immune response responded to the presence of *Varroa* mites with an increase in titre (Section 5.3.1)

1b. The ProPO immune response also showed an increase in titre with increasing *Varroa* numbers (Section 5.3.1)

2. Eliminating *Varroa* using an acaricide produced variation in both the a) PO and b) ProPO immune response (Section 5.3.2)

3. There was no difference in the a) PO and b) ProPO immune responses in an infested colony exhibiting different levels of *Varroa* infection and wing deformities (Section 5.3.3).

Extreme weather conditions experienced over the experimental seasons lead to favourable conditions in terms of parasite prevalence for the bees, but in turn, the inability to forage and expand the colony may have exerted pressures on the resources of the bees. As bees from mite-infested colonies have been shown to have a reduced immune response compared to mite-free bees, there is potentially some immune cost to infestation (Yang & Cox-Foster 2005). Whether this cost is in response to *Varroa* wounding, bacterial challenge or viral transmission remains to be seen.

Chapter 6

General Discussion

6.1 Synopsis of the thesis

This thesis has examined the ecological immunity of the honey bee in a life-history context. It has used immunological techniques to explore aspects of the constitutive and induced innate immune response in a model organism, and shown how these responses are affected by life-history and parasite infection. This chapter summarises the main findings of this thesis, expands on issues arising from this work, and highlights areas for potential future work.

6.2 Summary of the chapters

Chapter 1 introduced the innate immune system in insects, detailed the immune responses investigated in this work, and discussed the associated underlying mechanisms. Investment in immunity focussing on changes throughout development and aging was discussed, with particular reference to the social insects and their ecological constraints. Finally, the honey bee, *Apis mellifera*, was introduced as a model organism.

Chapter 2 described three immune assays encompassing both constitutive (PO, ProPO) and induced (AMPs) immune responses. The development and optimisation of these assays, including repeatabilities, was covered, and the importance of re-working assays for species-specificity was discussed. Protocols of the standardised assays developed for the three immune measures were recorded. All three assays were subsequently used in Chapters 3 and 4, and the PO and ProPO assays were used to assess response to parasite infestation in Chapter 5.

The change in immunity during development in honey bees has provoked little academic interest to date, despite bees being prone to pathogens during these stages. **Chapter 3** charted the course of the PO and ProPO immune responses throughout all developmental stages, and discovered a stage-specific increase in ProPO production between the red-eyed pupa and eclosed adult stages. The behavioural and physiological changes that accompany the switch from hive to forager in worker bees have been extensively studied (Neukirch, 1982; Robinson, 1987; Winston, 1987; Robinson,

1992; Hooper, 1997; Omholt & Amdam, 2004; Amdam *et al.*, 2005). This chapter found that, despite previous evidence to the contrary (Bendick *et al.*, 2001; Amdam *et al.*, 2005), PO, ProPO and AMP immune responses all increase with age, although AMP production shows signs of senescence following an immune challenge.

Chapter 4 covered the same experiments, this time looking at drones. Due to their low numbers and lack of foraging activity within a colony, drones are largely overlooked, but they are potentially subject to the same range of pathogens as workers (Hooper, 1997; Boomsma *et al.*, 2005; Cremer *et al.*, 2007), and in some cases, are favoured over workers for pathogen infection (Otten and Fuchs, 1988; Fuchs, 1992). ProPO was found to increase sequentially with developmental age, with PO activity following a similar pattern, but dropping during the red-eyed pupal stage, possibly due to its use in cuticle melanisation or sperm production. As with workers, PO activity was found to increase with age in adult drones, whilst ProPO levels remained constant throughout. AMP production was elicited only following an immune challenge, and, as with workers, the response showed signs of senescence in old drones.

The *Varroa* mite, *Varroa destructor*, is one of the major pathogens of honey bees and may be an important component of colony collapse (Shimanuki *et al.*, 1994; De Jong & De Jong, 1983; Akratanakul & Burgett, 1975; Hung *et al.*, 1995; Martin *et al.*, 1998; Sammataro *et al.*, 2000; Kralj *et al.*, 2007). **Chapter 5** investigated the impact of *Varroa* infestation on the immune responses within and between colonies. Levels of immune response decreased when the threat of parasite infestation was low, and increased following a peak in the mite populations. Rainfall was found to play a vital role in levels of colony infestation, and the impact of pesticide use on immunity was investigated. Physical deformities (most likely caused by Deformed Wing Virus) were found to have a negligible effect on the immune response in a *Varroa*-infested colony. The implications of mite infestation on bee immunity were discussed, with particular focus on external environmental constraints.

Appendix 1 expanded on Chapter 2, providing further information on the complexities of enzyme kinetics. The effects of freeze-thawing and lipid presence on PO and ProPO assays were investigated, with both found to have a negative effect. Finally, **Appendix 2** catalogued all results figures from this thesis, presenting the data from all four immune challenge treatment groups.

6.3 Discussions arising from the thesis

6.3.1 The importance of age

Although components of the immune response in the honey bee have been shown to vary with age, to date no work has comprehensively covered all developmental stages in such detail across two of the three castes (Amdam *et al.*, 2004; Amdam *et al.*, 2005; Schmid *et al.*, 2008; Wilson-Rich *et al.*, 2008). This work has shown that differences within what may be considered a single developmental stage, e.g. 'pupae', when split into white- and red-eyed groups in fact have fundamental differences in investment of immune effecter systems such as phenoloxidase. Indeed, a similar result has recently been shown within a single larval stage of the tobacco hornworm *Manduca sexta* (Eleftherianos *et al.*, 2008). However, this thesis provides evidence that during some developmental stages (larval and early pupal stages), PO and ProPO are most likely not employed directly as immune defence in either workers or drones. The stage-specific changes in activity levels are likely to be linked to either physiological process associated with development, such as cuticle melanisation, or in the case of drones, sperm development. The ProPO gene is expressed more strongly in older pupae and adults compared to larval and young pupae, suggesting that it is produced when required for cuticle melanisation (Lourenço *et al.*, 2005). In a similar pattern, the honey bee antimicrobial peptide hymenoptaecin has been found to be expressed in lower amounts in larvae compared to adult bees (Chan *et al.*, 2006). The reason for this apparent lack of an immune response in developing bees is unknown. Larval and pupal stages face parasite pressures, all be it different ones to those encountered as adults. Developing bees may not possess the resources to form an immune response, or their constant monitoring by nurse bees, and the hygienic behaviours associated with this may remove the necessity for immunity.

The immune response is, however, very much required in adult life, with PO, ProPO and AMPs all employed. Both workers and drones showed variation in immunity with age. PO responses were similar in both castes, but ProPO levels in drones were lower than workers and remained unaltered with age or immune challenge. Adult workers have been shown to have higher levels of expression of the gene encoding ProPO than drones, and it may be that drones maintain a maximum level of available ProPO throughout their adult life

(Lourenço *et al.*, 2005).

In adult workers, contrary to expectations, all three immune responses increased with age. In the case of PO and ProPO this has been shown to be a gradual increase, unrelated to task or pathogen encounters outside of the hive. In both workers and drones, AMP production increased following an immune challenge, and the maintenance of these elevated levels showed signs of senescence. This suggests that components of the induced immune response are more costly to maintain, and more susceptible to aging than constitutive immune responses. It is likely that the up-regulation in AMP production occurs only following a specific immune challenge; foragers may exhibit higher levels due to cumulative pathogen encounters, but not because of physiological processes directly associated with age polyethism. The increased levels of immunity seen in older bees highlights their importance to the colony: whether for their own survival and subsequent success as foragers or mates, or to prevent contamination of the hive on their return, it undoubtedly works for the greater good and survival of the colony as a whole.

6.3.2 Why do drones need immunity?

The presence of an immune response in adult drones was in some ways unexpected. PO only responded to an immune challenge in young drones, but its increase with age may represent an immune response that can be maintained throughout all challenges. However, the production of AMPs following a challenge with LPS shows that they have the resources available for specific immunity when needed. The question is why? The drones sampled at two weeks old were at the peak of sexual maturity, with maximum sperm viability and everything to play for (Page & Peng, 2001), yet showed a significant drop in AMP response compared to young drones. This disparity between the immune responses of the two age groups may well be due to resource allocation. The availability of high levels of AMP production in sexually immature drones will enable them to survive long enough to have a shot at reproduction, which is their sole purpose in life. Once sexually mature, the window of opportunity for mating is only approximately 9 days long (based on average life-expectancy (Hooper, 1997); there is no (negative) trade-off between energy expended for flight and lifespan (Rueppell *et al.*, 2004)). During this time they may choose to invest all their energy into mating flights and

chasing queens, and consequently this may lead to a reduction in investment in resource sinks, such as immunity.

6.3.3 Temporal changes in immunity

Chapter 5 highlights the temporal changes that can occur in colony immunity. Although the immune responses of honey bee colonies can be altered through artificial manipulation of the colony structure (Amdam *et al.*, 2005), seasonal variation in immunity within a single developmental stage of bees unparasitised by *Varroa*, as recorded in Section 5.3.1, has, I believe, not been recorded before. The adaptive value of this phenotypic plasticity, with colony immunity responding as one organism is unknown. The costs of maintaining an immune response have been widely reported (Schmid-Hempel, 2005; Siva-Jothy *et al.*, 2005) and the ability to be flexible with regard to immune investment must therefore be a benefit.

6.3.4 Parasites and immunity

This thesis has identified an up-regulation of immune investment in *Varroa*-infested hives, observed in uninfested individuals following a surge in the mite population. This response has prophylactic ramifications: previous experience with a pathogen can provide individuals, or in this case, the newly emerging brood, with an enhanced immunity (Little & Kraaijeveld, 2004; Sadd & Schmid-Hempel, 2007). Along with the additional cost of maintaining an elevated response, this represents important issues in the study of host-parasite evolution. Honey bee colonies represent a high density environment with ideal conditions for parasite spread. Consequently, bees have developed several behavioural mechanisms to deal with infection in the hive (Sammataro *et al.*, 2000; Spivak & Boecking, 2001). If these behavioural measures are not successful, how is the need for an increased immune response detected, and what pressures does the additional cost exert on the colony as a whole? Pheromones may well play a part: they allow nurse bees to detect and remove diseased larvae from the colony, and such messages are able to spread throughout the colony to elicit change in the colony response as a whole (Winston, 1987; Bailey & Ball, 1991). The fact that all bees sampled during this experiment were 24 hours old, and had no visible sign of parasitism or disease means that the level of immune investment must have been set either through

an alteration of egg physiology or brood food composition or altered nursing behaviour that resulted in an increased immune response.

6.3.5 Single colonies as a measure of general bee response

Colonies used in Chapters 2, 3 and 4 were *Varroa*-free in the build-up to, and during experimental periods. Results from all experiments in these chapters found that colonies showed qualitatively (though not quantitatively) identical responses within each experiment. However, the colonies used in Chapter 5 showed both quantitative and qualitative variation in their immune responses. With *Varroa* infestations sweeping through UK hives, the presence of *Varroa*-free colonies is a rarity, and only achievable in this thesis due to a combination of extreme weather conditions and strategic pesticide application.

Although the variation seen in Chapter 5 may be due to natural variation at the colony level over time, further investigation may show it to be a colony response to *Varroa* infestation. If this is the case, experimental designs which have previously sampled only one colony as representative of the honey bee population as a whole should perhaps be carefully reconsidered.

6.3.6 Is PO a good measure of the immune response?

PO undoubtedly plays many crucial roles in the organisation and efficacy of the innate immune system of insects, and it has long been used to measure an individual's immune defence ability (Brookman *et al.*, 1989; Söderhäll & Cerenius, 1998; Wilson *et al.*, 2001; Sugumaran, 2002). Nevertheless, contradictory evidence exists that increasing PO concentrations are indeed correlated with an increased resistance to pathogens, and the use of PO assays as a good measure of the immune response of an individual is not without controversy (Adamo, 2004; Mucklow *et al.*, 2004).

Variation in cuticle colour in the mealworm beetle, *Tenebrio molitor*, due to increased melanin production correlates with PO levels, with darker beetles having an elevated PO level and an increased resistance to pathogens (Barnes & Siva-Jothy, 2000; Armitage & Siva-Jothy, 2005). However, cuticle melanisation is a prophylactic response: although the majority of studies have shown that it confers an increased resistance to pathogen invasion, it is not a measure of the immune capabilities of PO in response to a direct immune challenge (Wilson *et al.*, 2001).

Whilst some studies have shown no correlation between PO concentration and pathogen susceptibility (Adamo, 2004; Mucklow *et al.*, 2004; Leclerc *et al.*, 2006; Schwarzenbach & Ward, 2007), others provide compelling evidence to suggest otherwise.

Active PO levels double in bumble bees (*Bombus terrestris*) infected with the trypanosome gut parasite *Crithidia bombi*, compared to non-infected individuals (Brown *et al.*, 2003). Although the trypanosomes are non-invasive, this suggests that a messenger system exists between the gut and the ProPO-AS, with PO being up-regulated in preparation for an immune response to invading pathogens (Wilson *et al.*, 2001; Brown *et al.*, 2003). In *Drosophila*, mutant strains devoid of blood phenoloxidase activity showed a decreased resistance to infection with fungal spores (Braun *et al.*, 1998). Haemolymph concentrations of ProPO and PO of tsetse fly species (*Glossina palpalis palpalis*) bred for refractoriness to *Trypanosoma brucei rhodesiens* were higher than in species susceptible to infection (Nigam *et al.*, 1997). Further evidence comes from *Photorhabdus luminescens*, a virulent bacterial pathogen of the tobacco hornworm, *Manduca sexta*, which secretes a compound to inhibit host PO production. Mutant bacteria that lack this compound are non-virulent, suggesting that PO is needed for host defence (Eleftherianos *et al.*, 2008).

6.3.7 Applied relevance of findings

During the period of this research, honey bees have been thrust into the global limelight, as Colony Collapse Disorder (CCD) arrived and continues to cause widespread devastation to bee populations in America and potentially, the UK (Cox-Foster *et al.*, 2007). Given their contribution to the British economy as crop pollinators (Carreck & Williams, 1998), it has never been more relevant to study the honey bee. Whilst ongoing research is attempting to identify the factors causing CCD, the work of this thesis to establish a grounding in honey bee immunity provides the opportunity of an additional perspective from which to address the issues associated with CCD and other bee diseases.

6.4 Future Research

6.4.1 Queen immunity

This thesis has not covered the immune responses of honey bee queens, although the selective pressures exerted on them as the sole producer of

offspring for the colony, and the consequences that this has on immune investment, make them an obvious choice for study. However, several problems arise when attempting this. Large numbers of queens can be obtained through artificial grafting of eggs. Several attempts were made during this thesis to graft queens. Whilst some were successful, they did not yield a workable sample size due to additional complications of incubator malfunctions and cell destruction by other queens (Winston, 1987). Once emerged, adult queens may be introduced into queenless colonies, where they can live for 3-4 years (Hooper, 1997), or can be kept in queen cages with a couple of worker attendants and successfully maintained in an incubator for at least a month (S. Martin, pers. comm.).

However, to gain a perspective of their ecological immunity, experimental queens should be returned to a colony so they can start reproduction. Queens do not show a trade-off with reproduction and longevity, and this is thought to be due to the yolk precursor vitellogenin (Corona *et al.*, 2007). In workers, a decrease in vitellogenin concentration with age is associated with haemocyte pycnosis (Amdam *et al.*, 2004). In queens however, vitellogenin titres increase with age, and is associated with increasing longevity and resistance to oxidative stress (Corona *et al.*, 2007). This suggests that high haemocyte numbers should be maintained throughout their life, and may lead in an increased ability for immune investment.

