

The development of *Galleria mellonella* as a model to test the toxicity of food additives



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Declaration

This Thesis has not been submitted in whole or in part to this or any other university for any degree, and is original work of the author except where otherwise stated.

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Table 6.2 Relative fold changes of proteins decreased in abundance in *G. mellonella* larvae administered monosodium glutamate and the number of matched peptides, sequence coverage and overall intensity

Table 6.3 LFQ intensities of proteins exclusively expressed in the haemolymph of larvae injected with monosodium glutamate.

Table 6.4 LFQ intensities of proteins exclusively expressed in the haemolymph of control larvae injected with PBS.

Abbreviations

ACN	Acetonitrile
AMP	Anti-microbial Peptide
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BP	Biological process
BSA	Bovine serum albumin
Cat	Catalase
Caff	Caffeine
°C	Degrees centigrade
CC	Cellular component
Cm	Centimetre
Cre	Creatine monohydrate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EST	Expressed sequence tag
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescence activated cell sorting
FDR	False discovery rate
g	grams
g	G-force
GO	Gene ontology
GST	Glutathione S transferase
h	Hours
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
k	Kilo

kDa	Kilodaltons
KEPP	Kyoto encyclopaedia of genes and genomics
LPS	Lipopolysaccharide
LC/MS	Liquid chromatography mass spectrometry
LFQ	Label free quantification
M	Molar
MF	molecular Function
mM	Millimolar
mg	Milligram
µg	Microgram
min	minute
µl	microlitre
MSG	Monosodium glutamate
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PNa	Potassium nitrate
PNi	Potassium nitrite
PS	Potassium nitrite
P value	Probability
ROS	Reactive oxygen species
SA	Sodium acetate
SB	Sodium benzoate
SC	Sodium chloride
SNa	Sodium nitrate
SNi	Sodium nitrate
SOD	Superoxide dismutase
TEMED	N,N,N',N'-Tetramethylethylenedimine
TFA	Trifluoroacetic acid
TLR	Toll-like receptor

U	Unit
UV	Ultraviolet
V	Volts
V	volume
v/v	volume per volume
w/v	weight per volume
YEPD	Yeast extract-peptone-D-glucose

Abstract

Traditionally the *in-vivo* assessment of novel therapeutics and food additive toxicity has relied strongly upon the use of a variety of vertebrate species, such as mice, rats, guinea pigs and birds. However the use of mammalian models incurs large costs and raises issues of ethical acceptance. In the past decade alternative models showing striking metabolic similarities to mammalian models have been widely utilized as mini models in biological research. These alternative model systems include; Zebrafish, *Caenorhabditis elagans*, *Drosophila melanogaster* and *Bombyx mori*.

A widely used alternative model is *Galleria mellonella* larvae. *G. mellonella* fulfil many of the basic requirements of a useful animal infection model and have many advantages over other invertebrate systems. There are a lot of similarities between the insect and mammalian gastrointestinal tracts. Several xenobiotic metabolism enzymes and anti-oxidant enzymes are highly conserved between species. The insect fat body functions in drug metabolism in a similar way to the mammalian liver. In addition the insect immune system and mammalian innate immune system show vast similarities in the cellular and Humoral responses. The response of *G. mellonella* larvae to food additives was ascertained.

In this study larvae of the greater wax moth were administered eight commonly used food additives by intra-haemocoel or force feeding. A strong correlation between the relative toxicity of the compounds was observed between HEP-2 cells and larvae force-fed or administered the compounds by intra-haemocoel challenge. In addition a positive correlation between the LD₅₀ values obtained for the preservatives in rats and in *G. mellonella* larvae administered the compounds by feeding was established.

The fungicidal ability of haemocytes from larvae administered commonly used food additives was ascertained. There were significant decreases seen in the fungicidal ability of haemocytes extracted from larvae administered sodium benzoate, sodium nitrate, potassium nitrate, however there was no significant changes seen in the fungicidal killing ability of haemocytes from larvae treated with potassium sorbate, potassium nitrite, caffeine, monosodium glutamate and creatine.

Significant increases in catalase activity was seen in the haemolymph of larvae administered sodium benzoate. Significant increases in superoxide dismutase activity were seen in larvae exposed to potassium sorbate, potassium nitrate and monosodium glutamate. A Significant increase in alkaline phosphatase activity was seen in larvae challenged with monosodium glutamate, but no significant changes were seen in larvae challenged with creatine monohydrate.

The metabolism of caffeine was analysed using RP-HPLC analysis. Caffeine levels in larval haemolymph were significantly reduced from $t = 0$ to $t = 48$. The metabolites theobromine and theophylline were detected in larvae administered caffeine. The effect of caffeine on larval movement and rate of pupation was also measured. Both processes saw significant reductions when compared to control larvae.

Three proteomic studies were conducted to determine the proteomic response to potassium nitrate, caffeine and monosodium glutamate. All three studies revealed findings that were comparable to those of vertebrates.

Chapter 1

Introduction

1.1 Food additive toxicity and the development of a novel model for toxicity screening

Conventional *in-vivo* assays to assess the toxicity of novel therapeutics and food additives have relied strongly upon the use of a variety of vertebrate species, such as mice, rats, guinea pigs and birds (Tsai *et al.*, 2016). The use of mammals for studying toxicity of compounds has contributed enormously to our knowledge of food additive toxicity primarily due to the striking homology between mammalian genomes and the many similarities in anatomy, cell biology and physiology (Perlman, 2016). The toxicity of a compound can be reduced *in-vivo* due to degradation by host enzymes, binding to host components, and the effects of other physiological conditions such as pH (Desbois and Coote, 2012). Up until 1965 in Japan, the food preservative AF-2 had been used, until it was banned because of carcinogenicity detected in experimental animals (Sasaki *et al.*, 2002). However, despite these advances, there is recognition that the number of mammals used in such tests must be reduced to the minimum and that alternative, but ethically acceptable systems, must be developed (Browne and Kavanagh, 2013).

In the past decade, insects have been widely utilised as model hosts in biological research (Wojda, 2017). Insects are a very successful group of invertebrates with approximately 1 million species that inhabit all ecological niches, apart from the sea. A highly versatile and efficient immune system has allowed insects to inhabit a wide variety of environments. Invertebrates and vertebrates diverged approximately 500 million years ago, but despite this there are striking similarities between their immune systems and gastrointestinal tracts. As a result of these conserved similarities a wide range of insects have been employed as models to replace conventional mammals for studying the virulence of pathogens (Jacobsen, 2014; Kavanagh and Fallon, 2010; Junqueira, 2012), the assessment of the activity of antifungal agents (Lionakis and Kontoyianis, 2005; MacCallum *et al.*, 2013; Maurer *et al.* 2015) and in determining the efficacy and toxicity of novel antimicrobial drugs (Homamoto *et al.*, 2004; Rowan *et al.*, 2009; Desbois and Coote, 2011; Browne *et al.*, 2014). Insects are also used as models to test the acute and chronic toxicity of pesticides (Buyukguzel *et al.*, 2013), solvents (Soos and Szabad, 2014) nanoparticles

(Carmona *et al.*, 2015) and food additives (Andretic *et al.*, 2008; Chen *et al.*, 2009; Grunwald *et al.*, 2013). In fact insect are becoming well established alternatives to mammalian models, providing results that are comparable to mammalian data (Cook and McArthur, 2013; Jander *et al.*, 2000; Maccallum *et al.*, 2013).

Insects provide a more ethically acceptable model, a means to reduce the number of vertebrate models employed and therefore minimizing the amount of suffering imposed on animals with similar neurological and sentient capacities to humans (Scully & Bidochka 2006). Mammalian models, although a necessity, have certain disadvantages such as time, cost, legal and ethical restrictions (McMillan *et al.* 2015). A key principal of scientific research is the 3R policy (replace, reduce and refine). The 3R policy requires the use of mammalian models to be reduced to a minimum and to employ alternative, more ethically acceptable systems where possible. A wide variety of systems has been evaluated as possible alternative models to mammalian testing (e.g. zebra fish embryos, nematodes, animal cell culture) and insects have emerged as very useful model systems for specific tests. In particular, insects belonging to the order Lepidoptera (the moths and butterflies) are now a popular choice for evaluating the virulence of fungal pathogens, for assessing the *in vivo* activity of antifungal agents and for measuring the *in vivo* toxicity of compounds (Maurer *et al.*, 2015). Insect infection models are cheaper and less laborious to establish and maintain than mammalian models and are more amenable to high-throughput screening, giving statistically sound data (Desbois & McMillan, 2015) (Cook and McArthur, 2013). A number of insect models have been established including *Drosophila melanogaster* (Sarikaya and Cakir, 2005; Lionakis and Kontoyiannis, 2005), *Galleria mellonella* (Kelly and Kavanagh, 2011; Cook and McArthur, 2013; Kavanagh and Fallon, 2010), *Bombyx mori* (Hamamoto *et al.*, 2004, 2009), *Manduca sexta* (Dean *et al.*, 2002) and *Romalea microptera* (Johny *et al.*, 2007). A number of insects have also been established as food toxicity models including *D. melanogaster* (Sarikaya and Cakir, 2005; Coelho *et al.*, 2015; Wu *et al.*, 2010) the red flour beetle *Tribolium castaneum* (Grunwald *et al.*, 2013)(Nakayama *et al.*, 2012) and *Apis mellifera* (Ishay & Painiry, 1979).

Larger mammals such as sheep or rats can have physiologies and organ sizes that are similar to humans. There is a high degree of functional conservation in basic cell biological processes between mammals and invertebrates, for example between the insect haemocyte and the mammalian neutrophil (Kavanagh and Reeves, 2004). Mammals and insects are both susceptible to predation by micro-organisms. Moreover, the mechanisms by which pathogens establish infection (i.e. adhesion, invasion, systemic spread and avoidance of immune response) is the same in both. In response to these infections, both mammals and insects have evolved a range of mechanisms to protect themselves. Although some of these mechanisms (i.e. the adaptive immune system) are restricted to higher order metazoans, physical barriers to infection and innate immune systems are both common to mammals and insects and show high functional homology (Kemp and Massey, 2007). Insects however are not applicable models for the study of all human pathogens (e.g. the malaria parasite), although if appropriately utilised, they provide a range of benefits to the researcher. Hence, employing insects as model systems for the determination of microbial pathogenesis, toxicity testing and antimicrobial therapies is extremely suitable and relevant.

1.2 Insect Immune system

Although vertebrates and invertebrates diverged over 500 million years ago many of the structures and functions of the immune systems have been retained (Kavanagh and Reeves, 2004). The use of insects, including *G. mellonella* larvae, also offers the possibility of studying a system equivalent to the innate immune system of mammals without 'interference' from the adaptive immune response. Thus insects, and *G. mellonella* larvae in particular, may be used to model innate immune responses and give a clearer understanding of the role of this system in immune defences of vertebrates. Insects rely on multiple innate immune defence mechanisms which show strong structural and functional similarities to those of mammals (Kavanagh and Reeves, 2004; Browne *et al.*, 2013). These immune functions have mainly been characterized in *D. melanogaster* which serves as a model for insect immunity (Schneider, 2000). The insect immune response consists of two tightly interconnected components known as the cellular and the humoral responses. The cellular response is mediated by haemocytes and involves responses such as phagocytosis, encapsulation and clotting. The humoral defences are composed of soluble effector molecules such as anti-microbial peptides, complement-like proteins, melanin, and products created by proteolytic cascades, such as the phenoloxidase (PO) pathway, which immobilize or kill pathogens in a way similar to the complement cascade in mammals (Kavanagh and Fallon, 2010).

1.2.1 Cuticle and haemolymph

The cuticle is the first line of defence against pathogens in insects and acts in a similar way to the skin of mammals by providing a physical barrier against infection. The epicuticle is covered in a waxy layer containing lipids and fatty acids, which may display anti-microbial properties (Kavanagh and Reeves 2004). The cuticle itself is composed of chitin embedded in a protein matrix. Injury to the cuticle leads to the production of ceropins and attacins (Browne *et al.*, 2013). Pathogens that breach the cuticle activate wound healing through the use of clottable lipophorin proteins (Kavanagh and Reeves 2004). The mammalian Von Willebrand's factor, functions similarly to these lipophorin proteins, and both clotting factors share a homologous domain (Vilmos and Kurrucz, 1998). The insect haemolymph is functionally similar to

mammalian blood and functions in the transport of nutrients, waste products and signal molecules; however, unlike mammalian blood, it does not function in respiration (Mellanby, 1939). The volume of haemolymph present in an insect differs between species and even within a species depending upon the developmental stage of the individual insect (Rathcliff, 1985).

1.2.2 Humoral response

Insects contain pathogen recognition receptors such as integrins, apolipoproteins and lectins, which are conserved between multiple species of mammals and insects (Mowlds *et al.*, 2010). Upon recognition of foreign material these pathogen recognition receptors can activate both the production of AMPs and the melanisation reaction. The systems that mediate *Drosophila* Toll and mammalian IL-1 receptor-mediated signalling are very similar in structure and function (Browne *et al.*, 2013). Both pathways lead to the activation of NF κ B transcription factors causing the degranulation of both neutrophils and haemocytes and the subsequent release of AMPs (Browne *et al.*, 2013, Kavanagh and Reeves, 2004). AMPs are highly conserved between species and can act on pathogens as immunomodulatory and/or bactericidal agents (Strand, 2008). AMPs are usually produced in the fat body but can also be produced and stored in haemocytes. The range of AMPs in insects include lysozyme; which breaks down bacterial cell walls, cecropins; which have been found to also attack bacterial cell walls, defensins; which attack Gram positive bacteria and apolipoprotein 3, which binds to components of bacterial cell walls such as lipopolysaccharides (LPS) (Ratcliff, 1985; Kavanagh and Reeves, 2004). The melanisation reaction, which is a common response to parasite entry in invertebrate animals, especially arthropods, is due to the activity of an oxidoreductase, phenoloxidase. Prophenoloxidase shows similar sequence regions to vertebrate complement proteins C3 and C4 (Söderhäll & Cerenius 1998). The prophenoloxidase activating system in a non-self-recognition system that leads to the deposition of melanin on microbial surfaces. The recognition of foreign material such as lipopolysaccharides by pathogen recognition receptors activates the phenoloxidase cascade. Monophenyl L-dopa: oxygen oxidoreductase, also known as phenoloxidase,

is an enzyme that catalyses the oxidization of phenols to quinones. These quinones are then polymerized non-enzymatically to melanin (Söderhäll & Cerenius, 1998).

1.2.3 The cellular response

Haemocytes are found circulating freely in the haemolymph or adhering to internal organs such as the fat body or the digestive tract of the insect and can be rapidly mobilised upon breach of the cuticle or entry of a pathogen. At least eight different subtypes of haemocyte have been found in insects: prohaemocytes, plasmatocytes, granular cells, coagulocytes, crystal cells, spherulocytes, oenocytoids and thrombocytoids (Pandey and Tiwari, 2012; Tanada and Kaya, 1993). At least six types of haemocytes have been identified in *G. mellonella*; prohaemocytes (can differentiate into a number of cell types), plasmatocytes (contain lysosomal enzymes and are involved in capsule formation), granulocytes (contain granules), coagulocytes (function in the clotting process), spherulocytes (have small spherical inclusions) and oenocytoids (may contain prophenoloxidase) (Price and Ratcliffe, 1974) (Fig 1.1). Lepidopteran haemopoietic organs are found in the meso and metathorax of the insect and haematopoiesis occurs during insect embryogenesis (Grigorian and Hartenstein, 2013). These organs are the primary sources of prohaemocytes and plasmatocytes (Ling *et al.*, 2005). Circulating granulocytes, oenocytes and the spherulocytes most likely derive from the prohaemocytes that are already in circulation in the haemolymph (Nardi *et al.*, 2003). In order for phagocytic cells to engulf and kill pathogens, haemocytes must first recognize invading pathogens and this pathogen recognition mechanism shows further similarities in both insects and mammals (Kavanagh and Reeves, 2004). Phagocytosis in insects is known to be lectin mediated and is similar to what occurs in human neutrophils (Kavanagh and Reeves, 2004). The insect haemocyte shows structural and functional similarities to the mammalian neutrophil in that both can phagocytose and neutralize engulfed pathogens through the generation of superoxide and the secretion of lytic enzymes in the process known as degranulation (Renwick *et al.*, 2007; Browne *et al.*, 2013) (Table 1.1). Bergin *et al.*, (2005) identified proteins in haemocytes homologous to a number of proteins essential for superoxide production in human neutrophils and demonstrated that significant regions of the 67-kDa and 47-kDa insect haemocyte

proteins are identical to regions of the p67^{phox} and p47^{phox} proteins of neutrophils (Fig. 1.2). The killing ability of both the mammalian neutrophil and insect haemocyte are inhibited when exposed to various toxins such as gliotoxin (Renwick *et al.*, 2007), fumagillin (Fallon *et al.*, 2011), cytochalasin b and nocodazole (Banville *et al.*, 2011).

Encapsulation occurs when haemocytes bind to large structures such as protozoa, nematodes and the eggs and larvae of parasitic insects, forming a layer of haemocytes. The process usually occurs within a half an hour of the invasion of foreign material. Granular cells initially form a layer around the invading object and release plasmatocyte spreading peptides which attracts plasmatocytes to the site and induces aggregation (Vilmos & Kurucz, 1998; Kavanagh and Reeves, 2004). The invading objects are then destroyed. Apolipophorin 3 an antimicrobial peptide has been identified to stimulate the process of encapsulation in *G. mellonella* (Whitten *et al.*, 2004). Nodulation is lectin mediated and occurs when multiple haemocytes bind to clusters of bacteria and fungi (Vilmos and Kurucz, 1998). The binding of haemocytes together allows for the formation of an overlapping sheath around the invading pathogen. This process results in the activation of the phenoloxidase cascade and the subsequent killing of the pathogen (Lavine and Strand, 2002).

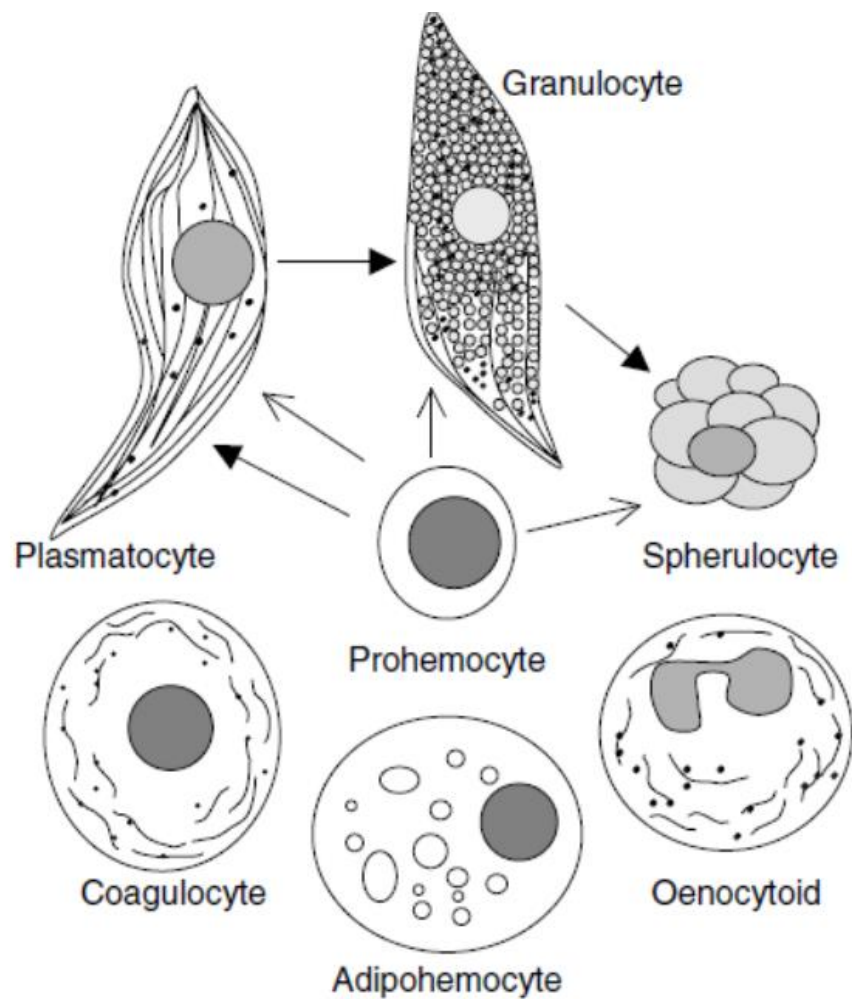


Fig 1.1 Haemocytes found within *G. mellonella* larvae (Kavanagh and Reeves, 2004). *G. mellonella* has 6 types of haemocytes granulocyte (granular cell) and plasmatocyte (phagocytosing cells), oenocytoid and coagulocytes (secondary roles involved in immune defence), spherulocyte and adipohaemocyte (energy storage).

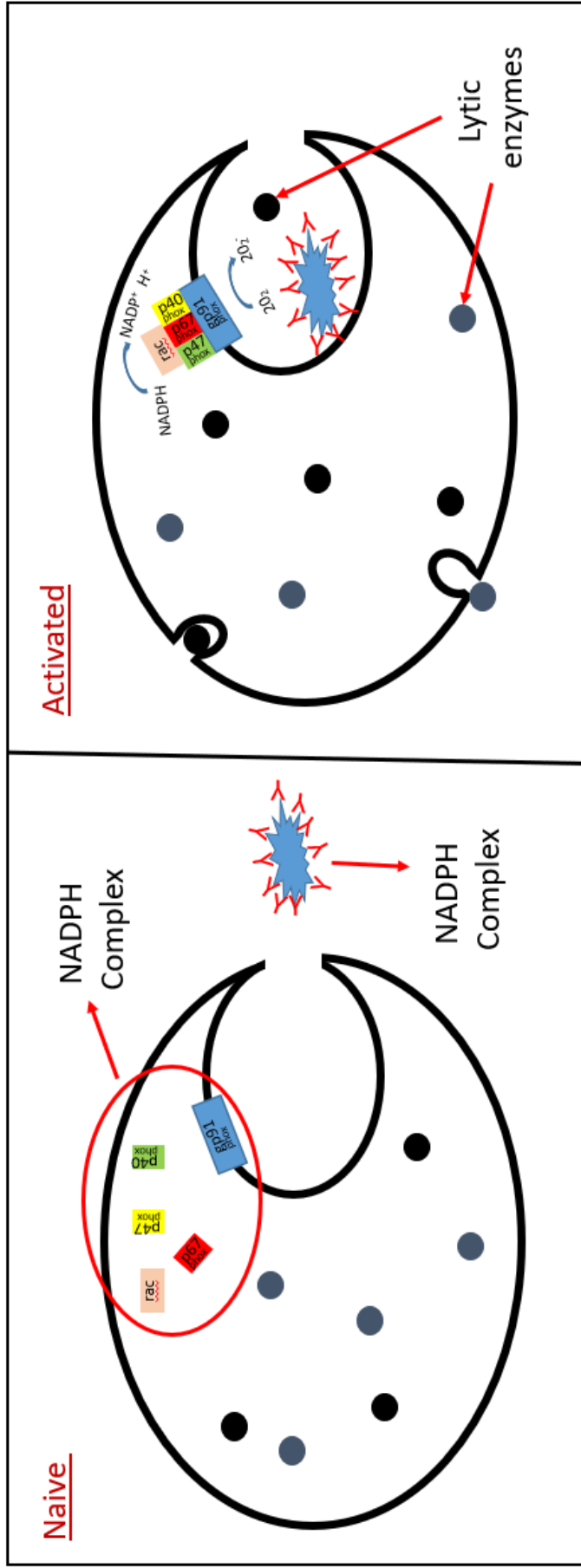


Fig 1.2 Mode of action of insect phagocytic immune cells (Browne et al. 2013). Following phagocytosis of opsonized particle by activated haemocytes a functional NADPH oxidase complex is formed by the translocation of cytosolic proteins (p40phox, p47phox, p67phox, and rac) to the membrane bound GP91phox which results in the formation of superoxide. Degranulation results in the release of lytic enzymes into the phagocytic vacuole and around the perimeter of the cell.

	Haemocytes	Neutrophils
Phagocytosis	Lectin-mediated	Lectin-mediated
ROS	O_2^- , H_2O_2 , NO^-	O_2^- , H_2O_2 , NO^-
Degranulation	Yes	Yes
AMPS	Peroxyneptin, transferrin, lysozyme, defensin	MPO, transferrin, lysozyme, defensin
Receptors	TLRs, B-1, 3-glucan, IL-IR	TLRs, B-1, 3-glucan, IL-IR
Transcription factors	NFκB, IκB	NFκB, IκB
Cascades	IMD, JNK, JAK-STAT	IMD, JNK, JAK-STAT
Kinases	p38 MAPK, ERK, PKC, PKA	p38 MAPK, ERK, PKC, PKA

Table 1.1 Comparison of processes in mammalian neutrophils and insect haemocyte (Browne et al. 2013).

Abbreviations: AMPs, antimicrobial peptides: ERK extracellular signal-regulated kinases: JAK-STAT, janus kinase-signal transducer and activator or transcription: MAPK, mitogen-activated protein kinases: MPO, myeloperoxidase: PKA, protein kinase A: PKC, protein kinase C: ROS, reactive oxygen species

1.3 Similarities between the insect and mammalian gastrointestinal tracts.

In addition to the similarities in the immune responses (Section 1.2), the insect and mammalian gastrointestinal tracts share similar tissue, anatomy and physiological function (Fedhila *et al.*, 2010). Plasma membranes from insect midgut cells are separated into apical and basolateral domains. The apical domain is usually modified into microvilli with a molecular structure similar to microvilli present in the gastrointestinal tract of mammals (Fedhila *et al.*, 2010). Intestinal micro-organisms can demonstrate several types of biotransformation reactions, such as hydrolysis, decarboxylation and deamination and play an important role in xenobiotic metabolism (Jourova *et al.*, 2016). Microvilli in the midgut of *G. mellonella* contain microbes that resemble those found in the intestinal microvilli of mammals (Mukherjee *et al.*, 2013). The insect fat body is an organ that also functions in drug metabolism like the liver in mammals (Büyükgüze *et al.*, 2013). A number of antioxidant enzymes produced by the fat body, such as superoxide dismutase, catalase and glutathione-s-transferase are highly conserved between species (Büyükgüze *et al.*, 2013). The phase I metabolism enzyme cytochrome p450 is conserved between *Homo sapiens* (ref), Zebrafish (Vliegenthart *et al.* 2014), *Caenorhabditis elegans* (Laing *et al.*, 2015), and multiple insect species (Coelho *et al.*, 2015). The phase II metabolism enzyme UGTs are conserved between species such as *H. sapiens* (ref) Zebrafish (Ouzzine *et al.* 2014), *C. elegans* (McElwee *et al.*, 2004), and multiple insect species (Luque *et al.*, 2002) (Table 1.2).

Metabolism enzymes	Present in insects
Phase I enzymes	
Cytochrome P-450	(Feyereisen, 1999)
FAD- containing monooxygenase	(Kulkarni and Hodgson, 1984)
Epoxide hydrolase	(Xu <i>et al.</i> , 2015)
Alcohol dehydrogenase	(Carvalho <i>et al.</i> , 2009)
Aldehyde dehydrogenase	(Leal and Barbancho, 1993)
Esterase	(Montella <i>et al.</i> , 2012)
Phase II enzymes	
Glucuronosyltransferase	(Montella <i>et al.</i> , 2012)
Sulfotransferase	(Vakiani <i>et al.</i> , 1998)
N-Acyl transferase	(Wierenga and Hollingworth, 1990)
Glutathione S-Transferases	(Kostaropoulos <i>et al.</i> , 2001)
Rhodanese	(Beesley <i>et al.</i> , 1985)

Table 1.2 List of phase I and phase II enzymes involved in xenobiotic metabolism in insects as well as mammals.

1.4 *Galleria mellonella*

The Greater (Lepidoptera: Pyralidae, *Galleria mellonella*) and lesser (Lepidoptera: Pyralidae, *Achroia grisella*) wax moths are pests of bee colonies globally. Their economic importance has led to a number of investigations on their life history, biology, behaviour, ecology, molecular biology, physiology, and control. In addition to their importance in apiculture, wax moth larvae are widely used as model organisms for studies in insect physiology and human pathogens (Browne and Kananagh, 2013). *G. mellonella* larvae have been employed to assess the virulence of a range of *Candida albicans* isolates and to quantify the relative virulence of pathogenic and non-pathogenic yeast species (Cotter *et al.*, 2000). Brennan *et al.*, (2002) established a positive correlation between the virulence of *C. albicans* mutants in *G. mellonella* larvae and in BalbC mice and a strong correlation was also established between the virulence of *Aspergillus fumigatus* mutants in larvae and mice (Slater *et al.*, 2011). *G. mellonella* larvae have been utilised to establish a correlation between toxin production and virulence of *A. fumigatus* (Reeves *et al.*, 2004) and the stage of *A. fumigatus* spore germination has been shown to affect fungal clearance in larvae (Renwick *et al.*, 2006). *G. mellonella* larvae are now utilised as an infection model for *Cryptococcus neoformans* (Mylonakis *et al.*, 2005) and London *et al.*, (2006) identified that a number of virulence-related genes important in *C. neoformans* mammalian infections were also involved in infection of *G. mellonella*. *Aspergillus flavus* is a pathogen of *G. mellonella* (St Leger *et al.*, 2000) and Scully and Bidochka (2006) demonstrated increased virulence of *A. flavus* when serially passaged in *G. mellonella* larvae. Antibiotics are effective in the wax moth larvae model for the treatment of infections caused by Gram-positive bacteria (Desbois and Coote, 2011). *G. mellonella* larvae have also been utilised for assessing the *in vivo* activity of amphotericin B, flucytosine and fluconazole following challenge with *C. neoformans* (Mylonakis *et al.*, 2005) and to evaluate the antifungal properties of novel silver-based compounds (Rowan *et al.*, 2009).

When using *G. mellonella* larvae, or other insects, for assessing the *in vivo* antimicrobial properties of compounds it is important to determine whether the introduction of the agent into the haemocoel of the insect provokes an inherent anti-

microbial immune response separate to the antimicrobial properties of the test agent. Administration of caspofungin (Kelly and Kavanagh, 2011), glucan (Mowlds *et al.*, 2010) or silver nitrate (Rowan *et al.*, 2009) to *G. mellonella* larvae induces a non-specific immune response which operates in parallel with the antifungal activity of the introduced agent. Thus, in cases where larvae are used to determine the antimicrobial properties of a novel compound it is important to be able to differentiate between the inherent antifungal properties of the agent and the antimicrobial responses induced by the agent.

Examples of the utilization of <i>Galleria</i> larvae as a model organism	
Author	Title
Yeasts	
Brennan <i>et al.</i> , 2002	Comparison of virulence of <i>C. albicans</i> mutants with virulence in mice
Mylonakis <i>et al.</i> , 2005	<i>Galleria mellonella</i> as a model system to study <i>Cryptococcus neoformans</i> pathogenesis
Bergin <i>et al.</i> , 2003	Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of <i>Galleria mellonella</i> .
Bacteria	
Mukherjee <i>et al.</i> , 2013	Brain infection and activation of neuronal repair mechanisms by the human pathogen <i>Listeria monocytogenes</i> in the lepidopteran model host <i>Galleria mellonella</i>
Fedhila <i>et al.</i> , 2009	Comparative analysis of the virulence of invertebrate and mammalian pathogenic bacteria in the oral insect infection model <i>Galleria mellonella</i>
Peleg <i>et al.</i> , 2009	<i>Galleria mellonella</i> as a Model System to Study <i>Acinetobacter baumannii</i> Pathogenesis and Therapeutics
Jander <i>et al.</i> , 2000	Positive correlation between virulence of <i>Pseudomonas aeruginosa</i> mutants in mice and insects
Desbois <i>et al.</i> , 2011	Wax moth larva (<i>Galleria mellonella</i>): an in vivo model for assessing the efficacy of antistaphylococcal agents
Efficacy of Novel Antimicrobial Agents	
Rowan <i>et al.</i> , 2008	Use of <i>Galleria mellonella</i> larvae to evaluate the in vivo anti-fungal activity of [Ag ₂ (mal)(phen) ₃]
Browne <i>et al.</i> , 2014	Assessment of in vivo antimicrobial activity of the carbene silver(I) acetate derivative SBC3 using <i>Galleria mellonella</i> larvae
Dolan <i>et al.</i> , 2016	Synthesis, antibacterial and anti-MRSA activity, <i>in vivo</i> toxicity and a structure–activity relationship study of a quinoline thiourea
Toxicity Testing	
Megaw <i>et al.</i> , 2015	<i>Galleria mellonella</i> as a novel in vivo model for assessment of the toxicity of 1-alkyl-3-methylimidazolium chloride ionic liquids
Büyükgüzel <i>et al.</i> , 2013	Effect of boric acid on antioxidant enzyme activity, lipid peroxidation, and ultrastructure of midgut and fat body of <i>Galleria mellonella</i>

Table 1.3 Uses of *G. mellonella* as model organism

1.4.1 Procedures for utilising *Galleria mellonella* larvae

G. mellonella larvae are a widely used model organism (Table 1.3), fulfilling many of the basic requirements of a useful animal infection model and have many advantages over other invertebrate systems (Fallon *et al.*, 2012). Legal and ethical issues restrict the number of mammals that may be used to the lowest possible number, however large numbers of *G. mellonella* larvae can be used in experiments and this can yield results demonstrating a high degree of statistical robustness. *G. mellonella* larvae are widely available and are relatively inexpensive to purchase (Desbois and McMillan, 2015). Larvae can be directly purchased from local suppliers in their preferred sixth instar stage, ready to use and do not incur husbandry costs. *G. mellonella* can be stored at temperatures between 4 and 37°C (Mowlds and Kavanagh, 2008) which makes *G. mellonella* a good model to study fungal pathogens at human body temperature. This is especially important when studying virulence of pathogens as temperature regulates expression of a range of virulence factors. Mowlds and Kavanagh, (2008) identified the induction of a protective cellular and humoral immune response mediated by increased numbers of haemocytes and elevated expression of antimicrobial peptides 24 h after incubation of larvae at 4 or 37°C. *G. mellonella* are a convenient size (2-3cm in length) and a large number of larvae can be inoculated in a short period of time. Larvae are easily maintained in wood shavings or on filter paper. Banville *et al.* (2012) showed that haemolymph from larvae deprived of food demonstrated reduced expression of a range of antimicrobial peptides (e.g., lipocalin) and immune proteins (e.g., apolipophorin and arylphorin) and starved *G. mellonella* larvae demonstrated an increased susceptibility to infection.

Quantifying the infecting inoculum is more accurate in *G. mellonella* than in *D. melanogaster* (Lionakis, 2011). *G. mellonella* larvae may be inoculated with pathogen by topical application – this is usually achieved by rolling the larvae on a layer of spores and can be used with entomopathogenic fungi (e.g. *A. flavus*) or by dipping in a solution of spores/cells. Direct inoculation of larvae with pathogen or aliquot of antifungal agent can be by injection into the haemocoel (body cavity) through one of the prolegs (Cotter *et al.*, 2000) or by force feeding (Mukherjee *et al.*,

2013) (Fig 1.3). Intra-haemocoel infection closely mimics systemic infection in mammals, while the feeding route can be used to replicate administration of an oral antimicrobial agent or an intestinal pathogen. Larvae can be incubated at temperature up to 37°C and meaningful results can be obtained in 24 – 48 hours.

A variety of end points is used to quantify the response of larvae to the inoculum. Larval death is easily assessed by gently probing larvae. The degree of melanisation can give an indication of the larval response to the pathogen or toxin as can changes in the density of the circulating haemocyte population. Assessing the density of circulating haemocytes is a useful indicator of whether an immune response has been triggered by the introduction of a pathogen or antifungal agent (Bergin *et al.*, 2003). Measuring the phagocytotic and killing ability of haemocytes gives an indication of the activity of this cell type (Fallon *et al.*, 2011). More in-depth analysis can quantify changes in gene expression or protein abundance to create a full picture of the larval response to a toxin (Fallon *et al.*, 2012). While the *G. mellonella* genome is not sequenced, the recent characterization of the *G. mellonella* immune gene repertoire and transcriptome by next generation sequencing and traditional Sanger sequencing has led to the design of gene microarrays which strengthen its development as a model organism (Lionakis, 2011). Antioxidant stress caused by a test compound can be assessed by measuring the activity of enzymes such as superoxide dismutase, catalase and glutathione-S-transferase (Büyükgüzel *et al.*, 2013). Developmental changes, such as inability to pupate and movement disorders can be used to measure the toxicity of a compound. The metabolism of a compound can be measured using RP-HPLC analysis.

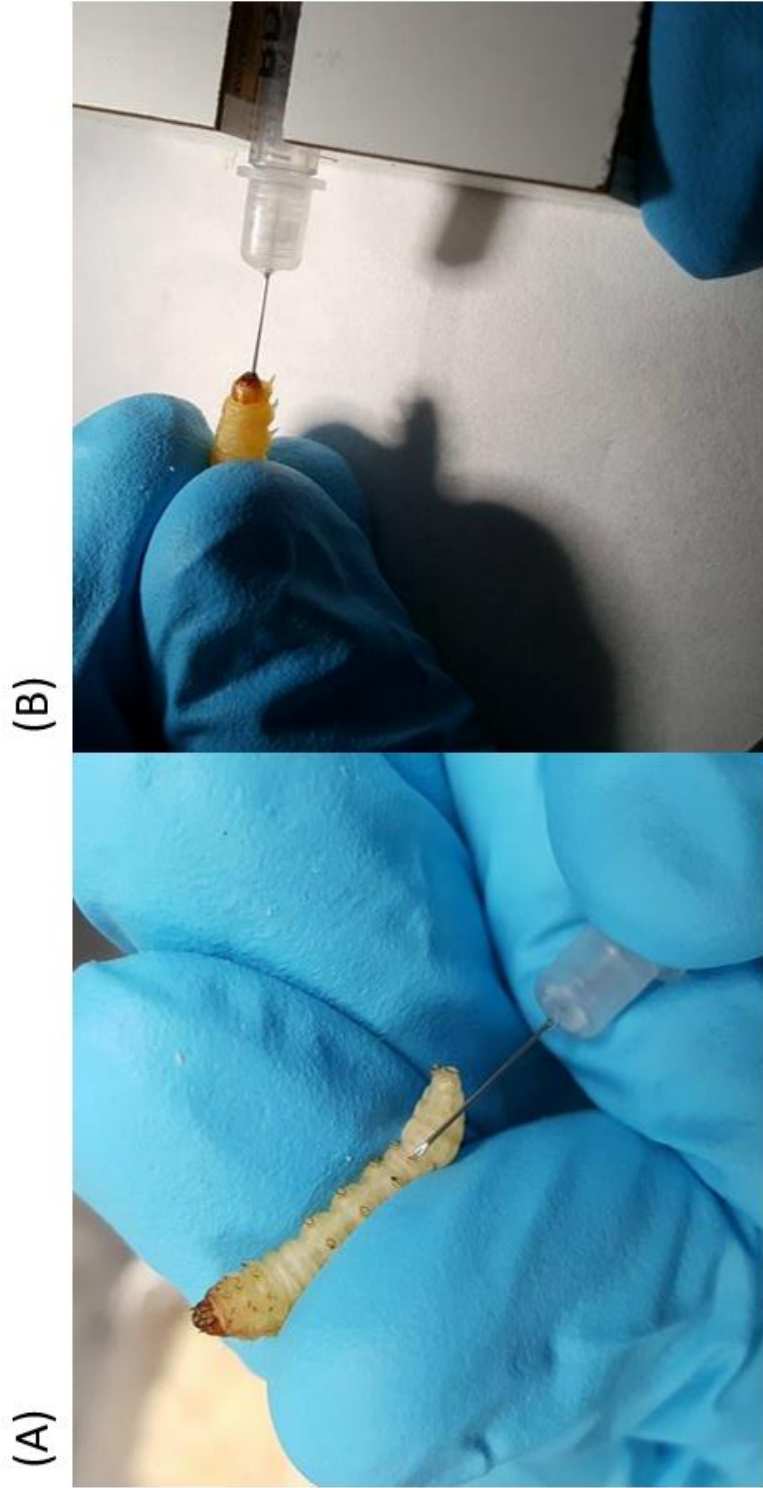


Fig 1.3 Administration of a solution to *G. mellonella* via (A) intra-haemocoel injection through the last left pro leg and (B) force feeding

1.5 Alternative model systems for toxicity testing of food additives.

Mammalian toxicity studies are expensive and time consuming and meta-analysis indicate that rodent models successfully predict adverse effects in humans only approximately 50% of the time (Hunt, 2017). Therefore, using more than one mammalian species can result in greater predictability of the toxicological effects of a compound. A wide range of mammalian cell lines have also been used for assessing the *in vitro* toxicity of compounds (Fotakis and Timbrell, 2006). In Vitro systems for organ toxicity evaluations have been developed for various organs and tissues (Tiffany-Castiglioni et al, 1996; Pfaller and Gstraunthaler, 1998; Gribaldo et al, 1996). Human epithelial type 2 (HEp-2) cells were originally derived from a human laryngeal carcinoma and have been utilized to determine the efficacy of anticancer drugs (Fujii., et al 1995), the cytotoxicity of nanoparticles (Ahamed et al, 2014) and the toxicity of food additives (De Angelis et al 1994) In addition, by utilising various other alternative models that show similarities to mammalian models, such as *C. elegans*, *D. melanogaster*, *G. mellonella* and *B. mori* (Fig 1.4), the researcher is provided with a cost effective approach to further increasing the toxicological predictability of a compound.

1.5.1 *Caenorhabditis elegans*

C. elegans (Fig. 1.4) is a nematode that feeds on fungi and bacteria in soil and rotting fruit with a typical length of 1 mm and a diameter of 50 µm. Immunity of *C. elegans* relies on three mechanisms: chemosensory neurons sense pathogens and promote avoidance behaviour, physical barriers and innate immunity (Trevijano-Contador and Zaragoza, 2014). The innate immunity of *C. elegans* relies solely on pattern recognition receptors and signal pathways to induce the production of antimicrobial peptides. The immune system of *C. elegans* does not possess phagocytic cells (Trevijano-Contador and Zaragoza, 2014). The small size of the nematode means it can be placed in 96 well plates for high throughput screening. *C. elegans* has an average life span of approximately 2–3 weeks and a reproduction time of 3.5 days (Murschiol *et al.*, 2009). Hermaphrodite nematodes produce both oocytes and sperm and have a reproductive capacity of about 300 progeny per

hermaphrodite by self-fertilization (Hunt, 2017). *C. elegans* can be easily maintained on nematode growth medium agar, fed on the non-pathogenic bacterium *Escherichia coli* OP50 strain, and preserved at $-80\text{ }^{\circ}\text{C}$. *C. elegans* are easy to store and handle, cost effective and can be utilized with both liquid and solid medium (Desalermos *et al.*, 2011). Unlike toxicity testing using cell cultures, *C. elegans* toxicity assays provide data from a whole animal with intact and metabolically active digestive, reproductive, endocrine, sensory and neuromuscular systems (Hunt, 2017). The nematode is transparent so processes such as axon growth, embryogenesis and fat metabolism can be easily studied, with the use of *in-vivo* fluorescence markers (Kaletta and Hengartner, 2006). *C. elegans* has a fully sequenced publicly available genome and can be genetically modified easily at minimal cost. Various striking similarities exist between *C. elegans* and higher organisms. Bioinformatics has identified *C. elegans* homologues present in 60-80% of human genes, with 12 of 17 known signal transduction pathways are conserved between humans and *C. elegans* (Kaletta and Hengartner, 2006). Some key biomedical discoveries were enabled by *C. elegans* research, for example mutations in presenilin-1 can cause early onset familial Alzheimers disease (Chan *et al.*, 2002). In 1993 the first presenilin gene was described in *C. elegans* (Sundaram & Greenwald, 1993).

C. elegans has been previously utilised to study the toxicity of food additives, the lethal dose 50 of monosodium glutamate, tannic acid and thiourea in *C. elegans* showed significant positive correlations with data obtained from rats ($R^2 = 0.8$) and mice ($R^2 = 0.8$) (Paul and Manoj, 2009). The toxicity of the food colouring tartrazine was studied in *C. elegans* (Himri *et al.*, 2013) and Wistar rats (Himri *et al.*, 2011) and both studies showed a good correlation for tolerability and lethality between rodents and *C. elegans*. *C. elegans* exposed to tartrazine did not result in death even at concentrations as high as 3mM and, in accordance with this, over a 90 day administration period no deaths occurred across animal groups (Himri *et al.*, 2011; Himri *et al.*, 2013). *C. elegans* has also been employed to assess the toxicity of heavy metals (Jiang *et al.* 2016; Chen *et al.* 2013; Hunt *et al.* 2012), organic solvents and pesticides (Leung *et al.*, 2008), with several toxicity end points such as reproduction (Boyd *et al.*, 2011), feeding (Hunt *et al.*, 2012), DNA damage (Feng *et al.*, 2017) and

gene expression (Kumar *et al.*, 2015). Despite the numerous advantages provided by *C. elegans* it is important to note its disadvantages. *C. elegans* toxicity assays consist of exposing the nematode to a test compound in an agar culture along with feeder bacteria, this poses two distinct disadvantages of the model. Firstly the feeder bacteria might modify the test compound and secondly there is no way to accurately measure the inoculum (Trevijano-Contador and Zaragoza, 2014). In addition, *C. elegans* development proceeds from embryo through four distinct larval stages to adult hermaphrodites in approximately 72 hours at 20°C (Boyd *et al.*, 2012); however, *C. elegans* does not tolerate high temperatures. Making them an inadequate model to study the virulence factors of human pathogens (Trevijano-Contador and Zaragoza, 2014)

1.5.2 *Drosophila melanogaster*

Short life cycle, fast reproduction, larval stages and fewer ethical issues associated with its use make *D. melanogaster* (Fig. 1.4) a suitable model of human disease (Prüßing *et al.*, 2013; Pandey & Nichols, 2011), fungal virulence (Lionakis & Kontoyiannis, 2012; Lionakis & Kontoyiannis, 2005), cancer (Miles *et al.*, 2011; Rudrapatna *et al.*, 2012), aging and metabolic disorders (Hoffmann *et al.*, 2013; Bharucha, 2009). Many basic biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*, and 60% of human disease-causing genes are believed to have a functional homolog in the fly (Schneider, 2000) so it is no surprise that *Drosophila* is the most widely used insect host for modelling pathogenic disease (Kemp & Massey, 2007). Initially *D. melanogaster* was utilised to study Mendelian genetics (Schneider, 2000). The genome of the fruit fly has been available since 2000 (Adams *et al.*, 2000), the fruit fly is amenable to forward and reverse genetics and with a large collection of *D. melanogaster* mutant and transgenic cell lines the insect mini model is a very effective model to study human genetics (Wolf & Rockman, 2008; Lionakis, 2011). To date *D. melanogaster* has been employed to test the toxicity of pesticides (Arain *et al.*, 2014; Parádi and Lovenyák, 1981), solvents (Soós & Szabad, 2014; Cvetkovic *et al.*, 2015), and nano particles (Araj *et al.*, 2015; Ong *et al.*, 2015). It has been well documented that *D. melanogaster* possess an efficient system to metabolise xenobiotics (Coelho *et al.*, 2015; Lasek *et*

al., 2011) resulting in the insect being adapted to measure the toxicity of common food additives such as caffeine (Mustard, 2009; Coelho *et al.*, 2015), sodium nitrate (Sarıkaya and Çakir, 2005), ethanol (Lasek *et al.*, 2011) and tartrazine (Tripathy *et al.*, 1994) and has provided data that correlate with that obtained from mammalian studies (Uysal and Ayar, 2015). Coelho *et al.*, (2015) identified some of the primary caffeine metabolites produced in the body of *D. melanogaster* males administered caffeine. These primary metabolites were theobromine, paraxanthine and theophylline, all of which are also produced in mammals during the metabolism of caffeine (Lelo *et al.*, 1986). In mammals the metabolisation of caffeine is catalysed by an enzyme called cytochrome P450. Caffeine metabolism was dramatically decreased in flies treated with a cytochrome P450 inhibitor, indicating that the cytochrome P450 is also involved in the breakdown of caffeine in *D. melanogaster*. These findings show strong correlation between the metabolic responses of mammals and *D. melanogaster* to caffeine.

A long established genotoxicity test is the Wing Somatic Mutation and Recombination test (SMART) or as it is also known as the “Wing Spot Test”. The somatic cells of *D. melanogaster* provide a means of developing a rapid and flexible short term assay to detect a wide spectrum of genotoxic substances (Graf and Singer, 1992). SMART supports a number of routes of exposure such as oral, injection and inhalation, while the most favoured route of administration is chronic feeding of three day old larvae for the rest of their larval development and the wings from the emerging adult flies are then scored for the presence of spots of mutant cells Graf and Singer, 1992). The presence of single spots are indicative of a somatic point mutation, deletion, somatic recombination or non disjunction appearing between two markers (Mademtoglou *et al.*, 2013). The wing spot test has been utilised to examine the genotoxicity of food additives (Tripathy *et al.*, 1994; Mademtoglou *et al.*, 2013).

There are several disadvantages to the use of *D. melanogaster* as a model organism, firstly its small size makes it difficult to inoculate and limits its ability for individuals to be biochemically analysed (Kemp and Massey, 2007). *D. melanogaster* requires a more significant commitment of time and resources than the *G. mellonella* model, the fruit fly requires considerable experience and specialized equipment

(Arvanitis *et al.*, 2013) . The fruit fly model must be propagated at approximately 22°C which is not an ideal temperature for investigating interactions that usually occur at 37°C (Kemp and Massey, 2007).

1.5.3 *Bombyx mori*

B. mori, commonly known as the silkworm, grows to approximately 5cm in length (Fig. 1.4). The silkworm's large body size is an advantage over other invertebrate models such as *Drosophila*. Its large body size makes haemolymph preparations and organ isolation easier, making the silkworm amenable to drug pharmacodynamics studies (Hamamoto *et al.*, 2005). Silkworms reproduce in a short period of time, larvae are easily maintained in a laboratory, and a large numbers of larvae with a fixed genetic background can be used at very low cost (Hamamoto *et al.*, 2009). The ability to administer an exact inoculum to silkworm larvae is essential for quantitative evaluation of pathogenicity and for assessing the efficacy and toxicity of antimicrobial drugs (Hamamoto *et al.*, 2009). *B. mori* has been evaluated as an *in-vivo* invertebrate model system for identifying substances that suppress sucrose-induced postprandial hyperglycaemia (Matsumoto *et al.*, 2016). In humans, excess intake of sucrose leads to an increase in blood glucose levels. An increase in glucose levels can lead to adverse health effects such as obesity and diabetes. Silkworm haemolymph glucose levels rapidly increase after intake of a sucrose-containing diet. Addition of acarbose or voglibose, α -glycosidase inhibitors clinically used for diabetic patients, suppressed the dietary sucrose-induced increase in silkworm haemolymph glucose levels. *B. mori* has been employed to measure the toxicity of fluoride (Li *et al.*, 2015) and pesticides (Muthusamy and Rajakumar, 2016).

1.5.4 Zebrafish

One widely used alternative vertebrate model is the zebrafish (Fig. 1.4) (Chen *et al.*, 2009; Dooley and Zon, 2000; Meeker and Nikolaus, 2008). In recent years, the zebrafish has been widely used to study developmental biology (Meeker and Nikolaus, 2008), molecular genetics (Kabashi *et al.*, 2011), human disease modelling (Dooley and Zon, 2000), and drug and toxicant analysis (Spitsbergen and Kent., 2003). There are many advantages of the zebrafish model including; a short generation time, external fertilization, fast development, transparent embryos, and a large and growing biological database (Lieschke and Currie, 2007). Zebrafish embryos develop most of the major organ systems present in mammals, including the cardiovascular, nervous and digestive systems in less than a week (Rubinstein, 2006). The highly studied brain and central nervous system of the zebrafish shares many similarities to the mammalian nervous system (Milan *et al.*, 2003). Zebrafish have also been employed to measure the ototoxicity of drugs (Milan *et al.*, 2003), because hair cells in zebrafish known as neuromasts are similar to the inner ear hair cells in mammals (Rubinstein, 2006). Neuromasts are easily visualised by staining with a fluorescent vital dye, allowing the investigators to quantify the loss of hair cells in response to toxic compounds (Rubinstein, 2006). Zebrafish have a two chambered heart and several mutations that cause phenotypes similar to human diseases have been described in the heart of zebrafish (Rubinstein, 2006). The measurement of heart rate in zebrafish is used as an indicator of cardiac toxicity and the cardiostimulatory effects of adrenaline, have been shown in zebrafish (Milan *et al.*, 2003). Zebrafish are a good model to test toxicity and teratogenicity since they have transparent eggs, making the organogenesis of zebrafish embryos easier to observe (Chen *et al.*, 2008). The exposure of zebrafish to ethanol during gastrulation has been shown to cause cyclopia and this phenotype is also observed in human babies that were exposed to high levels of alcohol during gestation (Rubinstein, 2006).

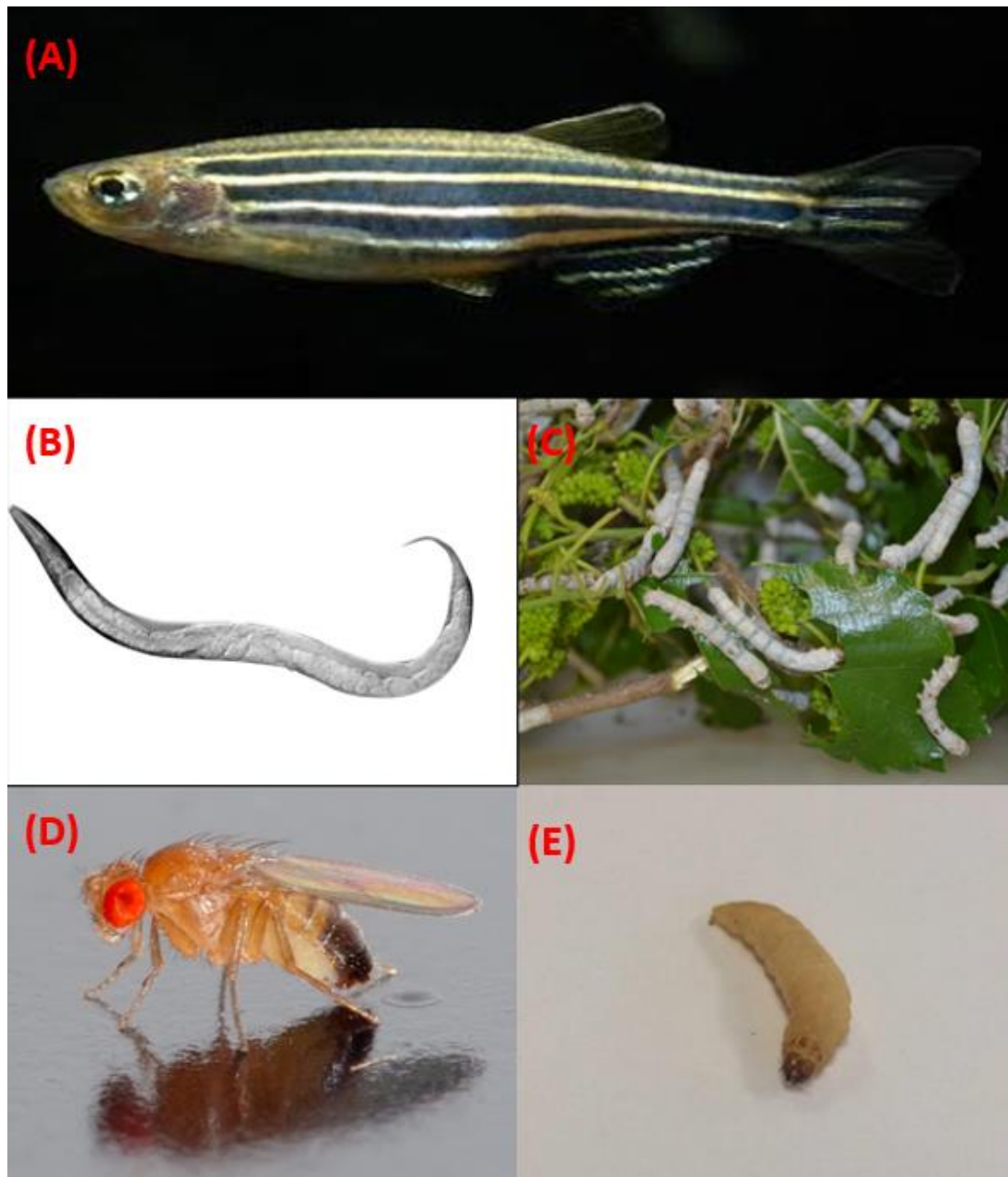


Fig 1.4 Alternative models of toxicity

(A) Zebrafish (2-7 cm in length), (B) *Caenorhabditis elegans* (1 mm in length) (C) *Bombyx mori* (20-70 mm in length), (D) *Drosophila melanogaster* (3 mm in length) and (E) *Galleria mellonella* (3-30 mm in length)

(A) <http://www.uniprot.org/taxonomy/7955>

(B) https://web.science.uu.nl/developmentalbiology/boxem/elegans_intro.html

(D) https://en.wikipedia.org/wiki/Drosophila_melanogaster

1.6 Xenobiotic metabolism

Metabolism is the term used to describe the biochemical changes that a compound undergoes *in-vivo*. The metabolism of a compound generally leads to its detoxification, for example the metabolism of cyanide with an LD50 value of 0.002g/kg to thiocyanide with an LD50 value of 0.4g/kg (Deshpande, 2002). However the metabolism of a compound can sometimes lead to the formation of a compound with increased toxicity, for example the metabolism of pyridine with an LD50 value of 1.2 g/kg to methylpyridine with an LD50 value of 0.2 g/kg (Deshpande, 2002). Generally lipophilic compounds are more readily absorbed through the gastrointestinal tract and will accumulate in an organism to toxic levels unless effective means of elimination are achieved (Jandacek and Tso, 2001). Enzymes responsible for metabolizing xenobiotics are classed into phase 1, phase 2 and transporter enzymes (Croom, 2012). In order to prevent a compound accumulating to a toxic level *in-vivo*, it must undergo chemical changes known as biotransformations. These biotransformations result in a more hydrophilic and less lipophilic molecule. This increase in water solubility decreases the renal tubular and intestinal reabsorption of the compound leading to its elimination by urinary and biliary fecal routes (Lin, 1995). Biotransformations can also lead to the production of reactive oxygen species (Nebbia, 2001). The biotransformation of a xenobiotic by phase 1 enzymes usually involves the adding or unmasking of a hydrophilic group, such as a hydroxyl, amine or sulfhydryl group and usually involves reactions such as oxidation, deamination, dehalogenation, desulfuration, epoxidation, peroxygenation, and reduction (Deshpande, 2002). Phase 1 enzymatic reactions result in the exposure of functional moieties on a compound, allowing for phase 2 metabolism and the conjugation of the molecule with sugars, peptides or amino acids (Snyder and Hedli, 1996). Not all xenobiotic compounds must undergo phase I and phase II metabolism and it may be adequate for a xenobiotic to undergo phase I metabolism and be excreted. It may also be sufficient for a xenobiotic that already contains a functional group capable of conjugation to undergo only phase II metabolism before excretion (Deshpande, 2002). There are two distinct types of

biotransformation systems that exist in mammals. The first system involves enzymes that occur normally in tissues functioning to transform normal endogenous chemicals in tissues, while also playing a role in the biotransformation of xenobiotics. An example of this system of enzymes is the hydrolyzing enzyme cholinesterase that in normal biological conditions is responsible for the hydrolysis of acetylcholine, but is also involved in the hydrolysis of the xenobiotic procaine. The second type of biotransformation systems exclusively metabolises xenobiotics having no known endogenous substrates (Jancova *et al.*, 2010). Phase I enzymes are chiefly located in the endoplasmic reticulum, allowing the membrane bound enzymes to come in contact with lipophilic xenobiotics, while the majority of phase II enzymes are located in the cytosol (Deshpande, 2002).

Some of the enzymes involved in xenobiotic metabolism are glutathione-S-transferases (Hayes and Pulford, 1995), sulfotransferases (Gamage *et al.*, 2006) and UDP-glucuronosyltransferase (Guillemette, 2003), flavin mono-oxygenases (Phillips and Shephard, 2018), alcohol dehydrogenases (Cedebaum, 2012) and aldehyde dehydrogenases (Leal and Barbancho, 1993). Cytochrome P450 enzymes are responsible for a vast amount of phase 1 reactions (Zanger and Schwab, 2013). The toxicity of xenobiotics can be altered by metabolic processes such as oxidation and conjugation reactions. Consequently the potential for the conversion of a toxic compound into a non-toxic derivative and vice versa (Deshpande, 2002).

Cytochrome p450 enzymes are involved in multiple cellular processes such as pigment synthesis (Ren *et al.*, 2016), protection against chemical insults (Gu and Manautou, 2013), synthesis of sex steroid synthesis (Praporski *et al.*, 2009), the oxidative metabolism of retinoic acid (Ross and Zolfaghari, 2013), mono-oxidation of compounds (Cankar *et al.*, 2011) and dehydration (Boucher *et al.*, 1994). Dehydrogenation, isomerisation and reduction of substrates are some of the many reactions that are catalysed by CYP enzymes (Meunier *et al.*, 2004). CYPs 3A4, 2C9, 2C8, 2E1, and 1A2, are the highest expressed in the liver while 2A6, 2D6, 2B6, 2C19 and 3A5 are less abundant and CYPs 2J2, 1A1, and 1B1 are mainly expressed extrahepatically (Zanger and Schwab 2013). Fifty seven functional genes and 58 pseudogenes make up the human CYP superfamily, with members of the 1, 2, and 3

families playing an important role in the metabolism of therapeutic drugs and other xenobiotics such as food additives (Zhanger *et al.*, 2013).

1.6.1 Phase II metabolic enzymes

Phase II biotransformations are anabolic reactions and therefore require energy to drive them. Glucuronidation is one of the major phase II drug-metabolizing reactions that contributes to drug biotransformation (Guillemette, 2003). The addition of a glucuronic acid moiety, from the endogenous precursor UDP-glucuronic acid is catalysed by the UDP-glucuronosyltransferases. This addition of a glucuronic acid moiety occurs at a range of different nucleophilic functional groups of endogenous and exogenous compounds, such as hydroxyl, carboxylic acid, amines, sulfhydryl and ester moieties (Testa and Kramer, 2007). The majority of phase II enzymes are present in the cytosol, however the enzymatic activity of UDP-glucuronosyltransferases is localised in the endoplasmic reticulum (Deshpande, 2002).

1.7 Food additives

With the world's population ever increasing there is now more than ever a need to increase food production to meet this demand. This increase food production must be met while still adhering to high quality standards that preserve taste, appearance, texture and microbiological safety. The integrity of food production is achieved through the use of food additives. Despite the many benefits and the vast amount of food additives in use there still remains a lot of debate of the health and safety of their use. Through risk benefit analysis a toxicological standard can be defined for a substance. Traditionally, health based recommendations or guide values are based on data obtained from toxicological studies carried out on experimental mammalian models. However, data obtained from mammalian models can sometimes give misleading information for the prediction human toxicity. For example, the toxicity of theobromine in humans is 1000mg/kg however it is 250-500mg/kg in dogs (Jansson *et al.*, 2001). Despite this, the use of mammals in toxicity testing is still the best way to predict human toxicity.

Several different toxicological units are used to express the toxicity potential of chemicals (Table 1.4). The most widely used method is the Lethal dose 50 (LD50) method as a measurement of acute toxicity. LD50 value is the amount of a compound that kills 50% of a test population and is expressed as mg/kg (Zbinden and Flury-Roversi, 1981). Several parameters are used to set acceptable human exposure levels to food additives (Table 1.5). The most widely used is the average daily intake (ADI) value. The definition in a World Health Organisation (WHO) report defines the ADI as, "an estimate of the amount of a food additive expressed on a body weight basis that can be ingested daily over a life time without appreciable health risks." The no-observed-adverse-effect-level (NOAEL) is used to estimate the ADI of a component (Lu, 1988). The highest dose that shows no observed adverse effect on an animal test population is then divided by a safety factor of 100 to allow for human interpretation. The safety factor takes into consideration the differences between animals and humans and the difference between human populations (Lu, 1998).

Food additives are divided into 26 functional classes in the EU as follows; sweeteners, colorants, preservatives, antioxidants, carriers, acids, acidity regulators,

anticaking agents, antifoaming agents, bulking agents, emulsifiers, emulsifying salt, firming agents, flavour enhancers, foaming agents, gelling agents, glazing agents, humectants, modified starches, packaging gases, propellants, raising agents, sequestrants, stabilizers, thickeners, and flavour treatment agents. In the USA there are over 3000 approved food additives grouped into 6 categories (Carocho *et al.*, 2014) (Fig 1.5)

Food preservatives can consist of antimicrobials, antioxidants and anti-browning agents. The preservative prevents natural spoilage and prevent or control micro-organism contamination. Acetic acid, potassium acetate, calcium acetate, lactic acid, carbon dioxide, and maleic acid are the main antimicrobials used in food with *quantum satis* status. Benzoic acids and benzoates, sorbic acids and sorbates, propionic acids and propionates, nitrites, nitrates and parabens are commonly used food additives that are restricted in their use (Carocho *et al.*, 2014).

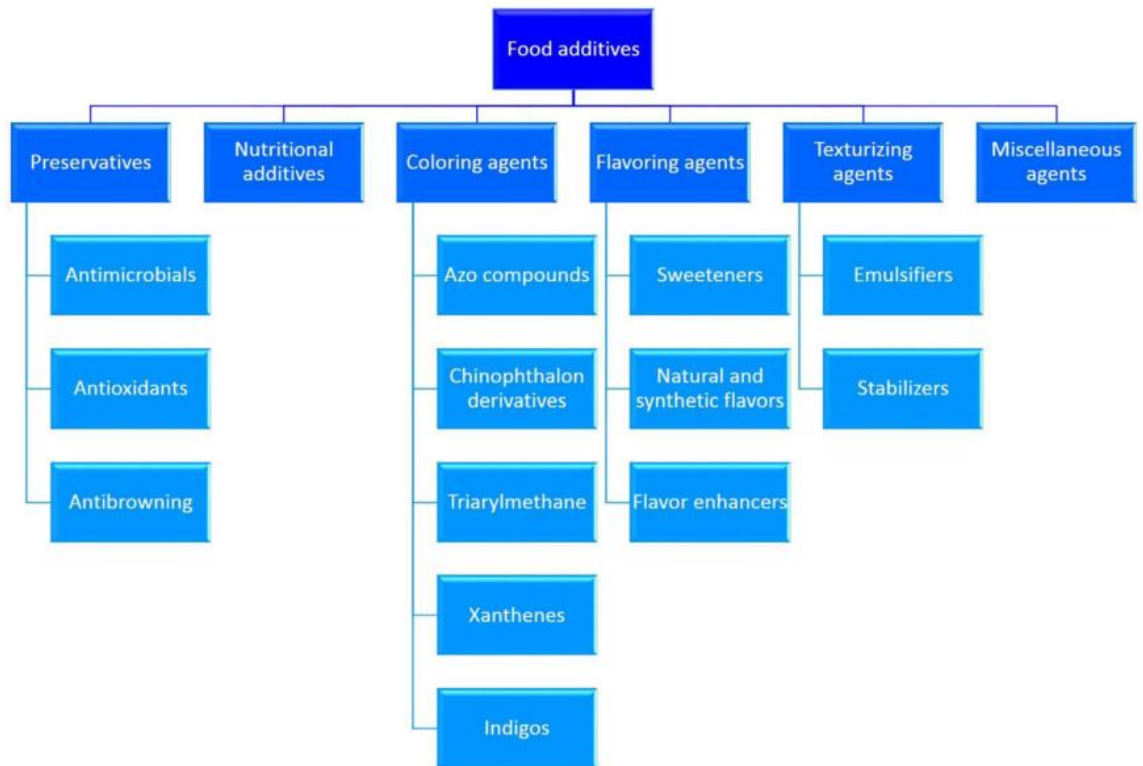


Fig 1.5 Groups and subgroups of food additives (Carocho *et al.*, 2014).

Abbreviation	Full name	Description
LD₅₀	Lethal dose/50% response	Calculated dose of a substance that is expected to cause the death of 50% of an entire defined experimental animal population
LD₀₁	Lethal dose/1% response	Maximal dose of a substance that is likely to be sublethal to an entire experimental animal population
LD₉₉	Lethal dose/99% response	Minimal dose of a substance that is likely to be lethal to an entire experimental animal population
LDL₀	Lowest lethal dose	Dose of a substance administered over any given period in one or more divided portions and reported to have caused death in humans and animals
ED₅₀	Effective dose/50% response	Median effective dose obtained from a dose versus percentage response curve that uses not lethality as end point but rather a graded response by identifying a value above which the response is defined as positive; may use any clearly defined measure of efficiency
ED₀₁	Effective dose/1% response	Dose effective in only 1 % of treated animals
ED₉₉	Effective dose/99% response	Dose effective in only 99 % of treated animals
TD₀₁, TD₅₀, TD₉₉	Toxic dose/1%, 50%, 99%	Similar to effective dose (ED) but uses a clearly defined measure of clinical toxicity
LC₅₀	Lethal concentration/50% response, inhalation exposure	Median lethal concentration of an inhaled chemical defined as a "statistically derived concentration of a substance that can be expected to cause death during exposure"
LCt₅₀	Lethal concentration x time/50% response, inhalation exposure	Statistically derived concentration of a substance that can be expected to cause death within a fixed time after exposure in 50% of the animals exposed for the specific time
LCL₀	Lowest lethal concentration, inhalation exposure	Dose of a substance inhaled over any given period in one or more divided portions reported to have caused death in humans or animals
LC₅₀	Lethal concentration/50% response, exposures other than inhalation	For aquatic toxicity: concentration of a chemical in water killing 50% of a test batch of fish within a particular period of exposure; using cultured hamster cells: concentration of a chemical that causes transformation of 50% cells in response to carcinogens without toxicity-induced selection of subpopulations
MTD	Maximal tolerated dose	Highest dose of the test agent given during the chronic study that can be predicted not to alter the animal's longevity from effects other than carcinogenicity; should not produce greater than 10% inhibition of weight gain, produce clinical evidence of toxicity of pathological lesions, or alter longevity except as a result of carcinogenesis
NEL	No effect level	Level of a substance that can be included in the diet of a group of animals without toxic effects; used interchangeably with no adverse effect level (NAEL), no observed effect level (NOEL), and no observed adverse effect level (NOAEL)
MED	minimal effective dose	Minimal effective dose used as an alternative to the NEL: minimal dose that produces an observed effect; used interchangeably with lowest effect level (LEL), lowest observed effect level (LOEL), and lowest observed adverse effect level (LOAEL)

Table 1.4 Several different toxicological units are used to express the toxicity potential of chemicals (Dehpande, 2002)

Abbreviation	Full name	Description
TLV	Threshold limit value	Recommended atmosphere concentrations of work place substances (ppm or mg/m ³) to which workers may be exposed to without adverse health effects
TLV-TWA	Threshold limit value-time weighted average	Same as TLV except that it represents a time weighted average concentration for an 8 hour workday and a 40 hour work week; can be exceeded for short periods during the workday without producing adverse health effects as long as the average concentration is at or below TLV
TLV-STEL	Threshold limit value-short term exposure limit	Used for chemicals that may produce adverse effects when TLV is exceeded for only a brief period; time weighted concentration limit for 15 min; no more four such exposure periods permitted per day, and maximal of 60 min must elapse between exposures
TLV-C	Threshold limit value ceiling	Stringent maximal permissible exposure that may not be exceeded even for short periods; similar to maximal acceptable (formerly allowable) concentrations (MACs); applied to fast acting highly toxic or extremely irritating substances for which even brief exposure periods may cause serious toxicity
ADI	Acceptable daily intake	Amount of food additive that can be taken daily in the diet, even over a lifetime, without risk; concept also applicable to contaminants in food, air and water
SNARL	Suggested no adverse response level	Level of drinking water contaminants below which no adverse effects are expected after specified exposure period, usually defined for 24-hr, 7 day, or chronic (lifetime) exposure

Table 1.5 Several different toxicological units are used to express the permissible exposure levels of chemicals (Deshpande, 2002)

1.7.1 Benzoic acids and Benzoate

Benzoic acids and benzoates are commonly coupled with either sodium calcium or potassium, for different anti-microbial targets. These antimicrobials are effective against yeast, bacteria and fungi through its ability to disrupt membranes, inhibit metabolic reactions, and cause the accumulation of toxic anions inside the microbial cell. Sodium benzoate is primarily used as a preservative of acidic foods, such as soft drinks and fruit juices. Benzoates are more effective against yeast and bacteria as opposed to molds. Studies on humans suggest that sodium benzoate is non-deleterious to human health (WHO, 2016). Benzoates do not appear to accumulate in the body and are absorbed by the intestines and metabolised and excreted as hippuric acid (Barshop *et al.*, 1989). The ADI for total benzoate in the human diet is established at 0-5mg/kg body weight (WHO, 2005).