6.4.2 The honey bee-*Varroa* mite system

The groundwork covered in this thesis has built up a comprehensive picture of honey bee immunity and investment in workers and drones throughout the whole life-cycle. It was initiated with the aim of looking to honey bees, *Varroa* mites and a virus (Deformed Wing Virus, DWV) they vector as an ideal model system with which to empirically study parasite competition and the evolution of virulence. Chapter 5 has started this work, exploring the effects of *Varroa* on the immune response. Other work on this system has so far focussed on the identification and quantification of viral strains within bees and mites (e.g. Chen *et al.*, 2005a; Chen *et al.*, 2006; Cox-Foster *et al.*, 2007), presence of multiple infections in hosts (e.g. Tentcheva *et al.*, 2004; Chen *et al.*, 2004b; Chen *et al.*, 2005b; Chen *et al.*, 2005c; Shen *et al.*, 2005a, Shen *et al.*, 2005b) and modes of transmission (Bowen-Walker *et al.*, 1999; Nordstrom, 2003; Chen *et al.*, 2004b), but due to the relatively recent introduction of *Varroa* and DWV to

European bees, this work is still in its infancy.

Of particular interest to me are the host responses to multiple virus infections, with a view to developing further assays and techniques to investigate the mechanism of viral infection. Within-host selection on the virus, incorporating transmission dynamics and virulence levels in the host and vector are also prime targets for investigation. DWV has been found in all developmental stages of all host castes, and its presence in queen ovaries and spermathecae suggest mechanisms for vertical as well as horizontal transfer (Chen *et al.*, 2004a; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Chen *et al.*, 2006).

DWV has been shown to replicate within the mite vector (Bowen-Walker *et al.*, 1999; Ongus *et al.*, 2004) which gives rise to the possibility that *Varroa* could vector the virus to a wider host range than previously imagined (Genersch *et al.*, 2006). Its recent detection in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) turns this possibility into a reality and provides a platform to study the evolution of host shift in a virus (Genersch *et al.*, 2006).

Multiple viral infections within a single individual have been well documented (Anderson & Gibbs, 1988; Martin *et al.*, 1998; Brodsgaard *et al.*, 2000; Benjeddou *et al.*, 2001; Evans, 2001; Tentcheva *et al.*, 2004; Chen *et al.*, 2004b; Chen *et al.*, 2005b; Chen *et al.*, 2005c; Shen *et al.*, 2005a, Shen *et al.*, 2005b), and development of multiplex RT-PCR allows these infections to be detected in a single test, but it is not known whether these co-infecting viruses are in direct competition (Chen *et al.*, 2004b). Increased replication in response to competition may effect transmission, and stimulate the replication of inapparent infections.

6.4.3 Understanding the immune system

One of the main findings of this thesis is that despite evidence showing that foraging workers have reduced numbers of functioning haemocytes (Flachuk, 1998; Amdam *et al.*, 2004; Amdam *et al.*, 2005), the cellular PO and ProPO immune responses are increased in foragers compared to hive bees. This suggests that we cannot simply infer what appears to be logical, and that this 'simple' immune system is in fact much more complex. The pycnotic effect on bee haemocytes is caused by apoptosis, and so irreversible (Amdam *et al.*, 2005). However, honey bees are highly plastic in their division of labour, and reversal of foragers into nurse bees is possible (Robinson, 1992; Amdam *et al.*,

2005). This switch is associated with an increase in numbers of haemocytes, meaning old bees have the ability to rapidly recruit new haemocytes (Amdam *et al.*, 2005). Investigation into correlations of the level of functional haemocytes in normal and reversed foragers and their cellular immune responses could provide an explanation for this disparity.

6.5 Final conclusions

When developing a novel system for immunity studies, it is vital to understand the basal immunity profile of the chosen animal for all the life-stages you wish to investigate. The findings of this thesis support this notion, with unexpected conclusions discovered within developmental, senescent, caste and colony level divisions. This knowledge, when placed in an ecological immunity context, opens new avenues with which to explore host-pathogen effects. In light of the current global crisis, this may be fundamentally important for the honey bee.

Appendix 1

Methods

A1.1 Enzyme kinetics

The Michaelis-Menten equation is used to describe the kinetic behaviour of many enzymes. It assumes a hyperbolic relationship between the velocity of an enzyme reaction and the substrate concentration described by the equation:

$$v = \frac{\mathbf{Vmax}\ [s]}{\mathbf{Km} + [s]}$$

v = velocity of the enzyme reaction

Vmax = reaction velocity when all substrates are at saturating concentration

[s] = substrate concentration

Km = Michaelis constant; equal to the substrate concentration at which the reaction rate is half the maximum velocity (Vmax/2) of the enzyme (Henderson, 1992)

If the reaction is not substrate limited then it should proceed at Vmax. However, this rarely happens due to limiting factors such as solubility restrictions (Tipton, 1992). As the value of Km is specific to the enzyme and substrate used, it must be found in order to calculate the concentration of enzyme needed to prevent substrate limitation. As Km is equal to the substrate concentration at which the reaction proceeds at half its maximum velocity, doubling Km should give the substrate concentration needed to achieve maximum velocity (Henderson, 1992).

Figure A1.1 shows a basic reaction curve of absorbance against time. The absorbency (measured photometrically) is proportional to the concentration of the product (dopachrome). By measuring the change in absorbency over time during the linear rate phase of the curve, the Vmax of the reaction can be found. The concentration of enzyme (PO) present in the sample is proportional to the Vmax (Tipton, 1992).

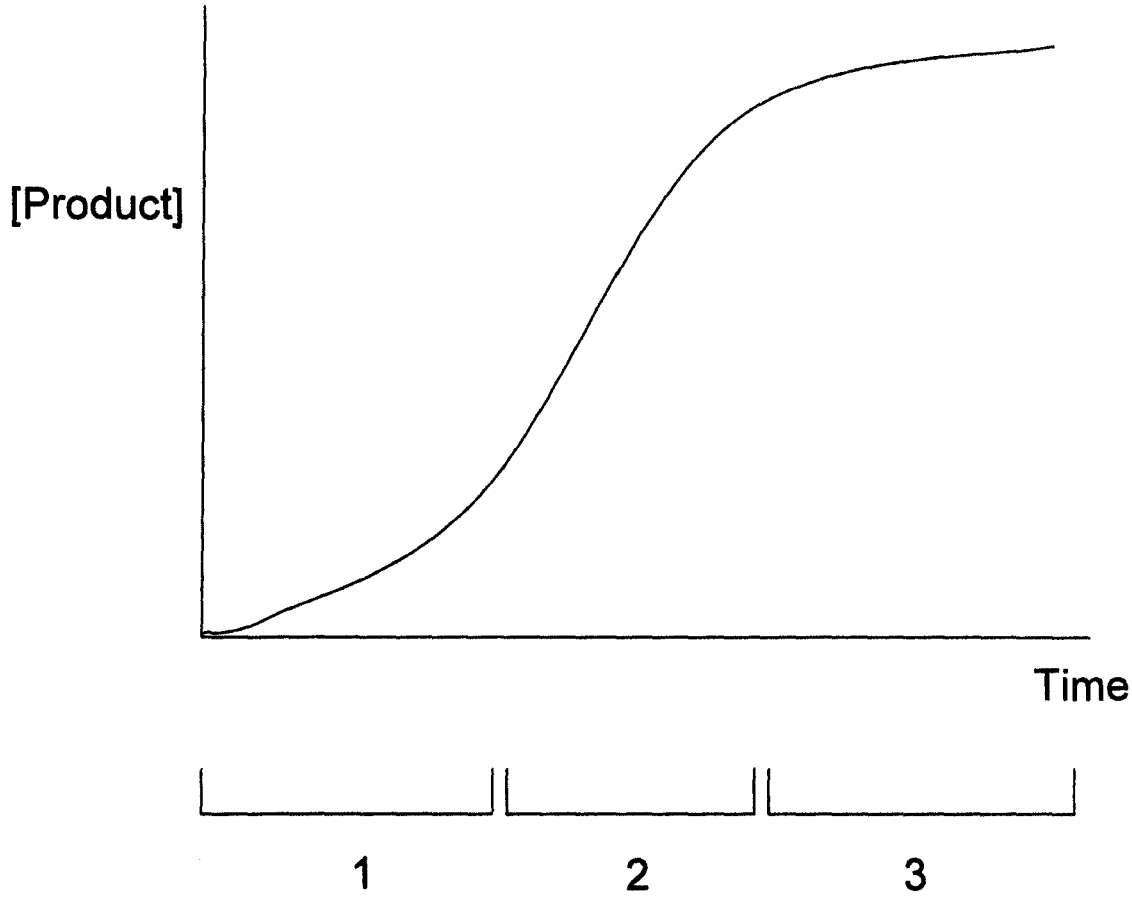


Figure A1.1. Typical enzyme-catalysed reaction curve showing an increase in product concentration over time. This curve is identical to that seen in the conversion of L-dopa to dopachrome by the action of the enzyme phenoloxidase.

1 - Initial Lag Phase

2 - Rate phase

3 - End Lag Phase

A1.2 The Effect of Repeated Defrosting on Haemolymph Samples

A1.2.1 Introduction

All samples collected for this PhD were assayed for two different PO values: the 'active' PO, and 'total immune potential' after the activation of ProPO. Previous studies have defrosted and refrozen the same sample on numerous occasions to take different measures. Doubt arose during preliminary experiments as to the value of defrosting the same sample when it was seen that PO levels increased with each defrosting event (pers. obs.). To determine whether this increase was due to extra activation of ProPO during periods out of the freezer, or additional cell breakdown caused by repeated freeze-thawing, the following experiment was carried out.

A1.2.2 Materials and methods

Worker bees were collected from a single colony in November 2005. Bees were split into two treatment groups:

Group A: Haemolymph collected with a standard perfusion bleed (n=18), and frozen at -90 °C for 24 hours. The sample was defrosted and assayed for PO and ProPO as standard (Sections 2.3.11 & 2.4.13). The sample was then spun and the supernatent removed and frozen separately. The supernatant was subsequently defrosted and assayed for PO and ProPO twice more.

Group B: Haemolymph collected as standard and frozen at -90 °C. Each sample was then defrosted and refrozen three times, with PO and ProPO assays taken after each defrosting (n=41). An initial PO and ProPO assay was run before the first freezing (n=21).

A1.2.3 Results

Group A: There was no significant effect of defrosting and re-freezing the samples once the supernatant had been separated from the pellet. This result was seen for both PO (Figure A1.2, $F_{1,55} = 2.758$, $p=0.1024$, data log10 transformed) and ProPO (Figure A1.3, $F_{1,55} = 1.373$, $p=0.2464$, data log10 transformed).

Group B: There was a significant increase in both PO (Figure A1.4, $F_{1,139} = 30.307$, $p<0.0001$, data log10 transformed) and ProPO (Figure A1.5, $F_{1,139} = 12.381$, $p<0.001$, data log10 transformed) concentrations with each defrosting event.

A1.2.4 Discussion

We conclude that the observed increase in PO and ProPO concentrations with assays taken from the same samples, repeatedly defrosted, is due to additional cellular breakdown during the freezing process. All subsequent samples taken in this thesis are frozen in aliquots to avoid this error.

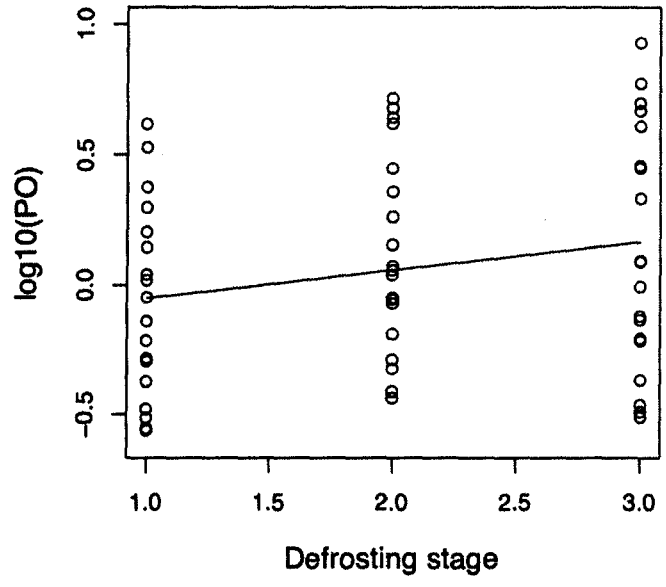


Figure A1.2. Group A PO, measured over 3 repeated defrosting/refreezing events. There was no significant difference between the repeats ($F_{1,55} = 2.758$, p=0.1024, data log10 transformed)

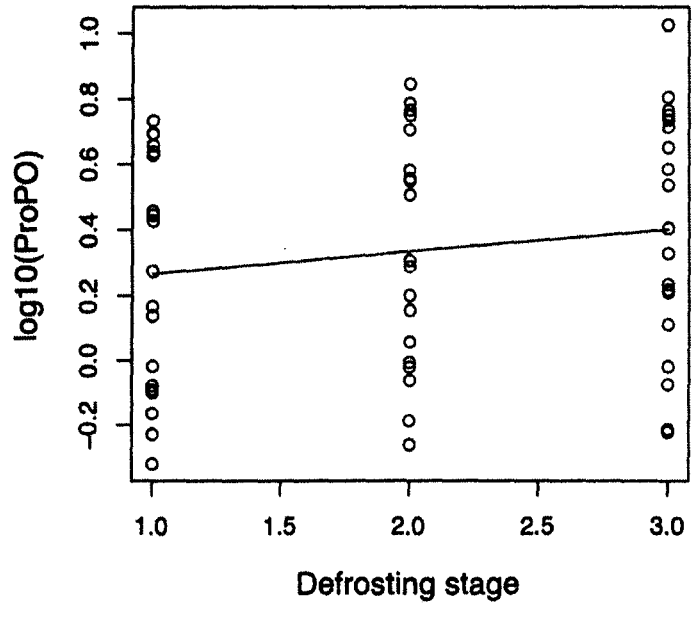


Figure A1.3. Group A ProPO, measured over 3 repeated defrosting/refreezing events. There was no significant difference between the repeats ($F_{1,55} = 1.373$, p=0.2464, data log10 transformed)

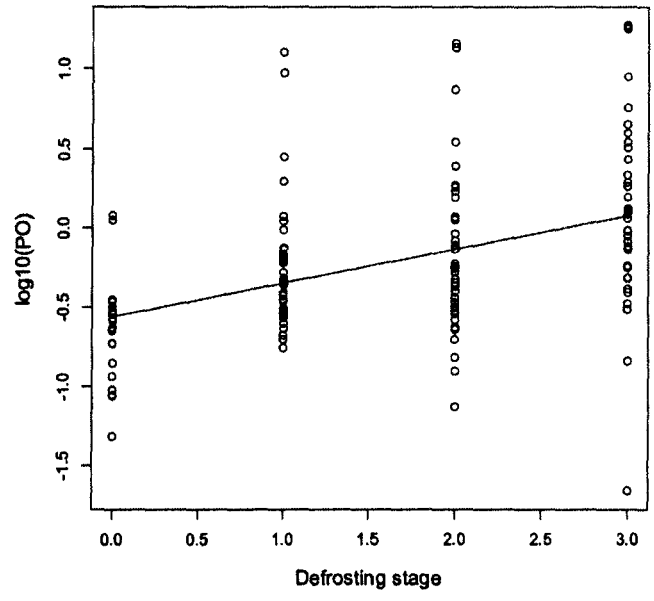


Figure A1.4. Group B PO, measured before freezing (stage 0), and the over 3 repeated defrosting/refreezing events. There was a significant increase in PO concentration between the defrosting events ($F_{1,139} = 30.307$, $p<0.0001$, data log10 transformed)

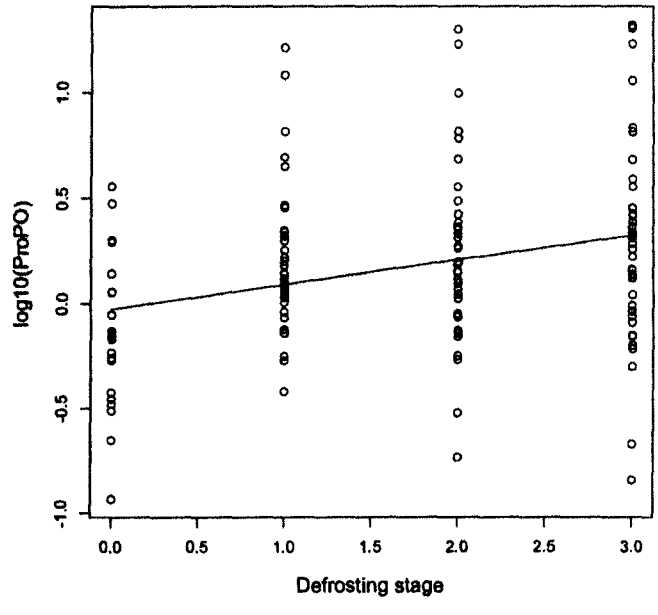


Figure A1.5. Group B ProPO, measured before freezing (stage 0), and the over 3 repeated defrosting/refreezing events. There was a significant increase in PO concentration between the defrosting events ($F_{1,139} = 30.307$, $p<0.0001$, data log10 transformed)

A1.3 The Effect of Lipid on PO and ProPO Assays

A1.3.1 Introduction

As seen in Chapter 3, Section 3.3.2, a high percentage of the samples assayed from earlier developmental stages returned a concentration of PO (and in some cases, ProPO), which was so low as to be unrecordable by this assay. A standard enzyme curve, as seen in the majority of PO and ProPO assays is shown in Figure A1.6. Many of the larval and pupal samples produced negative curves (Figure A1.7). This suggests that the sample is becoming a lighter colour over time. I highlighted three possible explanations for these results:

1. PO is being produced at such a rate that it is being rapidly degraded to prevent its cytotoxic effects, or it is aggregating into larger protein aggregates
2. Although samples were centrifuged twice to remove fat, there was still some fat present in the sample, which gave it a slightly cloudy colour. Lipases may be working to break down the fat, so making the solution clearer over time
3. Something is working to break down the L-dopa which is produced from the reaction with PO

A1.3.2 Materials and Methods

To test these hypotheses, 8 uncapped, unchallenged larval aliquots were defrosted from samples that had showed a negative enzyme curve in the developmental immunity experiment (Chapter 3, Section 3.3.2). Samples were defrosted on ice, and spun to remove the fat. Each sample was split into 4 different treatment groups:

1. Samples run as standard PO assay (Chapter 2, Section 2.3.11)
2. Samples run with the dH_2O replaced by dH_2O into which a protease inhibitor was added (1 x COMPLETE Mini EDTA tablet dissolved in 7ml dH_2O)
3. Samples run substituting L-dopa for 20µl dH_2O
4. Samples run substituting L-dopa for L-dopa which had been allowed to develop into dopachrome over 48 hours, so was already a dark colour

Controls for all four treatments were run, along with a positive control (adult haemolymph).

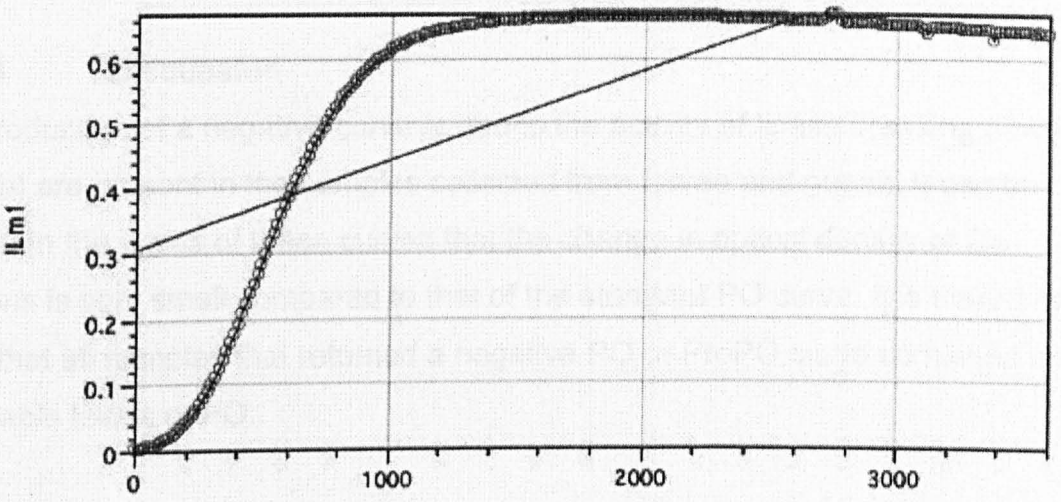


Figure A1.6. Standard PO enzyme curve

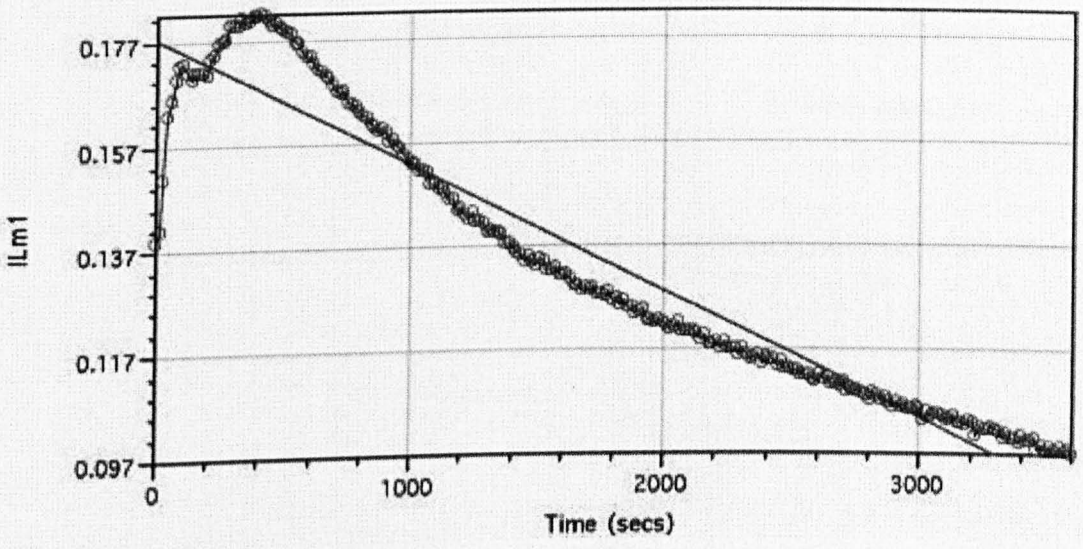


Figure A1.7. Negative PO enzyme curve

A1.3.3 Results

Treatment groups 2 to 4 showed no difference in the slope of the curves to that produced in the control (Treatment 1), i.e. in the case of Treatment 3, the same curve was produced without L-dopa being present. Treatment 4 produced the same curve, but shifted up the y-axis due to the darker colour of the sample.

A1.3.4 Discussion

The production of a negative curve is due to the activity of lipase breaking down fats that are present in the samples collected from larvae and pupae. It can be seen from the y-axis of these curves that the change in optical density of the solutions is very small compared to that of the standard PO curve. It is therefore taken that all samples that returned a negative PO or ProPO curve contained no recordable levels of PO.

Appendix 2

Figures

This appendix chapter provides full figures for all 4 treatment groups used throughout this thesis. Figures are divided up into the relevant Chapters, and are ordered as they appear in each Chapter.