1.7.2 Nitrates and nitrites

Nitrate has been used for centuries to pickle meat and as an additive for fish and cheese. The growth of yeast and fungi is not affected by nitrites and the action is almost exclusively anti-bacterial (Cammack *et al.*, 1999). Nitrates, such as potassium nitrate and sodium nitrate, have limited antimicrobial capacity, the main microbiological capacity of nitrates is its microbiological conversion to nitrites during the curing process (Jouve *et al.*, 1980). Human dietary nitrate may be reduced to nitrite by bacteria present in the mouth and sometimes in the stomach (Walker 1996). Nitrate added to cured meats and other food serves three functions. Through its ability to inhibit the development of rancid off flavours nitrites actively contribute to flavour (Cammack *et al.*, 1999). Interaction between nitrites and myoglobin produces mononitrosylhaemochrome and the characteristic pink colour of cured meats (Cammack *et al.*, 1999). Finally, and probably the most important function of nitrate addition to meat, is its ability to inhibit the growth of food spoilage bacteria, especially *Clostridium botulinum* (Sindelar & Milkowski, 2012). *C. botulinum* produces botulinum, a neurotoxin that blocks the release of acetylcholine causing muscle paralysis (Nigam & Nigam, 2010). Nitrites are the only food preservative that inhibit the growth of *C. botulinum*, thereby validating its use despite various

deleterious health problems associated with high levels of nitrites in food (Pierson & Smoot, 1982).

High doses of nitrates and nitrites (and their metabolic products) are carcinogenic in humans due to the formation of nitrosamines (Camargo *et al.*, 2005). Exposure of HEP-2 cells to nitrates results in increased expression of genes associated with a stress response, cell cycle control and DNA repair (Bharadwaj *et al.*, 2005). The acute toxicity of potassium nitrate was measured in juvenile blue swimmer crabs, histopathological changes to the anterior gill lamellae were observed, including lamellae swelling, epithelial thickening, pillar cell disruption, necrosis, and distortion (Romano & Zeng, 2007). Epidemiological studies have suggested that high nitrate concentrations in drinking water are associated with conditions such as teratogenicity, thyroid hypersensitivity and childhood diabetes (Bharadwaj *et al.*, 2005). The main toxic effect of chronic nitrate is due to the conversion of haemoglobin to methaemoglobin which leads to methaemoglobinemia (Van Dijk *et al.*, 1983). Patients administered nitrates display enhanced nitric oxide bioavailability in the vasculature, vasodilation effects, and inhibition of platelet aggregation. Administration of potassium nitrate (0.06–0.35 mmol kg/day) reduces diastolic and systolic blood pressure (Van Dijk *et al.*, 1983). Sodium nitrate also reduces blood pressure but leads to a reduction in oxygen consumption and increased blood flow (Van Dijk *et al.*, 1983).

1.7.3 Sorbates

Potassium sorbate is used to preserve cheeses, cakes and syrups. Sorbic acid and its salts are effective against a wide variety of yeast molds and bacteria and are considered to be among one of the safest antimicrobial agents to be used in foods (ADI of 25mg/kg body weight) (Deshpande, 2002). Toxicity tests over two generations in fed mice and rats with sorbic acid doses as high as 90mg/kg showed no abnormalities and no carcinogenic or mutagenic effects have been observed (Dickens *et al.*, 1968). The low toxicity of sorbic acids may be explained by the fact that they are metabolised rapidly by similar pathways to other fatty acids (Walker, 1990). Allergic type reactions in sensitive individuals can occur by the irritation of mucous membranes and skin in response to high level exposure of sorbates in

pharmaceutical or cosmetic products (Lueck, 1980). When in contact with nitrates, sorbic acids can form mutagenic compounds however, this only occurs in extreme conditions such as high concentrations or temperatures and is not detectable under normal conditions, even in curing brines (Walker, 1990).

1.7.4 Caffeine

Caffeine (1,3,7-trimethylxanthine) is the most widely used central nervous system stimulant in the world and along with its metabolites, theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), is found in a wide range of plants (Porciúncula *et al.* 2013). A third metabolite paraxanthine, (1,7-dimethylxanthine) is not found in food, but is the main metabolite of caffeine *in vivo* (Aresta *et al.*, 2005). Over 60 plant species contain caffeine with the most widely used being coffee (*Coffea arabica*), kola nuts (*Cola acuminata*), tea (*Thea sinensis*), and chocolate (*Cocoa bean*) (Vanderveen *et al.*, 2001); and it is also available in synthetic form. Caffeine competitively binds to adenosine receptors allowing it to act as an antagonist with inverse agonist activity (Rivera-Oliver and Díaz-Ríos, 2014). Caffeine affects cAMP signalling both by binding to adenosine receptors and by competitively inhibiting the activity of phosphodiesterases which function to degrade cAMP (Vanderveen *et al.*, 2001). Caffeine can stimulate dopaminergic activity by removing the negative modulatory effects of adenosine at dopamine receptors (Ferré, 2008). In addition to the effect caffeine has on adenosine receptors, studies have also suggested that paraxanthine, the primary metabolite of caffeine in humans, produces increased locomotive activity, as well as a phosphodiesterase inhibitory effect which in turn results in increased extracellular levels of dopamine (Orrú *et al.*, 2010). The consumption of low to moderate doses of caffeine is generally regarded as safe (GRAS) (daily intake of no more than 400 mg in healthy adults) however the consumption of higher doses by vulnerable individuals can result in an increased risk of negative health consequences such as cardiovascular and perinatal complications (Meredith *et al.*, 2013).

1.7.5 Monosodium glutamate

Monosodium glutamate (MSG) is a sodium salt of glutamate and it is widely used as a flavour enhancer in several meat preparations, being responsible for the umami sensation. MSG is found naturally in tomatoes cheese and other foods. Monosodium glutamate causes glutamate hypersensitivity, more commonly known as Chinese restaurant syndrome, of which the symptoms include headache, sweating, numbness, tickling or burning in the face, chest pain, nausea and weakness (Dixit *et al.*, 2014).

The incidence of obesity was measured in mice and rats of different ages that were administered MSG by various methods (Bunyan *et al.*, 1976). Sixteen per cent of new-born mice injected subcutaneously with 3mg MSG/g of body weight at 1,2,3,6,7 and 8 days of age died. Out of the remaining new-born mice 90% became markedly obese. Studies in animals have shown that MSG is toxic in organs such as the liver (Nakanishi *et al.*, 2008), brain (Xiong *et al.*, 2009), thymus (Hassan *et al.*, 2014) and kidneys (Sharma, 2015).

1.7.6 Creatine monohydrate

Creatine monohydrate is used by both professional and amateur athletes to increase muscle power output. The Lohmann reaction is a reversible reaction catalysed by creatine kinase in muscles, where the high energy phosphate bound to ATP is transferred to creatine, forming creatine phosphate (Kemp, 2007). Muscle cells use both adenosine tri phosphate (ATP) and creatine phosphate as quick access to energy. Aerobic respiration cannot produce energy as quickly as muscle cells can use it, therefore when muscle ATP levels are depleted creatine phosphate can donate a phosphate group to adenosine di phosphate forming adenosine phosphate (Clark, 1997). There has been long standing concern that creatine monohydrate supplementation could be associated with cancer. This has grown through the idea that creatine monohydrate can facilitate the formation of carcinogenic heterocyclic amines (HCA). However, dos Santos Pereira *et al.* (2015) provided evidence that low and high doses of creatine supplementation given either acutely or chronically, does not cause a significant increase in HCA formation.

1.8 Objectives of this study

The objectives of this study are summarized as follows;

1. Measure and compare the acute toxicity of food additives in *G. mellonella* larvae and a HEp-2 cell line.
2. Determine correlations between the mammalian/ Hep-2 cell line and insect response to food additives.
3. Evaluate the toxic effects of potassium nitrite, sodium nitrate, potassium sorbate, sodium benzoate, potassium nitrate, caffeine, monosodium glutamate and creatine monohydrate in *G.mellonella*.
4. Evaluate the potential of using *G. mellonella* larvae as a preliminary model to test the toxicity of commonly used food additives.

Chapter 2

Materials and Methods

2.1 General laboratory practice and sterilisation procedures

2.1.1 Chemicals and reagents

All reagents were of the highest purity and were purchased from Sigma Aldrich Ltd, Somerset, U.K unless otherwise stated.

2.1.2 Sterilisation procedures

All liquids were sterilised prior to use by autoclaving in a Systec 3170 ELV autoclave at 121°C and 15 lb/sq. inch for 15 minutes. Any chemicals unsuitable for autoclaving were filter-sterilised using a filter with a pore size of 0.22 µm (Sarstedt, Nümbrecht, Germany).

2.1.3 Phosphate buffered saline (PBS)

One PBS (NaCl 8.0 g/l; KCl 0.2 g/l; Na₂HPO₄ 1.15 g/l; KH₂PO₄ 0.5 g/l; pH 7.3) tablet (Oxoid) was dissolved in 100ml deionised water and autoclaved at 121°C for 15 minutes. PBS was stored at room temperature.

2.2 Statistical analysis.

All experiments were performed on three independent occasions and results are expressed as the mean ± SE. Changes in *G. mellonella* survival and HEp-2 cell growth were analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00. Analysis of significant changes in cell density and killing ability, enzymatic activity and protein expression was performed by two-way ANOVA using GraphPad Prism version 5.00 for Windows 8, GraphPad Software, San Diego California USA, (www.graphpad.com). For all experimentation a p-value of < 0.05 was deemed to indicate a statistically significant difference.

2.3 *Candida albicans* growth and harvest.

2.3.1 *C. albicans* liquid culture and cell harvest.

A single colony of *C. albicans* (MEN) (serotype B, wild-type originally isolated from an eye infection by Dr. D. Kerridge, Cambridge, UK) was transferred to sterile YEPD broth (section 2.3.2) using a sterile inoculating loop. The flask was re-plugged with cotton wool and incubated at 30°C at 200 rpm overnight. The cell suspension

was transferred to a sterile 50 ml tube, cells were diluted in sterile PBS, enumerated by the trypan blue cell exclusion assay and adjusted in PBS to the correct concentration prior to inoculation.

2.3.2 YEPD broth / agar

YEPD agar was prepared by dissolving glucose (2% w/v), yeast extract (1% w/v), and bacteriological peptone (2% w/v), in deionised water and autoclaving at 121°C for 30 minutes. For agar plates 2% (w/v) agar was added and autoclaved as described. In some cases erythromycin was added to the hand warm agar prior to pouring to control bacterial contamination. This was prepared by dissolving 0.1g erythromycin in 1 ml of deionised (di) H₂O and transferring the erythromycin solution into the hand warm agar solution. Once in the agar solution the plates were spread as per normal and stored at 4°C in the dark. All erythromycin supplemented plates were used within 3 days.

2.3.3 Maintaining long term microbial stocks.

Storage of fungal strains over the longer term was achieved by freezing stocks of liquid culture in 50% (v/v) glycerol and placing at -80°C.

2.4 *G. mellonella* larval storage and experimental conditions.

2.4.1 *G. mellonella* storage and food.

Sixth instar larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were purchased and stored in wood shavings in the dark at 15°C to prevent pupation (Cotter *et al.*, 2000; Hornsey and Wareham, 2011). Larvae weighed 0.20 ± 0.03 g and were excluded if there was evidence of localised melanisation or infection.

2.4.2 Inoculation of *G. mellonella* larvae.

Larvae were injected through the last left pro-leg, with a Myjector U-100 insulin syringe, (Terumo Europe N.V., 3001 Leuven, Belgium), while applying mild pressure to the insect to allow the opening of pro-legs (Fig 1.3). In order to administer compounds by the feeding route, a blunted Myjector syringe was gently

inserted into the mouth of larvae to force-feed 20 μ l of each solution (Mukherjee et al. 2013) (Fig. 1.3; Fig. 2.2). The larvae were kept in 9cm petri-dishes with 0.45 mm Whatmann filter paper inserted on the lids and some wood shavings for all experiments (Fig 2.1). Great care was taken with each injection to maintain a high accuracy of drug or pathogen delivery. All *in-vivo* assays were performed on three separate occasions using a group size of 10 larvae per dish (unless stated otherwise) and average results were calculated. For viability studies larvae were injected with sterile PBS as a control for the injection of larvae.



Fig. 2.1 Larvae were stored in 9 cm petri-dishes with 0.45 mm Whatmann filter paper inserted on the lids and some wood shavings



Fig. 2.2 Apparatus used to force fed larvae

2.5 *G. mellonella* haemocyte protocols

2.5.1 Extraction of haemocytes from *G. mellonella* larvae.

Larvae were pierced through the head with a sterile 23G needle and haemolymph was squeezed through the head region into a pre-chilled microcentrifuge tube. Haemocytes were pelleted by centrifugation at 500 x g for 5 minutes and washed in PBS to remove any excess haemolymph. Following a further centrifugation step at 500 x g for 5 minutes, the remaining supernatant was removed and the pelleted cells were resuspended gently in 1 ml of PBS (supplemented with 0.37% 2-mercaptoethanol)

2.5.2 Trypan blue cell exclusion assay for measuring cell viability

Cell counts for assays involving haemocytes and HEp-2 cells were achieved by performing a trypan blue cell exclusion assay (Eichner et al., 1986). A cell suspension (20 µl) was diluted with a trypan blue solution (0.4%, w/v) (60 µl), and PBS (pH 7.4) (20 µl) at a ratio of 1 : 5. An aliquot of this mixture was counted using a haemocytometer (Neubauer improved cell counting chamber). The number of cells stained, and unstained was recorded. From here, cells that excluded trypan blue were deemed viable, the percentage (%) cell viability was calculated, and the cell density per ml of the cell suspension solution was calculated.

2.5.3 Haemocyte mediated killing using *Candida albicans* as a target.

G. mellonella larvae were administered a food additive (Section 2.4.2) and incubated at 30°C for 24 hours. Ten larvae were bled into 10 ml of cold PBS. The haemocytes were pelleted by centrifugation at 500 x g. Cell free haemolymph was then poured into a separate container and left on ice. The pelleted haemocytes were washed in 10ml of cold sterile PBS, resuspended in 1 ml of PBS, enumerated (Section 2.5.2) and adjusted to 1×10^5 cells/ml. *C. albicans* were grown up to the stationary phase in YEPD broth (Section 2.3.2). The cell suspension was transferred to a sterile 50 ml universal and centrifuged at 1500 x g. The pellet of *C. albicans* cells was washed in sterile PBS, resuspended in 40ml of PBS, enumerated and adjusted to 2×10^5 cell/ml. The *C. albicans* cells were transferred into the container containing the cell

free haemolymph to allow for the opsonization of the cells. The solution of cell free haemolymph and *C. albicans* cells were incubated for 30mins at 30°C. The opsonized *C. albicans* cells were pelleted by centrifugation at 1500 x g and resuspended in 1 ml of PBS. Haemocytes (1×10^5 cells/ml) were mixed with opsonised *C. albicans* cells (2×10^5 cells/ml) in a ratio of 1:2 in a stirred chamber at 30°C (Bergin *et al.*, 2005). Aliquots were removed at $t = 0, 20, 40, 60$ and 80 minutes and serially diluted (1/100) in ice cold minimal essential medium to quench phagocytosis, prior to plating on YEPD agar plates to ascertain fungal viability. The percentage reduction in yeast cell viability was calculated based on viability of control which was defined as 100% as $t = 0$

2.5.4 Haemocyte cell preparation for Flow Cytometry analysis

Ten larvae were bled (Section 2.5.1) into a sterile pre-chilled centrifuge tube containing 800 μ l of chilled PBS. Haemolymph was mixed with the PBS by pipetting slowly several times. Larval haemolymph was centrifuged at 1500 x g for 5mins at 4°C, supernatant was removed and pelleted cells were re-suspended in 1ml of ice cold PBS. Cells were washed a further 2-3 times using the above method. Re-suspended cells were enumerated and viability was checked using the trypan blue exclusion assay (Section 2.5.2). Cells were adjusted to a cell density of 1×10^6 per 500 μ l. Cells were fixed in 3.7% formaldehyde in PBS for 10min at 4°C. The cells were centrifuged at 1500 x g for 5min and re-suspended in 1% bovine serum albumin (BSA) /PBS and washed 1 -2 times before final re-suspension in 500 μ l 1% (w/v) BSA/PBS. Cells can be stored at 4°C for approximately 3 days prior to flow cytometry analysis. Cells were transferred into a flow cytometry (FACs) tube with a 35 μ m cell strainer cap to reduce potential blockage of the instrument by cell clumps and debris.

2.5.5 Flow Cytometry analysis of haemocyte cell populations

Cells were analysed on a Becton Dickson® FACS ARIA, with cell populations grouped based on their forward scatter (size) and side scatter (granularity). This work was carried out in the National Institute of Cellular Biotechnology, Dublin City University under the supervision of Dr. Clair Gallagher.

2.6 Larval movement and development

2.6.1 Assessment of larval movement

Larvae were administered food additives (Section 2.4.2) and placed on their dorsal surface and the time for each to return to their normal orientation was measured at 0, 1, 4, 24, 48 and 72 hours.

For more detailed analysis about larval movement work was carried out in collaboration with Martin Kunc and Dr. Pavel Hyršl (Masarykova University Czech Republic) using the FIMTrack table method (Kunc *et al.*, 2017). Briefly, the method used FTIR (frustrated total internal reflection) of infrared light in acrylic glass. On the boundary between glass and larva the light is reflected down and captured by camera beneath the FIMTrack table (Risse *et al.*, 2013). Ten larvae (5 food additive treated and 5 PBS treated as the control) were placed into a square arena made from paper (28 x 28 cm), and behaviour response was recorded for 10 minutes. Experiment was repeated independently on two separate occasions. Images were captured via DMK 31AU03 camera (IMAGINGSOURCE) in a dark room without any additional light source except the built-in infrared light, which was generated by the FIMTrack. The size of the images was 1024x768 pixels. Images were captured with a frequency of 1 FPS (frame per second) for 600 seconds. The scale factor was 24 pixels/cm. Images were processed by FIMTrack v2 Windows (X86) software (downloaded from <http://fim.uni-muenster.de/>). All tracks were manually verified so that data for each track belonged to a given larval trajectory. Data gathered from software was processed and visualized in Prism 6 (USA GraphPad).

2.6.2 Analysis of effect of food additive on pupation

Larvae were administered food additives (Section 2.4.2) and were placed in sterile 9cm Petri dishes lined with Whatman filter paper containing some wood shavings and incubated at 30°C. Larval pupation (Fig. 2.3) was recorded every 24 hours over a twelve day period. All experiments were performed on three independent occasions.



Fig 2.3 The pupation step, larvae progressing to the next stage of development

2.7 Peptide extraction from *G. mellonella*.

2.7.1 Extraction of *G. mellonella* haemolymph.

Haemolymph was extracted from *G. mellonella* larvae by piercing and bleeding through the anterior end with a sterile 23G needle (Section 2.5.1) and haemolymph was squeezed into a pre-chilled microcentrifuge tubes tube containing a few grains of N-Phenylthiourea to prevent melanisation. A 1/10 dilution in cold PBS was carried out using a pipette tip with the tip cut off to enable uptake of the viscous haemolymph. Haemocytes were then pelleted by centrifugation at 1500 x g for 5mins and the protein supernatant was transferred to a fresh pre-chilled microcentrifuge tube and stored at -20°C. For protein enumeration and quantification, a further 1/10 dilution of the protein supernatant was carried out (1/100 of the original). Protein was then quantified using the Bradford assay (Section 2.8.1) and adjusted 100µg.

2.7.2 Extraction of brain and surrounding tissues from *G. mellonella*.

Larvae were anesthetized using CO₂. Following this larval heads were dissected and placed into a pre-chilled container containing cold PBS supplemented with 7M urea, 2M thiourea. Using a pellet pestle, the tissue was homogenized to a liquid consistency, resting on ice occasionally to avoid overheating. A further 500µl of cold PBS was added per sample. The tubes were centrifuged at 4°C at 9000 x g and the lipid layer removed from the top of the supernatant using a sterile fine tip spatula. The supernatant was removed and placed into a fresh microcentrifuge tube. The tubes were stored at -20°C until needed. Before use protein was quantified by carrying out a Bradford (Section 2.8.1) and acetone precipitated (Section 2.8.2) at a concentration of 100µg.

2.8 Protein methodology for shotgun label free proteomics.

2.8.1 Bradford assay for protein quantification.

Sterile PBS was used to make a serial dilution of bovine serum albumin and this was used to make a range of standards (0.05-1.5mg/ml). All samples used were diluted in sterile PBS. Biorad Bradford protein assay reagent was diluted in ddH₂O (ratio 1:5). Twenty microliters of sample were placed in a 1 ml cuvette. To this 980µl of diluted Biorad Bradford protein assay reagent was added. The cuvettes were inverted to mix the contents and then allowed to incubate for 5 minutes at room temperature before being read in a microcentrifuge tube Bio-photometer. The quantity of protein was based on the OD590 readings.

2.8.2 Acetone precipitation of protein samples.

Acetone precipitation was used to concentrate protein from a dilute sample and also to purify protein samples. The required volume of protein was calculated following Biorad Bradford assay quantification. The correct protein volume was aliquoted into a fresh pre-chilled microcentrifuge tube and 100% ice cold acetone was added to the tube at a volumetric ratio of 1:3 (sample: acetone). Protein was left at -20°C overnight and then precipitated at 13,000 x g for 10 minutes to pellet protein. All protein pellets were placed upside down to air dry for 5 mins following removal of acetone.

2.8.3 In solution digest protocol for overnight peptide digestion for label free proteomics .

The list of buffers used for the in solution digestion of proteins in preparation for label free proteomics are presented below. Buffers were made fresh daily. Protease Max (Promega) was used in order for the trypsin (Promega) to digest the protein when using urea and thiourea. All water was deionised and was taken fresh before use from the deionised water dispenser.

Sample Resuspension Buffer (pH to 8.0)

- 7.2g Urea
- 2M Thiourea
- 0.1M Tris-HCL
- 20mls diH₂O

200mM Ammonium Bicarbonate (AmBic)

- 0.394g Ammonium bicarbonate
- 25ml water

50mM AmBic

- 2.5ml 200mM AmBic
- 7.5ml water

0.5M Dithiothreitol (DTT)

- 0.077g DTT
- 1ml 50mM AmBic

0.55M Iodoacetamide (IAA) (Protect from light)

- 0.102g IAA
- 1ml 50mM AmBic

ProteaseMax solution (1mg/100µl)

- 1mg ProteaseMax™ Surfactant Trypsin Enhancer (Promega)
- 100µl 50mM AmBic

Trypsin solution (0.5µg/µl)

- 20µg Sequence grade modified trypsin (Promega)
- 40µl trypsin reconstitution buffer (Promega)

Following overnight acetone precipitation, the protein samples were centrifuged at 13000 x g for 10 minutes and allowed to air dry. Pellets were resuspended in 25µl of sample resuspension buffer. To aid in pellet resuspension samples were placed in a sonication bath for 5 minutes and vortexed for approximately 30 seconds. Protein enumeration was carried out using a Qubit fluorometer, and the Qubit protein assay kit (Thermo scientific). Protein concentrations for each sample determined by the qubit protein assay were later used to quantify the samples to 1µg/µl just before they were loaded onto the Q-exactive. One hundred and five microliters of 50mM ammonium bicarbonate was added to the samples. Following the addition of 1µl of 0.5M DTT samples were incubated at 56°C for 20 minutes. Samples were allowed cool to room temperature. The samples were alkylated by the addition of 2.7µl 0.55M IAA and incubated at room temperature in the dark for 15 minutes. One microliter of ProteaseMax solution and trypsin solution were added to the samples. The samples were wrapped in tinfoil, incubated for 24 hours at 37°C in an orbital shaker.

2.8.4 Sample clean-up prior to loading on Q-exactive.

The list of buffers used for the sample clean-up for use on Q-exactive using C18 spin columns (Thermo Scientific) are listed below. Buffers were made fresh directly before use. All water was deionised and was taken fresh before use from the deionised dispenser. The C18 columns are designed to trap only 30µg of protein and so is the final step of re-quantification before loading on the Q-exactive OrbiTrap. Care was taken to ensure the resin did not reach any flow through.

Sample buffer (2% TFA, 20% Acetonitrile)

- 200µL Acetonitrile
- 20µl TFA
- 780µl diH₂O

Equilibration Buffer (0.5% TFA, 5% Acetonitrile)

- 25µl TFA
- 250µl Acetonitrile

- 4.3ml diH₂O

Wash buffer (Same as equilibration buffer)

Elution buffer (70% Acetonitrile, 30% water)

- 700µL Acetonitrile
- 300µl diH₂O

Activation buffer (50% Acetonitrile, 50% water)

- 5ml Acetonitrile
- 5ml diH₂O

Loading buffer (0.05% TFA, 2% Acetonitrile)

- Taken straight from the Q-exactive buffer reservoirs

Digested protein samples (following digestion according to section 2.8.3) were briefly centrifuged in a microfuge to collect any condensate, straight from the 37°C incubator following overnight peptide digestion. TFA to a concentration of 0.75% of the total volume of sample was added (approximately 0.75µl), vortexed briefly, and incubated at room temperature for 5 minutes. Samples were centrifuged at 13000 x g for 10 minutes to remove any debris that may have formed overnight, and the supernatant transferred to a fresh tube. Samples were mixed at a ratio of 3 parts sample:1 part sample buffer.

Pierce™ C-18 spin columns (Thermo scientific) were tapped briefly to settle the resin, and the protective caps were removed from either end. Holes were pierced in the lid of sterile microcentrifuge tubes to place C-18 spin columns into (Fig. 2.4). Resin was activated using 200µl of activation buffer, added to the top of the resin, and centrifuged at 1500g for 1 minute. Flow through was discarded and the process repeated. Equilibration buffer (200µL) was added to the column, spun for 1 minute 1500 x g and the flow through discarded, and repeated once more. Samples were loaded to the top of the resin in the C18 column, and a fresh receiver tube placed underneath. Tubes were spun at 1500 xg for 1 minute, flow-through collected, and placed back onto the resin. This was repeated three times to ensure

complete peptide binding to the C18 resin. C18 columns were placed in a fresh receiver microcentrifuge tube, and 200 μ l of wash buffer added. This was then spun at 1500 x g for 1 minute, flow through discarded, and the process repeated a total of three times to remove contaminants such as Urea and Ammonium Bicarbonate. Column was placed over a fresh receiver tube, this time with the lid open and no hole pierced through the lid, and 20 μ l of elution buffer added to the top of the resin bed. The tubes were spun at 1500 x g for 1 minute, and the flow-through untouched. This was repeated a total of three times to obtain a final volume of 60 μ l in the receiver microcentrifuge tube. This is now the cleaned peptide sample. Samples were then dried down in a SpeedyVac and stored at 20 μ l until running on the Q-exactive.

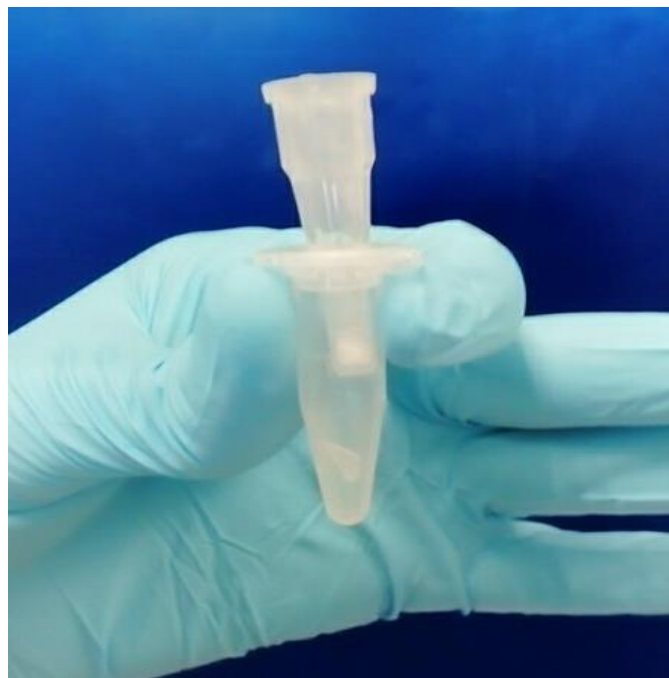


Fig. 2.4 Image shows the C-18 spin placed in a 1.5 ml microcentrifuge tubes.

2.8.5 Preparation of sample prior to loading on Q-exactive.

The protein concentration of each sample (from Qubit protein assay) was used to determine the concentration of sample loading buffer needed to achieve peptide resuspension at a concentration of 1 μ g/ μ l. The samples were vortexed for 30 seconds and placed in a water bath for sonication for 5 minutes. Following resuspension of the peptide pellet, samples were spun at 13000 x g for 5 minutes at room temperature to pellet any insoluble material, and 30 μ l of the supernatant transferred to vials (VWR).

2.8.6 Parameters for running samples on Q-exactive.

One microliter of peptide suspension was eluted onto the Q-Exactive, a high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system (Fig. 2.5). Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID), using a 180 minutes reverse phase gradient at a flow rate of 250 ml /min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

2.8.7 Parameters for analysing quantitative results and statistical analysis.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; <http://maxquant.org/>) to correlate the data against a database for *G. mellonella* (Galleria_6_frame_database), depending on the experiment. A combined database for the two organisms (*D. melanogaster* and *B. mori*) was also used. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5ppm with cysteine carbamidomethylation as a fixed modification and Nacetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and

proteins were only considered identified when more than one unique peptide for each protein was observed. Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. Label free quantification (LFQ) intensities were \log^2 -transformed and ANOVA of significance and t-tests between the proteomes of control and treated larvae was performed using a p-value of 0.05 and significance was determined using FDR correction (BenjaminiHochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least three of the four replicates in the second group (referred to as qualitatively differentially abundant proteins). The Blast2GO suite of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes (BP), molecular function (MF) and cellular component (CC). Enzyme commission (EC) numbers and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was performed as part of the Blast2GO annotation pipeline.

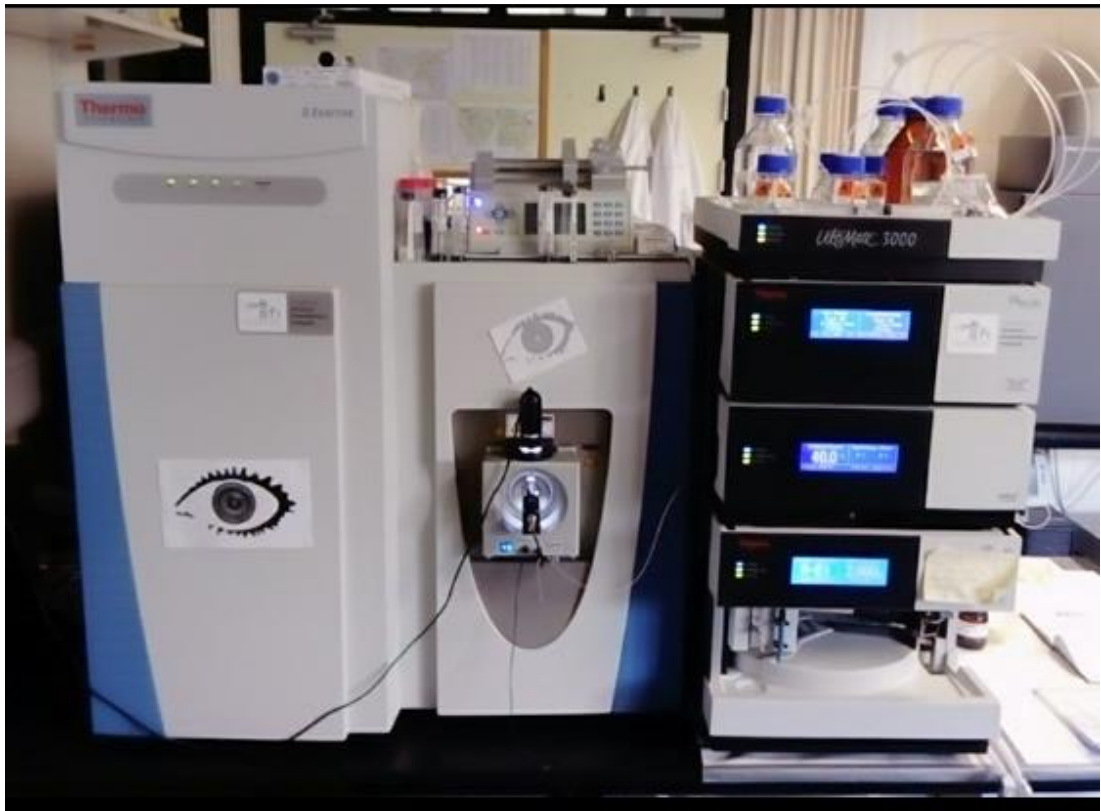


Fig. 2.5 Q-Exactive, a high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system.

2.9 Determination of enzymatic activities.

2.9.1 Determination of catalase activity in larval haemolymph.

Five larvae were administered a compound and catalase activity in haemocyte-free haemolymph was determined after 4 and 24 hours incubation as described previously (Rowan et al., 2009). Five larvae were bled (Section 2.5.1) into a prechilled microcentrifuge tubes with a few grains of N-Phenylthiourea to prevent melanisation. Haemolymph was spun at 10000 x g for 10 minutes at 4°C. Cell free haemolymph was pipetted into a fresh pre-chilled microcentrifuge tube and its protein content was enumerated using the Bradford Assay (Section 2.8.1). Haemolymph protein was corrected to 7mg/100mls of PBS. The 100 ml of haemolymph protein was then incubated with 1.8 ml of H₂O₂ (17mM) for 15 mins at room temperature in the dark. The samples were then centrifuged at 10000 x g for 1 min to stop the reaction. After this time, the supernatant was removed and placed in a clean quartz cuvette. The absorbance at 240nm was obtained using BeckmanDU640 spectrophotometer. A blank consisted of 17mM H₂O₂.

2.9.2 Determination of superoxide dismutase activity in larval haemolymph.

Homogenisation buffer

- 272mg potassium phosphate
- 100ml diH₂O

Assay Buffer

- 50ml homogenisation buffer
- 15mg Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)
- 4.6µL of N,N,N',N'-Tetramethylethylenediamine (TEMED)

Quercetin solution

- 1.5mg Quercetin
- 10ml Dimethyl sulfoxide

Five Larvae were administered a compound and superoxide dismutase activity in haemocyte-free haemolymph was determined after 4 and 24 hours. Five larvae were bled (Section 2.5.1) into a prechilled microcentrifuge tubes with a few grains of N-Phenylthiourea to prevent melanisation. Haemolymph was spun at 10000 x g for 10 minutes at 4°C. Cell-free haemolymph was pipetted into a fresh pre-chilled microcentrifuge tubes. Haemolymph was serially diluted (1/2) in homogenisation buffer from 100% haemolymph to 6.25% haemolymph (0x-16x). Each sample of haemolymph was pipetted in duplicate into a 96-well plate (Corning Incorporated Costar®). Homogenisation buffer (100%) was also pipetted in duplicate into the well as a control. One hundred and fifty µl of Assay buffer was added to each well. Immediately 40µl of quercetin solution was added to each well. Superoxide dismutase activity in haemocyte-free haemolymph was determined by assaying the oxidation of quercetin by N,N',N'-tetramethyl-ethylenediamine and the absorbance at 406 nm for 5 min was obtained using a microplate reader (Boi-Tec® Synergy HT) (Fig. 2.6). One-unit total SOD activity was calculated as the amount of protein causing 50% inhibition of quercetin oxidation (Buyukguzel *et al.*, 2013) and total SOD activity was expressed as units per micro gram of protein.

2.9.3 Determination of alkaline phosphatase activity in larval haemolymph.

Alkaline phosphatase buffer

- 104mg p-nitrophenyl phosphate
- 41mg Sodium acetate
- 5ml diH₂O

Sodium hydroxide buffer

- 200mg Sodium hydroxide
- 5ml of diH₂O

Five Larvae were administered a compound and catalase activity in haemocyte-free haemolymph was determined after 4 and 24 hours. Five larvae were bleed (Section 2.5.1) into a prechilled microcentrifuge tubes with a few grains of N-Phenylthiourea to prevent melanisation. Haemolymph was spun at 10000 x g

for 10 minutes at 4°C. Cell free haemolymph was pipetted into a fresh pre-chilled microcentrifuge tubes and its protein content was enumerated using the Bradford Assay (Section 2.8.1). Haemolymph protein was corrected to 7mg/100mls of PBS. The corrected protein sample was added to 100µl of alkaline phosphatase buffer and incubated at 30°C for 2 hours. The reaction was stopped with the addition of 100µl of sodium hydroxide buffer. The solution was thoroughly mixed by pipetting and 200µl was aliquoted into a 96 well plate (Corning Incorporated Costar®). Colour development was assayed at 405nm, using a microplate reader (Boi-Tec® Synergy HT) (Fig 2.6).



Fig. 2.6 microplate reader, Boi-Tec® Synergy HT.

2.10 Caffeine, theobromine and theophylline extraction from *G. mellonella* haemolymph.

Ten larvae were administered 20µl of 0.8M caffeine by intra-haemocoel injection and force feeding. Larvae were bled into a micro-centrifuge tube and the haemolymph was immediately centrifuged at 500 x g for 5 minutes at 4 °C to pellet haemocytes. Cell free haemolymph was centrifuged at 20,000 x g for 30 minutes to remove any remaining cellular debris. Haemolymph (100µl) was diluted in extraction buffer (900µl) (CH₃OH: H₂O: CH₃COOH ratio 90: 9: 1) and mixed thoroughly for 1 hour on a rocker. The contents of the micro centrifuge tubes were centrifuged at 20,000 x g for 10 minutes at 4°C and supernatant stored at 20°C overnight and subsequently centrifuged again at 20,000 x g for 10 minutes at 4°C. Samples were split into two 400µl aliquots and lyophilized until dryness. Lyophilized samples were stored at -20°C and resuspended in apyrogenic H₂O supplemented with trifluoacetic acid (TFA; 0.1% v/v).

2.10.1 RP-HPLC analysis of caffeine and metabolites in haemolymph.

Samples extracted from cell-free haemolymph for RP-HPLC (reverse phase high-performance liquid chromatography) analysis were loaded in a 20 µl volume on a C-18 Shimadzu HPLC column with diode array detection of 273 nm (Fig. 2.10). Samples were maintained at 4°C in thermally controlled sample tray. The elution profile was 5 minutes of Buffer B (HPLC grade acetonitrile supplemented with 0.1 % (v/v) TFA) at 5 % followed by a linear gradient mobile phase with Buffer B to 100 % acetonitrile for 24 minutes. The column was eluted fully with 100 % Buffer B for 3 minutes and was re-equilibrated with 95 % Buffer A (HPLC grade water supplemented with 0.1 % (v/v) TFA), 5 % Buffer B for 15 minutes prior to further analysis. The retention times of caffeine, theobromine and theophylline were 13.8 minutes, 9.5 minutes and 11.9 minutes respectively. Caffeine standard curve was constructed using concentrations from 2 - 200 µg/ml (Fig 2.7). Theophylline and theobromine standards were from 20 – 1 µg/ml (Fig 2.8: 2.9).

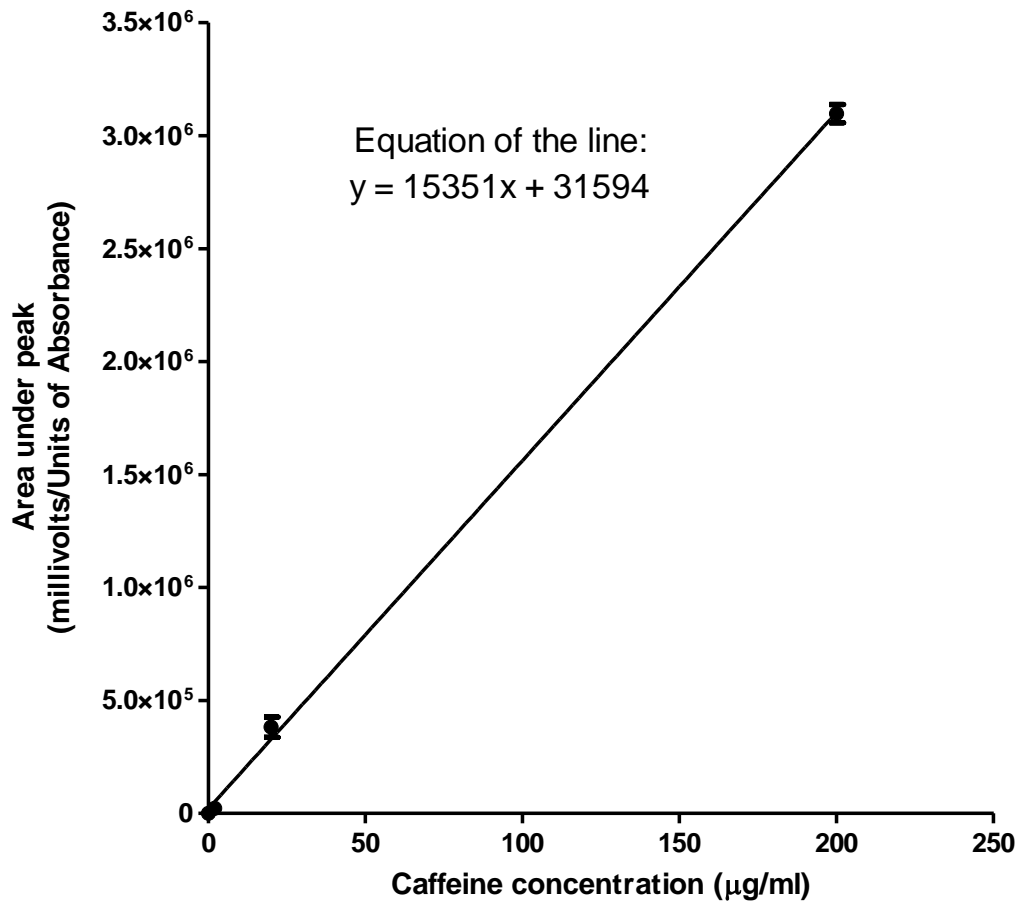


Fig 2.7 Standard curve of caffeine detected by RP-HPLC. All values are the mean \pm SE of three independent replicates.

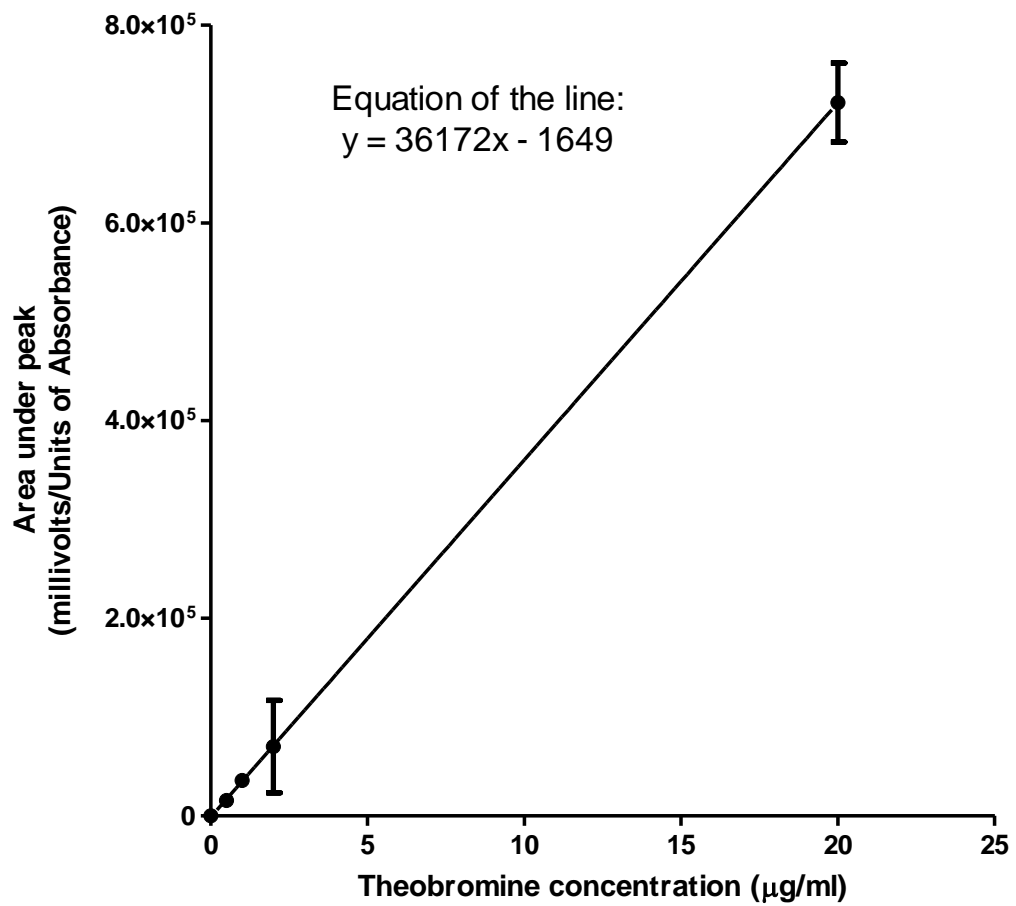


Fig 2.8 Standard curve of theobromine detected by RP-HPLC. All values are the mean \pm SE of three independent replicates.

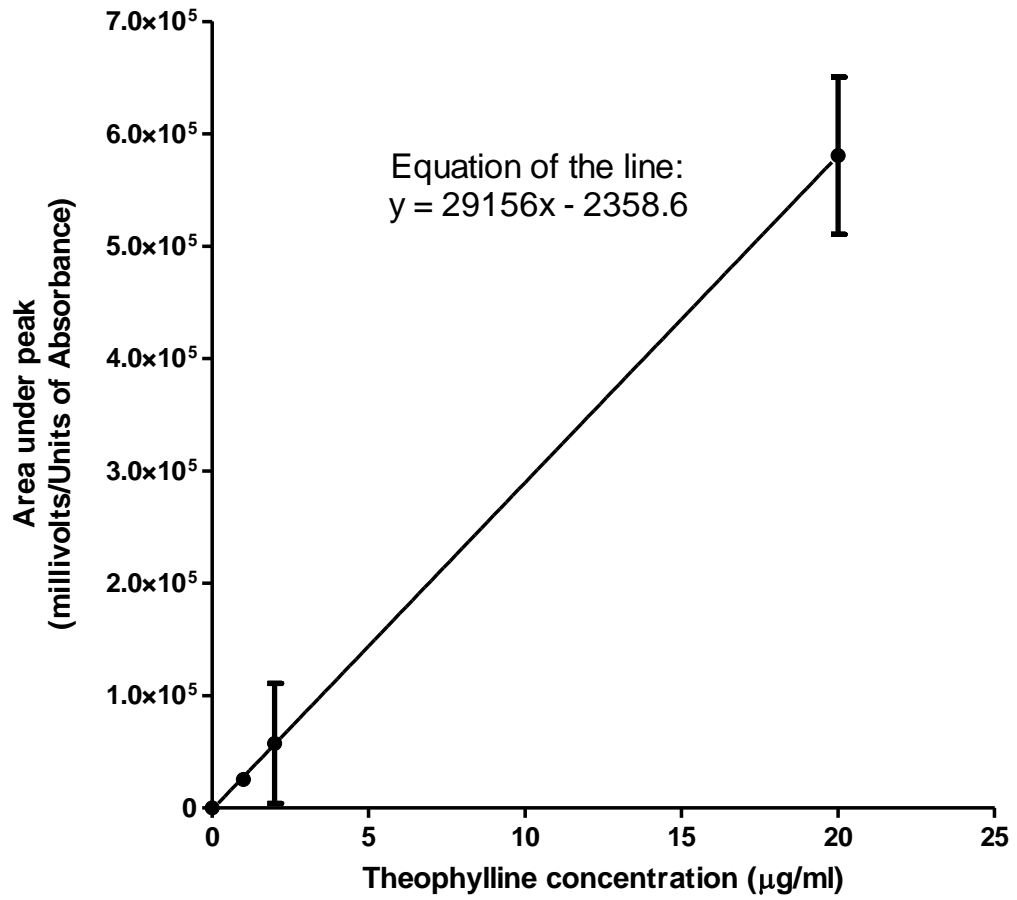


Fig. 2.9 Standard curve of theophylline detected by RP-HPLC. All values are the mean \pm SE of three independent replicates.

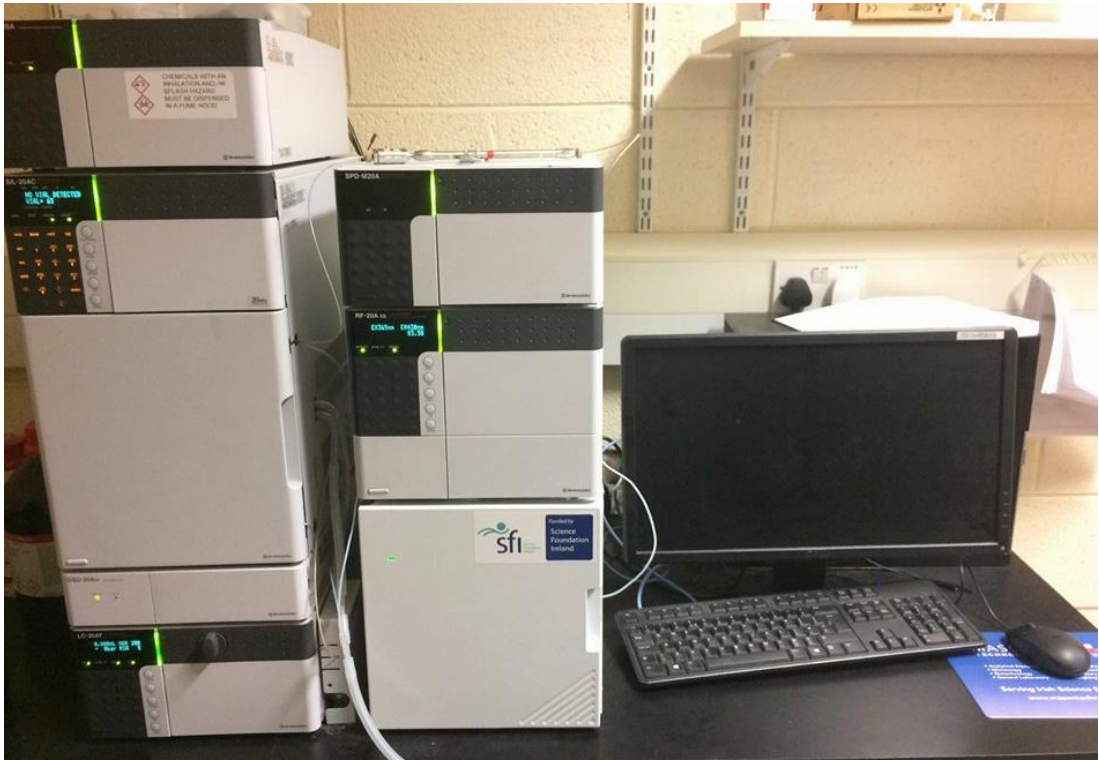


Fig. 2.10 C-18 Shimadzu high powered liquid chromatography

2.11 General cell culture methodology.

2.11.1 HEp-2 cell line.

HEp-2 cell line (ATCC CCL23, derived from an epidermoid carcinoma of the larynx) was obtained from the American type culture collection (Maryland, USA). The HEp-2 cells were grown in 25 cm² tissue culture flasks (Sarstedt) containing Eagle's minimum essential medium supplemented with 5 % (v/v) foetal calf serum (GIBCO Laboratories) and 2%(v/v) glutamine (GIBCO) and incubated at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were subcultured by trypsinisation every 3-4 days as described below.

2.11.2 Sub-culturing an adherent cell line.

Cell medium was poured into a waste bottle and 1 ml of trypsin solution (1ml Trypsin (GIBCO): 9ml PBS) was used too rinse out the remaining medium. Five millimetres of trypsin solution was added into the 25 cm² tissue culture flasks and placed in an incubator at 37°C, 5% CO₂ for 3-4 minutes. The flask was removed from the incubator and examined under an inverted microscope to ensure the successful dislodgement of HEp-2 cells from the flask surface into solution. The side of the flask was gently hit on the side 2-3 times to ensure full cell dislodgement. Five millimetres of culture medium was added to the flask to neutralise the trypsin solution. The solution of cells were gently poured into a sterile universal and harvested by centrifugation at 200 x g. Medium was poured off and the pellet of cells was gently resuspended in 5ml of pre-heated fresh culture medium. The cells were reseeded by pipetting 2 ml of cells and 10 ml of fresh preheated medium into a fresh flask.

2.11.3 Cryopreservation of HEp-2 cells in liquid nitrogen (N₂).

HEp-2 cells were cultured to the exponential phase of growth (approximately 60% confluency), and harvested by trypsinization (trypsin [0.25%, w/v]-EDTA (0.022%, w/v) in PBS). Cells counts were performed using the trypan blue cell exclusion method (Section 2.5.2) before resuspension of HEp-2 cells (2 x 10⁶ cells/ml) in cryopreservation buffer (Dulbecco's modified eagle medium

(DMEM) (Gibco®) (80%, v/v), supplemented with FCS (10%, v/v), and DMSO (10%, v/v) aliquoted to 1 ml volumes using cryovials, and transferred to a Mr Frosty™ freezing container (Nalgene®, Thermo Scientific) (contained ice-cold methanol (100 ml) and was stored at -80°C the night before use) and stored at -80°C overnight before the cryovials were placed in a liquid N₂ (-195.79°C) tank for long term storage. The cryopreservation buffer was kept at 4°C before use.

2.11.4 Recovery of HEp-2 cells from liquid N₂.

HEp-2 cells were recovered from liquid N₂ storage by rapid thawing of cells using pre-warmed cell recovery medium (DMEM (95%, v/v) supplemented with FCS (5%, v/v)) (10 ml of recovery medium for a 1 ml aliquot of cells per cryovial). The cell recovery process was performed quickly to maintain cell viability, and was achieved by swiftly pipetting cells into suspension with the recovery medium followed centrifugation at 200 x g for 3 minutes. Recovered cells were washed (x 2) in Eagle's minimum essential medium, and centrifuged again before transfer to a 25 cm² tissue culture flask (Sarstedt). The flasks were placed in a 5% CO₂ humidified atmosphere incubator at 37°C for 1 hour followed by a medium change to remove unattached cells (non viable).

2.11.5 Acid phosphatase assay for quantifying cell growth.

Confluent HEp-2 cells were trypsinized, enumerated (Trypan Blue Cell Exclusion Assay) and used to seed 96-well plates (Corning Incorporated Costar®) at a density of 1×10⁴ cells/well in 100µl minimum essential medium (MEM) culture medium. After 24 h, incubation cells were exposed to different concentrations of the food additives dissolved in MEM. The plates were incubated 37°C and 5% CO₂ for 7 days prior to the quantification of cell growth. The effect of the food additives on the growth of HEp- 2 cells was determined using the acid phosphatase assay (Yang *et al.*, 1996). Following incubation, medium containing test compounds was removed from each well, and the attached cells were washed with PBS. To each well, 100µl of buffer containing 0.1M sodium acetate (pH 5.0), 0.1 % Triton X-100 and 5 mM p-nitrophenyl phosphate was added, and the plates were incubated at 37 °C and 5 % CO₂ for 2 h. The reaction was stopped with the addition of 50µl of

1M NaOH, and colour development was assayed at 405nm, using a microplate reader (Boi-Tec® Synergy HT) (Fig. 2.6). The percentage growth of HEP-2 cells in the presence of food additives was determined. The inhibitory concentration 50 (IC50) was defined as the concentration of agent that inhibited growth of HEP-2 cells by 50 % over the period of the experiment.

Chapter 3

**Evaluation of *Galleria mellonella* larvae
as an *in-vivo* model for assessing the
relative toxicity of food preservative
agents**

3.1 Evaluation of *Galleria mellonella* larvae as an *in-vivo* model for assessing the relative toxicity of food preservative agents.

The aim of the first part of this Chapter was to evaluate the relative toxicity of potassium nitrate, potassium nitrite, potassium sorbate, sodium benzoate, sodium nitrate, sodium chloride, sodium nitrite and sodium acetate in *G.mellonella* larvae and human epithelial type 2 (HEp-2) cells. Larvae of *Galleria mellonella* are widely used for evaluating the virulence of microbial pathogens (Fedhila *et al.*, 2010) and for measuring the efficacy of anti-microbial agents (Desbois and Coote 2011) and produce results comparable to those that can be obtained using mammals (McMillan *et al.*, 2015). A wide range of mammalian cell lines have been utilized for assessing the *in-vivo* toxicity of compounds (Fotakis and Timbrell, 2006). HEp-2 cells were originally derived from a human laryngeal carcinoma and have been utilized to determine the efficacy of anti-cancer drugs (Rossi *et al.*, 2003), the cytotoxicity of nanoparticles (Ahamed *et al.*, 2015) and the toxicity of food additives (Angelis *et al.*, 1994).

The acute toxicity of a compound intended for use as a food additive or preservative is measured as the calculated dose of a substance that is expected to cause the death of 50 % of a defined experimental animal population and is known as the lethal dose 50 (LD₅₀) value. The inhibitory concentration 50 (IC₅₀) value is measured in HEp-2 cells, the value was calculated as the dose of a substance that is expected to cause 50 % inhibition of growth in the cell line.

The second part of this Chapter focused on a more in-depth study of the toxic effects of 4 food additives: potassium sorbate, sodium nitrate, sodium benzoate and potassium nitrite in *G. mellonella* larvae. The in-depth study focused on the effect these 4 food additives have on the immune system and enzymatic responses of *G. mellonella*. Despite divergence almost 500 million years ago striking similarities exist between insects and mammals. In particular human neutrophils and insect haemocytes display many similarities (Browne *et al.*, 2013) including the ability to produce superoxide by a functional NADPH oxidase complex (Bergin *et al.*, 2005). The metabolism of xenobiotics can lead to the production of reactive oxygen species causing oxidative stress, resulting in the production of antioxidant enzymes

(Büyükgüze *et al.*,2013). A number of antioxidant enzymes produced by the fat body such as superoxide dismutase, catalase and glutathione-s-transferase are highly conserved between species (Büyükgüze *et al.*,2013).

This work presented here aimed to characterise the similarities between the response of *G. mellonella* to pre-existing models of food additive toxicity therefore highlighting the insect's utility as a preliminary screening model for measuring the toxicity of food additives.

3.2 Assessment of effect of food preservative agents on *G. mellonella*

G. mellonella larvae were administered 20 µl of the food additive agents by intra-haemocoel injection (Fig. 3.1). or by force feeding (Fig. 3.2). as described, and the LD₂₀, LD₅₀ and LD₈₀ values of each compound were determined.

LD₂₀ values for Intra-haemocoel injected compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.07 M, sodium nitrite 0.07 M, sodium benzoate 0.17 M, potassium sorbate 0.15 M, sodium acetate 0.42 M, sodium nitrate 0.50 M, potassium nitrate 0.76 M and sodium chloride 0.85 M. LD₅₀ values for Intra-haemocoel injected compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.09 M, sodium nitrite 0.10 M, sodium benzoate 0.21 M, potassium sorbate 0.44 M, sodium acetate 0.50 M, sodium nitrate 0.66 M, potassium nitrate 0.89 M and sodium chloride 1.00 M. LD₈₀ values for Intra-haemocoel injected compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.11 M, sodium nitrite 0.13 M, sodium benzoate 0.25 M, potassium sorbate 0.47 M, sodium acetate 0.56 M, sodium nitrate 0.82 M, potassium nitrate 0.96 M and sodium chloride 1.20 M.

LD₂₀ values for force fed compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.14 M, sodium nitrite 0.07 M, sodium benzoate 0.38 M, potassium sorbate 0.40 M, sodium acetate 0.62 M, sodium nitrate 0.50 M, potassium nitrate 0.77 M and sodium chloride 0.70 M. LD₅₀ values for force fed compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.2 M, sodium nitrite 0.29 M, sodium benzoate 0.45 M, potassium sorbate 0.48 M, sodium acetate 0.90 M, sodium nitrate 0.81 M, potassium nitrate 0.90 M and sodium chloride 1.14 M. LD₈₀ values for force fed compounds in *G. mellonella* were recorded to be as follows: potassium nitrite 0.25 M, sodium nitrite 0.32 M, sodium benzoate 0.5 M, potassium sorbate 0.53 M, sodium acetate 1.20 M, sodium nitrate 0.95 M, potassium nitrate 0.98 M and sodium chloride 1.24 M.

The relative toxicity of each compound in larvae was greatest when administered by intra-haemocoel injection compared to the feeding route. For example, larvae administered potassium nitrite by intra-haemocoel injection showed

an LD₅₀ value of 0.09 M, compared to an LD₅₀ value of 0.20M when larvae were force-fed the compound. Larvae administered sodium benzoate into the haemocoel showed an LD₅₀ value of 0.21 M, compared to an LD₅₀ value of 0.45 M in larvae that were force-fed with the compound. A strong correlation between the LD₂₀, LD₅₀ and LD₈₀ values for each compound in larvae due to feeding or intra-haemocoel injection was established. The results show an R² value of 0.81 (p=0.0022) between the LD₂₀ values (Fig. 3.3), an R² value of 0.88 (p=0.0006) between the LD₅₀ values (Fig. 3.4) and an R² value of 0.76 (p=0.0046) between the LD₈₀ values (Fig. 3.5) obtained due to feeding and intra-haemocoel administration respectively.

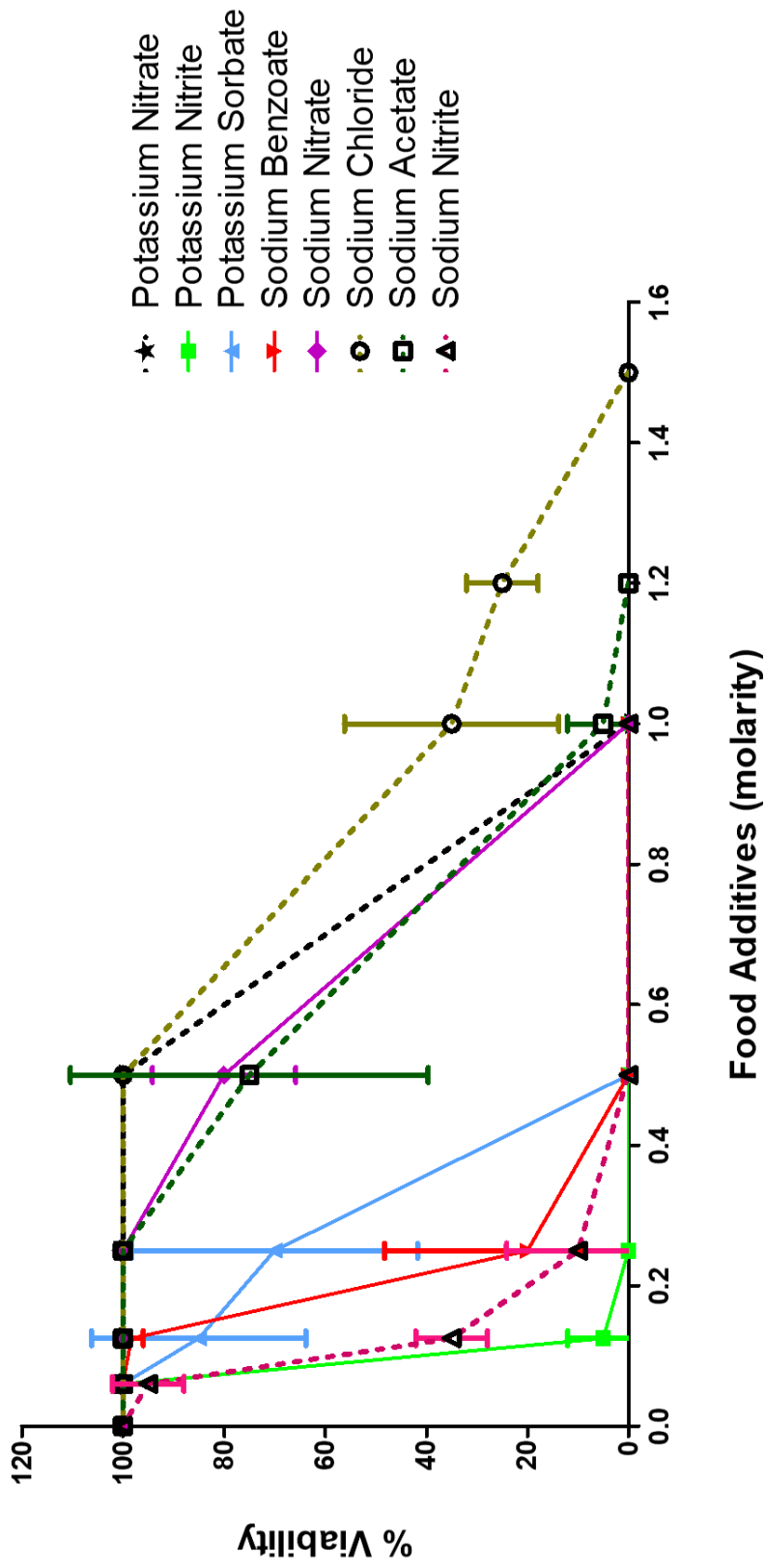


Fig. 3.1 Viability (%) of *Galleria mellonella* larvae following administration of food preservatives by intrahaemocoel injection at 48 hours. All values are the mean \pm SE of three independent determinations.

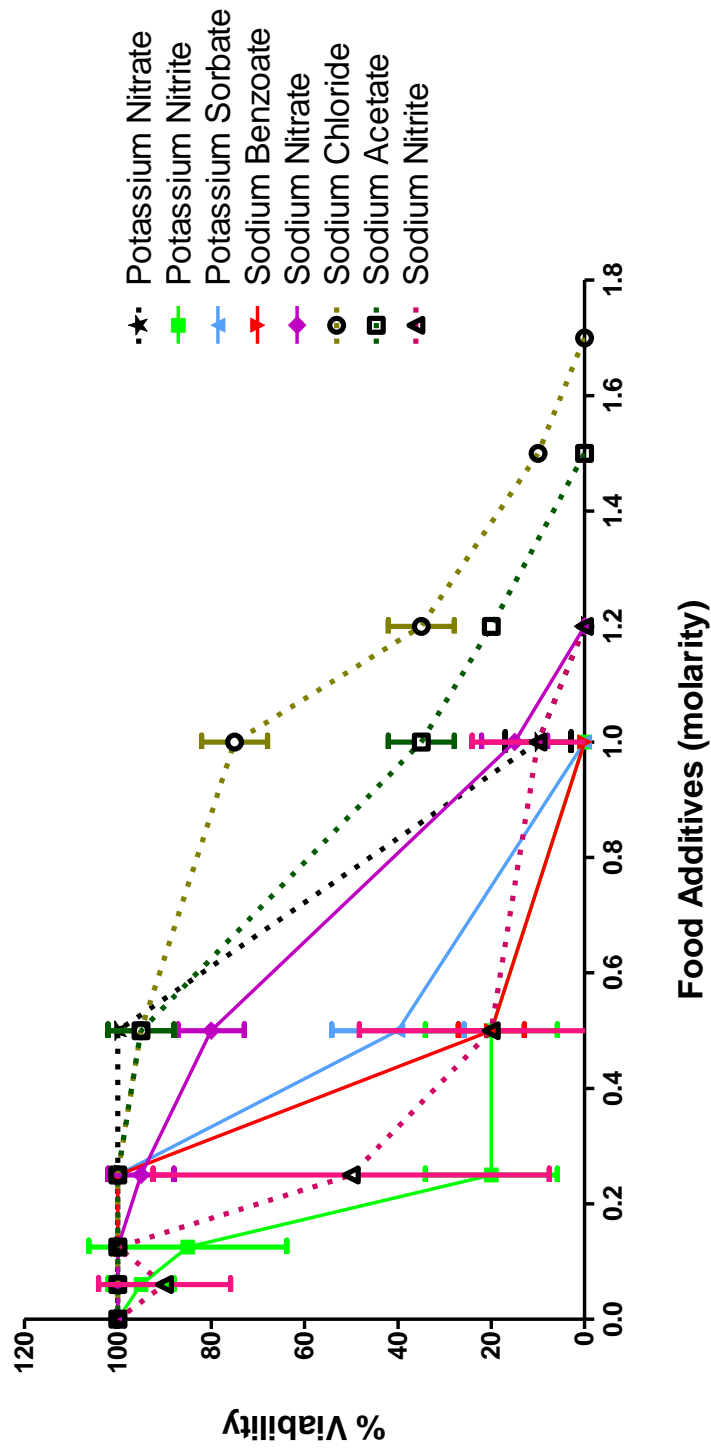


Fig. 3.2 Viability (%) of *Galleria mellonella* larvae following administration of food preservatives by force feeding at 48 hours. All values are the mean \pm SE of three independent determinations.