A2.1 Chapter 3: Ontogeny of worker immunity: Figures

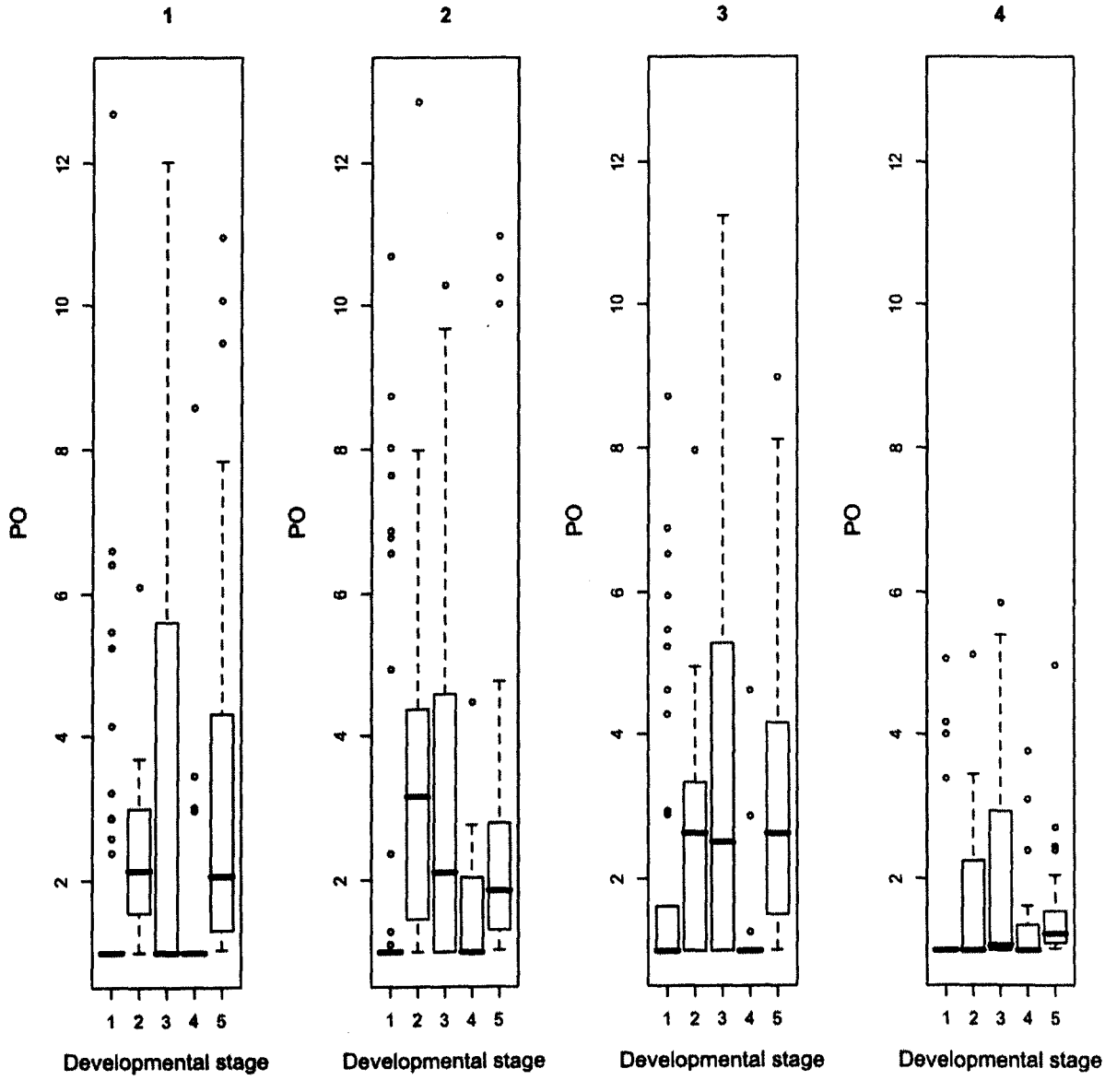


Figure A2.1.1. Hive A: PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

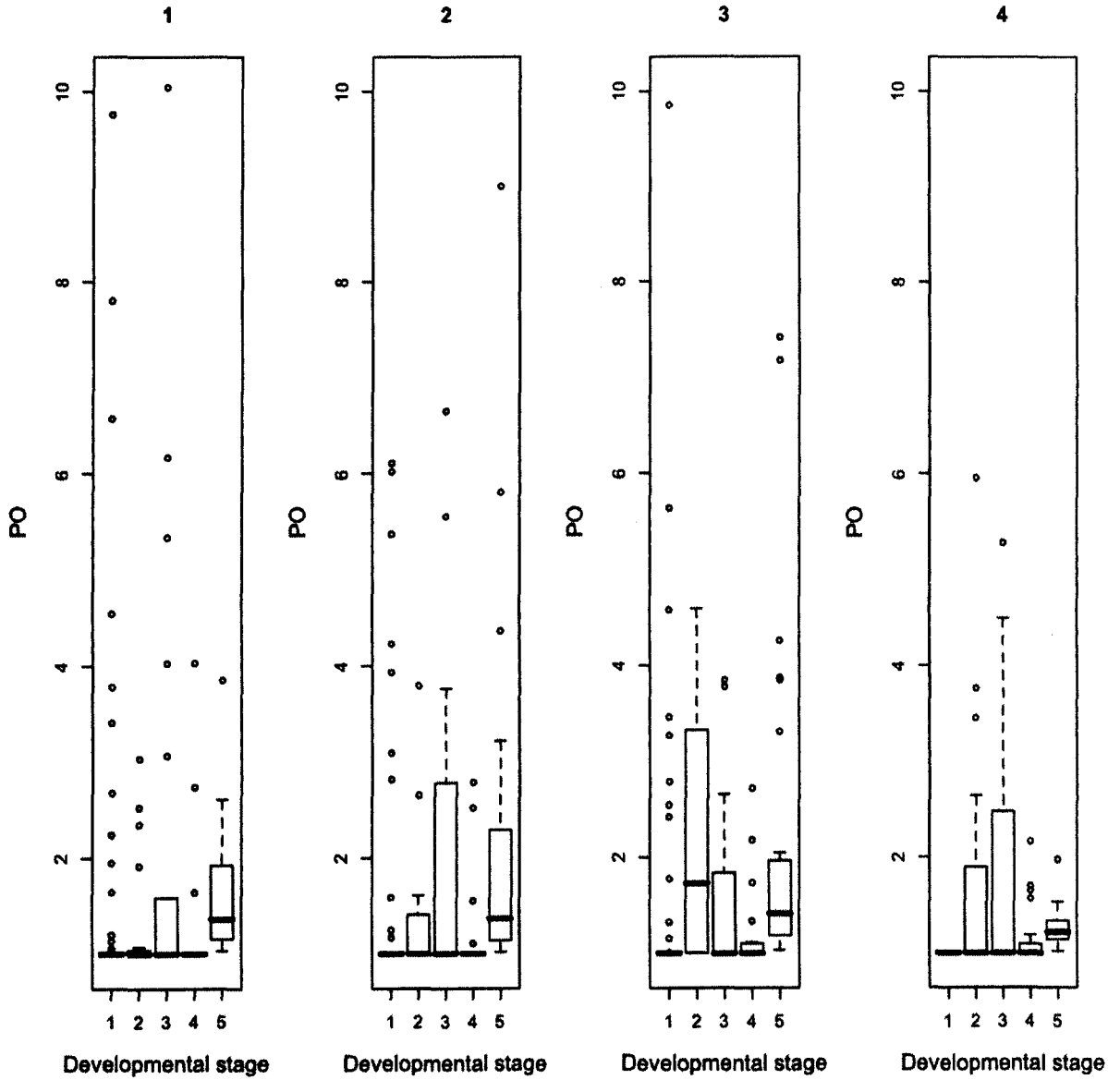


Figure A2.1.2. Hive B: PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

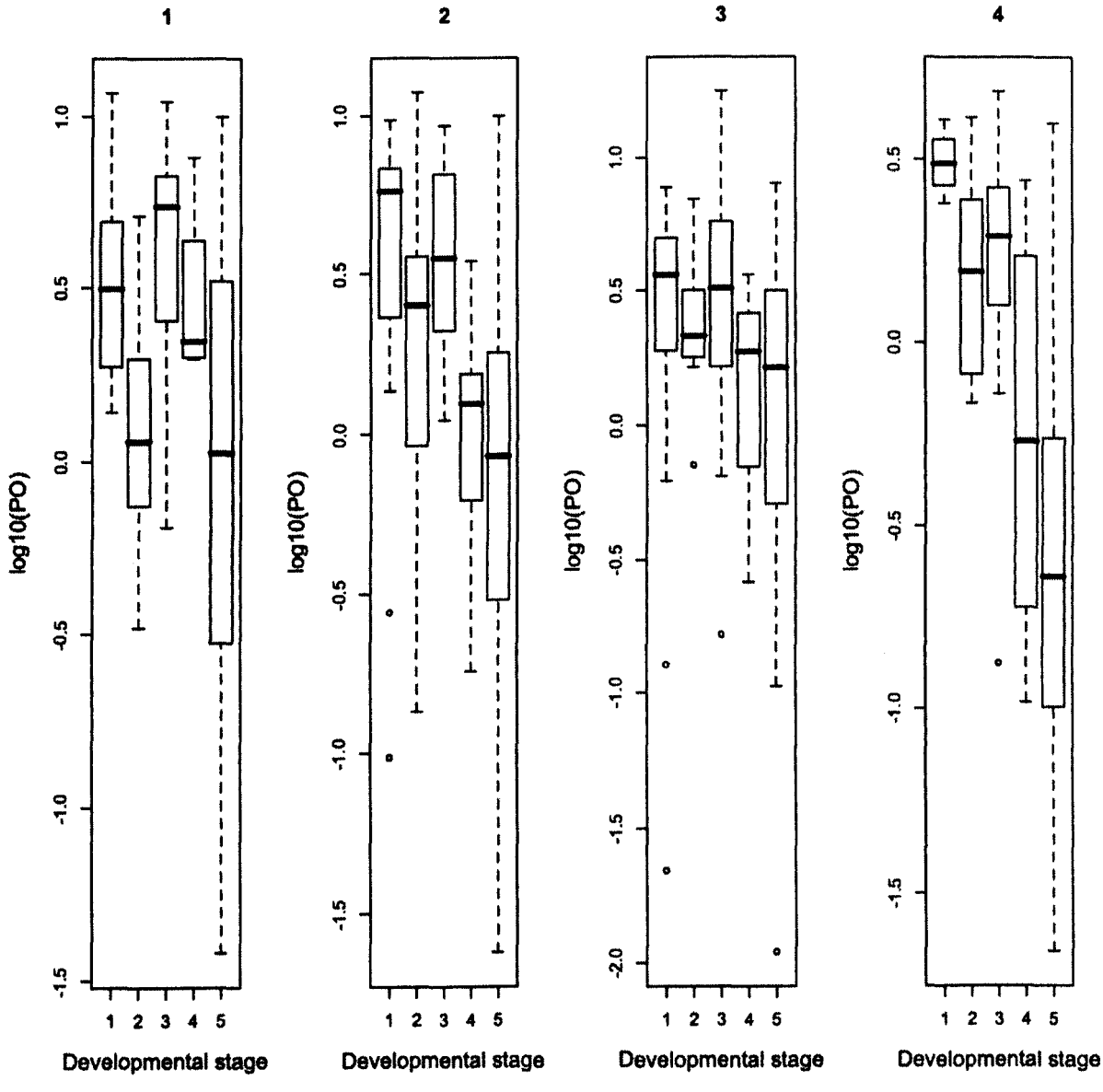


Figure A2.1.3. Hive A: PO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

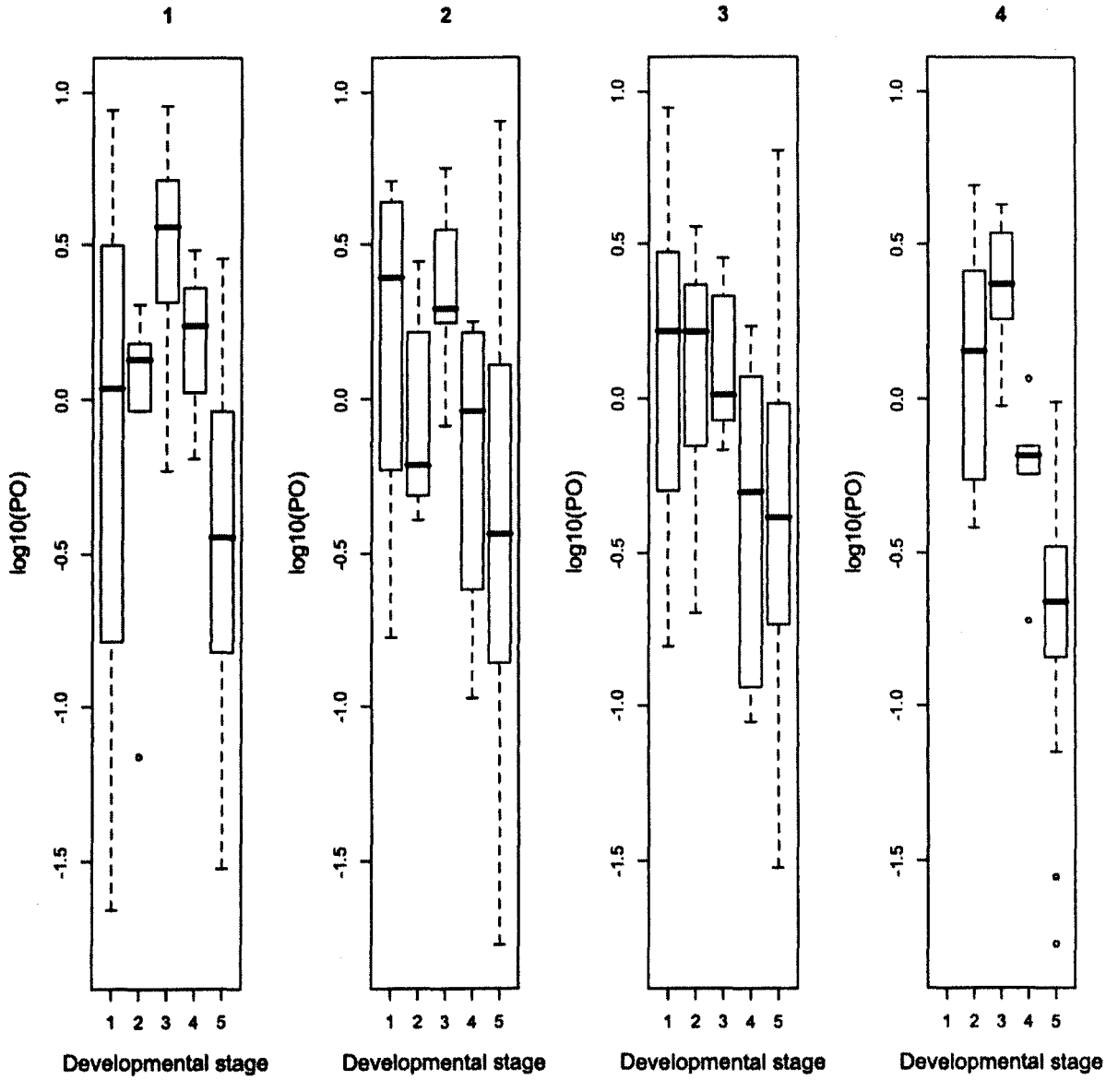


Figure A2.1.4. Hive B: PO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

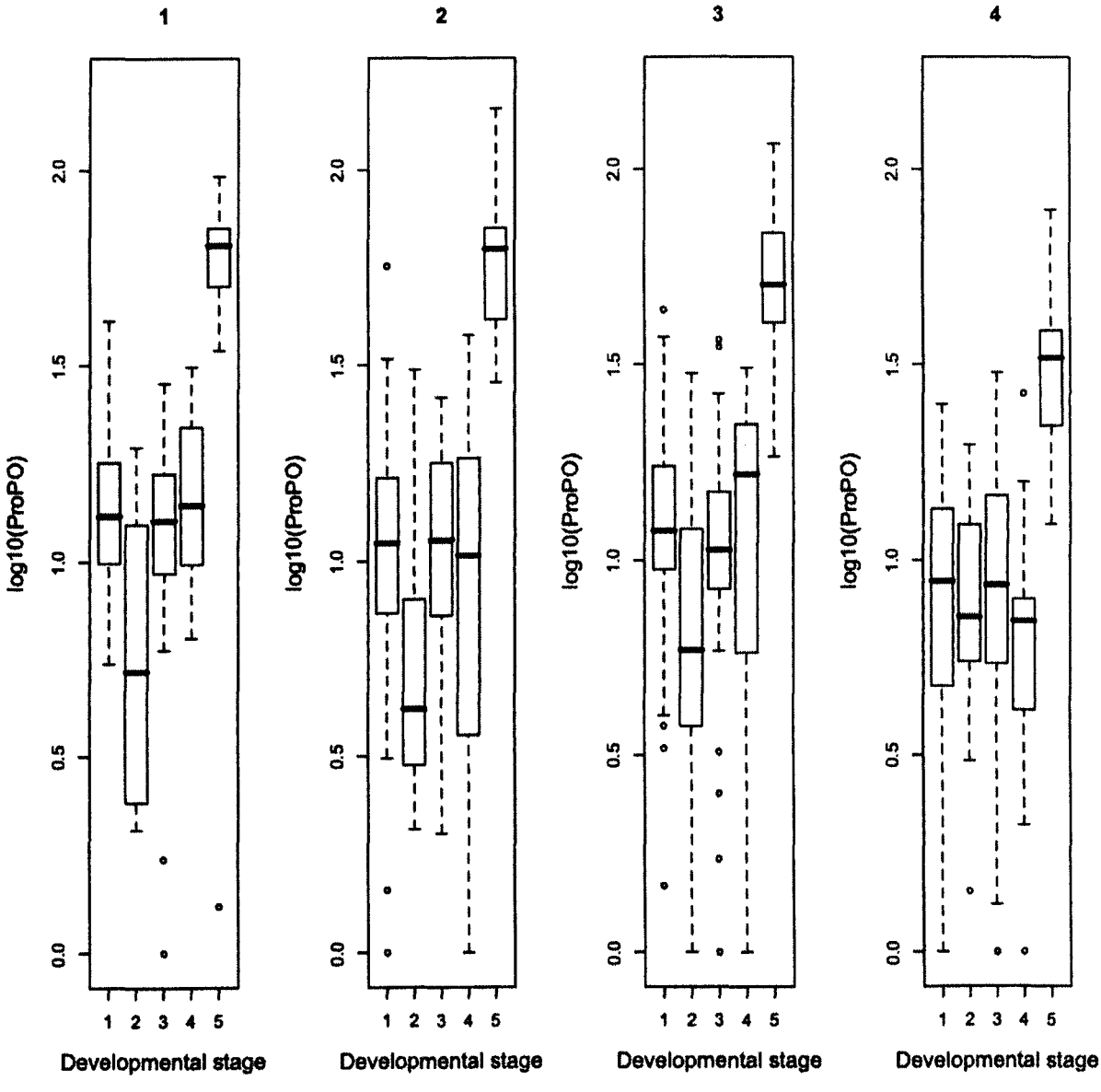


Figure A2.1.5. Hive A: ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

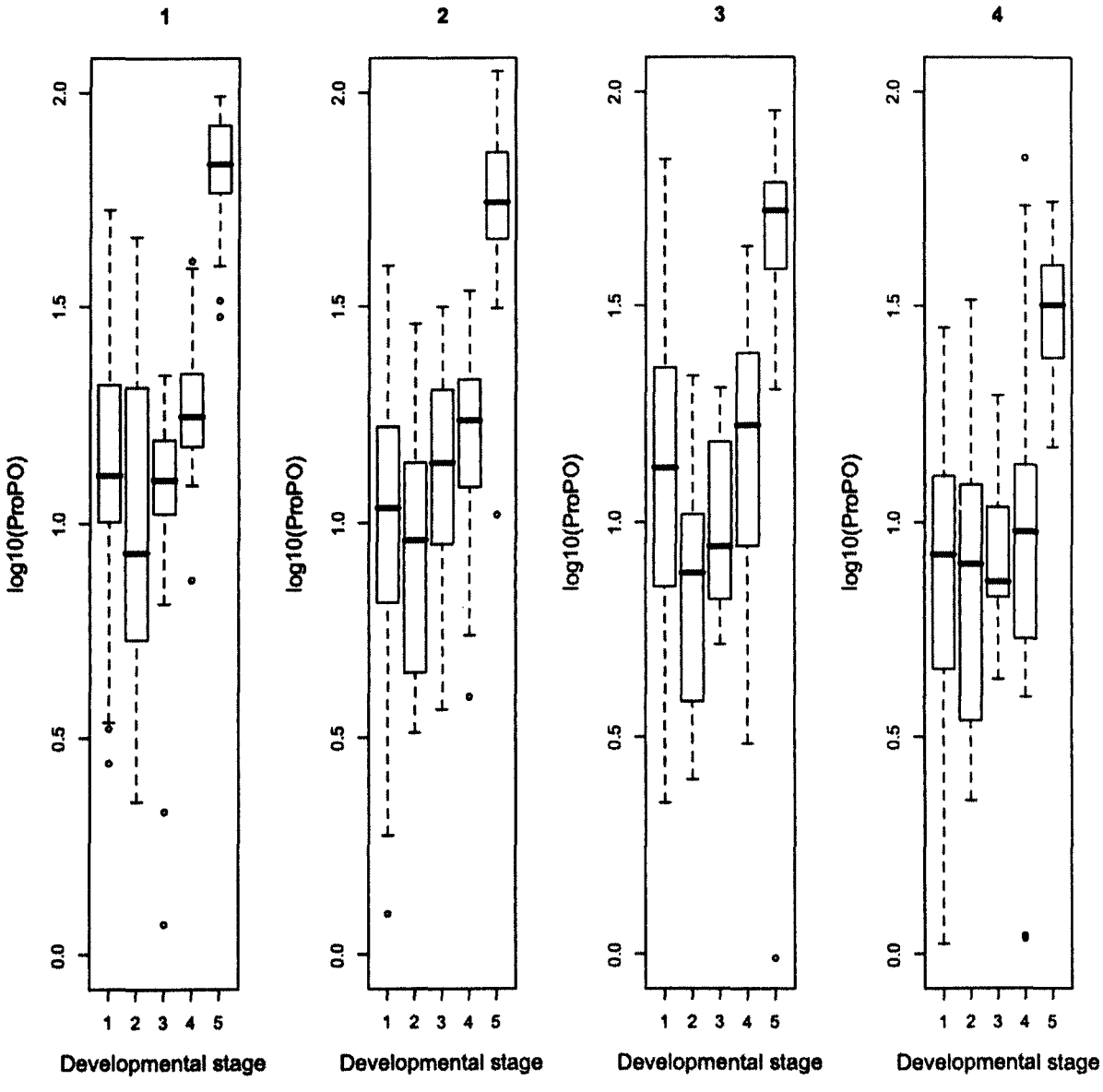


Figure A2.1.6. Hive B: ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

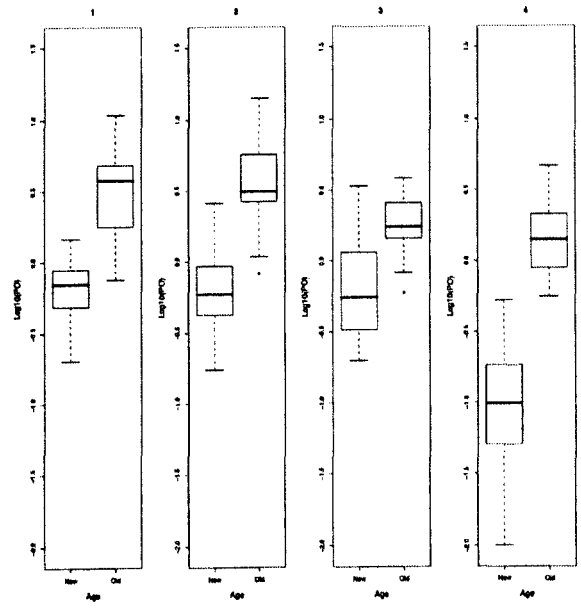


Figure A2.1.7. Hive A: PO concentrations for the two age groups, new and old, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