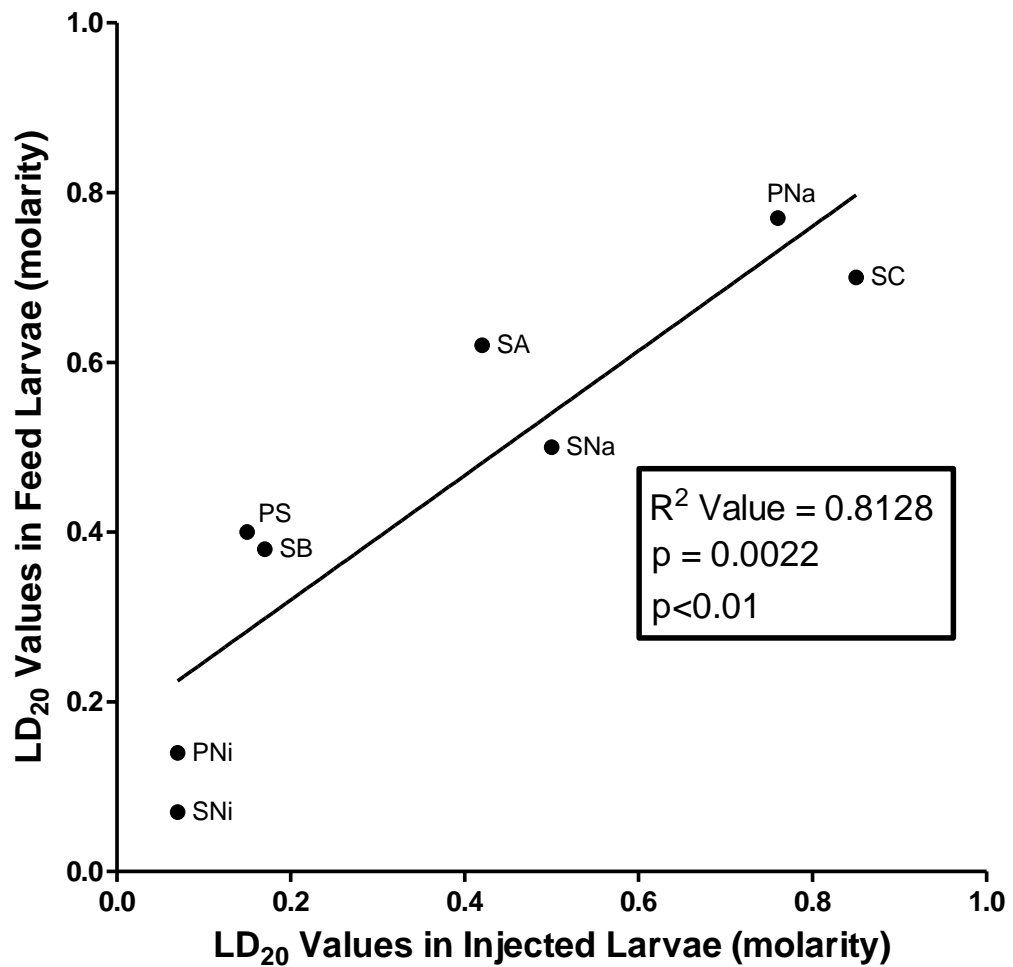


Fig. 3.3 Correlations between the LD₂₀ values of food preservatives when administered to *Galleria mellonella* larvae by feeding or by intra haemocoel injection. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa potassium nitrate, SC sodium chloride.

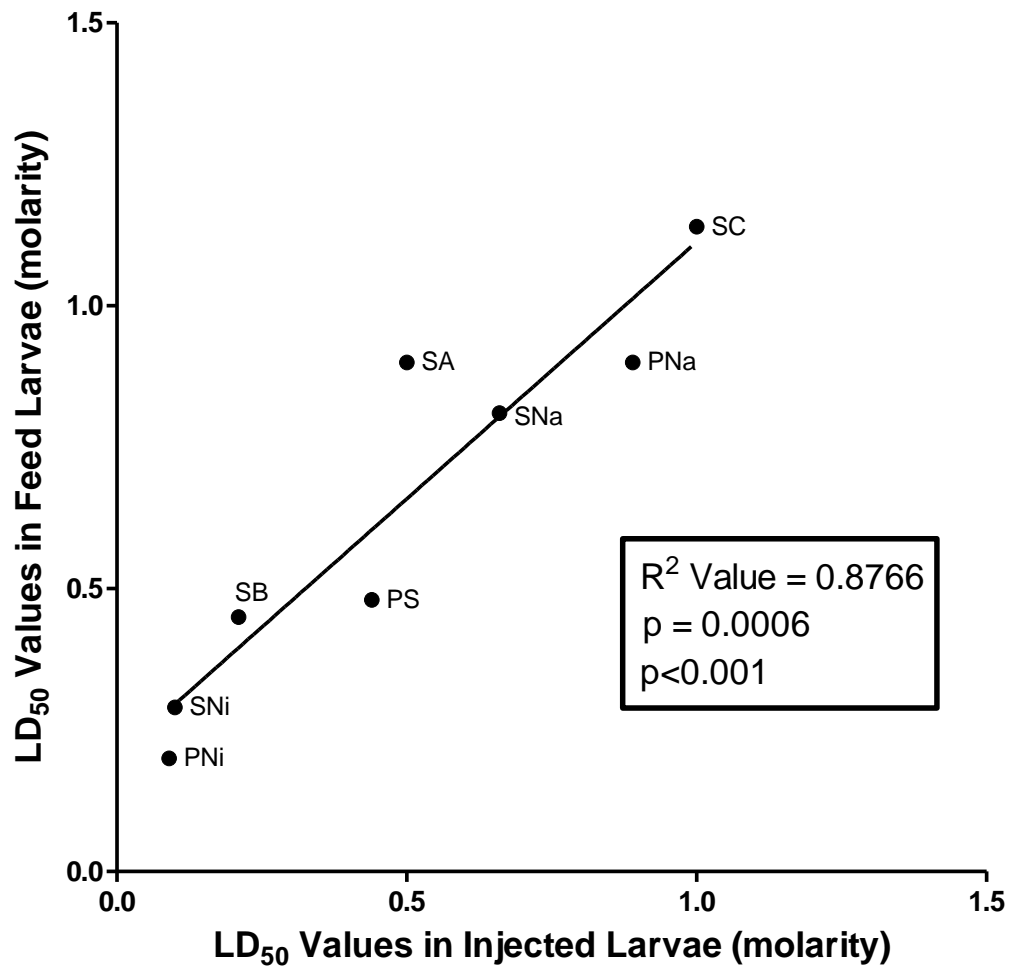


Fig. 3.4 Correlations between the LD₅₀ values of food preservatives when administered to *Galleria mellonella* larvae by feeding or by intra haemocoel injection. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa potassium nitrate, SC sodium chloride.

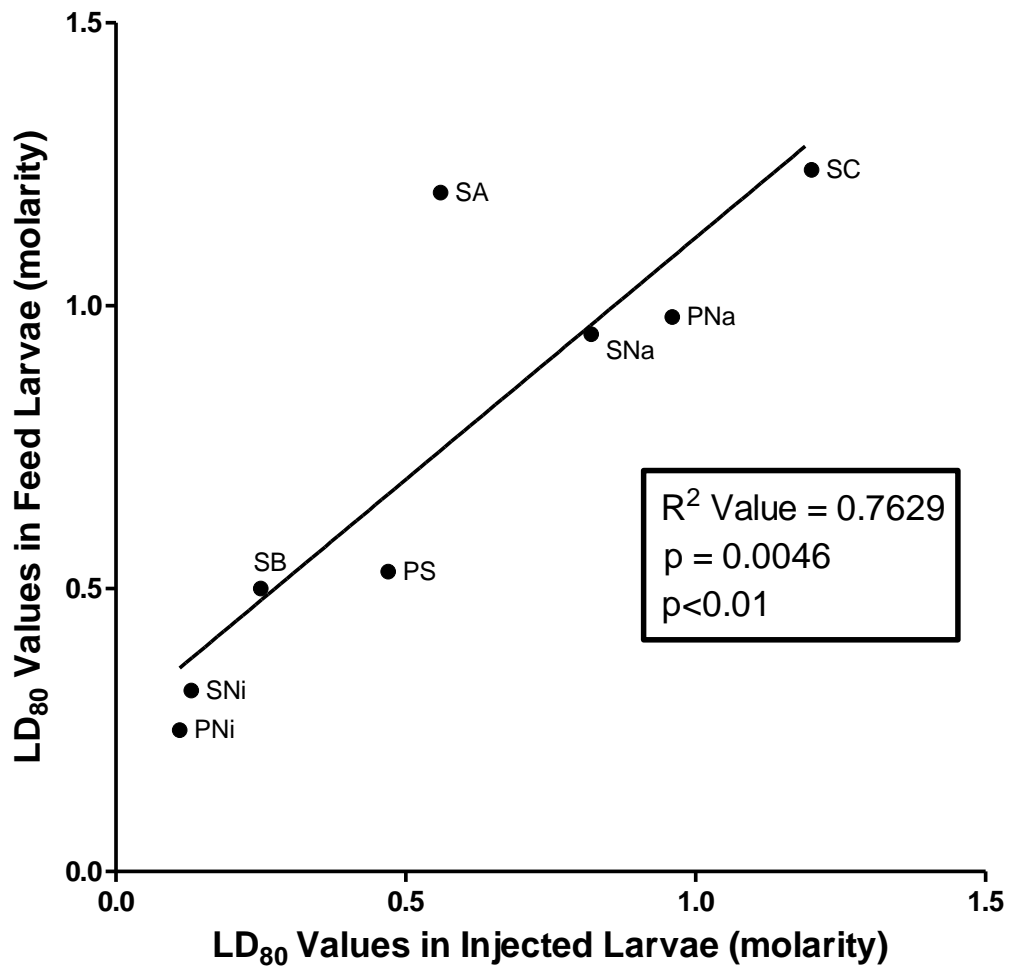


Fig. 3.5 Correlations between the LD₈₀ values of food preservatives when administered to *Galleria mellonella* larvae by feeding or by intra haemocoel injection. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa potassium nitrate, SC sodium chloride.

3.3 Response of HEp-2 cells to food preservative compounds.

HEp-2 cells were exposed to different concentrations of the food preservatives as described, and the effect on growth after 7 days of incubation was assessed using the acid phosphatase assay (Fig. 3.6). Higher concentrations of the compounds reduced growth of the HEp-2 cells. The IC₅₀ values for each compound in HEp-2 cells were determined.

IC₅₀ values for compounds in HEp-2 cells were recorded to be as follows: potassium nitrite 0.01 M, sodium nitrite 0.03 M, sodium benzoate 0.04 M, potassium sorbate 0.03 M, sodium acetate 0.14 M, sodium nitrate 0.11 M, potassium nitrate 0.05 M and sodium chloride 0.185 M.

The relative toxicity of some compounds when tested against HEp-2 cells was similar to the toxicity observed in *G. mellonella*. For example potassium nitrite and sodium nitrite were the two most toxic food preservatives in both model systems, whereas sodium chloride was the least toxic compound in both systems. An R² value of 0.7666 (p = 0.0076) and 0.5032 (p=0.0488) was obtained by plotting the LD₅₀ value for each compound obtained via force-feeding larvae (Fig. 3.7) or by intra-haemocoel (Fig. 3.8) challenge respectively, against the IC₅₀ value obtained using HEp-2 cells.

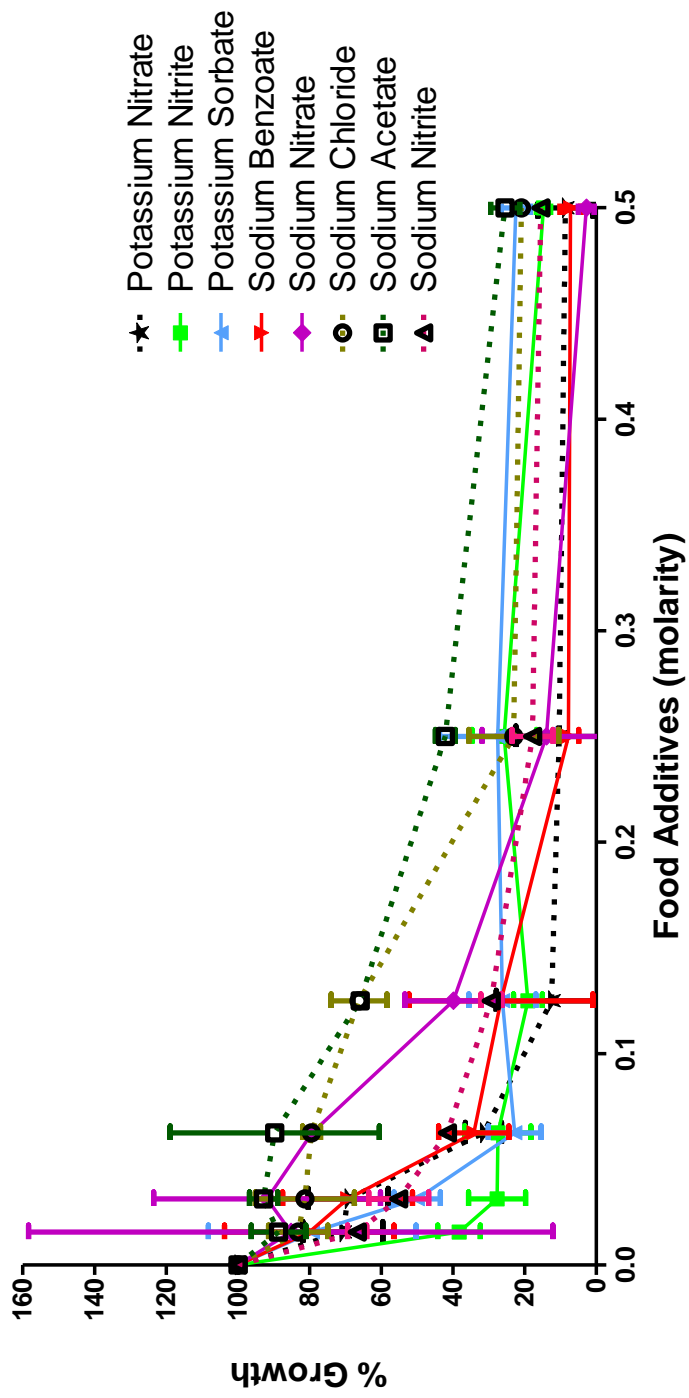


Fig. 3.6 Effect of food preservatives on growth of HEp-2 cells. HEp-2 cells were exposed to different concentrations of food preservatives, and the effect on growth was quantified using an acid phosphatase assay. All values are the mean \pm SE of three independent determinations.

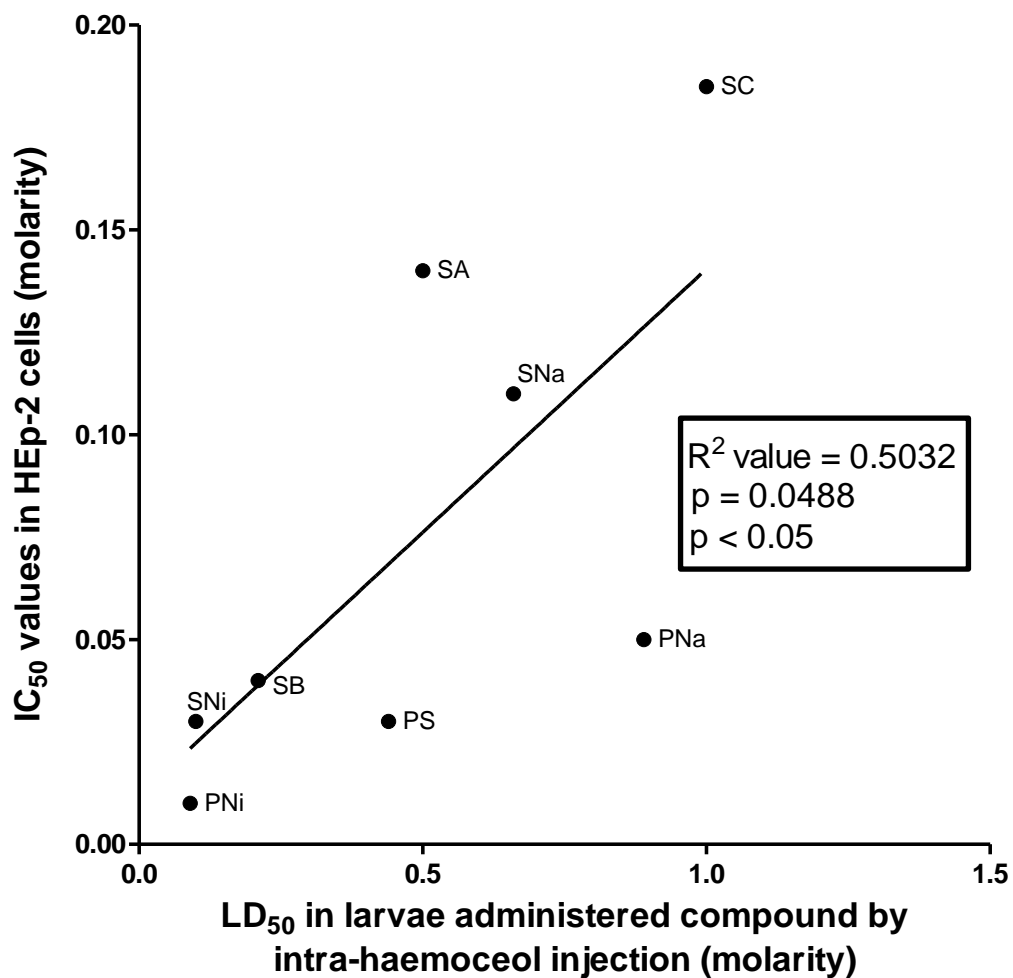


Fig. 3.7 Correlation between the IC₅₀ values of food preservatives in HEp-2 cells and LD₅₀ values of food preservatives when administered by intra-haemocoel injection to *Galleria mellonella* larvae. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa Potassium nitrate, SC sodium chloride.

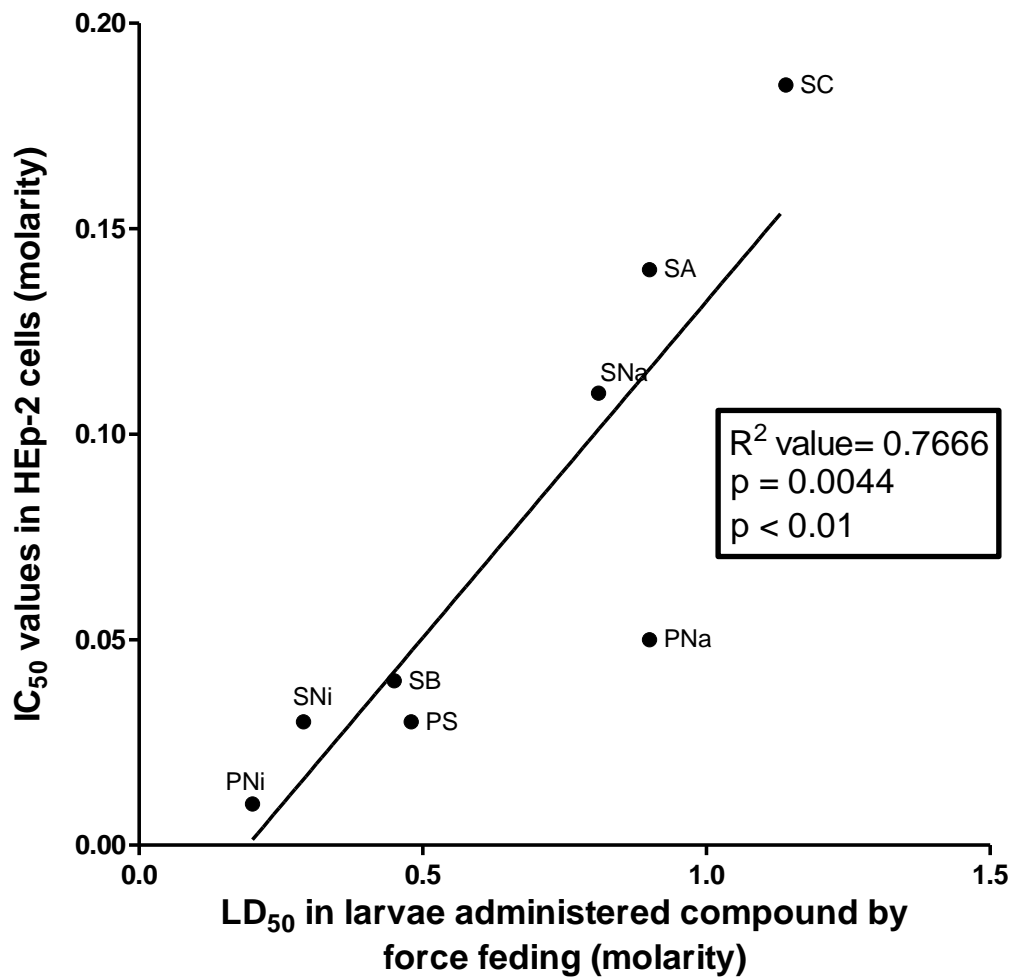


Fig. 3.8 Correlation between the IC₅₀ values of food preservatives in HEp-2 cells and LD₅₀ values of food preservatives when administered by feeding to *Galleria mellonella* larvae. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa potassium nitrate, SC sodium chloride

3.4 Correlation between response of larvae and mammals to food preservative compounds

LD₅₀ values for compounds in rats were obtained in the literature and previously recorded to be as follows: potassium nitrite 200 mg/kg, sodium nitrite 180mg/kg, sodium benzoate 4070 mg/kg, potassium sorbate 4340 mg/kg, sodium acetate 3530 mg/kg, sodium nitrate 1267 mg/kg, potassium nitrate 3750 mg/kg and sodium chloride 3000 mg/kg (Chemistry 2016; Pfizer 2007; Scholar 2009). The LD₅₀ values of each food preservative as determined in rats by feeding was plotted against the corresponding LD₅₀ for the compounds obtained by feeding *G. mellonella* larvae. The resulting graph shows a significant positive correlation between the LD₅₀ values obtained in both systems ($R^2=0.6506$, $p=0.0156$) (Fig. 3.9).

LD₅₀ values for compounds in rabbits were previously recorded to be as follows: potassium nitrite 200 mg/kg, sodium benzoate 2000 mg/kg, sodium nitrate 2680 mg/kg, potassium nitrate 1901, sodium nitrite 186 mg/kg (Chemistry 2016; Pfizer 2007; Scholar 2009). The LD₅₀ values of each food preservative as determined in rabbits by feeding was plotted against the corresponding LD₅₀ for the compounds obtained by feeding *G. mellonella* larvae. The resulting graph shows a non-significant positive correlation between the LD₅₀ values obtained in both systems ($R^2 = 0.7524$, $p = 0.0568$) (Fig. 3.10).

LD₅₀ values for compounds in mice were previously recorded to be as follows: sodium benzoate 1600 mg/kg, sodium nitrite 175 mg/kg, sodium chloride 4000mg/kg, sodium acetate 6891mg/kg (Chemistry 2016; Pfizer 2007; Scholar 2009). The LD₅₀ values of each food preservative as determined in mice by feeding was plotted against the corresponding LD₅₀ for the compounds obtained by feeding *G. mellonella* larvae. The resulting graph shows a non-significant positive correlation between the LD₅₀ values obtained in both systems ($R^2 = 0.7162$, $p = 0.1537$) (Fig. 3.11).

3.5 Summary

The response of larvae to eight commonly used food additives administered by feeding or by intra-haemocoel injection was measured. A significant correlation between the LD₅₀ ($R^2 = 0.8766$, $p = 0.0006$) and LD₈₀ ($R^2 = 0.7629$, $p = 0.0046$) values obtained due to oral or intra- haemocoel administration of compounds was established. The response of HEp-2 cells to the food additives was determined, and a significant correlation ($R^2=0.7217$, $p=0.0076$) between the LD₅₀ values of the compounds administered by feeding in larvae with the IC₅₀ values of the compounds in HEp-2 cells was established. A strong correlation between the LD₅₀ values of the eight food preservatives in *G. mellonella* larvae and rats ($R^2=0.6506$, $p=0.0156$) was demonstrated. A positive correlation between the LD₅₀ values of the 5 food preservatives in *G. mellonella* larvae and rabbits ($R^2 = 0.7524$, $p = 0.0568$) (Fig. 3.10). A positive correlation between the LD₅₀ values of the 4 food preservatives in *G. mellonella* larvae and mice ($R^2 = 0.7162$, $p = 0.1537$) (Fig. 3.11).

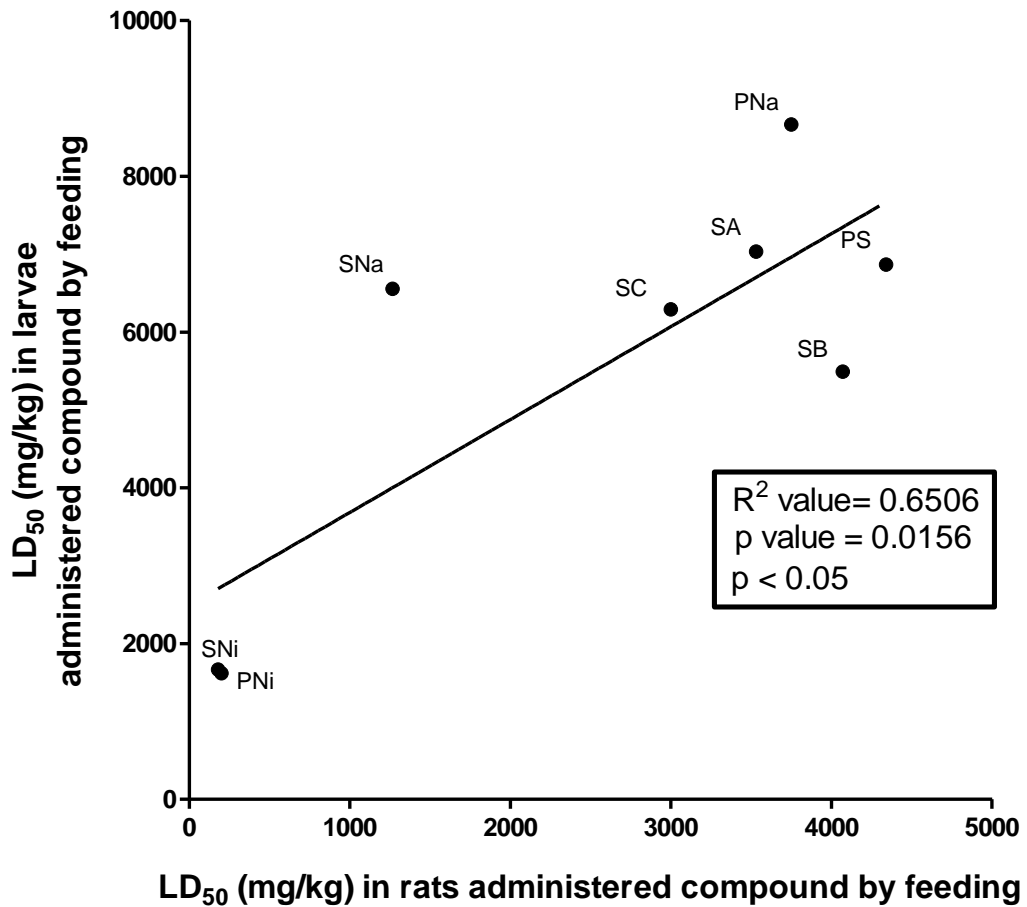


Fig. 3.9 Correlation between LD₅₀ values of eight food preservatives when administered to rats and *Galleria mellonella* larvae by feeding. PNi potassium nitrite, SNi sodium nitrite, SB sodium benzoate, PS potassium sorbate, SA sodium acetate, SNa sodium nitrate, PNa potassium nitrate, SC sodium chloride.

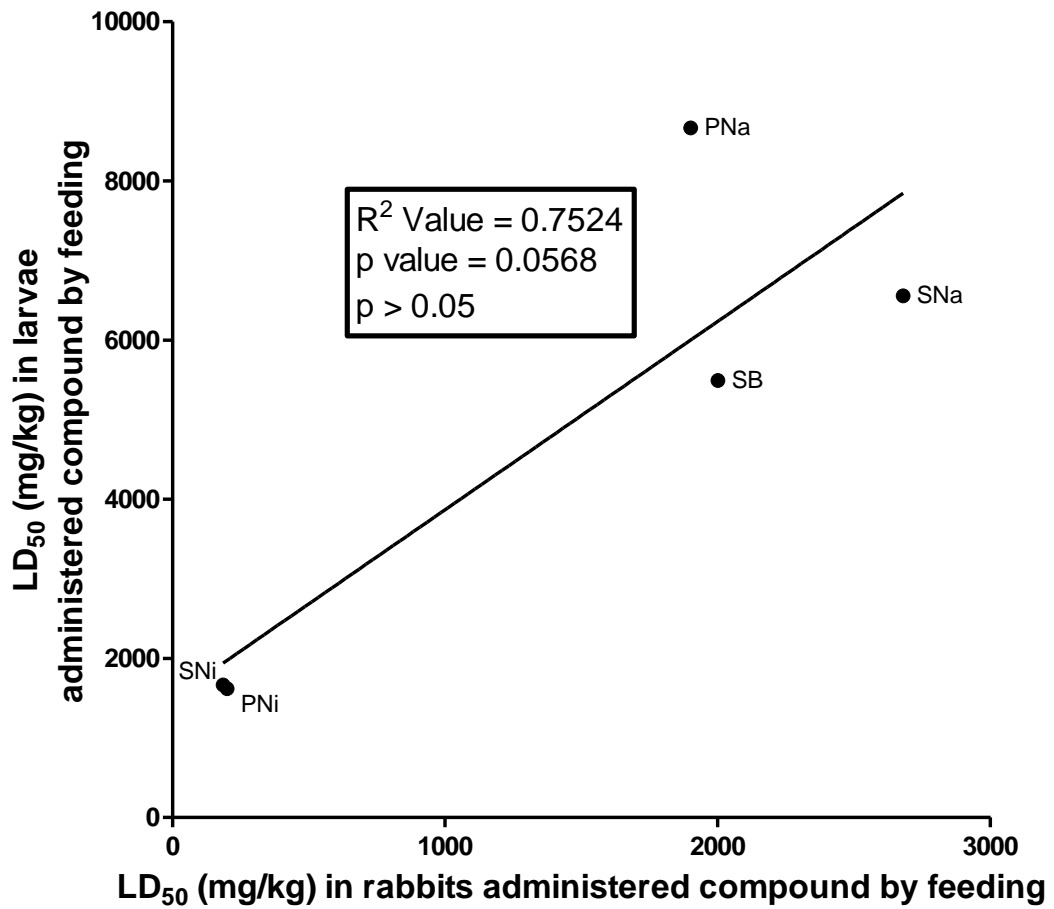


Fig. 3.10 Correlation between LD₅₀ values of five food preservatives when administered to rabbits and *Galleria mellonella* larvae by feeding. PNi potassium nitrite, SNa sodium nitrite, SB sodium benzoate, SNa sodium nitrate, PNa potassium nitrate.

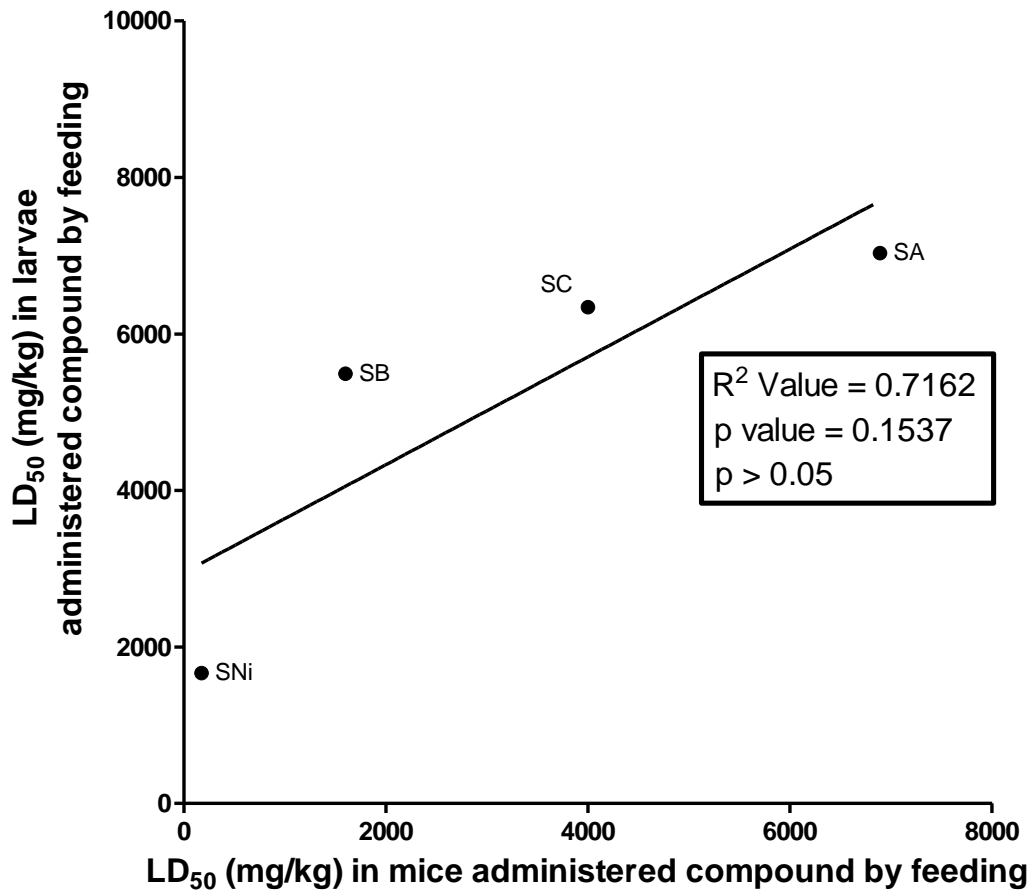


Fig. 3.11 Correlation between LD₅₀ values of four food preservatives when administered to mice and *Galleria mellonella* larvae by feeding. SNI sodium nitrite, SB sodium benzoate, SA sodium acetate, SC sodium chloride.

3.6 The effect of four commonly used food additives on the immune response of *G. mellonella*.

This part of this chapter aimed to determine the effect of 4 commonly used food additives (sodium benzoate, potassium sorbate, sodium nitrate, potassium nitrite) on the immune system of insects. It focused on the effect of food additives on both haemocyte density and haemocyte function.

Insects lack the adaptive immune response present in vertebrates, yet despite this insect possess an effective immune response composed of both the cellular and humoral immune systems. The insect's cellular immune response is mediated by haemocytes which can engulf, encapsulate, or neutralize pathogens (Pech and Strand, 1996; Ratcliffe, 1993). Circulating haemocytes in the haemocoel are the first to the site of infection and their numbers can be supplemented by the release of haemocytes bound to internal organs such as the fat body (Kavanagh and Reeves, 2004). Bergin *et al.* (2005) highlighted that *G. mellonella* haemocytes are able to consume oxygen and produce superoxide mediated by a NADPH oxidase complex that contains proteins homologous to p40^{phox}, p47^{phox}, p67^{phox} and gp91^{phox} of human neutrophils. It has also been shown that haemocyte p40, p47 and p67 proteins translocate from the cytosol to the membrane in a comparable manner to neutrophils (Renwick *et al.*, 2007). Both haemocytes and neutrophils also react in a similar way when challenged with the *Aspergillus fumigatus* toxins, gliotoxin and fumagillin. Nodulation is induced in the absence of microbial agents by saline injection in cockroaches and locusts (Gunnarsson and Lackie, 1985). This would suggest an alternative pathogen-independent mechanism of haemocyte activation, that might be induced by agents such as phospholipids released from or exposed on wounded cells (Scherfer, 2004). The aim of this work was to determine the effects commonly used food additives have on the cellular immune system of insects.

3.7 Effect of PBS administration on haemocyte densities.

Larvae were administered by intra haemocoel injection or force feeding three food additive concentrations corresponding to the LD₂₅, LD₅₀ and LD₈₀ values for sodium nitrate, sodium benzoate, potassium sorbate and potassium nitrite. The

effect of the food additives administration on the density of circulating haemocytes in larval haemolymph was ascertained after 4 and 24 hour incubation at 30°C. For experimental purposes control larvae were administered PBS by intra haemocoel injection or force feeding.

Untouched larvae at $t = 0$ hours showed $0.53 \pm 0.06 \times 10^7$ haemocytes/ml of haemolymph. After 4 and 24 hours control larvae injected with PBS showed $0.79 \pm 0.09 \times 10^7$ and $0.27 \pm 0.012 \times 10^7$ haemocytes/ml of haemolymph respectively. This corresponds to a 0.47 fold increase ($p < 0.05$) after 4 hours and a 0.51 fold decrease ($p < 0.05$) after 24 hours in haemocyte densities.

Larvae force fed with PBS and incubated for 4/24 hours at 30°C showed $0.86 \pm 0.15 \times 10^7$ and $0.99 \pm 0.02 \times 10^7$ haemocytes/ml of haemolymph respectively. This corresponds to a 0.60 fold increase ($p < 0.01$) and a 0.84 fold decrease ($p < 0.001$) when compared to untouched larvae at $t = 0$.

3.8 Effect of sodium benzoate on haemocyte densities in *G. mellonella* larvae.

Larvae were injected with sodium benzoate and incubated for 4 hours. When compared with control PBS injected larvae the administration of sodium benzoate by intra-haemocoel injection induced significant decreases in haemocyte densities (Fig. 3.12). The haemocyte densities of larvae injected with the LD₂₅ and LD₅₀ value of sodium benzoate were $0.6 \pm 0.07 \times 10^7$ and $0.39 \pm 0.02 \times 10^7$ haemocytes/ml. When compared to control injected larvae a 0.24 fold decrease ($p < 0.05$) in LD₂₅ treated larvae and a 0.51 fold decrease ($p < 0.001$) in LD₅₀ treated larvae was revealed. Interestingly the haemocyte density in larvae injected with the LD₈₀ value of sodium benzoate was $0.73 \pm 0.04 \times 10^7$ haemocytes/ml, showed no significant difference with the haemocyte densities shown in control larvae.

Larvae were injected with sodium benzoate and incubate for 24 hours. A significant increase was seen between the relevant PBS injected control group and larvae injected with the corresponding LD₅₀ value of sodium benzoate (Fig. 3.13). Control larvae showed $0.26 \pm 0.01 \times 10^7$ haemocytes/ml while larvae injected the LD₅₀ value of sodium benzoate showed $0.59 \pm 0.09 \times 10^7$ haemocytes/ml, revealing a 1.24 fold increase ($p < 0.01$) in LD₅₀ treated larvae. Larvae injected with the LD₂₅ and LD₈₀

values of sodium benzoate revealed $0.39 \pm 0.02 \times 10^7$ and $0.28 \pm 0.04 \times 10^7$ haemocytes/ml respectively, demonstrating slight increases when compared to PBS injected control larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of sodium benzoate and incubated for 4 hours. The resulting haemocyte densities were compared with control larvae force fed with PBS (Fig. 3.14). The haemocyte densities of larvae injected with the LD₂₅/LD₅₀/LD₈₀ value were $0.84 \pm 0.05 \times 10^7$, $0.9 \pm 0.46 \times 10^7$, $1.08 \pm 0.18 \times 10^7$ haemocytes/ml respectively. Larvae force fed PBS and incubated for 4 hours had $0.86 \pm 0.15 \times 10^7$ haemocytes/ml, showing no significant differences with the densities in sodium benzoate treated larvae.

The increase in incubation time to 24 hours following sodium benzoate feeding reveals significant decreases in haemocyte densities in control larvae vs. treated larvae (Fig. 3.15). Control larvae force fed PBS showed $0.99 \pm 0.02 \times 10^7$ haemocytes/ml. Larvae force fed with the corresponding LD₂₅/LD₅₀/LD₈₀ of sodium benzoate showed $0.83 \pm 0.07 \times 10^7$, $0.83 \pm 0.07 \times 10^7$ and $0.64 \pm 0.05 \times 10^7$ haemocytes/ml respectively. When compared with the control group these values showed a 0.16 fold decrease ($p < 0.05$) for LD₂₅ treated larvae, a 0.16 fold decrease ($p < 0.05$) for LD₅₀ treated larvae and finally a 0.35 fold decrease ($p < 0.001$) for LD₈₀ treated larvae.

3.9 Effect of potassium sorbate on haemocyte densities in *G. mellonella* larvae.

Larvae were administered potassium sorbate by intra-haemocoel injection and incubated for 4 hours. The administration of potassium sorbate induced a significant decrease in haemocyte density when compared to PBS injected controls (Fig. 3.12). The haemocyte density of larvae injected with the LD₅₀ value of potassium sorbate was $0.3 \pm 0.04 \times 10^7$ haemocytes/ml, equating to a 0.62 fold decrease ($p < 0.001$) when compared to control larvae. Surprisingly the haemocyte densities in larvae injected with the LD₂₅ and LD₈₀ value of potassium sorbate were $0.7 \pm 0.02 \times 10^7$ and $0.81 \pm 0.01 \times 10^7$ haemocytes/ml inducing no significant difference with the haemocyte densities shown in control larvae.

The incubation time of larvae injected with potassium sorbate was increased to 24 hours. A significant increase was seen between the relevant control group and larvae injected with the corresponding LD₈₀ value of potassium sorbate (Fig. 3.13). Control larvae showed $0.26 \pm 0.01 \times 10^7$ haemocytes/ml while larvae injected with the LD₈₀ value of potassium sorbate showed $0.93 \pm 0.07 \times 10^7$ haemocytes/ml, which was a 2.52 fold increase ($p < 0.05$). Following 24-hour incubation larvae injected with the LD₂₅ and LD₅₀ values of potassium sorbate showed $0.26 \pm 0.02 \times 10^7$ and $0.36 \pm 0.02 \times 10^7$ haemocytes/ml respectively. These values showed no significant differences when compared with control larvae.

Larvae were force fed potassium sorbate and incubated for 4 hours. A significant increase was evident between the relevant PBS force fed control group and larvae force fed with the corresponding LD₈₀ value of potassium sorbate (Fig. 3.14). Control larvae showed $0.86 \pm 0.15 \times 10^7$ haemocytes/ml while larvae injected with the LD₈₀ value of potassium sorbate showed $1.66 \pm 0.44 \times 10^7$ haemocytes/ml, indicating a 0.94 fold increase ($p < 0.05$) in LD₈₀ treated larvae. In addition, following 4 hours incubation larvae force fed with the LD₂₅ and LD₅₀ values of potassium sorbate revealed $0.83 \pm 0.11 \times 10^7$ and $1.36 \pm 0.09 \times 10^7$ haemocytes/ml respectively. These values show no significant changes when compared with control larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of potassium sorbate and incubated for 24 hours (Fig. 3.15). Haemocyte densities of larvae injected with the LD₂₅/LD₅₀/LD₈₀ values were $1.04 \pm 0.09 \times 10^7$, $1.04 \pm 0.08 \times 10^7$, $0.95 \pm 0.14 \times 10^7$ haemocytes/ml respectively. Control Larvae force fed PBS and incubated for 24 hours revealed $0.99 \pm 0.02 \times 10^7$ haemocytes/ml, showing no significant differences with the densities in potassium sorbate treated larvae.

3.10 Effect of sodium nitrate on haemocyte densities in *G. mellonella* larvae.

Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of sodium nitrate through intra-haemocoel injection and incubated for 4 hours (Fig. 3.12). Haemocyte densities of larvae injected with the LD₂₅/LD₅₀/LD₈₀ value were $0.36 \pm 0.09 \times 10^7$, $0.80 \pm 0.31 \times 10^7$, $0.44 \pm 0.02 \times 10^7$ haemocytes/ml respectively. Control larvae injected

with PBS revealed $0.79 \pm 0.09 \times 10^7$ haemocytes/ ml. No significant differences were recorded between control and sodium nitrate treated groups.

As the incubation time of larvae injected with sodium nitrate increased to 24 hours, significant increases were seen between the relevant PBS injected control group and larvae injected with the corresponding LD₅₀ and LD₈₀ values of sodium nitrate (Fig. 3.13). Control larvae injected with PBS showed $0.26 \pm 0.01 \times 10^7$ haemocytes/ ml while larvae injected with the LD₅₀ and LD₈₀ values of sodium nitrate showed $0.45 \pm 0.04 \times 10^7$ and $0.725 \pm 0.04 \times 10^7$ haemocytes/ml respectively. When compared with the control group the LD₅₀ treated larvae revealed a 0.71 fold increase ($p < 0.05$) and the LD₈₀ treated larvae showed a 1.76 fold increase ($p < 0.001$). Larvae injected with the LD₂₅ value of sodium nitrate showed $0.14 \pm 0.06 \times 10^7$ haemocytes per mil revealing no significant changes when compared with control larvae.

Larvae incubated for 4 hours following the administration of sodium nitrate via force feeding (Fig. 3.14). A significant increase was seen between the relevant PBS force fed control group and larvae force fed with the corresponding LD₈₀ value of sodium nitrate. Control larvae force fed PBS showed $0.86 \pm 0.15 \times 10^7$ haemocytes/ml while larvae injected with the LD₈₀ value of sodium nitrate showed $1.56 \pm 0.32 \times 10^7$ haemocytes/ml. This revealed a 0.83 fold increase ($p < 0.05$) in LD₈₀ treated larvae compared to control larvae. Larvae force fed with the LD₂₅ and LD₅₀ values of sodium nitrate revealed $1.01 \pm 0.27 \times 10^7$ and $1.14 \pm 0.19 \times 10^7$ haemocytes/ml respectively. These values show no significant changes when compared with control larvae.

Following 24 hours incubation the haemocyte densities of larvae force fed with the LD₂₅/LD₅₀/LD₈₀ values were $0.89 \pm 0.44 \times 10^7$, $0.89 \pm 0.44 \times 10^7$, $0.75 \pm 0.04 \times 10^7$ haemocytes/ml respectively. Larvae force fed PBS and incubated for 24 hours revealed $0.99 \pm 0.02 \times 10^7$ haemocytes/ml of haemolymph, showing no significant differences with the densities in sodium nitrate treated larvae (Fig. 3.15).

3.11 Effect of potassium nitrite on haemocyte densities in *G. mellonella* larvae.

Larvae were incubated for 4 hours following the administration of potassium nitrite via intra haemocoel injection (Fig. 3.12). Significant increases were seen between the relevant PBS injected control group and larvae injected with the corresponding LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrite. Control larvae injected with PBS showed $0.7875 \pm 0.09 \times 10^7$ haemocytes/ml while larvae injected with the LD₂₅/LD₅₀/LD₈₀ values of potassium nitrite showed $0.39 \pm 0.02 \times 10^7$, $1.08 \pm 0.02 \times 10^7$ and $0.95 \pm 0.07 \times 10^7$ haemocytes/ml respectively. When compared with control larvae a 0.51 fold decrease ($p < 0.001$) in LD₂₅ treated larvae, 0.37 fold increase ($p < 0.01$) in LD₅₀ treated larvae and a 0.21 fold increase ($p < 0.05$) in LD₈₀ treated larvae was revealed.

As the incubation time of larvae injected with potassium nitrite increased to 24 hours, a significant increase was seen between the relevant PBS injected control group and larvae injected with the corresponding LD₅₀ and LD₈₀ values of potassium nitrite (Fig. 3.13). Control larvae injected with PBS showed $0.26 \pm 0.01 \times 10^7$ haemocytes/ml while larvae injected with the LD₅₀ and LD₈₀ values of potassium nitrite showed $0.7 \pm 0.07 \times 10^7$ and $0.78 \pm 0.07 \times 10^7$ haemocytes/ml respectively. When compared with control larvae a 1.67 fold increase ($p < 0.001$) in LD₅₀ treated larvae, 1.95 fold increase ($p < 0.001$) in LD₈₀ treated larvae was revealed. Larvae injected with the LD₂₅ value of potassium nitrite revealed $0.28 \pm 0.04 \times 10^7$ haemocytes per mil revealing no significant changes when compared with control larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrite and incubated for 4 hours. Haemocyte densities of larvae injected with the LD₂₅/LD₅₀/LD₈₀ values were $0.53 \pm 0.04 \times 10^7$, $1.23 \pm 1.03 \times 10^7$, $1.23 \pm 0.60 \times 10^7$ haemocytes/ml respectively (Fig. 3.14). Larvae force fed PBS had $0.86 \pm 0.15 \times 10^7$ haemocytes/ml, showed no significant differences with the densities in potassium nitrite treated larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrite and incubated for 24 hours (Fig. 3.15). Haemocyte densities of larvae injected with the

LD₂₅/LD₅₀/LD₈₀ values was $0.5 \pm 0.18 \times 10^7$, $0.5 \pm 0.18 \times 10^7$, $1.09 \pm 0.37 \times 10^7$ haemocytes/ml respectively. Control larvae force fed PBS and incubated for 24 hours revealed $0.99 \pm 0.02 \times 10^7$ haemocytes/ml. Slight decreases in haemocyte densities were evident between control larvae and larvae force fed the corresponding LD₂₅ and LD₅₀ values of potassium nitrite, however no significant differences were observed between control groups and potassium nitrite treated groups.

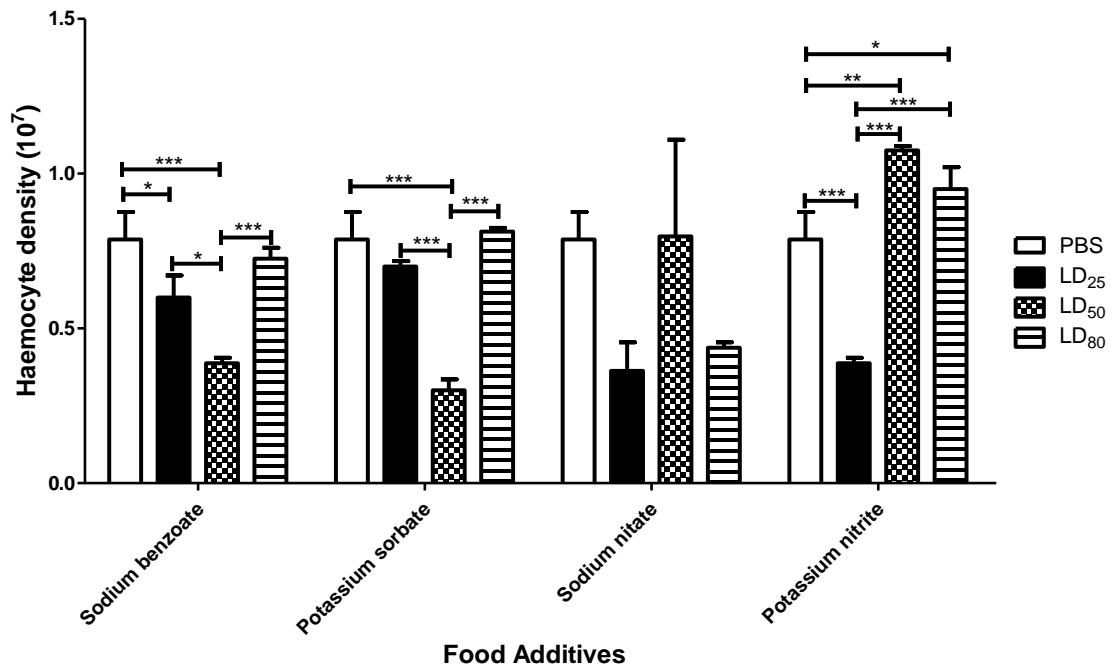


Fig. 3.12 Effect of food additives on haemocyte density in intra-haemocoel injected *G.mellonella* larvae at 4 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of; sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. The haemocyte density was calculated after 4 hours (*: p < 0.05) (**: p < 0.01) (***: p < 0.001). All values are the mean ± SE of 3 independent determinations.

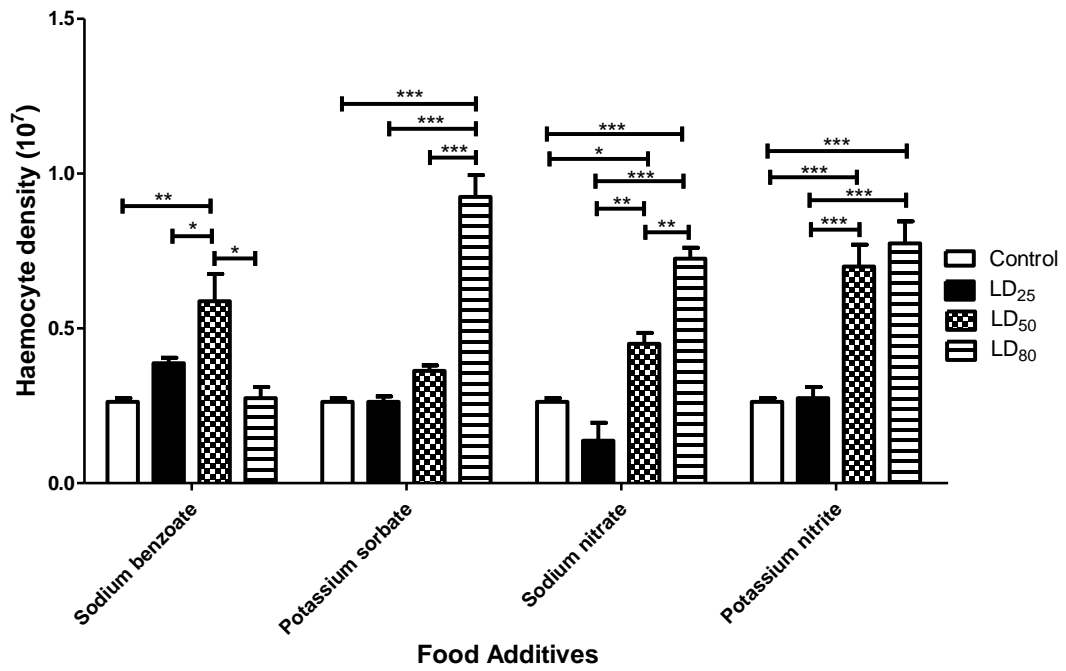


Fig. 3.13 Effect of food additives on haemocyte density in intra-haemocoel injected *G.mellonella* larvae at 24 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of; sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. The haemocyte density was calculated after 24 hours (*: p < 0.05) (**: p < 0.01) (***: p < 0.001). All values are the mean ± SE of 3 independent determinations.

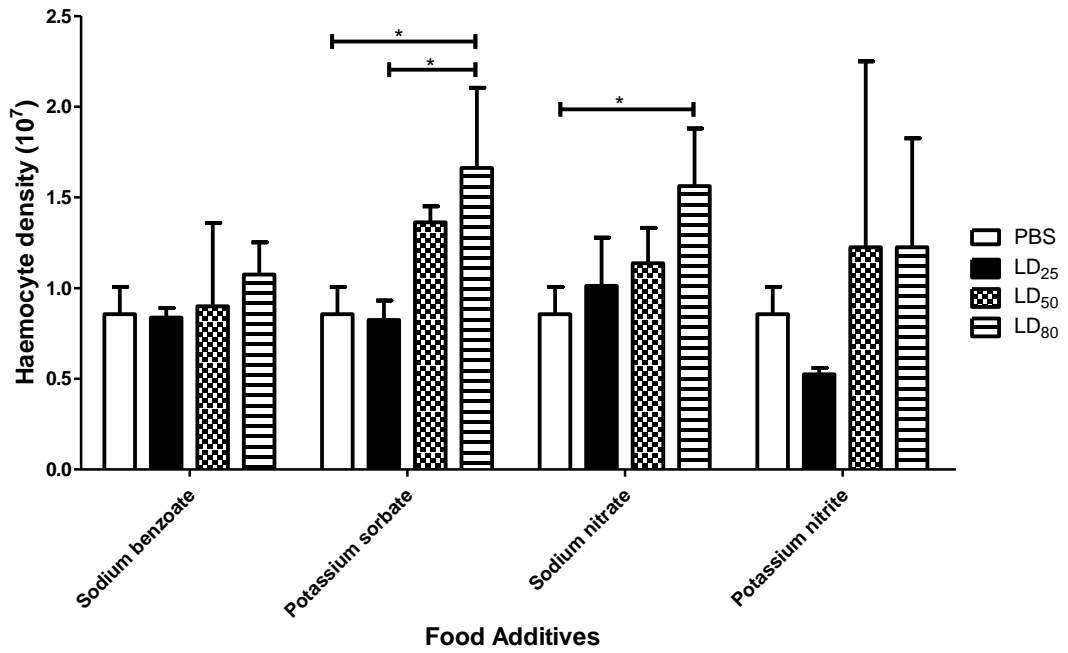


Fig. 3.14 Effect of food additives on haemocyte density in force fed *G.mellonella* larvae at 4 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of; sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. The haemocyte density was calculated after 4 hours (*: p < 0.05). All values are the mean ± SE of 3 independent determinations.

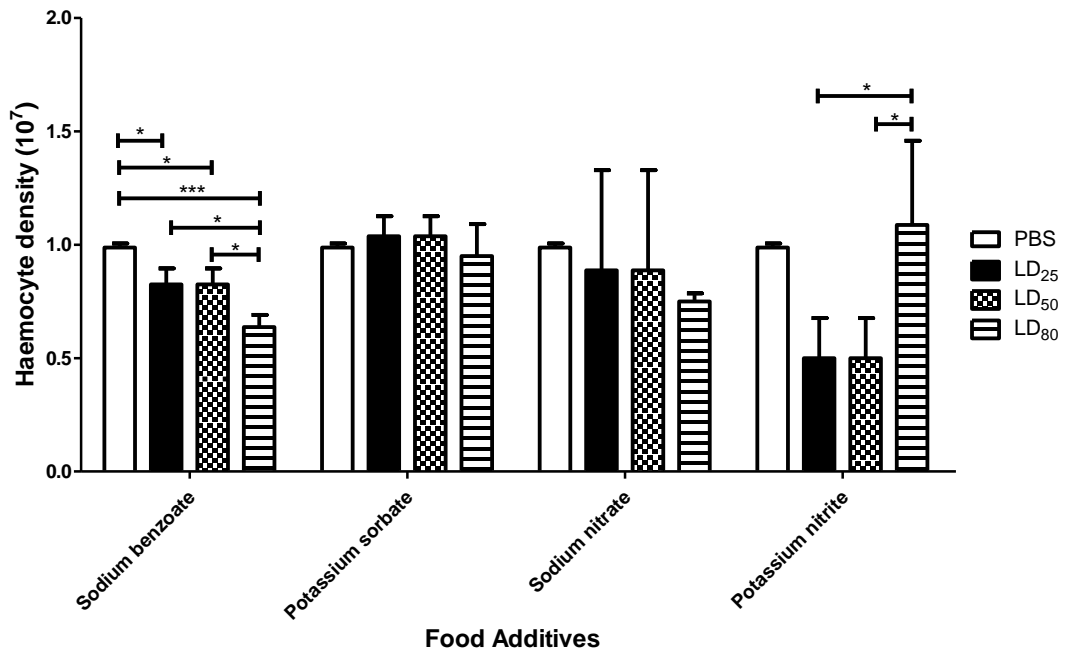


Fig. 3.15 Effect of food additives on haemocyte density in force fed *G.mellonella* larvae at 24 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of; sodium benzoate, potassium Sorbate, sodium Nitrate and potassium Nitrite. The haemocyte density was calculated after 24 hours (*: p < 0.05)(***: p < 0.001). All values are the mean ± SE of 3 independent determinations.

3.12 Analysis of the effect of commonly used food additives on haemocyte mediated pathogen killing.

G. mellonella have been employed in recent times for *in-vivo* studies of immunological pathways and functions that are common to both vertebrates and invertebrates (Kavanagh and Reeves, 2004; Fuchs and Mylonakis, 2006). The aim of this work is to ascertain the killing ability of haemocytes exposed to commonly used food additives and determine any conserved reaction between haemocytes and neutrophils.

3.13 Analysis of the effect of PBS administration on haemocyte mediated pathogen killing.

Larvae were administered by intra haemocoel injection a concentration corresponding to the LD₅₀ values for sodium nitrate, sodium benzoate, potassium sorbate and potassium nitrite. Haemocytes were extracted from control and test larvae following 24-hour incubation at 30°C and mixed with opsonised *C. albicans* for 80 minutes. By counting the percentage viability of *C. albicans* at 20 min intervals, the effect of these food additives on haemocyte mediated pathogen killing was ascertained.

Haemocytes from PBS injected control larvae killed 26 ± 3.4%, 55 ± 3.4% , 65 ± 5.9%, 79 ± 2.9% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. Whereas haemocytes from PBS force fed control larvae killed 21 ± 10.21%, 35 ± 7.94%, 50 ± 4.76%, 80 ± 17% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively.

3.14 Analysis of the effect of sodium benzoate on haemocyte mediated pathogen killing.

Haemolymph from larvae injected with the LD₅₀ value of sodium benzoate showed a significant increase ($p < 0.01$) in haemocyte density when compared with a relevant control group (Fig. 3.13). In addition haemocytes obtained from larvae incubated for 24 hours at 30°C following sodium benzoate injection killed 0 ± 9.68%, 25 ± 9.68% , 21.79 ± 12.36%, 38.47 ± 15.38% of *C. albicans* MEN cells after 20, 40, 60

and 80 minutes *in-vitro* incubation respectively (Fig. 3.16). This amounts to a 0.51 fold decrease ($p < 0.01$) in the killing ability of haemocytes extracted from sodium benzoate injected larvae when compared with the killing ability of haemocytes from PBS injected larvae.

Haemolymph from larvae force fed with the LD₅₀ value of sodium benzoate showed a significant decrease ($p < 0.05$) in haemocyte density when compared with a relevant control group (Fig. 3.15). Haemocytes obtained from larvae incubated for 24 hours at 30°C following sodium benzoate force feeding killed $26.04 \pm 8.09\%$, $47.9 \pm 3.49\%$, $63.5 \pm 7.26\%$, $71.1 \pm 5.71\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.17). These findings did not suggest any significant differences between the killing ability of control larvae force fed PBS and larvae force fed sodium benzoate.

3.15 Analysis of the effect of potassium sorbate on haemocyte mediated pathogen killing.

Haemolymph from larvae injected with the LD₅₀ value of potassium sorbate showed no significant changes in haemocyte density when compared with a relevant control group (Fig. 3.13). Haemocytes obtained from larvae incubated for 24 hours at 30°C following potassium sorbate injection killed $18.18 \pm 3.03\%$, $46 \pm 1.75\%$, $47 \pm 12.15\%$, $56 \pm 7.78\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.16). These findings did not suggest any significant differences between the killing ability of control larvae injected with PBS and larvae administered potassium sorbate by intra haemocoel injection however a slight decrease (0.29 fold) in the killing ability was observed in haemocytes from potassium sorbate injected larvae compared to haemocytes from the relevant control.

Haemolymph from larvae force fed with the LD₅₀ value of potassium sorbate showed no significant changes in haemocyte density when compared with a relevant control group (Fig. 3.15). Haemocytes obtained from larvae incubated for 24 hours at 30°C following potassium sorbate force feeding killed $55.1 \pm 15.45\%$, $50.01 \pm 23.19\%$, $73.64 \pm 6.86\%$, $82.74 \pm 7.87\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.17). These findings did not suggest

any significant differences between the killing ability of control larvae force fed PBS and larvae force fed potassium sorbate.

3.16 Analysis of the effect of sodium nitrate on haemocyte mediated pathogen killing.

Haemolymph from larvae injected with the LD₅₀ value of sodium nitrate showed a significant increase ($p < 0.05$) in haemocyte density when compared with a relevant control group (Fig. 3.13). In addition haemocytes obtained from larvae incubated for 24 hours at 30°C following sodium nitrate injection killed $3.12 \pm 16.38\%$, $22.92 \pm 7.15\%$, $25 \pm 34.10\%$, $46.88 \pm 15.48\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.16). This amounts to a 0.41 fold decrease ($p < 0.05$) in the killing ability of haemocytes extracted from sodium nitrate injected larvae after 80 mins *in-vitro* incubation with *C. albicans* when compared with the relevant control.

Haemolymph from larvae force fed the LD₅₀ value of sodium nitrate showed no significant changes in haemocyte density when compared with a relevant control group (Fig. 3.15). Haemocytes obtained from larvae incubated for 24 hours at 30°C following sodium nitrate force feeding killed $28.25 \pm 18.65\%$, $38 \pm 2.29\%$, $80.16 \pm 1.32\%$, $90.84 \pm 3.97\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.17). These findings did not suggest any significant differences between the killing ability of control larvae force fed PBS and larvae force fed sodium nitrate.

3.17 Analysis of the effect of potassium nitrite on haemocyte mediated pathogen killing.

Haemolymph from larvae injected with the LD₅₀ value of potassium nitrate showed a significant increase ($p < 0.001$) in haemocyte density when compared with a relevant control group (Fig. 3.13). In addition haemocytes obtained from larvae incubated for 24 hours at 30°C following potassium nitrite injection killed $25.72 \pm 3.16\%$, $20.74 \pm 16.99\%$, $52.26 \pm 4.77\%$, $60 \pm 0.56\%$ of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.16). These findings did

not suggest any significant differences between the killing ability of PBS injected control larvae and larvae force fed potassium nitrate.

Haemolymph from larvae force the LD₅₀ value of potassium nitrite showed no significant changes in haemocyte density when compared with a relevant control group (Fig. 3.15). Haemocytes obtained from larvae incubated for 24 hours at 30°C following potassium nitrite force feeding killed 23 ± 16.32%, 34 ± 16.32%, 42 ± 30.53%, 69 ± 2.72% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 3.17). These findings did not suggest any significant differences between the killing ability of control larvae force fed PBS and larvae force fed potassium nitrite.

3.18 Summary.

Larvae were administered the corresponding LD₂₅, LD₅₀ and LD₈₀ values of 4 commonly used food additives and the haemocyte densities in larvae challenged with the food additives were measured.

The administration of food additives had different effects at different concentrations and different incubation times. Generally, when compared to control, administering of food additives by intra-hemocoel injection lead to the significant decrease in haemocyte densities after 4-hour incubation. An exception to this trend was the administration of sodium nitrate which induced no significant differences to control larvae and the administration of a value corresponding to the LD₅₀ and LD₈₀ values of potassium nitrite which revealed significant increases when compared to controls.

Larvae incubated for 24 hours following administration of the food additives by injection revealed numerous significant increases in haemocyte densities when compared with control larvae. The largest increase in haemocyte density was recorded in larvae injected with the corresponding LD₈₀ value of potassium sorbate. The 4-hour incubation of larvae following the force feeding of food additives revealed only two significant increases in haemocyte densities when compared to control.

These increases were in larvae that had received the LD₈₀ value of potassium sorbate and sodium nitrate.

Larvae incubated for 24 hours following administration of the food additives by force feeding revealed numerous significant increases in haemocyte densities when compared with control larvae. An exception to this was the administration of sodium benzoate which revealed significant decreases in haemocyte densities when compared to control.

Haemocytes from larvae administered sodium benzoate and potassium sorbate by intra haemocoel injection revealed significant decreases in their ability to kill *C. albicans* cells when compared with a control. Haemocytes from larvae administered the 4 food additives by force feeding showed no significant changes in their ability to kill *C. albicans* when compared with a control.

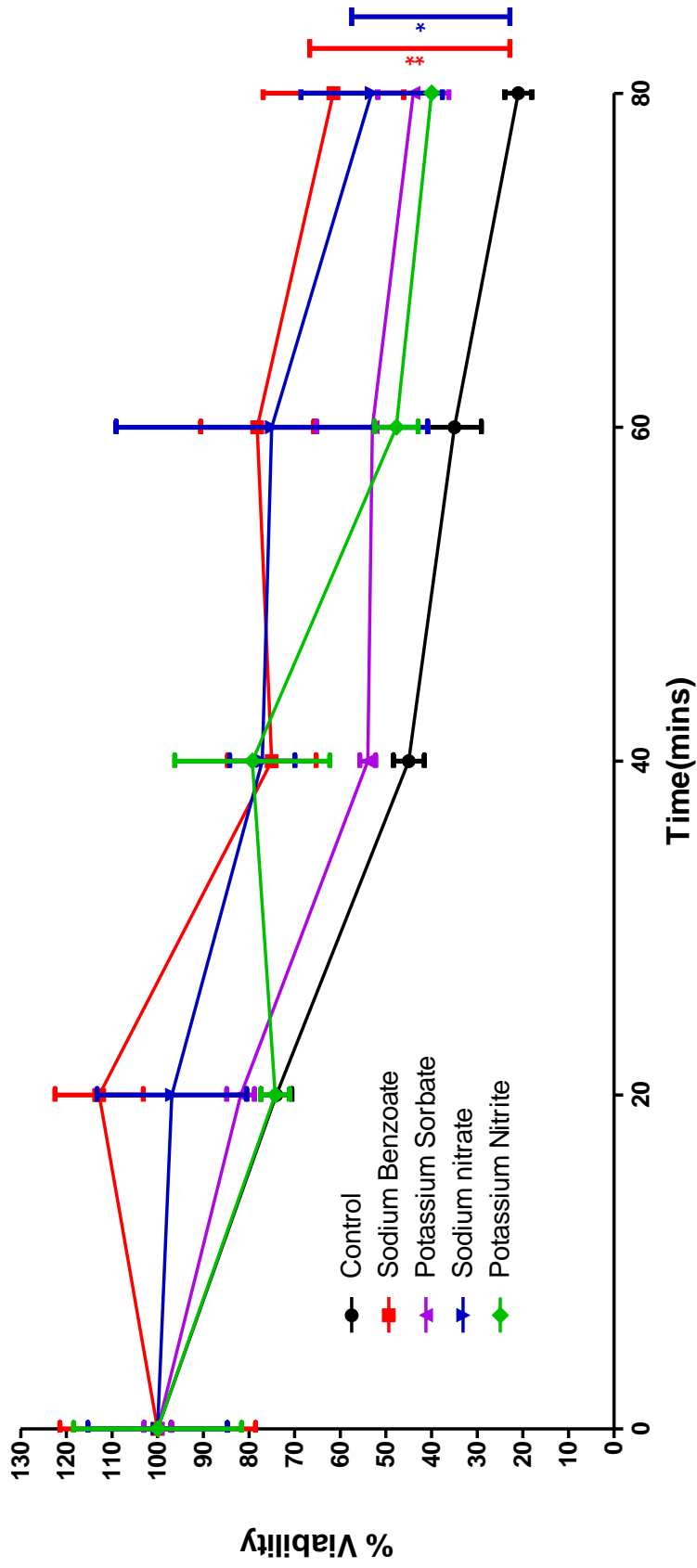


Fig. 3.16 Fungicidal activity of haemocytes from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite via intra-haemocoel injection at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined. All values are the mean \pm SE of 3 independent determinations.

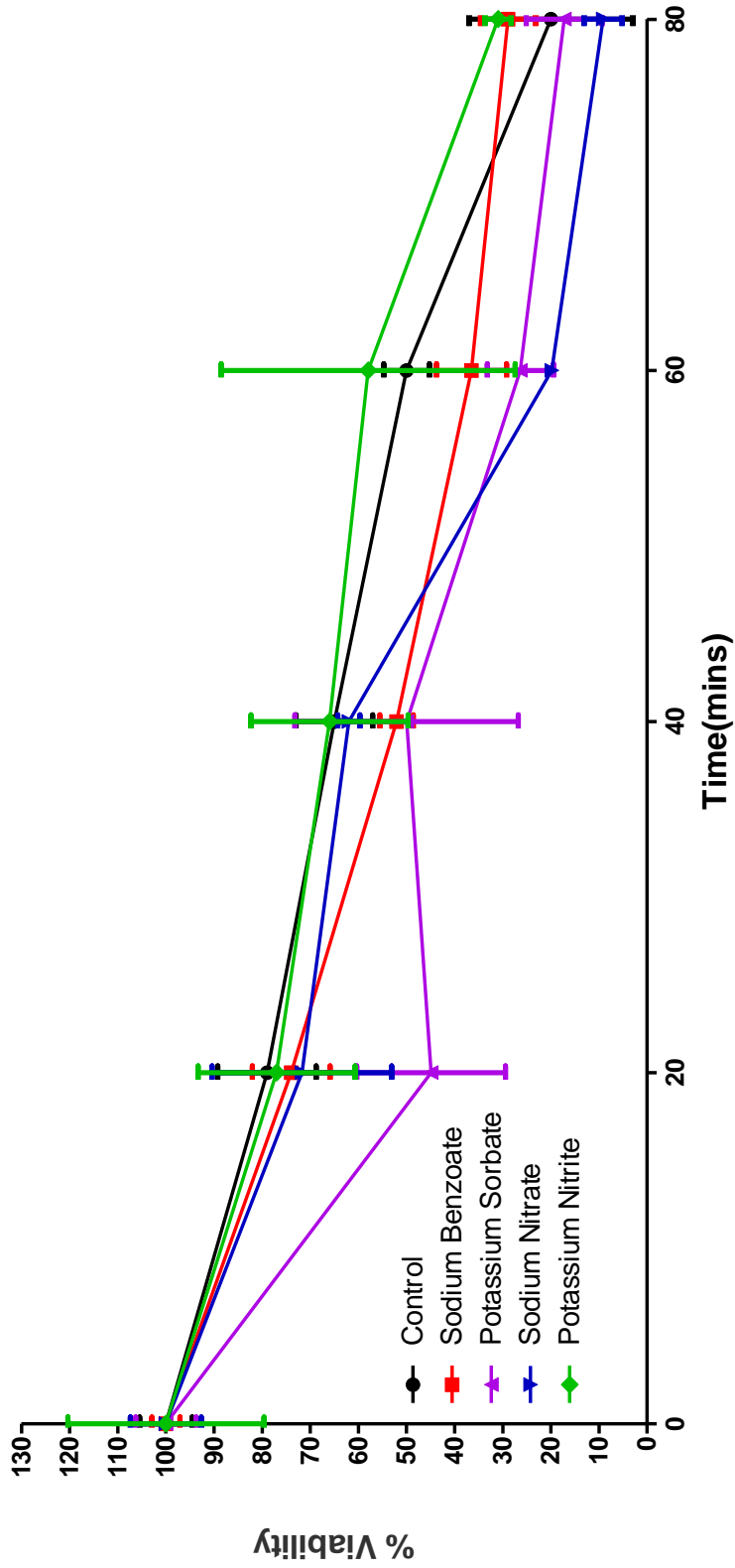


Fig. 3.17 Fungicidal activity of haemocytes from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite via force feeding at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined (*p < 0.05)(**p < 0.01). All values are the mean \pm SE of 3 independent determinations.