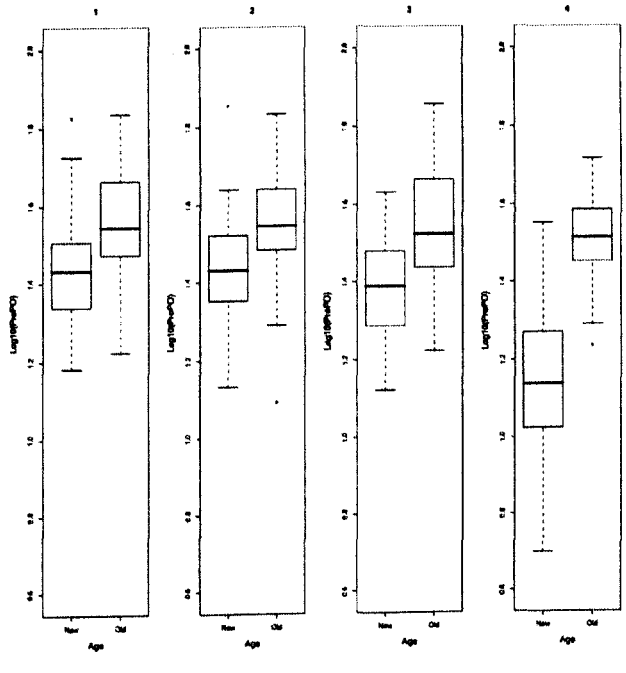


Figure A2.1.8. Hive A: ProPO concentrations for the two age groups, new and old, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

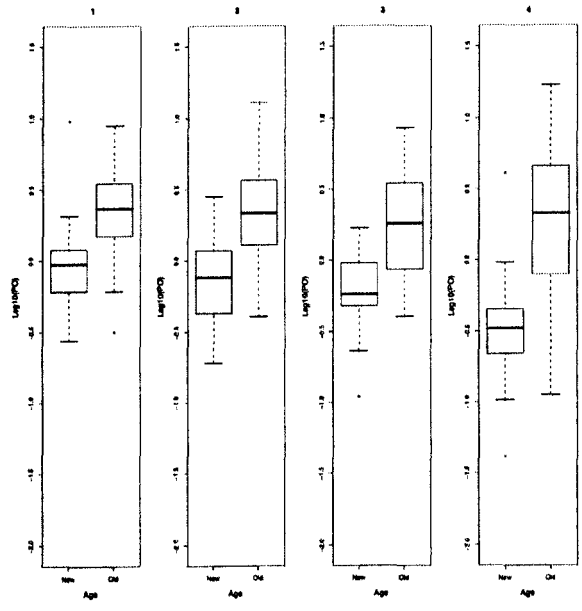


Figure A2.1.9. Hive B: PO concentrations for the two age groups, new and old, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

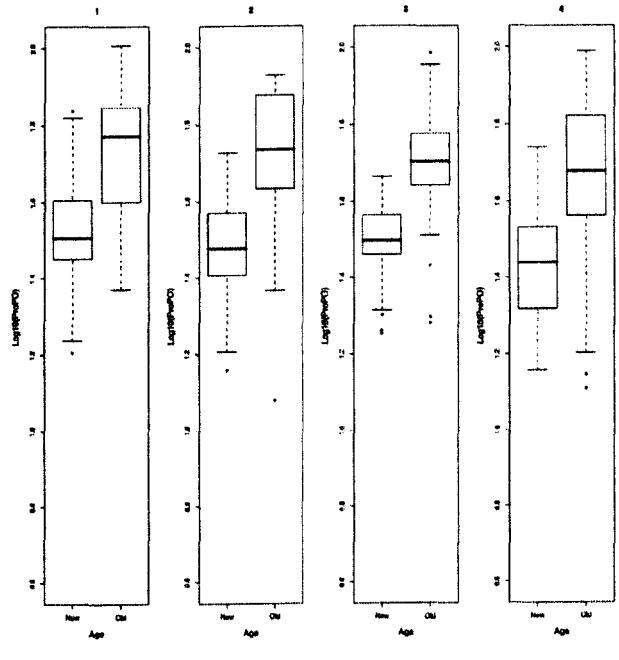


Figure A2.1.10. Hive B: ProPO concentrations for the two age groups, new and old, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

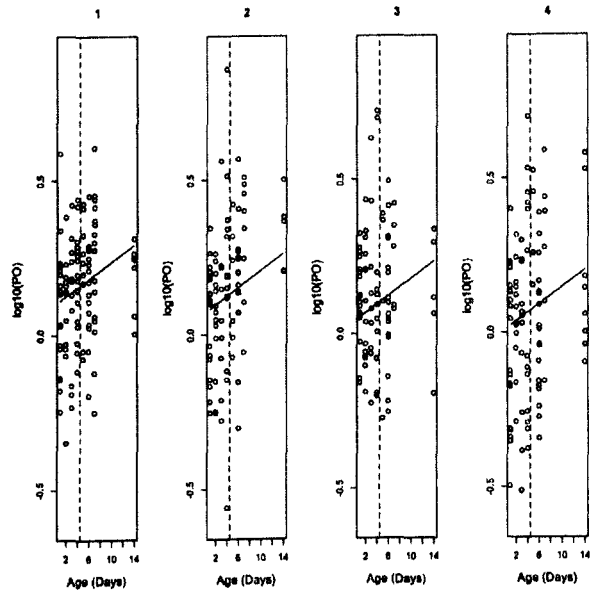


Figure A2.1.11. Hive G: Change in PO activity during the first 14 days of adult worker life, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed line represents the age at which test flights start.

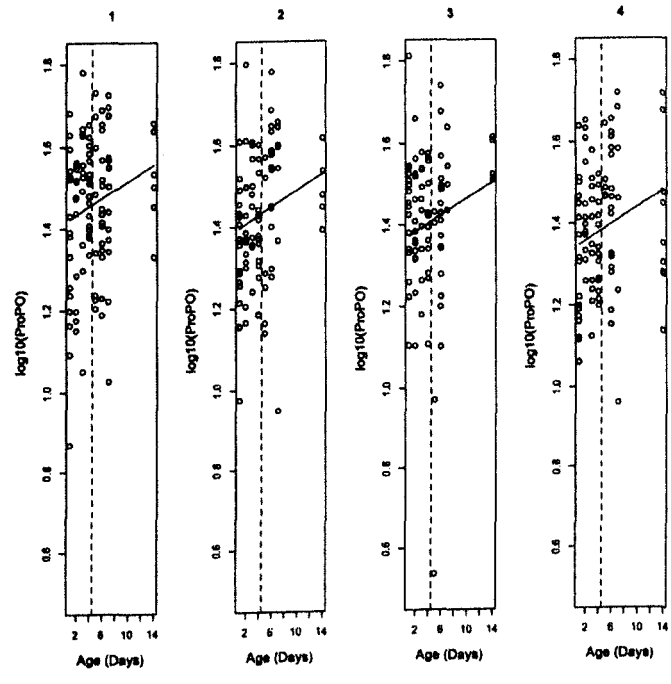


Figure A2.1.12. Hive G: Change in ProPO activity during the first 14 days of adult worker life, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed line represents the age at which test flights start.

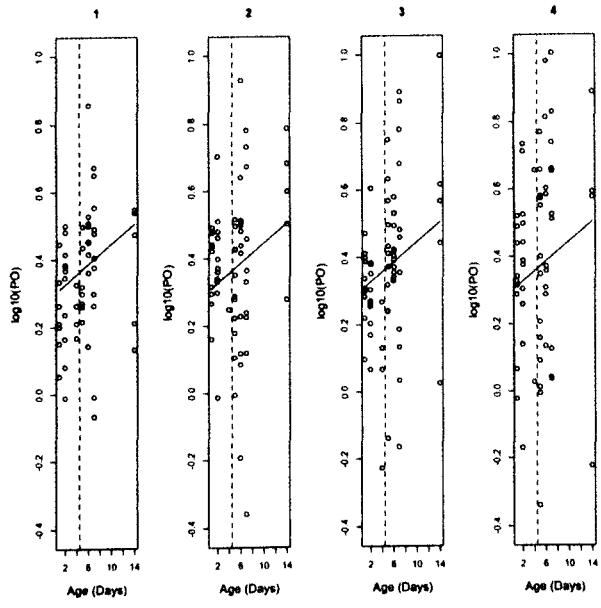


Figure A2.1.13. Hive H: Change in PO activity during the first 14 days of adult worker life, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed line represents the age at which test flights start.

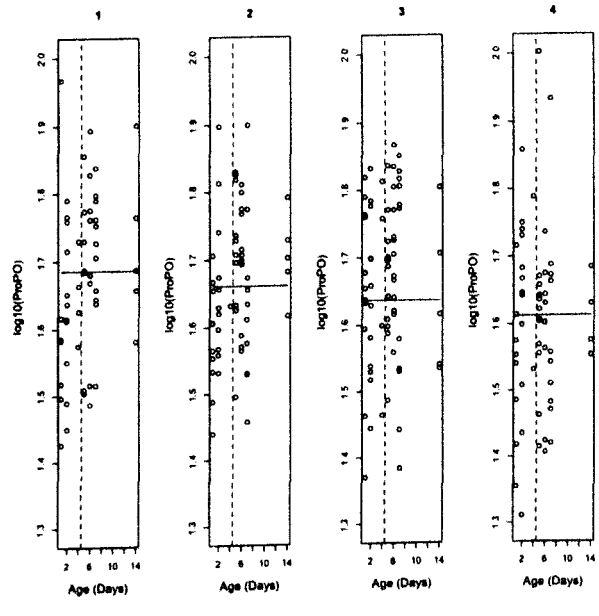


Figure A2.1.14. Hive H: Change in ProPO activity during the first 14 days of adult worker life, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed line represents the age at which test flights start.

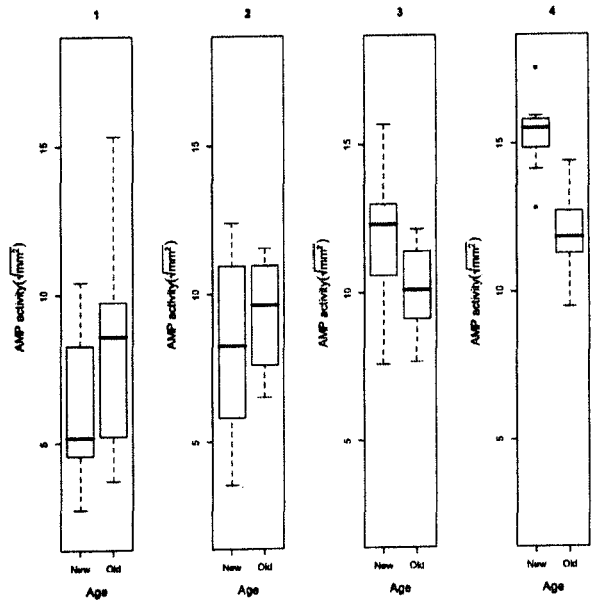


Figure A2.1.15. Antimicrobial peptide response of the two age groups, new and old, in Hive F for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

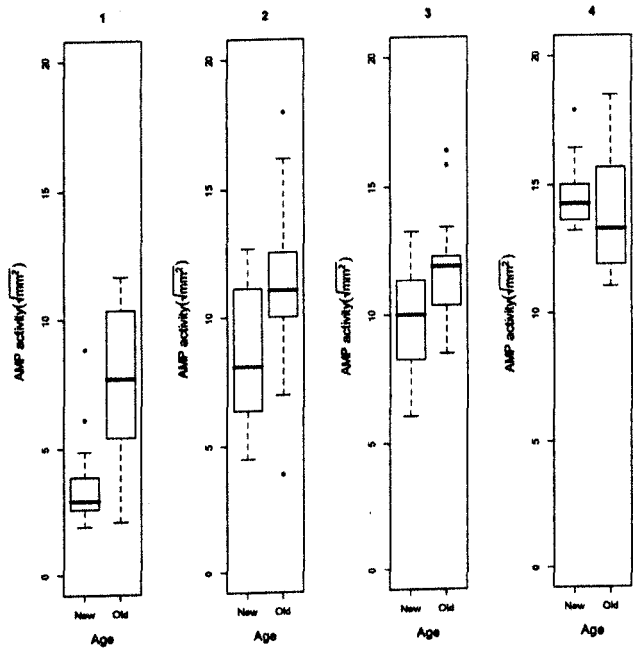


Figure A2.1.16. Antimicrobial peptide response of the two age groups, new and old, in Hive G for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