3.19 Effects of commonly used food additives on superoxide dismutase activity of the haemolymph.

Superoxide activity in the haemolymph of larvae administered 4 commonly used food additives was measured. Larvae were administered a concentration of food additive by intra-haemocoel injection or force feeding corresponding to its LD₅₀ value. Larvae were incubated at 30°C for 4 and 24 hours post administration of a food additive and the total superoxide dismutase activity was measured in larval haemolymph. Superoxide dismutase activity was quantified as units of activity, 1 unit of superoxide dismutase activity equated to the 50% inhibition of the oxidation of quercetin. The less µg of protein needed for one unit of SOD activity the greater the activity of the antioxidant enzyme *in-vivo*.

One unit of superoxide dismutase activity in PBS injected control larvae equated to 131.24 ± 12.1 µg/µl and 231.135 ± 41.98 µg/µl of haemolymph protein following 4 or 24 hours incubation respectively. This equates to a 0.76 fold increase ($p < 0.05$) in superoxide dismutase activity seen in larvae incubated for 4 hours when compared to larvae incubated for 24 hours.

One unit of superoxide dismutase activity in PBS force fed control larvae equated to 87.4 ± 11.3 µg/µl and 235.46 ± 35.86 µg/µl of haemolymph protein following 4 or 24-hours accordingly. This indicated a 1.69 fold increase ($p < 0.01$) in SOD activity seen in larval incubated for 4 compared to 24 hours.

3.20 Effects of sodium benzoate on superoxide dismutase activity of the haemolymph

Superoxide dismutase activity was measured in haemolymph from larvae administered sodium benzoate via intra haemocoel injection prior to incubation. One unit of superoxide dismutase activity equated to 124.95 ± 27.4 µg/µl and 230.01 ± 2.68 µg/µl of protein at 4 and 24 hours respectively (Fig. 3.18, 3.19).

Superoxide dismutase activity was also measured in haemolymph from larvae administered sodium benzoate via force feeding. At 4 and 24 hours one unit of

superoxide dismutase activity was $90.63 \pm 31.4 \mu\text{g}/\mu\text{l}$ and $199.52 \pm 15.54\mu\text{g}/\mu\text{l}$ of protein (Fig. 3.18, 3.19).

These findings revealed no significant differences between the superoxide dismutase activity in haemolymph extracted from test larvae and control larvae at 4 or 24 hours.

3.21 Effects of potassium sorbate on superoxide dismutase activity of the haemolymph.

A significant increase in superoxide dismutase activity was recorded in the haemolymph of larvae administered potassium sorbate. One unit of superoxide dismutase activity in larvae injected with potassium sorbate equated to $131.92 \pm 29.1 \mu\text{g}/\mu\text{l}$ and $131.16 \pm 6.31 \mu\text{g}/\mu\text{l}$ of protein following 4 and 24 hours incubation respectively (Fig. 3.18, 3.19). When comparing superoxide dismutase activity at 24 hours a 0.43 fold increase ($p < 0.05$) was seen in potassium sorbate injected larvae compared to PBS injected control larvae.

One unit of SOD activity in larvae force fed potassium sorbate equated to $118.43 \pm 16.3 \mu\text{g}/\mu\text{l}$ and $130.3 \pm 28.28 \mu\text{g}/\mu\text{l}$ of protein following 4 or 24 hours incubation respectively. No significant changes in superoxide dismutase activity was noted when comparing the data to relevant control groups (Fig. 3.18, 3.19).

3.22 Effects of sodium nitrate on superoxide dismutase activity of the haemolymph.

Larvae were administered sodium nitrate by intra-haemocoel injection prior to incubation for 4 or 24 hours. In these larvae 1 unit of superoxide dismutase activity was shown to be $124.8 \pm 31.2 \mu\text{g}/\mu\text{l}$ of protein when incubated for 4 hours and $142.28 \pm 21.2132 \mu\text{g}/\mu\text{l}$ of protein when incubated for 24 hours (Fig. 3.18, 3.19).

Haemolymph was collected from larvae force fed the LD₅₀ value of sodium nitrate, 1 unit of superoxide dismutase activity was measured to be $100.75 \pm 19.9 \mu\text{g}/\mu\text{l}$ and $183.1 \pm 15.13 \mu\text{g}/\mu\text{l}$ of protein following 4 and 24 hours respectively (Fig. 3.18, 3.19).

Slight increases in 24-hour superoxide dismutase activity is evident in sodium nitrate injected larvae when compared to PBS injected control larvae. With a

difference in mean values of $88.86 \pm 33.23 \mu\text{g}/\mu\text{l}$ between control and sodium nitrate treated groups. However no significant differences were revealed.

3.23 Effects of potassium nitrite on superoxide dismutase activity of the haemolymph.

Larvae were administered potassium nitrite via direct injection into the haemocoel and incubated for 4 or 24 hours. One unit of superoxide dismutase activity was $109.14 \pm 14.8 \mu\text{g}/\mu\text{l}$ and $149.14 \pm 25.8094 \mu\text{g}/\mu\text{l}$ of protein after 4 and 24 hours respectively (Fig. 3.18, 3.19).

One unit of superoxide dismutase activity in larvae force fed potassium nitrite equated to $102.6 \pm 15.8 \mu\text{g}/\mu\text{l}$ of protein after 4 hours and $156.04 \pm 37.42 \mu\text{g}/\mu\text{l}$ of protein after 24 hours (Fig. 3.18, 3.19).

A slight increase in superoxide dismutase activity at 24 hours was evident in potassium nitrite injected larvae compared to PBS injected control larvae, with an $82.00 \pm 34.85 \mu\text{g}/\mu\text{l}$ difference in protein needed for 1 unit of SOD activity. However, the slight increases observed did not yield any significant changes.

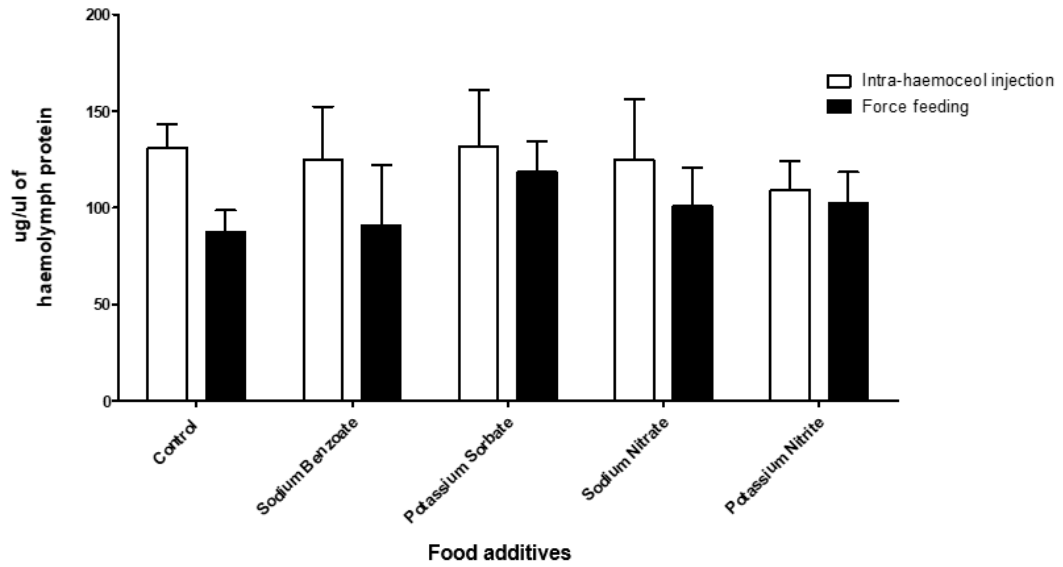


Fig. 3.18 Superoxide dismutase activity of haemolymph from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. After 4 hours haemolymph was extracted from larvae that were administered an LD₅₀ value corresponding to a food additive. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

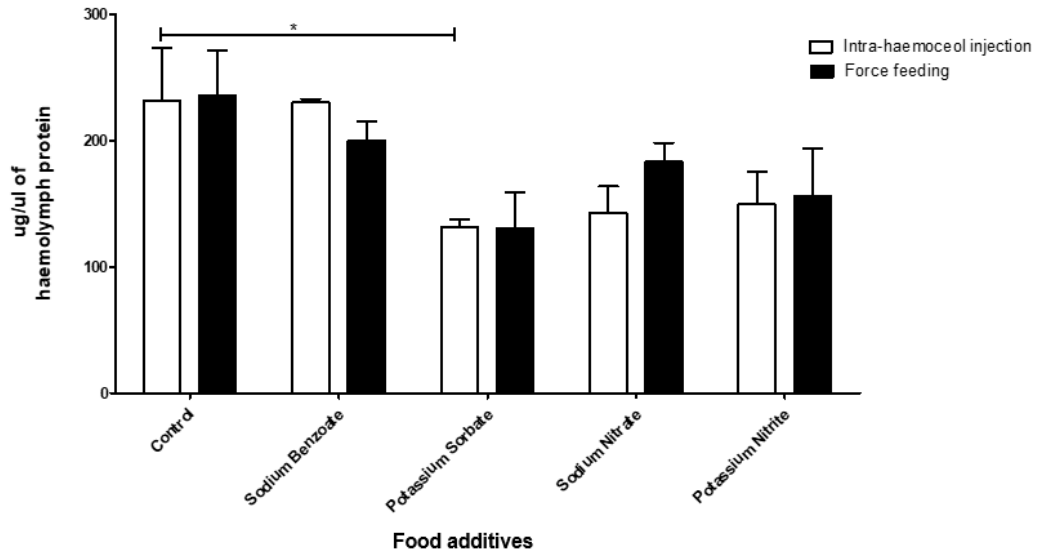


Fig. 3.19 Superoxide dismutase activity of haemolymph from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite via intra-haemocoel injection at 24 hours. Superoxide dismutase activity was determined as described (*: $p < 0.05$). All values are the mean \pm SE of 3 independent determinations (*: $p < 0.05$).

3.24 Effects of commonly used food additives on catalase activity in *G. mellonella* haemolymph.

Larvae were administered by intra haemocoel injection or force feeding a food additive concentration corresponding to the LD₅₀ values for sodium nitrate, sodium benzoate, potassium sorbate and potassium nitrite. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with hydrogen peroxide. The decomposition of hydrogen peroxide and thus rate of catalase activity was measured spectrometrically at 240nm. The rate of catalase activity in larvae administered food additives was expressed as the fold change compared with their relevant control groups administered PBS.

3.25 Effects of sodium benzoate on catalase activity in *G. mellonella* haemolymph.

The catalase activity in haemolymph of larvae challenged by injection or force feeding with sodium benzoate was measured. Haemolymph taken from larvae injected with sodium benzoate and incubated for 24 hours showed a non significant 0.66 ± 0.07 fold increase in catalase activity when compared to haemolymph from a relevant control (Fig. 3.21). However haemolymph extracted from larvae injected with sodium benzoate and incubated for 4 hours showed a significant ($p < 0.001$) 2.68 ± 0.78 fold increase when compared to haemolymph from a relevant control (Fig. 3.20).

Haemolymph from *G. mellonella* larvae force fed sodium benzoate, showed a 0.57 ± 0.37 and a 0.06 ± 0.04 fold increase when compared to relevant controls after 4 and 24 hour incubation respectively.

3.26 Effects of potassium sorbate on catalase activity in *G. mellonella* haemolymph.

Larvae were administered potassium sorbate by direct injection into the haemocoel or force feeding. Larvae injected with potassium sorbate showed a 0.37 ± 0.04 and a 0.18 ± 0.02 fold increase when compared to relevant controls after 4 and 24 hours respectively (Fig. 3.20, 3.21).

Larval force fed potassium sorbate, showed a 0.27 ± 0.31 and a 0.07 ± 0.09 fold increase when compared to relevant controls after 4 and 24 hours incubation

respectively (Fig. 3.20, 3.21). The administration of potassium sorbate by force feeding or direct injection revealed no significant changes in catalase activity between control and test groups.

3.27 Effects of sodium nitrate on catalase activity in *G. mellonella* haemolymph.

The catalase activity in haemolymph of larvae challenged by injection or force feeding with sodium nitrate was measured. Haemolymph taken from larvae injected with sodium nitrate showed a 0.61 ± 0.16 and a 0.04 ± 0.03 fold increase when compared to relevant controls after 4 and 24 hours incubation respectively (Fig. 3.20, 3.21).

Haemolymph from *G. mellonella* larvae force fed sodium nitrate, showed a 0.21 ± 0.13 and a 0.01 ± 0.06 fold increase when compared to relevant controls after 4 and 24 hours incubation respectively (Fig. 3.20, 3.21). The administration of sodium nitrate by either of the two routes of administration described above did not reveal any significant changes in catalase activity between control and test groups.

3.28 Effects of potassium nitrite on catalase activity in *G. mellonella* haemolymph.

G. mellonella larvae were administered the LD₅₀ value of potassium nitrite by direct injection into the haemocoel or by force feeding and the catalase activity in larval haemolymph was measured. Catalase activity showed fold changes of 0.30 ± 0.11 and a 0.75 ± 0.19 when compared with relevant controls following 4 and 24 hours incubation respectively (Fig. 3.20, 3.21).

Potassium nitrite force fed larvae showed a 0.26 ± 0.18 and a 0.03 ± 0.001 fold increase when compared to relevant controls after 4 and 24 hours incubation respectively (Fig. 3.20, 3.21). The findings presented here show no significant differences in catalase activity between control and potassium nitrite treated larvae.

3.29 Summary.

Larvae were administered 4 commonly used food additives and the effect on enzymatic activity in the haemolymph was measured.

The administration of the food additives to larvae by force feeding revealed no significant changes in superoxide dismutase and catalase activity when compared to the relevant controls.

Following 4 hours incubation administration of sodium benzoate to larvae by force feeding revealed a significant increase in catalase activity when compared to a relevant control.

After 24 hours incubation larvae administered potassium sorbate by intra haemocoel injection revealed a significant increase in superoxide dismutase activity when compared to control larvae.

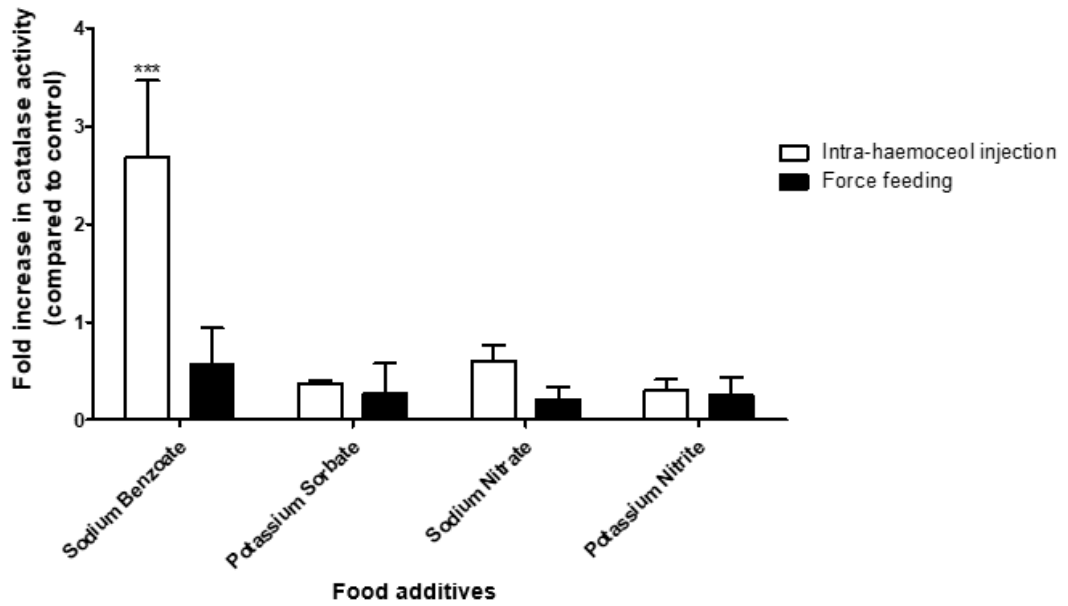


Fig. 3.20 Catalase activity of haemolymph from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. After 4 hours haemolymph was extracted from larvae that were administered an LD₅₀ value corresponding to a food additive. Catalase activity was determined as described (***:p < 0.001). All values are the mean ± SE of 3 independent determinations.

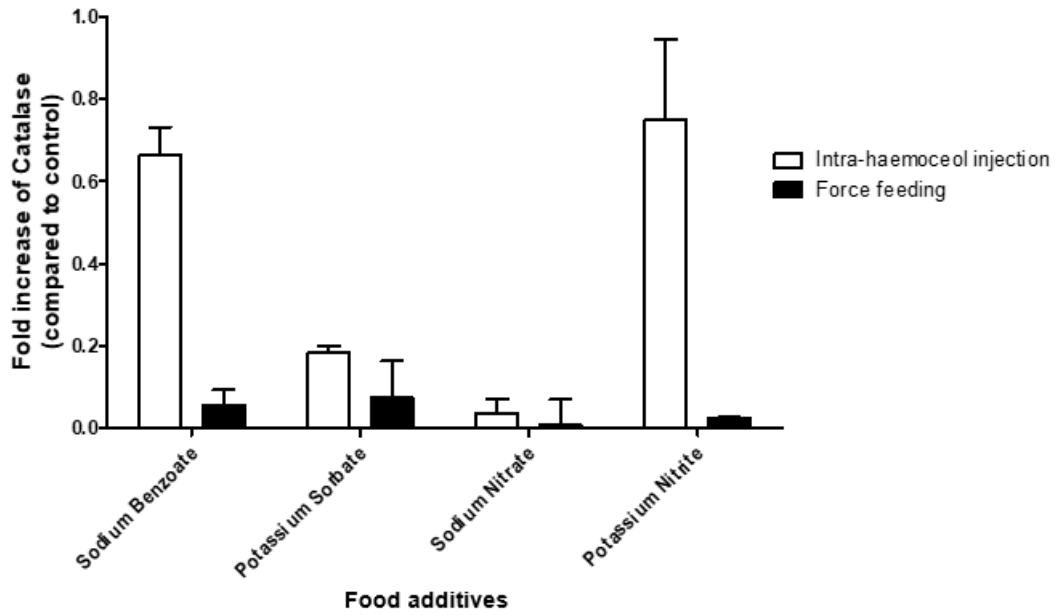


Fig. 3.21 Catalase activity of haemolymph from larvae administered sodium benzoate, potassium sorbate, sodium nitrate and potassium nitrite. After 24 hours haemolymph was extracted from larvae that were administered an LD₅₀ value corresponding to a food additive. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

3.30 Discussion.

The many structural and functional similarities between the immune response of insects and the innate immune system of mammals (Browne *et al.*, 2013) have been exploited to allow the use of insects as *in-vivo* models for screening microbial pathogens and for measuring the efficacy of anti-microbial drugs (Browne *et al.*, 2014). The similarities between the insect and the mammalian gastrointestinal and hepatic systems could also be exploited to allow the use of insects as models for measuring the relative toxicity of food preservatives and other food additives. *D. melanogaster* has been utilized to evaluate the toxicity of a wide range of products including pesticides (Arain *et al.*, 2014), solvents (Soos and Szabad, 2014) and nanoparticles (Carmona *et al.*, 2015). The *Drosophila* wing spot test is a well-established method for measuring the genotoxicity of compounds (Graf and Singer, 1992) and has been employed to measure the genotoxicity of four food preservatives (sodium nitrite, sodium nitrate, potassium nitrite and potassium nitrate) (Sarıkaya and Cakir, 2005). The red flour beetle (*Tribolium castaneum*) has been utilized to investigate the effect of the food contaminant acrylamide on fitness and survival (Grunwald *et al.*, 2013) and demonstrated a correlation with results obtained using rats (Wang *et al.*, 2010).

In the work presented here, *G. mellonella* larvae were administered doses of food preservative by force-feeding or by intra-haemocoel injection. The results demonstrated that the compounds were toxic irrespective of the route of administration, but the toxicity of the compounds were greatest when administered by direct injection into the haemocoel. The difference in the relative toxicity observed between the routes of administration may be explained by the structure of the insect digestive system. The insect foregut and hindgut are covered by cuticle, and the midgut epithelium is protected by the peritrophic membrane. Both the cuticle and the peritrophic membrane retard the entry of ingested microbes into the haemocoel (Vallet-Gely *et al.*, 2008), and these structures may limit the entry of the food preservatives administered by the feeding route into the haemocoel and therefore reduce the relative toxicity.

The relative toxicity (LD_{50}) of the compounds in larvae is virtually identical by the different routes of administration. The only discrepancy is the relative toxicity of sodium nitrate and sodium acetate in larvae administered the compounds by feeding or by intra- haemocoel challenge. Strong positive correlations between the LD_{20} (Fig. 3.3), LD_{50} (Fig. 3.4) and LD_{80} (Fig. 3.5) values for each compound obtained by the different administration routes in larvae were established.

HEp-2 cells are widely used for screening the *in-vivo* activity of anti-cancer drugs (Rossi *et al.*, 2003) and have previously been employed to measure the relative toxicity of food additives (Stefanidou *et al.* 2003). The effect of the compounds on the growth of HEp-2 cells was determined, and a significant correlation between the effect of the compounds on the growth of HEp-2 cells and the response of larvae to the compounds was established (Fig. 3.7, 3.8). A positive correlation ($R^2=0.6506$, $p=0.0156$) between the LD_{50} values obtained for the preservatives in rats and in *G. mellonella* larvae administered the compounds by feeding was also established (Fig. 3.9).

A positive correlation between the LD_{50} values of 5 food preservatives (potassium nitrite, sodium nitrite, potassium nitrate, sodium benzoate and sodium nitrate) in *G. mellonella* larvae and rabbits ($R^2 = 0.7524$, $p = 0.0568$) was established (Fig. 3.10). A positive correlation between the LD_{50} values of 4 food preservatives (sodium nitrite, sodium benzoate, sodium chloride and sodium acetate) in *G. mellonella* larvae and mice ($R^2 = 0.7162$, $p = 0.1537$) was demonstrated (Fig. 3.11). The two positive correlations observed were not deemed significant. With a greater availability of mammalian data a more significant correlation may be observed.

Larvae were administered by intra haemocoel injection or force feeding three food additive concentrations corresponding to the LD_{25} , LD_{50} and LD_{80} values for sodium nitrate, sodium benzoate, potassium sorbate & potassium nitrite. The effect of food additive administration on haemocyte densities was ascertained. Control larvae administered PBS via intra haemocoel injection after 4 hours incubation showed significant increases ($p < 0.05$) when compared to untouched larvae at $t = 0$ hours (Section 3.6). While control larvae administered PBS via intra haemocoel injection after 24 hours incubation showed significant decreases ($p <$

0.05) when compared to untouched larvae at $t = 0$ hours (Section 3.6). In cockroaches and locusts nodulation is induced in the absence of microbial agents by saline injection (Gunnarsson and Lackie, 1985). This might suggest why a significant increase in haemocyte densities is seen in control larvae after 4 hours. The significant decrease in haemocyte densities after 24 hours may be explained as a drop in haemocyte densities following a short peak.

Significant increases were seen in the haemocyte densities of control larvae that were administered PBS via force feeding after 4 ($p < 0.01$) and 24 ($p < 0.001$) hours incubation compared to untouched larvae (Section 3.6). Haemocytes are found circulating freely in the haemolymph or adhering to internal organs such as the fat body or the digestive tract of the insect and can be rapidly mobilised upon breach of the cuticle or entry of a pathogen (Browne *et al.*, 2013). In the process of force feeding a blunted needle is inserted into the mouth and down the gastrointestinal tract. The insertion of the needle into the gastrointestinal tract and the force of the PBS being expelled from the needle may result in the dislodgment of gastrointestinal attached haemocytes into the haemolymph thus explaining this increase.

Different concentrations of xenobiotics can have different biological effects (Schulz *et al.*, 2012) and this is evident in the haemocyte densities of larvae challenged with a food additive. Larvae were administered food additives by intra-haemocoel injection or force feeding and incubated for 4 and 24 hours. Significant reductions in haemocyte densities were observed in larvae that received a food additive. For example larvae that were injected with the LD_{25} and LD_{50} values of sodium benzoate showed significant reductions ($p < 0.05$) at 4 hours in their haemocyte densities when compared to a relevant control (Fig. 3.12). This may be due to the paralysation of the insect directly after the injection of a food additive. This paralysation in the insect could be preventing it from mounting a cellular immune response. Significant increases in haemocyte densities were also observed in larvae that received a food additive. For example significant increases ($p < 0.001$) were seen in the haemocyte densities of larvae injected with the LD_{50} and LD_{80} values of potassium nitrite following 4 hours incubation (Fig. 3.12). The increase in haemocyte densities in the absence of a pathogen would suggest an alternative

pathogen-independent mechanism of haemocyte activation, that might be induced by agents such as phospholipids released from or exposed on wounded cells (Schherfer, 2004)

In general there were less significant changes observed in the haemocyte densities of larvae that received a food additive by force feeding compared to intra-haemocoel injection. Perhaps this may also be explained by peritrophic membrane of the midgut retarding the entry of the food additives into the haemocoel. By doing this it could reduce the number of significant changes in haemocyte densities.

Larvae were administered by intra haemocoel injection or force feeding a food additive concentration corresponding to the LD₅₀ values for sodium nitrate, sodium benzoate, potassium sorbate and potassium nitrite. Following 24 hours incubation the effect of these food additives on haemocyte mediated pathogen killing was ascertained. Haemocytes from larvae force fed the food additives showed no significant decreases in their killing ability when compared with control larvae (Fig. 3.17). Perhaps this was also due to the peritrophic membranes ability to retard the entry of compounds from the midgut into the haemocoel. However there was a significant reduction in the fungicidal ability of haemocytes extracted from larvae injected with sodium benzoate and sodium nitrate (Fig. 3.16). Sodium benzoate has been shown to have a deleterious effect on the mammalian neutrophil, the food additive was shown to have a significantly effect on the oxidative respiratory burst and phagocytosis of isolated human neutrophils (Bano *et al.*, 2014). Human neutrophils and insect haemocytes display many similarities (Browne *et al.*, 2013) including the ability to produce superoxide by a functional NADPH oxidase complex (Bergin *et al.*, 2005). Haemocytes from larvae injected with sodium benzoate may also display a reduced ability to kill *C. albicans* cells due to a negative effect on their oxidative respiratory burst and ability to phagocytose caused by sodium benzoate. It has been demonstrated that sodium nitrate inhibits the formation of reactive oxygen species by activated murine neutrophils and macrophages (Deriagina *et al.*, 2003) and inhibition of reactive oxygen formation may be due to nitric oxide interference with the membrane component of the NADPH oxidase complex (Clancy *et al.*, 1992). Perhaps the significant reduction in the fungicidal ability of haemocytes from larvae

injected with sodium benzoate is also due to this nitric oxide interference with NADPH oxidase complex.

Various chemical (pesticides, drugs, metals, abnormal oxygen concentration, etc.), physical (radiation, temperature, noise, vibration) and physiological (diseases, injury, aging, inflammation) stressors can cause a stress situation that may result in oxidative stress (Kodrík *et al.*, 2015). Antioxidant enzymes such as superoxide dismutase, catalase, glutathione transferase, and glutathione reductase are highly conserved between mammals and insects (Felton and Summers, 1995) making *G. mellonella* a promising model organism of oxidative stress. Catalase and superoxidase dismutase are two enzymes that are believed to act in tandem and efficiently terminate oxygen radical reactions in insects (Stanic *et al.*, 2004). Larvae were administered a food additive by intra-haemocoel injection and force feeding and the activity of superoxide dismutase and catalase was measured in larval haemolymph. Control larvae were administered PBS by intra-hemocoel injection or force feeding and incubated for 4 and 24 hours. The superoxide dismutase activity in PBS injected and fed control larvae incubated for 24 hours was significantly reduced compared to PBS injected and fed control larvae incubated for 4 hours. Larvae are stored at 15°C prior to use and incubated at 30°C when in use (Section 3.19). The reduction in superoxide dismutase activity as time progresses from 4 hours incubation to 24 hours might be explained by the normalisation of superoxide activity in larvae after being stored at the colder temperature of 15°C. Colder temperatures are associated with altered metabolic activity. Altered metabolic activity can lead to oxidative stress and increases in superoxide dismutase activity (Stanic *et al.*, 2004). This could perhaps explain the significant decrease in superoxide activity in control larvae incubated for 24 hours at 30°C.

A significant increase in catalase activity was seen in larvae injected with sodium benzoate following 4 hours incubation. A significant increase in superoxide dismutase activity was seen in larvae injected with potassium sorbate following 24 hours incubation. Both benzoates and Sorbates can have a disruptive effect on membrane structure (Stratford *et al.*, 2013). This disruption can have an effect on mitochondria function, causing a decreased electron flow from substrate

dehydrogenases to ubiquinone, resulting in the leakage of free electrons from the respiratory chain. These free electrons can then combine with molecular oxygen resulting in the production of superoxide (Piper and Piper, 2017). The increase seen in anti-oxidant enzymes in larvae exposed to sodium benzoate and potassium sorbate might also be due to the affect these weak acids have on mitochondria function. It must be also noted that the administration of the organic nitrate nitroglycerin has been reported to cause the increased production of reactive oxygen species by mitochondria in rat aorta (Sydow *et al.*, 2004). However no significant increases in antioxidant enzymes were observed as a response to sodium nitrate administration in larvae.

The results presented here indicate that larvae of *G. mellonella* may be a useful model system for assessing the relative *in-vivo* toxicity of food preservative agents and generate results that show a strong correlation to those that can be obtained using a cultured cell line and mammals. While an alternative system such as *G. mellonella* larvae will never replace the need to use mammals for evaluating the relative toxicity of food preservatives, their use may lead to a reduction in the number of mammals required for such testing and enables the rapid testing of the toxicity of novel food preservatives

Chapter 4

Analysis of the acute response of *Galleria mellonella* larvae to potassium nitrate

4.1 Analysis of the acute response of *Galleria mellonella* larvae to potassium nitrate.

Having previously determined the LD₂₀/LD₅₀/LD₈₀ values of potassium nitrate in *G. mellonella* larvae (Section. 3.2) and shown significant correlations between the results obtained in larvae and those from pre-existing models of food additive toxicity, the aim of the work presented here was to characterize the acute effect of potassium nitrate on *G. mellonella* larvae in order to establish similarities with effects in mammals.

Potassium nitrate (KNO₃) is widely used as fertilizer in agriculture and, due to its oxidative properties, in many industrial processes. Potassium nitrate is found in drinking water, vegetables and, as E252, is widely utilized in the food industry as a preservative in the curing of meat (Sebranek and Bacus, 2007). However, high doses of nitrates and nitrites (and their metabolic products) are carcinogenic in humans due to the formation of nitrosamines (Camargo *et al.*, 2005). Exposure of HEp-2 cells to nitrates results in increased expression of genes associated with a stress response, cell cycle control and DNA repair (Bharadwaj *et al.*, 2005). The acute toxicity of potassium nitrate was measured in juvenile blue swimmer crabs, histopathological changes to the anterior gill lamellae were observed, including lamellae swelling, epithelial thickening, pillar cell disruption, necrosis, and distortion (Romano and Zeng, 2007). Epidemiological studies have suggested that high nitrate levels in drinking water are associated with conditions such as teratogenicity, thyroid hypersensitivity and childhood diabetes (Bharadwaj *et al.*, 2005). The main toxic effect of chronic nitrate is due to the conversion of haemoglobin to methaemoglobin which leads to methaemoglobinemia (Van Dijk *et al.*, 1983). Patients administered nitrates display enhanced nitric oxide bioavailability in the vasculature, vasodilation effects, and inhibition of platelet aggregation (Lonberg *et al.*, 2008). Administration of potassium nitrate (0.06–0.35 mmol kg⁻¹ day⁻¹) reduces diastolic and systolic blood pressure (Lonberg *et al.*, 2008). Sodium nitrate also reduces blood pressure but leads to a reduction in oxygen consumption and increased blood flow (Lonberg *et al.*, 2008).

The development of an insect based screening system for measuring the relative toxicity of preservatives, such as potassium nitrate, could contribute to reducing the need to use mammals for this type of testing.

4.2 Effect of potassium nitrate administration on haemocyte densities.

Larvae were administered by intra haemocoel injection or force feeding potassium nitrate concentrations corresponding to the LD₂₅, LD₅₀ and LD₈₀ values. The effect of potassium nitrate administration on the density of circulating haemocytes in larval haemolymph was ascertained after 4 and 24-hour incubation at 30°C. For experimental purposes control larvae were administered PBS by intra haemocoel injection or force feeding.

Untouched larvae at t = 0 hours showed $0.53 \pm 0.06 \times 10^7$ haemocytes/ml of haemolymph. After 4 and 24 hours control larvae administered PBS via intra-haemocoel injection showed $0.79 \pm 0.09 \times 10^7$ and $0.27 \pm 0.01 \times 10^7$ haemocytes/ml respectively. This corresponds to a 0.47 fold increase ($p < 0.05$) after 4 hours and a 0.51 fold decrease ($p < 0.05$) after 24 hours in haemocyte densities. Larvae force fed with PBS and incubated for 4/24 hours at 30°C showed $0.86 \pm 0.15 \times 10^7$ and $0.99 \pm 0.02 \times 10^7$ haemocytes in 1 ml of haemolymph respectively. This corresponds to a 0.60 fold increase ($p < 0.01$) and a 0.84 fold decrease ($p < 0.001$) when compared to untouched larvae at t = 0 hours (Fig. 4.1).

Larvae were incubated for 4 hours following the administration of potassium nitrate via intra haemocoel injection (Fig. 4.1). Significant changes were seen between the relevant PBS injected control group and larvae injected with the corresponding LD₂₅ and LD₅₀ values of potassium nitrate. Control larvae injected with PBS showed $0.79 \pm 0.09 \times 10^7$ haemocytes/ml while larvae injected with the LD₂₅/LD₅₀/LD₈₀ values of potassium nitrite showed $0.2 \pm 0.04 \times 10^7$, $0.94 \pm 0.016 \times 10^7$ and $0.68 \pm 0.04 \times 10^7$ haemocytes/ml respectively. When compared with control larvae a 0.75 fold decrease in haemocyte densities ($p < 0.001$) in LD₂₅ treated larvae, 0.19 fold increase ($p < 0.05$) in LD₅₀ treated larvae and a 0.14 fold decrease in LD₈₀ treated larvae was observed.

As the incubation time of larvae injected with potassium nitrate increased to 24 hours, a significant increase was seen between the relevant PBS injected control group and larvae injected with the corresponding LD₅₀ and LD₈₀ values of potassium nitrate (Fig. 4.1). Control larvae injected with PBS showed $0.26 \pm 0.01 \times 10^7$

haemocytes/ml while larvae injected with the LD₅₀ and LD₈₀ values of potassium nitrate showed $0.78 \pm 0.07 \times 10^7$ and $0.93 \pm 0.07 \times 10^7$ haemocytes/ml respectively. When compared with the control group the LD₅₀ treated larvae revealed a 1.95 fold increase ($p < 0.01$) and the LD₈₀ treated larvae showed a 2.5 fold increase ($p < 0.01$). Larvae injected with the LD₂₅ value of potassium nitrate showed $0.34 \pm 0.06 \times 10^7$ haemocytes/ml revealing no significant changes when compared with control larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrate and incubated for 4 hours. The resulting haemocyte densities were compared with control larvae force fed with PBS (Fig. 4.2). The haemocyte densities of larvae force with the LD₂₅/ LD₅₀/ LD₈₀ value were $1.41 \pm 0.93 \times 10^7$, $1.36 \pm 0.16 \times 10^7$, $1.18 \pm 0.04 \times 10^7$ haemocytes/ml respectively. Larvae force fed PBS and incubated for 4 hours revealed $0.86 \pm 0.15 \times 10^7$ haemocytes/ml, showing no significant differences with the densities in potassium nitrate treated larvae.

Larvae were force fed the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrate and incubated for 24 hours (Fig. 4.2). Haemocyte densities of larvae injected with the LD₂₅/ LD₅₀/ LD₈₀ values were $1.09 \pm 0.27 \times 10^7$, $1.09 \pm 0.27 \times 10^7$, $1.01 \pm 0.55 \times 10^7$ haemocytes/ ml respectively. Control Larvae force fed PBS and incubated for 24 hours revealed $0.99 \pm 0.02 \times 10^7$ haemocytes/ ml, showing no significant differences with the densities in potassium nitrate treated larvae.

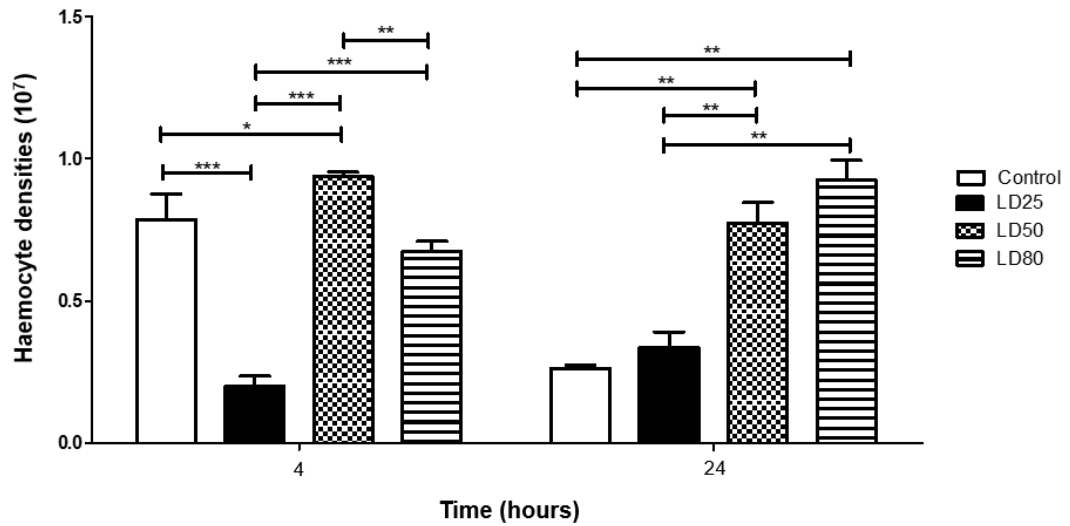


Fig.4.1 Effect of potassium nitrate on haemocyte density in intra-haemocoel injected *G.mellonella* larvae at 4 and 24 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrite. (*: p < 0.05) (**: p < 0.01) (***: p < 0.001). All values are the mean ± SE of 3 independent determinations.

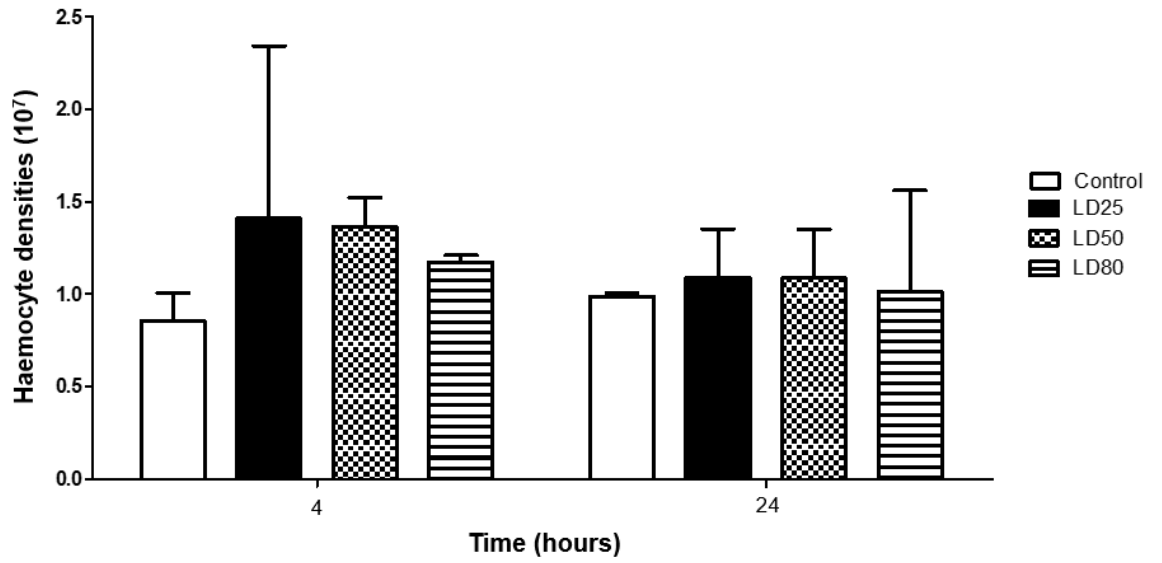


Fig. 4.2 Effect of potassium nitrate on haemocyte density in force fed *G.mellonella* larvae at 4 and 24 hours. Larvae were administered the LD₂₅, LD₅₀ and LD₈₀ values of potassium nitrite. All values are the mean \pm SE of 3 independent determinations.

4.3 Analysis of the effect of potassium nitrate administration on haemocyte mediated pathogen killing.

Larvae were administered by intra haemocoel injection a potassium nitrate concentration corresponding to the LD₅₀ value of potassium nitrate. Haemocytes were extracted from control and test larvae following 24-hour incubation at 30°C and mixed with *C. albicans* for 80 minutes. By counting the percentage viability of *C. albicans* at 20-minute intervals, the effect of potassium nitrate on haemocyte mediated pathogen killing was ascertained.

Haemocytes from PBS injected control larvae killed 26 ± 3.4%, 55 ± 3.4% , 65 ± 5.9%, 79 ± 2.9% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. Whereas haemocytes from PBS force fed control larvae killed 21 ± 10.21%, 35 ± 7.94% , 50 ± 4.76%, 80 ± 17% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively.

Haemolymph from larvae injected with the LD₅₀ value of potassium nitrate showed a significant increase ($p < 0.01$) in haemocyte density when compared with a relevant control group (Fig. 4.1). In addition haemocytes extracted from larvae incubated for 24 hours at 30°C following potassium nitrate injection killed 9.07 ± 10.96% , 39 ± 23.86%, 30.69 ± 17.15% and 40 ± 3.94%, *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 4.3). This amounts to a 0.49 fold decrease ($p < 0.01$) in the killing ability of haemocytes extracted from potassium nitrate injected larvae when compared with the killing ability of haemocytes from PBS injected larvae.

Haemolymph from larvae force with LD₅₀ value of potassium nitrate showed no significant changes in haemocyte density when compared with a relevant control group (Fig. 4.2). Haemocytes obtained from larvae incubated for 24 hours at 30°C following potassium nitrate force feeding killed 23 ± 8.82%, 30 ± 14.46% , 42 ± 19.22%, 70 ± 2.21% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes *in-vitro* incubation respectively (Fig. 4.3). These findings did not suggest any significant differences between the killing ability of control larvae force fed PBS and larvae force fed potassium nitrate.

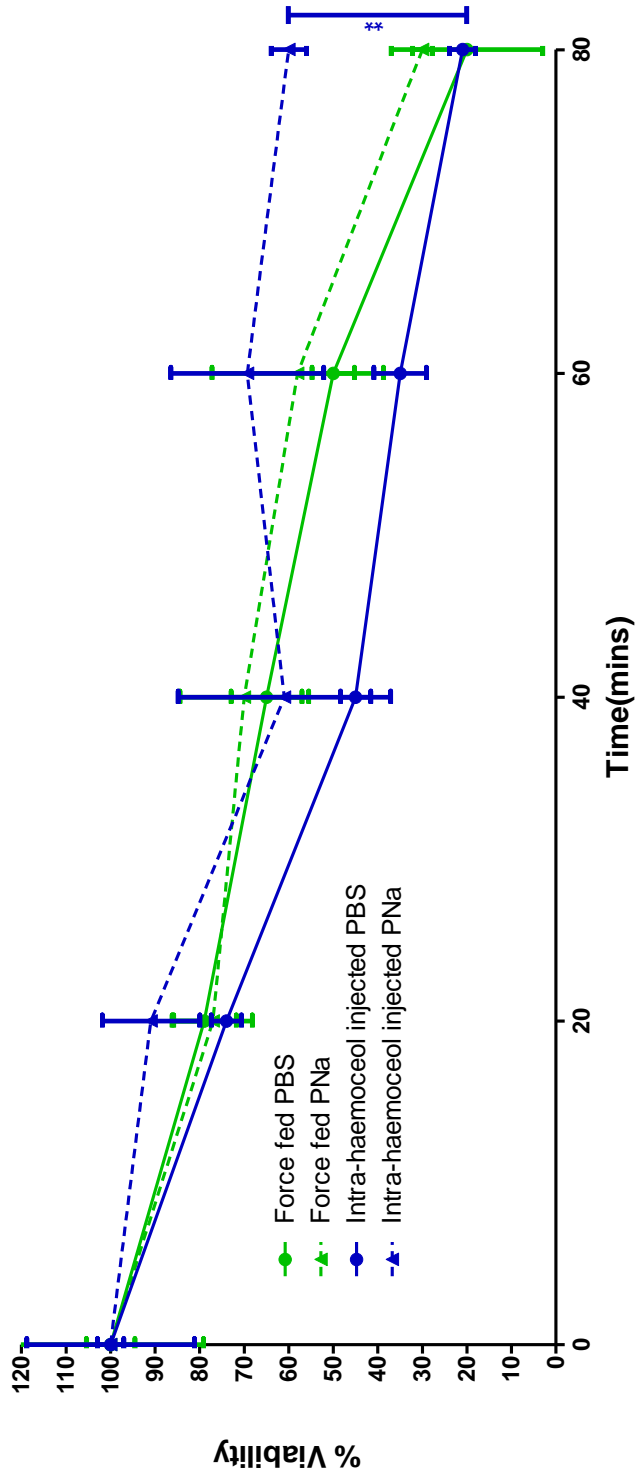


Fig. 4.3 Fungicidal ability of haemocytes from larvae administered potassium nitrate by intra-haemocoel injection or force feeding at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined (**:p < 0.01). All values are the mean \pm SE of 3 independent determinations.

4.4 Effect of potassium nitrate on the composition of haemocyte populations in *G. mellonella*.

FACs analysis was employed to establish if there was a change in the relative proportions of each haemocyte sub-population in control larvae and larvae injected with potassium nitrate and incubated for 24 hours. Haemocyte populations were differentiated on the basis of size and granularity and at least 6 distinct subpopulations, labelled P1, P2, P3, P4, P5 and P6 were identified (Fig. 4.4). In control larvae the relative abundance of P1, P2, P3, P4, P5 and P6 were $58.42 \pm 0.82\%$, $12.91 \pm 3.85\%$, 5.84 ± 0.51 , $8.72 \pm 1.35\%$, 9.04 ± 2.69 and $5.07 \pm 1.29\%$ respectively. In potassium nitrate injected larvae the relative abundance of P1, P2, P3, P4, P5 and P6 were 50.06 ± 3.56 , 15.58 ± 2.20 , 5.78 ± 0.88 , 13.75 ± 2.36 , 11.70 ± 3.97 and 3.13 ± 1.21 respectively. The results demonstrated a significant decrease ($p < 0.05$) in the relative abundance of P1 haemocytes (small granular cells) in the total haemocyte populations of larvae injected with potassium nitrate compared to control larvae. The abundance of the other haemocyte populations (P2-P6) remained relatively constant in both control and potassium nitrate treated larvae (Fig. 4.5).

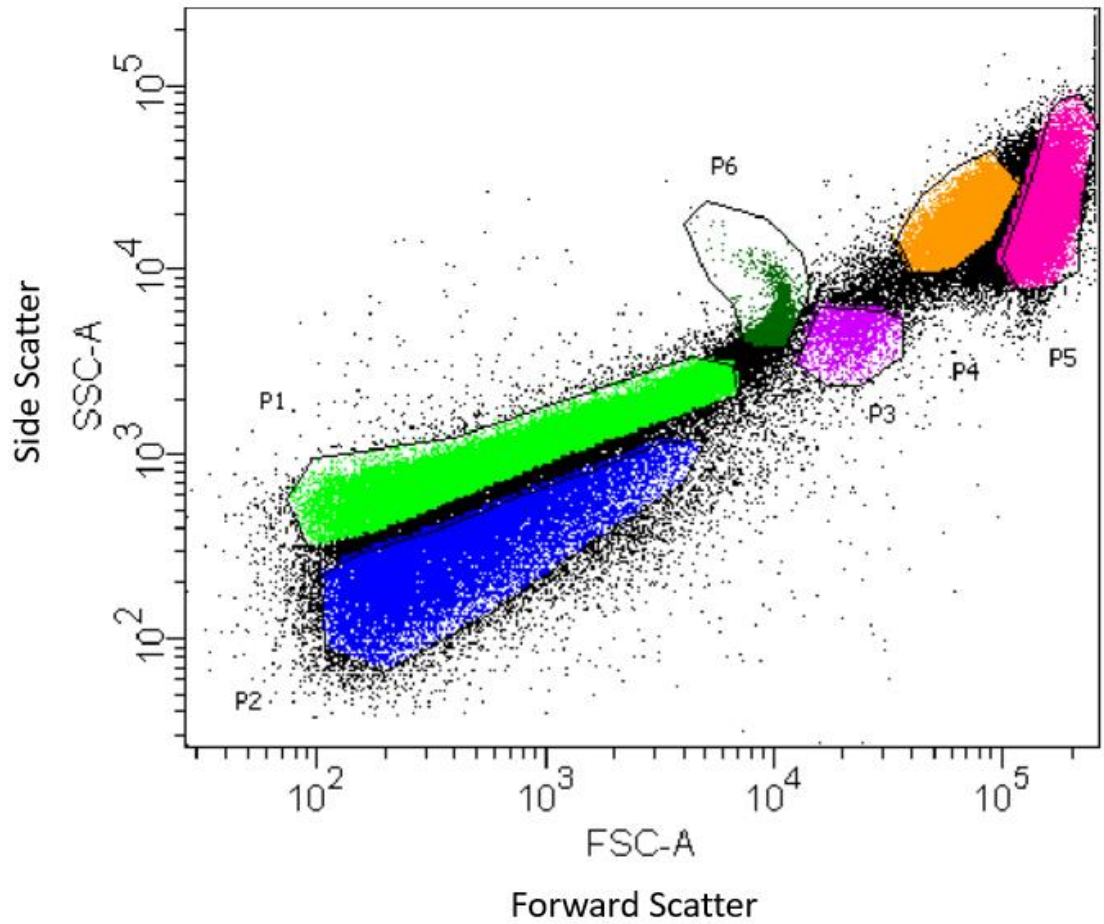


Fig. 4.4 FACS Scatterplot image of haemocyte subpopulations P1-P6 in larvae injected with potassium nitrate at 24 hours.

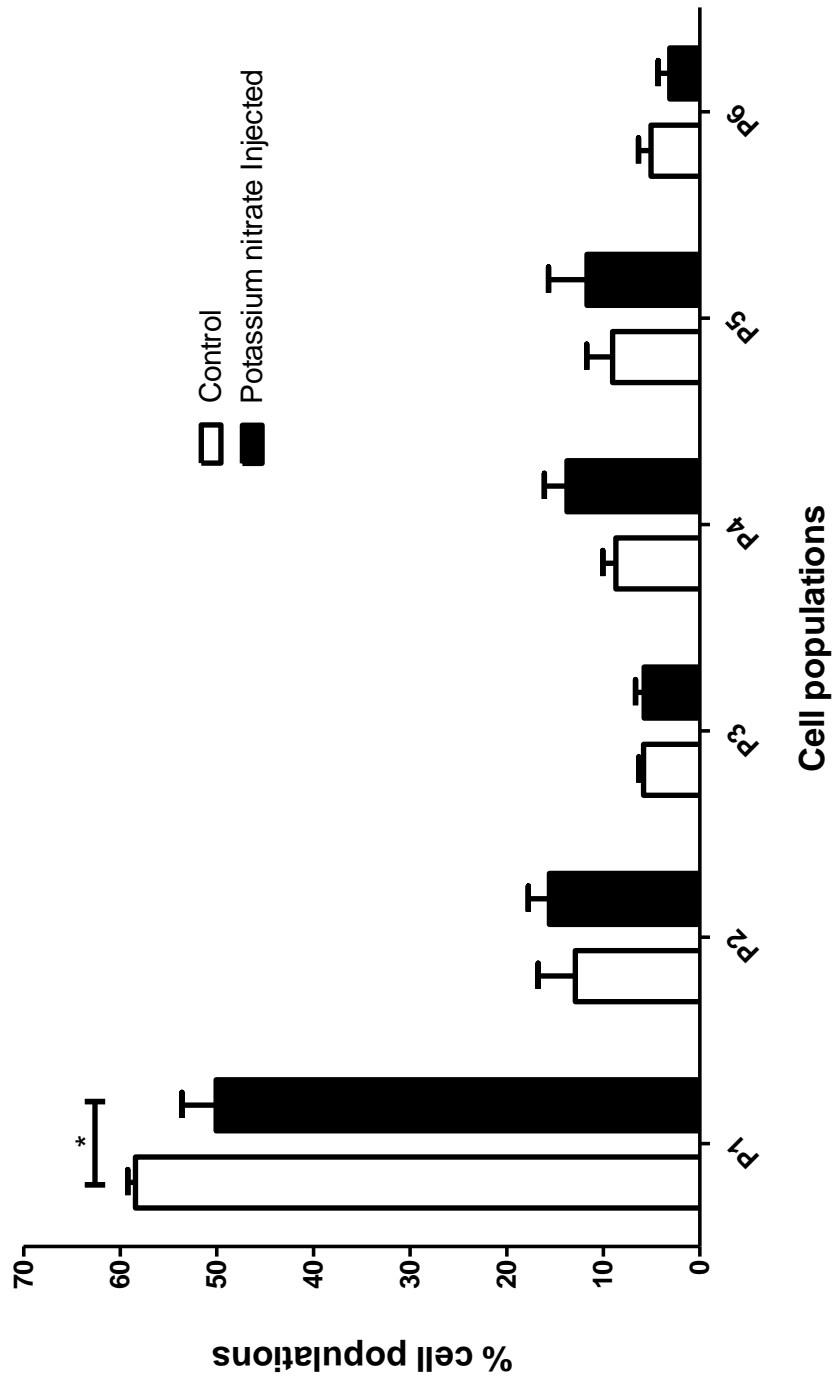


Fig. 4.5 FACS analysis of haemocyte sub-populations. Fluctuations in haemocyte sub-populations in control larvae and larvae injected with potassium nitrate. Haemocyte sub-populations were measured based on size and granularity as described (*: $p < 0.05$). All values are the mean \pm SE of 3 independent determinations.

4.5 Summary.

The effect of potassium nitrate on haemocyte densities was ascertained. Larvae force fed potassium nitrate showed no significant changes in their haemocyte densities when compared to controls (Fig. 4.2). Larvae injected with the LD₂₅ value of potassium nitrate at 4 hours showed significant decrease ($p < 0.001$) in haemocyte densities when compared to controls. Larvae injected with the LD₅₀ value of potassium nitrate at 4 hours showed significant increases ($p < 0.05$) in haemocyte densities when compared to controls. In addition larvae injected with the LD₅₀ and LD₈₀ value of potassium nitrate 24 hours showed significant increases ($p < 0.01$) in haemocyte densities when compared to controls (Fig. 4.1).

Haemocytes from larvae force fed potassium nitrate showed no significant differences in their fungicidal ability when compared to controls. Haemocytes from larvae administered potassium nitrate via direct injection in the haemocoel showed a significant reduction ($p < 0.01$) in their fungicidal ability (Fig. 4.3).

Larvae administered potassium nitrate showed a significant decrease in the relative abundance of P1 haemocytes (small, granular cells). There was a small increase in the P2 (very small granular cells), P4 and P5 (large and granular) populations and a small decrease in the population of P6 (large granular) population (Fig. 4.5).

4.6 Effects of potassium nitrate on superoxide dismutase activity of the haemolymph.

Superoxide activity in the haemolymph of larvae administered potassium nitrate was measured. Larvae were administered potassium nitrate by intra-haemocoel injection or force feeding of a concentration of food additive corresponding to its LD₅₀. Larvae were incubated at 30°C for 4 and 24 hours post administration of potassium nitrate and the total superoxide dismutase activity was measured in larval haemolymph. Superoxide dismutase activity was quantified as units of activity, 1 unit of SOD activity equated to the 50% inhibition of the oxidation of quercetin.

One unit of superoxide dismutase activity in PBS injected control larvae equated to $131.24 \pm 12.1 \mu\text{g}/\mu\text{l}$ and $231.135 \pm 41.98 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 4 and 24 hours incubation respectively. This equates to a 0.76 fold increase ($p < 0.05$) in superoxide dismutase activity in larvae incubated for 4 hours when compared to larvae incubated for 24 hours.

One unit of superoxide dismutase activity in PBS force fed control larvae equated to $87.4 \pm 11.3 \mu\text{g}/\mu\text{l}$ and $235.46 \pm 35.86 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 4 and 24-hours. This indicated a 1.69 fold increase ($p < 0.01$) in superoxide dismutase activity seen in larvae incubated for 4 compared to 24 hours.

A significant increase in superoxide dismutase activity was recorded in the haemolymph of larvae administered potassium nitrate via intra-haemocoel injection. One unit of superoxide dismutase activity in larvae injected with potassium nitrate equated to $145.26 \pm 24.40 \mu\text{g}/\mu\text{l}$ and $100.57 \pm 27.75 \mu\text{g}/\mu\text{l}$ of protein following 4 and 24 hour incubation respectively. When comparing 24 hour superoxide dismutase activity a 0.56 fold increase ($p < 0.05$) was seen in potassium nitrate injected larvae compared to PBS injected control larvae (Fig. 4.6).

Superoxide dismutase activity was also measured in haemolymph from larvae administered potassium nitrate via force feeding prior to incubation. At 4 and 24 hours one unit of superoxide dismutase activity was $102.60 \pm 15.8 \mu\text{g}/\mu\text{l}$ and $156.04 \pm 37.42 \mu\text{g}/\mu\text{l}$ of protein respectively (Fig. 4.7). These findings did not suggest any significant changes between control and potassium nitrate fed larvae.

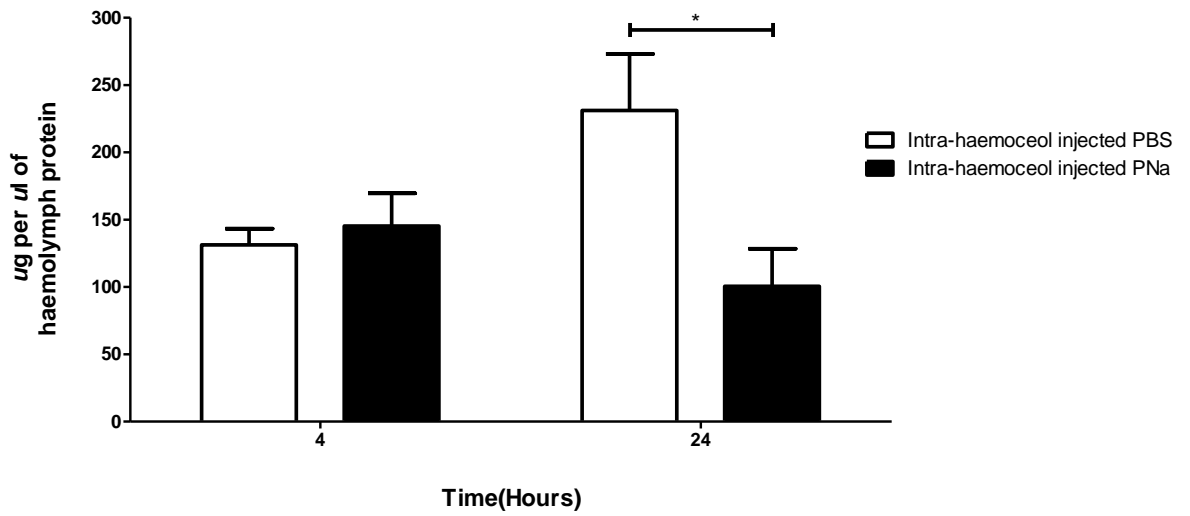


Fig. 4.6 Superoxide dismutase activity of haemolymph from larvae administered potassium nitrate via intra-haemocoel injection. After 4 and 24 hours haemolymph was extracted from larvae that were administered an LD₅₀ value of potassium nitrate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

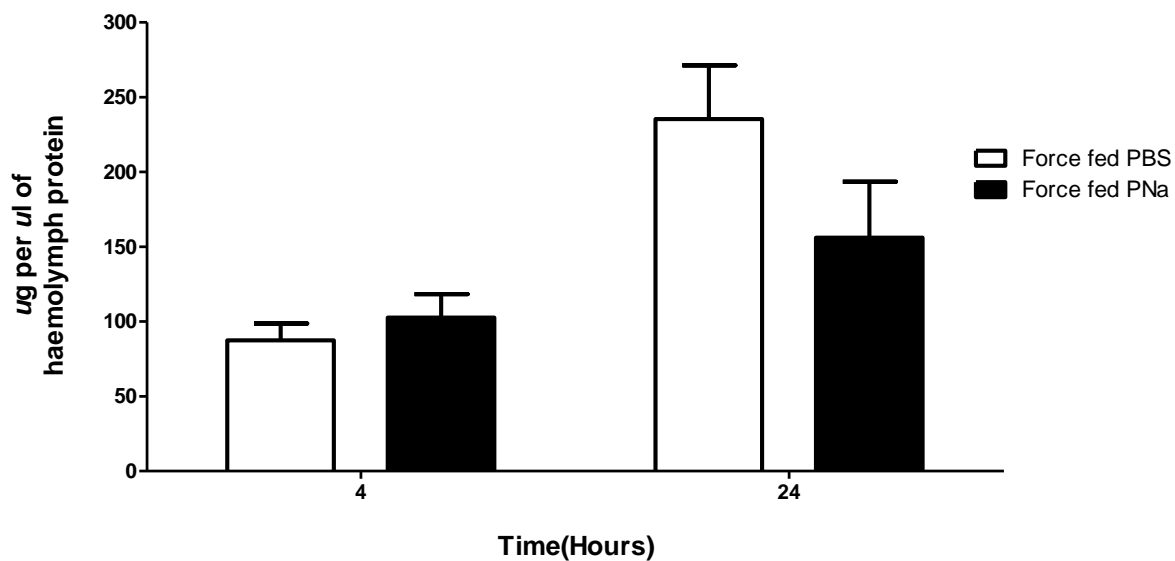


Fig. 4.7 Superoxide dismutase activity of haemolymph from larvae administered potassium nitrate via force feeding. After 4 and 24 hours haemolymph was extracted from larvae that were administered an LD₅₀ value of potassium nitrate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

4.7 Effects of potassium nitrate on catalase activity in *G. mellonella* haemolymph.

Larvae were administered by intra haemocoel injection or force feeding a food additive concentration corresponding to the LD₅₀ values of potassium nitrate. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with hydrogen peroxide. The decomposition of hydrogen peroxide and thus rate of catalase activity was measured spectrometrically at 240nm. The rate of catalase activity in larvae administered potassium nitrate was expressed as the fold change compared with their relevant control groups administered PBS.

Larvae injected with potassium nitrate showed a 0.63 ± 0.30 and a 0.54 ± 0.19 fold increase when compared to relevant controls after 4 and 24 hour respectively (Fig. 4.8). Larvae force fed potassium nitrate, showed a 0.31 ± 0.18 and a 0.44 ± 0.06 fold increase when compared to relevant controls after 4 and 24 hour incubation respectively (Fig. 4.8). The administration of potassium nitrate by force feeding or direct injection revealed no significant changes in catalase activity between control and test groups.

4.8 Summary.

Haemolymph from larvae force fed or injected with the LD₅₀ value of potassium nitrate was extracted and the activity of two antioxidant enzymes (superoxide dismutase and catalase) was measured. Haemolymph from larvae administered potassium nitrate by the two routes of administration showed no significant changes in catalase activity compared with controls at 4 and 24 hours (Fig. 4.8). Haemolymph from larvae injected with potassium nitrate showed no significant changes in superoxide dismutase activity at 4 hours however after 24 hours a significant increase ($p < 0.05$) in activity was recorded (Fig. 4.6, 4.7).

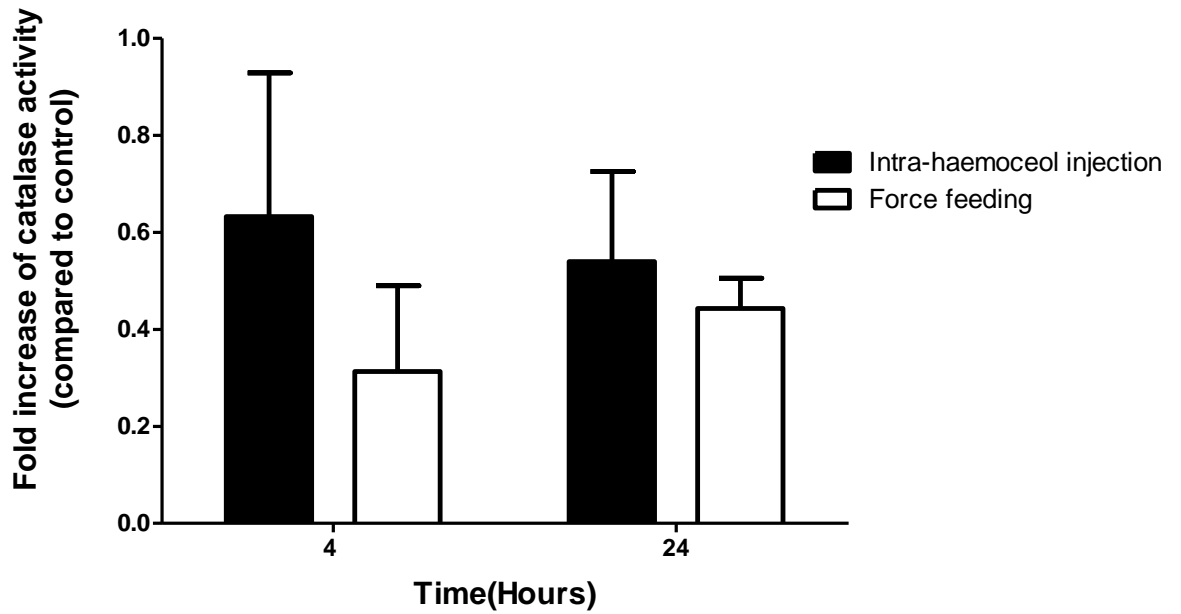


Fig. 4.8 Catalase activity of haemolymph from larvae administered potassium nitrate via force feeding and intra-haemocoel injection. After 4 and 24 hours haemolymph was extracted from larvae that were administered an LD₅₀ value corresponding to a food additive. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

4.9 Effects of potassium nitrate on the proteome in *G. mellonella* haemolymph

Label free quantitative proteomics was conducted on haemolymph from larvae administered via intra-haemocoel injection the LD₅₀ of potassium nitrate for 24 hours. Principal component analyses was employed showing clustering of control and treatment replicates (Fig. 4.9). In total 525 peptides were identified representing 167 proteins with two or more peptides and 47 proteins differentially expressed (Fig. 4.10). Forty proteins (31 proteins imputed and 9 proteins non-imputed) were significantly increased in abundance in larvae administered potassium nitrate compared to control larvae. (Table 4.1, Fig. 4.11, 4.12). Seven proteins were significantly decreased in abundance in larvae administered potassium nitrate compared to control larvae (Table 4.2, Fig. 4.13). The exclusively expressed hits showed 25 proteins only expressed in potassium nitrate injected larvae (Table 4.3).

The protein showing the highest increase in abundance in larvae exposed to potassium nitrate was beta actin with a fold increase of 47.9 ($p < 0.05$), while the proteins highest in abundance with an imputed value were mitochondrial aldehyde dehydrogenase (288.6 fold increase), beta-tubulin (283.1 fold increase), aliphatic nitrilase (132.5 fold increase), triosephosphate isomerase enolase (79.8 fold increase) and alcohol dehydrogenase precursor (52.8 fold increase) (Table 1). A number of related proteins were observed at increased abundance in larvae administered potassium nitrate, including a number of dehydrogenases and transferases (mitochondrial aldehyde dehydrogenase (288.6 fold increase), alcohol dehydrogenase precursor (52.8 fold increase), cytosolic malate dehydrogenase (46.2 fold), glyceraldehyde-3-phosphate dehydrogenase (20.3 fold increase), zinc-containing alcohol dehydrogenase (16.9 fold increase), serine hydroxymethyltransferase (12.1 fold increase) short-chain dehydrogenase/reductase (12.2 fold increase), isocitrate dehydrogenase (2.3 fold increase), glutathione S-transferase (20.6 fold increase)) and serine hydroxymethyltransferase (12.1 fold increase). Numerous proteins involved in mitochondrial function, glycolysis and the TCA cycle (e.g. mitochondrial aldehyde dehydrogenase, putative mitochondrial Mn superoxide dismutase (15.2 fold increase increase), cytosolic malate dehydrogenase (46.2 fold increase),

triosephosphate isomerase (37.1 fold increase), enolase (62.4 fold increase) and glyceraldehyde-3-phosphate dehydrogenase (9.7 fold increase) were present at a higher abundance in larvae exposed to potassium nitrate (Fig. 4.14).

Proteins decreased in abundance in larvae that received potassium nitrate included putative protease inhibitor 4 (1.9 fold decrease), imaginal disc growth factor (1.7 fold decrease), kazal-type proteinase inhibitor precursor (1.6 fold decrease), twelve cysteine protein 1 (1.5 fold decrease), carboxylesterase CarE-7 (1.5 fold decrease) (Table 4.2).

Blast2GO annotation software was used to group proteins based on conserved GO terms in order to identify processes and pathways potentially associated with potassium nitrate metabolism. GO terms were categorized by biological processes (BP) and molecular function (MF) and cellular components (CC).

The increases in BP in larvae administered potassium nitrate included proteins labelled as cellular metabolic process (11 proteins in control – 18 proteins in treated larvae), biosynthetic process (5-9), catabolic process (4-7), cellular component biogenesis (3-5), cellular component organization (3-4), nitrogen compound metabolic process (8-13), organic substance metabolic process (10-21), primary metabolic process (9-18), regulation of biological quality (5-7), regulation of cellular process (3-4), response to stress (2-4), single-organism cellular process (9-16), single-organism metabolic process (8-19), establishment of localization (3-0), negative regulation of cellular process (3-0), positive regulation of cellular component biogenesis (2-0), positive regulation of cellular process (2-0), regulation of cellular component biogenesis (2-0) (Fig. 4.15).

The increases in MF included proteins labelled as oxidoreductase activity (4 proteins in control – 12 proteins in treated larvae), carbohydrate derivative binding (4-7), heterocyclic compound binding (7-11), hydrolase activity (7-13), ion binding (7-10), organic cyclic compound binding (7-11), protein binding (3-5), small molecule binding (8-12), transferase activity (3-5), cofactor binding (3-0), lipid binding (2-0), pigment binding (4-0) (Fig. 4.16).

The increases in CC included proteins labelled as intracellular (2 proteins in control – 6 proteins in treated larvae) anchoring junction (1-2), cell leading edge (1-1), cell projection (1-1-), cell-substrate junction (1-2), extracellular space (2-4), intracellular organelle (2-4), intracellular part (2-6), membrane-bounded organelle (2-1), blood microparticle (0-1), cell periphery (0-1), extracellular exosome (0-1), extracellular organelle (0-1), extracellular vesicle (0-1), intracellular organelle part (0-2), myelin sheath (0-1), non-membrane-bounded organelle (0-4), plasma membrane (0-1), protein complex (0-2), supramolecular polymer (0-2) (Fig. 4.17).

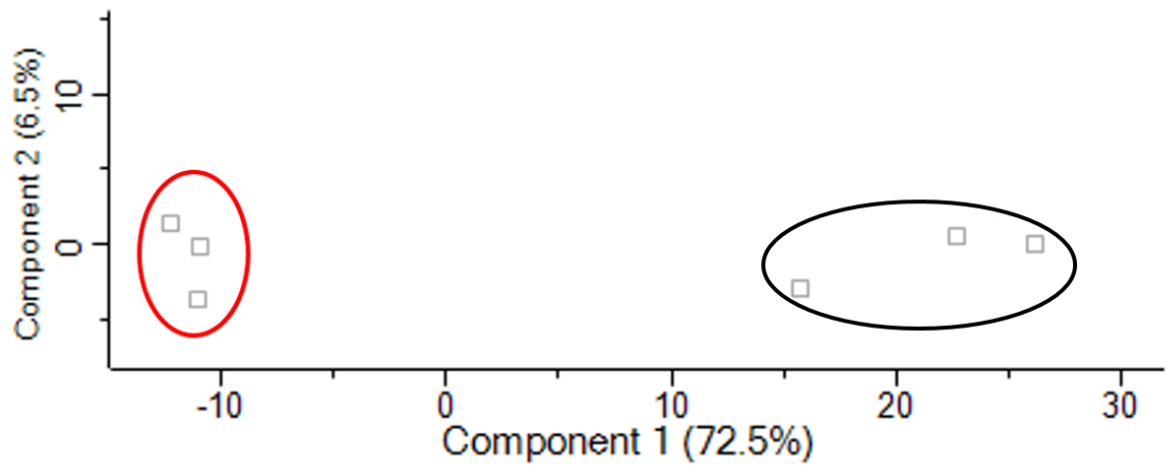


Fig. 4.9 Principal component analyses of the proteome of control larvae (red circle) and larvae injected with potassium nitrate (black circle).

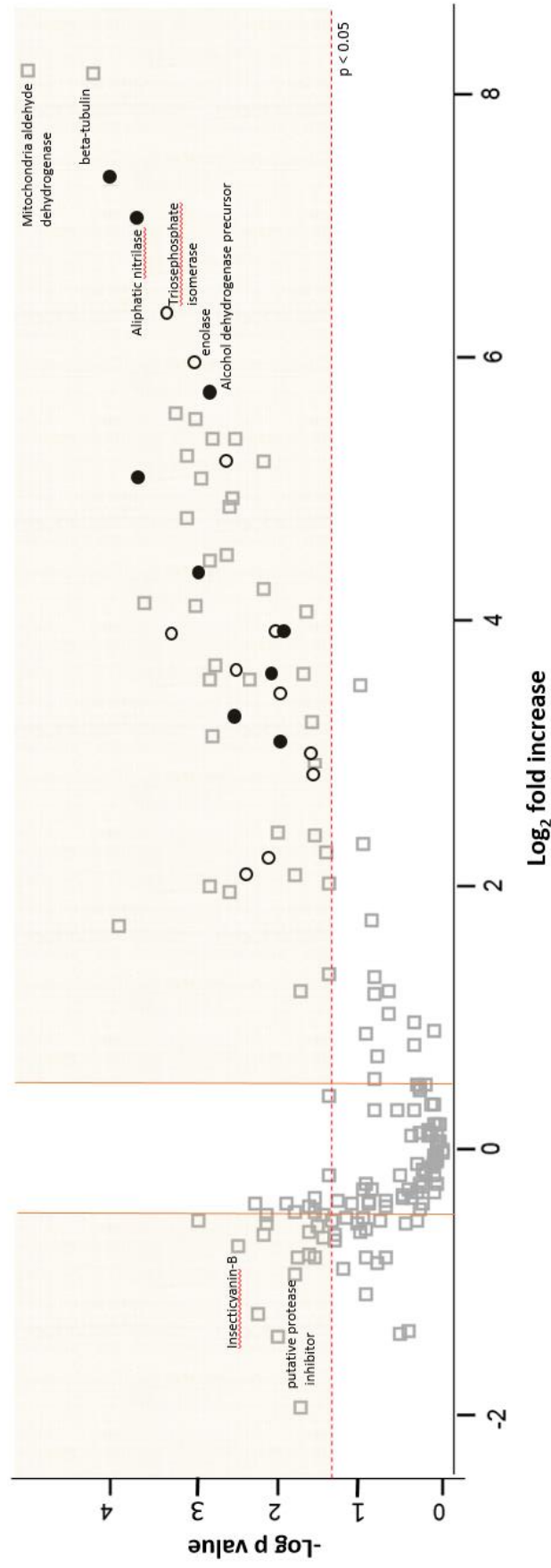


Fig. 4.10 Volcano plot showing proteins altered in abundance in *G.mellonella* larvae treated with potassium nitrate. Proteins above the line are statistically significant ($p < 0.05$) and those to the right and left of the vertical lines indicate fold changes 1.5 fold positive and 1.5 fold negative in the potassium nitrate treated larvae. Open circles: protein associated with metabolism and oxidoreductase activity, closed circles proteins associated with metabolism and oxidoreductase activity, squares all other proteins.

Protein Name (* = imputed protein)	Peptides	Sequence coverage [%]	PEP	Mean LFQ Intensity	Fold difference
*mitochondrial aldehyde dehydrogenase	30	64.5	0	1.20E+11	288.64
*beta-tubulin	17	31.7	0	5.92E+10	283.14
*aliphatic nitrilase	19	53	0	7.06E+10	132.52
*triosephosphate isomerase	18	30.1	0	1.97E+10	79.80
*enolase	18	43	0	1.40E+10	62.48
*alcohol dehydrogenase precursor	13	45.2	1.4106E-167	1.32E+10	52.88
beta actin	11	52.7	0	8.66E+10	47.90
*cytosolic malate dehydrogenase	15	34.4	2.1709E-143	1.17E+10	46.20
*AAEL005062-PA	9	26.5	3.5948E-74	1.14E+10	41.93
*alpha 2-tubulin, partial	4	47.2	0	1.84E+10	38.11
*thioredoxin-like protein	5	20.4	3.5015E-59	7.57E+09	33.97
*actin 3	8	29.1	0	1.04E+10	29.17
*elongation factor 1 alpha	9	34	1.6052E-198	1.03E+10	21.98
*glutathione S-transferase	10	41.1	7.1188E-140	8.03E+09	20.63
glyceraldehyde-3-phosphate dehydrogenase	13	49.4	0	7.07E+10	20.36
*glutathione-S-transferase-like protein	10	47.2	0	9.43E+09	17.68
*chemosensory protein	4	14	1.7008E-91	7.56E+09	17.29
*zinc-containing alcohol dehydrogenase	11	44.4	0	8.68E+09	16.97
*putative mitochondrial Mn superoxide dismutase	9	45.5	2.9836E-281	7.23E+09	15.20
*phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	19	37.2	4.6317E-271	1.29E+10	15.13
*cyclophilin-like protein	6	24.7	4.1192E-168	5.47E+09	15.02
cellular retinoic acid binding protein	15	35.6	7.0508E-292	7.31E+10	12.77
arginine kinase, partial	10	71.1	3.7151E-203	3.40E+10	12.35
*short-chain dehydrogenase/reductase	8	30	8.537E-302	3.43E+09	12.22
*serine hydroxymethyltransferase	21	43.3	3.6909E-238	1.27E+10	12.15

*heat shock protein hsp21.4	6	15.4	6.3966E-115	5.09E+09	11.74
*serine protease inhibitor 5 precursor	11	31.2	2.416E-49	3.12E+09	10.98
*glyceraldehyde-3-phosphate dehydrogenase	11	39.2	0	7.57E+09	9.74
*muscle protein 20-like protein	12	39.7	1.7499E-185	4.96E+09	8.70
*AAEL000132-PA	6	47.8	4.0548E-86	2.57E+09	8.51
*Tropomyosin 2	8	35.9	2.7637E-91	2.08E+09	7.58
RecName: Full=Seroin; AltName: Full=Silk 23 kDa glycoprotein; Flags: Precursor	3	16.2	1.0038E-121	6.29E+09	5.33
*14-3-3zeta	3	4.6	1.2498E-295	2.20E+09	5.21
*prophenol oxidase activating enzyme 3	10	25.4	1.4735E-51	1.46E+09	4.76
*cathepsin B-like cysteine proteinase	6	19.6	8.7962E-35	1.99E+09	4.63
abnormal wing disc-like protein	9	46.9	8.0549E-169	1.05E+10	4.22
AGAP010733-PA, partial	4	15.3	3.6022E-27	1.87E+09	3.90
*heat shock-like protein, partial	3	17.7	2.0202E-20	1.81E+09	3.24
heat shock protein 25.4 precursor	11	47.2	0	4.92E+10	2.51
isocitrate dehydrogenase	17	48.2	0	1.78E+10	2.31

Table 4.1 Relative fold changes of proteins increased in abundance in *G. mellonella* larvae administered potassium nitrate and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 fold were considered to be in significantly variable abundances between control and treated larvae.

Protein Name (* = imputed protein)	Peptides	Sequence coverage [%]	PEP	Mean LFQ Intensity	Fold difference
putative protease inhibitor 4	7	25.7	9.239E-288	7.00E+10	-1.91
Anopheles gambiae str. PEST AGAP012658-PA	11	47.3	7.915E-85	1.28E+10	-1.75
imaginal disc growth factor	16	51.9	0	7.00E+11	-1.75
RecName: Full=Insecticyanin-B; Short=INS-b; AltName: Full=Blue biliprotein; Flags: Precursor	14	45.5	0	1.96E+11	-1.65
kazal-type proteinase inhibitor precursor	8	21.4	1.4788E-224	5.80E+10	-1.61
twelve cysteine protein 1	10	43.4	0	3.10E+10	-1.59
carboxylesterase CarE-7	6	42.9	1.096E-232	2.00E+10	-1.55

Table 4.2 Relative fold changes of proteins decreased in abundance in *G. mellonella* larvae administered potassium nitrate and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 fold were considered to be in significantly variable abundances between control and treated larvae.

Protein Annotation	LFQ Intensity Control 1	LFQ Intensity Control 2	LFQ Intensity Control 3	LFQ Intensity PNa inj 1	LFQ Intensity PNa inj 2	LFQ Intensity PNa 3
elongation factor 1 alpha	0	0	0	1.43E+09	5E+08	7.29E+08
zinc-containing alcohol dehydrogenase	0	0	0	1.74E+09	1.78E+08	1.61E+09
glutathione S-transferase	0	0	0	1.1E+09	6.55E+08	1.38E+09
14-3-3zeta	0	0	0	4.11E+08	1.44E+08	2.99E+08
triosephosphate isomerase	0	0	0	4.29E+09	1.9E+09	1.15E+09
short-chain dehydrogenase/reductase	0	0	0	6.51E+08	2.1E+08	5.04E+08
alpha 2-tubulin, partial	0	0	0	3.25E+09	1.1E+09	1.82E+09
beta-tubulin	0	0	0	1.05E+10	4.14E+09	5.89E+09
Tropomyosin 2	0	0	0	2.67E+08	1.62E+08	3.99E+08
enolase	0	0	0	2.56E+09	1.06E+09	1.8E+09
AAEL000132-PA	0	0	0	8.28E+08	4.36E+08	3.07E+08
cyclophilin-like protein	0	0	0	1.02E+09	4.42E+08	7.96E+08
alcohol dehydrogenase precursor	0	0	0	2.86E+09	7.8E+08	1.73E+09
glutathione-S-transferase-like protein	0	0	0	1.95E+09	8.82E+08	1.22E+09
uncharacterized protein Dyak_GE20538	0	0	0	2.52E+08	1.24E+08	2.37E+08
mitochondrial aldehyde dehydrogenase	0	0	0	2.2E+10	1.23E+10	1.49E+10
chemosensory protein	0	0	0	51226000	35067000	87836000
prophenol oxidase activating enzyme 3	0	0	0	2.55E+08	1.3E+08	2.32E+08
thioredoxin-like protein	0	0	0	9.73E+08	5.7E+08	1.43E+09
actin 3	0	0	0	1.73E+09	7.81E+08	2.12E+09
AAEL005062-PA	0	0	0	3.26E+09	1.16E+09	1.64E+09
serine hydroxymethyltransferase	0	0	0	2.17E+09	2.54E+08	1.28E+09
muscle protein 20-like protein	0	0	0	4.8E+08	4.86E+08	8.57E+08
serine protease inhibitor 5 precursor	0	0	0	7.09E+08	3.11E+08	1.96E+08
putative mitochondrial Mn superoxide dismutase	0	0	0	9.52E+08	5.77E+08	1.38E+09

Table 4.3 LFQ intensities of proteins exclusively expressed in the haemolymph of larvae injected with potassium nitrate. A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”. (PNa, potassium nitrate)

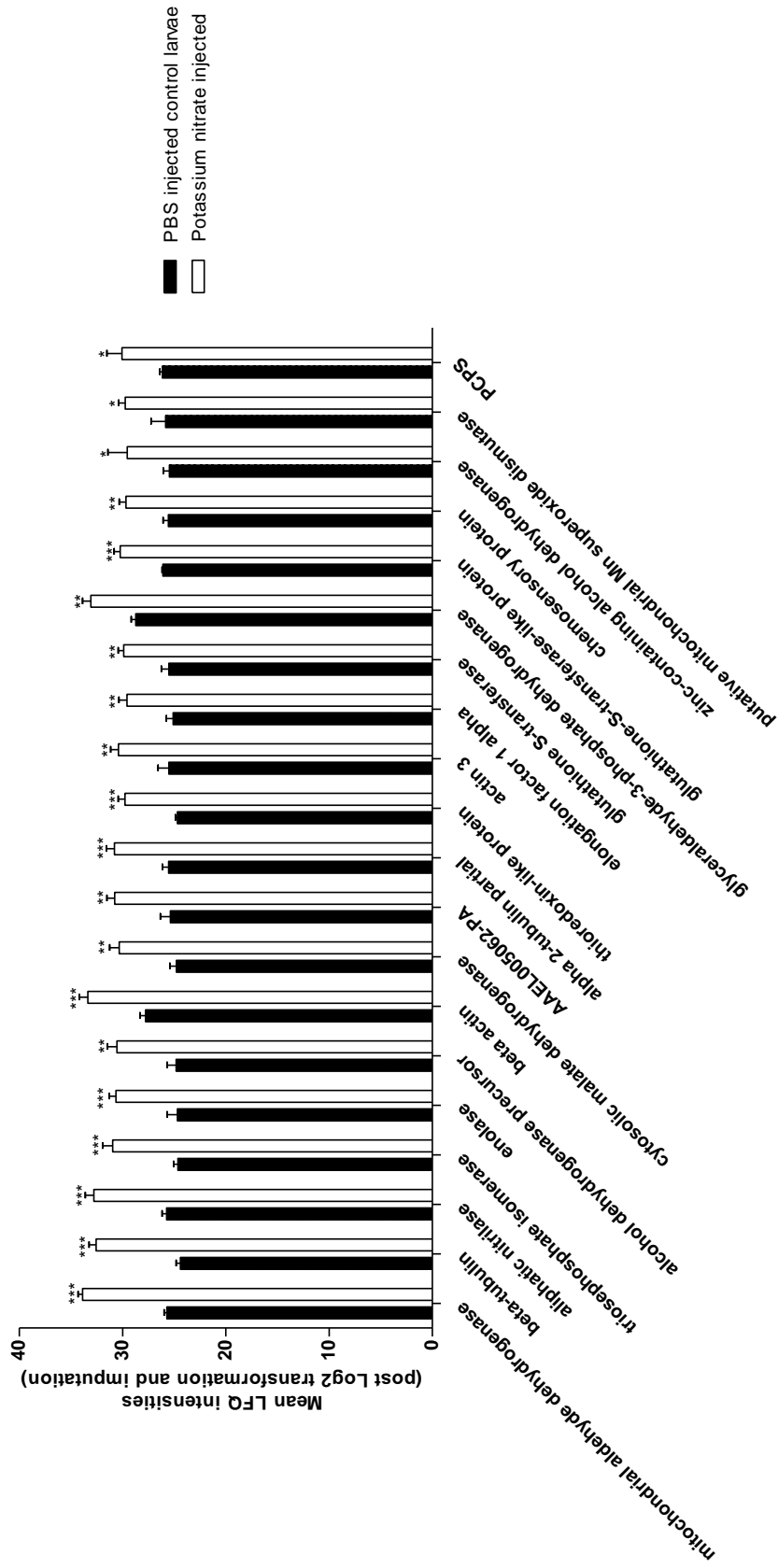


Fig 4.11 Bar chart showing average LFQ values of proteins significantly increased in expression in potassium nitrate injected larvae compared to control larvae (*: $p < 0.05$) (**: $p < 0.01$) (***: $p < 0.001$).

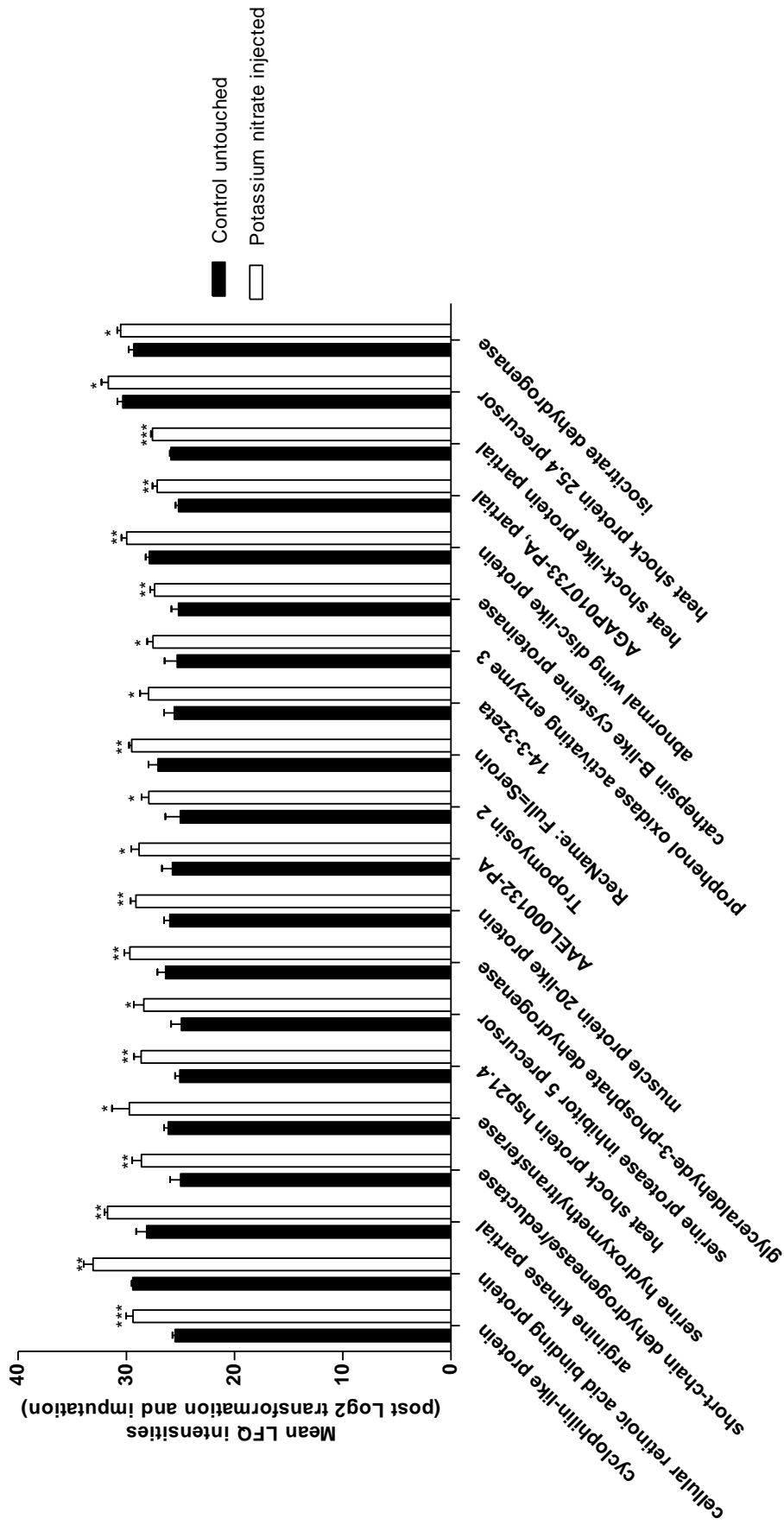


Fig. 4.12 Bar chart showing average LFQ values of proteins significantly increased in expression in potassium nitrate larvae compared to control larvae (*: $p < 0.05$) (**: $p < 0.01$) (***: $p < 0.001$).

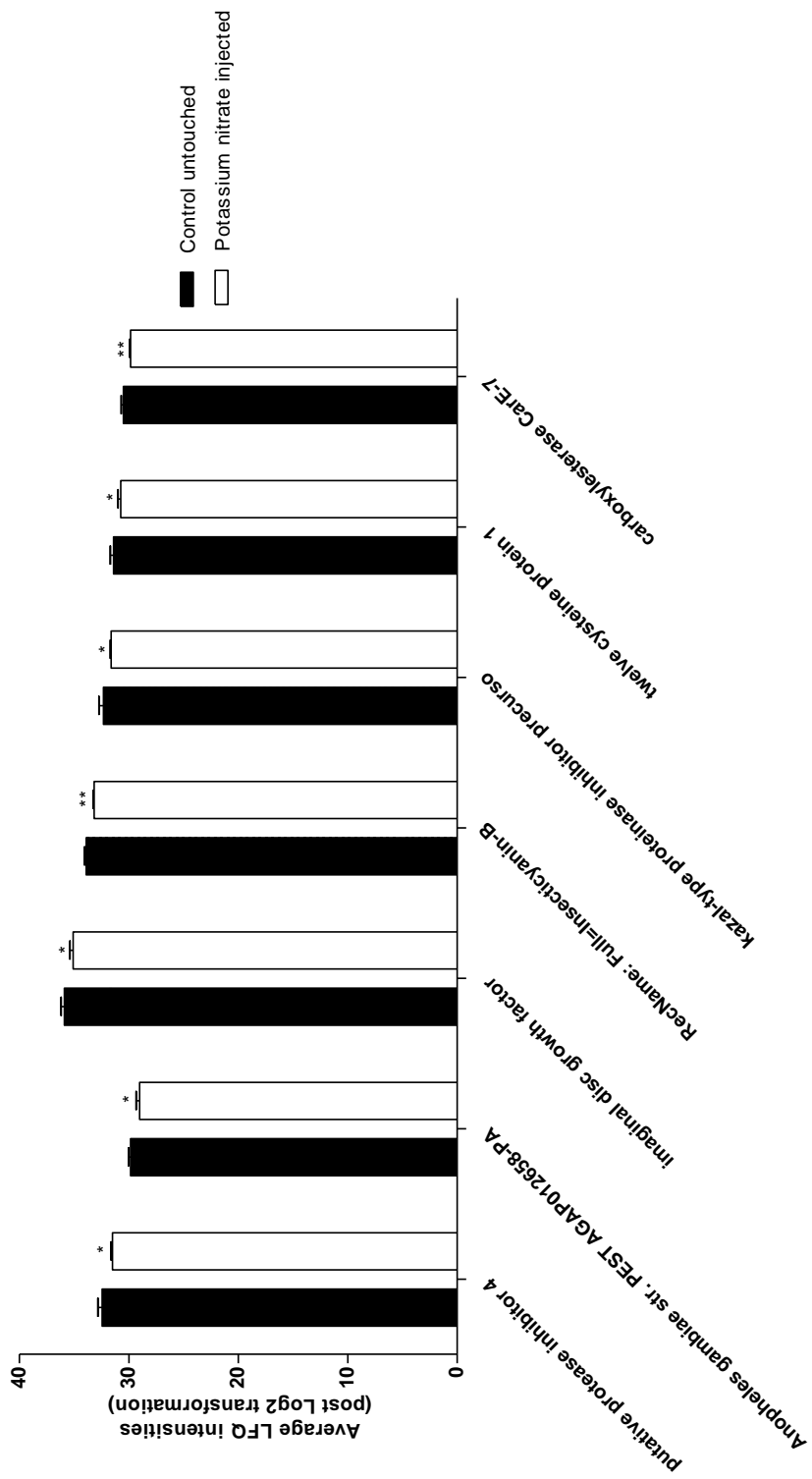


Fig. 4.13 Bar chart showing average LFQ values of proteins significantly reduced in expression in potassium nitrate larvae compared to control larvae (*: $p < 0.05$) (**: $p < 0.01$).

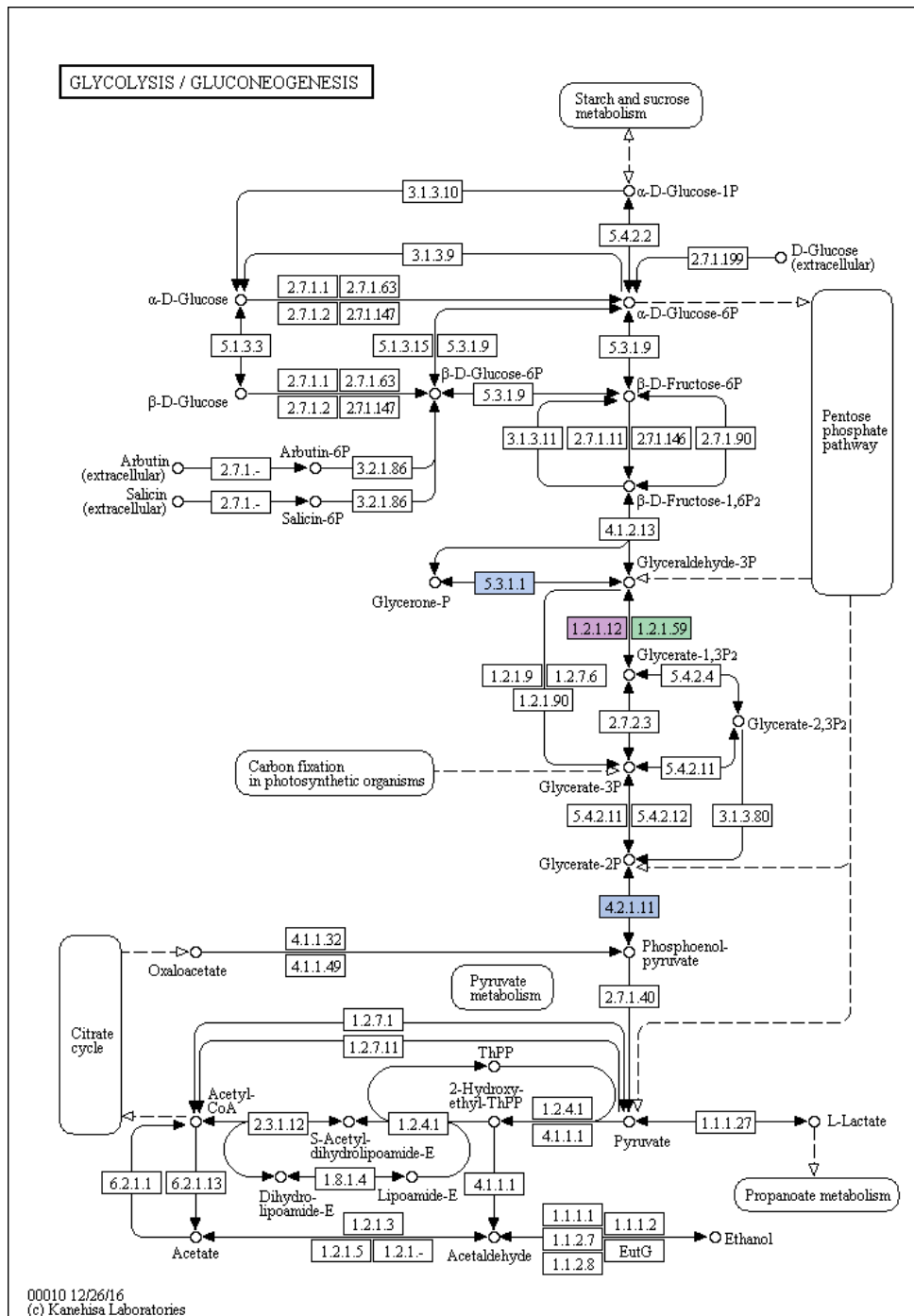


Fig. 4.14 KEGG pathway analysis of glycolysis/glycogenesis pathway showing a number of proteins significantly increased in potassium nitrate injected larvae compared to control larvae. (ec:5.3.1.1 - triosephosphate isomerase; ec:1.2.1.12 - glyceraldehyde-3-phosphate dehydrogenase; ec:1.2.1.59 - glyceraldehyde-3-phosphate dehydrogenase; ec:4.2.1.11 – enolase)

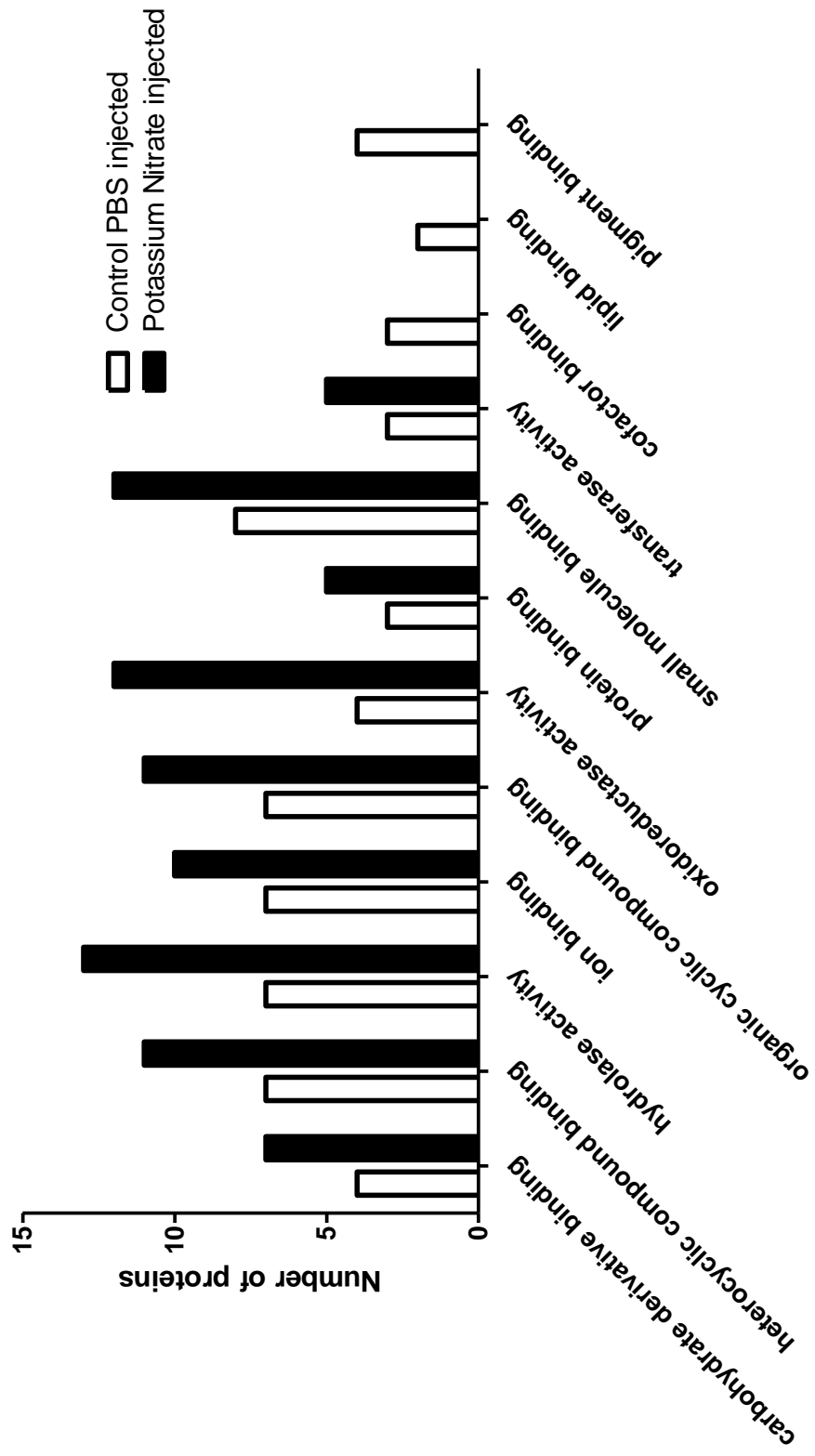


Fig. 4.16 Bar chart showing changes to number of proteins given various molecular functions at level 3 ontology. Proteins were assigned groups based on involvement in molecular function for control and potassium nitrate treated larvae. Open bar: control, closed bar: potassium nitrate treated larvae.

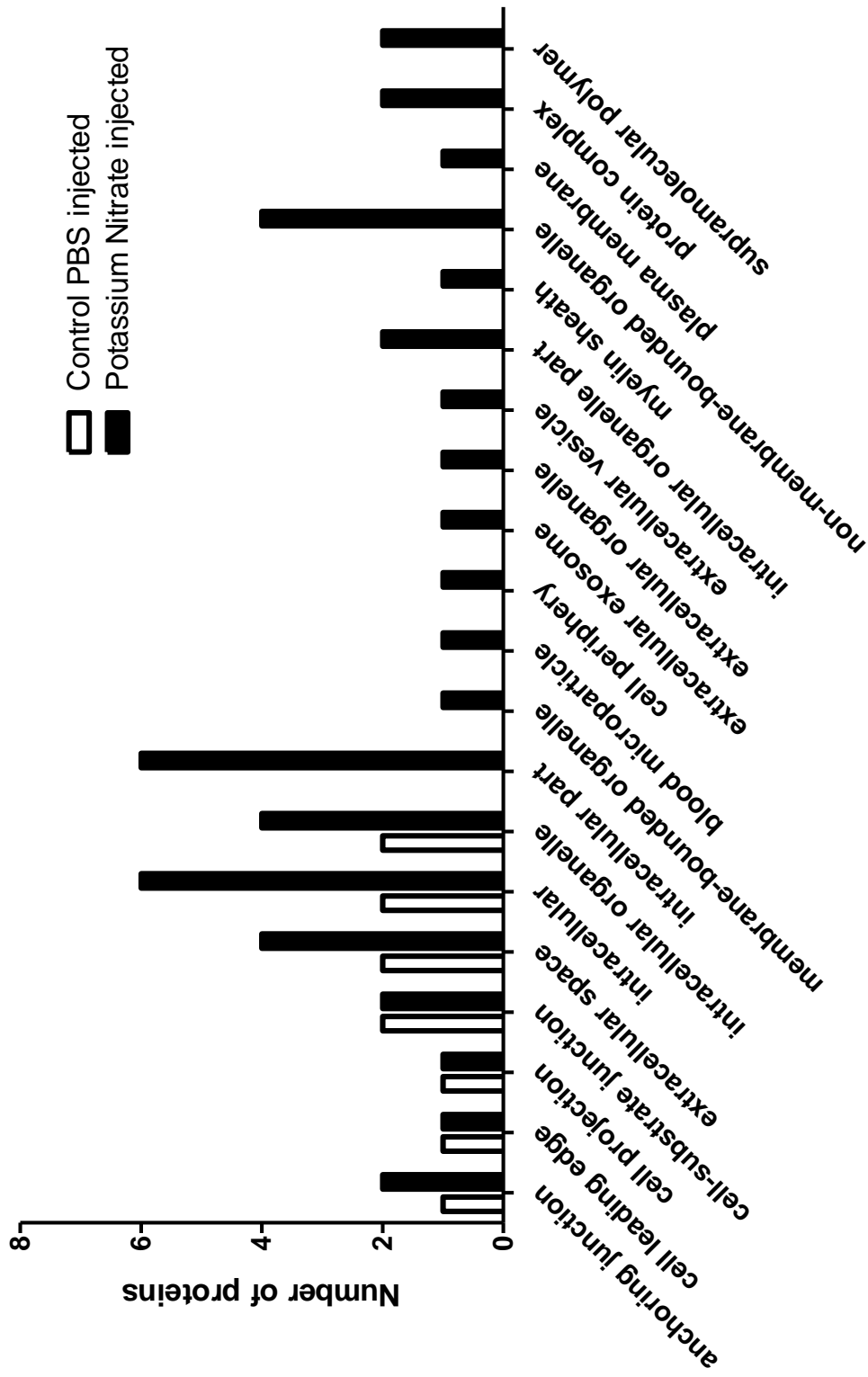


Fig. 4.17 Bar chart showing changes to number of proteins in various cellular components at level 3 ontology. Proteins were assigned groups based on involvement in cellular components in the total proteome for control and potassium nitrate treated larvae.

4.10 Discussion.

The Insect immune system displays interconnected cellular and humoral components despite lacking an adaptive immune system. The cellular immune response of insects is mediated by haemocytes which can engulf, encapsulate or neutralize pathogens (Pech and Strand, 1996). The first haemocytes to a site of infection are those free floating in the haemolymph and the haemocyte densities are soon supplemented by the release of haemocytes bound to internal organs (Kavanagh and Reeves, 2004). A variety of mechanisms including anti-microbial peptide production, melanisation, haemolymph clotting (Kavanagh and Reeves, 2004) mediate the insect humoral response. Various enzymes involved in xenobiotic metabolism including superoxide dismutase and catalase are highly conserved between species (Büyükgüzel *et al.*, 2013). The aim of this chapter was to determine the effect of potassium nitrate on the immune, enzymatic and proteomic responses, of *G. mellonella* larvae.

Control larvae administered PBS via intra haemocoel injection after 4 hours incubation showed significant increases ($p < 0.05$) in haemocyte densities when compared to untouched larvae at $t = 0$ hours. While control larvae administered PBS via intra haemocoel injection after 24 hour incubation showed significant decreases ($p < 0.05$) when compared to untouched larvae at $t = 0$ hours. Nodulation is induced in the absence of microbial agents by saline injection in cockroaches and locusts (Gunnarsson and Lackie, 1985). This might suggest why a significant increase in haemocyte densities was seen in control larvae after 4 hours. The significant decrease in haemocyte densities after 24 hours may be explained as a drop in haemocyte densities following a short peak.

Significant increases were seen in the haemocyte densities of control larvae that were administered PBS via force feeding after 4 ($p < 0.01$) and 24 ($p < 0.001$) hours incubation compared to untouched larvae at $t = 0$ hours. Haemocytes are found circulating freely in the haemolymph or adhering to internal organs such as the fat body or the digestive tract of the insect and can be rapidly mobilised upon breach of the cuticle or entry of a pathogen (Browne *et al.*, 2013). In the process of force feeding a blunted needle is inserted into the mouth and down the gastrointestinal

tract. The insertion of the needle into the gastrointestinal tract and the force of the PBS being expelled from the needle may result in the dislodgment of gastrointestinal attached haemocytes into the haemolymph thus explaining this increase.

Larvae were administered potassium nitrate by intra-haemocoel injection and incubated for 4 and 24 hours (Fig. 4.1). After 4 hours incubation, larval administration of the LD₂₅ of potassium nitrate by injection resulted in a significant reduction ($p < 0.001$) in haemocyte densities when compared to control larvae. However after 4 and 24 hours significant increases were also seen when larvae were administered potassium nitrate by intra-haemocoel injection. Different concentrations of xenobiotics can have different biological effects (Schulz *et al.*, 2012). Nitrates are broken down to nitric oxide *in-vivo* and the level of nitric oxide produced is thought to determine whether nitric oxide acts as a proinflammatory or anti-inflammatory mediator in mammals (Connelly *et al.*, 2017). Nitric oxide activates NF- κ B and induces the generation of proinflammatory cytokines such as tumour necrosis factor alpha (TNF α) (Connelly *et al.*, 2017). However, nitric oxide can also cause the inhibition of NF- κ B by upregulating the production of its inhibitor I κ B (Kuo *et al.*, 2000). The administration of varied concentrations of potassium nitrate can lead to varied production of nitric oxide. This therefore might suggest that nitric oxide production following potassium nitrate injection was having both a positive and negative effect on the haemocyte densities.

Larvae that were administered potassium nitrate via force feeding showed no significant differences in haemocyte counts when compared to controls (Fig. 4.2). Perhaps this maintenance of haemocyte density homeostasis in force fed larvae is due to the peritrophic membrane of the midgut, retarding the entry of the food additives into the haemocoel.

Administration of potassium nitrate to *G. mellonella* larvae by intra-haemocoel injection leads to significant changes in the density of circulating haemocytes but only small changes in the relative proportions of the haemocyte subpopulations. Larvae administered potassium nitrate by intra-haemocoel injection showed a significant decrease in the relative abundance of P1 haemocytes (small, granular cells). There was a small increase in the P2 (very small granular cells), P4 and

P5 (large and granular) populations and a small decrease in the population of P6 (large granular) population (Fig 4.5). Haemocytes from larvae administered potassium nitrate by intra-haemocoel injection showed a significant ($p < 0.05$) reduction in their fungicidal ability compared with haemocytes extracted from control larvae. The changes in the relative proportion of haemocyte sub-populations would be insufficient to account for this decline in fungicidal ability. It has been demonstrated that sodium nitrate inhibits the formation of reactive oxygen species by activated murine neutrophils and macrophages (Deriagina *et al.*, 2003) and inhibition of reactive oxygen species formation may be due to nitric oxide interference with the membrane component of the NADPH oxidase (Clancy *et al.*, 1992). In addition potassium nitrate was shown to have a significantly inhibitory effect on the oxidative respiratory burst and phagocytosis of isolated human neutrophils (Bano *et al.* 2014). Human neutrophils and insect haemocytes display many similarities (Browne *et al.*, 2013) including the ability to produce superoxide by a functional NADPH oxidase complex (Bergin *et al.*, 2005). Haemocytes from larvae injected with potassium nitrate may also display a reduced ability to kill *C. albicans* cells due to suppressed superoxide production.

Haemocytes from larvae administered potassium nitrate by force feeding showed no reduction in their fungicidal ability compared with haemocytes from control larvae. This again might be explained by the ability of the peritrophic membrane to retard the entry of compounds into the haemocoel (Fig. 4.3).

Nitrates interact with mitochondrial function and modulate oxidative stress (Lundberg *et al.*, 2011). A balanced antioxidant enzyme system against reactive oxygen/nitrogen species is present in cells; catalase reduces H_2O_2 to water and oxygen and superoxide dismutase catalyses the dismutation of superoxide radicals to H_2O_2 and oxygen, and appears to be the main response to dietary pro-oxidant exposure. There was no statistically significant change in the catalase activity in haemolymph of larvae administered potassium nitrate by intra hemocoel injection or force feeding and no statistically significant changes in the superoxide dismutase activity observed in larvae force fed potassium nitrate. In contrast, a significant increase in superoxide dismutase activity ($p < 0.05$) was observed in larvae

administered potassium nitrate by intra-haemocoel injection (Fig. 4.6). Sydow *et al.* (2004) reported that the administration of the organic nitrate nitroglycerin (GTN) increased the production of reactive oxygen species by mitochondria. In mammals the binding of nitric oxide to cytochrome c oxidase causes a depolarization of the inner mitochondrial membrane and combined with an increase in the reduction state of the electron transport chain results in the generation of superoxide anions, which are subsequently converted to hydrogen peroxide by superoxide dismutase (Moncada and Erusalimsky, 2002). Proteomic analysis also showed the increased abundance of a putative mitochondrial Mn superoxide dismutase (15.2 fold increase) in larvae that received potassium nitrate.

Proteomic analysis revealed the increased abundance of a mitochondrial aldehyde dehydrogenase (288.6 fold) ($p < 0.05$) in larvae challenged with potassium nitrate. Chen *et al.* (2002) purified a nitrate reductase from mouse RAW264.7 cells that specifically catalyses the formation of 1,2-glyceryl dinitrate (GDN) 1,3-GDN, inorganic nitrite and nitric oxide from organic nitrate (GTN), which mediates vasorelaxation. This nitrate reductase was identified as the redox sensitive enzyme mitochondrial aldehyde dehydrogenase and it was also demonstrated that rabbit aorta made tolerant to GTN, showed a significantly decrease in GTN reductase activity (Chen *et al.*, 2002). It has been previously shown that rabbits chronically treated with GTN demonstrate greater degrees of tolerance to GTN after three days exposure due to increased steady state concentrations of vascular O_2^- (Munzel *et al.*, 1995). The finding presented here showed not only a 300-fold ($p < 0.05$) increase of mitochondrial aldehyde dehydrogenase abundance but the significant increase ($p < 0.05$) of superoxide dismutase activity in larvae challenged with potassium nitrate. These findings would suggest that both mammals and insects metabolise nitrates in a similar manner.

In *G. mellonella* larvae treated with potassium nitrate there was an increase in the abundance of a number of mitochondrial-associated proteins (e.g. mitochondrial aldehyde dehydrogenase (288.6 fold increase), putative mitochondrial Mn superoxide dismutase (15.2 fold increase), isocitrate dehydrogenase (2.3 fold increase) and cytosolic malate dehydrogenase (46.2 fold increase). Multiple proteins

involved in glycolysis and the Krebs cycle were also shown to be increased in abundance such as triosephosphate isomerase (79.80 fold increase), glyceraldehyde 3 phosphate dehydrogenase (20.36 fold increase), enolase (62.8 fold increase) and isocitrate dehydrogenase (2.3 fold increase). Lundberg *et al.* (2011) reported that nitric oxide binds to cytochrome c oxidase, the terminal respiratory complex in the mitochondrial electron transport chain, in competition with oxygen, reducing oxygen costs and extending time to exhaustion, perhaps due to a reduction in ATP cost of muscle force production or as a direct effect of the improved metabolic efficiency. Nitric oxide, through its interaction with components of the electron transport chain, may act as a physiological regulator of cell respiration and the inhibition of cytochrome c oxidase by nitric oxide leads to an increase in the rate of glycolysis, ensuring a sufficient supply of glycolytic ATP to fuel the ATPase (Moncada and Erusalimsky, 2002). *G. mellonella* larvae challenged with potassium nitrate demonstrated an increase in proteins involved in oxidoreductase activity, together with significant increases in mitochondria associated proteins and proteins involved in glycolysis and the Krebs cycle suggesting that the response to potassium nitrate in larvae share similarities with the cell respiration regulatory responses of mammals challenged with potassium nitrate.

Endogenous levels of nitrates are necessary for the production of a diverse group of metabolites including nitric oxide, nitrosothiols and nitroalkenes and the subsequent regulation of vasodilation, blood pressure, inflammatory cell recruitment, and platelet aggregation (Hord, 2011). Dietary nitrate, nitrite and the amino acid L-arginine can serve as sources for the production of these metabolites via ultraviolet light exposure to skin, mammalian nitrate/nitrite reductases in tissues, and nitric oxide synthase enzymes (Hord, 2011). In larvae that received potassium nitrate there was an increase in the abundance of proteins associated with nitrate metabolism including mitochondrial aldehyde dehydrogenase (288.6 fold increase), aliphatic nitrilase (132.5 fold increase), enolase (62.4 fold increase) and glutathione S-transferase (20.6 fold increase). Chen *et al.*, (2002) showed that mitochondrial aldehyde dehydrogenase specifically catalyses the formation of 1,2-glyceryl dinitrate (GDN) 1,3- GDN, inorganic nitrite and NO from the organic nitrate nitroglycerin.

This chapter has demonstrated numerous similarities between the response of an insects and mammals to potassium nitrate challenge. However the dissimilarities must also be acknowledged. The main toxic effect of chronic nitrate is due to the conversion of haemoglobin to methaemoglobin which leads to methaemoglobinemia (Van Dijk *et al.*, 1983). The insect haemolymph is functionally similar to mammalian blood and functions in the transport of nutrients, waste products and signal molecules however, unlike mammalian blood, it does not function in respiration (Mellanby, 1939). Therefore the primary toxic effect of nitrates in mammals cannot be modelled in insects as they do not contain haemoglobin. In addition nitrates are almost 100% absorbed through the gastrointestinal tract in mammals (Ahluwalia *et al.* 2016). However the findings presented in this chapter suggested that potassium nitrate absorption through the gastrointestinal tract is retarded by peritrophic membrane in contrast to what happens in mammals.

There are numerous similarities between response of *G. mellonella* and mammals to potassium nitrate. The findings presented here validate the potential of *G. mellonella* as a candidate to test the toxicity of food potassium nitrate and other food additives.

Chapter 5

Analysis of the effects of caffeine administration on the behaviour and development of *Galleria mellonella* larvae

5.1 Analysis of the effects of caffeine administration on the behaviour and development of *Galleria mellonella* larvae.

The aim this Chapter was to characterise the effect of caffeine on the behaviour of insects as this might give an insight into the effect of the compound in mammals. The development of an insect based system for measuring the relative toxicity and mode of action of caffeine could contribute to reducing the need to use mammals for this type of testing and give a greater insight into its mode of action.

Caffeine (1,3,7-trimethylxanthine) is the most widely used central nervous system stimulant in the world and along with its metabolites, theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), is found in a wide range of plants (Porciúncula *et al.*, 2013). A third metabolite paraxanthine (1,7-dimethylxanthine) is not found in food, but is the main metabolite of caffeine *in vivo* (Aresta *et al.*, 2005). Over 60 plant species contain caffeine with the most widely used being coffee (*Coffea arabica*), kola nuts (*Cola acuminata*), tea (*Thea sinensis*), and chocolate (Cocoa bean) (Vanderveen *et al.*, 2001) and it is also available in the synthetic form. Caffeine competitively binds to adenosine receptors allowing it to act as an antagonist with inverse agonist activity (Vanderveen *et al.*, 2001). Caffeine affects cAMP signalling both by binding to adenosine receptors and by competitively inhibiting the activity of phosphodiesterases which function to degrade cAMP (Vanderveen *et al.*, 2001). Caffeine can also bind to intracellular calcium-channel ryanodine receptors resulting in the release of intracellular Ca^{2+} (Bhat *et al.*, 1997). Caffeine can stimulate dopaminergic activity by removing the negative modulatory effects of adenosine at dopamine receptors (Ferré, 2008). In addition to the effect caffeine has on adenosine receptors, studies have also suggested that paraxanthine, the primary metabolite of caffeine in humans, produces increased locomotive activity as well as a phosphodiesterase inhibitory effect which in turn results in increased extracellular levels of dopamine (Orrú and Guitart, 2013). The consumption of a low to moderate doses of caffeine is generally regarded as safe (daily intake of no more than 400 mg in healthy adults) however the consumption of higher doses by vulnerable individuals can result in an increased risk of negative health consequences such as cardiovascular and perinatal

complications (Meredith *et al.*, 2013). The most dominant route of consumption is by oral intake but injection of caffeine or caffeine citrate is also available.

The effect of caffeine on physiology and behaviour of animals is widely studied in invertebrates as well as vertebrates (Cruz *et al.*, 2017). In *Apis mellifera* caffeine modulates learning and memory; it specifically affects performance during acquisition but not the processes involved in the formation of long term memory (Mustard *et al.*, 2012). In *Drosophila* chronic administration of caffeine reduced and fragmented sleep and also lengthened the circadian period (Wu *et al.*, 2009). Administration of caffeine to Zebrafish (*Danio rerio*) embryos at concentrations above 300 ppm proved lethal, but lower concentrations resulted in shorter body length and reduced tactile sensitivity (Chen *et al.*, 2008). This latter effect was due to the misalignment of muscle fibres and motor neurone defects in treated embryos. Zebra fish embryos also showed reduced mobility when exposed to caffeine possibly as a result of decreased expression of adenosine receptors (Cruz *et al.*, 2017).

The aim of the work presented here was to characterise the effect of caffeine on the behaviour of insects as this might give an insight into the effect of the compound in mammals and vertebrate species.

For experimental purposes larvae were administered 20 µl of 0.1 M of caffeine, as this was the highest concentration that could be achieved in solution without resulting in larval death.

5.2 Effect of caffeine administration on haemocyte densities.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.1 M of caffeine. The effect of the food additives administration on the density of circulating haemocytes in larval haemolymph was ascertained after 4 and 24 hour incubation at 30°C (Fig. 5.1). For experimental purposes control larvae were administered PBS by intra haemocoel injection or force feeding.

Larvae force fed PBS and incubated for 4 or 24 hours at 30°C showed $0.96 \pm 0.2 \times 10^7$ and $0.97 \pm 0.05 \times 10^7$ haemocytes/ml respectively. Larvae force fed caffeine and incubated for 4 hours showed $1.5 \pm 0.43 \times 10^7$ haemocytes/ml. This equated to a 0.56 fold increase in haemocyte densities between control larvae and caffeine force fed larvae. Larvae force fed caffeine and incubated for 24 hours showed $1.3 \pm 0.22 \times 10^7$ haemocytes/ml. This equated to a 0.34 fold increase ($p < 0.05$) in haemocyte densities between control larvae and caffeine force fed larvae (Fig. 5.1).

Larvae injected with PBS and incubated for 4 or 24 hours at 30°C showed $1.16 \pm 0.1 \times 10^7$ and $0.71 \pm 0.13 \times 10^7$ haemocytes/ml respectively. Larvae injected caffeine and incubated for 4 hours showed $1.7 \pm 0.08 \times 10^7$ haemocytes/ml. This equated to a 0.47 fold increase ($p < 0.01$) in haemocyte densities between control larvae and caffeine injected larvae. Larvae injected with caffeine and incubated for 24 hours showed $1.2 \pm 0.18 \times 10^7$ haemocytes/ml. This equated to a 0.69 fold increase ($p < 0.05$) in haemocyte densities between control larvae and caffeine injected larvae (Fig. 5.1).

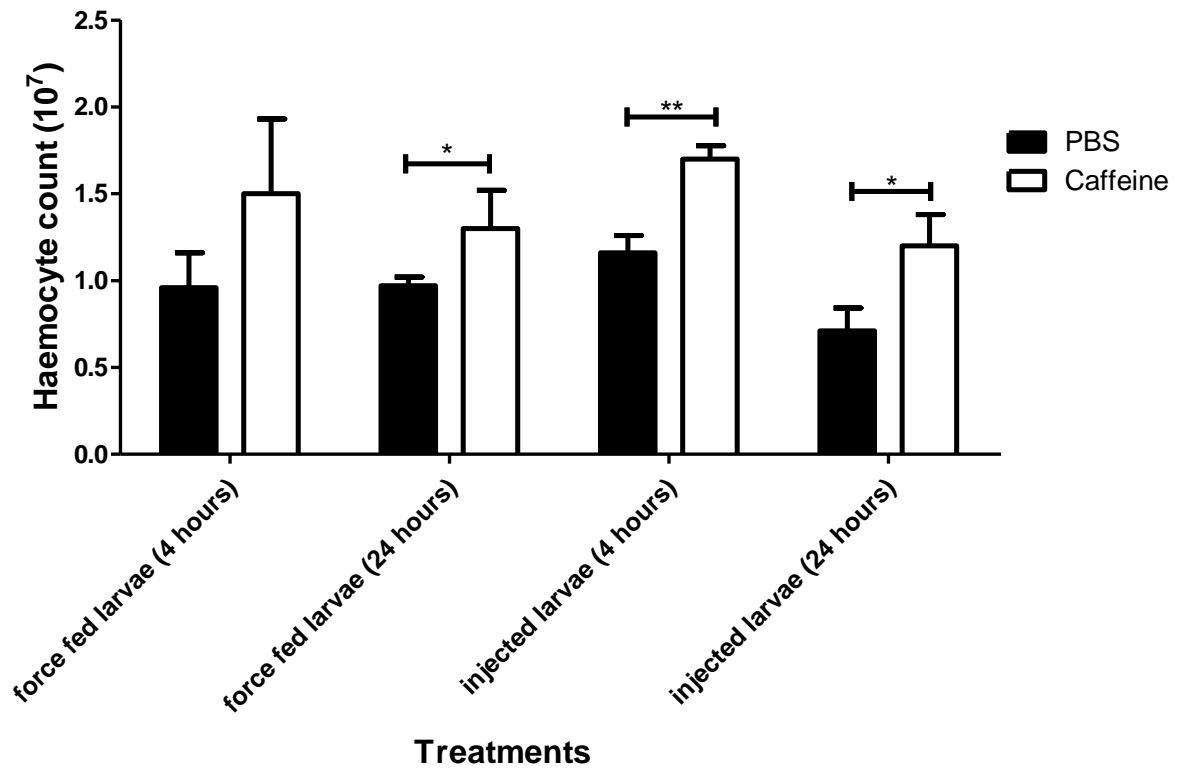


Fig. 5.1 Effect of caffeine on haemocyte density in force fed and intra-haemocoel injected *G. mellonella* larvae at 4 and 24 hours. (*: $p < 0.05$) (**: $p < 0.01$). All values are the mean \pm SE of 3 independent determinations.

5.3 Analysis of the effect of caffeine administration on haemocyte mediated pathogen killing.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.1 M of caffeine. Haemocytes were extracted from control and treated larvae following 24-hour incubation at 30°C and mixed with opsonised *C. albicans* for 80-minute (Fig. 5.2). By counting the percentage viability of *C. albicans* at 20 min intervals, the effect of these food additives on haemocyte mediated pathogen killing was ascertained.

Haemocytes from PBS force fed control larvae killed 22.52 \pm 18.5%, 43.76 \pm 21.81% , 43.01 \pm 28.29%, 67.23 \pm 17.96% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. Haemocytes from caffeine force fed larvae killed 39.97 \pm 16.48%, 54.48 \pm 27.10%, 61.75 \pm 29.36%, 75.69 \pm 23.12% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 5.2).

Haemocytes from PBS injected control larvae killed 34.33 \pm 21.14%, 50 \pm 17.79%, 67.93 \pm 21.26%, 76.33 \pm 18.29% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. In addition haemocytes from caffeine injected larvae killed 34.09 \pm 20.84%, 36.57 \pm 14.68%, 58.17 \pm 6.26%, 62.59 \pm 4.37% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 5.2).

The results indicated no significant changes between the killing ability of haemocytes from control larvae and larvae administered caffeine by intra-haemocoel injection or force feeding.

5.4 Summary

Larvae were administered caffeine by force feeding and intra-haemocoel injection. Larvae that were administered caffeine by intra-haemocoel injection showed significant increases in haemocyte densities after 4 ($p < 0.05$) and 24 ($p < 0.01$) hour incubation. Larvae that were administered caffeine by force feeding showed significant increases ($p < 0.05$) in haemocyte densities after 24 hour incubation when compared to their relevant controls.

There were no significant changes between the fungicidal ability of haemocytes from control or treated larvae (Fig. 5.2).

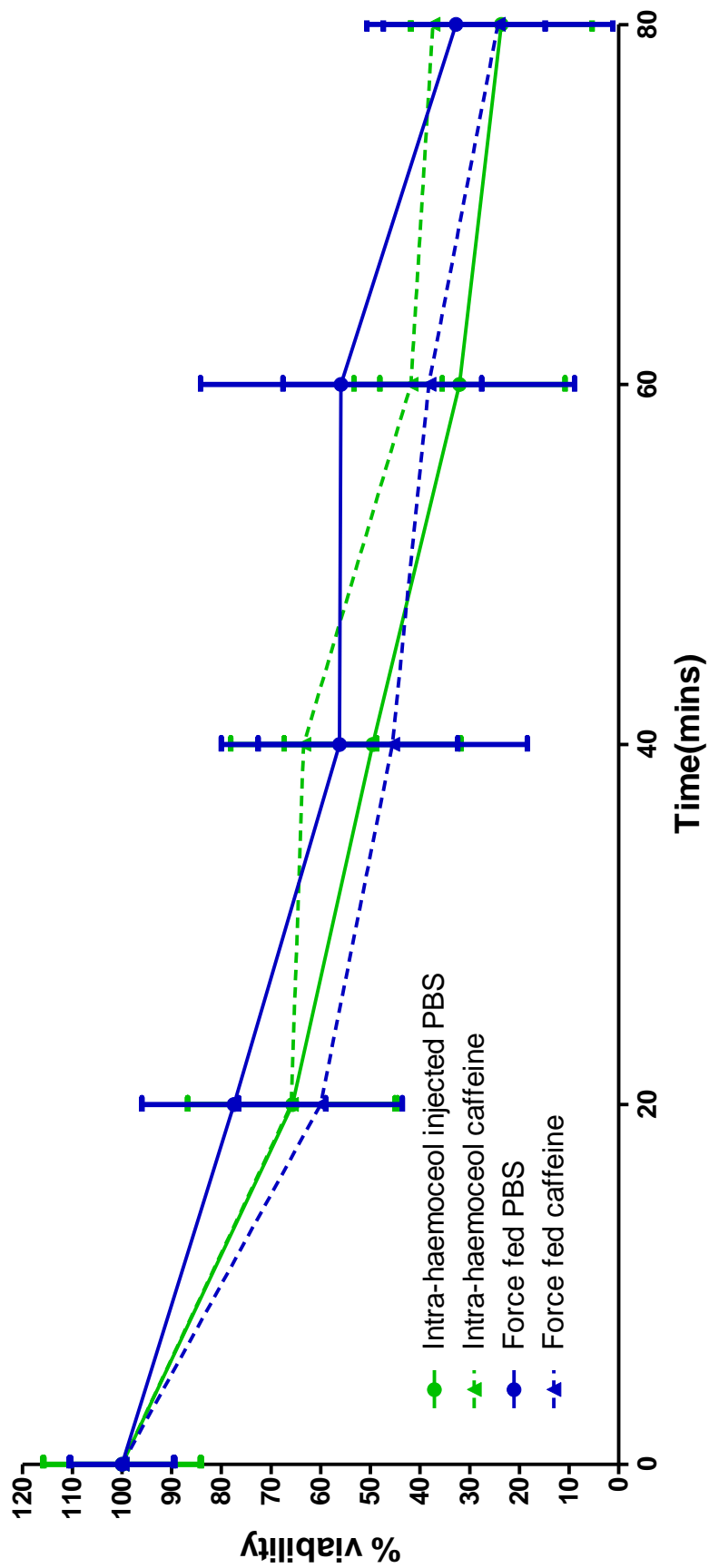


Fig. 5.2 Fungicidal activity of haemocytes from larvae administered caffeine by intra-haemocoel injection or force feeding at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined. All values are the mean \pm SE of 3 independent determinations.

5.5 Effects of caffeine on superoxide dismutase activity of the haemolymph.

Superoxide dismutase activity in the haemolymph of larvae administered caffeine was measured. Larvae were administered 20 μl of 0.1 M of caffeine by intra-haemocoel injection or force feeding. Larvae were incubated at 30°C for 4 and 24 hours post administration of caffeine and the total superoxide dismutase activity was measured in larval haemolymph (Fig. 5.3, 5.4). Superoxide dismutase activity was quantified as units of activity, 1 unit of superoxide dismutase activity equated to the 50% inhibition of the oxidation of quercetin.

One unit of superoxide dismutase activity in PBS force fed control larvae equated to $109.03 \pm 35.54 \mu\text{g}/\mu\text{l}$ and $122.78 \pm 5.35 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 4 or 24-hours incubation. In addition 1 unit of superoxide dismutase activity in PBS injected control larvae equated to $99.43 \pm 24.50 \mu\text{g}/\mu\text{l}$ and $137.68 \pm 32.24 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 4 or 24 hours incubation (Fig. 5.3, 5.4).

Superoxide dismutase activity was also measured in haemolymph from larvae administered caffeine via force feeding prior to incubation. At 4 and 24 hours one unit of superoxide dismutase activity was represented as $92.42 \pm 28.88 \mu\text{g}/\mu\text{l}$ and $145.63 \pm 40.83 \mu\text{g}/\mu\text{l}$ of protein. Superoxide dismutase activity was also measured in larvae administered caffeine via intra-haemocoel injection prior to incubation. At 4 and 24 hours one unit of superoxide dismutase activity was represented as $119.48 \pm 28.31 \mu\text{g}/\mu\text{l}$ and $152.64 \pm 30.64 \mu\text{g}/\mu\text{l}$ of protein (Fig. 5.3, 5.4).

These findings revealed no significant differences between the superoxide dismutase activity in haemolymph extracted from test larvae and control larvae at 4 or 24-hours.

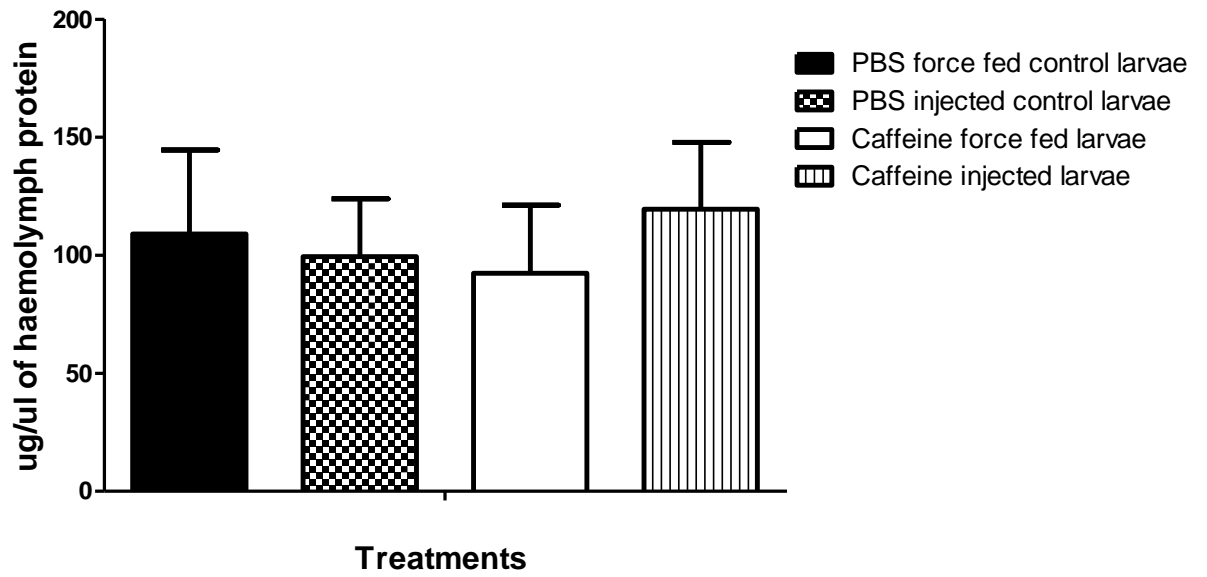


Fig. 5.3 Superoxide dismutase activity of haemolymph from larvae administered Caffeine via intra-haemocoel injection and force feeding. After 4 hours haemolymph was extracted from larvae that were administered caffeine. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

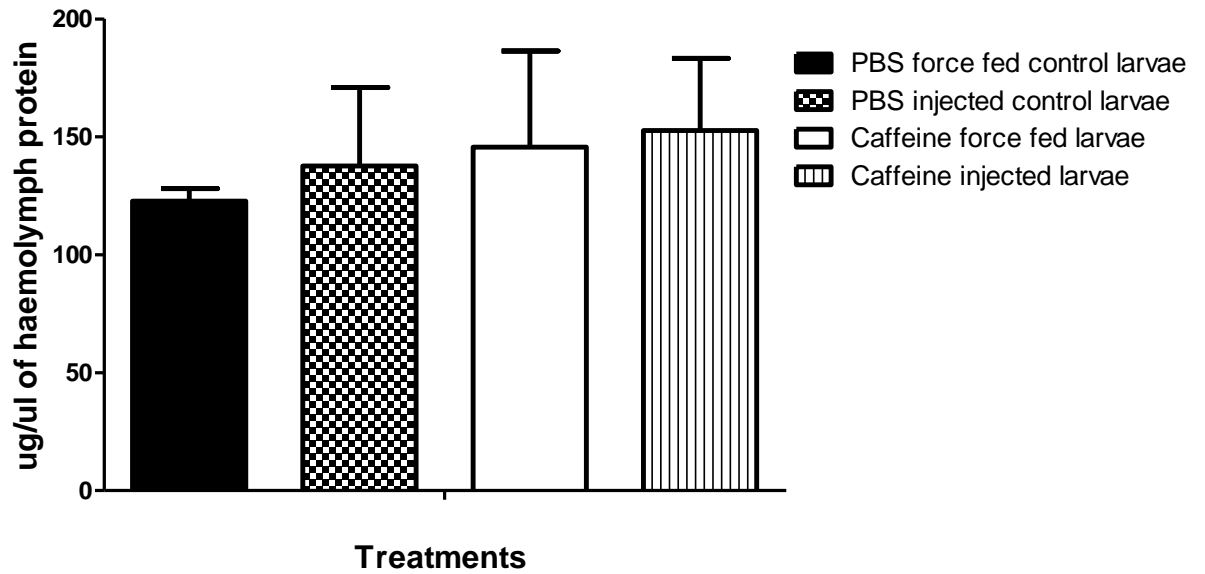


Fig. 5.4 Superoxide dismutase activity of haemolymph from larvae administered caffeine via intra-haemocoel injection and force feeding. After 24 hours haemolymph was extracted from larvae that were administered caffeine. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

5.6 Effects of caffeine on catalase activity in *G. mellonella* haemolymph

Larvae were administered by intra haemocoel injection or force feeding 20 μ l 0.1 M of caffeine. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with hydrogen peroxide. The decomposition of hydrogen peroxide and thus rate of catalase activity was measured spectrometrically at 240 nm (Fig. 5.5, 5.6). Catalase activity in larvae administered caffeine was expressed as the fold change compared with their relevant control groups administered PBS.

The catalase activity in larvae force fed PBS was measured as 0.36 ± 0.04 Abs and 0.31 ± 0.05 Abs following 4 and 24 hours incubation respectively. In addition the catalase activity in larvae administered PBS by intra-haemocoel injection was measured as 0.39 ± 0.04 Abs and 0.28 ± 0.07 Abs following 4 and 24 hour incubation respectively.

The catalase activity in caffeine fed larvae was measured as 0.37 ± 0.03 Abs and 0.30 ± 0.06 Abs after 4 and 24 hours incubation respectively. These results showed no significant changes between control and treated groups (Fig. 5.5)

Larvae were administered caffeine via intra-haemocoel injection. The catalase activity in these larvae was measured as 0.42 ± 0.01 Abs and 0.35 ± 0.02 Abs after 4 and 24 hours incubation respectively. Similar to the previous route of administration these results showed no significant changes between control and treated groups (Fig 5.6).