A2.2 Chapter 4: Ontogeny of drone immunity: Figures

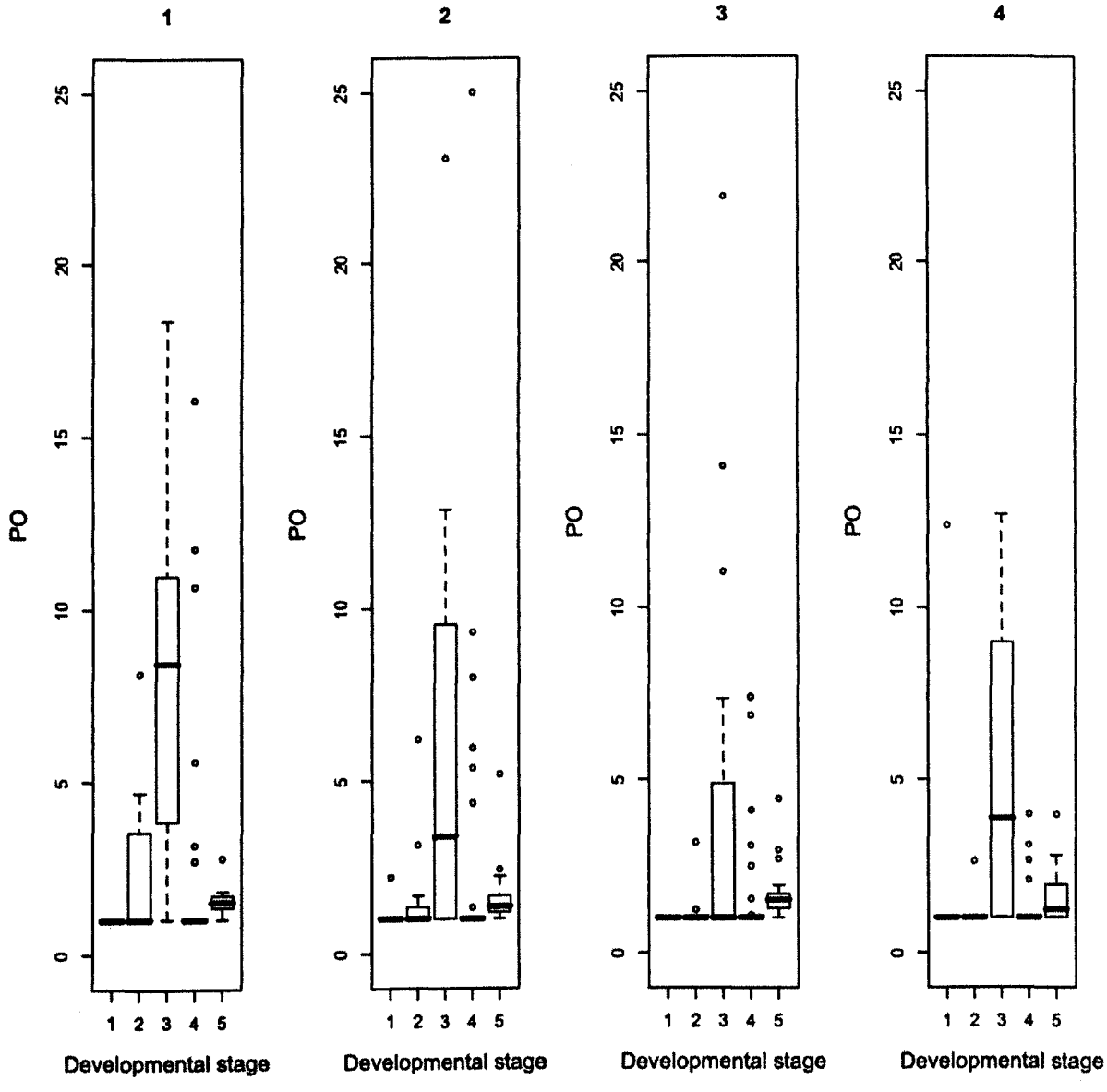


Figure A2.2.1. Hive D: Drone PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

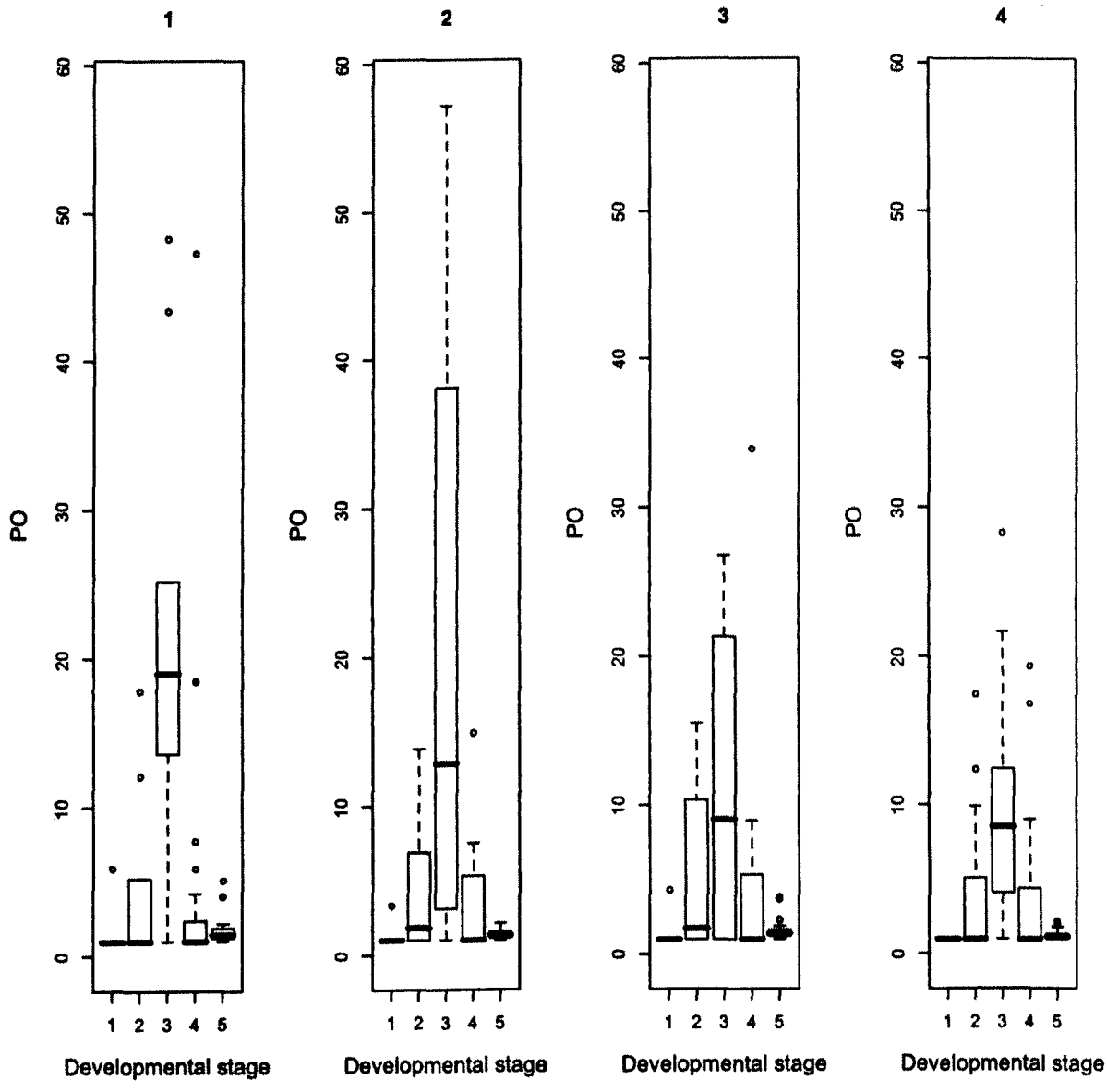


Figure A2.2.2. Hive E: Drone PO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

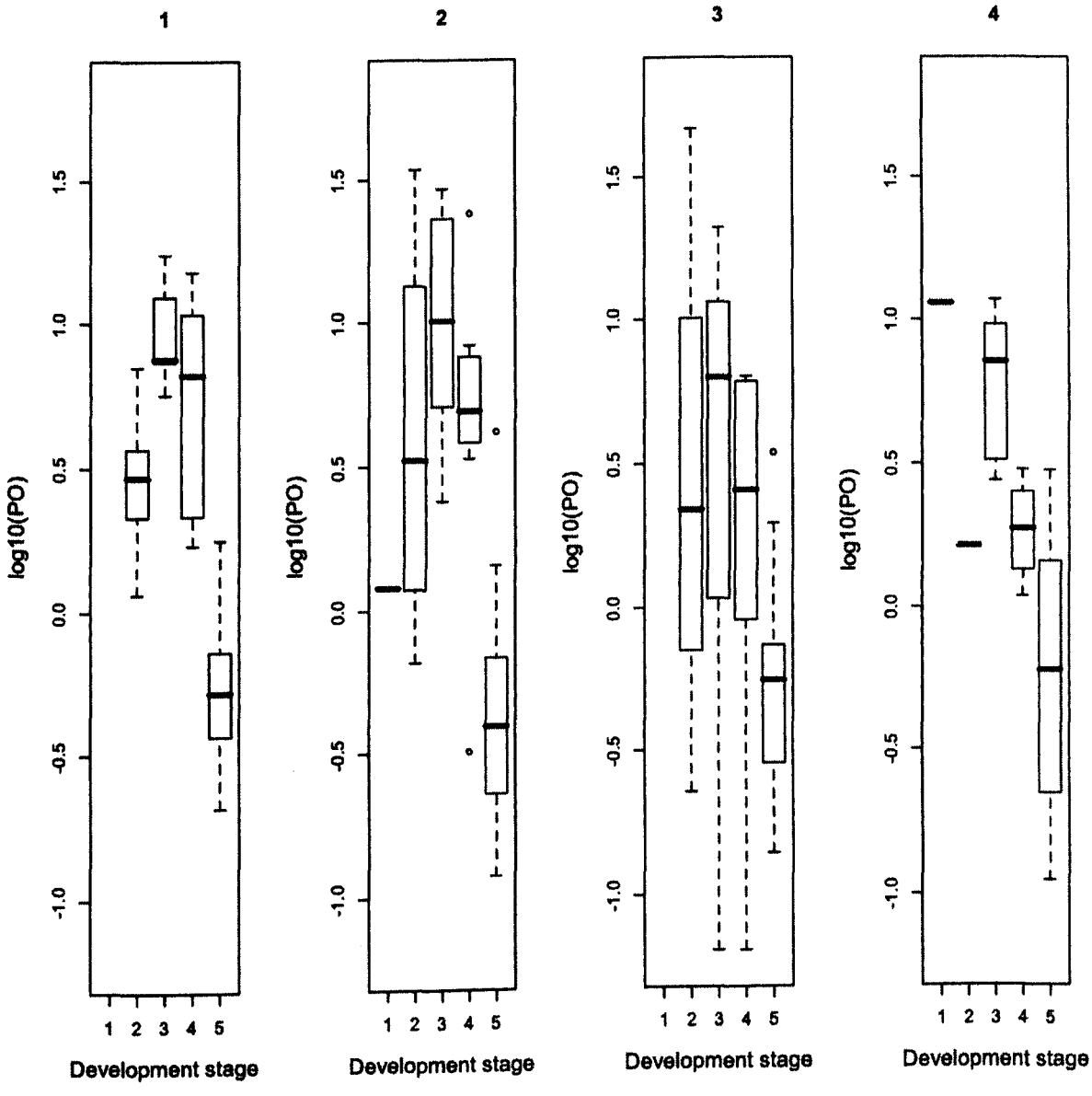


Figure A2.2.3. Hive D: Drone PO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

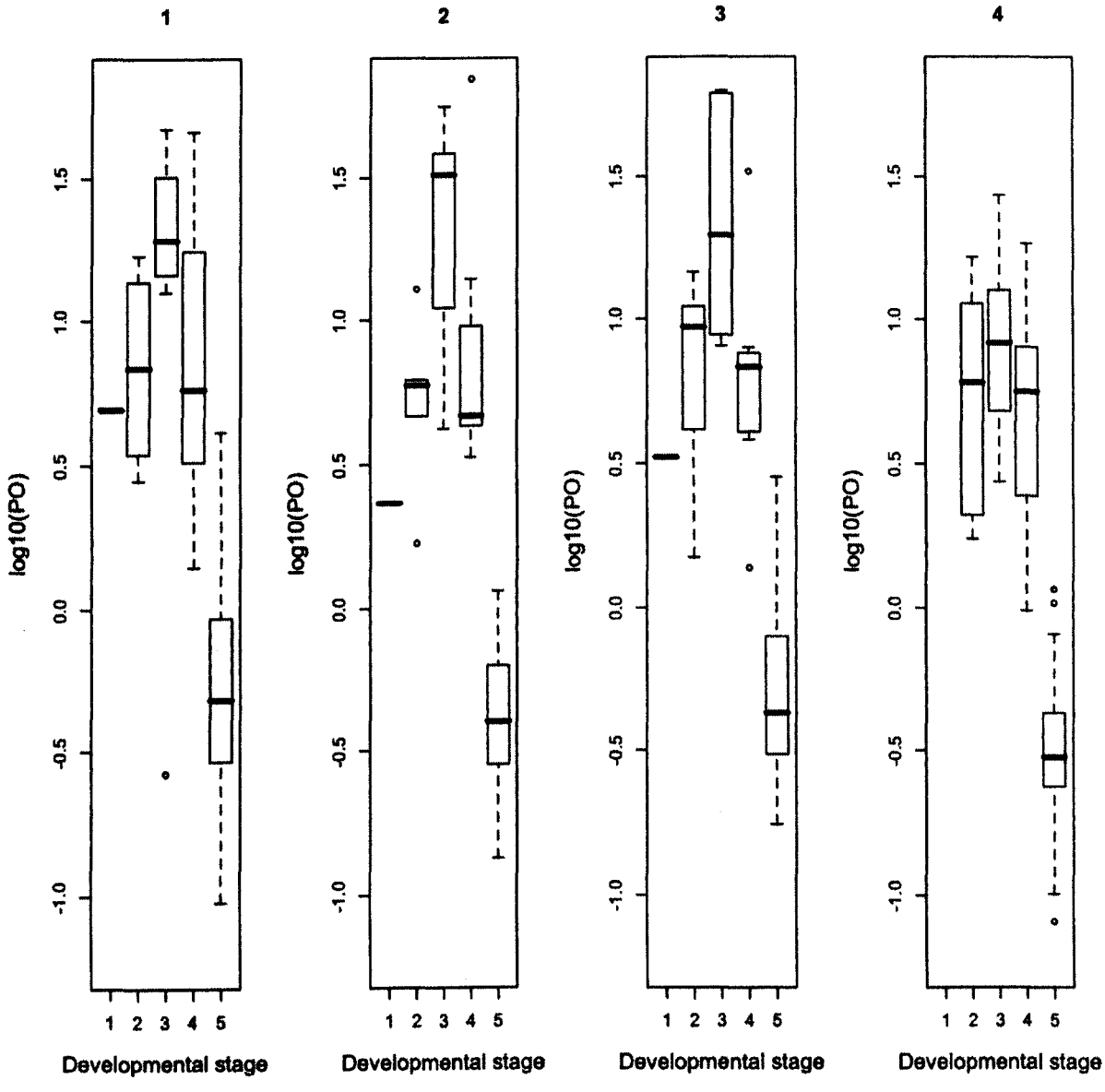


Figure A2.2.4. Hive E: Drone PO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