5.7 Summary

The activity of two anti-oxidant enzymes, superoxide dismutase and catalase was measured in the haemolymph of larvae challenged with caffeine via force feeding or direct injection into the haemocoel. No significant changes were recorded in the activity of either anti-oxidant enzyme between test and control larvae administered PBS.

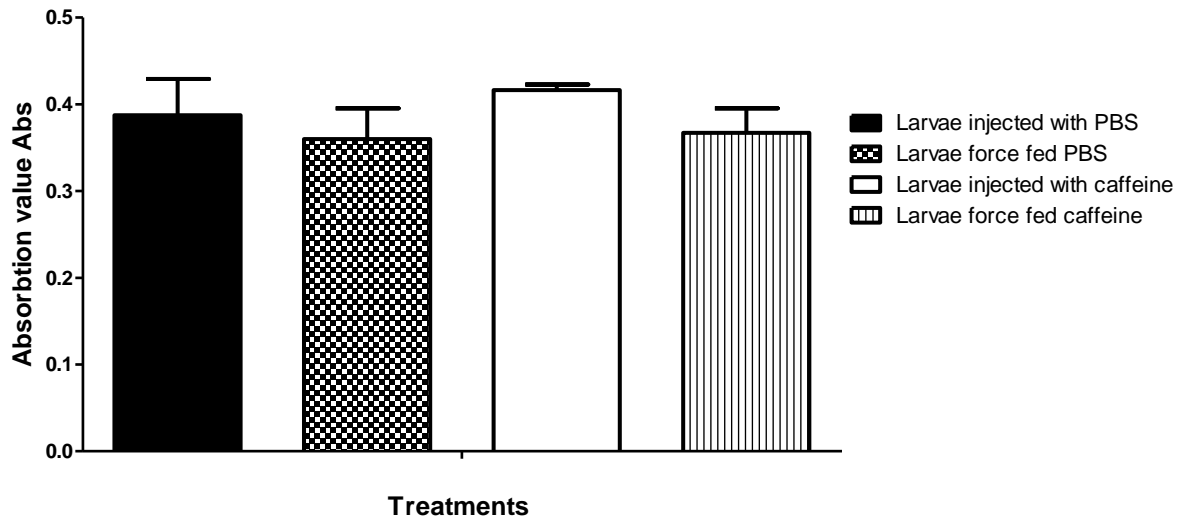


Fig. 5.5 Catalase activity of haemolymph from larvae administered caffeine via force feeding and intra-haemocoel injection. After 4 hours haemolymph was extracted from larvae that were administered caffeine. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

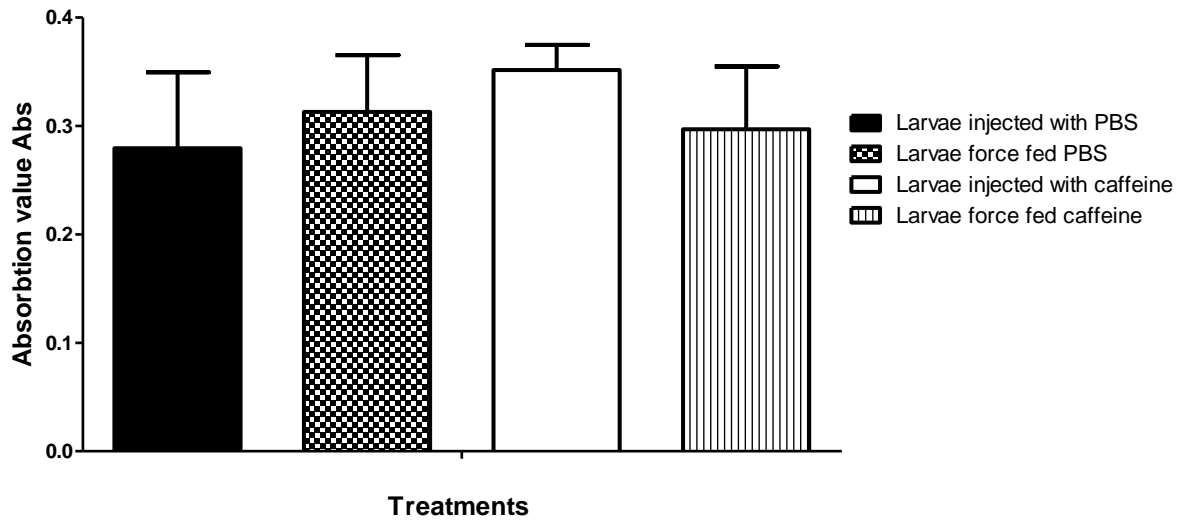


Fig. 5.6 Catalase activity of haemolymph from larvae administered caffeine via force feeding and intra-haemocoel injection. After 24 hours haemolymph was extracted from larvae that were administered caffeine. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

5.8 Analysis of caffeine metabolism in *G. mellonella* larvae.

Larvae were administered 20 µl of 0.08 M of caffeine via direct injection into the haemocoel or force feeding and haemolymph was extracted at 0, 4, 24 and 48 hours. Caffeine and its associated metabolites were extracted from haemolymph at each time point, samples were loaded on an Agilent C-18 column and quantified as described by RP-HPLC

The caffeine level in force fed larvae were 150.89 ± 36.33 µg/ml, 142.33 ± 11.75 µg/ml, 73.52 ± 0.34 µg/ml and 75.66 ± 12.66 µg/ml at t = 0, 4, 24 and 48 hours respectively (Fig. 5.7). This amounts to a 0.06 fold decrease at 4 hours, a 0.51 fold decrease ($p < 0.01$) at 24 hours, and a 0.50 fold decrease ($p < 0.01$) at 48 hours when compared to the caffeine levels of larvae at t = 0 hours. At t = 0 hours theobromine and theophylline were not detected. However theophylline levels at t = 4 hours were 18.94 ± 2.17 µg/ml, at t = 24 hours were 2.88 ± 0.25 µg/ml, and at t = 48 were 0.87 ± 0.16 µg/ml (Fig. 5.8). This amounts to a significant increase in theophylline levels at t = 4 hours ($p < 0.001$) and at t = 24 hours ($p < 0.05$) when compared to levels at t = 0 hour. Theobromine levels at t = 4 were 4.06 ± 0.45 µg/ml, at t = 24 were 5.76 ± 0.46 µg/ml, and at t = 48 were 9.11 ± 1.82 µg/ml. This equated to significant increases in theobromine levels at t = 4 hours ($p < 0.01$), at t = 24 hours ($p < 0.01$) and at t = 48 hours ($p < 0.001$) when compared to levels at t = 0 hours (Fig. 5.11).

The caffeine level in intra-haemocoel injected larvae were 161.91 ± 50.64 µg/ml, 144.92 ± 55.13 µg/ml, 35.91 ± 30.93 µg/ml and 18.86 ± 5.55 µg/ml at t = 0, 4, 24 and 48 hours respectively (Fig. 5.10). This amounts to a 0.10 fold decrease at 4 hours, a 0.78 fold decrease ($p < 0.05$) at 24 hours, and a 0.88 fold decrease ($p < 0.05$) at 48 hours when compared to the caffeine levels of larvae at t = 0 hours. At t = 0 hours theobromine and theophylline were not detected. However theophylline levels at t = 4 hours were 15.53 ± 4.70 µg/ml, at t = 24 hours were 4.56 ± 0.29 µg/ml, and at t = 48 hours were 3.83 ± 4.09 µg/ml. This amounts to a significant increase in theophylline levels at t = 4 hours ($p < 0.01$) when compared to levels at t = 0 hours (Fig. 5.11). Theobromine levels at t = 4 hours were 18.59 ± 13.57 µg/ml, at t = 24 hours were 19.94 ± 1.26 µg/ml, and at t = 48 hours were 7.31 ± 0.64 µg/ml.

This equated to significant increases in theobromine levels at t = 4 hours ($p < 0.05$) and at t = 24 hours ($p < 0.05$) when compared to levels at t = 0 hours (Fig. 5.12).

5.9 Summary

Larvae were administered 20 μl of 0.08 M of caffeine via intra-haemocoel injection or force feeding. Caffeine and its associate metabolites, theobromine and theophylline were measured by RP-HPLC analysis in the haemolymph of caffeine treated larvae at t = 0, 4, 24 and 48 hours.

Haemolymph from larvae that were administered caffeine by force feeding showed significant decreases in caffeine levels at t = 24 hours ($p < 0.01$) and t = 48 hours ($p < 0.01$) when compared to t = 0 hours. Theobromine and theophylline were not detected in haemolymph at t = 0 hours. However Theophylline and theobromine was detected in haemolymph at t = 4, 24 and 48 hours. The concentration of theophylline and theobromine peaked at t = 4 and t = 48 hours respectively.

Haemolymph from larvae that were administered caffeine by intra-haemocoel injection showed significant decreases in caffeine levels at t = 24 hours ($p < 0.05$) and t = 48 hours ($p < 0.05$) when compared to t = 0 hours. Theobromine and theophylline were not detected in haemolymph at t = 0 hours. However theophylline and theobromine were detected in haemolymph at t = 4, 24 and 48 hours. The concentration of theophylline and theobromine peaked at t = 4 hours and t = 48 hours respectively.

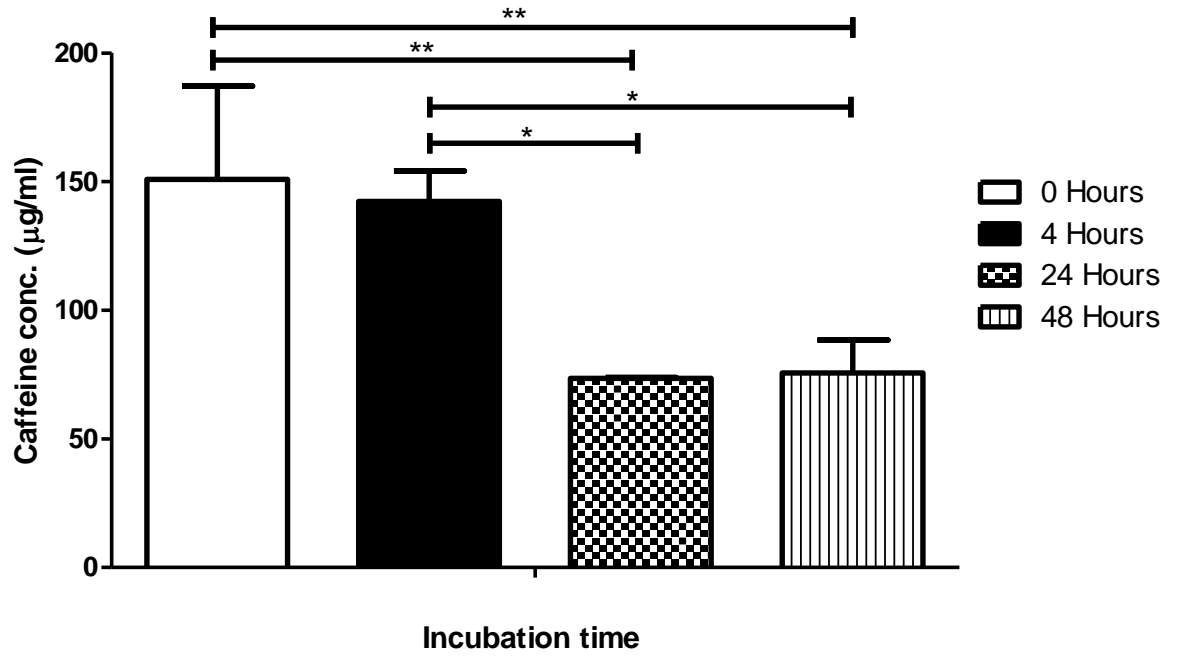


Fig 5.7 Analysis of the metabolism of caffeine in force fed *G. mellonella* larvae. The presence of caffeine in haemolymph was detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05)(** :p < 0.01). All values are the mean \pm SE, n = 15.

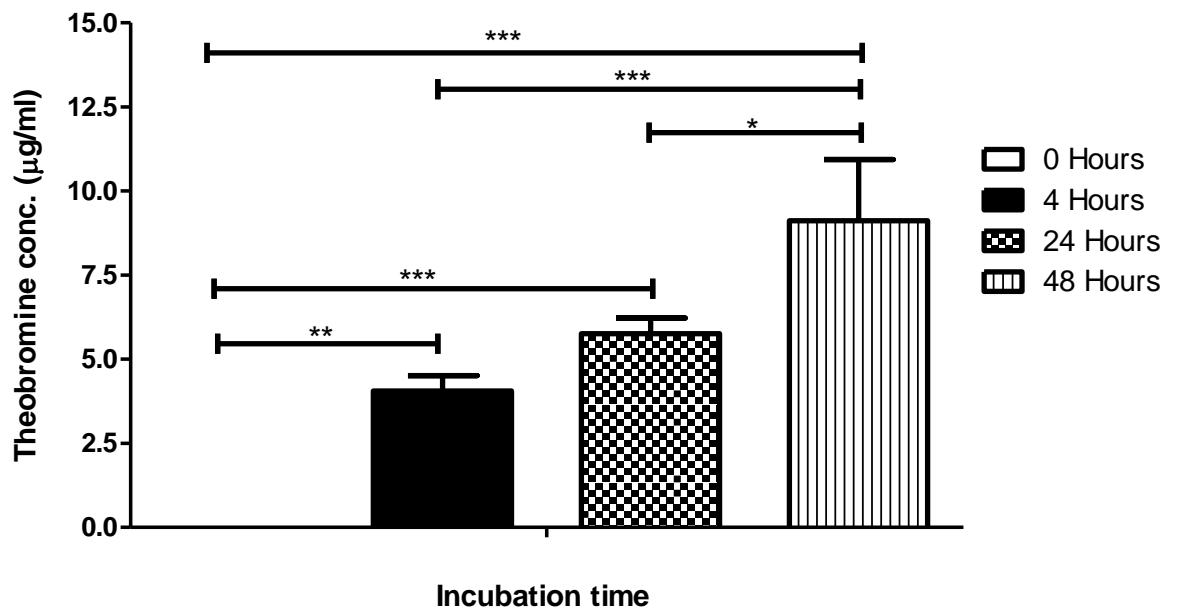


Fig. 5.8 Presence of theobromine in caffeine fed larvae. The presence of theobromine in haemolymph were detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05)(** :p < 0.01)(***:p < 0.001). All values are the mean \pm SE, n = 15.

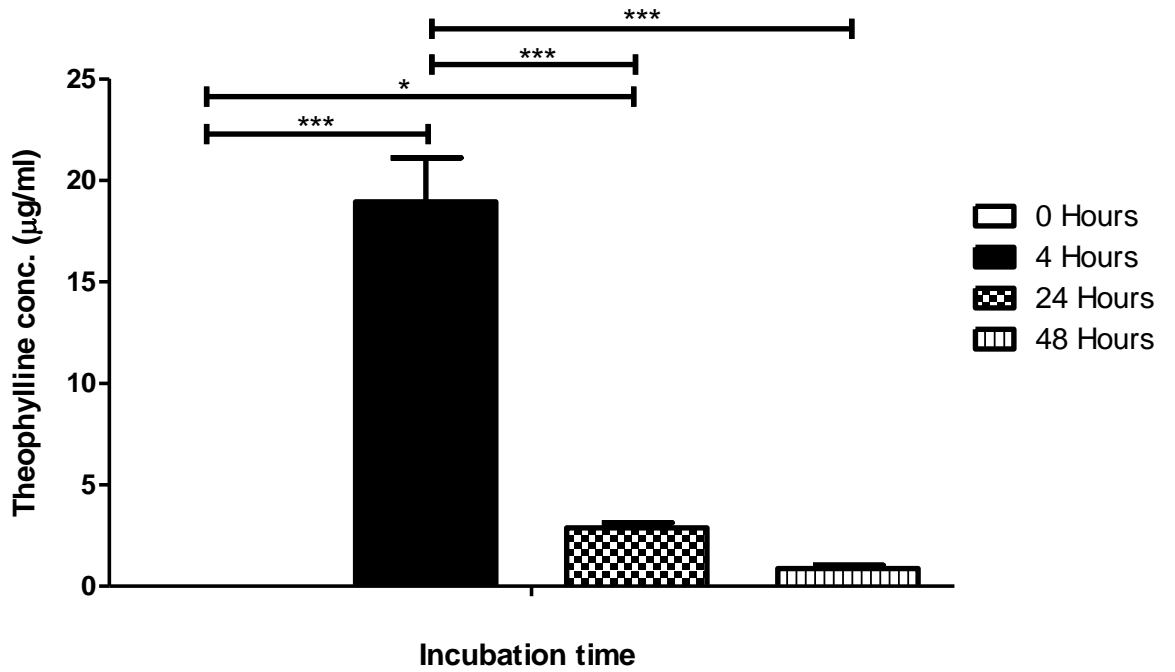


Fig 5.9 Presence of theophylline in caffeine fed larvae. The presence of theophylline in haemolymph were detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05)(***:p < 0.001). All values are the mean \pm SE, n = 15.

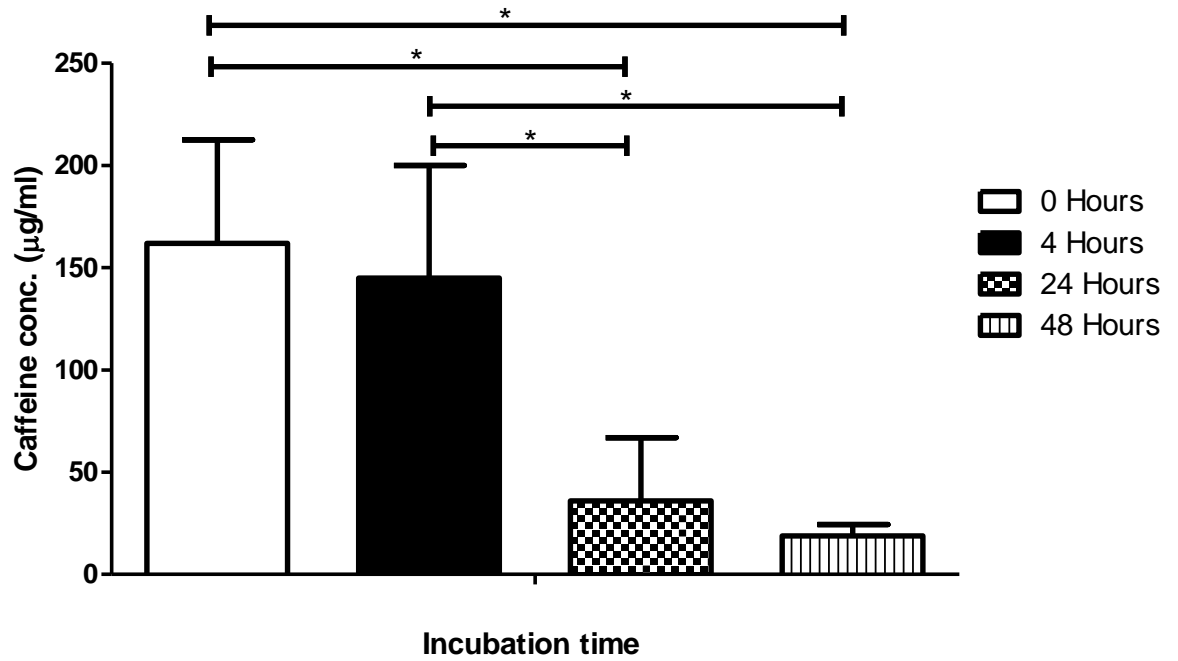


Fig. 5.10 Analysis of the metabolism of caffeine in intra-haemocoel injected *G. mellonella* larvae. The presence of caffeine in haemolymph were detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05). All values are the mean \pm SE, n = 15.

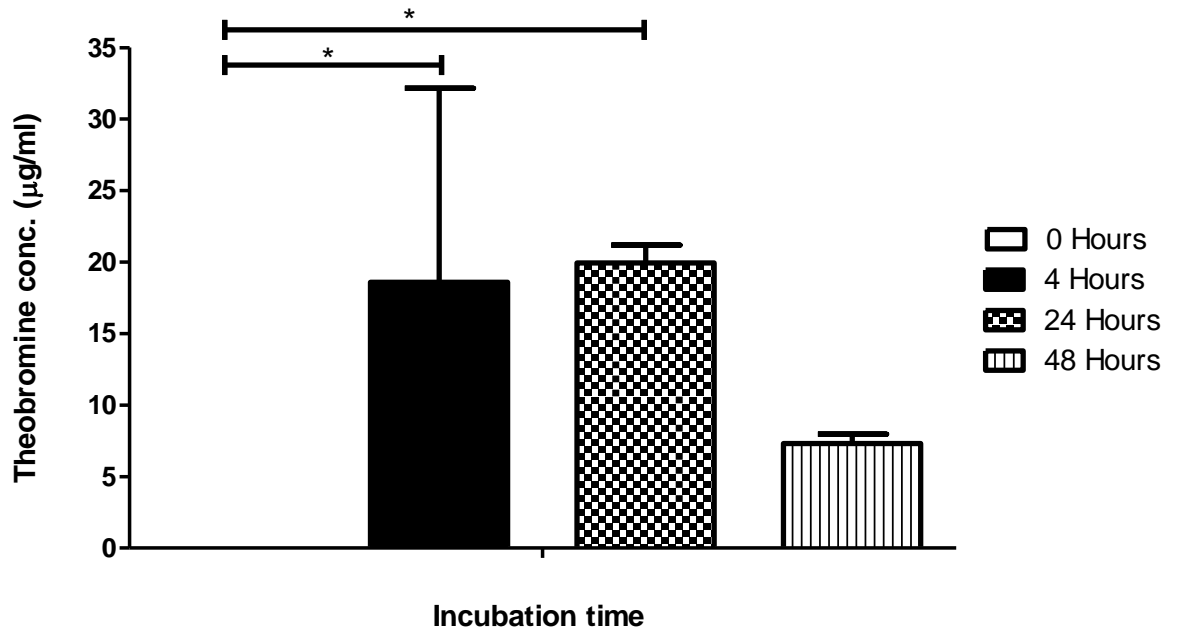


Fig. 5. 11 Presence of theobromine in caffeine injected larvae. The presence of theobromine in haemolymph were detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05). All values are the mean \pm SE, n = 15.

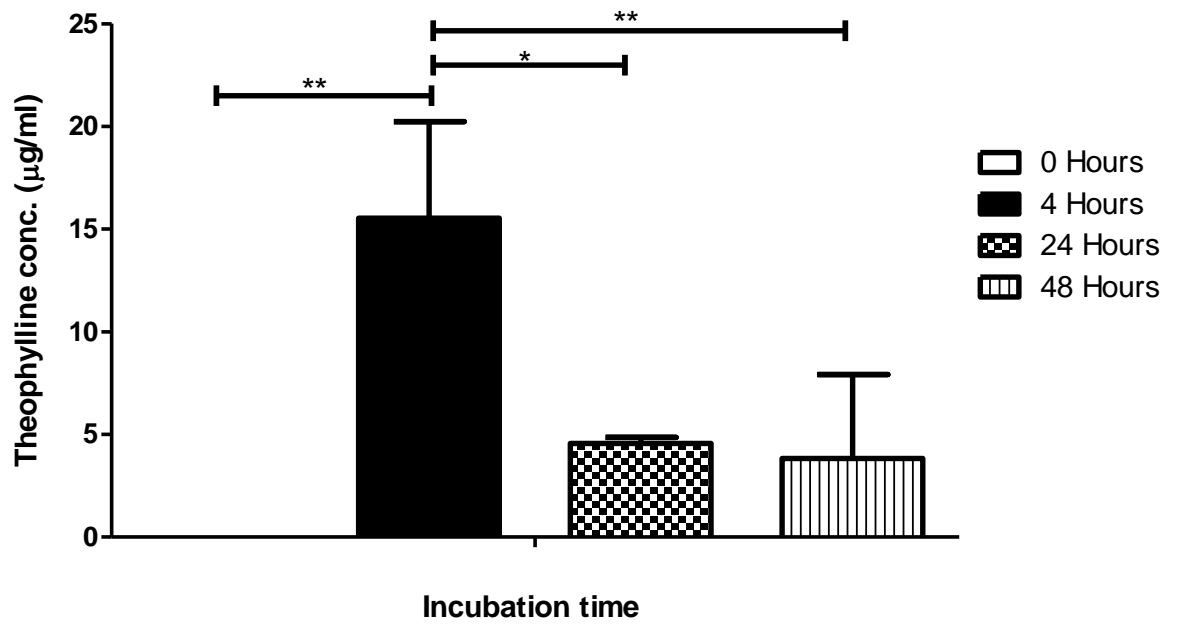


Fig 5.12 Presence of theophylline in caffeine injected larvae. The presence of theophylline in haemolymph were detected by RP-HPLC at 0, 4, 24 and 48 hours (*:p < 0.05) (**:p < 0.01). All values are the mean \pm SE, n = 15.

5.10 Effect of caffeine on larval movement.

Larvae were administered 20 μ L of 0.1 M of caffeine by force feeding and the effect on movement was monitored as described. Larvae were placed on their dorsal surface and the time to reposition correctly was recorded.

Control larvae force fed PBS took 3 ± 0 seconds to reposition and larvae administered caffeine at $t = 0$ hours took 3 ± 0 seconds to reposition. However larvae administered caffeine at $t = 1$ hour took 254 ± 251 seconds to reposition. This increased to 319 ± 247.61 seconds at 4 hours and to 309 ± 299 seconds at 24 hours. The time to reposition declined at 48 hours to 126 ± 243 seconds and to 35.53 ± 136 seconds at 72 hours. Significant increases were observed between larvae force fed PBS and larvae incubated for 4 ($p < 0.001$) and 24 hours ($p < 0.001$) post caffeine administration. Significant increases were also observed between larvae incubated for 1 hour and 72 hours ($p < 0.05$) (Fig. 5.13).

Control larvae injected with PBS took 3 ± 0 seconds to reposition and larvae administered caffeine at $t = 0$ took 81.64 ± 160.06 seconds to reposition. However larvae administered caffeine at $t = 1$ hour took 246.37 ± 244.76 seconds to reposition. This increased to 318 ± 265.90 seconds at 4 hours and decreased to 217.07 ± 288.72 seconds at 24 hours. The time to reposition declined at 48 hours to 40.68 ± 135.92 seconds and to 35.11 ± 136 seconds at 72 hours. Significant increases were observed between larvae injected with PBS and larvae incubated for 1 ($p < 0.01$), 4 ($p < 0.001$) and 24 hours ($p < 0.05$) post-caffeine administration. Significant increases were also observed when larvae incubated for 1 hour where compared to larvae incubated for 48 ($p < 0.05$) and 72 hours ($p < 0.05$) (Fig. 5.14).

For more detailed analysis work was carried out in collaboration with Martin Kunc and Dr. Pavel Hyršl (Masarykova University Czech Republic), larvae were placed on the FIMTrack table surface, images were acquired (Fig. 5.15, 5.16). and distance travelled and velocity were recorded.

Larvae were administered 20 μ l of 0.1 M caffeine by force feeding and placed on the FIMTrack table surface the distance travelled was recorded. Significant decreases in velocity and distance travelled were observed at 1, 4, 24

and 48 hours after administration of caffeine compared with control larvae. The distance travelled by control larvae force fed PBS was 108.02 ± 45.60 cm, 160.31 ± 56.60 cm, 215.20 ± 61.47 cm, 215.03 ± 67.81 cm, 177.70 ± 58.29 cm and 125.33 ± 44.60 cm at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. The distance travelled by larvae force fed caffeine was 99.23 ± 46.10 cm, 67.91 ± 24.36 cm, 64.53 ± 21.73 cm, 52.38 ± 13.26 cm, 51.49 ± 21.24 cm and 99.23 ± 46.1 cm at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. Significant decreases in distance travelled were observed at $t = 1, 4, 24$ and 48 hours ($p < 0.001$) after administration of caffeine compared with control larvae (Fig. 5.17).

Larvae were administered $20 \mu\text{L}$ of 0.1 M of caffeine by force feeding and placed on the FIMTrack table surface and the velocity of larvae was recorded. The velocity of larvae force fed PBS was 6.22 ± 2.27 mm/s, 9.11 ± 2.88 mm/s, 9.42 ± 3.67 mm/s, 12.12 ± 3.01 mm/s, 7.53 ± 3.32 mm/s and 7.03 ± 2.05 mm/s at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. The velocity of larvae force fed caffeine was 5.52 ± 1.53 mm/s, 5.30 ± 1.99 mm/s, 2.65 ± 1.52 mm/s, 4.01 ± 1.00 mm/s, 2.41 ± 1.30 mm/s and 5.15 ± 2.15 mm/s at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. Significant decreases in velocity were observed at 1 ($p < 0.01$), 4 ($p < 0.001$), 24 ($p < 0.001$) and 48 hours ($p < 0.001$) after administration of caffeine compared with control larvae (Fig. 5.18).

Larvae were administered $20 \mu\text{L}$ of 0.1 M of caffeine by intra-haemocoel injection and placed on the FIMTrack table surface the distance travelled and velocity were recorded. The distance travelled by control larvae injected with PBS was 170.90 ± 70.17 cm, 238.94 ± 53.60 cm, 251.26 ± 55.53 cm, 215.43 ± 66.67 cm, 190.97 ± 38.96 cm and 150.18 ± 57.34 cm at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. The distance travelled by larvae injected with caffeine was 37.81 ± 12.50 cm, 59.12 ± 25.12 cm, 57.00 ± 19.07 cm, 54.78 ± 17.16 cm, 84.26 ± 57.4 cm and 133.92 ± 54.03 cm at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. Significant decreases in distance travelled were observed at $t = 0, 1, 4, 24$ and 48 hours ($p < 0.001$) after administration of caffeine compared with control larvae (Fig. 5.19).

Larvae were administered $20 \mu\text{l}$ 0.1 M caffeine by intra-haemocoel injection and were placed on the FIMTrack table surface and the velocity of larvae was

recorded. The velocity of larvae injected with PBS was 4.26 ± 2.63 mm/s, 10.90 ± 3.69 mm/s, 5.76 ± 2.68 mm/s, 10.97 ± 2.28 mm/s, 7.78 ± 1.69 mm/s and 5.42 ± 2.12 mm/s at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. The velocity of larvae injected with caffeine was 0.40 ± 0.14 mm/s, 2.47 ± 1.21 mm/s, 0.98 ± 0.65 mm/s, 4.13 ± 1.10 mm/s, 3.64 ± 2.23 mm/s and 5.32 ± 2.49 mm/s at $t = 0, 1, 4, 24, 48$ and 72 hours respectively. Significant decreases in velocity were observed at $t=0$ ($p < 0.01$), 1 ($p < 0.001$), 4 ($p < 0.001$), 24 ($p < 0.001$) and 48 hours ($p < 0.001$) after administration of caffeine compared with control larvae (Fig. 5.20).

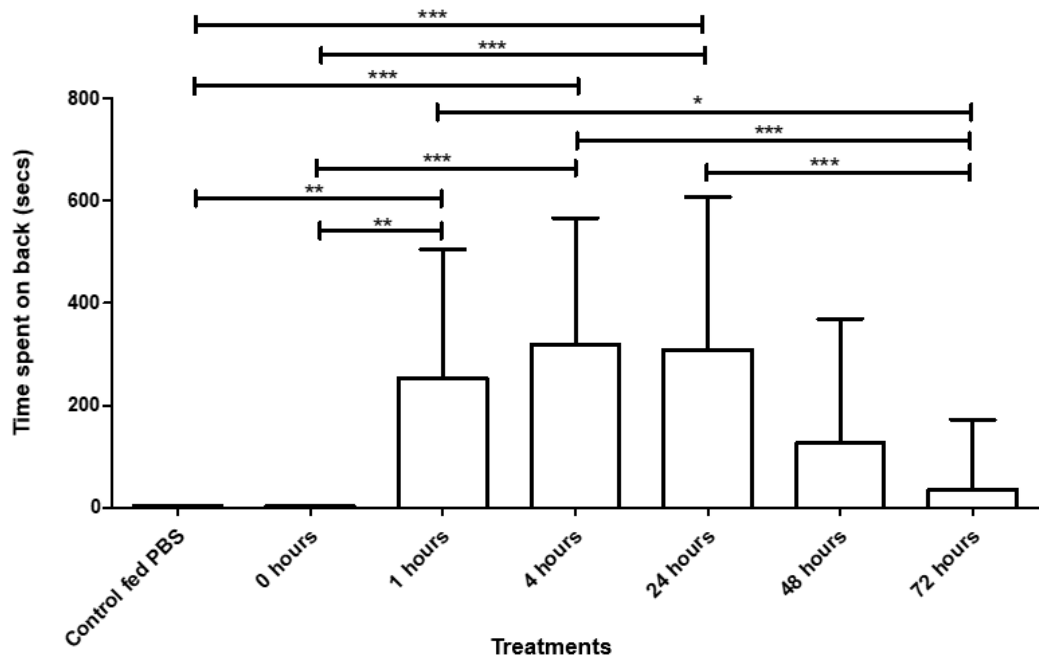


Fig. 5.13 Effect of caffeine on ability of *G. mellonella* larvae to move from their ventral to dorsal surface. Larvae were positioned on their ventral surface after administration of caffeine via force feeding and the time to reposition correctly was recorded (*:p < 0.05) (**:p < 0.01) (***:p < 0.001). All values are the mean \pm SE, n = 20.

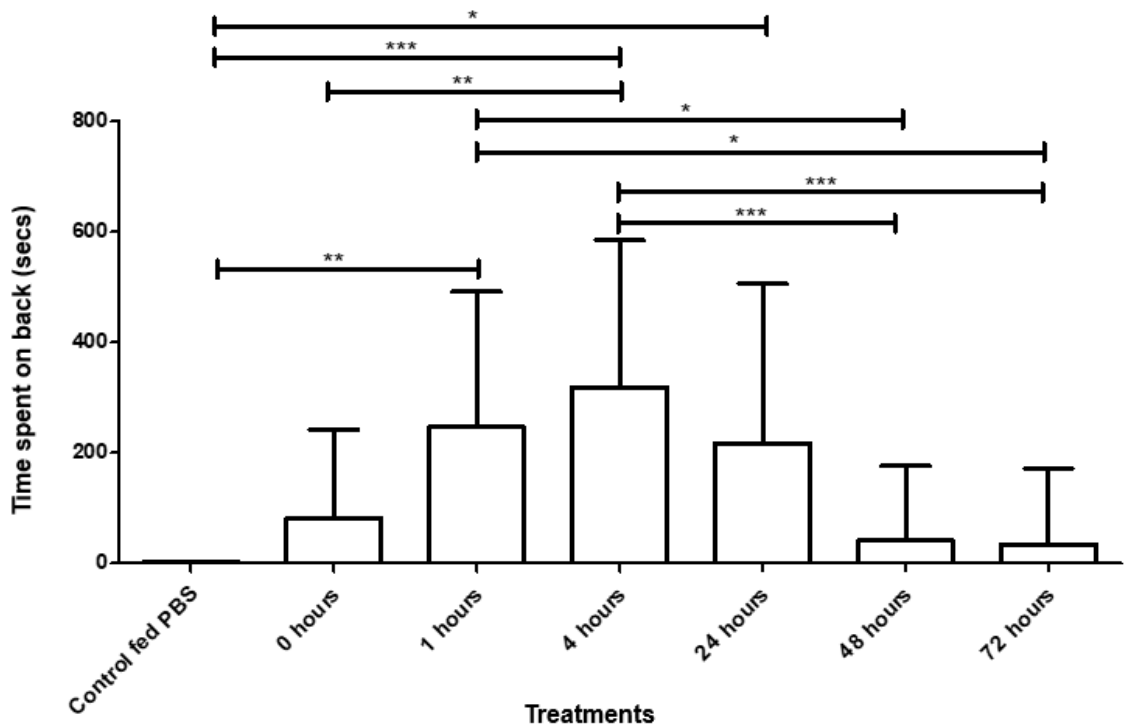


Fig. 5.14 Effect of caffeine on ability of *G. mellonella* larvae to move from their ventral to dorsal surface. Larvae were positioned on their ventral surface after administration of caffeine by intra-haemocoel injection and the time to reposition correctly was recorded (*:p < 0.05) (**:p < 0.01) (***:p < 0.001). All values are the mean \pm SE, n = 20.

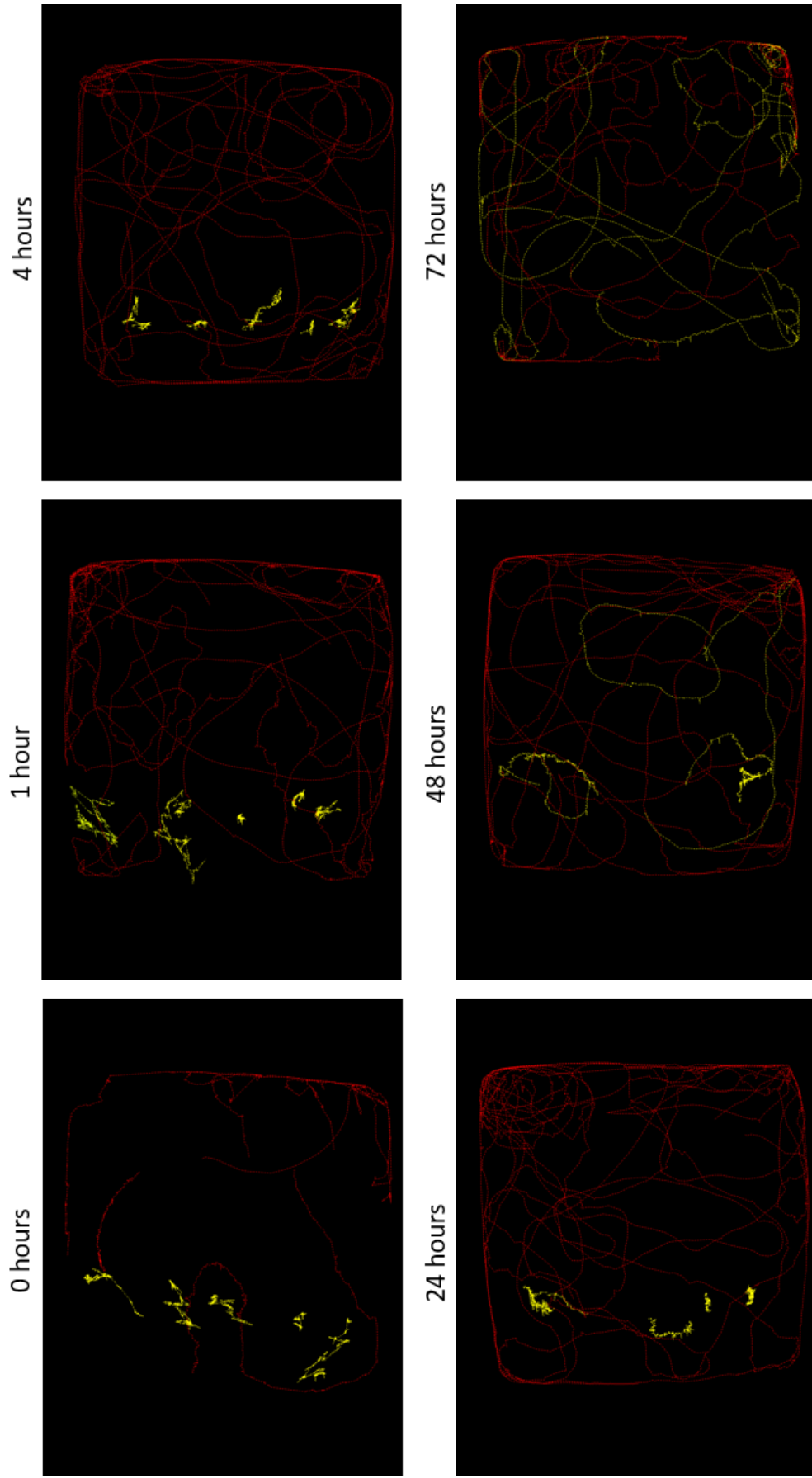


Fig. 5.15 Examples of individual tracks of *G. mellonella* larvae in different time points - five caffeine force fed larvae (yellow) and five control larvae (red) are presented on each image.

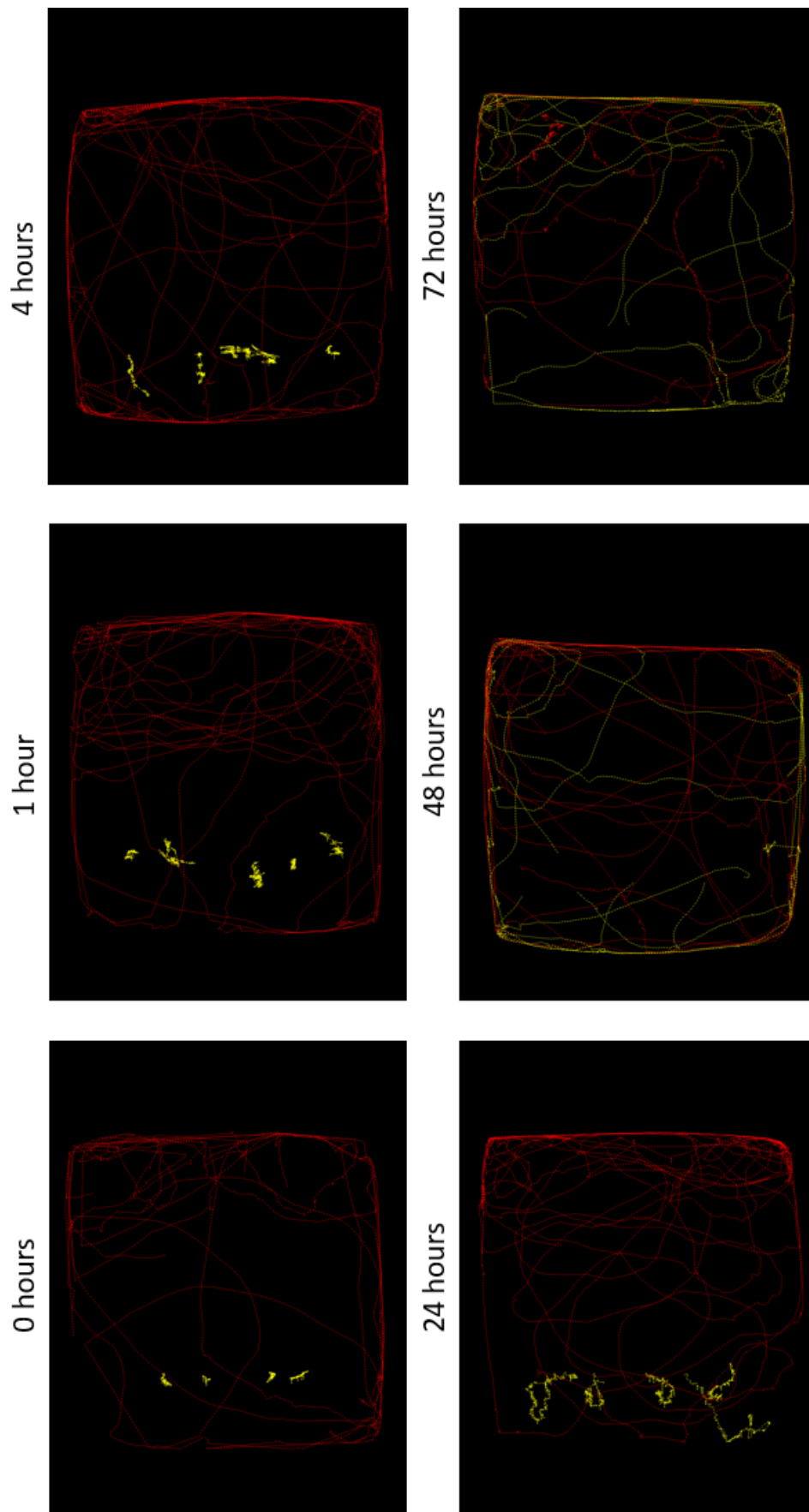


Fig. 5.16 Examples of individual tracks of *G. mellonella* larvae in different time points - five caffeine intra-haemocoel injected larvae (yellow) and five control larvae (red) are presented on each image.

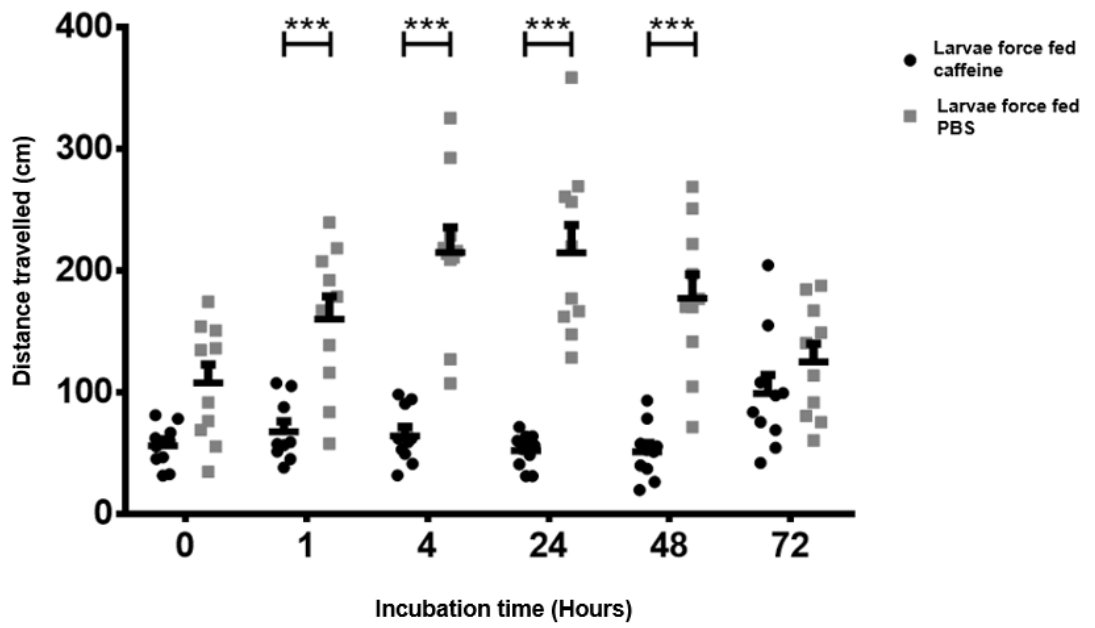


Fig. 5.17 Effect of caffeine on movement distance of force fed *G. mellonella* larvae. Each dot represents individual larva and the line is mean \pm SE, n = 10 (***:p < 0.001).

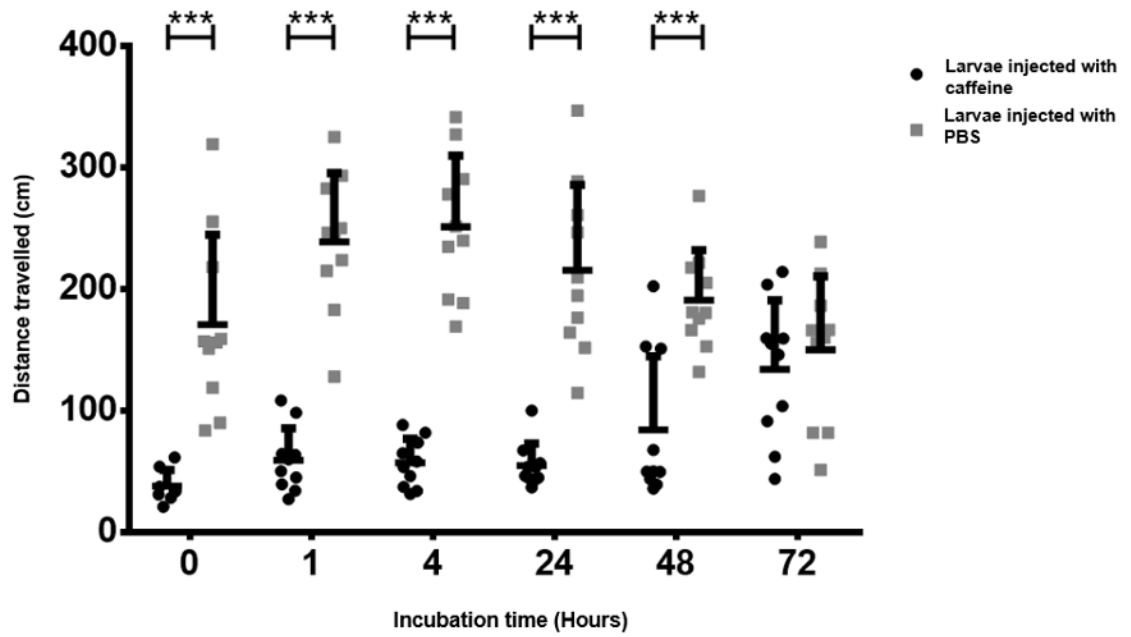


Fig. 5.18 Effect of caffeine on movement distance of intra-haemocoel injected *G. mellonella* larvae. Each dot represents individual larva and the line is mean \pm SE, n = 10 (***:p < 0.001).

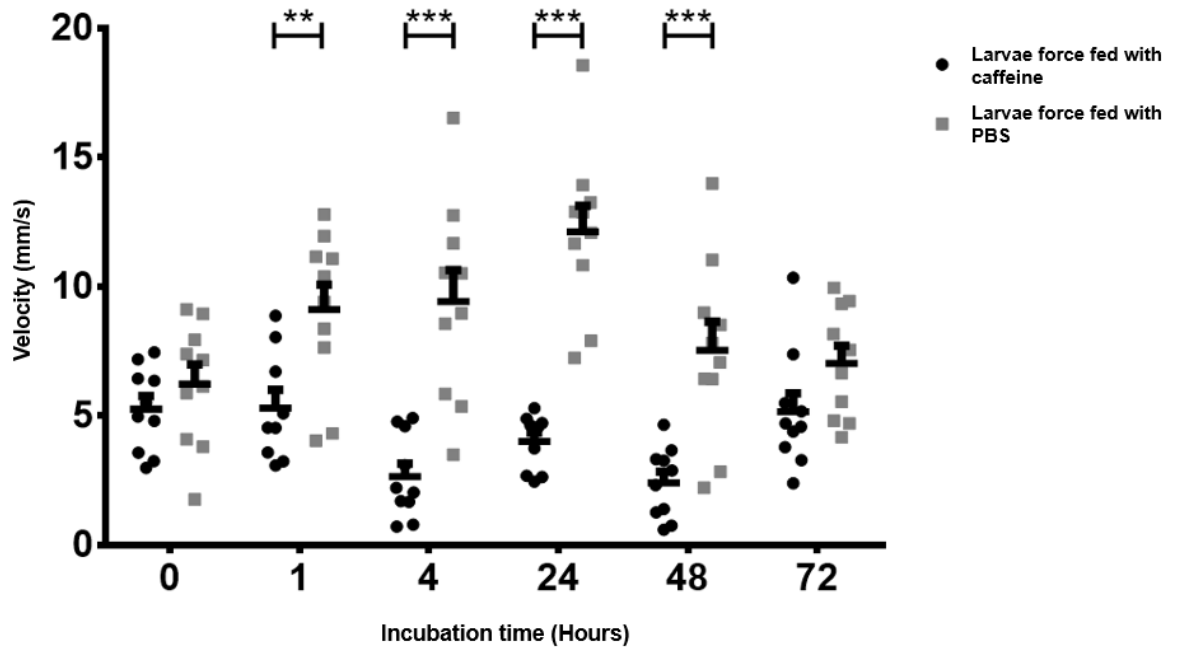


Fig. 5.19 Effect of caffeine on movement velocity of force fed *G. mellonella* larvae. Each dot represents individual larva and the line is mean \pm SE, n = 10 (**:p < 0.01) (***:p < 0.001).

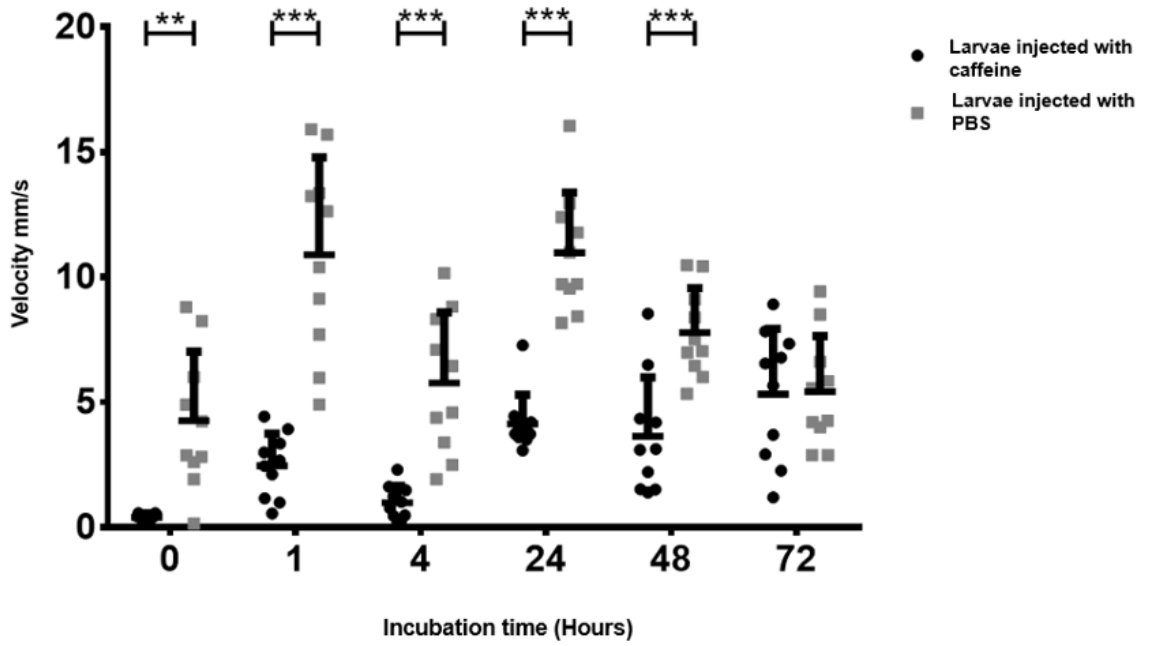


Fig. 5.20 Effect of caffeine on movement velocity of intra-haemocoel injected *G. mellonella* larvae. Each dot represents individual larva and the line is mean \pm SE, n = 10 (**:p < 0.01) (***:p < 0.001).

5.11 Effect of caffeine on formation of pupae.

The rate of pupae formation at 30°C was recorded from days 0 – 12 in larvae administered 20 µl of 0.1 M caffeine via intra-haemocoel injection or force feeding. No mortality was observed during experiments. The rate of pupae formation in control larvae was 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 5 ± 7.07%, 50 ± 7.07%, 70 ± 14.14%, 75 ± 7.07%, 80 ± 14.14%, 85 ± 14.07%, 90 ± 0%, 90 ± 0% and 90 ± 0% after 0-12 days incubation respectively

The rate of pupae formation in larvae force fed caffeine was 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 5 ± 7.07%, 10 ± 7.07%, 20 ± 0%, 25 ± 7.07%, 27 ± 0%, 30 ± 7.07% and 32.5 ± 10.6% after 0-12 days incubation respectively. There was a significant decrease ($p < 0.001$) in the ability of caffeine treated larvae to form pupae when compared with naive control larvae (Fig. 5.21). Pupation in control larvae commenced at day 4 whereas pupation commenced at day 6 in larvae force fed caffeine. In addition to a delay in the commencement of pupation, the rate of pupation was also decreased in treated larvae. Larvae that were force fed had an average pupation rate of 0.54 larvae per day over a 12 day period, however control larvae had an average pupation rate of 1.5 larvae per day over the same period (Fig. 5.24).

The rate of pupae formation in larvae injected with caffeine was 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 0 ± 0%, 5 ± 0%, 7.5 ± 3.53%, 17.5 ± 3.53%, 20 ± 0%, 20 ± 0% and 25 ± 7.07% after 0-12 days incubation respectively There was a significant decrease ($p < 0.001$) in the ability of caffeine injected larvae to form pupae when compared with naive control larvae. Pupation in control larvae commenced at day 4 whereas pupation commenced at day 7 in larvae force fed caffeine. In addition to a delay in the commencement of pupation, the rate of pupation was also decreased in treated larvae. Caffeine injected larvae had an average pupation rate of 0.33 larvae per day over a 12 day period, however control larvae had an average pupation rate of 1.5 larvae per day over the same period (Fig. 5.21).

5.12 Summary

Larvae were administered 20 μ l of 0.1 M of caffeine by force feeding or intra-haemocoel injection and the effect on movement and rate of pupation was monitored as described.

Significant increases in the inability to reposition correctly were observed between larvae force fed PBS and larvae incubated for 4 ($p < 0.001$) and 24 hours ($p < 0.001$) post-caffeine force feeding. Significant increases were also observed between larvae incubated for 1 hour and 72 hours ($p < 0.05$). The inability to reposition correctly peaked in larvae tested 4 hours post force feeding of caffeine. FIMTrack table surface was used to record the distance travelled and velocity of larvae force fed caffeine. Significant decreases in velocity and distance travelled were observed at $t = 1, 4, 24$ and 48 hours after administration of caffeine compared with control larvae

Significant increases were observed between larvae injected with PBS and larvae incubated for 1 ($p < 0.01$), 4 ($p < 0.001$) and 24 hours ($p < 0.05$) post-caffeine administration. Significant increases were also observed when larvae incubated for 1 hour where compared to larvae incubated for 48 ($p < 0.05$) and 72 hours ($p < 0.05$). FIMTrack table surface was used to record the distance travelled and velocity of larvae injected with caffeine. Significant decreases in velocity and distance travelled were observed at 0, 1, 4, 24 and 48 hours after administration of caffeine compared with control larvae.

There was a significant decrease ($p < 0.001$) in the ability of caffeine force fed and injected larvae to form pupae over a 12 day period when compared with naive control larvae.

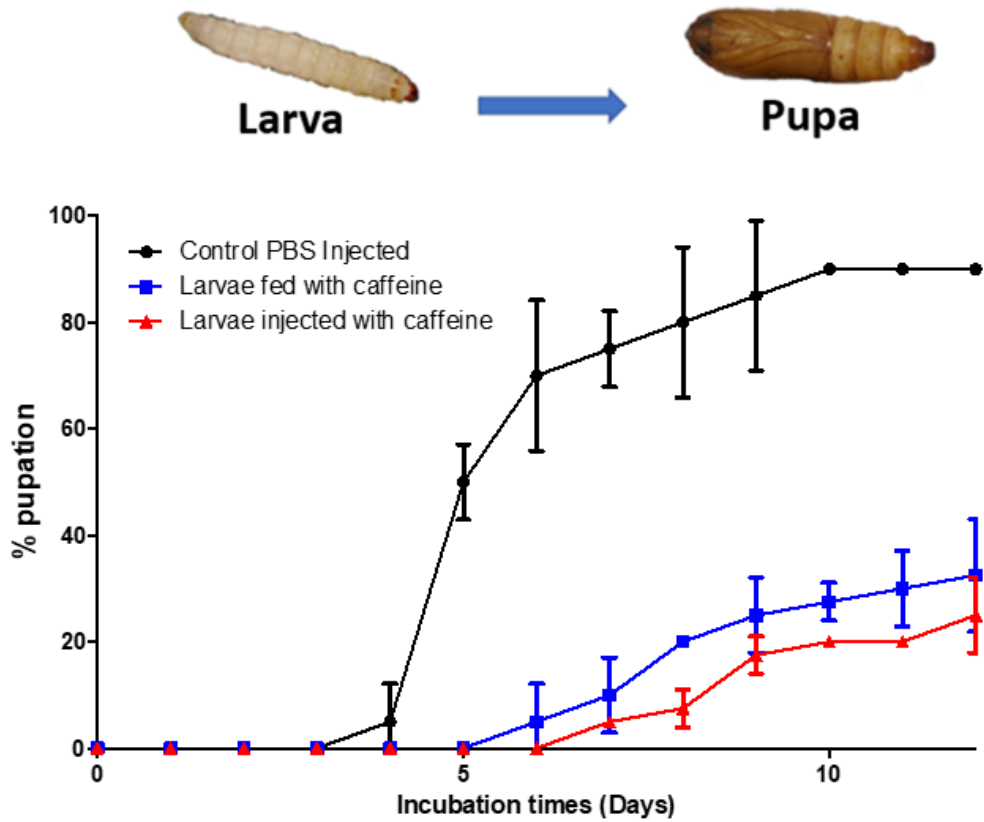


Fig. 5.21 Caffeine administration inhibits pupation in *G. mellonella* larvae. The formation of pupae was recorded from days 0 – 12 (***: $p < 0.001$). All values are the mean \pm SE, $n=20$.

5.13 Effect of caffeine on larval brain proteome

Label free quantitative proteomics was performed on the brain and surrounding tissue of larvae force fed 20 μ l of 0.1 M caffeine for 24 hours. Principal component analyses was employed showing clustering of control and treatment replicates (Fig. 5.22). In total 480 peptides were identified representing 471 proteins with two or more peptides and 32 differentially expressed proteins (Fig. 5.23). Seventeen proteins (7 proteins imputed and 10 proteins non-imputed) were significantly increased ($p < 0.05$) in abundance in larvae administered caffeine compared to controls (Table 5.1). Fifteen proteins (10 proteins imputed and 5 proteins non-imputed) were significantly decreased in abundance in larvae administered caffeine compared to controls (Table 5.2). The exclusively expressed hits showed 11 proteins only expressed in control larvae (Table 5.3) and 3 proteins only expressed in caffeine administered larvae (Table 5.4)

The protein showing the highest increase in abundance in larvae exposed to caffeine was larval cuticle protein 1 at a fold increase of 2.76 ($p < 0.05$), while the proteins highest in abundance with an imputed value were immune-related Hdd1 (6.28 fold increase), AAEL003067-PA (3.11 fold increase), GG11101 (2.58 fold increase) and ras protein (1.91) (Table 5.1).

Proteins showing the highest decrease in abundance included uncharacterized protein Dvir_GJ22788, isoform A (4.23 fold decrease), C-type lectin 21 precursor (2.25 fold decrease) and chemosensory protein (1.54 fold decrease), while the proteins lowest in abundance with an imputed value were proteasome beta-subunit (4.86 fold decrease), chitin deacetylase, partial (3.67 fold decrease), putative neuropeptide precursor protein precursor (3.14 fold decrease), AAEL003067-PA (3.11 fold decrease) and GG11101 (2.58 fold decrease) (Table 5.2).

Blast2GO annotation software was used to group proteins based on conserved GO terms in order to identify processes and pathways potentially associated with caffeine metabolism. GO terms were categorized by biological processes (BP) and molecular function (MF) and cellular components (CC).

The increases in BP in larvae administered caffeine included proteins labelled as catabolic process cellular (99 proteins in control – 99 proteins in treated larvae), cellular component biogenesis (45-44), single-organism metabolic process (62-62), biosynthetic process (168-166), cellular metabolic process (28-28), nitrogen compound metabolic process (133-132), single-organism cellular process (187-184), organic substance metabolic process (176-173), primary metabolic process (28-28), establishment of localization (87-87) and regulation of cellular process (94-92) (Fig. 5.24). No significant changes were observed.

The increases in MF in larvae administered caffeine included proteins labelled small molecule binding (57 proteins in control – 56 proteins in treated larvae), organic cyclic compound binding (93-91), ion binding (76-73), carbohydrate derivative binding (103-101), hydrolase activity (93-91), heterocyclic compound binding (50-48), oxidoreductase activity (72-70) and structural constituent of ribosome (52-52) (Fig. 5.25). No significant changes were observed.

The increases in CC in larvae administered caffeine included proteins labelled intracellular organelle (32 proteins in control – 31 proteins in treated larvae), ribonucleoprotein complex (172-169), membrane-bounded organelle (123-121), intracellular organelle part (41-41), catalytic complex (165-162), intracellular (49-47), protein complex (77-77), non-membrane-bounded organelle (53-51) and intracellular part (65-65) (Fig. 5.26). No significant changes were observed.

5. 14 Summary

Analysis of the changes in proteome of the brain and surrounding tissues of caffeine force fed larvae revealed an increase in the abundance of immune related proteins such as immune-related Hdd1 (6.28 fold increase) and hemolin (1.68 fold increase), ATPase associated proteins such as H⁺ transporting ATP synthase O subunit isoform 1 (1.87 fold increase) and H⁺ transporting ATP synthase delta subunit (1.53 fold increase) and proteins indicative of brain trauma such as troponin T transcript variant B, partial (1.55 fold increase). Proteins involved in development and protein degradation such as SUMO-activating enzyme subunit 1

(3.08 fold decrease) and chitin deacetylase, partial (3.67 fold decrease) were decreased in abundance.

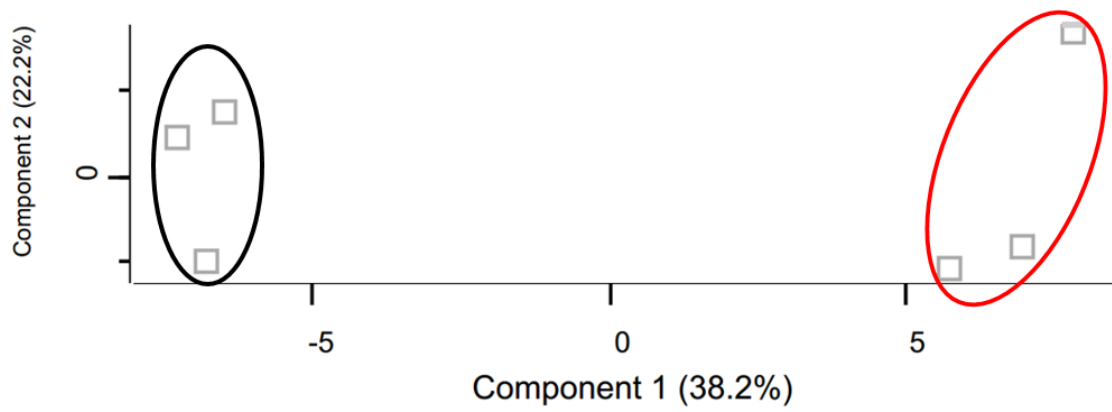


Fig. 5.22 Principal component analyses of the proteome of control larvae (black circle) and larvae force fed caffeine (red circle).

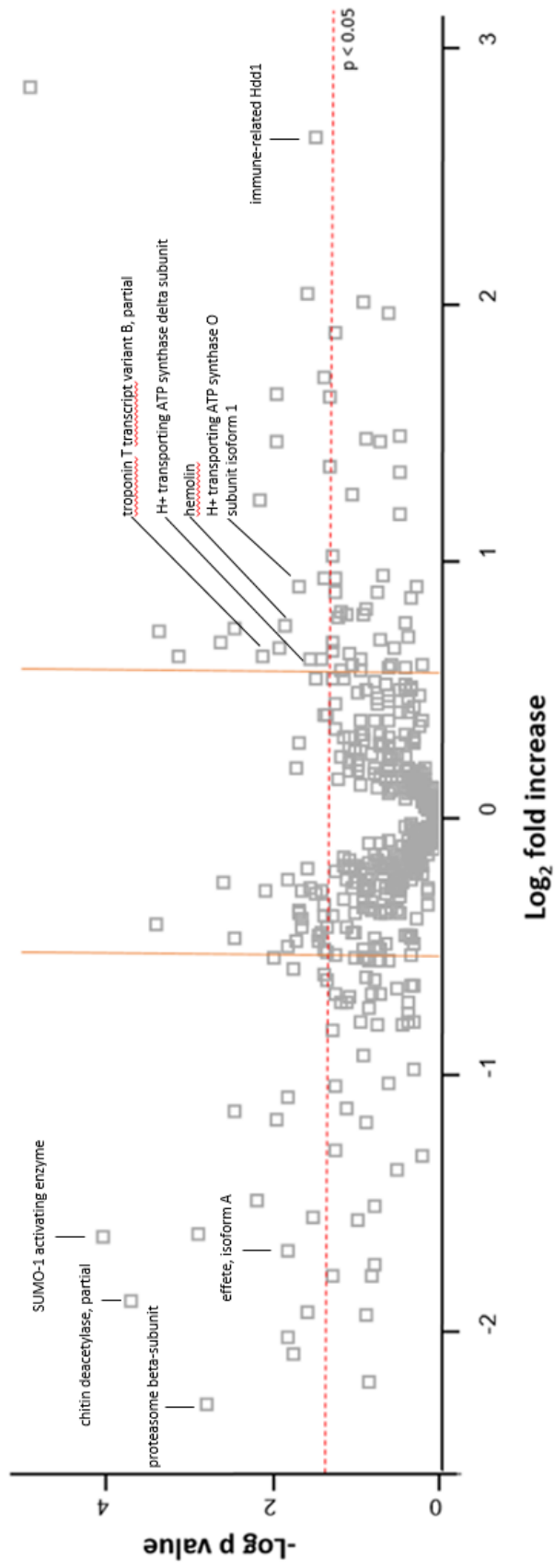


Fig. 5.23 Volcano plot showing proteins altered in abundance in *G. mellonella* larvae force fed caffeine. Proteins above the line are statistically significant ($p < 0.05$) and those to the right and left of the vertical lines indicate fold changes 1.5 fold positive and 1.5 fold negative in the caffeine force fed larvae and control larvae.

Protein Name (*=imputed protein)	Peptides	Sequence Coverage %	PEP	Mean LFQ Intensity	Fold difference
*PREDICTED: adenylyl cyclase-associated protein 1 isoform X2 [Tribolium castaneum]	2	8.9	1.71E-13	2.42E+09	7.19
*immune-related Hdd1 [Hyphantria cunea]	10	15.7	2.45E-140	7.12E+09	6.28
*PREDICTED: protein CREG1 [Tribolium castaneum]	4	20.6	2.99E-59	1.83E+09	4.11
*AAEL003067-PA [Aedes aegypti]	5	16.2	4.81E-71	7.58E+08	3.11
RecName: Full=Larval cuticle protein 1; Flags: Precursor	4	19	1.38E-108	4.87E+09	2.76
*GG11101 [Drosophila erecta]	6	20.3	2.22E-34	7.91E+08	2.58
myofilin variant A [Bombyx mandarina]	6	24.3	1.09E-59	8.96E+09	2.35
*ras protein [Bombyx mori]	3	4.3	1.20E-17	1.40E+09	1.91
H+ transporting ATP synthase O subunit isoform 1 [Bombyx mori]	12	42.7	4.65E-99	9.55E+09	1.87
hemolin [Heliothis virescens]	3	47.9	1.55E-83	8.13E+09	1.68
*hypothetical protein, partial [Bombyx mori]	2	16.7	2.42E-84	1.07E+09	1.66
AGAP008115-PA [Anopheles gambiae str. PEST]	4	11.7	5.25E-145	8.39E+09	1.65
bifunctional purine biosynthesis protein, putative [Pediculus humanus corporis]	10	27.4	1.70E-77	3.93E+09	1.61
troponin T transcript variant B, partial [Bombyx mandarina]	6	25.6	0	3.96E+10	1.55
heat shock 70 kD protein cognate precursor [Bombyx mori]	3	76.2	1.52E-69	6.44E+09	1.54
heat shock cognate 70 protein [Spodoptera frugiperda]	4	14.8	1.89E-13	4.72E+09	1.54

H ⁺ transporting ATP synthase delta subunit [Bombyx mori]	7	41.8	7.98E-106	9.07E+09	1.53
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Table 5.1 Relative fold changes of proteins increased in abundance in *G. mellonella* larvae administered caffeine and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 were considered to be in significantly variable abundances between control and treated larvae.

Protein Name (*=imputed protein)	Peptides	Sequence Coverage %	PEP	Mean LFQ intensity	Fold difference
*proteasome beta-subunit [Bombyx mori]	8	41.7	3.23E-50	2.24E+09	-4.86
uncharacterized protein Dvir_GJ22788, isoform A [Drosophila virilis]	13	28.6	6.71E-182	1.18E+10	-4.23
*PREDICTED: probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial [Tribolium castaneum]	2	14.9	1.66E-45	1.61E+09	-3.78
*chitin deacetylase, partial [Helicoverpa armigera]	5	15	3.93E-37	9.53E+08	-3.67
*effete, isoform A [Drosophila melanogaster]	4	10.5	2.65E-31	1.71E+09	-3.21
*SUMO-1 activating enzyme [Bombyx mori]	8	32.4	9.40E-72	1.82E+09	-3.08
*chitin binding protein [Papilio xuthus]	6	9.1	1.64E-37	1.39E+09	-3.08
*PREDICTED: similar to multiple inositol polyphosphate phosphatase [Nasonia vitripennis]	6	32.1	5.68E-81	1.19E+09	-2.93
*acyl-coenzyme A dehydrogenase [Bombyx mori]	5	7.9	5.19E-21	1.06E+09	-2.80
C-type lectin 21 precursor [Bombyx mori]	9	38.1	1.89E-73	5.00E+09	-2.25
*PREDICTED: protein BCCIP homolog [Nasonia vitripennis]	3	15.7	1.90E-18	7.25E+08	-2.20
*similar to CG3590-PA [Papilio xuthus]	8	25.4	1.54E-73	1.52E+09	-2.12
chemosensory protein [Papilio xuthus]	6	16.7	1.87E-206	5.50E+10	-1.54

unknown [Picea sitchensis]	10	36.8	3.52E-178	2.34E+10	-1.52
RecName: Full=Inducible serine protease inhibitor 2; Short=ISPI-2	3	9.8	2.87E-87	3.17E+10	-1.50

Table 5.2 Relative fold changes of proteins decreased in abundance in *G. mellonella* larvae administered caffeine and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 were considered to be in significantly variable abundances between control and treated larvae.

Protein Annotation	LFQ Intensity Control 1	LFQ Intensity Control 2	LFQ Intensity Control 3	LFQ Intensity Caff 1	LFQ Intensity Caff 2	LFQ Intensity Caff 3
acyl-coenzyme A dehydrogenase	27.2704	27.7843	27.4955	NaN	NaN	NaN
chitin deacetylase, partial	27.7001	28.1569	27.7596	NaN	NaN	NaN
PREDICTED: prefoldin subunit 5	26.7372	26.8253	26.5938	NaN	NaN	NaN
PREDICTED: probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	28.0088	28.4985	28.0911	NaN	NaN	NaN
PREDICTED: protein BCCIP homolog	27.3057	27.5698	27.5029	NaN	NaN	NaN
proteasome beta-subunit	28.6598	28.7928	28.8288	NaN	NaN	NaN
putative neuropeptide precursor protein precursor	24.8122	25.8067	25.1918	NaN	NaN	NaN
uncharacterized protein Dere_GG18643	25.2109	25.5083	24.8352	NaN	NaN	NaN
effete, isoform A	28.2484	27.8466	28.2797	NaN	NaN	NaN

Table 5.3 LFQ intensities of proteins exclusively expressed in the haemolymph of control larvae. A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”.

Protein Annotation	LFQ Intensity Control 1	LFQ Intensity Control 2	LFQ Intensity Control 3	LFQ Intensity Caff 1	LFQ Intensity Caff 2	LFQ Intensity Caff 3
PREDICTED: adenylyl cyclase- associated protein 1 isoform X2	NaN	NaN	NaN	29.2461	28.9088	29.0392
AAEL003067-PA	NaN	NaN	NaN	28.69	26.9949	27.1828
PREDICTED: protein CREG1	NaN	NaN	NaN	28.9519	28.812	27.7894

Table 5.4 LFQ intensities of proteins exclusively expressed in the haemolymph of larvae administered caffeine. A zero value indicates a protein that was absent or undected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”. (Caff, potassium nitrate)

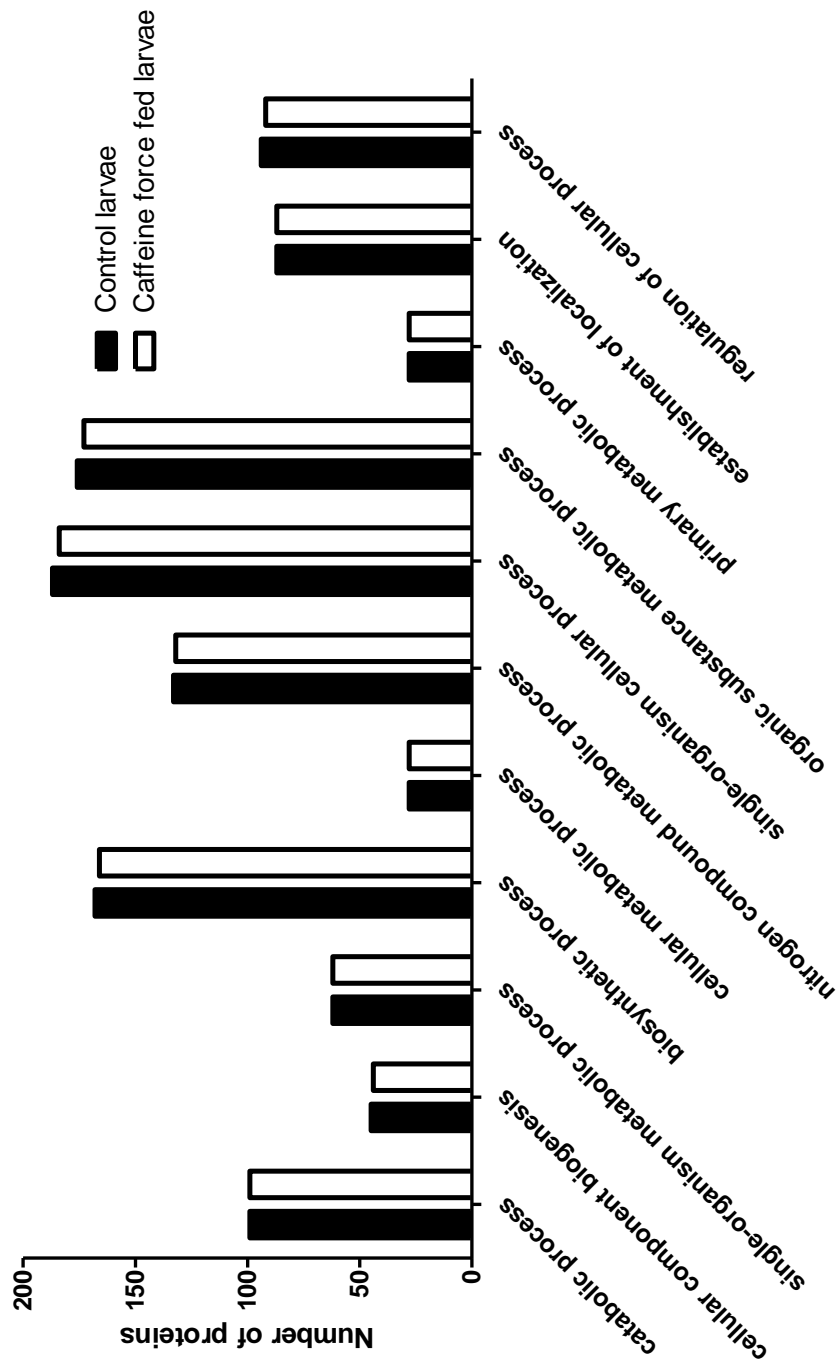


Fig. 5.24 Bar chart showing changes to number of proteins involved in various biological processes at level 3 ontology. Proteins were assigned groups based on involvement in biological processes for control and caffeine treated larvae. Closed bar: control, Open bar: caffeine treated larvae

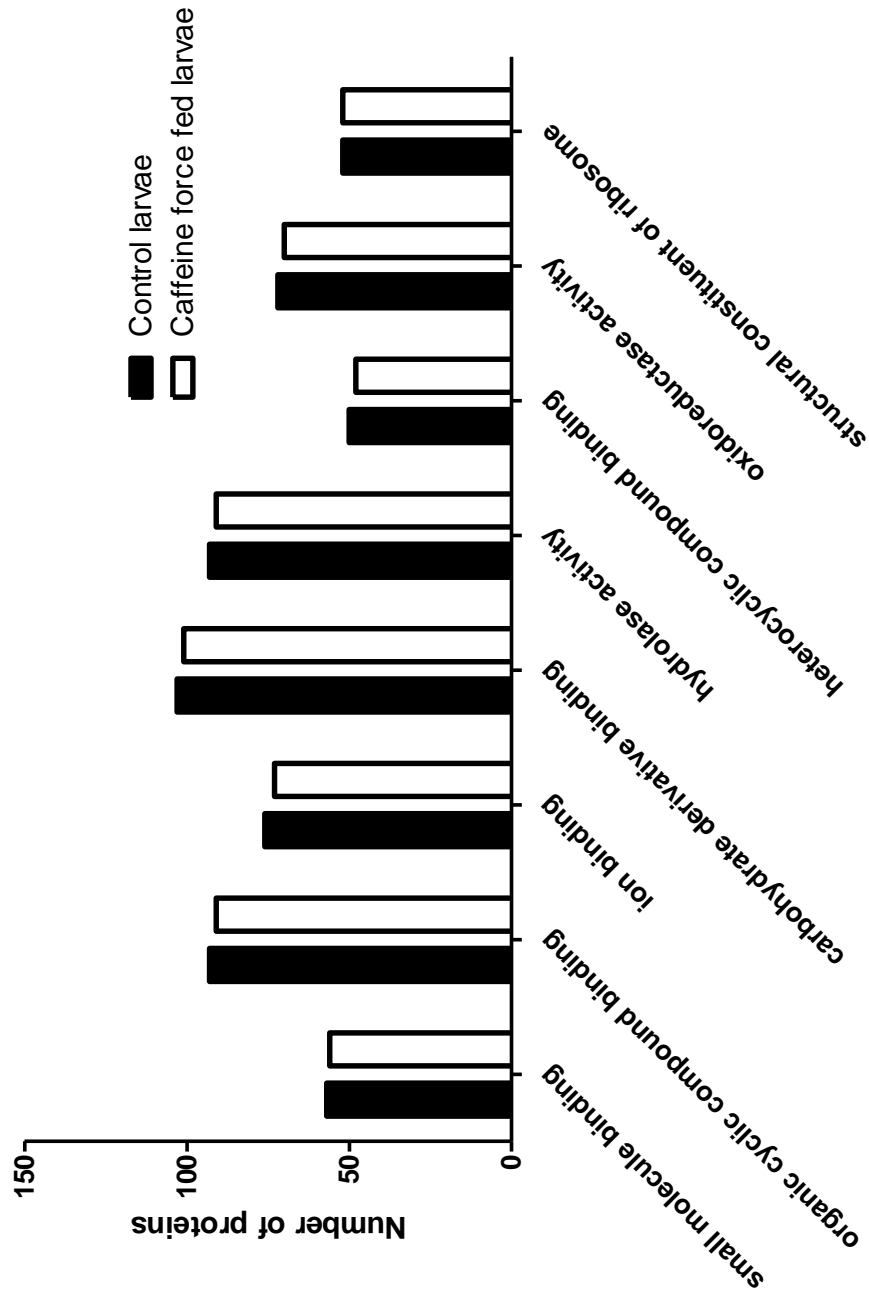


Fig. 5.25 Bar chart showing changes to number of proteins given various molecular functions at level 3 ontology. Proteins were assigned groups based on involvement in molecular function for control and caffeine treated larvae. Closed bar: control, Open bar: Caffeine treated larvae.

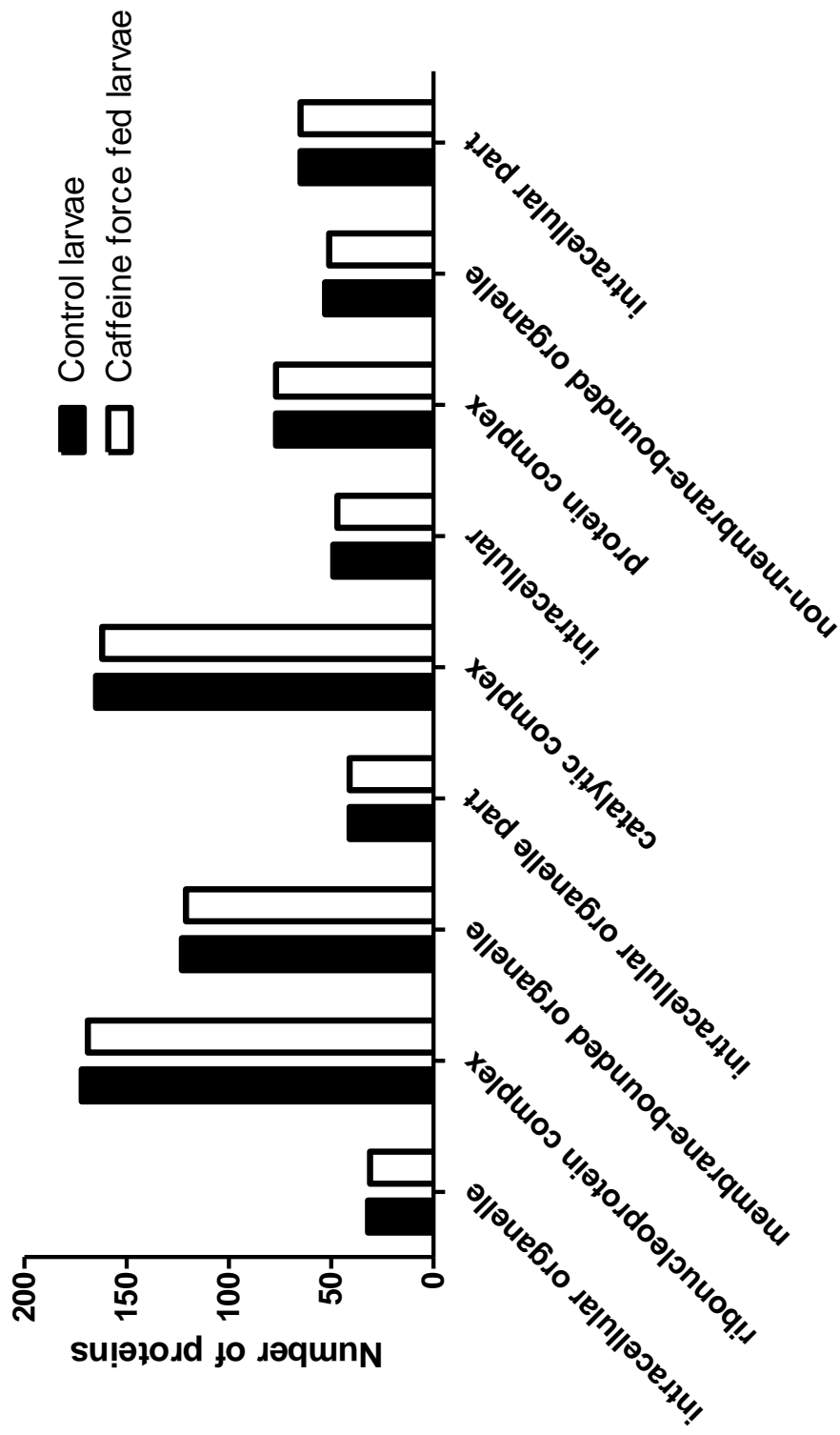


Fig. 5.26 Bar chart showing number of proteins in various cellular components at level 3 ontology. Proteins were assigned groups based on involvement in cellular components in the total proteome for control and caffeine treated larvae. Closed bar: control, Open bar: caffeine treated larvae

5.15 Discussion.

Caffeine is the most widely consumed stimulant in the world and is ingested in a variety of beverages and food stuffs. It has a variety of physiological effects in humans including increasing alertness and performance, and reducing fatigue (Smith, 2002). Various histological, anatomical and physiological functions in insects share similarities with the mammalian gastrointestinal tract and microbes found in the midgut of *G. mellonella* larvae resemble those found in the intestinal microvilli of mammals (Fedhila *et al.*, 2010). The fat body of insects functions in xenobiotic metabolism in a similar way to the liver of mammals and contains a number of cytochrome P450 and sulfo-, glutathione- or glucose- conjugation enzymes which are involved in drug metabolism (Büyükgüzel *et al.* 2013) . *Drosophila* treated with metyrapone, an inhibitor of cytochrome P450s enzyme, showed dramatically decreased caffeine metabolism suggesting the highly conserved enzymes cytochrome P450s are involved in the metabolism of caffeine in both mammals and insects (Coelho *et al.*, 2015) . The results presented here indicate that caffeine is metabolised in *G. mellonella* larvae in a similar manner to that in mammals and that the caffeine metabolites theobromine and theophylline were detected four hours post administration of caffeine.

Larvae that were administered caffeine by intra-haemocoel injection showed significant increases in haemocyte densities after 4 ($p < 0.05$) and 24 ($p < 0.01$) hour incubation when compared to their relevant controls. In addition, larvae that were administered caffeine by force feeding showed significant increases ($p < 0.05$) in haemocyte densities after 24 hours incubation when compared to their relevant controls. The slight increase in haemocyte density at 4 hours and significant increase in haemocyte density at 24 hours in larvae force fed caffeine would suggest that caffeine is readily absorbed into circulation in insects. However despite the increase in haemocyte densities the administration of caffeine by force feeding or intra-haemocoel injection showed no significant changes between the fungicidal ability of haemocytes from control or treated larvae. Caffeine and its major metabolite paraxanthine suppress neutrophil and monocyte chemotaxis, and also suppress production of the pro-inflammatory cytokine tumour necrosis factor

(TNF)- α from human blood (Horrigan *et al.*, 2006). In addition caffeine administration to *Macrobrachium rosenbergii* significantly reduced the respiratory burst in haemocytes (Sung *et al.*, 2008). The findings presented here however do not suggest any inhibition of the cellular immune system when larvae are administered 20 μ l of 0.1M caffeine.

The activity of two anti-oxidant enzymes, superoxide dismutase and catalase was measured in the haemolymph of larvae challenged with caffeine via force feeding or direct injection into the haemocoel. No significant changes were recorded in the activity of either anti-oxidant enzyme between test larvae administered caffeine and control larvae administered PBS. These findings are in disagreement with the antioxidant properties of caffeine displayed in mammals (Prasanthi *et al.*, 2010). However further investigation is necessary to determine if caffeine reduces oxidative stress in larvae that are already pre-exposed to an agent that causes oxidative stress.

Larvae incubated for 4 hours following administration of caffeine showed a significant reduction in their ability to move and reposition correctly, FIMTrack software also revealed a significant decrease ($p < 0.001$) in distance travelled and velocity of larvae force fed caffeine following 4 hour incubation compared to control larvae. This inhibition of movement corresponded to a peak in theophylline concentration of 18.94 μ g/ml. The ability to move and reposition correctly gradually recovered by 48 hours corresponding to a theophylline concentration of 0.87 μ g/ml. Theophylline is a muscle relaxant in mammals and human airways the EC₅₀ for airway smooth muscle relaxation by theophylline is approximately 1.5×10^{-4} M (Barnes, 2010). The presence of theophylline in the haemolymph of larvae force fed caffeine after 4 hours incubation might suggest that it is acting as a muscle relaxant in larvae. Previous work on Zebra fish embryos has demonstrated administration of caffeine also reduced mobility (Chen *et al.*, 2008).

Both the rate of pupation and commencement of pupation were reduced in larvae force fed caffeine compared with control larvae. Zebra fish embryos administered caffeine displayed developmental alterations including reduced body length, reduced tactile responses and muscle fibre formation (Chen *et al.*, 2008).

Developmental changes are also seen in the human foetus where caffeine doses of ≥ 300 mg per day during pregnancy were associated with reductions in infant birth weight that may be especially detrimental to premature or low-birth-weight infants (Hinds *et al.*, 1996).

Proteomic analysis revealed the increased abundance of troponin T transcript variant B, partial (1.55 fold) ($p < 0.05$) in larvae challenged with caffeine. Elevated serum troponin is frequently observed after traumatic brain injury in patients (Salim *et al.* 2008). The increase in abundance of troponin T transcript variant B, partial in *G. mellonella* larvae would suggest that caffeine is having an adverse effect on the brain. A number of immune related proteins were increased in abundance in larvae administered caffeine including immune-related Hdd1 (6.28 fold) and hemolin (1.68 fold). The brain's immune system, which consists mainly of astrocytes, microglia and infiltrating immune cells is activated in response to pathophysiological events such as ischemia, trauma, inflammation and infection (Haskó *et al.*, 2005). Ischemia, head injury, seizure activity and inflammation induce rapid increases in extracellular adenosine concentrations to 30–100-times that of the resting concentration (Von Lubitz, 1999). Adenosine interacts with specific G-protein-coupled receptors on astrocytes, microglia and infiltrating immune cells to regulate the function of the immune system in the brain (Haskó *et al.*, 2005). This suggests that caffeine is inducing brain injury in the larvae and thereby leading to increased abundance of troponin and various immune related proteins.

H⁺ transporting ATP synthase O subunit isoform 1 (1.87 fold) and H⁺ transporting ATP synthase delta subunit (1.53 fold) were also increased in abundance in larvae that had received caffeine. Blayney *et al.* (1978) studied the effects of caffeine on calcium transport by subcellular organelles isolated from rabbit myocardium and reported that caffeine increased myofibrillar basic and calcium-activated ATPase activity.

In *G. mellonella* larvae treated with caffeine there was a decrease in the abundance of proteins associated with cellular protein degradation (e.g. proteasome beta-subunit (4.86 fold), effete, isoform A (3.21)). The reduced formation of pupae in caffeine administered larvae may be explained by the

decrease of SUMO-activating enzyme subunit 1 (3.08 fold). Smt3 is the only homologue to SUMO in *Drosophila melanogaster* and plays a role in the regulation of ecdysteroid levels during post-embryonic development (Talamillo *et al.*, 2008). The SUMO homologue causes an ecdysteroid peak in the prothoracic glands which is required for the larval to pupal transition (Talamillo *et al.*, 2013). Proteomic analysis also revealed decreases in cuticle proteins such as chitin deacetylase, partial (3.67 fold decrease). Chitin deacetylases are mainly expressed in the integument and play critical roles in molting, cuticle degradation, and new cuticle formation .

The results presented here indicate that caffeine is metabolised in *G. mellonella* larvae producing theobromine and theophylline. Caffeine administration results in reduced larval movement possibly due to the presence of the muscle relaxant theophylline. Proteomic analysis revealed decreased abundance of a range of proteins associated with development including SUMO-activating enzyme subunit 1 and chitin deacetylase, partial which may play a role in reducing pupation. *G. mellonella* larvae are now widely used as an *in vivo* model for assessing the virulence of microbial pathogens and for determining the activity of antimicrobial drugs (Browne *et al.*, 2014). The results presented here show a strong correlation with the effect of caffeine on Zebra fish embryos and offer the possibility of utilising *Galleria* larvae as a model to study the *in vivo* activity of caffeine and related neuroactive compounds.

Chapter 6

Analysis of the acute response of *Galleria mellonella* larvae to monosodium glutamate and creatine monohydrate

6.1 Analysis of the acute response of *Galleria mellonella* larvae to Monosodium glutamate and Creatine monohydrate

The aim of the first part of this Chapter was to evaluate the relative toxicity of monosodium glutamate in *Galleria mellonella* larvae. The lethal doses 20/50/80 were determined for monosodium glutamate in *G. mellonella*. In addition the first part of this chapter focused on a more in-depth study of the toxic effects of monosodium glutamate in *G. mellonella* larvae. This study focused on the effect of monosodium glutamate on the immune system and enzymatic system of *G. mellonella*. The second part of this chapter focused on the toxic effects of creatine monohydrate on the immune and enzymatic systems of *G. mellonella* larvae.

Monosodium glutamate is a sodium salt of glutamate and has been widely used as a flavour enhancer in several meat preparations and is responsible for the “umami” sensation. Monosodium glutamate is found naturally in tomatoes cheese and other foods. Monosodium glutamate causes glutamate hypersensitivity, more commonly known as Chinese restaurant syndrome, of which the symptoms include, headache, sweating, numbness, tickling or burning in the face, chest pain nausea and weakness (Dixit *et al.*, 2014). The incidence of obesity was measured in mice and rats of different ages that were administered MSG by various methods (Bunyan *et al.*, 1976). Sixteen per cent of new-born mice injected subcutaneously with 3mg of monosodium glutamate per gram of body weight at 1, 2, 3, 6, 7 and 8 days of age died. Out of the remaining new-born mice 90% became markedly obese.

Creatine monohydrate is a widely used supplement in the sports industry. It has been shown to increase intramuscular phosphocreatine as well as increasing the high intensity of various high-powered work tasks (Earnest *et al.*, 1995). A long standing concern is that creatine monohydrate supplementation could be associated with cancer. This has grown through the idea that creatine monohydrate can facilitate the formation of carcinogenic heterocyclic amines (HCA). However dos Santos Pereira *et al.* (2015) provided evidence that low and high doses of creatine supplementation given either acutely or chronically, does not cause a significant increase in HCA formation.

This work aimed to characterise the similarities between the response of *G. mellonella* to monosodium glutamate and creatine monohydrate and therefore highlight the insect's utility as a preliminary screening model for measuring the toxicity of these compounds.

6.2 Assessment of effect of monosodium glutamate on viability in *G. mellonella*.

G. mellonella larvae were administered 20 μ l of 0.12 M monosodium glutamate by intra-haemocoel injection (Fig 6.1). or by force feeding (Fig 6.1). as described, and the LD₂₀, LD₅₀ and LD₈₀ values were determined. The LD₂₀/LD₅₀/LD₈₀ values for Intra-haemocoel injected monosodium glutamate in *G. mellonella* were 0.5M, 0.66M and 0.82M respectively (Fig 6.1). The LD₂₀/LD₅₀/LD₈₀ values for Intra-haemocoel injected monosodium glutamate in *G. mellonella* were 0.7M, 0.92M and 1.01M respectively (Fig. 6.1).

Section 3.2 established a strong correlation between the LD₂₀, LD₅₀ and LD₈₀ values for eight commonly used food additives compound in larvae due to feeding or intra-haemocoel injection. The LD₂₀, LD₅₀ and LD₈₀ values from section 3.1 was combined with the LD₂₀, LD₅₀ and LD₈₀ values of monosodium glutamate. This showed an R² value of 0.79 (p = 0.0014) between the LD₂₀ values, an R² value of 0.87 (p = 0.0002) between the LD₅₀ values and an R² value of 0.77 (p = 0.0018) between the LD₈₀ values obtained due to feeding and intra-haemocoel administration.

LD₅₀ values for monosodium glutamate in rats was obtained in the literature and previously recorded to be between 15,000/18,000mg/kg (Walker & Lupien, 2000). Section 3.4 obtained the LD₅₀ values for eight commonly used food additives in rats from the literature. The LD₅₀ values of the 8 commonly used food additives as determined in rats by feeding was plotted against the corresponding LD₅₀ values for the compounds obtained by feeding *G. mellonella* larvae (Section 3.4). The resulting graph showed a significant positive correlation between the LD₅₀ values obtained in both systems (R²=0.6506, p=0.0156) (Fig. 3.6). However when the LD₅₀ value of monosodium glutamate in *G. mellonella* larvae and rats was combined with data from Section 3.4 no significant correlation is observed (R²=0.0032, p=0.8853).

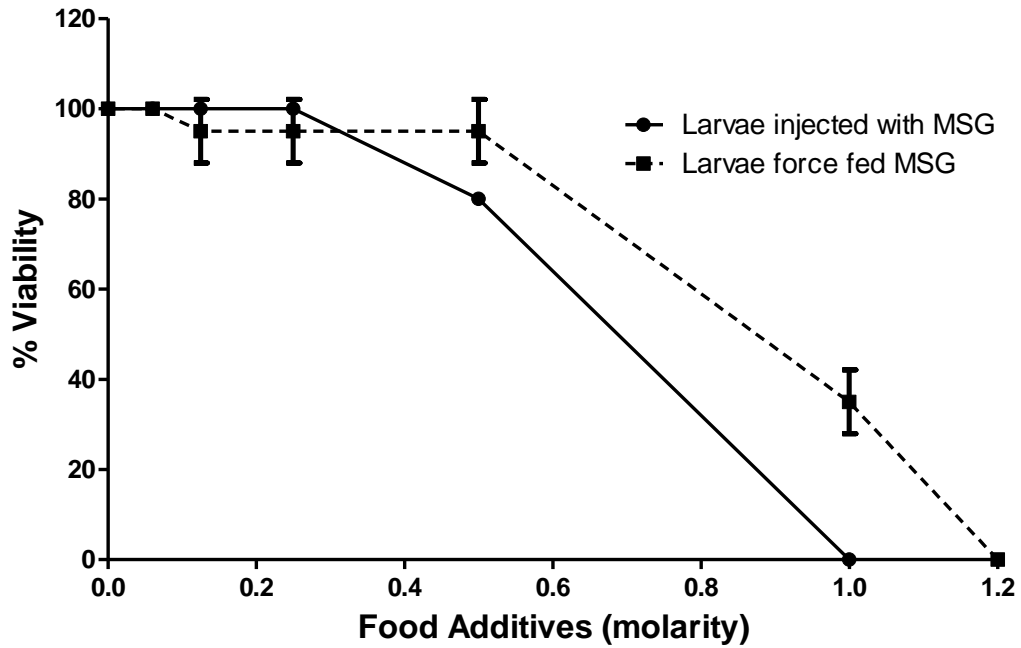


Fig 6.1 Viability (%) of *Galleria mellonella* larvae following administration of monosodium glutamate by intra-haemocoel injection (closed circles) and force feeding (closed boxes). Larvae (n = 10) were administered compounds by intra-haemocoel injection. All values are the mean \pm SE of three independent determinations.

6.3 Effect of monosodium glutamate administration on haemocyte densities.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.12M monosodium glutamate. This concentration of monosodium glutamate was the highest concentration that did not result in larval death. The effect on the density of circulating haemocytes in larval haemolymph was ascertained after 4 and 24-hours incubation at 30°C. For experimental purposes control larvae were administered PBS by intra haemocoel injection or force feeding.

Larvae force fed PBS and incubated for 4/24 hours at 30°C showed $0.89 \pm 0.07 \times 10^7$ and $0.66 \pm 0.06 \times 10^7$ haemocytes/ml respectively. Larvae force fed monosodium glutamate and incubated for 4 hours showed $0.99 \pm 0.47 \times 10^7$ haemocytes/ml. Larvae force fed monosodium glutamate and incubated for 24 hours showed $0.94 \pm 0.35 \times 10^7$ haemocytes/ml. These findings showed no significant changes between control and treatment groups (Fig 6.2).

Larvae injected with PBS and incubated for 4/24 hours at 30°C showed $1.25 \pm 0.15 \times 10^7$ and $0.93 \pm 0.09 \times 10^7$ haemocytes/ml respectively. Larvae injected with monosodium glutamate and incubated for 4 hours showed $1.41 \pm 0.33 \times 10^7$ haemocytes/ml. Larvae injected with monosodium glutamate and incubated for 24 hours showed $1.59 \pm 0.27 \times 10^7$ haemocytes/ml. This equated to a 0.70 fold increase ($p < 0.05$) in haemocyte densities between control larvae and monosodium glutamate injected larvae following 24 hour incubation (Fig 6.2).

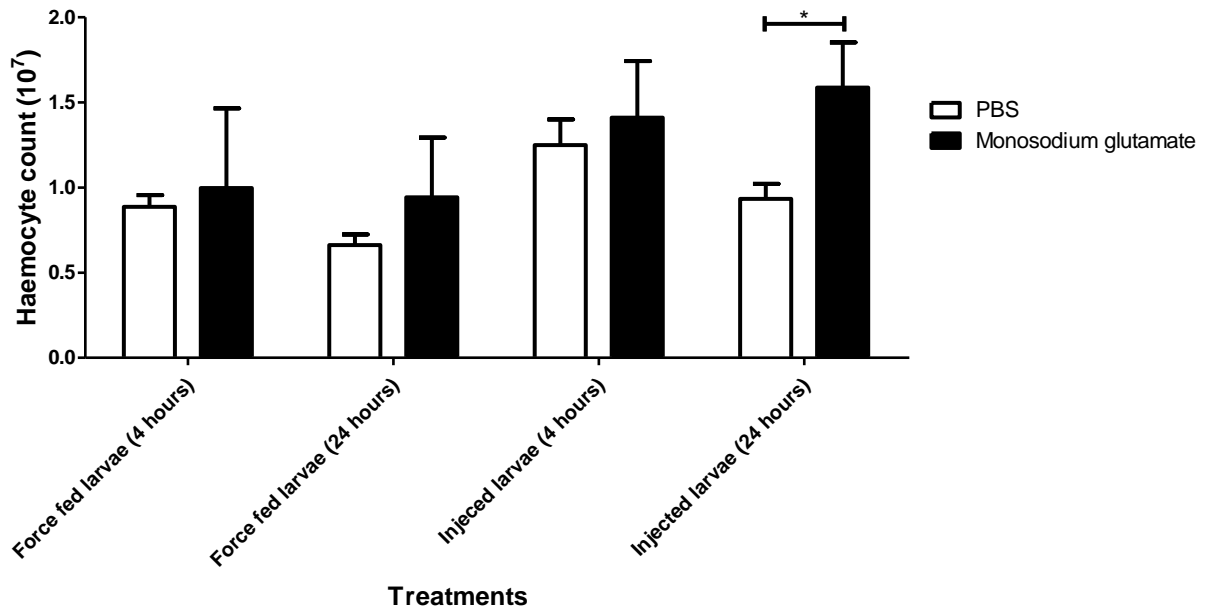


Fig. 6.2 Effect of monosodium glutamate on haemocyte density in force fed and intra-haemocoel injected *G.mellonella* larvae at 4 and 24 hours. (*: $p < 0.05$). All values are the mean \pm SE of 3 independent determinations.

6.4 Analysis of the effect of monosodium glutamate administration on haemocyte mediated pathogen killing.

Larvae were administered 20 µl of 0.12 M monosodium glutamate by intra haemocoel injection or force feeding. Haemocytes were extracted from control and test larvae following 24-hour incubation at 30°C and mixed with opsonised *C. albicans* for 80-minutes (Fig. 6.2). By counting the percentage viability of *C. albicans* at 20 min intervals, the effect of monosodium glutamate on haemocyte mediated pathogen killing was ascertained.

Haemocytes from force fed control larvae killed 22.52. ± 18.5%, 43.76 ± 21.81% , 43.01 ± 28.29%, 67.23 ± 17.96% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. Haemocytes from PBS injected control larvae killed 34.33 ± 21.14%, 50 ± 17.79%, 67.93 ± 21.26%, 76.33 ± 18.29% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 6.3).

Haemocytes from monosodium glutamate force fed larvae killed 39.97 ± 16.48%, 54.48 ± 27.10%, 61.75 ± 29.36%, 75.69 ± 23.12% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. In addition haemocytes from monosodium glutamate injected larvae killed 34.09 ± 20.84%, 36.57 ± 14.68%, 58.17 ± 6.26%, 62.59 ± 4.37% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 6.3).

The results indicated no significant changes in haemocyte mediated killing between control larvae and larvae administered monosodium glutamate by intra-haemocoel injection or force feeding.

6.5 Summary

The LD₂₀/LD₅₀/LD₈₀ values for monosodium glutamate in injected and force feed *G. mellonella* larvae were ascertained. A significant correlation between the LD₂₀ (R² = 0.7896, p = 0.0014), LD₅₀ (R² = 0.8727, p = 0.0002) and LD₈₀ (R² = 0.7743, p = 0.0018) values obtained due to oral or intra- haemocoel administration of compounds was established. No correlation between the LD₅₀ values of the 9 food preservatives in *G. mellonella* larvae and rats (R²=0.0032, p=0.8853) was demonstrated.

Larvae were administered monosodium glutamate by force feeding and intra-haemocoel injection. Haemocyte densities were measured in larvae administered monosodium glutamate via one of the two routes of administration following 4 and 24 hours incubation. Larvae that were administered caffeine by intra-haemocoel injection showed significant increases in haemocyte densities after 24 ($p < 0.05$) hours incubation when compared to their relevant controls. When compared with relevant controls there were no significant changes in the fungicidal ability of haemocytes extracted from monosodium glutamate challenged larvae.

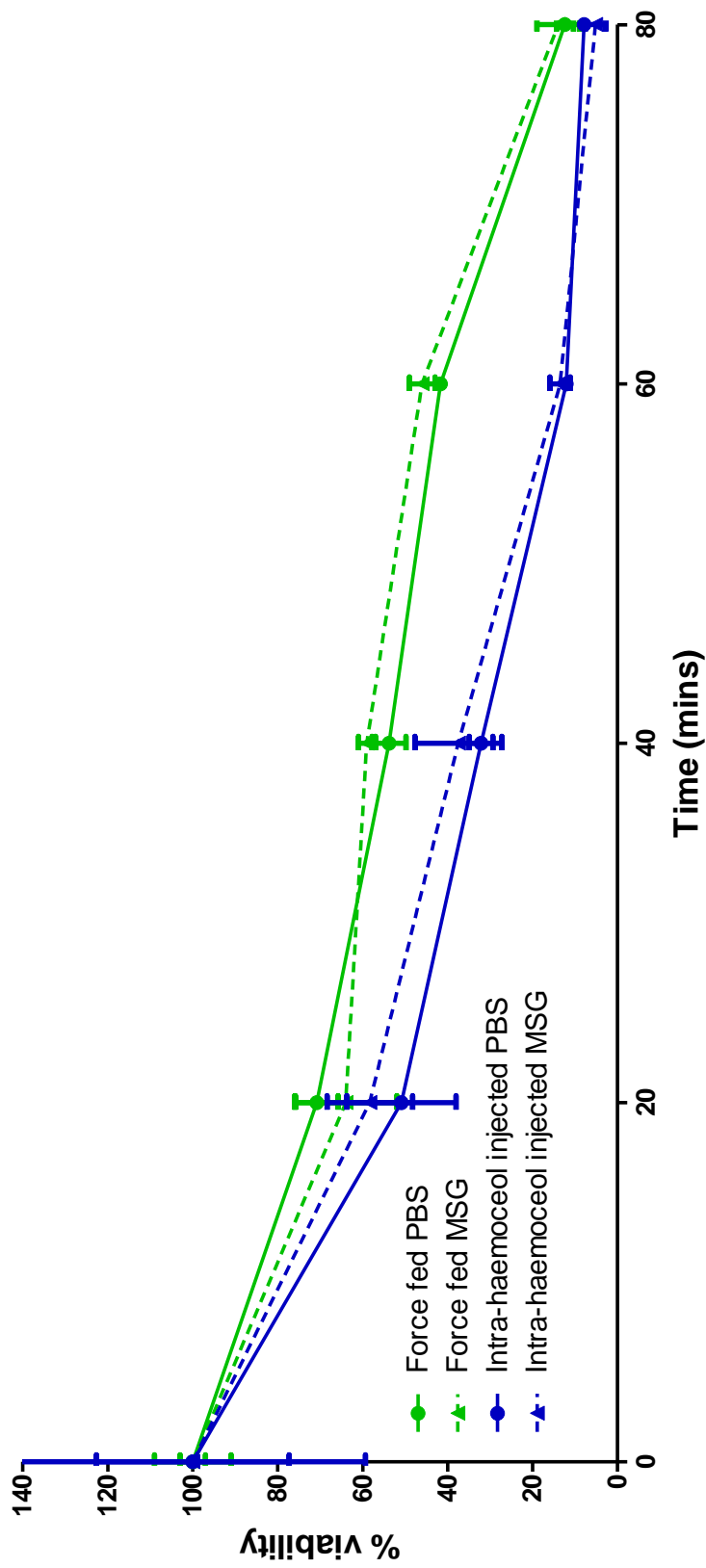


Fig. 6.3 Fungicidal activity of haemocytes from larvae administered monosodium glutamate by intra-haemocoel injection or force feeding at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined. All values are the mean \pm SE of 3 independent determinations.

6.6 Effects of monosodium glutamate on alkaline phosphatase activity in *G. mellonella* haemolymph

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.12M monosodium glutamate. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with p-nitrophenyl phosphate for 2 hours at 30°C. The hydrolysis of p-nitrophenyl phosphate by alkaline phosphatases was measured spectrometrically at 406 nm (Section 2.9.3).

As a positive control, larvae were physically shaken for 1 minute. Following this the alkaline phosphatase activity was measured at 4 and 24 hours at 30°C. The alkaline phosphatase activity in naive unshaken larvae was measured as 1.06 ± 0.01 Abs and 0.99 ± 0.06 Abs at 1 and 4 hours incubation respectively. The alkaline phosphatase activity in larvae shaken for 1 min was measured as 1.70 ± 0.03 Abs and 1.46 ± 0.10 Abs at 1 and 4 hours incubation respectively. When compared to their relevant controls this indicated a 0.59 fold increase ($p < 0.001$) after 1 hour and a 0.48 fold increase ($p > 0.01$) after 4 hours in the alkaline phosphate activity of larvae shaken for 1 min (Fig 6.4).

The alkaline phosphatase activity in injected control larvae was measured as 1.02 ± 0.11 Abs and 0.87 ± 0.08 Abs at 1 and 4 hours incubation respectively. The alkaline phosphatase activity in larvae force fed monosodium glutamate was measured as 1.55 ± 0.07 Abs and 0.9 ± 0.05 Abs at 1 and 4 hours incubation respectively. When compared to their relevant controls this indicated a 0.53 fold increase ($p > 0.01$) after 4 hours and a 0.03 fold increase after 24 hours in the alkaline phosphate activity of larvae force fed monosodium glutamate (Fig 6.5).

The alkaline phosphatase activity in force fed control larvae was measured as 1.05 ± 0.11 Abs and 1.32 ± 0.04 Abs at 1 and 4 hours incubation respectively. The alkaline phosphatase activity in larvae force fed monosodium glutamate was measured as 1.16 ± 0.02 Abs and 1.50 ± 0.06 Abs at 1 and 4 hours incubation respectively. When compared to their relevant controls this indicated a 0.11 fold increase after 4 hours and a 0.14 fold increase ($p > 0.05$) after 24 hours in the alkaline phosphate activity of larvae force fed monosodium glutamate (Fig 6.6).

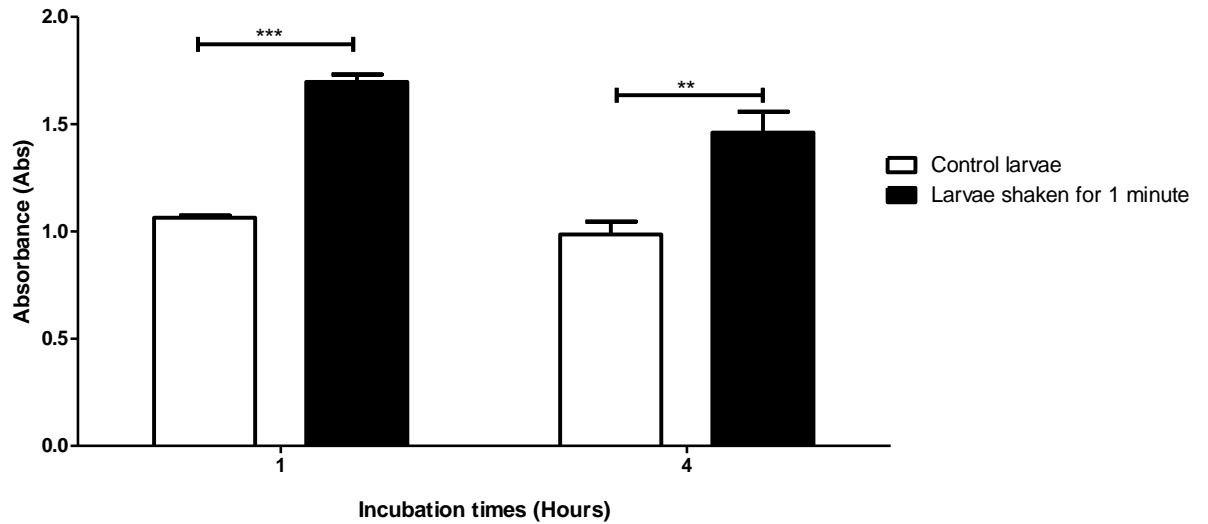


Fig. 6.4 Alkaline phosphatase activity of haemolymph from physically shaken larvae. After 4 and 24 hours haemolymph was extracted from larvae that were physically shaken. Alkaline phosphatase activity was determined as described. All values are the mean \pm SE of 3 independent determinations (**: $p < 0.01$) (***: $p < 0.001$).

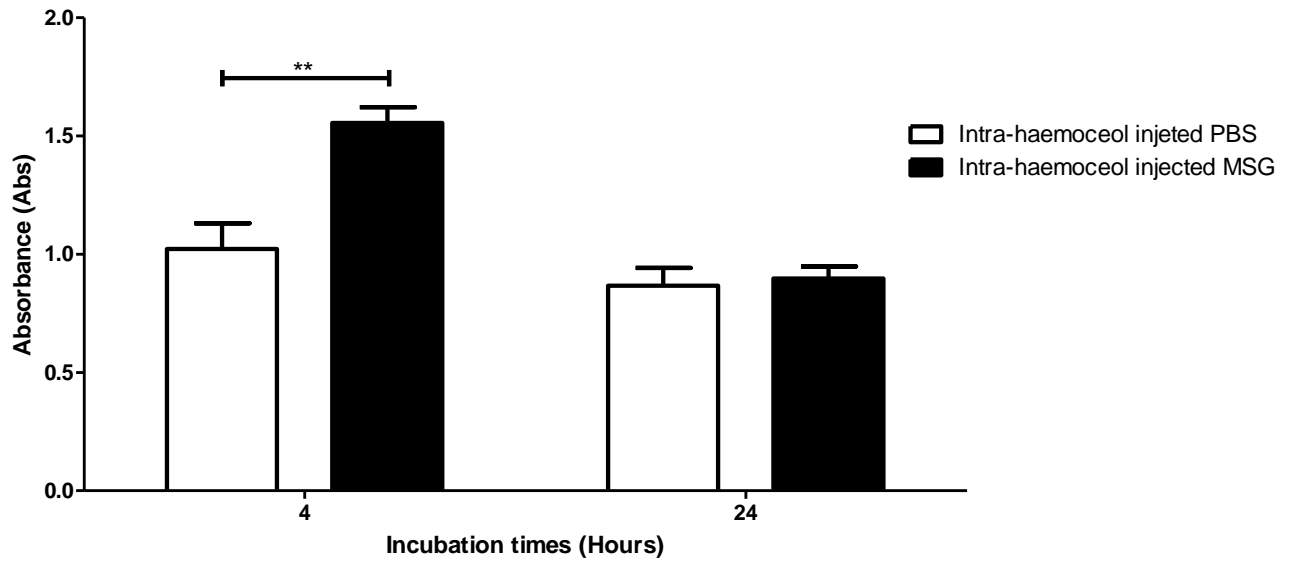


Fig. 6.5 Alkaline phosphatase activity of haemolymph from larvae administered monosodium glutamate via intra-haemocoel injection. After 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Alkaline phosphatase activity was determined as described. All values are the mean \pm SE of 3 independent determinations (**: $p < 0.01$).

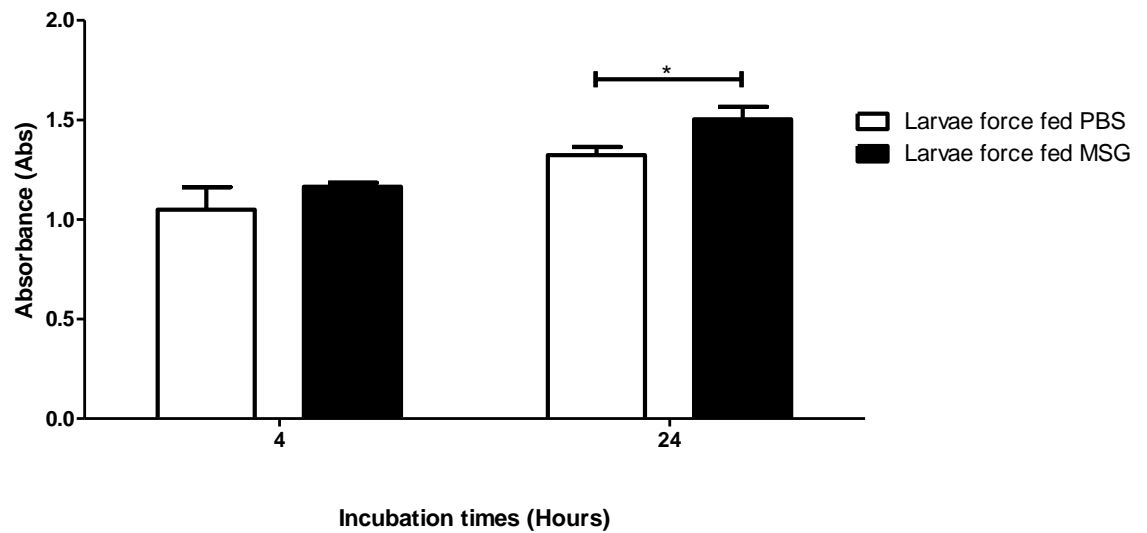


Fig. 6.6 Alkaline phosphatase activity of haemolymph from larvae administered monosodium glutamate via force feeding. After 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Alkaline phosphatase activity was determined as described. All values are the mean \pm SE of 3 independent determinations (*: $p < 0.05$).

6.7 Effects of monosodium glutamate on superoxide dismutase activity in *G. mellonella* haemolymph.

Superoxide dismutase activity in the haemolymph of larvae administered monosodium glutamate was measured. Larvae were administered 20 μl of 0.12 M monosodium glutamate by intra-haemocoel injection (Fig 6.6) or force feeding (Fig 6.7). Larvae were incubated at 30°C for 1, 4 and 24 hours and the total superoxide dismutase activity was measured in larval haemolymph. Superoxide dismutase activity was quantified as units of activity, 1 unit of superoxide dismutase activity equated to the 50% inhibition of the oxidation of quercetin (Section 2.9.2)

One unit of superoxide dismutase activity in PBS injected control larvae equated to $124.59 \pm 14.72 \mu\text{g}/\mu\text{l}$, $91.36 \pm 4.72 \mu\text{g}/\mu\text{l}$ and $106.04 \pm 18.54 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 1, 4 and 24 hours incubation respectively. One unit of superoxide dismutase activity in PBS force fed control larvae equated to $87.87 \pm 4.43 \mu\text{g}/\mu\text{l}$, $255.05 \pm 7.71 \mu\text{g}/\mu\text{l}$ and $283.93 \pm 33 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 1, 4 and 24-hours accordingly.

One unit of superoxide dismutase activity in larvae injected with monosodium glutamate equated to $73.46 \pm 12.91 \mu\text{g}/\mu\text{l}$, $88.29 \pm 26.32 \mu\text{g}/\mu\text{l}$ and $146.41 \pm 6.29 \mu\text{g}/\mu\text{l}$ of protein following 1, 4 and 24 hours incubation respectively. When comparing 1 hour superoxide dismutase activity a 0.41 fold increase ($p < 0.05$) was seen in monosodium glutamate injected larvae compared to PBS injected control larvae (Fig. 6.7).

Superoxide dismutase activity was also measured in haemolymph from larvae administered monosodium glutamate via force feeding prior to incubation. At 1, 4 and 24 hours one unit of superoxide dismutase activity was $73.82 \pm 3.63 \mu\text{g}/\mu\text{l}$, $266.33 \pm 62.41 \mu\text{g}/\mu\text{l}$ and $147.39 \pm 62 \mu\text{g}/\mu\text{l}$ of protein respectively. When comparing 24 hours superoxide dismutase activity a 0.48 fold increase ($p < 0.05$) was seen in monosodium glutamate injected larvae compared to PBS injected control larvae (Fig 6.8).

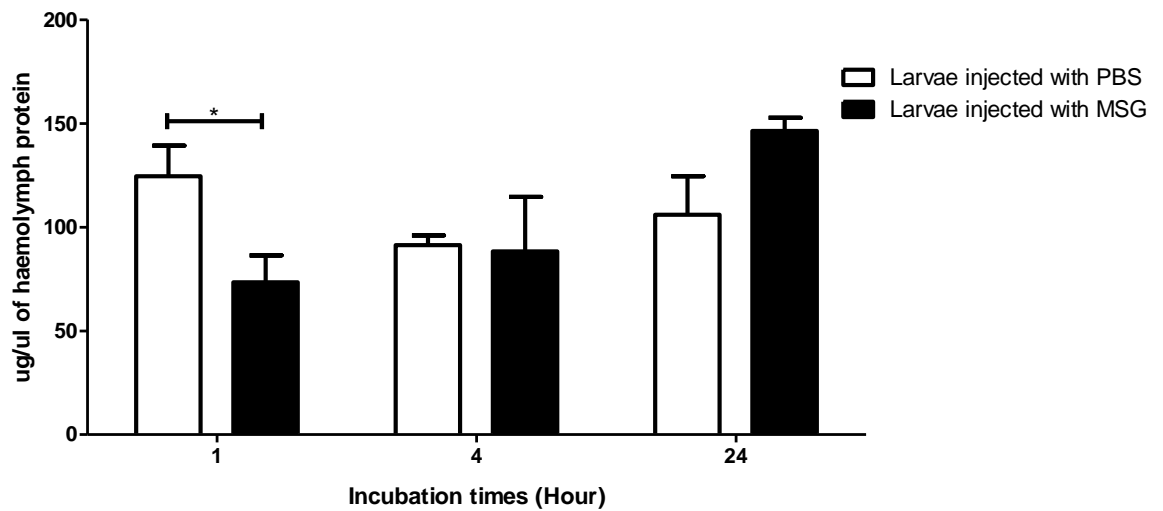


Fig. 6.7 Superoxide dismutase activity of haemolymph from larvae administered monosodium glutamate via intra-haemocoel injection. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations (*: $p < 0.05$).

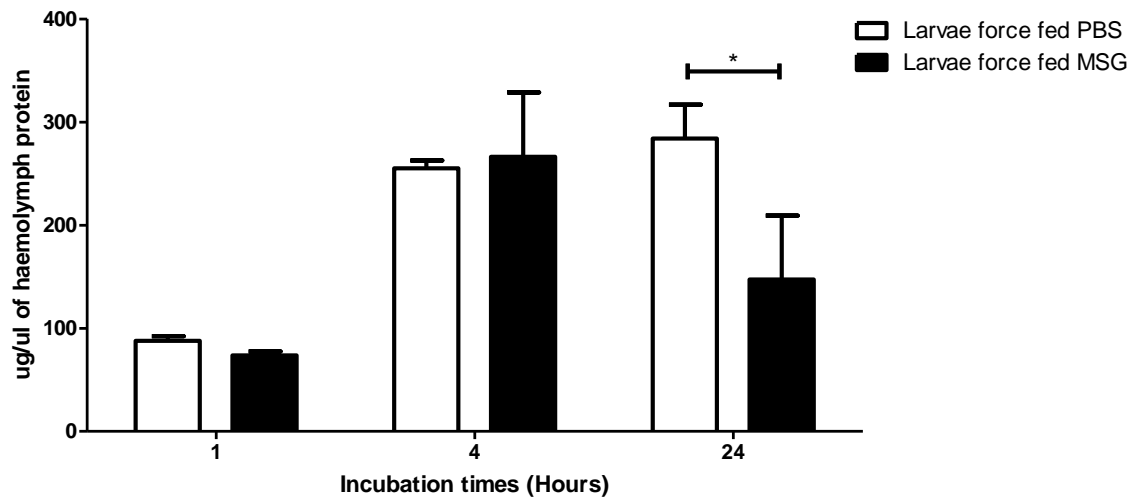


Fig. 6.8 Superoxide dismutase activity of haemolymph from larvae administered monosodium glutamate via force feeding. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered 0.12M of monosodium glutamate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations (*: $p < 0.05$).

6.8 Effects of monosodium glutamate on catalase activity in *G. mellonella* haemolymph.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.12M of monosodium glutamate. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with hydrogen peroxide. The decomposition of hydrogen peroxide and thus rate of catalase activity was measured spectrometrically at 240nm (Section 2.9.1).

The catalase activity in larvae force fed PBS was measured as 0.16 ± 0.04 Abs and 0.10 ± 0.03 Abs following 4 and 24 hours incubation respectively. In addition the catalase activity in larvae administered PBS by intra-haemocoel injection was measured as 0.20 ± 0.06 Abs and 0.15 ± 0.05 Abs following 4 and 24 hour incubation respectively.

Larvae were administered monosodium glutamate by force feeding. The catalase activity in these larvae was measured as 0.13 ± 0.03 Abs and 0.11 ± 0.02 Abs after 4 and 24 hours incubation respectively. These results showed no significant changes between control and treated groups (Fig 6.9).

Larvae were administered monosodium glutamate via intra-haemocoel injection. The catalase activity in these larvae was measured as 0.17 ± 0.06 Abs and 0.10 ± 0.06 Abs after 4 and 24 hours incubation respectively. Similar to the previous route of administration these results showed no significant differences between control and treated groups (Fig 6.10).

6.9 Summary.

The activity of alkaline phosphatase, superoxide dismutase and catalase were measured in the haemolymph of larvae challenged with monosodium glutamate via force feeding or direct injection into the haemocoel. When comparing 1hour superoxide dismutase activity a 0.41 fold increase ($p < 0.05$) was seen in monosodium glutamate injected larvae compared to PBS injected control larvae. In addition when comparing 24 hour superoxide dismutase activity a 0.48 fold increase ($p < 0.05$) was seen in monosodium glutamate fed larvae compared to force fed

control larvae. When compared to relevant controls no significant changes were seen in the catalase activity of larvae administered monosodium glutamate. When compared to relevant controls a 0.14 fold increase ($p > 0.05$) in the alkaline phosphate activity of larvae force fed monosodium glutamate was observed at 24 hours and a 0.53 fold increase ($p > 0.01$) in the alkaline phosphate activity of larvae injected with monosodium glutamate was observed at 4 hours.

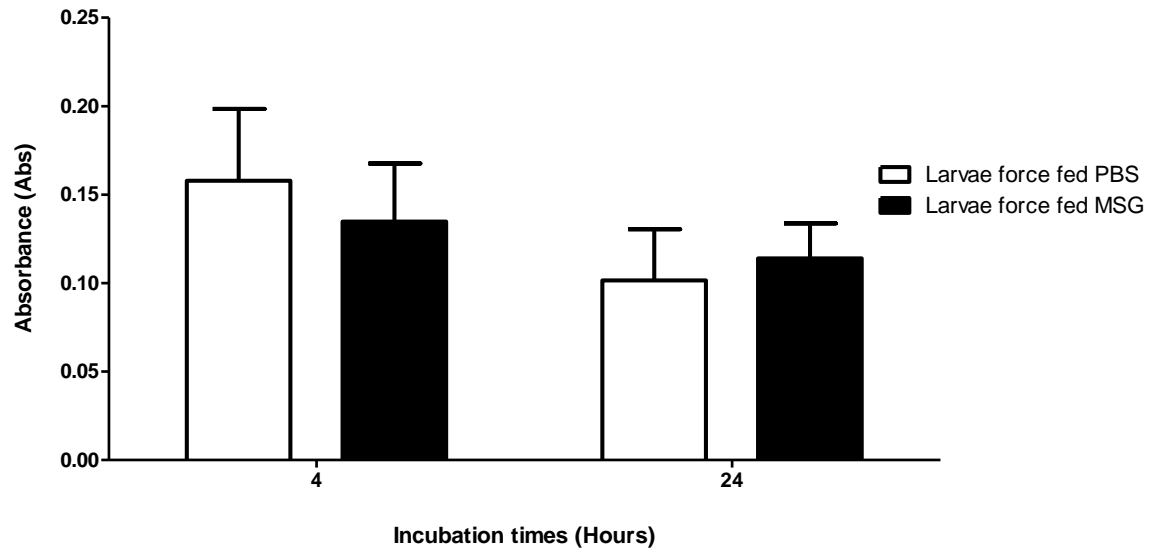


Fig. 6.9 Catalase activity of haemolymph from larvae administered monosodium glutamate via force feeding. After 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

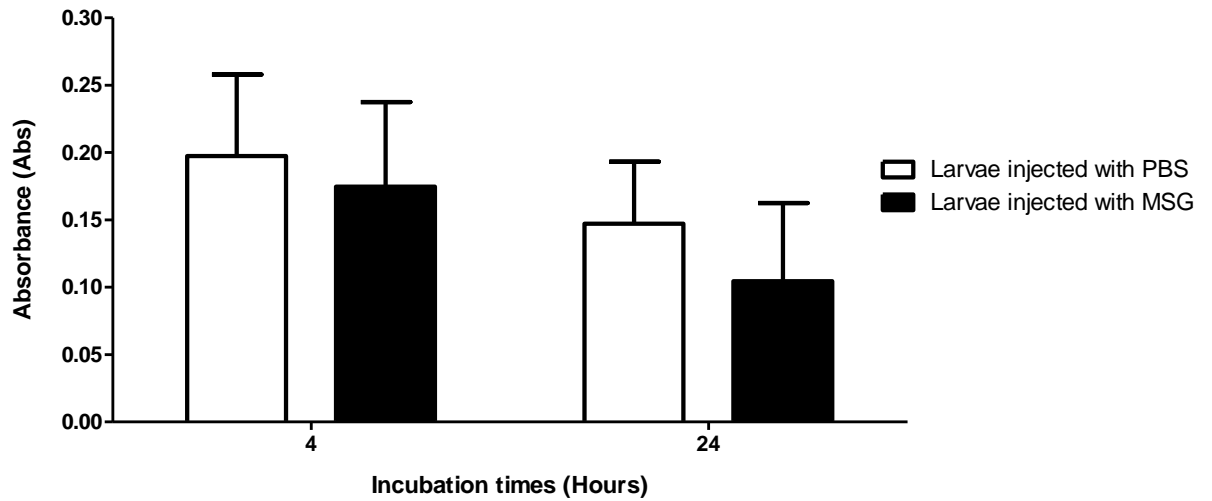


Fig. 6.10 Catalase activity of haemolymph from larvae administered monosodium glutamate intra-haemocoel injection. After 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

6.10 Effects of monosodium glutamate on the proteome in *G. mellonella* haemolymph

Label free quantitative proteomics was conducted on haemolymph from larvae injected with 20µl of 0.12M monosodium glutamate and incubated for 24 hours. Principal component analyses was employed showing clustering of control and treatment replicates (Fig. 6.11). In total 368 peptides were identified representing 292 proteins with two or more peptides and 24 proteins differentially expressed (Fig. 6.12). Seventeen proteins (9 proteins imputed and 8 proteins non-imputed) were significantly increased in abundance in larvae injected with monosodium glutamate compared to control larvae (Table 6.1). Seven proteins (2 proteins imputed and 5 proteins non-imputed) were significantly decreased in abundance in larvae injected with monosodium glutamate compared to control larvae (Table 6.2). The exclusively expressed hits showed 7 proteins only expressed in monosodium glutamate injected larvae (Table 6.3) and 2 proteins only expressed in control larvae (Table 6.4).

The protein showing the highest increase in abundance in larvae exposed to monosodium glutamate was peptidoglycan recognition-like protein B, partial with a fold increase of 33.16 ($p < 0.05$), while the proteins highest in abundance with an imputed value were gloverin (129.60), RecName: Full=Cecropin-D-like peptide (68.78), gloverin-like protein, partial (58.49) and peptidoglycan-recognition proteins-LB, partial (42.43). Numerous proteins involved in the immune response were significantly increased in abundance in larvae administered monosodium glutamate by intra-haemocoel injection (e.g. gloverin (129.60), gloverin-like protein, partial (58.49), peptidoglycan-recognition proteins-LB, partial (42.43), peptidoglycan recognition-like protein B, partial (33.12), RecName: Full+Lysozyme; AltName: Full=1,4-beta-N-acetylmuramidase (7.46), prophenol oxidase activating enzyme 3 (2.37) and peptidoglycan recognition protein precursor (2.29) (Table 6.1)

Proteins decreased in abundance in larvae that received monosodium glutamate included cathepsin B-like cysteine proteinase (3.68 fold decrease), promoting protein precursor (3.65 fold decrease), 3-dehydroecdysone 3beta-

reductase (1.93 fold decrease) and hexamerin storage protein PinSP2 (1.65 fold decrease) (Table 6.2).

Blast2GO annotation software was used to group proteins based on conserved GO terms in order to identify processes and pathways potentially associated with monosodium glutamate metabolism. GO terms were categorized by biological processes (BP) and molecular function (MF), cellular components (CC) and enzyme function (EF).

The increases in BP included proteins labelled as catabolic process (7 proteins in control – 8 proteins in treated larvae), cellular component organization (4-4), cellular metabolic process (11-11), establishment of localisation (5-5), negative regulation of cellular process (4-4), nitrogen compound metabolic process (11-12), organic substance metabolic process (23-23), primary metabolic process (18-17), regulation of biological quality (8-9), regulation of cellular process (7-7), response to stress (4-7), single organism cellular process (12-12), single organism metabolic process (13-13), response to external stimulus (0-5), response to biotic stimulus (0-5), immune response (0-5) (Fig 6.13). No significant changes were observed.

The increases in MF included proteins labelled as oxidoreductase activity (9 proteins in control – 9 proteins in treated larvae), small molecule binding (7-7), organic cyclic compound binding (8-8), ion binding (15-16), hydrolase activity (29-30) and heterocyclic compound binding (8-8) (Fig. 6.14). No significant changes were observed.

The increases in CC included proteins labelled as intracellular organelle (1 proteins in control – 1 proteins in treated larvae), cell projection (1-1), anchoring junction (1-1), cell-substrate junction (1-1), extracellular space (4-5), catalytic complex (1-1), non-membrane-bounded organelle (1-1), intracellular (2-2), cell leading edge (1-1), intrinsic component of membrane (1-1), intracellular part (2-2). (Fig. 6.15). No significant changes were observed.

The increases in EF included proteins labelled as oxidoreductases (5 proteins in control – 5 proteins in treated larvae), transferases (2-2), hydrolases (10-11), lyases

(1-1), isomerases (1-1) and ligases (1-1) (Fig. 6.16). No significant changes were observed.

6.11 Summary

Label free quantitative proteomics was conducted on haemolymph from larvae injected with 20µl of 0.12M monosodium glutamate and incubated for 24 hours. In total 368 peptides were identified. Sixteen proteins were significantly increased in abundance in larvae injected with monosodium glutamate compared to control larvae. Seven proteins (2 proteins imputed and 5 proteins non-imputed) were significantly decreased in abundance in larvae injected with monosodium glutamate compared to control larvae. Numerous proteins involved in the immune response were significantly increased in abundance in larvae administered monosodium glutamate by intra-haemocoel injection.

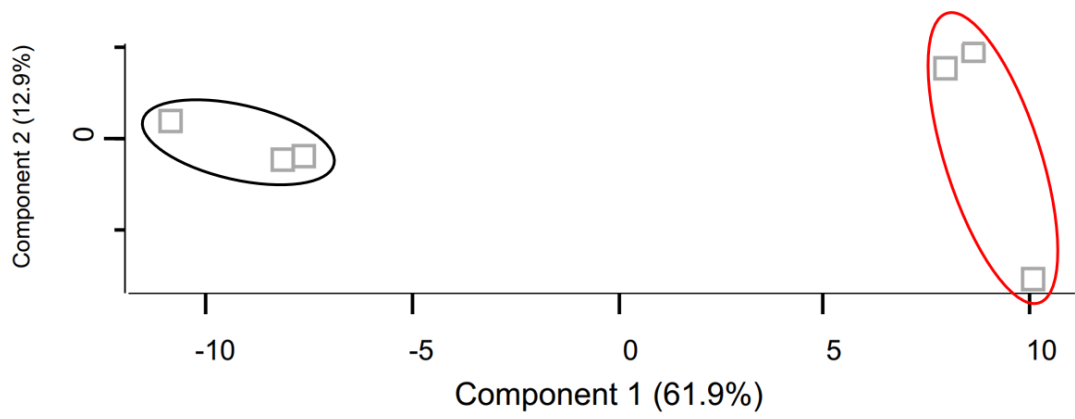


Fig. 6.11 Principal component analyses of the proteome of control larvae (black circle) and larvae injected with monosodium glutamate (red circle).

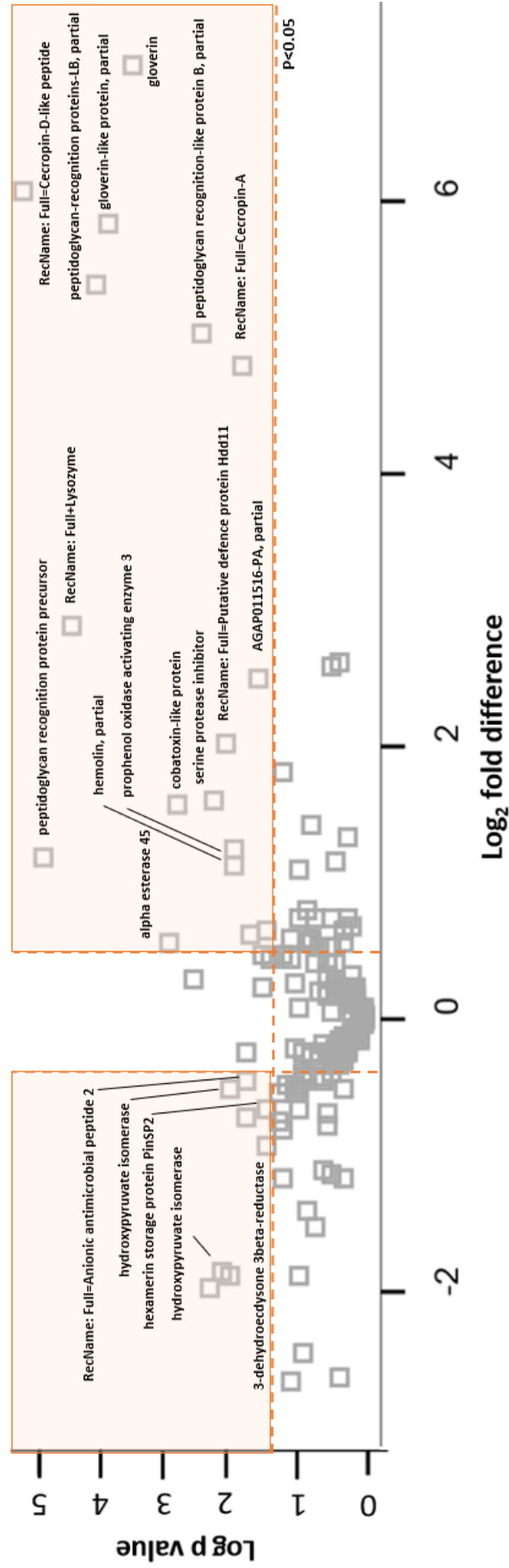


Fig. 6.12 Volcano plot showing proteins altered in abundance in *G. mellonella* larvae treated with monosodium glutamate. Proteins above the line are statistically significant ($p < 0.05$) and those to the right and left of the vertical lines indicate fold changes 1.5 fold positive and 1.5 fold negative in the monosodium glutamate treated larvae.

protein name (*=imputed vale)	Peptides	Sequence coverage %	Mean LFQ Intensity	Fold Difference
*gloverin	5	59.6	4.63E+09	129.60
*RecName: Full=Cecropin-D-like peptide	2	14.5	2.87E+09	68.78
*gloverin-like protein,partial	4	18.5	1.28E+09	58.47
*peptidoglycan-recognition proteins-LB, partial	7	89.5	1.04E+09	42.43
peptidoglycan recognition-like protein B, partial	13	32.6	2.35E+09	33.12
*RecName: Full=Cecropin-A	2	12.7	1.35E+09	28.00
*RecName: Full+Lysozyme	6	16.7	2.74E+08	7.46
AGAP011516-PA, partial	5	44.4	3.48E+08	5.66
*RecName: Full=Putative defense protein Hdd11	4	42.9	98276000	4.06
*serine protease inhibitor	6	18	1.54E+08	3.02
*cobatoxin-like protein	2	15.8	1.71E+08	2.96
prophenol oxidase activating enzyme 3	15	30.7	6.36E+08	2.37
peptidoglycan recognition protein precursor	11	52.6	4.14E+09	2.29
hemolin, partial	22	60.7	9.15E+09	2.20
heat shock protein hsp21.4	10	19.2	5.48E+08	1.57
RecName: Full=Beta-1,3-glucan-binding protein	2	14.5	6.06E+08	1.55
alpha esterase 45	13	15.7	1.42E+09	1.46

Table 6.1 Relative fold changes of proteins increased in abundance in *G. mellonella* larvae administered monosodium glutamate and the number of matched peptides, sequence coverage and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 were considered to be in significantly variable abundances between control and treated larvae.

protein name (*=imputed vale)	Peptides	Sequence coverage %	Mean LFQ Intensity	Fold Difference
*cathepsin B-like cysteine proteinase	7	18	2.17E+08	-3.68
*promoting protein precursor	6	18.1	96762000	-3.65
3-dehydroecdysone 3beta- reductase	11	31.5	8.29E+08	-1.93
Unknown	16	37.4	1.63E+10	-1.70
hexamerin storage protein PinSP2	14	63.2	1.2E+11	-1.65
hydroxypyruvate isomerase	20	38.4	2.31E+09	-1.58
RecName: Full=Anionic antimicrobial peptide 2	11	32.9	7.48E+10	-1.47

Table 6.2 Relative fold changes of proteins decreased in abundance in *G. mellonella* larvae administered monosodium glutamate and the number of matched peptides, sequence coverage and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than ± 1.5 were considered to be in significantly variable abundances between control and treated larvae.