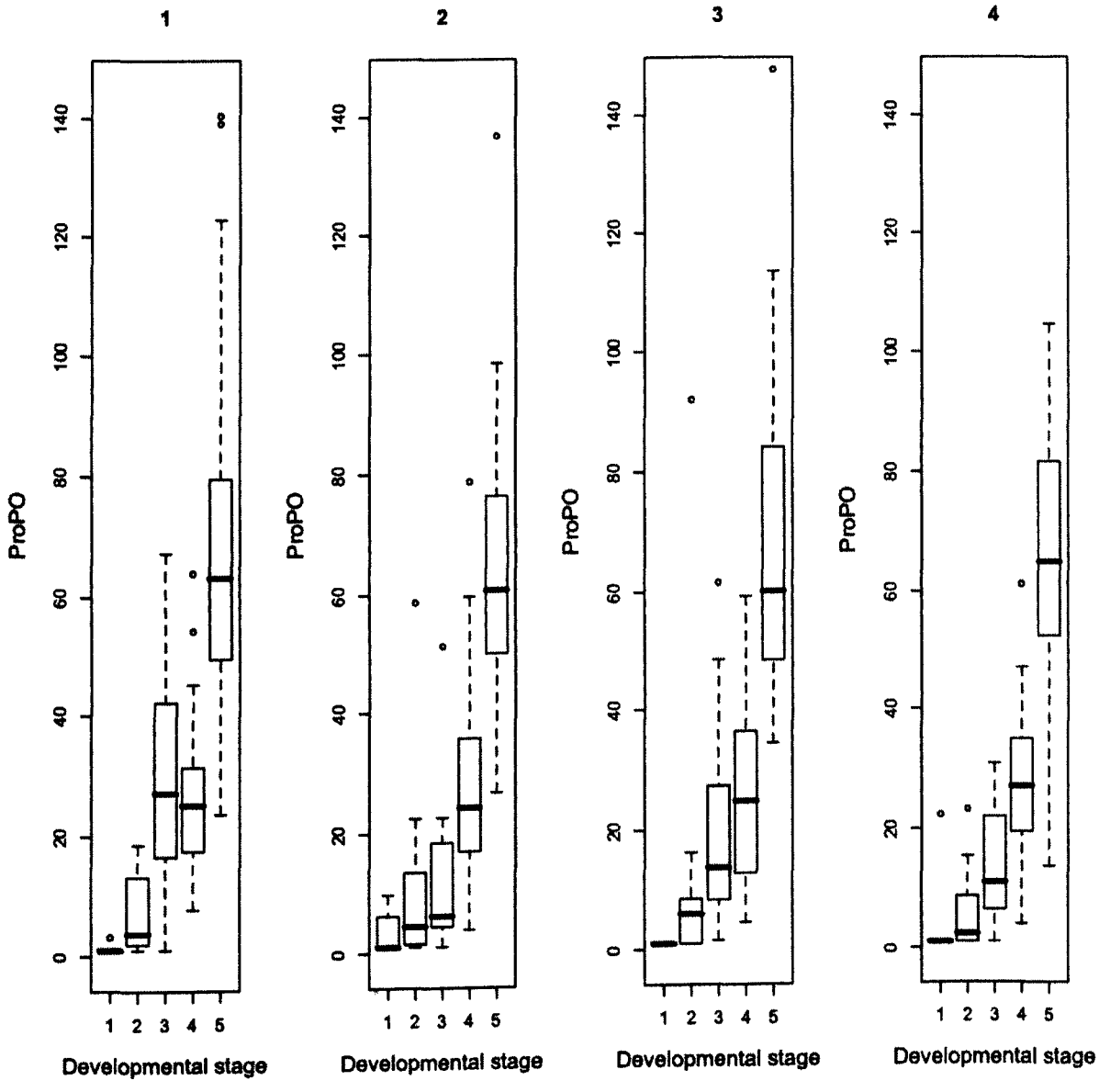


Figure A2.2.5. Hive D: Drone ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

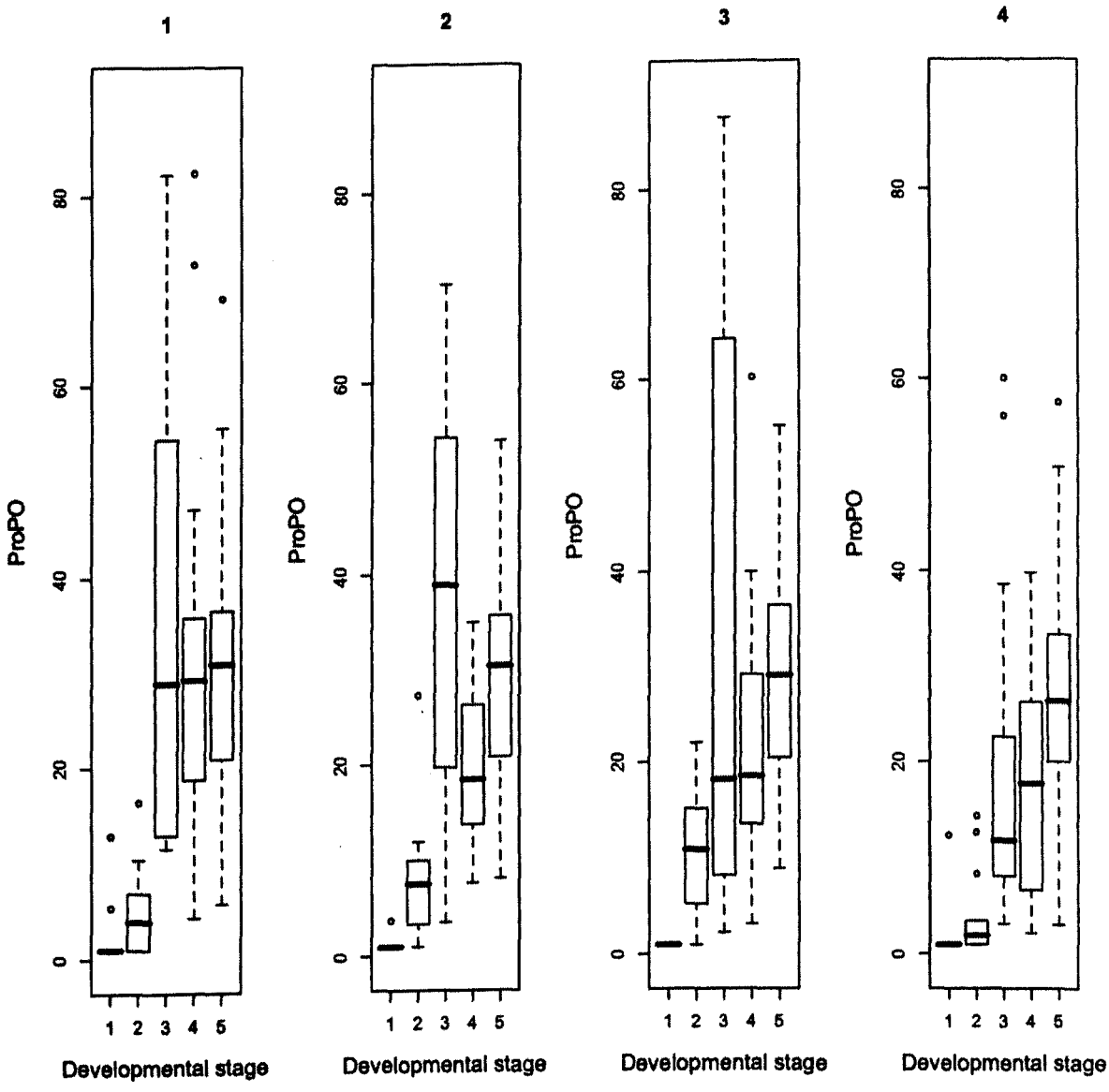


Figure A2.2.6. Hive E: Drone ProPO concentrations for all samples for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

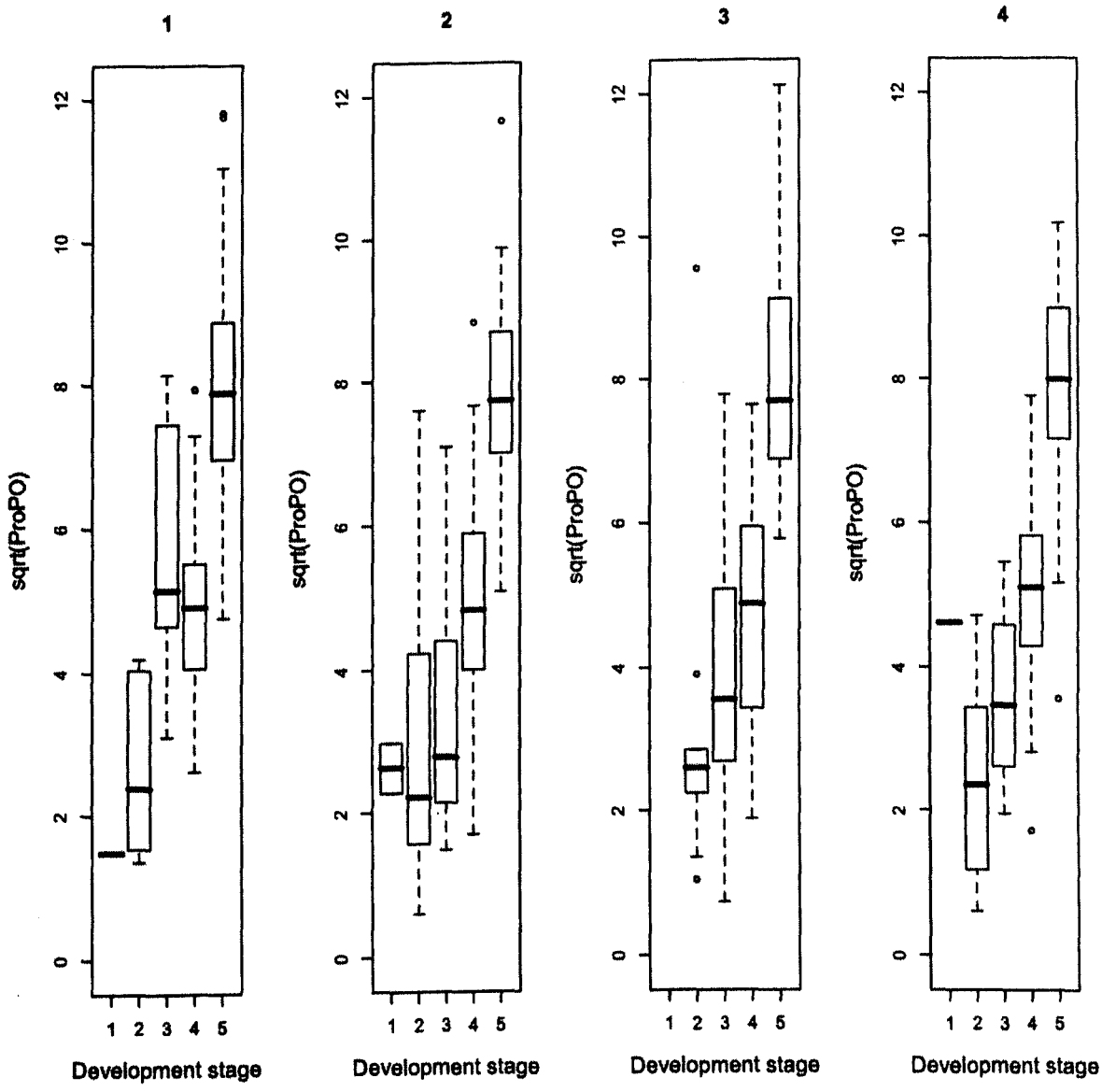


Figure A2.2.7. Hive D: Drone ProPO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

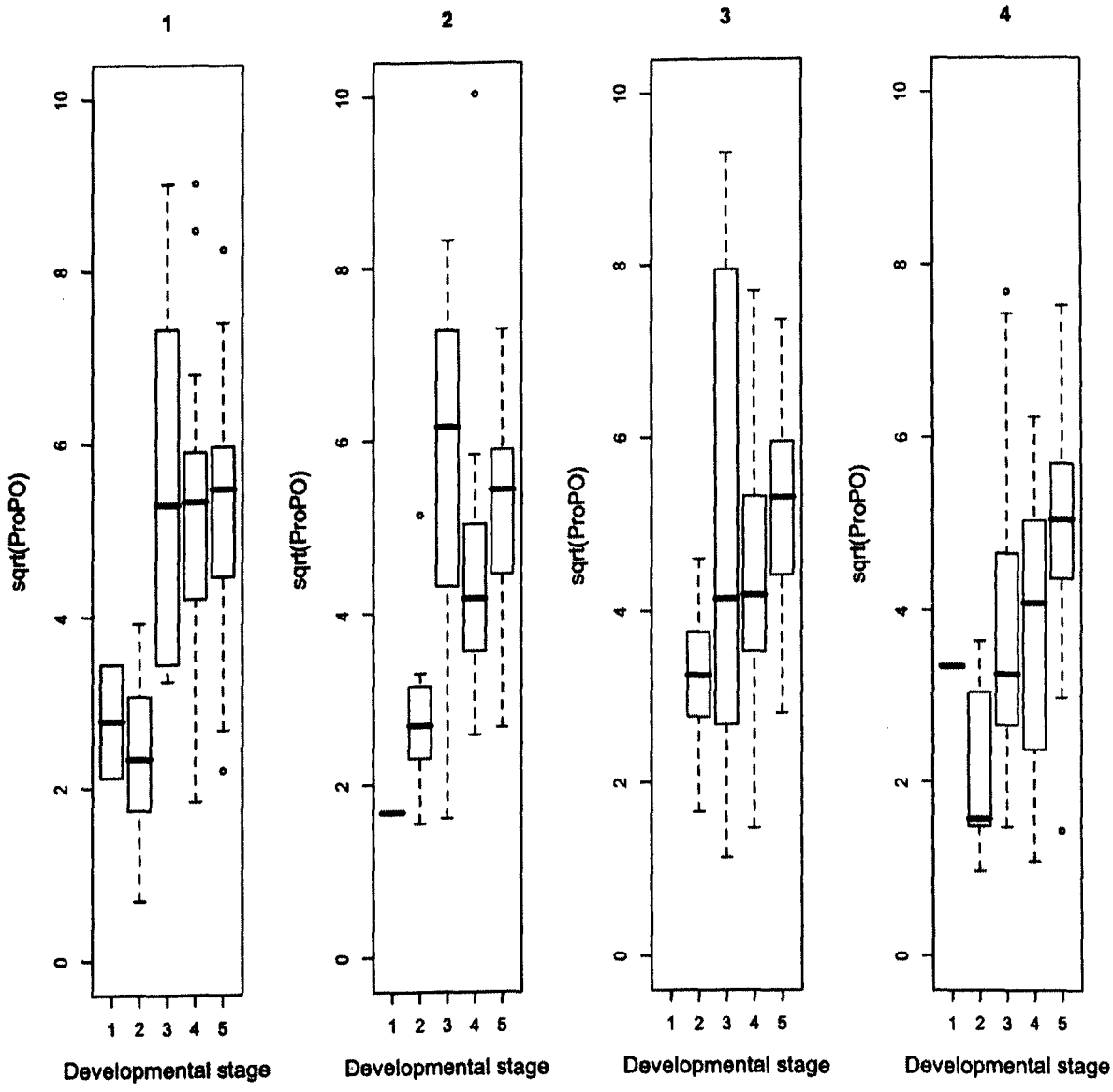


Figure A2.2.8. Hive E: Drone ProPO concentrations for samples that returned a recordable value, for all 5 developmental stages (1 = uncapped larvae, 2 = capped larvae, 3 = white-eyed pupae, 4 = red-eyed pupae, 5 = adults), for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

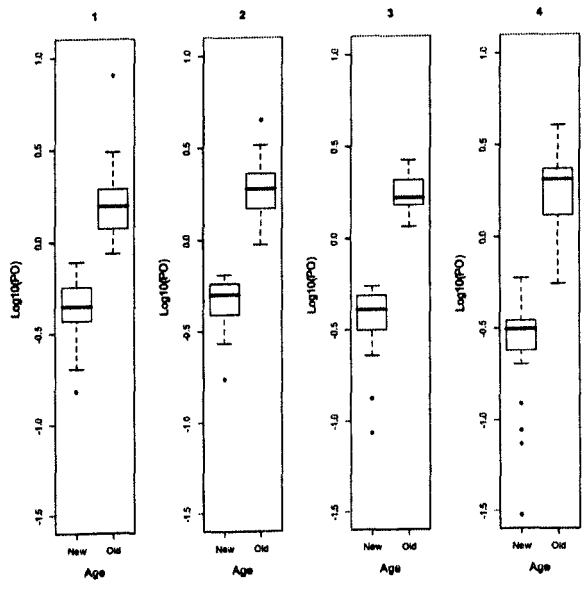


Figure A2.2.9. PO response of the two age groups, new and old drones, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