Protein Annotation	LFQ Intensity Control 1	LFQ Intensity Control 2	LFQ Intensity Control 3	LFQ Intensity MSG inj 1	LFQ Intensity MSG inj 2	LFQ Intensity MSG inj 3
serine protease inhibitor	NaN	NaN	NaN	26.11	25.59	25.15
RecName: Full=Cecropin-D-like peptide	NaN	NaN	NaN	29.85	29.35	29.22
RecName: Full+Lysozyme	NaN	NaN	NaN	26.28	26.54	26.45
RecName: Full=Putative defense protein Hdd11	NaN	NaN	NaN	25.00	25.08	24.96
peptidoglycan-recognition proteins-LB, partial	NaN	NaN	NaN	28.67	28.73	28.13
gloverin-like protein,partial	NaN	NaN	NaN	28.99	29.16	27.95
gloverin	NaN	NaN	NaN	30.53	30.71	30.34

Table 6.3 LFQ intensities of proteins exclusively expressed in the haemolymph of larvae injected with monosodium glutamate. A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”

Protein Annotation	LFQ Intrnsity Control 1	LFQ Intensity Control 2	LFQ Intensity control 3	LFQ Intensity MSG inj 1	LFQ Intensity MSG inj 2	LFQ Intensity MSG inj 3
promoting protein precursor	24.91	24.93	24.71	NaN	NaN	NaN
cathepsin B-like cysteine proteinase	25.24	25.50	25.88	NaN	NaN	NaN

Table 6.4 LFQ intensities of proteins exclusively expressed in the haemolymph of control larvae injected with PBS. A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”

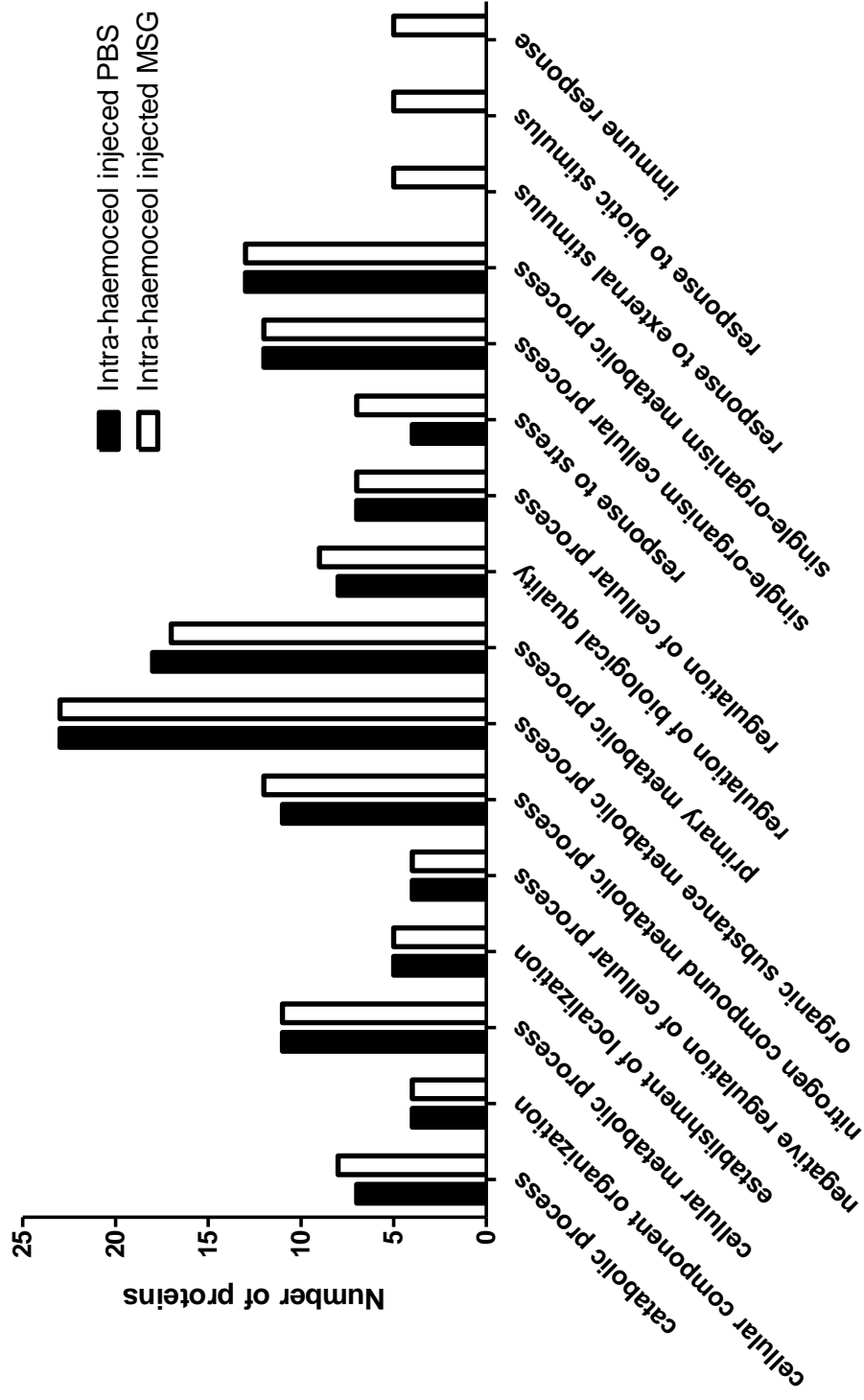


Fig. 6.13 Bar chart showing changes to number of proteins involved in various biological processes at level 3 ontology. Proteins were assigned groups based on involvement in biological processes for control and monosodium glutamate treated larvae. Closed bar: control, open bar: monosodium glutamate treated larvae

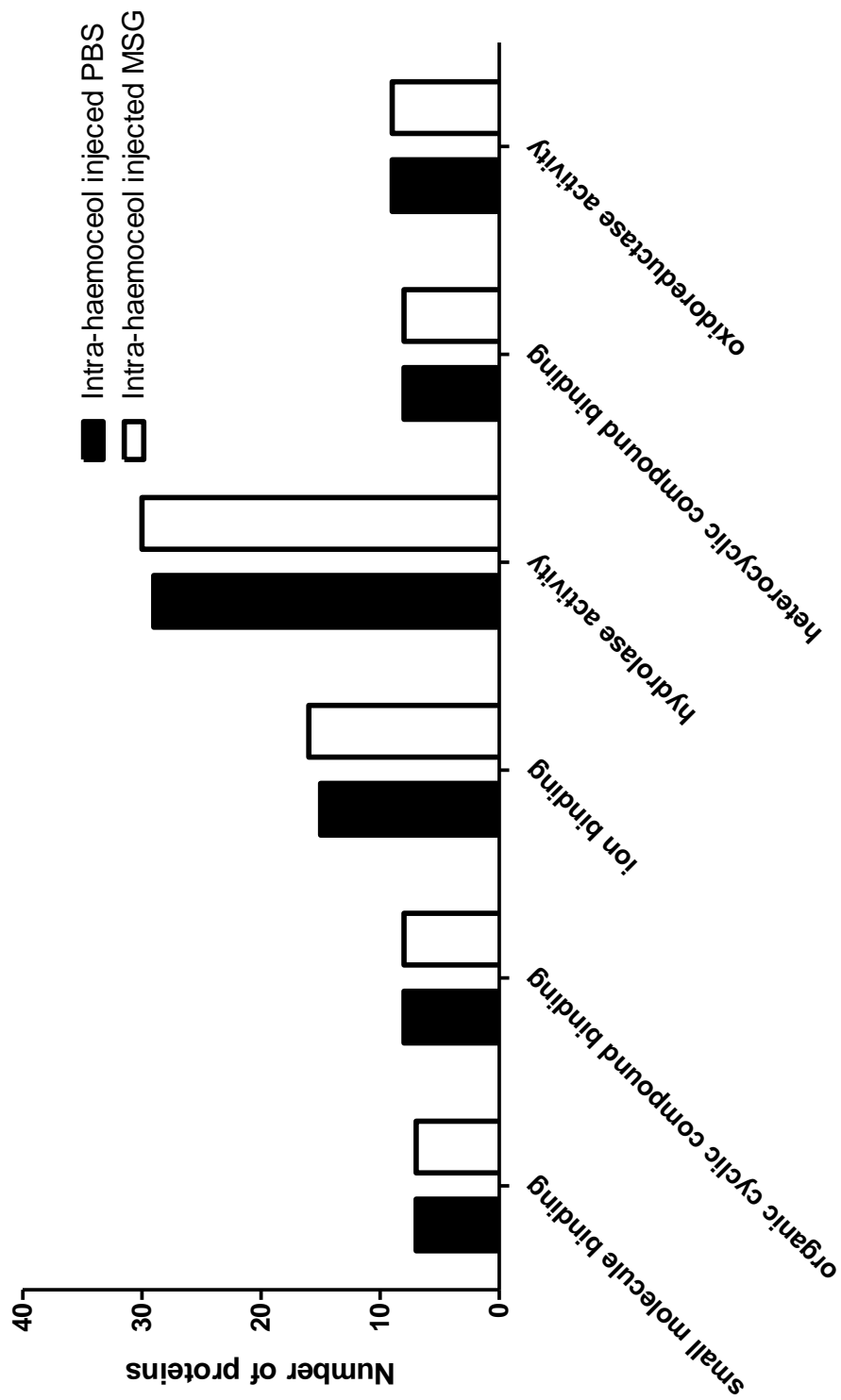


Fig. 6.14 Bar chart showing changes to number of proteins given various molecular functions at level 3 ontology. Proteins were assigned groups based on involvement in molecular function for control and monosodium glutamate treated larvae. Closed bar: control, open bar: monosodium glutamate treated larvae.

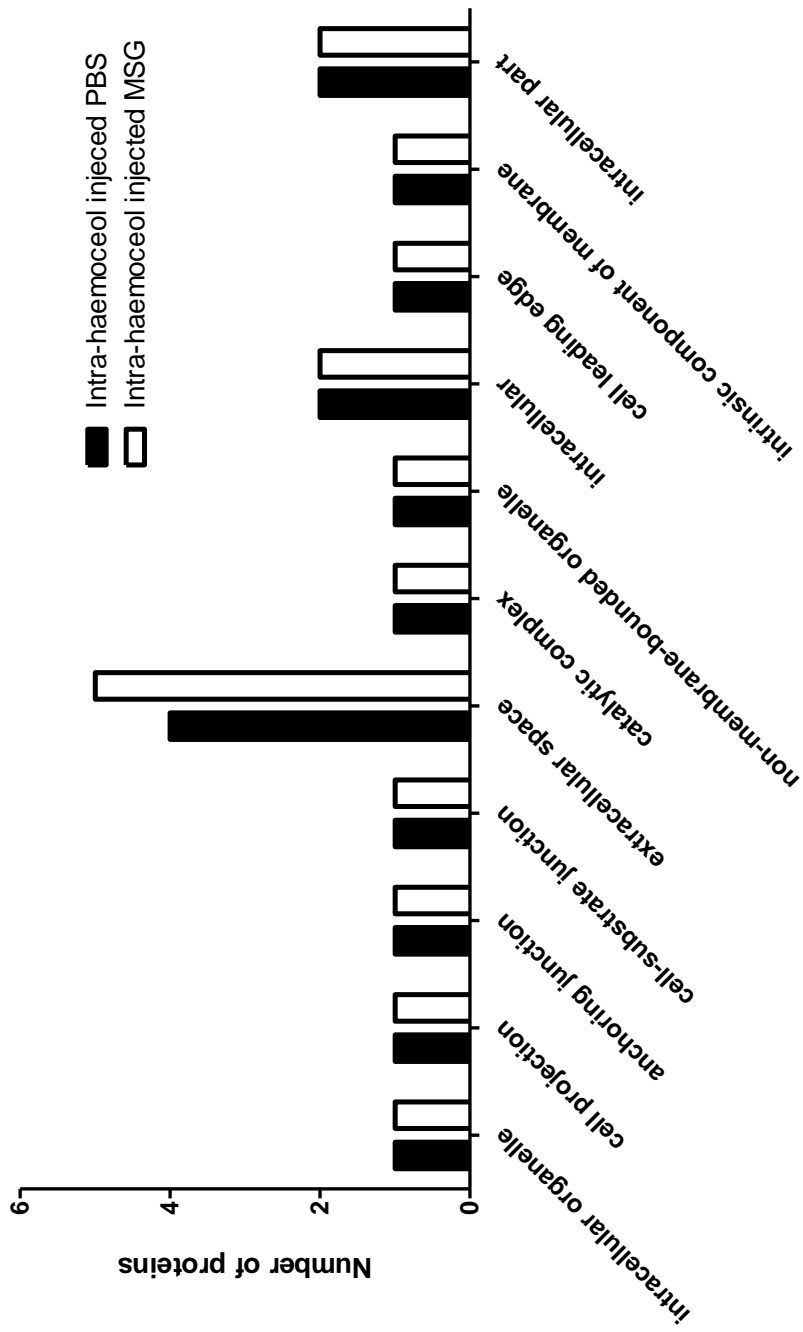


Fig. 6.15 Bar chart showing changes to number of proteins in various cellular components at level 3 ontology. Proteins were assigned groups based on involvement in cellular components in the total proteome for control and monosodium glutamate treated larvae. Closed bar: control, open bar: monosodium glutamate treated larvae

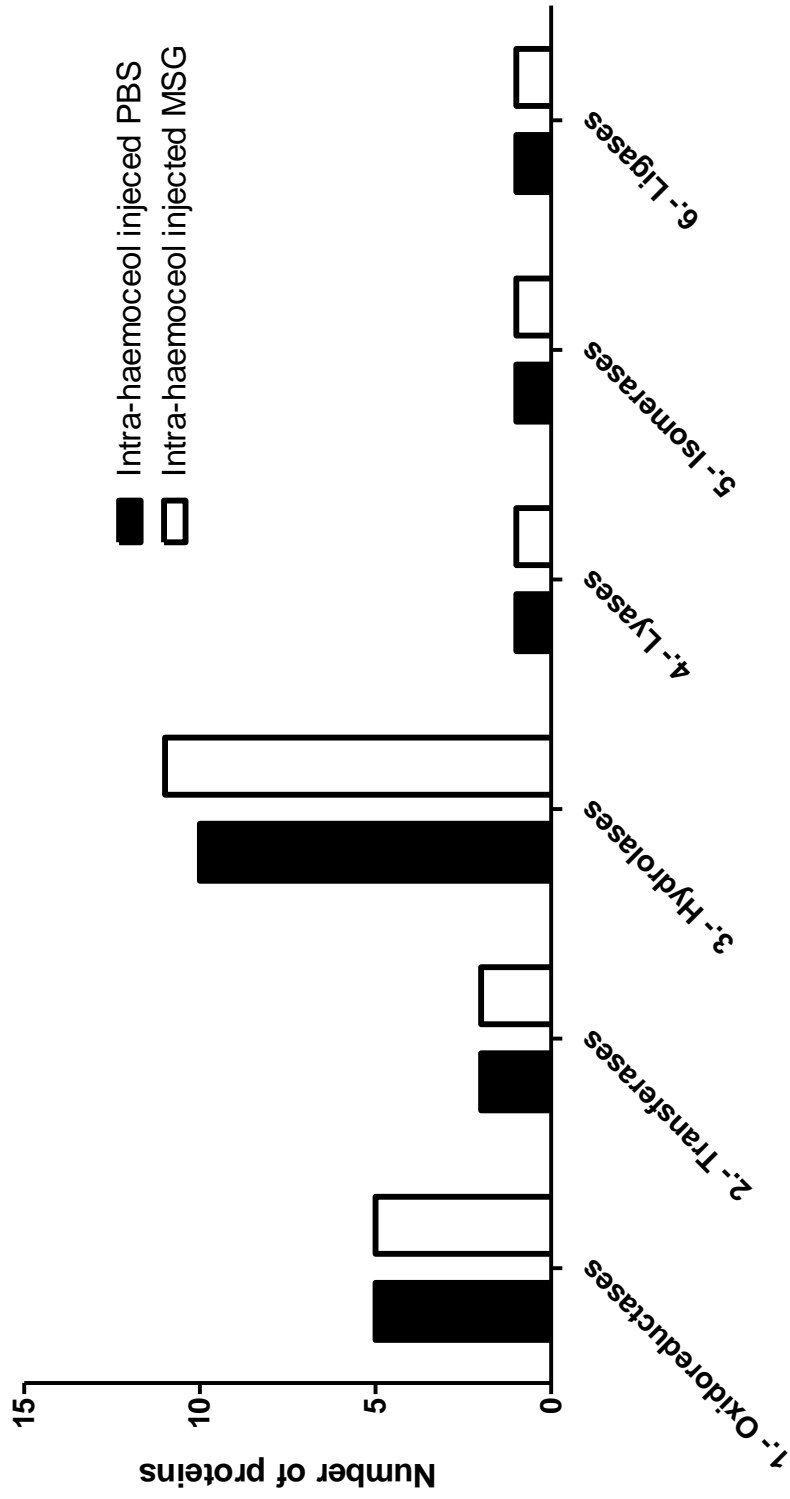


Fig. 6.16 Bar chart showing changes to number of proteins grouped into their enzymatic function. Proteins were assigned groups based on involvement in enzymatic processes in the total proteome for control and monosodium glutamate treated larvae. Closed bar: control, open bar: monosodium glutamate treated larvae

6.12 Effect of creatine monohydrate administration on haemocyte densities.

Larvae were administered by force feeding or intra-haemocoel injection 20 μ l of 0.15 M creatine monohydrate, as this was the highest concentration that could be achieved in solution without resulting in larval death. The effect of creatine monohydrate administration on the density of circulating haemocytes in larval haemolymph was ascertained after 4 and 24-hour incubation at 30°C. For experimental purposes control larvae were administered PBS by intra haemocoel injection or force feeding.

Larvae force fed PBS and incubated for 4 or 24 hours at 30°C showed $0.89 \pm 0.07 \times 10^7$ and $0.66 \pm 0.06 \times 10^7$ haemocytes/ml respectively. Larvae force fed creatine monohydrate and incubated for 4 hours showed $0.64 \pm 0.15 \times 10^7$ haemocytes/ml. Larvae force fed creatine monohydrate and incubated for 24 hours showed $0.93 \pm 0.12 \times 10^7$ haemocytes/ml. These findings showed no significant differences between control and treatment groups (Fig 6.17).

Larvae injected with PBS and incubated for 4/24 hours at 30°C showed $1.25 \pm 0.15 \times 10^7$ and $0.93 \pm 0.09 \times 10^7$ haemocytes/ml respectively. Larvae injected with creatine monohydrate and incubated for 4 hours showed $1.45 \pm 0.23 \times 10^7$ haemocytes/ml. Larvae injected with creatine monohydrate and incubated for 24 hours showed $1.53 \pm 0.35 \times 10^7$ haemocytes/ml. This equated to a 0.63 fold increase ($p < 0.05$) in haemocyte densities between control larvae and creatine monohydrate injected larvae following 24 hour incubation (Fig 6.17).

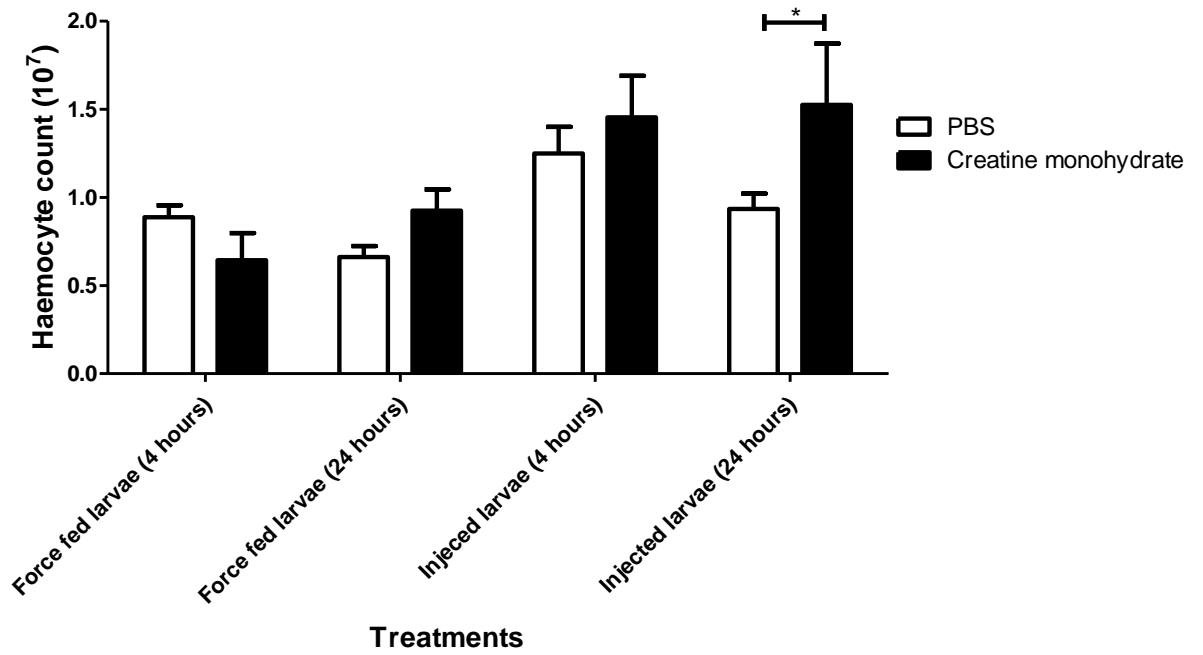


Fig. 6.17 Effect of creatine monohydrate on haemocyte density in force fed and intra-haemocoel injected *G. mellonella* larvae at 4 and 24 hours. (*: $p < 0.05$). All values are the mean \pm SE of 3 independent determinations.

6.13 Analysis of the effect of creatine monohydrate administration on haemocyte mediated pathogen killing.

Larvae were administered by intra haemocoel injection or force feeding 20 µl of 0.15 M creatine monohydrate. Haemocytes were extracted from control and test larvae following 24-hour incubation at 30°C and mixed with opsonised *C. albicans* for 80-minutes time (Fig. 6.18). By counting the percentage viability of *C. albicans* at 20 min intervals, the effect of creatine monohydrate on haemocyte mediated pathogen killing was ascertained.

Haemocytes from PBS force fed control larvae killed 45.12 ± 4.84%, 76.22 ± 10.18%, 86.59 ± 4.22%, 93.29 ± 3.80% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. Haemocytes from injected control larvae killed 50 ± 10.71%, 57.14 ± 12.37%, 65.48 ± 5.46%, 77.38 ± 7.43% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 6.18).

Haemocytes from creatine monohydrate force fed larvae killed 38.81 ± 4.63%, 60.74 ± 3.39%, 80.74 ± 3.39%, 85.92 ± 10.96% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively. In addition haemocytes from creatine monohydrate injected larvae killed 41.67 ± 10.05%, 62.5 ± 10.25%, 86.46 ± 2.39%, 94.79 ± 2.38% of *C. albicans* MEN cells after 20, 40, 60 and 80 minutes respectively (Fig. 7.18).

The results indicated no significant changes in haemocyte mediated killing between control larvae and larvae administered caffeine by intra-haemocoel injection or force feeding.

6.14 Summary

Larvae were administered creatine monohydrate by force feeding and intra-haemocoel injection. Larvae that were administered creatine monohydrate by intra-haemocoel injection showed significant increases in haemocyte densities after 24 (p < 0.05) hours incubation when compared to their relevant controls. In addition when compared with relevant controls there was no significant changes in the fungicidal ability of haemocytes extracted from monosodium glutamate challenged larvae.

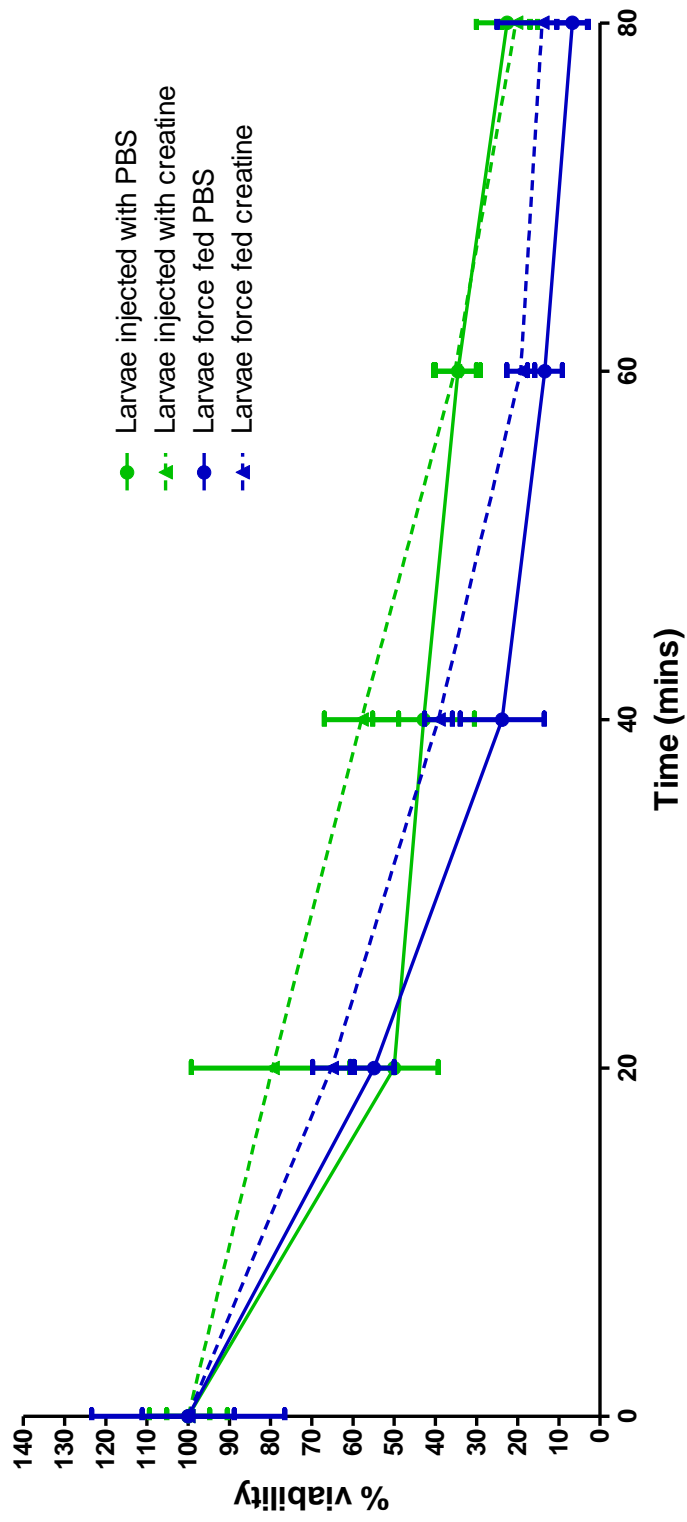


Fig. 6.18 Fungicidal activity of haemocytes from larvae administered creatine monohydrate by intra-haemocoel injection or force feeding at 24 hours. Haemocytes ability to kill *Candida albicans* cells was determined. All values are the mean \pm SE of 3 independent determinations.

6.15 Effects of creatine monohydrate on alkaline phosphatase activity in *G. mellonella* haemolymph.

Larvae were administered 20 µl of 0.15M creatine monohydrate by intra haemocoel injection or force feeding. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with p-nitrophenyl phosphate for 2 hours at 30°C. The hydrolysis of p-nitrophenyl phosphate by alkaline phosphatases is measured at 406nm (Section 2.9.3).

As a positive control, larvae were physically shaken for 1 minute. Following this alkaline phosphatase activity was measured at 1 and 4 hours at 30°C. Alkaline phosphatase activity in unshaken larvae was measured as 1.06 ± 0.01 Abs and 0.99 ± 0.06 Abs after 1 and 4 hours incubation respectively. Alkaline phosphatase activity in larvae shaken for 1 min was measured as 1.70 ± 0.03 Abs and 1.46 ± 0.10 Abs at 1 and 4 hours incubation respectively. When compared to their relevant controls this indicated a 0.59 fold increase ($p < 0.001$) after 1 hour and a 0.48 fold increase ($p < 0.01$) after 4 hours in the alkaline phosphate activity.

Alkaline phosphatase activity in injected control larvae was measured as 1.47 ± 0.30 Abs, 1.36 ± 0.36 Abs and 1.25 ± 0.24 Abs after 1, 4 and 24 hours incubation respectively. The alkaline phosphatase activity in larvae force fed creatine monohydrate was measured as 1.89 ± 0.44 Abs, 0.62 ± 0.50 Abs and 1.54 ± 0.35 Abs after 1, 4 and 24 hours incubation respectively. There were no significant changes seen between controls and creatine monohydrate force fed larvae (Fig 6.19).

The alkaline phosphatase activity in force fed control larvae was measured as 1.26 ± 0.30 Abs, 1.04 ± 0.57 Abs and 1.25 ± 0.54 Abs after 1, 4 and 24 hours incubation respectively. The alkaline phosphatase activity in larvae force fed creatine monohydrate was measured as 1.54 ± 0.44 Abs, 1.21 ± 0.47 Abs and 1.28 ± 0.33 Abs after 1, 4 and 24 hours incubation respectively. There were no significant changes seen between controls and creatine monohydrate force fed larvae (Fig 6.20).

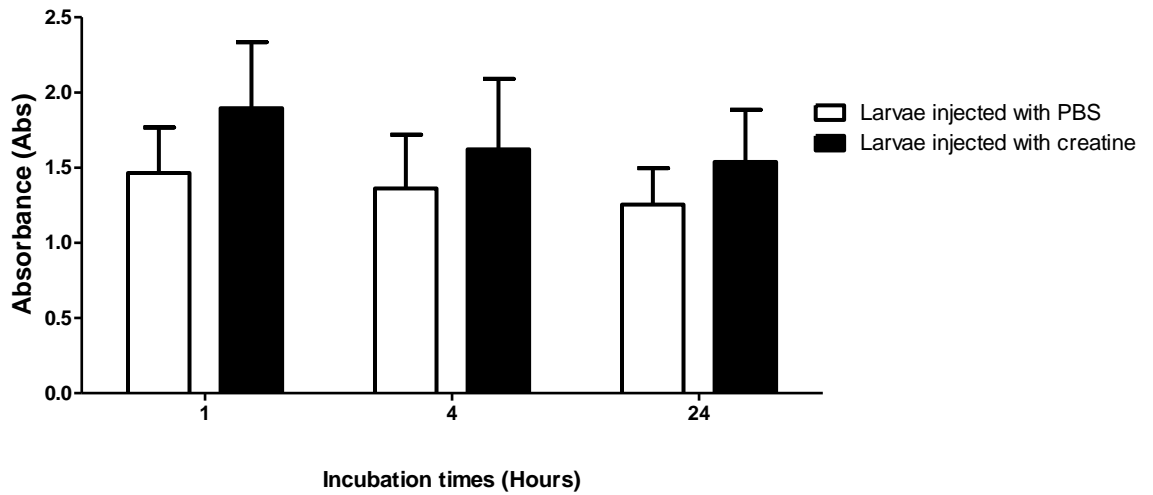


Fig. 6.19 Alkaline phosphatase activity of haemolymph from larvae administered creatine monohydrate via intra-haemocoel injection. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered creatine monohydrate. Alkaline phosphatase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

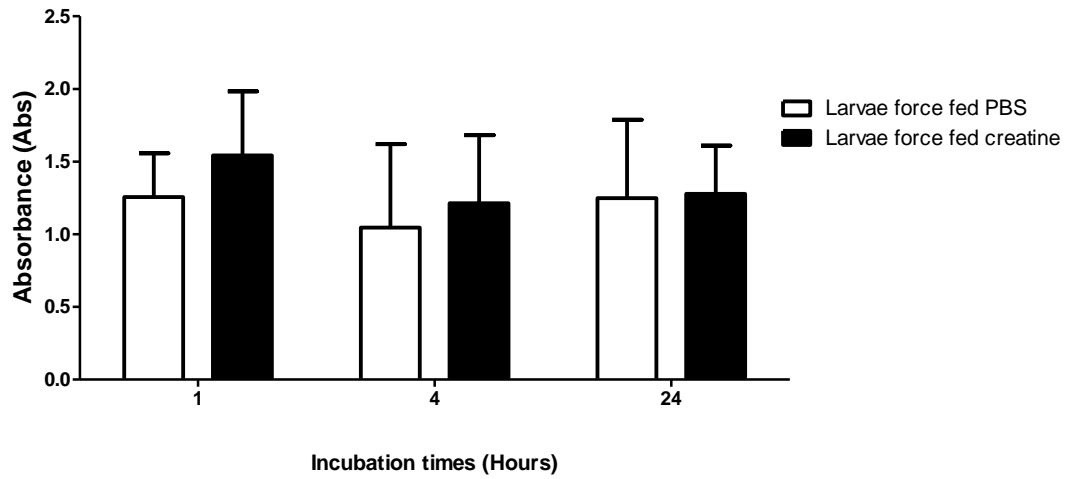


Fig. 6.20 Alkaline phosphatase activity of haemolymph from larvae administered creatine monohydrate via force feeding. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered creatine monohydrate. Alkaline phosphatase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

6.16 Effects of creatine monohydrate on catalase activity in *G. mellonella* haemolymph.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.15 M creatine monohydrate. Following 4 and 24 hours incubation haemolymph was extracted from larvae and incubated with hydrogen peroxide. The decomposition of hydrogen peroxide and thus rate of catalase activity was measured spectrometrically at 240nm (Section 2.9.1).

The catalase activity in larvae force fed PBS was measured as 0.24 ± 0.10 Abs, 0.34 ± 0.09 Abs and 0.49 ± 0.09 Abs following 1, 4 and 24 hours incubation respectively. In addition the catalase activity in larvae administered PBS by intra-haemocoel injection was measured as 0.28 ± 0.11 Abs, 0.24 ± 0.12 Abs and 0.33 ± 0.13 Abs following 1, 4 and 24 hour incubation respectively.

Larvae were administered creatine monohydrate by force feeding. The catalase activity in these larvae was measured as 0.28 ± 0.05 Abs, 0.30 ± 0.08 Abs and 0.41 ± 0.07 Abs after 1, 4 and 24 hours incubation respectively. These results showed no significant changes between control and treated groups (Fig 6.21).

Larvae were administered creatine monohydrate via intra-haemocoel injection. The catalase activity in these larvae was measured as 0.22 ± 0.07 Abs, 0.29 ± 0.08 Abs and 0.32 ± 0.19 Abs after 1, 4 and 24 hours incubation respectively. Similar to the previous route of administration these results showed no significant changes between control and treated groups (Fig 6.21).

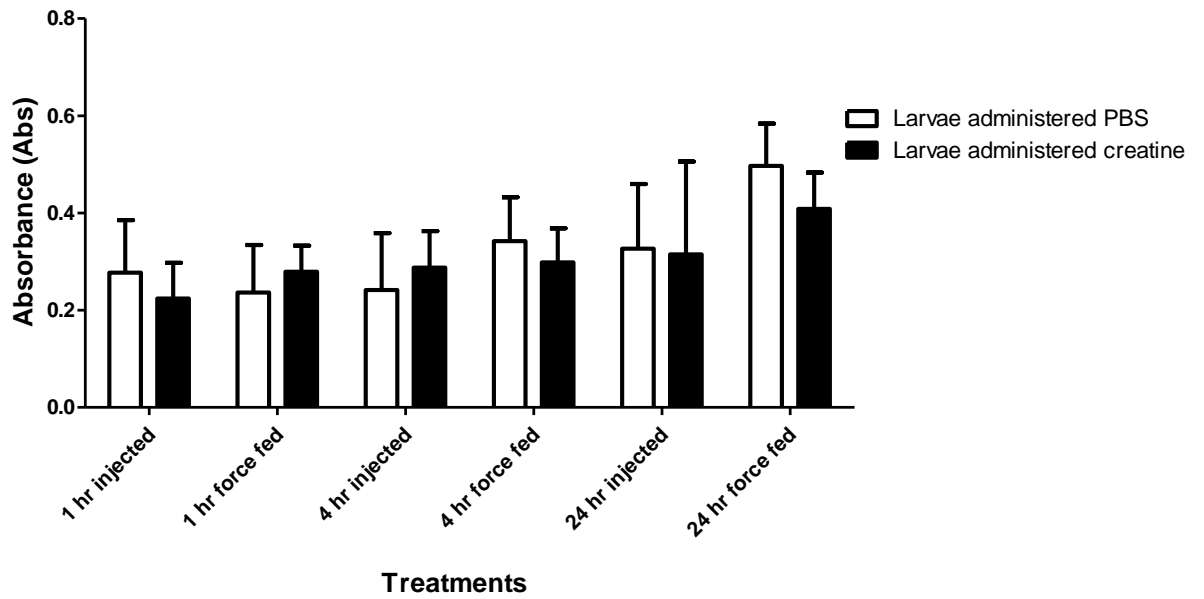


Fig. 6.21 Catalase activity of haemolymph from larvae administered creatine monohydrate via force feeding and intra-haemocoel injection. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered monosodium glutamate. Catalase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

6.17 Effects of creatine monohydrate on superoxide dismutase activity in *G. mellonella* haemolymph.

Superoxide dismutase activity in the haemolymph of larvae administered creatine monohydrate was measured. Larvae were administered 20 μ l 0.15M creatine monohydrate by intra-haemocoel injection or force feeding. Larvae were incubated at 30°C for 1, 4 and 24 hours post administration of creatine monohydrate and the total superoxide dismutase activity was measured in larval haemolymph. Superoxide dismutase activity was quantified as units of activity, 1 unit of SOD activity equated to the 50% inhibition of the oxidation of quercetin (Section 2.9.2)

One unit of superoxide dismutase activity in PBS injected control larvae equated to $130.96 \pm 36.92 \mu\text{g}/\mu\text{l}$, $164.84 \pm 25.56 \mu\text{g}/\mu\text{l}$ and $136.22 \pm 46.53 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 1, 4 and 24 hours incubation respectively. One unit of superoxide dismutase activity in PBS force fed control larvae equated to $170.47 \pm 48.15 \mu\text{g}/\mu\text{l}$, $145.46 \pm 34.56 \mu\text{g}/\mu\text{l}$ and $165.71 \pm 67.52 \mu\text{g}/\mu\text{l}$ of haemolymph protein following 1, 4 and 24-hours accordingly.

One unit of superoxide dismutase activity in larvae injected with creatine monohydrate equated to $110.86 \pm 31.55 \mu\text{g}/\mu\text{l}$, $138.04 \pm 6.47 \mu\text{g}/\mu\text{l}$ and $131.89 \pm 31.95 \mu\text{g}/\mu\text{l}$ of protein following 1, 4 and 24 hour incubation respectively (Fig 6.22). Superoxide dismutase activity was also measured in haemolymph from larvae administered creatine monohydrate via force feeding (Fig 6.23). At 1, 4 and 24 hours one unit of superoxide dismutase activity was $114.83 \pm 29.77 \mu\text{g}/\mu\text{l}$, $132.88 \pm 32.98 \mu\text{g}/\mu\text{l}$ and $172.22 \pm 39.02 \mu\text{g}/\mu\text{l}$ of protein respectively. No significant changes were observed between control larvae and larvae administered creatine monohydrate.

6.18 Summary

The activity of alkaline phosphatase, superoxide dismutase and catalase was measured in the haemolymph of larvae challenged with creatine monohydrate via force feeding or direct injection into the haemocoel. When compared to their relevant controls no significant changes were seen in the activity of the three enzymes in larvae challenged with creatine monohydrate.

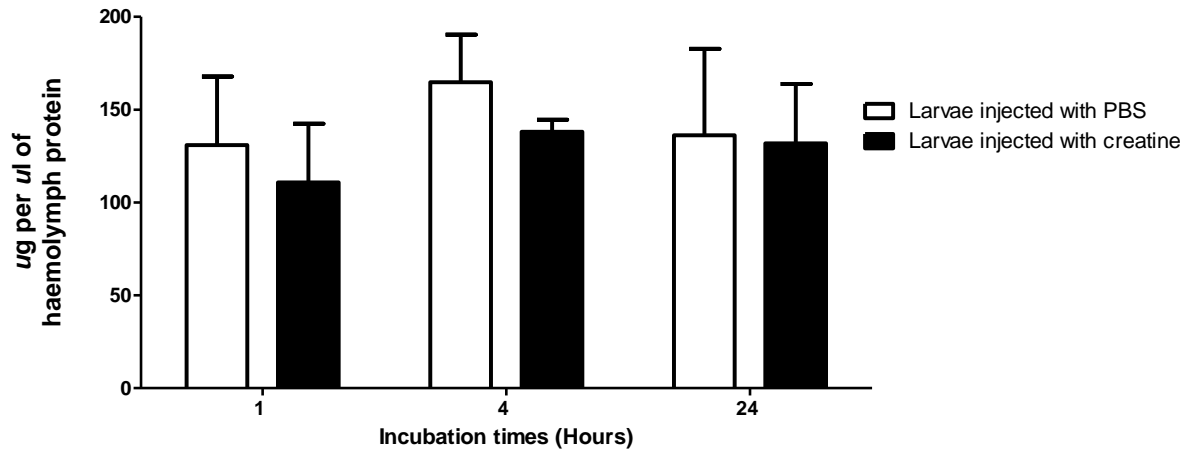


Fig. 6.22 Superoxide dismutase activity of haemolymph from larvae administered creatine monohydrate via intra-haemocoel injection. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administered of creatine monohydrate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

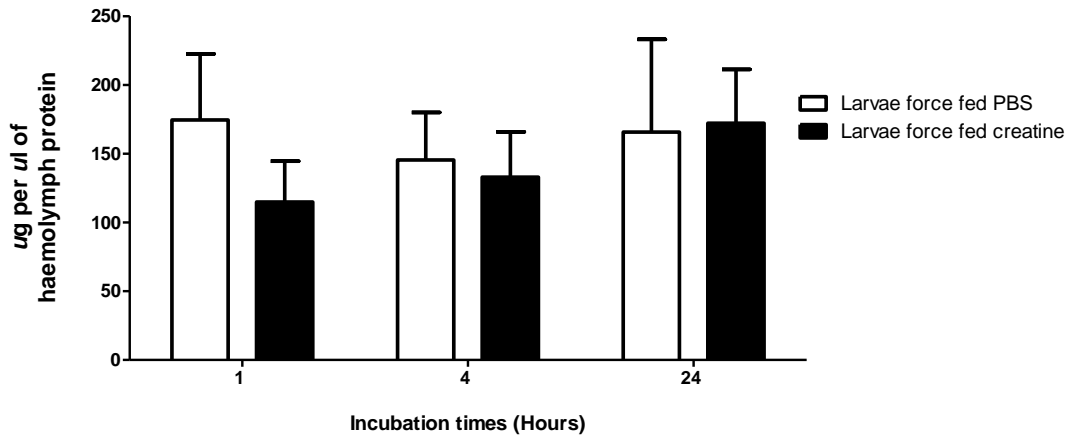


Fig. 6.23 Superoxide dismutase activity of haemolymph from larvae administered creatine monohydrate via force feeding. After 1, 4 and 24 hours haemolymph was extracted from larvae that were administration of creatine monohydrate. Superoxide dismutase activity was determined as described. All values are the mean \pm SE of 3 independent determinations.

6.19 Discussion

G. mellonella larvae were administered 20µl of 0.12 M monosodium glutamate by intra-haemocoel injection or by force feeding as described, and the LD₂₀, LD₅₀ and LD₈₀ values were determined. Section 3.2 established a strong correlation between the LD₂₀, LD₅₀ and LD₈₀ values for eight commonly used food additives in larvae due to feeding or intra-haemocoel injection. The LD₂₀, LD₅₀ and LD₈₀ values from section 3.2 were combined with the LD₂₀, LD₅₀ and LD₈₀ values of monosodium glutamate. This showed an R² value of 0.79 (p=0.0014) between the LD₂₀ values an R² value of 0.87 (p=0.0002) between the LD₅₀ values and an R² value of 0.77 (p=0.0018) between the LD₈₀ values obtained due to feeding and intra-haemocoel administration respectively. Similar to section 3.2 the results demonstrated that monosodium glutamate was toxic irrespective of the route of administration, but the toxicity of monosodium glutamate was greatest when administered by direct injection into the haemocoel. The difference in the relative toxicity observed between the routes of administration may be explained by the structure of the insect digestive system. The insect foregut and hindgut are covered by cuticle, and the midgut epithelium is protected by the peritrophic membrane. Both the cuticle and the peritrophic membrane retard the entry of ingested microbes into the haemocoel (Vallet-Gely *et al.*, 2008), and these structures may also retard the entry of monosodium glutamate into the haemocoel and therefore reduce the relative toxicity. The results however demonstrate a significant positive correlation between the toxicity of the food additives in larvae force fed and injected.

A positive correlation (R²=0.6506, p=0.0156) between the LD₅₀ values for eight preservatives in rats and in *G. mellonella* larvae administered the compounds by feeding was established in section 3.4 however the inclusion of monosodium glutamate data from both mammals and *G. mellonella* resulted in a non-significant correlation in the data. The phenylpyrazole insecticide fipronil is known to act on the γ-aminobutyric acid receptor to block the chloride channel (Narahashi *et al.*, 2010). Fipronil has been found to be much more toxic to insects than to mammals (Narahashi *et al.*, 2010). In recent times fipronil has been reported to block a glutamate-activated chloride channel, a chloride channel that is absent in mammals.

Fipronil blockage of the glutamate-activated chloride channel is deemed responsible, at least partially, for the higher selective toxicity to insects over mammals (Narahashi *et al.*, 2010). In addition while there have been a wide range of glutamate receptor antagonists found in spider venoms thus far only 1 spider venom toxin CNTX-Pn1a from the Brazilian armed spider has been found to inhibit vertebrate glutamate receptors (Pavlovic and Sarac, 2010). Insects and mammals show different responses to glutamate receptor antagonists in pesticides and spider venom, this might suggest that they also show a different receptor mediated response to glutamate and thus monosodium glutamate.

The fungicidal ability of haemocytes extracted from monosodium glutamate administered larvae were measured after 24 hours incubation. The results indicated no significant changes between control larvae and larvae administered monosodium glutamate by intra-haemocoel injection or force feeding. Food additives have been shown to have a deleterious effect on the mammalian neutrophil, vanillin, monosodium L- glutamate, sodium benzoate, and potassium nitrate, were shown to have a significant effect on the oxidative burst and phagocytosis of isolated human neutrophils (Bano *et al.*, 2014). The mammalian neutrophil and insect haemocyte share striking similarities in the way they recognize, phagocytose and kill pathogens (Browne *et al.*, 2013). Despite these similarities the results presented here suggest that the mammalian neutrophil and insect haemocyte react differently to monosodium glutamate exposure.

Alkaline phosphatase activity was determined in the haemolymph of larvae administered monosodium glutamate. When compared to control larvae significant increases in alkaline phosphatase activity was observed in larvae administered monosodium via direct injection into the haemocoel and force feeding. Damaged liver cells release increased amounts of alkaline phosphatase into the blood (Nagino *et al.*, 1999). Alkaline phosphatase is found in the fat body of insects (George and Eapen, 1959) and is conserved between insects and mammals (Eguchi, 1995). Therefore, an increase in alkaline phosphatase activity in the haemolymph could be indicative of fat body damage and thus it is hypothesized that this could serve as a preliminary model of liver damage. Adult male Wistar rats were administered

0.04mg/kg and 0.08mg/kg of monosodium glutamate thoroughly mixed with the grower's mash, on a daily basis for 42 days. Histological findings on liver samples from treated groups showed changes such as dilatation of the central vein, which contained lysed red blood cells, cyto-architectural distortions of the hepatocytes, atrophic and degenerative changes on the liver. This chronic study indicates liver damage in rats exposed to monosodium glutamate daily over a 42 day period (Eweka *et al.*, 2011). The findings presented here revealed fat body damage following a once off administration of monosodium glutamate via direct injection into the haemocoel or force feeding.

Superoxide activity in the haemolymph of larvae administered monosodium glutamate was measured. Significant increases in superoxide dismutase activity was recorded in the haemolymph of larvae administered monosodium glutamate by intra-haemocoel injection and force feeding. It has been demonstrated that administration of high concentrations of monosodium glutamate induce oxidative stress in different organs in rats (Pavlovic *et al.*, 2007). There were no significant increases in catalase activity in the haemolymph of larvae challenged by monosodium glutamate however the significant increase in superoxide dismutase activity would suggest that monosodium glutamate is inducing oxidative stress in the haemolymph of larvae.

Multiple immune related proteins were significantly increased in abundance in larvae injected with monosodium glutamate. These proteins included gloverin (129.59 fold increase), peptidoglycan recognition-like protein B, partial (33.12 fold increase), Lysozyme (7.46 fold increase) and prophenol oxidase activating enzyme 3 (2.37 fold increase). Gloverin is an inducible antibacterial insect protein, that interacts with lipopolysaccharide in the bacterial envelope, specifically inhibiting the synthesis of vital outer membrane proteins, resulting in an increased permeability of the outer membrane (Axen *et al.*, 1997). Peptidoglycan recognition proteins are highly conserved between insects and mammals, they recognize peptidoglycan of the bacteria cell wall (Dziarski, 2004). Lysozyme catalyzes the hydrolysis of a polysaccharide component of the cell wall of Gram-positive bacteria (Dziarski, 2004). Hemolin belongs to the immunoglobulin superfamily, binding to

bacterial surfaces (Sun *et al.*, 1990). These antimicrobial proteins that are increase in abundance would suggest that although there was no significant increases observed in the fungicidal ability of haemocytes from larvae challenged with monosodium glutamate perhaps there may be an increase in the bactericidal ability of these haemocytes. In addition perhaps the administration of monosodium glutamate has an inhibitory effect on the cellular immune response but a beneficial effect on the humoral response.

Glutamine and glutamate are not considered to be essential amino acids however they play an important role in a wide range of biological process (Phillips, 2007). A healthy adult human contains over 80g of free glutamine (Curthoys and Watford, 1995). Glutamine is made via the action of glutamine synthetase from glutamate and ammonia primarily in skeletal muscle, lungs, adipose tissue and liver (Curthoys and Watford, 1995). Under conditions of metabolic stress, including injuries or illness, the level of glutamine in the body declines markedly, which is thought to adversely influence resistance to infectious diseases (de Oliveira *et al.*, 2006). Plasma concentration of 420 $\mu\text{mol/l}$ has repeatedly been reported as a cut-off for a low plasma glutamine concentration associated with a higher risk of mortality in adults (de Oliveira *et al.*, 2006). Supplementation of diets with glutamine, glutamate or both at 0.5 to 1.0% to both suckling and recently weaned piglets improves intestinal and immune function and results in better growth (Curthoys and Watford, 1995). The increase in immune related proteins might be explained by the conversion of glutamate to glutamine.

Larvae were administered by intra haemocoel injection or force feeding 20 μl of 0.15 M creatine monohydrate and the activity of superoxide dismutase and catalase was measured. These results showed no significant changes between the superoxide dismutase and catalase activity in control and treated groups. Exercise training can lead to the induction of oxidative stress due to the over production of reactive oxygen species including superoxide anion, hydroxyl and peroxy radical (Yavari *et al.*, 2015). Studies have been carried out in humans to determine if creatine administration post exercise has an indirect or direct antioxidant effect. Kingsley *et al.* (2009) found that creatine supplementation post high intensity exercise in

humans was not effective in attenuating oxidative stress and did not influence the concentrations of non-enzymatic antioxidant vitamins. These findings presented here showed that creatine monohydrate administration did not lead to any significant changes in the activity of the antioxidant enzymes catalase and superoxide dismutase. Therefore, the findings presented here are in agreement with findings from human studies.

Larvae were administered by intra haemocoel injection or force feeding 20 μ l of 0.15M creatine monohydrate. There were no significant changes in alkaline phosphatase activity between controls and creatine monohydrate challenged larvae. These findings would suggest that creatine monohydrate does not damage the fat body of insects a homologue to the mammalian liver. Despite numerous publications on creatine monohydrate, there is little information on the possible adverse effects of this supplement. In a long-term placebo controlled creatine supplementation trial, 175 individuals were randomly assigned to receive 10g of creatine monohydrate daily or placebo during an average period of 310 days. At the end of the trial no adverse effects were recorded in liver or kidney function (Groeneveld *et al.*, 2005). Twenty-three members of an NCAA Division II American football team were divided into two groups in which they ingested 5 to 20 g of creatine monohydrate for 0.25 to 5.6 years, and a control group in which they took no supplements. Venous blood analysis for serum albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, bilirubin, urea, and creatinine produced no significant differences between groups (Mayhew *et al.* 2002). The findings presented here are indicative of no fat body damage. The fat body in insects is a homologue to the mammalian liver and its response to a compound could be an indication of the liver's response.

No *in-vivo* model is totally accurate in predicting toxicity however by employing multiple models the overall predictability of toxicity can be best described. In this study *G. mellonella* larvae have been employed to determine the acute toxicity of creatine monohydrate and monosodium glutamate. The results presented here provide data that with in combination with other model systems can greatly improve our understanding of the adverse effects these compounds pose to humans.

Chapter 7

General Discussion

7.1 General Discussion

Traditionally the *in-vivo* assessment of novel therapeutics and food additive toxicity has relied strongly upon the use of a variety of vertebrate species, such as mice, rats, guinea pigs and birds (Wojda, 2017). Meta-analysis indicate that rodent models successfully predict adverse effects in humans only 50% of the time (Hunt, 2017). In addition the use of mammalian models incurs large costs and raises issues of ethical acceptance (Browne and Kavanagh, 2013). In the past decade alternative models showing striking metabolic similarities to mammalian models have been widely utilized as mini models in biological research (Wojda, 2017). These alternative model systems include; Zebrafish (Rubinstein, 2006), *C. elegans* (Himri *et al.*, 2013), *D. melanogaster* (Sarikaya and Çakir, 2005) and *B. mori* (Hamamoto *et al.*, 2009).

G. mellonella larvae are a widely used alternative model. *G. mellonella* fulfil many of the basic requirements of a useful animal infection model and have many advantages over other invertebrate systems. Legal and ethical issues restrict the number of mammals that may be used to the lowest possible number, however large numbers of *G. mellonella* larvae can be used in experiments and this can yield results demonstrating a high degree of statistical robustness. *G. mellonella* larvae are widely available and are relatively inexpensive to purchase (Desbois and McMillan, 2015). Larvae can be directly purchased from local suppliers in their preferred sixth instar stage, ready to use and do not incur husbandry costs. *G. mellonella* can be stored at temperatures between 4 and 37°C (Mowlds and Kavanagh, 2008) which makes *G. mellonella* a good model to study fungal pathogens at human body temperature. Despite diverging almost 500 million years ago the insect immune system shows striking similarities to the mammalian innate immune system (Kavanagh & Reeves 2004). Insect gastrointestinal tracts share similar tissue, anatomy and physiological function (Mukerjee *et al.*, 2013). Both phase I and phase II metabolic enzymes are highly conserved between insects and mammals (Feyereisen, 1999; Montella *et al.*, 2012). *Galleria* larvae have been utilized as a model organism to study bacteria (Debois *et al.*, 2011), fungi (Mylonakis *et al.*, 2005), the efficacy and toxicity of novel antimicrobial drugs (Dolan

et al., 2016) and the toxicity of compounds (Büyükgüzel *et al.*, 2013). The aim of this study was to develop *G. mellonella* as a model to test the toxicity of commonly used food additives.

Various studies have demonstrated the possibility of using a less developed alternative model systems to obtain results comparable to vertebrate data. *C. elegans* has been previously utilised to study the toxicity of food additives, the LD₅₀ of monosodium glutamate, tannic acid and thiourea in *C. elegans* showed a significant positive correlation with data obtained from rats ($R^2 = 0.8$) and mice ($R^2 = 0.8$) (Paul and Manoj, 2009). There was a significant positive correlation ($R^2 = 0.924$) between virulence of different wild-type *Vibrio anguillarum* isolates in *Salmo salar* (native) and *G. mellonella* (alternative) infection models (McMillan *et al.*, 2015). Cotter *et al.* (2000) demonstrated results showing the existence of a hierarchy among *Candida* species in terms of their killing ability in *G. mellonella* larvae, i.e. *C. albicans* > *C. tropicalis* > *C. parapsilosis* > *C. pseudotropicalis* > *C. krusei* > *C. glabrata*, which reflects the hierarchy observed in the ability of these species to cause disease in a variety of mammals (Samaranayake and Samaranayake, 2017).

In this study larvae of the greater wax moth were administered eight commonly used food additives by intra-haemocoel or force feeding. The relative toxicity (LD₂₀/LD₅₀/LD₈₀) were determined in larvae administered the food additives by the two routes of administration. Strong positive correlations between the LD₂₀, LD₅₀ and LD₈₀ values for each compound obtained by the different administration routes in larvae were established.

The effect of eight commonly used food additives on the growth of HEP-2 cells was determined. A strong correlation between the relative toxicity of the compounds was observed between HEP-2 cells and larvae force-fed or administered the compounds by intra-haemocoel challenge. HEP-2 cells are widely used for screening the *in-vivo* activity of anti-cancer drugs (Rossi *et al.*, 2003) and have previously been employed to measure the relative toxicity of food additives (Stefanidou *et al.*, 2003). The significant correlation between compound toxicity in *G. mellonella* and a well-established preliminary model of toxicity would suggest *G. mellonella* can be used in the place of the less cost effective cell line.

A positive correlation ($R^2=0.6506$, $p=0.0156$) between the LD₅₀ values obtained for the preservatives in rats and in *G. mellonella* larvae administered the compounds by feeding was established. However the use of *G. mellonella* larvae to measure the acute toxicity of monosodium glutamate resulted in a significantly lower LD₅₀ value than in mammals. To determine the acute toxicity of an unknown compound in mammals a wide concentration range of that compound must be administered. This leads to a large number of experimental mammals being used, which is both ethically and financially unacceptable. Determining the acute toxicity of a compound in *G. mellonella* alone is not a satisfactory prediction of human toxicity (e.g. monosodium glutamate). However the values recorded for the acute toxicity of compounds in larvae may be used to narrow the compound concentration range used in mammalian studies thereby adhering to the three R policy of research.

The immune response of insects shares a number of structural and functional similarities to the innate immune system of mammals (Banville *et al.*, 2011), consequently insects may be used to predict the likely innate immune response of mammals to a variety of pathogens (Junqueira, 2012; Cook and McArthur, 2013) and toxins (Renwick *et al.*, 2007). Perhaps the most striking similarity between the two immune systems is the insect haemocyte and mammalian neutrophil. Both cells share structural and functional similarities to the mammalian neutrophil in that both can phagocytose and neutralize engulfed pathogens through the generation of superoxide and the secretion of lytic enzymes in the process known as degranulation (Renwick *et al.*, 2007). Previous work showed that mammalian neutrophils and insect haemocytes are inhibited when exposed to various toxins such as gliotoxin (Renwick *et al.*, 2007), fumagillin (Fallon *et al.*, 2010), cytochalasin b and nocodazole (Banville *et al.*, 2011). Food additives have been shown to have a deleterious effect on the mammalian neutrophil, vanillin, monosodium l- glutamate, sodium benzoate, and potassium nitrate, were shown to have a significant effect on the oxidative respiratory burst and phagocytosis of isolated human neutrophils (Bano *et al.* 2014). It has been demonstrated that sodium nitrate inhibits the formation of ROS by activated

murine neutrophils and macrophages (Deriagina *et al.*, 2003) and inhibition of ROS formation may be due to NO interference with the membrane component of the NADPH oxidase (Clancy *et al.*, 1992). Flavonoids have also been shown to inhibit the respiratory burst of neutrophils in mammals (Ciz *et al.*, 2012). The aim of this study was to determine if these deleterious effects are also seen in haemocytes. The fungicidal ability of haemocytes from larvae administered commonly used food additives was ascertained. There were significant decreases seen in the fungicidal ability of haemocytes extracted from larvae administered sodium benzoate, sodium nitrate, potassium nitrate, however there was no significant changes seen in the fungicidal ability of potassium sorbate, potassium nitrite, caffeine, monosodium glutamate and creatine. Sodium benzoate, sodium nitrate, potassium nitrate and monosodium glutamate all have deleterious effects on the mammalian neutrophil and all but monosodium glutamate appear to have a deleterious effect on haemocytes. These findings would suggest that haemocytes from *G. mellonella* are a relatively good model of the deleterious effects of food additives on the mammalian neutrophil.

Free radicals, reactive oxygen species and reactive nitrogen can be generated by an organism when exposed to physiochemical conditions, such as xenobiotic exposure (Lobo *et al.*, 2010). A balance between antioxidants and free radicals is necessary to protect an organism from oxidative stress and the resulting damage it may incur (Lobo *et al.*, 2010). A number of antioxidant enzymes produced by the fat body such as superoxide dismutase, catalase and glutathione-S-transferase are highly conserved between species (Büyükgüze *et al.*, 2013). Previously larvae exposed to the insecticide boric acid showed increased lipid peroxidation and altered activity of catalase, superoxide dismutase, glutathione S-transferase, and glutathione peroxidase (Büyükgüzel *et al.*, 2013). Significant increases in catalase activity were seen in the haemolymph of larvae administered sodium benzoate. Significant increases in superoxide dismutase activity were seen in larvae exposed to potassium sorbate, potassium nitrate and monosodium glutamate. In accordance with the findings potassium sorbate, sodium benzoate, potassium nitrate and monosodium glutamate have all been reported to cause

oxidative stress in mammals (Lundberg *et al.*, 2011; Pavlovic *et al.*, 2007; Stratford *et al.*, 2013). Sodium nitrate has been reported to cause oxidative stress in mammals (Lundberg *et al.*, 2011), however it does not cause any increase in superoxide dismutase or catalase activity in the haemolymph of larvae. The majority of these findings support mammalian data. The insect fat body is an organ that also functions in drug metabolism like the liver in mammals (Büyükgüze *et al.*, 2013). This could lead to the possibility to develop the insect fat body as a preliminary model to test the toxicity of food additives in the mammalian liver.

The metabolism of a compound can sometimes lead to the increased toxicity of a compound for example the metabolism of pyridine with an LD50 value of 1.2g/kg to methylpyridine with an LD50 value of 0.2g/kg (Dehpande, 2002). Therefore in this instance the toxicity of a compound is the sum of the toxicity of itself and its metabolites. Paramount to the understanding of the toxicity of a compound is knowledge of the compounds metabolites. Both phase I and phase II enzymes are highly conserved between insects and mammals (Kulkarni and Hodgson, 1984; Vakiani *et al.*, 1998). Previously the metabolism of caffeine to theobromine, theophylline and paraxanthine has been reported in *D. melanogaster* (Coelho *et al.*, 2015). In mammals caffeine is also metabolized to theobromine, theophylline and paraxanthine (Coelho *et al.*, 2015). In this study *G. mellonella* were administered caffeine via intra-haemocoel injection and force feeding. Haemolymph was extracted at t = 0, 4, 24 and 48 hours and analysed for the presence of caffeine and its two metabolites (theobromine and theophylline) using RP-HPLC analysis. Caffeine was detected and at t = 0 hours and at progressively lower concentrations as the time points progressed indicative of its metabolism. Theophylline and theobromine were not detected at t = 0 however both metabolites were detected at t = 4, 24 and 48 hours. The detection of paraxanthine was not possible due to the unavailability of standards. Future work can be done to detect the presence of paraxanthine in caffeine challenged larvae. The findings of this study are highly supportive for the development of *G. mellonella* larvae as a preliminary model of compound metabolism.

Damaged liver cells release increased amounts of alkaline phosphatase into the blood (Nagino *et al.*, 1999). Alkaline phosphatase is found in the fat body of insects (George and Eapen, 1959) and is conserved between insects and mammals (Eguchi, 1995). Therefore, an increase in alkaline phosphatase activity in the haemolymph could be indicative of fat body damage and thus it is hypothesized that this could serve as a preliminary model of liver damage. A chronic study reveals liver damage in rats exposed to monosodium glutamate daily over a 42 day period (Eweka *et al.* 2011). Twenty-three members of an NCAA Division II American football team were divided into a Cr monohydrate group in which they ingested 5 to 20g of creatine monohydrate for 0.25 to 5.6 years, and a control group which took no supplements. Venous blood analysis for serum albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, bilirubin, urea, and creatinine produced no significant differences between groups (Mayhew *et al.*, 2002). A significant increase in alkaline phosphatase activity was seen in larvae challenged with monosodium glutamate, but no significant changes were observed in larvae challenged with creatine monohydrate. The *G. mellonella* model is limited to an acute study and cannot be used in a chronic study, however the findings were in agreement with those from mammalian chronic studies. This would suggest *G. mellonella* as a useful preliminary acute model for screening possible hepatotoxicity.

The life cycle of *G. mellonella* proceeds through 4 life stages: egg, larvae, pupae and adult (Kwadha *et al.*, 2017). Monitoring the developmental progression of larvae into pupae could be used as an indicator of possible adverse effects to development in invertebrates. Zebra fish embryos administered caffeine displayed developmental alterations including reduced body length, reduced tactile responses and muscle fibre formation (Chen *et al.*, 2008). Developmental changes are also seen in the human foetus where by caffeine doses of ≥ 300 mg per day during pregnancy were associated with reductions in infant birth weight that may be especially detrimental to premature or low-birth-weight infants (Hinds *et al.*, 1996). In this study both the rate of pupation and commencement of pupation were reduced in larvae force fed and injected caffeine compared with control larvae. The findings of

this study agreed with vertebrate studies therefore suggesting *G. mellonella* as a biological developmental model.

In nature the greater wax moth larvae moves and burrows into the edge of midrib of honeybee comb. Larvae were administered caffeine and the effect of larval movement was measured. Following caffeine administration larvae were placed on their dorsal surface and the time to reposition correctly was measured. For more in-depth analysis caffeine challenged larvae were placed on the FIMTrack table surface and the distance travelled and velocity were recorded. Previous work on Zebra fish embryos has demonstrated administration of caffeine also reduced mobility (Chen *et al.*, 2008). In this study larvae that were challenged with caffeine showed a significant reduction in their ability to move and reposition correctly, FIMTrack software also revealed a significant decrease in distance travelled and velocity of larvae challenged with caffeine. These findings are in agreement with previous vertebrate findings and would suggest *G. mellonella* larvae as a possible model to test toxic effects on movement in invertebrate species such as zebrafish.

Both phase I and phase II enzymes are highly conserved between insects and mammals and in addition several antimicrobial pathogens are highly conserved between species (Kavanagh and Reeves, 2004). This would suggest *G. mellonella* as proteomic model to assess the humoral immune and metabolic response of compounds. In total three Q-exactive proteomic studies were carried out.

The first proteomic study was conducted on the hemolymph of larvae injected with potassium nitrate. This study revealed numerous changes in protein abundance of mitochondrial aldehyde dehydrogenase, mitochondria associated proteins and proteins involved in glycolysis and the Krebs cycle. The study reported that proteins that are involved in the metabolism (Lundberg *et al.*, 2011) and response to nitrates (Moncada and Erusalimsky, 2002) in mammals are also present and upregulated in *G. mellonella* challenged with potassium nitrate.

The second proteomic study was conducted on the brain and surrounding tissue of larvae force fed caffeine. Significant changes in the abundance of immune related proteins, proteins involved in ATPase synthase and proteins involved in larval

development. These changes in protein abundance were also seen in invertebrate studies (Haskó *et al.*, 2005; Blayney *et al.*, 1978; Talamillo *et al.*, 2013).

The third and final proteomic study was conducted on the haemlyphm of larvae injected with monosodium glutamate. Multiple immune related proteins were significantly increased in abundance in larvae injected with monosodium glutamate. Supplementation of diets with glutamine, glutamate or both at 0.5 to 1.0% to both suckling and recently weaned piglets improves intestinal and immune function and results in better growth (Watford 2015). This increase in immune function in mammals appears to be similar to the increase in humoral immune function in larvae challenged with monosodium glutamate. All three proteomic studies showed significant similarities with the response of various invertebrates to the three food additives.

The aim of this study was to develop *G. mellonella* as a model to test the toxicity of a range of food additives. This study has shown the use of *G. mellonella* as a preliminary model to measure the acute toxicity of food additives. The immune system of *G. mellonella* has in previous studies been utilised to test the virulence of pathogens (Fedhila *et al.*, 2009). In this study a strong correlation between the adverse effects of food additives on the immune system of insects and mammals was established. Various antioxidant enzymes are highly conserved between species and results obtained from this study showed significant increases in antioxidant enzymes in response to food additives that have been reported to cause oxidative stress in mammals. This study reported the metabolism of a food additive in *G. mellonella* larvae to be similar to mammalian metabolism. Developmental changes and movement disorders that were reported in vertebrate models were also observed in *G. mellonella* challenged with food additives. The insect fat body shows many similarities to the mammalian liver, an assay to measure fat body damage in *G. mellonella* larvae produced comparable results to mammalian studies of hepatotoxicity. Proteomic studies conducted on larvae challenged with food additives produced results that show similarities to the proteomic response of both mammals and vertebrates to food additives. In summary the larvae of the greater wax moth will never be a standalone model to measure the toxicity of food additives. However

G. mellonella larvae are a very strong preliminary model of food additive toxicity, and the data provided by this model could help to reduce the number of mammals and more neurologically developed vertebrates in biological toxicity testing. This work opens up the potential future applications of larvae in the study of toxicity.

Chapter 8

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