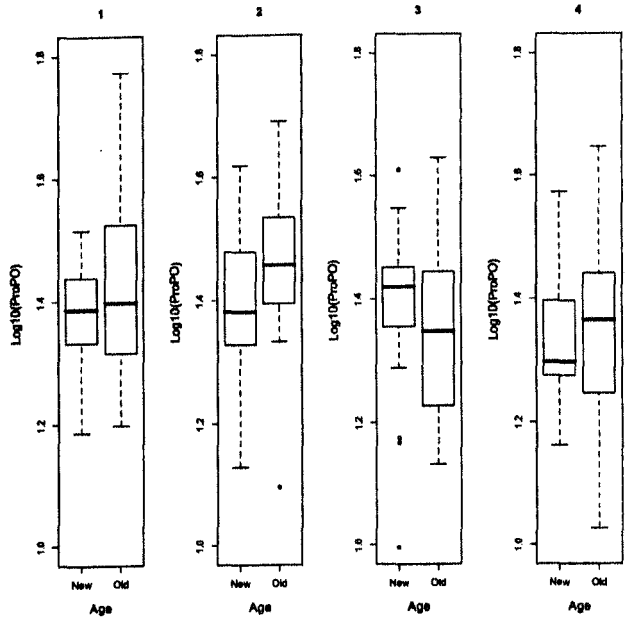


Figure A2.2.10. PO response of the two age groups, new and old drones, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

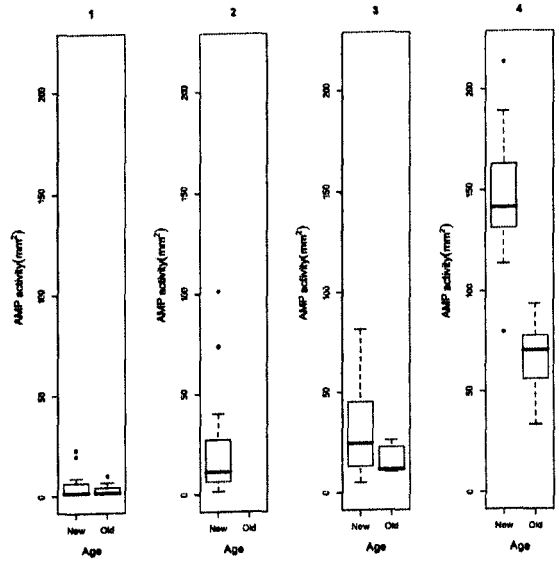


Figure A2.2.11. Antimicrobial peptide response of the two age groups, new and old drones, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). The figure shows the results for all samples.

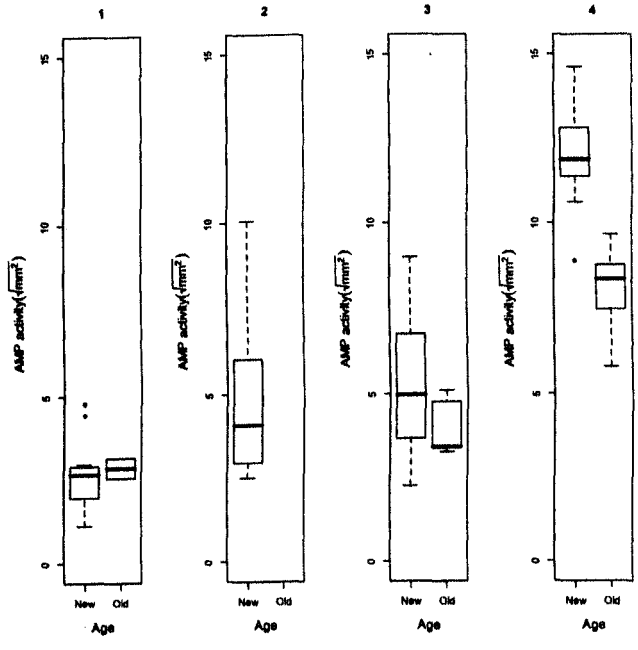


Figure A2.2.12. Antimicrobial peptide response of the two age groups, new and old drones, for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)), for those samples that contained a recordable level of AMPs.

A2.3 Chapter 5: *Varroa destructor* and the honey bee immune response: Figures

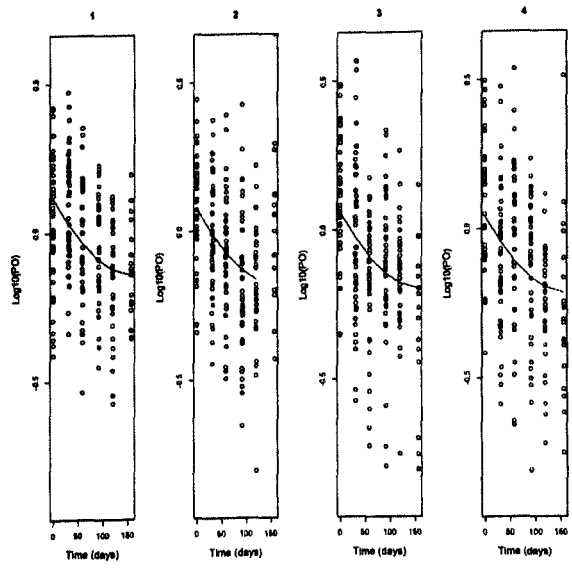


Figure A2.3.1. PO response of bees in low level infected hive for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

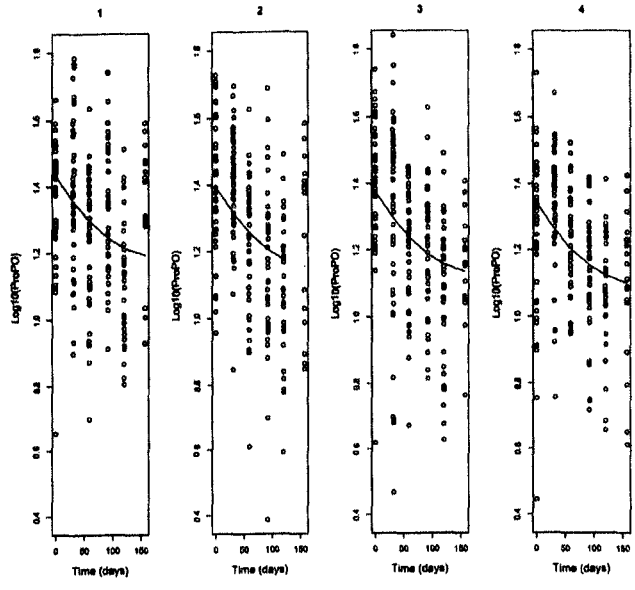


Figure A2.3.2. ProPO response of bees in the low level infected hive for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

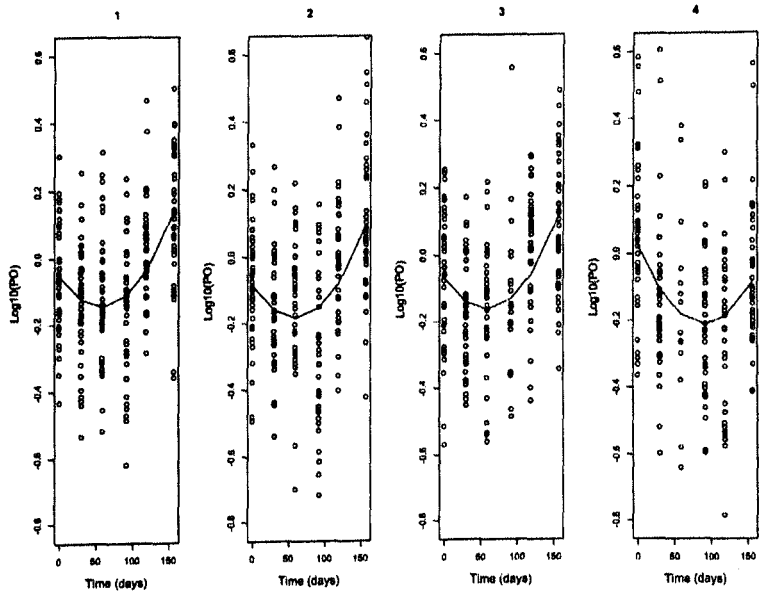


Figure A2.3.3. PO response of bees in high level infected hive for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

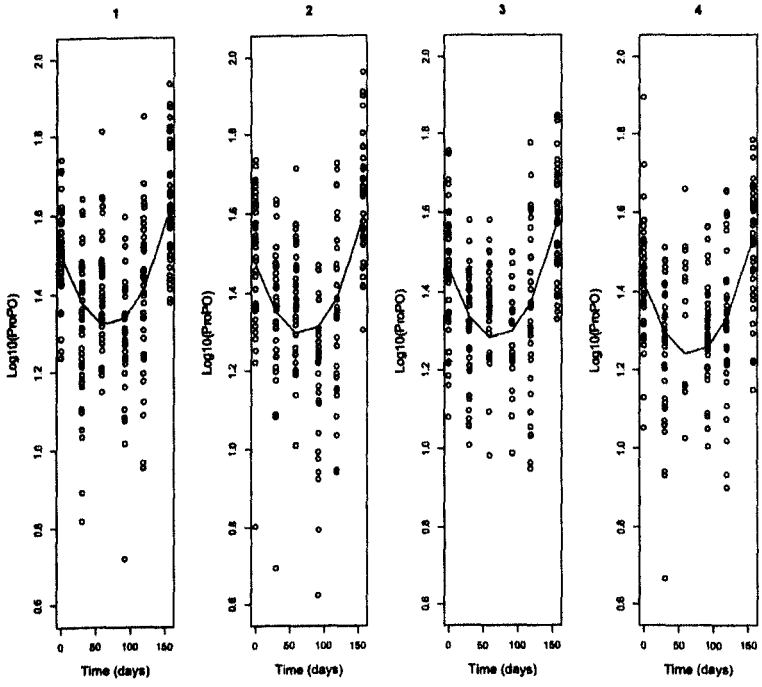


Figure A2.3.4. ProPO response of bees in the high level infected hive for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)).

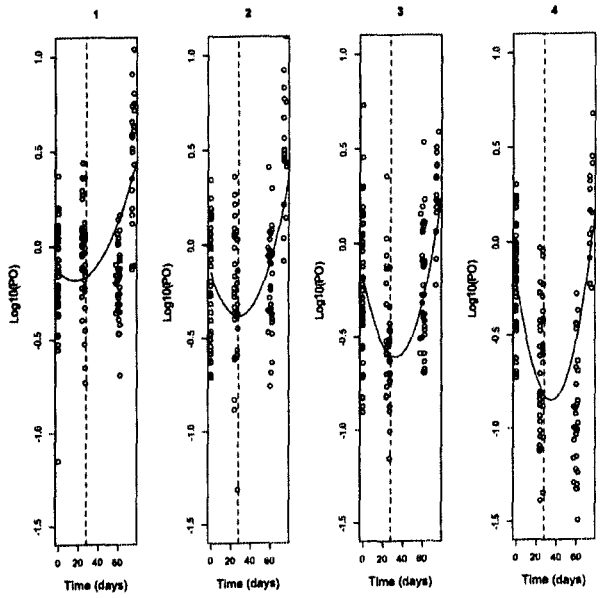


Figure A2.3.5. PO response of bees in Hive A for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed lines indicate the point at which Apistan was added.

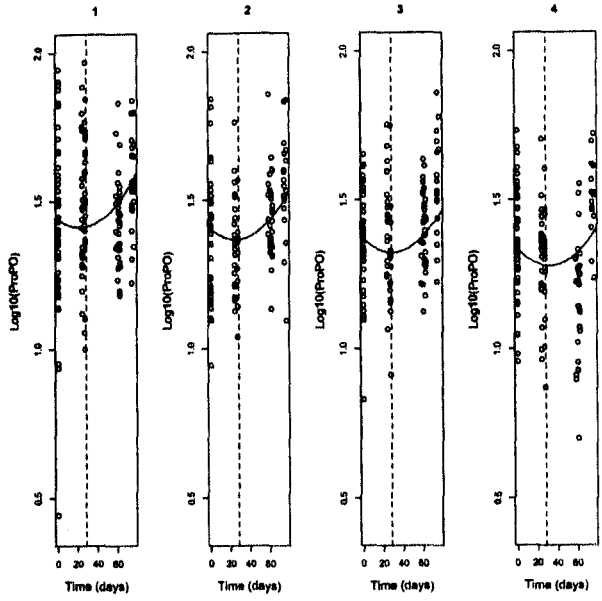


Figure A2.3.6. ProPO response of bees in Hive A for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed lines indicate the point at which Apistan was added.

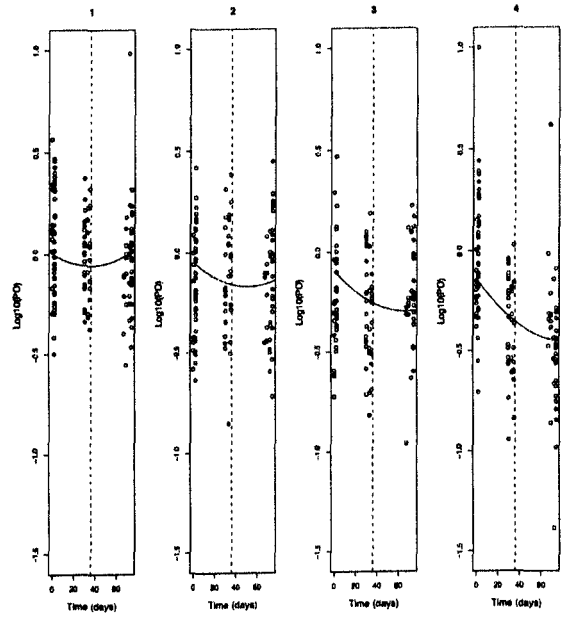


Figure A2.3.7. PO response of bees in Hive B for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed lines indicate the point at which Apistan was added.

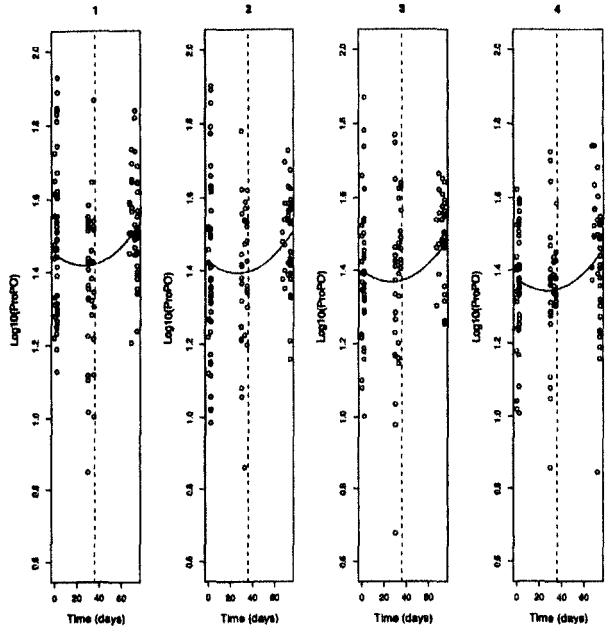


Figure A2.3.8. ProPO response of bees in Hive B for all 4 treatment groups (1 = No challenge, 2 = Needle (wounding control), 3 = 1µl Ringer (vehicle control), 4 = 1µl LPS (immune challenge)). Dashed lines indicate the point at which Apistan was added.

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